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1

CAR T cell-mediated cytotoxicity and cytokine release in response to varying levels of antigen expression on target cells.

S. Barnes[1], Z. Yu[2], D. Sullivan[1], D. Lazar[3], K. Haupt[4], D. Califano[5], S. Chvatal[5], D. Millard[1]
[1]Axion BioSystems, Applications, Atlanta, United States of America, [2]Axion BioSystems, Commercial, Atlanta, United States of America, [3]Promega, Cell Analysis, Madison, United States of America, [4]Promega, Product Management, Madison, United States of America, [5]Axion BioSystems, Product Management, Atlanta, United States of America

The development of immunotherapies relies on the use of in vitro potency assays, which are crucial for understanding the complex interactions between immune cells (effectors) and cancer cells (targets). Chimeric antigen receptor (CAR) T-cell therapy is an immunotherapeutic approach that exhibits promise; however, varying levels of antigen expression on the surface of tumor cells can influence the efficacy of CAR T cell-mediated killing. To fully understand the impact of target antigen density on CAR T cell activity, we utilized a matrix approach to assess both target cell death, using an impedance-based in vitro potency assay to quantify CAR T cell-mediated cytotoxicity, as well as cytokine release with homogeneous LumitTM immunoassays. SKOV3 (high HER2 expression), A549 (low HER2 expression), or MDA-MB-231 (no HER2 expression) target cells were seeded into a 96-well microplate with embedded electrodes in the substrate that detect the attachment and proliferation of target cells. HER2 CAR T cells were added after 24 hours at effector-to-target (E:T) ratios of 1:5 or 1:1 and target cell cytolysis was recorded continuously by the Maestro Z for 72 hours. Cytolysis of the target cells was calculated by comparing the treated wells to no treatment control wells and full lysis wells (1% TritonX). At 72 hours post CAR T cell addition, complete killing was observed in SKOV3 cells at the 1:1 ratio, while A549 cells exhibited only 80% cytolysis. MDA-MB-231 cells showed 20% cytolysis, likely due to nonspecific killing by non-engineered T cells, as approximately only 78% of the CAR T cell population was CAR positive. Because the cytolysis assay was label-free, cytokine analysis was able to be multiplexed with the same plates. To assess release of pro-inflammatory cytokines, TNF-α and IFN-γ, we collected supernatant post-antigen exposure at 24, 48, and 72 h. At the 1:1 E:T ratio, CAR T cells co-cultured with target cells expressing low (A549) or high (SKOV3) levels of HER2 had the highest TNF-α production at 24 hours, with CAR T cells co-cultured with SKOV3 cells releasing 30.6% more TNF-α when compared to A549 cells. The highest levels of IFN-γ were detected in both A549 and SKOV3 groups at the 1:1 E:T ratio, with the highest levels observed from CAR T cells co-cultured with SKOV3 cells at 72 hours, releasing approximately 1459.6 +/- 357.3 pg/mL of IFN-γ, compared to 1093.8 +/- 387.5 pg/mL IFN-γ released by CAR T cells co-cultured with A549 cells. As expected, CAR T cells co-cultured with MDA-MB-231 cells did not release any detectable TNF-α or IFN-γ at either E:T ratios during the duration of the experiment Future work will continue to explore multiplexed potency assays for the evaluation of CAR T cell therapies.

2

Enhancement of T cell therapy effects in human solid tumor explants by modulation of key chemokine networks

A. Berthel[1], P. Schmidt[2,3], F. Klupp[4], M. Schneider[4], M. Wallwiener[5], D. Jäger[2], N. Halama[1]
[1]Helmholtz Institute for Translational Oncology (HI-TRON), German Cancer Research Center, Department of Translational Immunotherapy, Mainz, Germany, [2]National Center for Tumor Diseases, Department of Medical Oncology, Heidelberg, Germany, [3]German Cancer Research Center, Department of GMP and T cell Therapy, Heidelberg, Germany, [4]University Hospital Heidelberg, Department of Surgery, Heidelberg, Germany, [5]University Hospital Heidelberg, Department of Obstetrics and Gynecology, Heidelberg, Germany

Cellular therapies are a promising immunotherapy strategy in hematological malignancies. In solid tumors, however, optimization of approaches is essential to enhance therapy effects. T cell infiltration highly depends on microenvironmental factors such as the extracellular matrix composition including chemokines. In colorectal cancer liver metastases (CRC-LM), we recently observed highest T cell numbers in the invasive margin and not in the tumor epithelium, which could be associated with key chemokines, that were similarly highest concentrated in the invasive margin and not in the tumor. Here we show that T cell infiltration into the tumor epithelium of solid tumors can be enhanced by chemokine network modulation. By utilizing our fully human T cell tracking explant model, we identified enhanced autologous and allogeneic T cell infiltration by combined modulation of key chemokines in approximately 50% of CRC-LM patients. We observed decreased tumor cell numbers and enhanced contact between T cells and tumor cells in the chemokine modulation-reactive patient group. Moreover, multiplex cytokine analysis revealed a strong reduction and reorganization of cytokines, especially myeloid cell related factors, in the tumor microenvironment of these patients. We observed effects on CD163-positive macrophages and their contact with T cells underscoring an important role of innate immune cells for effective treatment. Finally, we extended our approach utilizing autologous and allogeneic chimeric antigen receptor (CAR)-T cells and CRC-LM as well as ovarian cancer patient samples revealing similar effects of combined chemokine network modulation. In summary, modulation of key chemokines in the tumor microenvironment can enhance T cell therapy effects in solid tumors. Moreover, the identification of recurring patterns and patient stratification by treatment approaches in combination with spatial analyses highlights the strength of our fully human T cell tracking explant model as a promising tool for preclinical cellular therapy analyses.

3

Selection of superior KRAS G12V mutation-specific T cell receptors with unique characteristics for 3rd generation armored and enhanced T cell therapy

J. Bittmann, M. Salvermoser, D. Alterauge, A.-W. Mohr, D. Brechtefeld, M. Catarinella, P. U. Prinz, M. Buerdek, K. Davari, D. Schendel, G. Longinotti

Medigene Immunotherapies GmbH, Planegg/Martinsried, Germany

The presence of activating mutations within the Kirsten rat sarcoma (KRAS) gene represents a hallmark feature in a variety of hard-to-treat solid cancer indications, such as pancreatic, colorectal and non-small

cell lung cancer. KRAS mutations (mKRAS) are dominated by single amino acid substitutions, with exchanges at position 12 (G12D/V/C) being the most common. Owing to their unique presence in cancer cells and being described as driver mutations, such neoantigens represent prime targets for T cell receptor (TCR)-transgenic T cell (TCR-T) therapies. Despite promising clinical data, TCR-T therapies still face several challenges such as lack of durability and efficacy, potentially due to an immunosuppressive tumor microenvironment (TME), including T cell inhibition via the PD-L1/PD-1 axis. Armoring TCR-T cells with the PD1-41BB costimulatory switch protein (CSP) aims to mitigate the inhibitory effects by enhancing T cell function and counteracting T cell exhaustion. Here, we combined the PD1-41BB CSP with three mKRAS G12V-specific HLA-A*11-restricted TCRs and showcase their highly specific, sensitive and safe (3S) recognition profile.

mKRAS G12V-specific TCR sequences were isolated from healthy donor T cell repertoires using a high-throughput process and early functional screens. Selected TCRs were co-expressed with PD1-41BB CSP in recipient T cells and vetted for IFN-γ release and cytotoxicity in response to mKRAS G12V+ and KRAS wild type tumor cells, functional avidity, HLA-allogeneic cross-recognition, off-target toxicity as well as for additional attributes such as CD8 co-receptor independency.

Through our in-depth vetting process, we identified three candidate TCRs matching our 3S criteria. All three TCRs showed exclusive specificity for the KRAS G12V mutation across various HLA-A*11 subtypes and demonstrated high peptide sensitivity with strong tumor cell recognition as well as a favorable safety profile. Additional analyses unveiled CD8 co-receptor independency of one of the TCRs, as it showed comparable effector functions upon expression in $CD4^+$ T cells. Armoring of TCR-T cells with the PD1-41BB CSP yielded increased and sustained IFN-γ release and enhanced tumor spheroid killing in response to mKRAS G12V+ PD-L1+ tumor cells.

In summary, Medigene´s vetting assay algorithm delivered a selection of superior mKRAS G12V-specific HLA-A*11-restricted TCRs with optimal specificity, sensitivity and safety. Armoring mKRAS G12V-specific 3S TCR-T cells with PD1-41BB CSP resulted in enhanced effector functions to address the challenges of a hostile TME by its dual mode-of-action. This 3rd generation TCR-T cell product has the potential to show superior efficacy in hard-to-treat tumors and thus to improve clinical outcome of TCR-T cell therapies in solid tumor indications.

4

Tertiary lymphoid structure-related immune infiltrates in NSCLC tumor lesions correlate with low tumor-reactivity of TIL products

S. Castenmiller[1], N. Kanagasabesan[1], A. Guislain[1], B. Nicolet[1], M. van Loenen[1], K. Monkhorst[2], A. Veenhof[2], E. Smit[2], K. Hartemink[2], J. Haanen[2], R. de Groot[1], M. Wolkers[1]

[1]Sanquin Research, Amsterdam, The Netherlands, [2]Antoni van Leeuwenhoek hospital/Netherlands Cancer Institute, Amsterdam, The Netherlands

Adoptive transfer of tumor infiltrating lymphocytes (TIL) has shown great potential for the treatment of solid cancers, including non-small cell lung cancer (NSCLC). However, not all patients benefit from this therapy, and the parameters that define the likelihood of TIL products to be tumor reactive are to date unknown. Defining prognostic markers that correlate with high level of tumor-reactivity is key for achieving

better tailored immunotherapies.
To determine whether the composition of immune cell infiltrates correlates with the tumor-reactivity of expanded TIL products, we employed multi-parameter flow cytometry to characterize the immune cell infiltrates from 26 freshly isolated early-stage, and 20 late-stage NSCLC tumor lesions. Unbiased flow cytometry analysis with Cytotree and Spearman's Rank Correlation was used to correlate immune infiltrates with the expansion rate, immune cell activation and T cell differentiation stage, and the anti-tumor response of TIL products generated from the same lesions.
The composition of tumor immune infiltrates was highly variable between patients, irrespective of the disease stage. High percentages of B cell infiltrates positively correlated with the presence of conventional CD4 T cells, and an overall increase of naïve T cell infiltrates. In contrast, high B cell infiltrates negatively correlated with the tumor-reactivity of expanded TIL products, as defined by cytokine production upon exposure to autologous tumor digest. Tumors with high B cell infiltrates contained $BCL6^+$ B cells and $CXCR5^+BLC6^+$ CD4 T cell infiltrates and an increased percentage of naïve CD8 T cells, indicative of the presence of tertiary lymphoid structures (TLS) in tumors with high B cell infiltrates.
This study reveals that the composition of immune cell infiltrates in NSCLC tumors associates with the functionality of expanded TIL products from NSCLC tumor lesions. Importantly, the tumor-responsiveness of TIL products negatively correlated with the presence of TLS-associated immune infiltrates in tumors. Our finding may thus help improve patient selection for TIL therapy.

5

Anti-Sialyl-di-Lewis^a CAR T cells for effective tumor therapy of gastrointestinal tumors

I. Charles[1], K. Cook[1], G. Cane[2], B. Kaira[1], A. Skinner[1], A. Wright[1], M. Vankemmelbeke[1], R. Metheringham[1], V. Brentville[1], L. Durrant*[1]
[1]Scancell Limited, Biodiscovery Institute Phase 3, University of Nottingham, Nottingham, United Kingdom, [2]Scancell Limited, Bellhouse Building, Sanders Road, Oxford Science Park, Oxford, OX4 4GD, United Kingdom

Targeting cancer-associated glycans is a viable option for cancer immunotherapy. We previously described an antibody (SC129) that specifically targets Sialyl-di-Lewis^a which is overexpressed on many cancer types including pancreatic, colorectal, gastric, ovarian, and non-small cell lung with extremely limited normal expression. Chimeric antigen receptor (CAR) T cell therapy has the potential to target tumors with all the advantages of an antigen-specific T cell response, but without the dependence on MHC-presentation. We investigated the feasibility of Sialyl-di-Lewis^a CAR T cells as a therapeutic modality for the treatment of gastrointestinal (GI) tumors.
Murine SC129 antibody was humanised to produce h129 which showed similar Sialyl-di-Lewis^a binding and bound pancreatic and colorectal cancer cell lines. The SC129 and h129 binding sequences (scFv) in either the light-heavy (LH) or heavy-light (HL) orientations were assessed by ELISA, SPR and flow cytometry to determine the optimal configuration for CAR T cells.
In vitro assessment demonstrated Sialyl-di-Lewis^a CAR T activation and tumor killing in a glycan-dependent manner as measured by flow cytometry, ELISA and LDH release assays. In vivo efficacy of CAR T cells in a tumor therapy model was determined in NSG mice implanted with COLO205 tumor cells. The Sialyl-di-Lewis^a -targeting CAR T cells mediated tumor regression compared to treatment with non-transduced T

cells (p<0.0012) with complete regression of 70% of established tumours. This data showed that anti-Sialyl-di-Lewis^a CAR T cells induce effective control of Sialyl-di-Lewis^a expressing solid tumors. Our results suggest that targeting cancer-associated glycans using CAR T cell technology has potential and may lead to new avenues for cancer immunotherapy.

6

Targeting glioblastoma stem cells with an HLA-A*02-restricted TCR specific to cancer-associated antigen, PTPRZ1

Y.-C. Chih[1,2,3], A. Dietsch[1], P. Koopmann[1], D. Agardy[1,2,3], A. De Roia[1,2,3,4], A. Kourtesakis[2,5,6], M. Kilian[1,3], C. Krämer[1,3], M. Ratliff[6,7], E. Green[1], H. Bönig[8], M. Stenzinger[9], A. Blum[10], V. Pieńkowski[10], A. Suwala[11], F. Sahm[11], W. Wick[5,6], M. Platten[1,3,12,13,14], L. Bunse[1,3,14]

[1]German Cancer Research Center (DKFZ), Clinical Cooperation Unit (CCU) Neuroimmunology and Brain Tumor Immunology, Heidelberg, Germany, [2]Heidelberg University, Faculty of Biosciences, Heidelberg, Germany, [3]Heidelberg University, Department of Neurology, Mannheim Center for Translation Neuroscience (MCTN), Medical Faculty Mannheim, Mannheim, Germany, [4]DKFZ, DNA Vector Laboratory, Heidelberg, Germany, [5]Heidelberg University Hospital, Neurology Clinic, Heidelberg, Germany, [6]DKFZ, CCU Neurooncology, Heidelberg, Germany, [7]Heidelberg University, Neurosurgery Clinic, Mannheim, Germany, [8]IKTZ, Heidelberg, Germany, [9]Blood Donation Serice, Frankfurt, Germany, [10]Ardigen, Kraków, Poland, [11]Heidelberg University, Institute for Pathology, Department of Neuropathology, Heidelberg, Germany, [12]National Center for Tumor Disease (NCT), Immune Monitoring Unit, Heidelberg, Germany, [13]Helmholtz Institute of Translational Oncology (HI-TRON), Mainz, Germany, [14]DKFZ Hector Cancer Institute at the University Medical Center Mannheim, Mannheim, Germany

Cell therapy has proven effective against hematopoietic cancers, but its efficacy in solid tumors, particularly glioblastoma (GB), faces challenges such as the blood-brain barrier (BBB), limited antigens, and an immunosuppressive microenvironment. T cell receptor (TCR) engineered T cell therapy provides a circumvention by broadening the potential antigen repertoire. It targets both extracellular and intracellular antigens and has shown encouraging results in phase 1 and 2 trials, yet it has never been practiced for GB in the clinic. Here, we leveraged vaccine-induced T cell responses from the GAPVAC trial, where GB patients were immunized with a pool of peptides covering unmutated GB-associated antigens (APVAC1), including protein tyrosine phosphatase type Z1 (PTPRZ1). PTPRZ1 is expressed only in malignant cells, particularly in GB stem cells. PTPRZ1-reactive CD8+ T cells were sorted and subject to single-cell VDJ sequencing from an HLA-A*02+ GAPVAC patient responding to examined PTPRZ1 peptides. The dominant TCR clonotypes were functionally validated using Jurkat reporter cells. One TCR was validated to be highly PTPRZ1-reactive to both exogenously loaded peptide and naturally processed antigen. The safety of the identified TCR was further assured as it did not cross react with in silico artificial intelligence-predicted off-targets. Primary human T cells engineered with the TCR were activated and able to execute cytotoxicity in an antigen-specific manner. Moreover, the process of TCR engineering favored CD8+ T cell expansion and maintenance of the stem cell-like memory (T_{SCM}) phenotype. Although TCR-CD8+ T cells were the main cytotoxic effectors, the presence of TCR-CD4+ T cells improved maximal cytotoxic capacity. In a subcutaneous tumor model, intravenous (i.v.) transfer of TCR-T cells led to tumor regression in tumor-bearing mice, with 30% of them experiencing complete regression. Examining the TCR potency against experimental brain tumors, we

found that i.v. TCR-T cell transfer could not control intracranial (i.c.) tumors. Remarkably, with the addition of intracerebroventricular (i.cv.) transfer of TCR-T cells, i.c. tumors were potently controlled; 5 out of 7 mice showed radiographic response assessed by preclinical MRI, of which 4 (57%) survived until the experimental endpoint. The transferred CD8+ TCR-T cells in the host retained durable T_{SCM} phenotype. To investigate whether the identified TCR was universally therapeutic to HLA-A*02+ GB, we established primary GB spheroid cell lines. Intriguingly, TCR-T cells were only activated by and lysed all HLA-A*02+ GB cell lines (5 out of 5) with a preference for slow-cycling cells. Encouraged by these findings, we are preparing a phase 1 clinical trial, Intraventricular T cell receptor transgenic T cell therapy to treat glioblastoma (INVENT4GB), to assess the feasibility and safety of i.v. and i.cv. PTPRZ1-TCR-T cell therapy in patients with recurrent GB.

7

Broad T-cell receptor repertoires in TIL and differential Th1/2/17 functional phenotypes associated with the tumor microenvironment

E. de Sousa[1], J. Lérias[1], C. Gorgulho[1], S. Pineda[2], L. Lordello[1], W. Pan[3], M. Byrne-Steele[3], D. Ligeiro[1], V. Balan[1], Z. Wang[4,5], J. Han[3], M. Gama-Carvalho[6], M. Maeurer[1]
[1]Champalimaud Foundation, Lisbon, Portugal, [2]Complutense University of Madrid, Madrid, Spain, [3]iRepertoire Inc., Huntsville, United States of America, [4]Guangdong Provincial Hospital of Chinese Medicine, Guangzhou, China, People's Republic of, [5]Guangdong Provincial Academy of Chinese Medical Sciences, Guangzhou, China, People's Republic of, [6]Faculty of Sciences, University of Lisbon, Biosystems and Integrative Sciences Institute (BioISI), Lisbon, Portugal

Tumor Infiltrating Lymphocytes (TIL) therapy is a highly personalized active cell therapy (ACT), where TIL are removed from tumor-tissue, expanded and manipulated ex vivo to large numbers and subsequently re-infused into the patient, usually along with IL-2 and after lymphodepletion. TIL therapy has mediated clinically relevant regressions in patients with melanoma, cervical cancer, lung cancer and in some individual patients with epithelial cancer. Although the tumor-microenvironment (TME) suppresses effective cellular anti-tumor immune responses, TIL may exhibit a particular epigenetic imprint imposed by the TME which facilitates precursor T-cells to effectively home and remain in cancer lesions. The standard approach to characterize the TIL product prior to ACT is either phenotypic classification gauging immune potency with a broader examination of cytokine production, usually IFN-γ production.
High-throughput sequencing of the T-cell receptor (TCR) repertoire is a robust and molecularly defined way to identify the clonal composition of a T-cell population which may be used to potentially predict cancer-associated epitopes, it also aids to trace over time a complex autologous transplant after re-infusion associated with clinical outcome. Dispensable tumor tissue from 5 different patients with epithelial cancer were immediately segregated after surgery into individual zones based on the presence of tumor cells, immune cells, tertiary lymphoid structures (TLS), tumor core, invasion front or desmoplastic zones with sparse tumor and T-cell infiltrates. T-cells from these different zones were expanded separately. High-throughput sequencing of the TCR α, β, γ or δ CDR3 regions and a panel of 170 immune marker genes was performed in the parallel tumor section, the corresponding expanded TIL and in peripheral blood. TIL expansion results in a broad TCR repertoire distinct from non-expanded TIL in the parallel tumor tissue. Although TIL from different tumor zones exhibited between 5% and 30% shared TCRs in the TCR α, β and

0% and 50% shared in the TCR γ or δ repertoire, we identified differences in the antigen-specific Th1/2/17 cytokine production pattern which was associated with the location of TIL, i.e. TIL harvested from the invasion front of the tumor exhibited a Th1 production profile in response to synthetic tumor-associated target antigens and T-cells harvested from TLS and the tumor core exhibited a mixed Th1/2/17 pattern based on IFN-γ, IL-4 and IL-17 production in response to molecularly defined tumor targets.
The epigenetic imprint imposed by the TME shapes the quality of an anti-cancer directed immune responses which i) warrants a more selected analysis of TIL harvested from cancer lesions and ii) calls for a viable TME-context dependent therapeutic genetic engineering of cell products to 'ignore' immune-suppressive signals.

8

TanCAR design for PDAC enhances antitumor effects and reduces antigen escape

C. Dourlens[1,2,3], K. Vanderliek[3], D. Schäfer[3]
[1]University Medical Center Göttingen, Translational Molecular Imaging, Institute for Diagnostic and Interventional Radiology & Clinic for Hematology and Medical Oncology, Göttingen, Germany, [2]Max Planck Institute for Experimental Medicine, Translational Molecular Imaging, Göttingen, Germany, [3]Miltenyi Biotec B.V. & Co. KG, R&D Reagents, Bergisch Gladbach, Germany

Genetically engineered cellular products have become a newly established pillar of cancer therapy. This has dramatically revolutionized treatment paradigms for several leukemic diseases, especially in regard to B cell malignancies. While cellular therapies have been unprecedentedly successful against leukemia, they still lack efficacy when it comes to solid tumors and remain an area of active research. A major obstacle is the limited spectrum of T cell specificity in the face of tumour heterogeneity and potentially dynamic antigen escape variants, which can lead to tumour recurrence after initial treatment with CAR T cells directed to a single tumor antigen. Given the heterogeneous expression of antigens on many solid malignancies and the very poor prognosis of patients with pancreatic ductal adenocarcinoma (PDAC), we hypothesised that a bispecific CAR molecule with an OR-gated system (tandem CAR, TanCAR) can mitigate antigen escape and improve the antitumour activity of CAR T cells. To this end, we generated TanCARs varying in spacer length (short vs. extra short), myc-tag position (N- vs. C-terminal), and scFv combinations for three previously identified PDAC targets. We assessed CAR functionality based on transduction potential, tag-detection, cytotoxic potential, marker upregulation and cytokine release. CAR T cell were also co-cultured with cell lines reflecting target escape variants. We observed as a general pattern, that tagged CARs were less expressed and had lower efficiencies as compared to non-tagged CARs, leading to the hypothesis that tags can interfere with proper CAR folding. This inspired us to develop detection reagents specific to our TanCARs, to enable robust CAR detection without the need for additional tags. These reagents facilitated fast and efficient verification of CAR presence, while not impeding CAR folding. Although, several constructs exhibited satisfactory cytotoxicity, we could not deduce a clear correlation between TanCAR design and functionality. In conclusion, this study demonstrates that TanCAR T cells specific for PDAC can enhance immunotherapeutic effects by reducing chances for tumour antigen escape and increasing T cell functionality, which can be extended to other target combinations and other solid tumour entities. Future studies should investigate if our results generated in vitro can be replicated in murine models. In addition,

using CARs specific for two antigens, TanCARs come along with increased risk for on-target/off-tumor toxicities, which should be addressed carefully. If successful, TanCARs could be a valid option to remedy some of the current issues CAR T cell therapy is facing in the context of solid malignancies.

9

TCR-engineered T cells recognizing a shared β-catenin mutation presented on two prevalent HLA-alleles eradicates solid tumors in vivo

M. S. Eggebø[1,2,3], H. Palashati[1,2,3], N. Chandler[1,2,3], T. T. Tran[2,3,4], Y. Li[1,2,3], W. Yang[1,2,3], F. Lund-Johansen[2,3,4], M. Laos[1,2], I. Blaas[1,2], E. H. Rustad[1,2,3], R. C. Bollineni[1,2], M. Delic-Sarac[1,2,3], M. M. Nielsen[1,2,3], J. Olweus[1,2,3]
[1]Oslo University Hospital Radiumhospitalet, Deparment of Cancer Immunology, Oslo, Norway, [2]University of Oslo, Institute of Clinical Medicine, Oslo, Norway, [3]Precision of Immunotherapy Alliance, University of Oslo, Institute of Clinical Medicine, Oslo, Norway, [4]Oslo University Hospital, Department of Immunology, Oslo, Norway

We aimed to identify T-cell receptors (TCRs) capable of recognizing recurrent mutations within β-catenin, a protein encoded by the CTNNB1 gene. β-catenin is mutated in 3.1% of cancers, with several recurrent gain-of-function mutations in the N-terminal domain. These mutations are known to contribute to solid cancer progression by driving cell cycle induction and proliferation, whereby offering attractive targets for TCR therapy. By performing immunopeptidomics on human leukocyte antigen (HLA)-monoallelic cell lines transduced to express CTNNB1 hotspot mutations, we found that a recurrent mutation in the CTNNB1 gene ($CTNNB1^{Mut}$) was presented in the context of the two frequent HLA-alleles, HLA-A*02:01 and HLA-A*24:02. Targeted immunopeptidomics was performed to confirm and quantify neopeptide processing and presentation on cell lines endogenously expressing the mutation. To investigate whether the identified peptides could induce neoantigen-reactive TCRs from healthy donor naïve T cell repertoires we utilized an in vitro screening pipeline to identify T cell clones capable of reacting to the peptide antigens. Here, we identified two T-cell receptors recognizing the recurrent CTNNB1 mutation, one restricted by HLA-A*02:01 and the other restricted by HLA-A*24:02. The neoantigen-reactive TCRs were sequenced to enable retroviral expression of TCRs in healthy donor T cells. Both the HLA-A*02:01 and the HLA-A*24:02 restricted $CTNNB1^{Mut}$ TCRs recognize their cognate peptides at high sensitivity while showing no reactivity to the corresponding $CTNNB1^{WT}$ peptides. T cells modified to express the $CTNNB1^{Mut}$ TCRs demonstrate promising efficacy, effectively eliminating endogenous $CTNNB1^{Mut}$ cell lines in vitro and inducing tumor eradication and prolonged survival in an in vivo melanoma mouse model. These results suggest that the identified TCRs could represent a promising therapeutic opportunity for treatment of CTNNB1-mutated solid cancers in patients expressing HLA-A*02:01 or HLA-A*24:02, using TCR-modified T cells.

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Enhancing CAR-T cell therapy in chronic lymphocytic leukemia: overcoming limitations and augmenting efficacy

J. Hartmann[1,2], A. Floerchinger[1,3], B. Brinkmann[3,4,5,6,7], N. Mack[1], M. Coelho[1,3], S. Dietrich[5,6,7,8,9], M. Seiffert[1]

[1]German Cancer Research Center (DKFZ), Molecular Genetics, Heidelberg, Germany, [2]University of Heidelberg, Faculty of Medicine, Heidelberg, Germany, [3]University of Heidelberg, Faculty of Biosciences, Heidelberg, Germany, [4]German Cancer Research Center (DKFZ), Clinical Cooperation Unit Molecular Hematology/Oncology, Heidelberg, Germany, [5]Molecular Medicine Partnership Unit (MMPU), Heidelberg, Germany, [6]European Molecular Biology Laboratory (EMBL), Heidelberg, Germany, [7]University of Heidelberg, Department of Medicine V, Hematology, Oncology and Rheumatology, Heidelberg, Germany, [8]University Hospital Düsseldorf, Department of Hematology, Oncology and Clinical Immunology, Düsseldorf, Germany, [9]Center for Integrated Oncology Aachen Bonn Cologne Düsseldorf (CIO ABCD), Aachen/Bonn/Cologne/Düsseldorf, Germany

Anti-CD19 chimeric antigen receptor T cell (aCD19-CAR-T cell) therapy has emerged as an effective treatment for B cell leukemia and lymphoma for which it has been approved in patients after failure of standard therapies. However, in chronic lymphocytic leukemia (CLL) its efficacy remains limited with a majority of the patients not achieving sustained remission. Using the Eμ-TCL1 mouse model, a fully immunocompetent model of CLL, we observed a dose-dependent reduction of CLL development with CAR-T cell monotherapy, whereas untransduced T cells were not able to control the disease. Notably, when injecting aCD19-CAR-T cells at a dose with suboptimal treatment efficacy, disease progression often recurred after initial treatment response, mirroring the potential but also main clinical challenges.

In the setting of a survival study we assessed potential benefits of combining aCD19-CAR-T cells at a suboptimal dose with bispecific antibodies which target CD3 on T cells and CD20 on B cells (CD20-BsAb). Hence, we were aiming to enhance immunotherapeutic efficacy by targeting two antigens, CD19 and CD20, on malignant B cells and engaging endogeneous T cells additionally to CAR-T cells to deplete CLL cells. CAR-T cells were injected following lymphodepletion via sublethal irradiation. Subsequently, a titrated dose of CD20-BsAb was injected weekly which resulted in an enhanced treatment response and a marked reduction of CLL counts in blood. This ultimately led to 80% of the mice in the combination group (n=10) having no detectable malignant B cell population eight weeks after treatment initiation suggesting a potential cure. Co-administration of CD20-BsAb supported total T cell expansion including endogeneous as well as CAR-T cells, induced an increased expression of activation markers on both and enhanced degranulation and cytotoxicity of endogenous CD8+ T cells.

As dysfunction of T cells from CLL patients is considered as one reason for the failure of successful CAR-T cell therapy, we are now investigating potential differences in treatment efficacy and persistence of CAR-T cells generated from T cells isolated from tumor-naïve versus CLL-bearing mice. To address this, we will compare both CAR-T cell types in a killing assay, infuse them separately at varying doses into leukemic mice post-irradiation to evaluate their ability to control disease progression and finally, by injecting the CAR-T cells at a 50:50 ratio into the same recipients we aim to examine if one type dominates over time, indicating a superior proliferation capacity. We plan to overcome potential deficits of the latter by rationale combination therapy which will be tested in CLL culture systems as well as in the mouse model.

In summary, our findings highlight the enhanced therapeutic efficacy of combining aCD19-CAR-T cells with CD20-BsAb in CLL. The synergistic effect induces improved activation and proliferation in endogenous and infused CAR-T cells, contributing to enhanced disease control.

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Pre-clinical development and verification of a novel tumor-infiltrating lymphocyte product for clinical testing in patients with cervical cancer

C. Friese[1], C. Heeke[1], N. Kirketerp-Møller[1,2], S. Færch[1], I. Akdemir[1], U. Cordes[1]
[1]Cbio A/S, Soeborg, Denmark, [2]University of Copenhagen, Biotech Research & Innovation Centre, Copenhagen, Denmark

Cervical cancer is the fourth most prevalent cancer in women with more than 600,000 being diagnosed globally every year and 341,000 dying. Late-stage cervical cancer patients have few and ineffective treatment options leading to a high unmet medical need.
Adoptive cell transfer (ACT) using tumor-infiltrating lymphocytes (TILs), which recently has been approved by the FDA for treatment of advanced melanoma, has also shown promising efficacy in patients with cervical cancer. Shortly, TILs naturally present in the tumor are expanded using a two-step manufacturing process. In the first step, TIL cultures are established from tumor fragments in media containing high-dose interleukin-2 (IL-2). In the second step, the TILs are expanded massively in the presence of allogeneic feeder cells, anti-CD3 and high-dose IL-2 before being reinfused into the lymphodepleted patient followed by IL-2 infusions. Contrasting melanoma, establishing TIL cultures has proven to be more challenging in non-melanoma cancers such as cervical cancer. Additionally, the standard TIL manufacturing process was not scalable due to the number of manufacturing steps and especially the number of open process operations. Therefore, we have improved the standard TIL manufacturing protocol by adding monoclonal antibodies to the tumor fragment culture. Additionally, we have made the manufacturing process more scalable by transferring the young TIL expansion from 24-well plates into G-Rex vessels. Also, the first part of the rapid expansion protocol (REP) is performed in 1 G-Rex vessel instead of 20 T flasks.
Young TILs from ten resected cervical cancer tumors were expanded in a small-scale setup using high-dose IL-2 alone or in combination with monoclonal antibodies. The success rate, yield, expansion time, phenotype and functionality of the TILs were assessed. Some cultures were further expanded using the REP and fold expansion, phenotype and functionality were assessed.
Adding monoclonal antibodies to the tumor fragment cultures during the young TIL expansion could increase the culture success rate to 100% compared to 73.9% when using IL-2 alone. The average yield/fragment could be increased three times while reducing the average culture time from 31 days to 20 days when adding monoclonal antibodies to the cultures compared to cultures with IL-2 alone. Adding monoclonal antibodies to the cultures increased the frequency of $CD8^+$ T cells compared to those only cultured in IL-2. Finally, the entire production process was successfully verified using full-scale young TIL cultures and 20% REP batches. Using this improved manufacturing process, we could produce batches responding to $100x10^9$ TILs in a full-scale production in up to 26 days.
We are currently preparing a clinical trial treating 20 patients with advanced or persistent cervical cancer at Karolinska Institute. Enrollment will commence in Q3 2024.

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Moderate signal strength increases resilience in adoptively transferred neoantigen-specific TCR-transgenic T cells: transient inhibition for fine-tuning adoptive cellular therapies

F. Füchsl[1], J. Untch[1], A. Schwanzer[1], V. Kavaka[2,3], G. Zuleger[1], S. Braun[4], S. Jarosch[4], C. Vogelsang[1], N. de Andrade Krätzig[5,6], D. Gosmann[1], R. Öllinger[5], M. Hiltensperger[1,7], R. Rad[5,6], D. H. Busch[4,8,9], E. Beltrán[2,3,10], E. Bräunlein[1,7], A. M. Krackhardt[1,7,11,12]

[1]Technische Universität München, Klinikum Rechts der Isar, Klinik und Poliklinik für Innere Medizin III, München, Germany, [2]Ludwig-Maximilians-Universität München, Institute of Clinical Neuroimmunology, München, Germany, [3]Ludwig-Maximilians-Universität München, Faculty of medicine, Biomedical Center (BMC), München, Germany, [4]Technische Universität München, Institute for Medical Microbiology, Immunology and Hygiene, München, Germany, [5]Technische Universität München, Institute of Molecular Oncology and Functional Genomic, München, Germany, [6]Technische Universität München, Center for Translational Cancer Research (TranslaTUM), München, Germany, [7]German Cancer Consortium (DKTK)and German Cancer Research Center (DKFZ), München, Germany, [8]Technische Universität München, Focus Group "Clinical Cell Processing and Purification", Institute for Advanced Study, München, Germany, [9]German Center for Infection Research (DZIF), München, Germany, [10]Munich Cluster of Systems Neurology (SyNergy), München, Germany, [11]Technische Universität München, Center for Translational Cancer Research (TranslaTUM), München, Germany, [12]Malteser Krankenhaus St. Franziskus-Hospital, Flensburg, Germany

Strong T cell activation and high functional avidity in T cell receptor (TCR)-based adoptive therapies are generally associated with increased anti-tumor responses upon adoptive transfer. However, when it comes to the longevity and resilience of transgenic (tg) T cells, the factors determining long-term persistence of tumor-targeting T cells and therefore therapy responses remain ill-understood.

In this study, we first profiled the neoantigen-specific TCR (neoTCR)-repertoire of a melanoma patient detected by a mass spectrometry-based neoepitope identification pipeline combined with single-cell TCR- and RNA-sequencing analyses. In T cells transgenic for these neoTCRs we detected a spectrum from moderate to strong activation appearing in opposite direction to the original TCR-clonotype frequency in the patient: the higher the neoTCR frequency, the lower the activation level in vitro. While all neoTCRs potently rejected a neoepitope-expressing tumor cell line upon first encounter in an in vivo xenograft model, second in vivo rechallenge of neoTCR-tg tumor infiltrating lymphocyte-products demonstrated clearly improved persistence and killing capacity for initially more moderately stimulated T cells. Subsequently, we aimed to decrease signal strength of initially strongly activated neoTCR-tg T cells and modulated their activation profile by transient inhibition of the Mitogen activated-protein kinase (MAPK)-cascade adding MEK-inhibitor Cobimetinib in vitro. Indeed, we could show that within a narrow therapeutic window transiently decreased signal strength in TCR-tg T cells increased the persistence of CD8+ T cells and thereby tumor control. However, this effect strongly depended on the level of stimulation the T cells experienced and thus on factors like tumor cell line, number of target cells or the recognized amount of antigen.

Overall, we conclude that slightly lowered activation signals in T cells bear potential to improve fitness and persistence. A sensitive balance of T cell activation is essential to on the one hand prevent T cell

overstimulation due to overly strong anti-tumor response and on the other hand preserve T cell fitness without compromising tumor control. Therefore, a fine-tuned modulation will be key for the future success of patient-individualized adoptive cellular therapies. However, our data also outline additional factors involved in fine-tuning adoptively transferred T cells. Finally, these findings help understand some of the multiple characteristics of potent, long-term preservation of anti-tumor T cell responses upon adoptive transfer which surely exceed the selection based on highest avidity alone.

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The therapeutic potential of Vδ1+ γδ T-cells in primary colorectal cancer of microsatellite stable patients

L. C. Gatti[1], M. J. Nicolasen[1], T. Aarts-Riemens[2], M. Van der Wijst[2], W. Brandwijk[2], L. Gasull Celades[2], Z. Sebestyen[1], J. Kuball[1,3]

[1]UMC Utrecht, Clinical Translational Immunology, UTRECHT, The Netherlands, [2]UMC Utrecht, UTRECHT, The Netherlands, [3]UMC Utrecht, Department of Hematology, UTRECHT, The Netherlands

γδ T cells have been described to have a major role in cancer immune surveillance and thereby provide an immunotherapeutic option against solid tumors with a low mutational load. The clinical translation of using adoptive expanded or activated γδ T-cells or their receptors remains however challenging, which is partially explained by the functional heterogeneity of γδ T-cells and the diversity in their γδ T-cell receptor (TCR) repertoire. In order to develop therapeutic strategies based on γδ T cells and their receptors, we characterized the properties of γδ T cell populations in different compartments, namely peripheral blood, adjacent healthy colon tissue, and primary tumor of 24 microsatellite stable colorectal cancer (CRC) patients. We first observed in CRC patients a decrease in total numbers of circulating γδ T-cells compared to healthy donor (HD) and higher numbers of colon resident γδ T-cells compared to patient peripheral blood. In addition, Vδ1+ γδ T-cells derived from both intraepithelial lymphocytes (IEL) and tumor infiltrating lymphocytes (TILs) highly expressed activating NK co-receptors. Interestingly, TILs-derived Vδ1+ γδ T-cell bulks showed a significantly stronger IFNγ release against CRC cell lines. No correlation between surface expression of activating co-receptors and Vδ1+ potential to produce IFNγ was observed. In order to substantiate our findings and further investigate molecular mechanisms of tumor recognition by tumor infiltrating Vδ1+ γδ TCRs, we expanded and characterized multiple Vδ2- single cell clones. We confirmed in Vδ1+ clones derived from TILs a high frequency of tumor reactive clones with strong IFNγ release against CRC cell lines. As observed at polyclonal level, the Vδ1+ potential to produce IFNγ did not correlate with the expression of innate co-receptors. Gene transfer of a defined antitumor reactive Vδ1+ γδ TCR derived from γδ TIL into healthy donor αβ T-cells allowed to confirm that the tumor reactivity observed for Vδ1+ clones is at least partially mediated by their γδ TCR. In conclusion, our data emphasize the diversity and power of γδ T cells in different compartments in heath and disease and identify Vδ1+ γδ TCRs as main drivers of tumor reactivity of γδ TIL in microsatellite stable primary CRC patients.

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Arming CAR T cells with a combination of single chain bispecific antibody and IL-18 to improve antitumor activity

A. Glander, S. Stamova, A. Wurzel, B. Ott-Rötzer, P. Beckhove

Leibniz Institute for Immunotherapy, Interventional Immunology, Regensburg, Germany

Chimeric antigen receptor (CAR) T cell-therapy has proved to be effective in treating liquid tumors. However, the efficacy of this immunotherapeutic approach is limited in solid tumors. Tumor cells utilize a plethora of different immune escape mechanisms to avoid an immune response such as immune checkpoint molecules (ICM) and the generation of an immunosuppressive tumor microenvironment (TME). Systemic application of drugs targeting these mechanisms can induce severe systemic autoimmune reactions. To overcome this issue, 4th generation CAR T cells (TRUCKS), containing an additional transgene, have been developed. The transgene is induced via CAR activation and thus restricts the effect of any given effector molecule to the target tissue.
Our aim is to generate TRUCKs , that secrete two immunomodulatory molecules: the cytokine IL-18 and a single chain bispecific tandem antibody (scBsAb) binding to CD3 and a recently identified ICM. Both transgenes are organized in a bicistronic expression cassette downstream of a 6xNFAT response element to ensure activation induced expression. Binding of the scBsAb to the ICM ligand on tumor cells results in immune checkpoint blockade and its anti-CD3 arm activates bystander T cells to target the respective tumor cell. IL-18 as a pro-inflammatory cytokine on the other hand has several beneficial effects. It is associated with the differentiation of T cells into T-bet^{high} FoxO1^{low} effector cells, increases persistence and causes IFNγ secretion by several types of immune cell. Combining both the scBsAb and IL-18 in a TRUCK concept aims to counteract several dimensions of immune escape mechanisms to improve T cell functionality in the TME.
Western blot analyses demonstrate successful expression and secretion of the scBsAb after transient transfection of Hek 293T cells and the subsequent affinity purification of the protein. FACS staining of tumor or T cells using secondary antibodies targeting the His- and/or Myc-tag on the scBsAb confirm that each of the antibody domains is able to bind to their respective targets. In addition, ELISAs and cytotoxicity assays verify the induction of T cell activation by addition of the scBsAb to T cell-tumor cell co-cultures. The scBsAb and IL-18 transgenes are combined in the expression cassette of a lentiviral vector for transduction and generation of scBsAb-IL-18 TRUCK T cells.
In summary, our results suggest that T cells transduced with this expression cassette can specifically target several immune-escape mechanisms simultaneously to ameliorate antitumor activity. This proof-of-principle study combines different approaches of Immunotherapy in one TRUCK concept to increase efficacy and safety in treatment of solid tumors.

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Allogenic CAR-T cells armored with IL-12 generated from umbilical cord blood

A. Gruca[1,2], J. Misiak[1], M. Murzyn[1], N. Rozwadowska[1,2], T. Kolanowski[1,2]
[1]FamicordTX Gene Therapies, Warsaw, Poland, [2]Institute of Human Genetics, Polish Academy of Sciences, Poznan, Poland

Adoptive CAR-T (chimeric antigen receptor T) cell therapy is growing both in the number of clinical trials as well as approved therapeutic applications. The vast majority of existing products are autologous where the CAR-T cells are derived from the T cells of a given patient. As a consequence, companies developing CAR-T therapies face difficulties such as extended time of manufacturing, quality control issues and a risk of failure to acquire a sufficient therapeutic dose of the final product. To overcome these limitations we

focused on an allogenic approach, taking advantage of a readily available source of immunopermissive T cells obtained from cryopreserved umbilical cord blood (UCB) samples. We transduced UCB-derived T cells with a lentiviral vector delivering third generation armored-CAR construct that enables autocrine interleukin 12 signaling. With this approach, we managed to improve the yield of functional CAR-T cells compared to a second generation construct alleviating the problem of relatively low cellular content of banked cord blood samples. Following activation, transduction and expansion, CAR-T cells were cryopreserved prior to testing their effector function upon thawing, imitating the application of an off-the-shelf allogenic cell therapy product. CAR-T cells obtained with this approach performed significantly better than the unmodified T cells both in a 24h cytotoxicity assay against a B-cell precursor leukemia cell line (REH) at 5:1 and 2:1 effector to target ratios as well as in terms of the levels of produced cytotoxic cytokines upon an encounter with the target cells. In comparison to CAR-T cells generated from peripheral blood of an adult donor, UCB-derived CAR-T cells displayed a comparable anti-tumoral efficacy even though the cytostatic potency of non-transduced UCB T cells is significantly lower than that of T cells derived from the peripheral blood of an adult donor. In conclusion, our approach demonstrates the feasibility of utilizing UCB samples together with a 3rd generation armored-CAR construct and enabling generation of an off-the-shelf allogenic cell therapy product.

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Mesothelin-directed CAR-T cells combined with biogenic selenium nanoparticles exert potent anti-tumor function in the 4T1 triple-negative breast cancer model

M. Hosseini[1], H. R. Mirzaei[2], A. R. Shahverdi[1], J. Hadjati[2], M. A. Faramarzi[1], M. H. Yazdi[3]
[1]Tehran University of Medical Sciences, Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Tehran, Iran, Islamic Republic of, [2]Tehran University of Medical Sciences, Department of Medical Immunology, School of Medicine, Tehran, Iran, Islamic Republic of, [3]Tehran University of Medical Sciences, Biotechnology Research Center, Tehran, Iran, Islamic Republic of

Triple-negative breast cancer (TNBC) is the subtype of breast cancer lacking the overexpression of hormone and HER2 receptors, rendering limited therapeutic options along with the worst prognosis among breast cancer subtypes. Mesothelin (MSLN) is a tumor-associated antigen (TAA) with limited expression in normal tissues but an established overexpression in several solid tumors, including mesothelioma, pancreatic, ovarian, and breast cancers. Among breast cancer subtypes, TNBC has been associated with a notable MSLN overexpression, thus making it a suitable target for the development of targeted therapies, including immunotherapy. Chimeric antigen receptor-T (CAR-T) cells have emerged as one of the most effective immunotherapeutic approaches, with 5 FDA-approved products indicated in hematological malignancies to date. Yet, challenges exist in their clinical translation of in solid tumors. One of the barriers to the successful application of CAR-T cells in solid tumors is the immunosuppressive tumor microenvironment (TME), attenuating the immune response against tumor cells. Selenium nanoparticles (SeNPs) produced through biological systems have shown immunostimulatory properties in various cancer models, serving as potential adjuvants to cancer immunotherapies. We here describe the production of MSLN-specific CAR-T cells directed against a mesothelin-positive, murine TNBC cell line, 4T1. The aforementioned cells demonstrated potent cytotoxic activity against 4T1 cells in vitro, along with notable proliferation capacity

when co-cultured with 4T1 cells compared to a MSLN -negative cell line, B16/F10. ELISA assessment of co-culture supernatants for immunostimulatory cytokines IL-2 and IFN-γ also showed an elevated pattern as compared to mock-T cells. After production and purification of SeNPs in a biocompatible, probiotic host of Lactobacillus spp., MSLN-specific CAR-T cells were administered along with biogenic SeNPs in the 4T1 TNBC model in BALB/c mice preconditioned with single IP injection of cyclophosphamide. While CAR-T cells were injected as a single intra-tumoral dose, administration of biogenic SeNPs continued weekly for 3 weeks. Results showed significantly smaller tumor sizes in mice treated with MSLN-specific CAR-T cells after 3 weeks. Moreover, some mice receiving SeNPs along with CAR-T cells demonstrated a notable shrinkage of the tumor mass. Pathological studies of tumor samples are currently being conducted. These results suggest (1) MSLN as a potential target for effective CAR-T cell therapy in TNBC; and (2) Biogenic SeNPs as potent adjuvants along with CAR-T cell therapy for ameliorating the harsh immunosuppressive TME of solid tumors.

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Early induction of cytokine release syndrome by rapidly generated CAR T cells in preclinical models

A. Jamali[1], N. Ho[1], A. Braun[1], E. Adabi[1], C. J. Buchholz[1], F. B. Thalheimer[1,2]
[1]Paul-Ehrlich-Institut, FG1 – Molekulare Biotechnologie und Gentherapie, Langen, Germany, [2]Paul-Ehrlich-Institut, HZG Hämatologie / Zell- und Gentherapie, Langen, Germany

Cytokine release syndrome (CRS) is a significant side-effect of conventional chimeric antigen receptor (CAR) T-cell therapy. To facilitate patient accessibility, short-term (st) CAR T cells, which are administered to patients only 24 h after vector exposure, are in focus of current investigations. Their impact on the incidence and severity of CRS has been poorly explored. Here, we evaluated CD19-specific stCAR T cells in preclinical models. In co-culture with tumor cells and monocytes, stCAR T cells exhibited anti-tumoral activity and potent release of CRS-related cytokines (IL-6, IFN-γ, TNF-α, GM-CSF, IL-2, IL-10). When administered to NSG-SGM3 mice, stCAR T cells, but not conventional CAR T cells, induced severe acute adverse events within 24 h, including hypothermia and weight loss, as well as high body scores, independent of the presence of tumor target cells. Human (IFN-γ, TNF-α, IL-2, IL-10) and murine (MCP-1, IL-6, G-CSF) cytokines, typical for severe CRS, were systemically elevated. Our data highlight potential safety risks of rapidly manufactured CAR T cells and suggest NSG-SGM3 mice as sensitive model for their preclinical safety evaluation.

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Specific enrichment of HPV-reactive tumor infiltrating lymphocytes for adoptive cell transfer therapy in patients with cervical cancer

N. Jayashankar[1,2], C. Heeke[2], C. Friese[2], M. Kadivar[1], S. A. Tvingsholm[1], N. Kirketerp-Møller[2], S. Faerch[2], U. Cordes[2], S. R. Hadrup[1]
[1]Technical University of Denmark, Experimental and Translational Immunology, Department of Health Technology, Kongens Lyngby, Denmark, [2]Cbio A/S, Research and development, Søborg, Denmark

Cervical cancer is the fourth most common cancer amongst women worldwide. Annually, about 604,127 patients are diagnosed, among which 341,000 patients die from the disease. Although the standard-of-care may be effective in the initial stage of the disease, it is inadequate in patients with advanced disease. The introduction of cell-based immunotherapy may greatly improve the treatment of advanced cervical cancer. In an ongoing phase II trial of adoptive cell therapy with tumor infiltrating lymphocytes (TIL-ACT) treating patients with cervical cancer, an overall response rate of 44% was reported. However, it is currently not possible to generate a TIL infusion product for all patients, and existing protocols favor massive expansion of unspecific bystander T cells that do not contribute towards anti-tumor response.
In this study, we aim to improve the anti-tumor capacity of T-cell infusion products to treat cervical cancer, by increasing the frequency of tumor-reactive T cells. Cervical cancer is often induced by an infection of human papillomavirus (HPV). In order to screen for relevant epitopes, an HPV peptide library was generated using the bioinformatic peptide prediction tool, NETMHCpan, along with previously reported peptides in the Immune Epitope Database and Tools (IEDB). A cut-off elution ligand rank of 2 was used to select peptides with high affinity to their respective human leukocyte antigen (HLA) molecule. TILs generated from resected tumors from patients with cervical cancer will be screened for HPV-specific T cells using DNA-barcoded peptide-MHC (pMHC) multimers with an HLA-matched peptide panel.
In a preliminary screen of 4 cervical cancer patients, 41 unique pMHC responses were identified from a peptide panel of around 700 peptides covering 15 different HLA haplotypes. Interestingly, specific peptides could be recognized in the context of more than one HLA haplotype and across multiple patients, indicating the presence of immunodominant HPV-specific T cell clones. The identification of HPV-specific T cell clones in patients will allow their selective enrichment from peripheral blood, tumor fragment or young TILs using T-cell engaging scaffolds. These scaffolds can deliver T-cell stimulatory signals to selective T-cell clones of interest, which could be expanded further to generate a high-dose infusion product. In preliminary tests, further expansion of virus-specific T-cells resulted in a 600-fold expansion. These scaffold-enriched T cells were mainly effector memory, which is important for prolonged in-vivo survival. Hence, with the selective enrichment and expansion process, we can generate an infusion product with a high frequency of HPV-specific T cells with an improved overall phenotype. Thus, this strategy may provide a therapeutic alternative for patients that failed to respond to standard TIL-ACT.

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Macrophages with M2-like phenotype predominate the tumor microenvironment of osteosarcoma at various disease sites and increase during disease progression

S. Kailayangiri[1], M. Hebel[1], B. Altvater[1], L. Greune[1], K. Urban[1], J. Meltzer[1], S. S. Bielack[2], O. Witt[3], W. Hartmann[4], C. Rossig[1]
[1]University Children's Hospital Muenster, Department of Pediatric Hematology and Oncology, Muenster, Germany, [2]Klinikum Stuttgart – Olgahospital, Zentrum für Kinder-, Jugend- und Frauenmedizin, Stuttgart, Germany, [3]Heidelberg University Hospital, Department of Pediatric Hematology and Oncology, Heidelberg, Germany, [4]University of Muenster, Gerhard-Domagk-Institute of Pathology, Muenster, Germany

Immunotherapy with T cells engineered to recognize cancer cells via chimeric antigen receptors (CAR T cells) has impressive clinical efficacy against B-cell derived malignancies while solid tumors ultimately resist CAR T cell therapy. This is explained by immune-inhibitory components in the tumor microenvironment

(TME) that act as local barriers to prevent T cell infiltration and impede their antitumor effector functions. A key population in the TME of many human tumors, especially in osteocarcoma (OS), are tumor-associated macrophages (TAM) with immune-suppressive functions.
We hypothesize that tumor-associated macrophages with immunosuppressive phenotype (coexpressing CD68 and CD163) dominate the TME of OS throughout therapy and tumor progression, while scarcity of T cell infiltration remains a hallmark of this cancer. To this end, we performed systematic longitudinal studies of matched material from pre- and posttherapeutic primary tumors, metastatic disease at various sites and subsequent relapses in five individual patients receiving uniform first-line treatment. We compared and quantified the immune cell components of M1-like (CD68+CD163-) and M2-like (CD68+CD163+) macrophages and CD3+ T cells with cytotoxic (CD8+), T helper (CD4+FoxP3-) and regulatory (CD4+FoxP3+) T cell phenotypes in tumor biopsies using multiplex immunofluorescence/TSA panels. M2-like macrophages and T helper cells predominated in all patients and at all timepoints, while M1-macrophages, CD8+ T cells and CD4+FoxP3+ T cells are scarce in OS regardless of site of disease. Direct comparison of treatment-naïve tumors and post-therapeutic tumors showed a significant increase of M2-like macrophages (pre-therapeutic: median 57 (range 14-94); post-therapeutic: median 247 (range 21-426); p=0.01) as well as CD4+ T cells (pre-therapeutic: median 2, (range 1-3), post-therapeutic: range 70 (range 42-72); p=0.03), indicating that standard cytotoxic chemotherapy enhances the inflammatory nature of the TME. To understand the impact of disease recurrence on tumor infiltration, we compared numbers of infiltrating M2-macrophages and CD4+ T cells in primary tumors with multiple consecutive relapse manifestations. A significantly higher M2-infiltration (p=0.03) was seen in pulmonary relapse manifestations, while CD4+ T cell numbers were highly variable both within and among patients and did not significantly vary between primary and relapsed disease (p=0.16). Compared to first pulmonary relapses, subsequent pulmonary relapses across patients did not significantly differ with regard to either M2-like macrophages (p=0.49) or CD4+ T cell (p=0.45) infiltration, indicating that later stages of tumor progression are not associated with increased immune cell infiltration.
This work suggests that M2-like macrophages have an important role in the TME of OS and should be taken into consideration in the development of effective immune-targeting strategies

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Single-cell profiling and validation of tumor-specific TCR repertoires to study immune escape in small cell lung cancer

K. Khinvasara[1], E. Diken[1], M. Schork[1], A. Kübler[1], M. Diken[1], L. Merz[1], B. Schrörs[1], F. Lang[1], J. D'Rozario[2], J. George[2], R. Thomas[2], E. Stanganello[1], L. Launhardt[1], L. Kolb[1], U. Sahin[1,3,4]
[1]TRON-Translational Oncology at the University Medical Center of the Johannes Gutenberg University, Mainz, Germany, [2]University of Cologne, Cologne, Germany, [3]BioNTech, Mainz, Germany, [4]Research Center for Immunotherapy (FZI), Johannes Gutenberg University, Mainz, Germany

Small-cell lung cancer stands out as among the most aggressive forms of cancer, characterized by exceptionally high proliferative rate, strong predilection for early metastasis and poor prognosis. Our goal is to deploy transcriptome-based methods to identify tumor-specific T cell receptors (TCRs) and employ them in adoptive T cell immunotherapy to study immune escape mechanisms in small cell lung cancer. For this purpose, we plan to analyze at single-cell-resolution transcriptome (scRNA seq) and paired TCR α/β chain

profiles (scTCR seq) of tumor-infiltrating lymphocytes (TILs) obtained from biopsies of individual naïve patients. Deploying the advantage of scTCR seq data, we are able to obtain matching αß TCR chains, which would have been lacking with bulk TCR seq profiles. Relevant TCRs cloned from potential tumor-reactive TILs will be reconstituted in reporter T cells and analyzed for recognition and killing of autologous cell lines using a Jurkat-NFAT assay. With our data analysis pipeline we are also able to integrate, gene expression with TCR profiles and identify the phenotype of the cells with a TCR of interest. For our future direction, once confirmed for their tumor-specificity, relevant TCRs will be used to trace back the frequency, distribution and transcriptome profile of tumor-reactive T cells in patient samples, as well as to study the clonality and expression of recognized mutations in tumor samples and engrafted cell lines. Finally, T cells transduced with tumor-reactive TCRs would be used for in vitro and in vivo assays to study the mechanisms underlying outgrowing immune escape variants. These studies will help generate insights into T cell immune recognition and escape in small cell lung cancer patients.

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Expanding reach of tumor-infiltrating lymphocytes by substituting PBMCs with artificial feeder cells

N. Kirketerp-Møller[1,2], C. Friese[2], D. Rasic[3], S. Færch[2], L. Lund[4,5,6], L. Kjeld Petersen[7,8], K. Madsen[3,4], S. Detlefsen[3], M. Bau Mortensen[3], M. Rabjerg[3], N. Marcussen[9,10], K. Wennerberg[1], U. Cordes[2]
[1]University of Copenhagen, Biotech research and innovation centre, Copenhagen, Denmark, [2]Cbio A/S, Copenhagen, Denmark, [3]Odense University Hospital, Department of Pathology, Odense, Denmark, [4]Odense University Hospital, Department of Cardiovascular and Renal Research, Odense, Denmark, [5]Odense University Hospital, Department of Urology, Odense, Denmark, [6]Odense University Hospital, Institute of Clinical Research, Odense, Denmark, [7]Odense University Hospital, Department of Gynecology, Odense, Denmark, [8]University of Southern Denmark, Open Patient Data Explorative Network, Odense, Denmark, [9]University of Southern Denmark, Institute of Clinical Research, Odense, Denmark, [10]Hospital of Southern Jutland, Department of Pathology, Aabenraa, Denmark

Adoptive cell therapy (ACT) using tumor-infiltrating lymphocytes (TILs) is entering a new chapter following FDA approval in advanced melanoma. In this setting, between 39% and 72% of patients achieve a response, with up to 20% of the patients experiencing long-lasting complete responses. The treatment has also been investigated in other solid cancer types such as head and neck squamous cell carcinoma and cervical cancer where response rates of 44% have been reported. Despite these very promising results, TIL treatment still faces several inherent challenges that hinder its widespread adoption. Since TILs are autologous, creating a good product requires a meticulous process that must be carried out for each individual patient. First, the TILs need to be expanded from the tumor tissue, which has varying success depending on the malignancy. After outgrowth, the T cells are further expanded using the rapid expansion protocol (REP) which involves the use of allogeneic peripheral blood mononuclear cells (PBMCs) as feeder cells and stimulation with αCD3. The availability of buffy coats is limited and will increase due to a growing number of cell therapies as a single production of TILs requires the PBMCs from at least 8 donors. Furthermore, they are expensive and pose a risk of contaminating cancer patients with undiagnosed infectious diseases.
In this study, we present an engineered cell line as an alternative to the donor-derived feeder cells that can be expanded on demand. The artificial feeder cells were transduced to express immunostimulatory molecules and subsequently cloned to obtain a uniform expression. Our results show that the artificial feeder cells are stable for at least 45 passages making it a robust platform for TIL expansion. Additionally,

we have shown that the artificial feeder cells can engage and activate the T cells resulting in an expansion of >4000 fold which responds to a high-dose TIL product.
In conclusion, we have shown that it is feasible to replace the donor-derived feeder cells with our engineered cell line while maintaining a high level of expansion in the REP. The artificial feeder cells represent a scalable alternative and have potentially solved one of the critical bottlenecks for the widespread use of TIL therapy.

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Using CRISPR activation applications to identify novel tumour resistance mechanisms of aggressive lymphomas in CAR-T cell therapy.

C. Koenig[1,2,3], S. Diepstraten[3,4], P. A. Beavis[5,6], G. Kelly[3,4], M. J. Herold[1,2,3,4]
[1]Olivia Newton-John Cancer Research Institute, Genome Engineering and Cancer Modelling Program, Heidelberg, VIC, Australia, [2]La Trobe University, School of Cancer Medicine, Melbourne, VIC, Australia, [3]Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, Australia, [4]University of Melbourne, Department of Medical Biology, Melbourne, VIC, Australia, [5]Peter MacCallum Cancer Centre, Cancer Immunology Program, Melbourne, VIC, Australia, [6]The University of Melbourne, Sir Peter MacCallum Department of Oncology, Parkville, VIC, Australia

A particularly aggressive subtype of Diffuse Large B Cell Lymphoma (DLBCL) called Double Hit Lymphoma (DHL), expresses high levels of the pro-survival protein BCL-2 in addition to high cMYC. For patients presenting with DLBCL that have relapsed or are refractory to standard therapies the standard of care is CD19directed Chimeric Antigen Receptor (CAR) mediated T cell therapy. However, many patients respond poorly or not at all, and the mechanisms of resistance are not well understood.
To address this, we are using mouse DHL cells generated by unique CRISPR activator (dCas9A) technology in CD19$^+$CAR-T cell mediated killing assays to identify novel resistance mechanisms of DHL cells to CAR-T cell therapy. Therefore, we transduced dCas9A DHL cells with genome wide lentiviral CRISPRa sgRNA libraries able to induce robust expression of genes when introduced into dCas9A expressing cells. Successfully transduced dCas9A DHL cells were co-cultured with CD19$^+$CAR-T cells for a period of 16h and surviving DHL cells resistant against CAR-T cell killing mechanisms isolated. Since CAR-T cell mediated killing is mainly induced in a cytokine dependent manner post 4 hours of co-culture with their target cells, we also cultured sgRNA-library transduced dCas9A DHL cells with CD19$^+$CAR-T cells in sequential shorter killing cycles. This allows the identification of novel resistance mechanisms evolved specifically towards contact-dependent CAR-T killing mechanisms. Surviving DHL cells were recovered, genomic DNA isolated, and resistance-promoting sgRNAs identified by next generation sequencing. Top candidate hits were validated in CD19$^+$CAR-T killing assays in vitro. Finally, in vitro validated resistance genes are currently being tested in an in vivo setting by transplanting dCas9A DHL cells expressing these genes into immunocompromised RAG1 knockout animals. Upon lymphoma development, CD19$^+$CAR-T cells will be transplanted and their efficiency of clearing the tumour evaluated.
We expect that the findings upon completion of this project will enhance our understanding of tumour resistance mechanisms and provide us with novel targets to be used in future CAR-T therapies against DHL.

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Abstract has been withdrawn

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Utilization of biotin-guided CAR-T cells for the treatment of Glioblastoma

A. Kourtesakis[1], E. Bailey[1], H. N. H. Chow[1], D. A. Agardy[2], R. Will[3], Y.-C. Chih[2], L. Kaulen[1], M. Hahn[1], H. Rohdjeß[1], D. Reibold[1], S. Pusch[1], R. Wagener[1], D. C. F. Hoffmann[1], T. Sauer[4], M. Schmitt[4], W. Wick[1], T. Kessler[1]

[1]German Cancer Research Center (DKFZ), Clinical Cooperation Unit Neurooncology, Heidelberg, Germany, [2]German Cancer Research Center (DKFZ), Clinical Cooperation Unit Neuroimmunology and Brain Tumor Immunology, Heidelberg, Germany, [3]German Cancer Research Center (DKFZ), Cellular Tools Core Facility, Heidelberg, Germany, [4]University Hospital Heidelberg, Medicine, Hematology, Oncology and Rheumatology, Heidelberg, Germany

Although chimeric antigen receptor (CAR)-T cell therapy has shown impressive efficacy in treating hematological cancers, it has not yielded significant survival advantages for patients with glioblastoma (GB), the deadliest form of brain tumor. This is partially attributed to the non-universal tumor antigen expression patterns, also known as tumor heterogeneity. This necessitates not only the discovery of new targets but also the development of pipelines to test the binding capacity of different single chain variable fragments (scFVs), leading to optimal CAR design. In this study we generated CAR-T cells which encompass monomeric streptavidin (mSA2) instead of a specific target recognition domain. This allows to fully tailor the CAR-T cell tropism against any membrane-associated tumor antigen by utilization of the respective biotinylated antibody. First, we screened a panel of primary and conventional GB cell lines for expression of antigens which have been explored as CAR-T cell therapy candidates. To check the specificity of our approach we induced overexpression of CD70 and CD213a2 (aka IL13Ra2), as well as genetically ablated EPHA2 and CD276 using the CRISPR/Cas9 machinery, in $CD70^-$/$IL13Ra2^-$/$EPHA2^+$/$CD276^+$ primary GB cells. For our immunotherapeutic intervention we developed mSA2-featuring CAR-T cell constructs bearing different co-stimulatory domains (CD28 or 4-1BB) and successfully transduced primary T-Cells from healthy donors. In an in vitro co-culture, mSA2 CAR-T cells recognized tumor cells in a target-expressing and antibody-dependent manner, secreting significantly higher levels of Granzyme-B and IFN-γ compared to the control counterparts. This recognition was accompanied by a specific upregulation of T-Cell activation marker CD137 on the CAR-T cell surface as well as prominent reduction of tumor cell signal, demonstrated by live-cell confocal microscopy. Notably, these CAR-T cells showed potency in engaging selected antigen-expressing tumors in vivo in the brains of immunodeficient mice. Namely, NSG mice orthotopically implanted with patient-derived $CD70^+$/$CD276^+$ GB cells showed tumor regression after treatment with mSA2 CAR-T cells that had been pre-armed with biotinylated antibodies against either of these antigens, as shown by bioluminescence imaging (BLI). To corroborate these findings, immunofluorescence (IF) analysis on these brains revealed increased effector cell presence and elevated cleaved caspase-3 levels, trends which were not observed in the absence of CAR-T cell antibody pre-arming. In conclusion, we are the first to showcase the efficiency of fully switchable CAR-T cells to treat brain tumors using biotinylated antibodies targeting multiple different tumor antigens, highlighting their potential use as a rapid and cost-effective

platform to test scFVs for optimal CAR design, but also as a tool to overcome tumor heterogeneity by simultaneous tumor antigen targeting.

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T-cell receptor engineered T cell therapy in solid cancers: an antigen-agnostic approach to the identification of therapeutic TCRs from tumor-infiltrating lymphocytes

V. Lennerz[1], C. Doppler[2], M. Fatho[2], A. Dröge[3], S. Schaper[3], K. Gennermann[1], N. Genzel[1], S. Plassmann[1], D. Weismann[4], S. W. Lukowski[4], D. Bents[3], H. Herbst[5], V. Seitz[3], C. Beushausen[6], R. Hammer[3], P. J. Adam[4], C. Schmees[7], S. Eggeling[6], C. Wölfel[2], T. Wölfel[2], S. Hennig[3]
[1]TheryCell GmbH, Berlin, Germany, [2]University Medical Center (UMC) of the Johannes Gutenberg University, Internal Medicine III, Mainz, Germany, [3]HS Diagnomics GmbH, Berlin, Germany, [4]Boehringer Ingelheim RCV, GmbH & Co KG, Vienna, Austria, [5]Vivantes Clinic Neukölln, Vivantes Pathology, Berlin, Germany, [6]Vivantes Clinic Neukölln, Vivantes Thoracic surgery, Berlin, Germany, [7]NMI Natural and Medical Sciences Institute, Reutlingen, Germany

The unprecedented efficacy of CAR-T cell therapy in patients with B cell malignancies and Multiple Myeloma has unleashed a surge of preclinical and clinical research testing CARs against a multitude of antigens in hematological and solid cancers. Although first clinical trials of CAR-T cell therapy were conducted in solid cancers (ovarian, renal cell cancer) and despite encouraging preclinical studies, the clinical activity of CAR-T in patients with solid cancers is still unsatisfactory. TCR-engineered T cells are regarded a promising therapeutic alternative. The selection of all therapeutic CARs/TCRs in clinical development so far has been based on the antigens they target. For TCRs, epitopes from shared antigens (cancer/germline, overexpressed) associated with frequent HLA alleles have been prioritized. Yet, only a minority of patients is eligible for therapy and study results so far have fallen short of expectations. Direct identification of therapeutic TCRs from blood or TILs would make more patients eligible for therapy but requires personalization. We have developed a method for the direct selection of tumor-specific T cell clonotypes from TILs through comparative TCR repertoire profiling of clonotypes from TILs and adjacent normal tissue and scRNA-Seq of the TIL-clonotypes. Tumor-specific clonotypes are determined by (1) high abundance among TILs, (2) a high TIL versus normal tissue clonotype frequency ratio, and (3) a sc-gene expression profile indicative of a differentiation trajectory ranging from tumor-reactivity to exhaustion. We have produced predicted tumor-specific TCRs from different NSCLC patients. Ectopically expressed by retroviral transduction in (endogenous) TCR-depleted donor T cells, we show that the recombinant TCR-T cells recognize the corresponding patients' tumor cells and/or allogeneic HLA-matched tumor lines. In one patient a comprehensive antigen screening resulted in the identification of a neoepitope processed from a common KRAS driver mutation as target of three of four TCRs tested. In addition to the personalized strategy, the TCR profiling of >100 tumor patient samples enabled us to build and screen a database comprising >650.000 TCRs using a proprietary algorithm that effectively gathers paired TRAV/TRBV-CDR3 sequences with tumor specificity and high sequence homologies. Donor T cells transduced with several of the TCRs per specificity group showed identical recognition patterns against HLA-matched NSCLC cell lines. These "cluster TCRs" are candidates for off-the-shelf adoptive cell therapies with TCR-engineered TCR-T cells of HLA- and TCR-matched patients.

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Increased anti-tumor activity in transgenic anti-HER2 CAR-TIL with inducible IL-12 production

J. R. Lérias[1], E. de Sousa[1], J. Kamiki[1], P. A. António[1], R. Eduardo[1], S. Cascais[1], C. M. Gorgulho[1], D. Ligeiro[1], F. M. Marincola[2], V. Balan[1], M. Maeurer[1,3]

[1]Champalimaud Foundation, Lisbon, Portugal, [2]Sonata Therapeutics, Watertown, United States of America, [3]I Medical Clinic, University of Mainz, Mainz, Germany

Most patients with solid tumors do not respond at all or respond only transiently to standard therapies, as deploy mechanisms of action (MOAs) are centered on precise target presence: efficacy is limited due to evolving genetics of neoplasia generating therapy-resistant cell clones. Advanced smart treatment modalities engage endogenous immune cells that deploy distinct MOAs by decreasing treatment-resistance. Cell therapy is more successful if immune cells (i) are able to home into cancer lesions, (ii) survive longer in cancer lesions, (iii) exhibit increased cell fitness, while ignoring immunosuppression and (iv) produce anti-cancer directed cytokines (e.g. IL-12). We combined the 4 points listed above using transgenic TIL (from HER2-neg epithelial cancer) that expressed an HER2-tag and IL-12 production upon HER2-engagement. Autologous tumor was used to gauge TIL recognition (IFN-y production) and HER2 was provided using HER2 beads; BSA beads served as a control. Transgenic TIL lines were created using a CRISPRa system, which expresses dCas9 fused to transcriptional activator domains VP64-p65-Rta and 2 sgRNAs targeting 2 endogenous genes, IL12A (p35) and IL12B (p40), where HER2 engagement induces IL-12 and subsequently IFN-y. 3 different TIL lines were created, using dCas9-VPR as a control, the HER2 target receptor alone or IL-12 inducible in combination with the HER2 target receptor. The effectiveness of transduction was evaluated by genes associated with HER2 target receptor transcription or the dCas9 system by flow cytometry. IL-12 production potency upon target binding was tested by ELISA. TIL expressing IL12-HER2-CAR stimulated with HER2 beads (no tumor cells) leads to IL-12 production, showing efficient transduction. HER2 beads pre-activated TIL demonstrated stronger reactivity against autologous tumor (3775 pg/mL, IFN-y production) as compared to controls, which included non-infected TIL (1612 pg/mL), dCas9-VPR-TIL (2014 pg/mL) and HER2-CAR-TIL (1838 pg/mL). This effect was not observed using BSA-coated beads, highlighting the role of IL-12 production. RNAseq for tumor-reactive TIL was performed 72h after tumor:TIL incubation in non-infected TIL, dCas9-VPR-infected, HER2-CAR-TIL and IL-12-HER2-CAR-TIL. Remarkably, TIL reactivity against autologous tumor cells, including IL-12-HER2-CAR-TIL could be blocked by the anti-MHC I antibody (w6/32), but not with a control antibody, even in the presence of the nominal HER2 target, underlining the role of TCR signaling and downstream convergence of TCR:CAR-HER2 tag signaling events. We show that TIL can serve as recipients for CAR-cytokine constructs which increases anti-cancer efficacy (ex vivo) and combines endogenous immune effector mechanisms, tumor homing, T-cell fitness with smart epigenetic reprograming using a lentiviral dCas9 vector system.

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4th-generation CAR-T cells with inducible cytokine expression targeting CD176 to overcome the immunosuppressive tumor milieu in lung cancer

C. Malinconico[1], A. C. Dragon[1], M. Umland[1], P. Rahmati[1], A. Bonifacius[1], J. Weber[2], K. Zimmermann[3], O. Danov[4], M. Hudecek[2], P. Kehler[5], A. Braun[4], H. Abken[6], A. Schambach[3], R. Blasczyk[1], B. Eiz-Vesper[1]

[1]Hannover Medical School (MHH), Institute of Transfusion Medicine and Transplant Engineering, Hannover, Germany, [2]University Hospital of Würzburg, Chair of Cellular Immunotherapy, Department of Internal Medicine II, Würzburg, Germany, [3]Hannover Medical School (MHH), Institute of Experimental Hematology, Hannover, Germany, [4]Fraunhofer ITEM, Division of Preclinical Pharmacology and In vitro Toxicology, Hannover, Germany, [5]Glycotope GmbH, Research and Development Department, Berlin, Germany, [6]Leibniz Institute for Immunotherapy, Department Genetic Immunotherapy, Regensburg, Germany

In the development of effective CAR-Ts against solid tumors, both choosing an appropriate tumor-associated target and overcoming the immunosuppressive tumor microenvironment (TME) remain major challenges. The oncofetal carbohydrate CD176 (Thomsen-Friedenreich antigen), hidden in adult benign tissues by sialylation or prolongation with carbohydrates, is unmasked in 90% of carcinomas, predicting low "on-target/off-tumor"-toxicity. To target CD176 and overcome the immunosuppressive TME of lung cancer, we developed 4th-generation CD176-specific CAR-Ts, also known as T cells redirected for universal cytokine-mediated killing (TRUCKs). In addition to the constitutive CD176-CAR expression, these cells were engineered with an inducible cassette expressing either IL-12 (iIL12_CD176_TRUCKs), IL-18 (iIL18_CD176_TRUCKs), or EGFP as a control (iEGFP_CD176_TRUCKs). Functionality of CD176_TRUCKs was tested by co-cultivation with cell lines expressing different CD176 levels, A549 spheroids or human lung cancer tissue, after which activation and cytotoxicity of CD176-TRUCKs was characterized using multicolor flow cytometry, (real-time) fluorescence microscopy and multiplex assays. Following co-culture with different CD176^{+} lung carcinoma cell lines, all CD176_TRUCKs increased NF-κB activity, became activated, released effector molecules (e.g. IFN-γ), and mediated effective cytotoxicity. They did not react towards CD176^{-} control cells. The inducible cytokines IL-12 and IL-18 were released by respective TRUCKs in a target-specific manner and clearly improved particular effector functions in comparison with iEGFP_CD176_TRUCKs, which was specifically evident upon usage of CD176^{+} A549 spheroids as three-dimensional target model. Precision-cut lung sections (PCLS) were generated from explanted human lung adenocarcinoma tissue and shown to express CD176. Using PCLS as ex vivo model, specific cytotoxicity of CD176_TRUCKs against tumor tissue but not against healthy tissue of the same lung was demonstrated. In conclusion, CD176_TRUCKs equipped with inducible cytokines were shown to be highly functional, suggesting them as promising strategy to overcome the TME. Based on the tumor-selective expression of CD176, CD176_TRUCKs have a high potential to effectively control lung carcinoma while minimizing "on-target/off-tumor"-toxicity.

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LMP2a-targeting TCR-engineered T cells with inducible Interleukin-18 expression to treat Epstein-Barr Virus-associated malignancies

P. Mausberg[1], A. Bonifacius[1], A. C. Dragon[1], S. Stoll[1], P. Spieler[2], S. Thoelke[1], M. F. Lammoglia Cobo[3], T. Nerreter[2], S. Tischer-Zimmermann[1], R. Blasczyk[1], M. Hudecek[2], A. Schambach[4], L. Hansmann[3], B. Maecker-Kolhoff[5], B. Eiz-Vesper[1]

[1]Hannover Medical School, Institute of Transfusion Medicine and Transplant Engineering, Hannover, Germany, [2]University Hospital Würzburg, Chair of Cellular Immunotherapy, Department of Internal Medicine II, Würzburg, Germany, [3]Charité-Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Department of Hematology, Oncology and Tumor

Immunolog, Berlin, Germany, [4]Hannover Medical School, Institute of Experimental Hematology, Hannover, Germany, [5]Hannover Medical School, Department of Pediatric Hematology and Oncology, Hannover, Germany

Epstein-Barr virus (EBV) infects more than 90% of the population and remains in B-cell compartments life-long. While in healthy individuals strong immune responses control EBV reactivation, in immunocompromised patients, infections and reactivations can lead to severe EBV-associated malignant complications, such as post-transplant lymphoproliferative disorder (PTLD). In latency stages II/III, latent membrane protein 2a (LMP2a) is expressed, which is associated with PTLD as well as various malignancies. Recently, a clinically protective TCR recognizing an LMP2a-derived peptide in context of HLA-A*02 was identified. Based on this TCR, we developed TCR-engineered T cells (LMP2a_TCR-Ts) and further equipped these with an inducible cassette for locally restricted release of IL-18 (iIL18_LMP2a_TCR-Ts), which was shown to convert T cells into pro-inflammatory effector cells, preventing function loss and exhaustion, and reshaping the immunosuppressive tumor microenvironment. Using peptide-loaded HLA-A*02^{+} and HLA-A*02^{-} K562-derived target cells, no signs of HLA cross-reactivity or recognition of an irrelevant HLA-A*02-restricted peptide by (iIL18)_LMP2a_TCR-Ts were observed. Specific binding of (iIL18)_LMP2a_TCR-Ts to HLA-A*02^{+} EBV-infected B lymphoblastoid cell lines (B-LCLs), serving as in vitro EBV-associated PTLD model, was confirmed by avidity measurements. Next, functionality of the generated (iIL18)_LMP2a_TCR-Ts was evaluated using flow cytometry- and live cell imaging-based assays. Of note, iIL18_LMP2a_TCR-Ts showed superior cytotoxicity towards HLA-A*02^{+} B-LCLs when compared to LMP2a_TCR-Ts. This was further confirmed in a 3D tumor spheroid model of HLA-A*02^{+} B-LCLs using a live-cell imaging approach. In conclusion, ex vivo isolated protective TCRs were redirected into T cells from third-party donors with the potential to attract innate immune cells and alter the tumor environment, thereby widening the applicability of T-cell therapy to refractory viral infections.

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CD39 negative tumor infiltrating lymphocytes (TIL) from patients with pancreatic cancer are associated with a broader tumor antigen-specific recognition profile

C. Mendonça Gorgulho*[1], L. Lordello*[1], E. de Sousa[1], J. Lérias[1], J. Kamiki[1], P. Antonio[1], S. Cascais[1], R. Eduardo[1], D. Ligeiro[1], V. Balan[1], T. Jing[2], D. An[2], Z. Wang[3], M. Maeurer[1,4]
[1]Champalimaud Foundation, Immunosurgery / Immunotherapy, Lisbon, Portugal, [2]Immunonet Co, Beijing, China, People's Republic of, [3]Guangdong Provincial Hospital of Chinese Medicine & Guangdong Provincial Academy of Chinese Medical Sciences, Guangzhou, China, People's Republic of, [4]I Medizinische Klinik, Johannes Gutenberg University Mainz, Mainz, Germany

*Contributed equally

The low mutational burden in pancreatic ductal adenocarcinoma (PDAC) has led to the belief that biologically relevant immune responses in tumor-infiltrating lymphocytes (TIL) from patients with PDAC were not frequent. However, recent reports suggest that PDAC is infiltrated with a diverse T-cell receptor repertoire shaped by the tumor microenvironment (TME). This study reports the expansion of TIL from 30 patients with PDAC. The patient group was divided into patients who underwent surgery directly after

diagnosis or those who received neoadjuvant therapy. This allowed us to compare the TIL phenotype and antigen-specific T-cell response quality and quantity between the two groups. TIL reactivity was defined by ex vivo reactivity testing using a panel of 59 synthetic molecularly defined target epitopes commonly shared amongst gastrointestinal (GI) cancers, e.g. peptides representing 27 KRAS mutations, T-cell hotspots from mesothelin and 6 mutant plus 6 wildtype epitopes from MUC4, a protein expressed in PDAC but not in healthy tissue. We also tested non-tumor-associated targets, including targets from cytomegalovirus, Epstein-Barr virus, Influenza-M1, and a dominant epitope from the Mycobacterium tuberculosis-associated antigen ESAT. After 7 days in culture, we quantified interferon (IFN)-y production from the supernatant. This provides a matrix for future TIL analysis from patients with GI malignancies without necessarily analyzing private target epitopes on a case-by-case basis. We used flow cytometry to test TIL for activation and differentiation markers and analyzed the frequency of distinct phenotypes associated with target recognition. The frequency of $CD39^-$ TIL strongly correlates with the breadth of the target peptide repertoire for both the $CD4^+$ as well as $CD8^+$ TIL defined by IFN-gamma production. Next, we performed the same correlation coefficient calculation, but with the stratification of our cohort between treatment naïve versus post-chemotherapy patients. The correlation was maintained for the patients who did not receive chemotherapy, i.e. $CD39^-$ TIL frequency was strongly correlated with antigen recognition. In the post-chemotherapy patient group, we observed a shift towards the TEMRA ($CD45RA^+$ $CCR7^-$) phenotype in TIL correlating with anti-tumor reactivity. These results show that i) chemotherapy selects for a different subset of TIL harvested from cancer lesions after neoadjuvant therapy and ii) that the ectonucleotidase CD39, the limiting enzyme that converts an ATP-driven pro-inflammatory milieu to an anti-inflammatory state mediated by adenosine, is a key cell surface molecule that does not only affect the quality of an immune response, yet also the diversity of target epitopes being recognized by TIL. We are currently developing a lentiviral vector to create $CD39^-$ T-cells under an HIF1 alpha promotor to maintain immune-fitness either in TIL or in transgenic CAR-T-cells which are at risk to express CD39 imposed by the TME

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Ex vivo expanded, autologous NKG2C+ natural killer cells from blood of HCMV- seropositive patients for immunotherapy of glioblastoma

S. Michen[1], A. N. J. Sagerer[2], W. Jugel[1], L. Rupp[3], I. Eyüpoglu[2,4,5], A. Temme[1,4,5]

[1]TU Dresden, Medical Faculty and University Hospital Carl Gustav Carus, Neurosugery, Division of Experimental Neurosurgery and Tumor Immunology, Dresden, Germany, [2]TU Dresden, Medical Faculty and University Hospital Carl Gustav Carus, Neurosurgery, Dresden, Germany, [3]TU Dresden, Medical Faculty Carl Gustav Carus, Institute of Immunology, Dresden, Germany, [4]German Cancer Consortium (DKTK), partner site Dresden, Dresden, Germany, [5]National Center for Tumor Diseases (NCT/UCC), Dresden, Germany

This study focuses on NKG2C+ natural killer (NK) cells, a small NK cell subpopulation in the blood of human cytomegalovirus (HCMV) seropositive glioblastoma (GBM) patients. Besides recognition of peptides derived from classical HLA alleles, these NKG2C+ NK cells are able to recognize a processed HLA-G signal peptide presented by HLA-E molecules on the surface of GBM cells. Consequently, NKG2C+ NK cells have a higher intrinsic capability to kill GBM cells. In our study, we investigated an ex vivo expansion of NKG2C+ NK cells derived from 11 elderly patients suffering from GBM, a primary brain cancer frequently accompanied by an immune suppressive tumor microenvironment. The study furthermore focused on anti-tumor responses of

NKG2C+ NK cells against autologous and allogeneic primary GBM cells. NK cells were isolated from the blood of HCMV-seropositive GBM patients and the NKG2C+ NK cell subpopulation was specifically expanded for two weeks via a PC-3 feeder cell line genetically engineered with interleukin 2 (IL-2), membrane-bound IL-15, as well as a first-generation single chain trimeric HLA-E molecule. The NK cells were characterized for expression of NKG2A/C, activating/inhibitory receptors, and for appearance of maturation and exhaustion markers by flow cytometry analysis before and after expansion. Simultaneously, primary GBM cell cultures were established and tumor tissue was analyzed for GBM-associated immune cell populations and HLA-E expression by immunohistochemistry and flow cytometry. Subsequently, the cytotoxicity of ex vivo expanded NKG2C+ NK cells was evaluated by cytotoxicity assays employing autologous and allogeneic primary GBM cells. Co-cultivation of isolated NK cells with PC-3 feeder cells for 14 days resulted in a median 121-fold expansion of NKG2C+ NK cells, with a median NKG2C+ NK cell percentage of 71.6 % (range: 42.1 % - 93.8 %). Expanded NKG2C+ NK cells exhibited significantly increased numbers of CD16+, CD25+, TIM-3+ and KIR+ cells, but only a low number of PD-1+ and TIGIT+ cells. Of note, GBM tumor tissue showed very low infiltration of NK cells and T lymphocytes, but interestingly, a median of 49.9% of NK cells exhibited an activated phenotype compared to 8.5% of T lymphocytes. Expanded NKG2C+ NK cells demonstrated cytotoxicity towards allogeneic HLA-E+ GBM cells, but not against autologous GBM cells, which all exhibited a low median HLA-E expression of 13.5 % (range: 1.3 % - 46.6 %). Our results show a highly efficient ex vivo expansion of NKG2C+ NK cells from peripheral blood of HCMV-seropositive GBM patients. Expanded cells exhibited strong cytotoxicity against allogeneic HLA-E+ GBM cells irrespective of HLA/KIR setting, but remained inactive when encountering autologous tumor cells. Therefore, it might be concluded that an allogeneic setting is advantageous for NK cell immunotherapy of glioblastoma.

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PD-L1 and FOXP3 expression in a cohort of HIV positive and negative cervical cancer

R. Naidoo[1], O. Abdulmalik[2], P. Govender[1], H.-T. Wu[1]

[1]University of Cape Town, Pathology, Cape Town, South Africa, [2]University of Ibadan, Pathology, Ibadan, Nigeria

Cervical cancer is a major cause of morbidity and mortality in women globally. Despite advances in prevention and treatment, mortality remains high, particularly in developing countries. HIV is a major disease burden associated with worse outcomes in cervical cancer. Programmed death ligand 1 (PD-L1) has been shown to play a significant role in the pathology of several cancers. PD-L1 affects the proliferation and differentiation of T lymphocytes. Furthermore, therapies directed towards this pathway have shown promising results.

Our study investigates the differences in PDL1 expression and FoxP3 positive T lymphocytes distribution, in HIV positive and HIV negative cervical squamous cell carcinoma using immunohistochemical methods. The expressions were correlated with clinicopathological parameters.

Eighty FFPE tissue samples comprising of HIV positive, and HIV negative tissue blocks were used in our study The H&E slides were reviewed by a pathologist and the selected tissue blocks were stained using immunohistochemical methods with the DAKO PD-L1 clone 22C3 and Invitrogen FoxP3 clone 236A/E7.

The PDL1 expression was done using the Combined Positive Score (CPS) which was greater than 1 in 51.3% of cases and, HIV status was not significantly correlated with the expression of PDL1. In addition, the

distribution of PD-L1 expression was heterogenous in the 80% of samples with homogenous distribution in 2.5% of the sample whilst the remaining samples revealed no PD-L1 expression. Survival analysis showed that different expression levels did not significantly affect survival in this cohort. Tissues with zero and very low levels of PDL1 expression (CPS 0 and CPS <1) correlated with significant higher population of FoxP3 positive cells within tumour nests but not have significant association with the expression of FoxP3 in adjacent stroma.
The PDLI expression was similar in both our study cohorts, with no significant difference in the two groups and the expression of PDL1 was not prognostic in our study. Furthermore, the pattern of PD-L1 expression was heterogenous in most samples. Significantly higher FoxP3 positive cells were present in samples with low PD-L1 expression.

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Leveraging CRISPR screening and gene knockout technology to enhance the potency of next generation T cell therapies.

D. Nowakowska, Y. Huang, R. Jain, D. Israni, D. Manandhar, S. Hymson, F. La-Deur, T. Chen, J. Soundararajan, J. Xie, K. Foley, O. Cinar, C. Arieta, M. Rooney, M. van Buuren
BioNTech US Inc., Cambridge, United States of America

Adoptive T cell therapies have demonstrated clinical efficacy in hematological cancers and have the potential to transform the therapeutic landscape for patients with solid tumors. However, limited cell persistence and a suppressive tumor microenvironment (TME) can lead to loss of cell functionality and result in poor clinical outcomes. To address these shortcomings, an in vitro loss-of-function genetic screening platform was established, and several model systems and functional readouts were developed to identify and validate new targets enhancing the therapeutic potential of T cells. A CRISPR guide library was generated with gene targets curated for their relevance in modulating T cell functionality based on evidence collected from the literature. This custom library was screened in a pooled fashion in three primary T cell models: CD3/CD28-activated T cells, neoantigen (neoAg)-specific T cells (BioNTech's NEO-STIM™ platform) and TCR T cells across two in vitro assays that addressed the following areas of improvement: i. cell stemness and proliferation after acute antigen exposure or during long antigen-driven manufacturing process, and ii. cell survival under the stress of chronic antigen stimulation mimicking the TME. Top ranking targets were validated using Cas9 ribonucleoprotein-mediated editing across orthogonal in vitro assays evaluating T cell proliferative capacity, cytokine secretion, stemness/exhaustion profile, metabolism, and cytotoxicity, as well as anti-tumor activity in syngeneic and xenograft models. Screening revealed 11 high ranking targets associated with stem-like highly proliferative cells and/or cells surviving stress of chronic stimulation that were advanced to the validation stage. When target GenT01 was edited early during neoAg-specific T cell or TCR T cell generation, 4 to 25-fold improvement in cell expansion, proportion of central memory T cells and expression of stem-associated markers such as CD127 and TCF1 was observed. In vitro functional validation studies demonstrated that targets GenT07 and GenT09 significantly extended ability of edited cells to control tumor cell line growth in 3D repetitive killing assays in addition to enhancing their cytokine secretion and increasing cell spare respiratory capacity. Moreover, in vivo studies conducted in B16.OVA syngeneic and TCR T xenograft models showed that GenT07 and GenT09-edited cells suppressed tumor growth more effectively compared to unedited cells. In summary,

through the screening platform we identified potent targets that are expected to enhance T cell persistence and functionality in the TME. We are now evaluating the mechanism of action of single and selected combinations of targets to augment T cell potency and aim to translate these findings into therapeutic benefit by implementing knockout editing into our T cell therapy platforms.

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Deciphering cellular origin and molecular features of CIK cells using single-cell approach

P. Palmerini*[1,2], E. Vigolo*[1], H. Ngo[1], G. D'Accardio[3], E. Cappuzzello[1], R. Sommaggio[3], A. Rosato[1,3]
[1]Veneto Institute of Oncology, Padova, Italy, [2]University of Padova, Department of Cardiac, Thoracic, Vascular Sciences and Public Health, Padova, Italy, [3]University of Padova, Department of Surgery Oncology and Gastroenterology, Padova, Italy

*Contributed equally

The increasing rate in cancer mortality has augmented the need to develop more effective therapeutic options. Among available strategies, adoptive cell therapy with cytokine induced-killer (CIK) cells represents a promising field. CIK cells are characterized by the co-expression of CD3 and CD56, and share functional and phenotypical properties of both T and natural killer cells. CIK cells can be easily expanded ex vivo from peripheral blood lymphocytes in clinically relevant numbers, and can be redirected against specific tumor targets when combined with monoclonal antibodies. Clinical application of CIK cells has proven to be safe and efficient, but further improvements are needed to reach complete response in patients. To our knowledge, a detailed understanding of CIK cell differentiation dynamics into effector cells at the molecular and cellular level is still missing. In this study, we aim to dissect the molecular complexity of CIK cells, and to understand the differentiation trajectories which shape their cytotoxic phenotype. To achieve that, we expanded CIK cells from 8 healthy donors according to a serum-free, good manufacturing practice-grade protocol, using the G-Rex® devices. Throughout the 2-weeks culture, we analyzed the heterogeneous composition of cells by flow cytometry and single-cell RNA sequencing (scRNA-seq) with feature barcode technology for cell multiplexing (10X Genomics). Our data have proven high technical and biological quality, as confirmed by the identification of peripheral blood cell populations already characterized in literature. Interestingly, time-course analysis revealed an enrichment of proliferating effector $CD8^+$ T cells clusters, which we further sub-clustered to dissect additional population types. Among them, we identified fully differentiated $CD3^+CD56^+$ CIK cells, that we selectively purify to perform bulk RNA-seq to increase the robustness of our scRNA-seq analysis. Moreover, the identified CIK cells express genes associated to cytotoxicity (GZMA, GZMH, NKG7), migratory phenotype (CCL5), and metabolic reprogramming (LPAR6), underlining their anti-tumor potential. To trace the cellular origins of CIK cells, we followed changes in gene expression over time. Preliminary analysis shows that, at earlier timepoints of culture, CIK cells express T cell stem-like markers (TCF7, IL7R, TNFAIP3), suggesting a less differentiated cellular state. In conclusion, our preliminary results show that CIK cells have a unique transcriptional profile, in line with their cytotoxic potential, and exhibit an immature phenotype at early timepoints of culture. Ongoing investigation by employing RNA velocity and pathway analyses will reveal critical molecular insights, which will both maximize expansion of the effector CIK cell population, and improve their therapeutic efficacy against several cancer histotypes.

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Isolation and characterisation of TCRs that recognise Citrullinated and Homocitrullinated post translationally modified peptides

S. Paston[1], R. Choudhury[2], S. Shah[2], G. Cane[1], J. Chadwick[1], R. Metheringham[2], F. Master[1], R. Herbertson[3], L. Durrant[1,2]

[1]Scancell Ltd, Oxford, United Kingdom, [2]Scancell Ltd, Nottingham, United Kingdom, [3]Brighton and Sussex University Hospitals, Brighton, United Kingdom

Under conditions of cellular stress, proteins can be post translationally modified causing them to be recognised by the immune system. One such stress induced post translational modification (siPTM) modification is citrullination, the conversion of arginine residues to citrulline by peptidylarginine deiminase (PAD) enzymes. We have previously shown that targeting citrullination can induce CD4 responses that provides efficient tumour therapy in vivo in a process mediated by autophagy. We are currently running A Phase 1/2, Multicentre, Open-Label Study of Modi-1 in Patients with Breast, Head and Neck, Ovarian, or Renal Cancer (The ModiFY Study) study. The Moditope® vaccine incorporates two citrullinated vimentin peptides (Vim28cit and Vim415cit), and a citrullinated a-enolase peptide (Eno241cit), each conjugated to the toll-like receptor (TLR)1/2 ligand adjuvant AMPLIVANT®. Sixty percent of patients receiving Modi-1 monotherapy show stable disease and one patient showed a partial response. Currently we are recruiting patients receiving Modi-1 in combination with checkpoint inhibitors.

Eighty three percent of patients make a T cell response to Eno241cit, we have isolated a TCR from one of these patients. Single-cell RNA- & TCRseq was performed on sorted CD4+ IFNγ+. RNAseq analysis revealed these are Cytotoxic CD4 T cells, using Lentivirus-TCR transduced T cells we have successfully shown that two isolated TCRs react specifically to Eno241cit peptide with little or no recognition of the wild type peptide. The epitope has been mapped and the presenting HLA allele identified.

Another post translational modification is the carbamylation of lysine to homocitrulline. This reaction occurs when isocyanic acid reacts with the amine (NH_2) groups on lysine to yield homocitrulline. The carbamylation of amine groups leads to a change in molecular charge, which in turn alters antigenic properties and can lead to the generation of unique T cell and antibody epitopes.

We have successfully isolated a CD8 TCR that specifically recognises a post translationally modified peptide from Aldolase. The TCR recognises a 9mer peptide that has been modified with the conversion of lysine to homocitrulline at position 7 (VLAAVY-Hcit-AL). Using Lentivirus-TCR transduced T cells we have shown that the homocitrulline peptide is recognised and not wildtype, the epitope has been mapped and the HLA restriction confirmed.

T-cell based immunotherapy has achieved remarkable clinical responses in cancer patients. Our own data in preclinical mouse models have shown that T cells that recognise post translational modified peptides induce tumour regression.

Our data demonstrates the successful isolation of post translational-specific TCRs, these TCRs can be used to provide cancer therapy as either a cellular therapy or in a soluble T cell bispecific format.

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Vitamin C conditioning creates CAR-T cells with superior cytotoxic capacity and fitness to combat the

immunosuppressive tumor micromilieu

P. Rahmati[1], A. Bonifacius[1], A. C. Dragon[1], C. Malinconico[1], R. Blasczyk[1], M. Hudecek[2], S. Floess[3], J. Huehn[3], B. Eiz-Vesper[1]

[1]Hannover Medical School, Institute of Transfusion Medicine and Transplant Engineering, Hannover, Germany, [2]University Hospital Würzburg, Chair of Cellular Immunotherapy, Department of Internal Medicine II, Würzburg, Germany, [3]Helmholtz-Centre for Infection Research, Department Experimental Immunology, Braunschweig, Germany

Despite the promising results of CAR-T-cell therapy in hematological malignancies, poor in vivo persistence and low potency especially in the treatment of solid tumors are challenges that remain to be addressed. The overall survival rate after CD19 CAR-T-cell therapy for EBV-associated post-transplant lymphoproliferative disorder (PTLD), a major complication after solid organ transplantation, was below 50%. It has been shown that the efficacy of adoptively transferred and genetically modified T cells can be modulated by ex vivo culture conditions. Vitamin C (vitC) is a micronutrient that influences the immune system by mechanisms such as the regulation of epigenetic processes and oxidative stress. In this proof-of-concept study, we investigated the impact of vitC pre-conditioning on the functionality of CD19 CAR-T cells (vitC-CAR19-Ts vs. CAR19-Ts). vitC pre-conditioning resulted in a higher yield of CAR19-Ts. Moreover, vitC-CAR19-Ts showed significantly enhanced specific cytotoxicity towards $CD19^+$ Nalm-6 cells as well as towards EBV-infected B lymphoblastoid cell lines (B-LCLs), serving as in vitro model of EBV-associated PTLD. Transcriptomic profiling of vitC-CAR19-Ts and CAR19-Ts revealed upregulation of genes involved in T-cell activation, motility and epigenetic reprograming in vitC-CAR19-Ts after target recognition when compared to CAR19-Ts. Moreover, higher levels of GNLY mRNA, encoding for granulysin, were observed in vitC-CAR19-Ts when compared to CAR19-Ts, which was in line with significantly higher concentrations of secreted granulysin. To evaluate the role of granulysin for the cytotoxicity of vitC-CAR19-Ts, CRISPR/Cas9-mediated knockout of GNLY was performed ($GNLY^{KO}$). While the overall cytotoxicity of $GNLY^{KO}$ (vitC)-CAR19-Ts was reduced compared to (vitC)-CAR19-Ts, no difference between $GNLY^{KO}$ vitC-CAR19-Ts and $GNLY^{KO}$ CAR19-Ts was observed, suggesting that higher granulysin production is largely responsible for the enhanced cytotoxicity of vitC-CAR19-Ts. Based on the upregulation of cell motility genes in vitC-CAR19-Ts, 3D tumor spheroids of B-LCLs and fibroblasts were generated. Live cell imaging revealed significantly faster elimination of B-LCLs within the spheroids by vitC-CAR19-Ts compared to CAR19-Ts. Further, in an in vitro stress assay, the improved cytotoxic capacity of vitC-CAR19-Ts towards B-LCLs was found to be durable, indicating superior long-term functionality even in the presence of an immunosuppressive tumor milieu. In conclusion, we demonstrated that vitC preconditioning enhances CAR19-T-cell yield, improves their cytotoxic potential, motility and long-term functionality in presence of the immunosuppressive tumor milieu, suggesting vitC pre-conditioning as a promising strategy to improve CAR T-cell therapy for solid tumors.

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Automated Manufacturing of Tumor-Reactive T cells for Immunotherapy of Glioblastoma

A. Riabinska, E. Balta, E. Criado-Moronati, D. Frison, T. Telatar, D. Maciaczyk, J. Engeln, M. Domdey, L. Fischer, M. Ott, A. Dzionek, I. R. Hardy

Miltenyi Biotec, Personalized Immunotherapy, Bergisch Gladbach, Germany

In the current study, we sought to develop a clinical strategy focused on the use of tumor-infiltrating lymphocytes (TILs) to treat GBM. Glioblastoma multiforme (GBM) is the most common primary brain tumor with median overall survival of approximately 15 months from diagnosis. GBM will inevitably relapse following surgical resection and radiotherapy, and therefore has a high unmet need for new therapeutic strategies, particularly those which can target multiple antigens due to the high degree of intra- and interpatient heterogeneity. TILs have been used for decades in solid tumors with a good safety profile and polyreactive specificities and the first TIL-based drug “Amtagvi” was recently approved by FDA. To this end, we characterized the immune and non-immune cellular constituents of GBM tumor digests. Further, we established a workflow for optimal isolation of TILs out of primary GBM tumors and translated this to a clinical-scale process for GBM-derived TIL manufacturing. Briefly, GBM material was dissociated to generate a single-cell suspension and analyzed for tumor antigens as well as immune cell types by flow cytometry and single-cell sequencing. The digest, fresh or frozen, was used for TIL manufacturing within the automated and closed CliniMACS Prodigy® Tumor-reactive T cell (TRT) system. Reactive T cells were enriched based on expression of CD137, and entered into a two week rapid expansion protocol (REP) to reach clinically relevant numbers.

In total, tumor material from 30 glioblastoma patients was analyzed and processed. Most tumor cells expressed known GBM-associated tumor antigens, such as GD2 and CD276. In line with reported findings, the myeloid compartment was a dominant component of the infiltrating immune cells. Despite reports of low T cell frequencies within GBM tumors, we were able to consistently obtain adequate starting numbers of TIL (~5-10e6) sufficient for the enrichment of tumor-reactive TILs and the initiation of REP culture. We were able to use the CliniMACS Prodigy® to generate clinically-relevant amounts of autologous GBM-TIL product. The final product was highly pure for CD3+ T cells, displayed a generally central and effector memory T cell phenotype. Notably, we were able to detect significant autologous tumor-lysis with TIL derived from GBM tumor digests. In line with the importance of CD137 as a marker of tumor-reactive TIL, we observed significantly increased tumoricidal activity when TIL were enriched for CD137 and a complete loss of activity when CD137 was depleted prior to the initiation of the REP.
In conclusion, we provide a comprehensive analysis of primary GBM tumors including single-cell analysis and demonstrate the feasibility of automated and closed manufacturing of GBM-derived TRTs. These findings extend the applicability of TILs to poorly-infiltrated tumors and represent a novel therapeutic strategy for addressing the complex heterogeneity of primary brain malignancies.

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Identification and functional analysis of tumor-reactive CD4^{+} effector and regulatory T-cell subsets in human pancreatic cancer

A. Rodriguez Ehrenfried[1,2,3], L. Steffens[2,3], Z. Meng[2,4,5], L. Herzel[2], O. Strobel[4,6], S. Tenzer[7,8], I. Poschke[9,10], M. Volkmar[1], R. Offringa[2,4,5]
[1]Helmholtz-Institute for Translational Oncology by DKFZ (HI-TRON), TCR discovery platform, Mainz, Germany, [2]German Cancer Research Center, Molecular Oncology of Gastrointestinal Tumors, Heidelberg, Germany, [3]Heidelberg University, Faculty of Biosciences, Heidelberg, Germany, [4]University Hospital,

Department of General, Visceral and Transplantation Surgery, Heidelberg, Germany, [5]Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Sino-German Laboratory of Personalized Medicine for Pancreatic Cancer, Wuhan, China, People's Republic of, [6]Medical University Vienna, General Surgery, Vienna, Austria, [7]Helmholtz-Institute for Translational Oncology by DKFZ (HI-TRON), Immunoproteomics, Mainz, Germany, [8]University Medical Center Mainz, Institute of Immunology, Mainz, Germany, [9]German Cancer Research Center, Clinical Cooperation Unit Neuroimmunology and Brain Tumor Immunology, Heidelberg, Germany, [10]National Center for Tumor Diseases (NCT), Immune Monitoring Unit, Heidelberg, Germany

We recently reported on the analysis of the tumor-infiltrating T-cell repertoire in human pancreatic cancer by means of single cell RNA-sequencing (scRNA-seq), demonstrating the presence of expanded tumor-reactive CD8+ TCR clonotypes in these tumors. In the context of these studies, we developed a gene signature that accurately predicts tumor-reactive versus non-reactive bystander TCR clonotypes in scRNA-seq data sets of various solid tumor types, as confirmed by in vitro functional testing of TCR-reactivity against autologous tumor cells.

Given the importance of $CD4^+$ T-cell help in durable therapeutic anti-tumor immunity, we expanded our analyses to this T-cell subset. By means of the aforementioned gene signature, we also identified tumor-reactive CD4+ T-cell clonotypes, the TCRs of which mediate recognition of autologous tumor cells. This recognition requires IFNγ-pretreatment of the tumor cells to achieve enhanced surface HLA class II expression.
Our search also revealed tumor-reactive CD4+Foxp3+ Treg clonotypes, the TCRs of which mediate very strong T-cell reactivity to autologous tumor cells. These TCRs and their cognate antigens may offer formidable starting points for personalized immunotherapy, but could also reflect Treg-mediated suppression of immune responses against auto-antigens that – when activated in the context of cancer immunotherapy – would be harmful to the patient.

Analysis of HLA-restriction showed that the majority of the aforementioned CD4+ Th and Treg clonotypes are HLA-DR-restricted. Based on this information, we are currently working towards identification of the tumor-associated T-cell epitopes, and evaluating the processing and presentation of the corresponding antigens by tumor cells and HLA-DR-matched dendritic cells.

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Activation of PD-1^{K78A} CAR transduced T-cells via clinical grade anti-PD-1 monoclonal antibodies.

J. Schaukens[1], Y. De Vlaeminck[2], X. Geeraerts[1,3], L. Stevens[1,3], K. Breckpot[2], B. Neyns[1,3], S. Tuyaerts[1,3]
[1]Vrije Universiteit Brussel (VUB), Translational Oncology Research Center (TORC), team Laboratory for Medical and Molecular Oncology (LMMO), Brussel, Belgium, [2]Vrije Universiteit Brussel (VUB), Translational Oncology Research Center (TORC), team Laboratory for Molecular and Cellular Therapy (LMCT), Brussel, Belgium, [3]Vrije Universiteit Brussel (VUB), Universitair Ziekenhuis Brussel (UZ Brussel), Translational Oncology Research Center (TORC), team Laboratory for Medical and Molecular Oncology (LMMO), Brussel, Belgium

Malignant tumors elicit adaptive spontaneous T-cell responses and PD-1 immune checkpoint blocking monoclonal antibodies (ICB mAb, e.g. nivolumab) can reinvigorate such responses by counteracting the exhaustion of cytotoxic T-lymphocytes to the benefit of patients. Adoptive tumor-infiltrating lymphocyte (TIL) treatment has demonstrated activity in a minority of patients with different solid tumor types. Improvement of the anti-tumor activity of adoptively transferred TIL products via provision of additional T-cell stimulatory signals through transduction with chimeric antigen receptors (CARs) may result in improved tumor eradication. We investigated whether a novel CAR construct with a mutant PD-1 extracellular domain can be selectively activated by clinical-grade PD-1 blocking mAbs. This could potentially provide a "tunable" TIL product. Novel CAR lentiviral vectors with an extracellular wild-type PD-1 (PD-1^{wt}) or mutated PD-1 (PD-1^{K78A}, K78A point mutation) domain capable of binding with anti-PD-1 mAbs were manufactured. The PD-1^{K78A} CAR was generated to avoid clinical on-target, off-tumor toxicity interactions by binding to PD-$L1^{+}$ cells. Model-T-cells (that allow for read-out of CAR-mediated CD3ζ-signaling by intracellular GFP), expressing the novel CAR-T constructs were generated. A 2D co-culture assay with a 1:1 (CAR transduced model-T-cell/PD-$L1^{+}$ tumor cell) ratio was performed to evaluate their PD-L1 binding capacity via flow cytometry. Anti-PD-1 mAb binding and intracellular CD3ζ-signal activation were demonstrated by addition of various concentrations of plate-bound (immobilized) and soluble anti-PD-1 mAbs to a CAR-transduced model-T-cell monoculture using flow cytometry and Incucyte live-cell imaging. The PD-L1 co-culture assay confirmed the loss of PD-L1 binding capacity and retention of the anti-PD-1 mAb binding capacity for the PD-1^{K78A} CAR. A positive correlation between CAR expression and mAb binding was found, as well as a significant difference between nivolumab binding to the PD-1^{wt} and PD-1^{K78A} CAR with 90 µg/mL and 150 µg/mL nivolumab. Intracellular CD3ζ-signal activation was quantified, for both plate-bound and soluble nivolumab and pembrolizumab. Here, intracellular CD3ζ-signal activation was noticeable after addition of immobilized anti-PD-1 mAbs, while cross-linkage between CAR and soluble mAbs remained insufficient to obtain full activation. Our data shows the ability of both the PD-1^{wt} and PD-1^{K78A} CAR constructs to be activated by crosslinked (immobilized) anti-PD-1 mAbs. However, soluble mAbs were unable to fully trigger CAR-mediated CD3ζ-signaling.

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Targeting of immune checkpoints with multiplexed zinc finger-repressors improve anti-tumor activity of CAR-T cells and TILs

P. Schiele[1], D. Monteferrario[2], M. David[2], Y. Zhou[3], S. K. Tadi[2], C. F. Dupont[2], C. Jeanneau[2], G. Saviane[2], I. Marchetti[2], A. E. Martelli[2], G. D. Davis[3], A. Reik[3], I.-K. Na[1], M. Frentsch[1], M. de la Rosa[2], D. Fenard[2]
[1]BIH Center for Regenerative Therapies, Berlin, Germany, [2]Sangamo Therapeutics France, Valbonne, France, [3]Sangamo Therapeutics Inc, Richmond, United States of America

Cellular therapies with engineered T cells, such as CAR-T cells, are revolutionizing the treatment of hematological malignancies. However, their translation to solid tumors is impeded by the immunosuppressive tumor microenvironment (TME) and T cell exhaustion rendering CAR-T therapy ineffective. Moreover, the large varieties of tumor target antigens within solid tumors complicate the identification of universally applicable targets for CAR-T cells that might be circumvented by applying reinvigorated TME-resistant tumor-infiltrating lymphocytes (TILs).
Here, we describe the generation of Zinc Finger Repressors (ZFRs) capable of efficient and durable

epigenetic gene silencing of immune checkpoint molecules (ICs) in T cells without the need of DNA double-strand breaks. This innovative approach, specifically targeting T cells, is very attractive as it would potentially be more efficacious and reduce the side effects associated with the systemic administration of IC inhibitors. The compact size of ZFRs enables their multiplexing in a single bidirectional lentiviral vector (LV) in combination with a tumor-specific CAR. Through optimal zinc finger array engineering, we were able to express highly active ZFRs in CAR-T cells, with an efficient proliferation index, viability, and T cell functionality. Using an in vivo B cell lymphoma model, we showed superior anti-tumor activity and improved survival of mice treated with a CD19-CAR in combination with a PD1 ZFR repressor compared to the CAR alone. We also demonstrate that our approach is functional using TILs isolated from secondary tumor tissue, namely colorectal liver metastases from CRC patients. TILs were effectively transduced to stably express ZFRs targeting PD1 alone or in combination with either LAG3 or TIM3 ZF-Rs. While substantially reducing ICs surface expression, ZF-Rs had no negative impact on T cell expansion or phenotype alterations. Importantly, downregulation of ICs in TILs resulted in improved anti-cancer activity in an in vitro killing assay highlighting the potential of our ZF-Rs to refine cellular therapies.
Overall, our study describes the successful development of a ZF-R platform that could be used to potentiate the anti-tumor activity of CAR-T and TIL therapies.

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Density and ratio of stimulatory antibodies on immunofilaments influence ex vivo T cell expansion and phenotype

M. Schluck, L. Weiss, R. Classens, C. Figdor, R. Hammink
Radboudumc, Medical Biosciences, Nijmegen, The Netherlands

The development of immunotherapy has revolutionized the treatment of cancer by influencing the anti-tumour immune response. Numerous immunotherapies focus on expanding and activating tumour-reactive T cells, for instance by re-invigorating existing T cell responses by immune checkpoint blockade or by expanding the tumour-reactive T cell pool ex vivo through adoptive T cell therapies (ACT). With ACT high numbers of tumour-reactive T cells are infused into a patient, consisting of autologous tumour-infiltrating lymphocytes, T cell receptor engineered T cells, or chimeric antigen receptor T cells. ACT has shown promising effects in the treatments of various cancers, however, for ACT to succeed high numbers of T cells are required.
In recent years, biomaterial-based artificial antigen presenting cells (aAPCs) have been developed for the activation and expansion of T cells. aAPC can mimic T cell activation signals by using agonistic anti-CD3 (αCD3) and anti-CD28 (αCD28) antibodies, respectively. Rigid synthetic beads presenting these agonistic antibodies are widely used in expansion protocols. However, with these standard beads the signals given to the T cells cannot be tuned. Here, we used polyisocyanopeptide based immunofilaments as nanosized aAPCs to study the effects of the ratio and density of αCD3 and αCD28 on human T cell expansion and phenotype.
We cultured human total $CD3^+$ T cells for up to 14 days with the immunofilaments and investigated the effects on T cell expansion, cytokine production, and phenotype. We observed differences in T cell expansion depending on the density and ratio of the stimulatory signals. Additionally, the ratio and density of the stimulatory antibodies also influenced the production of the cytokines IFNγ, TNF-α, and IL-2 in the

first 3 days of culture. Moreover, differences in effector phenotype but in Treg phenotype could be observed.
Taken together these results suggest that careful finetuning of the density and ratio of stimulatory antibodies can significantly impact T cell expansion and phenotype. As such our findings can be used to optimize T cell expansion protocols for ACT.

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Dasatinib-mediated inhibition of CAR signaling during ex-vivo expansion enhances AML-specific CAR-NK cells by preventing fratricide and activation-induced cell death

D. Sedloev, J. Unglaub, Q. Chen, Y. Hao, G. Jiang, A. Schmitt, C. Müller-Tidow, M. Schmitt, T. Sauer
University Clinic Heidelberg, Heidelberg, Germany

Introduction: The prognosis of patients with acute myeloid leukemia (AML) remains poor. Chimeric antigen receptor expressing NK cells (CAR-NKs) are emerging as a promising platform for the immunotherapy of AML. Peripheral blood (PB) derived CD70 CAR-NKs have potent anti-leukemic activity against AML, but their endogenous CD70 expression causes significant fratricide during ex-vivo expansion. This leads to low NK cell yields during production and negatively impacts their long-term performance. We hypothesized that the addition of Dasatinib during ex-vivo expansion may inhibit CAR-mediated signaling and subsequently fratricide, hereby improving the production yield and functionality of CD70 CAR-NK.
Methods: Primary NK cells were isolated from PB, magnetically purified, and activated using irradiated K562 feeder cells. Viability, proliferation, phenotype and transduction efficacy during production were monitored by flow cytometry. In vitro performance against AML cells was evaluated using short-term cytotoxicity, ELISA and long-term serial co-culture assays. In-vivo efficacy was tested using an Molm-13 xenograft model with weekly IVIS.
Results: Activated PB-derived NK cells express CD70 on their surface. Transduction with a CD70-specific CAR construct was possible but yielded significantly lower cell counts than non-transduced NK controls (527.7±315.3 vs 2151±532.8, p=0.039).
The addition of Dasatinib during ex-vivo expansion improved viability and total yield of CD70-CAR-NKs (fold-change 527,7±315.3-fold vs 2151±532.8-fold, p=0.039). Interestingly, CD33 CAR-NKs also benefited from exposure to Dasatinib with significantly higher viability and production yield than non-Dasatinib treated CD33 CAR-NKs even though CD33 is not expressed on NKs. This suggests further protective mechanisms of Dasatinib treatment during ex-vivo expansion beyond the prevention of fratricide such as the inhibition of activation-induced cell death (AICD) and terminal differentiation.
Indeed, Dasatinib-treated CAR-NKs exhibited a significantly altered phenotype with lower expression of the activating receptors NKG2C/D and death ligands FasL/TRAIL than non-treated CAR-NKs. IFNg secretion by Dasatinib-treated CAR-NKs was also significantly stronger compared to the controls (535.5±39.9pg/ml vs 2961±122.5pg/ml, p<0.0001). Furthermore, in-vitro proliferation and antigen-dependent cytotoxicity were significantly enhanced for CAR-NKs treated with Dasatinib as demonstrated in our serial coculture assay and these cells also led to improved anti-tumor efficacy and prolonged overall survival compared to non-Dasatinib treated CAR-NKs and non-CAR transduced NKs in-vivo (17 vs18 vs 21 days p=0.0005) .
Conclusion: The addition of Dasatinib during ex-vivo expansion enhances the anti-leukemic efficacy of CD70-CAR NKs not only by reducing fratricide but also preventing AICD and terminal differentiation, making

them a promising off-the-shelf cell product for the treatment of AML.

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Identification of a potent tumor-reactive T-cell repertoire in microsatellite stable colorectal cancers by means of single cell RNA sequencing

L. K. Steffens[1,2], C. Brüggen[1,2], A. Paul[1,2], A. Rodriguez Ehrenfried[1,2,3], M. Volkmar[3], N. Halama[4], S. Tenzer[5], N. Schleußner[6], R. Jackstadt[6], R. Offringa[1,7]
[1]German Cancer Research Center (DKFZ), Molecular Oncology of Gastrointestinal Tumors, Heidelberg, Germany, [2]Heidelberg University, Faculty of Biosciences, Heidelberg, Germany, [3]Helmholtz-Institute for Translational Oncology (HI-TRON), TCR Discovery Platform, Mainz, Germany, [4]Helmholtz-Institute for Translational Oncology (HI-TRON), Translational Immunotherapy, Mainz, Germany, [5]Helmholtz-Institute for Translational Oncology (HI-TRON), Immunoproteomics, Mainz, Germany, [6]Heidelberg Institute for Stem Cell Technology and Experimental Medicine (HI-STEM), Cancer Progression and Metastasis, Heidelberg, Germany, [7]University Hospital Heidelberg, Department of General, Visceral and Transplantation Surgery, Heidelberg, Germany

Whereas immune checkpoint blockade can result in therapeutic benefit in microsatellite unstable (MSI) colorectal cancer (CRC), microsatellite stable (MSS) CRC cases are mainly refractory to this treatment. In order to explore the reason underlying this lack of responsiveness, we compared the tumor-infiltrating T-cell repertoire in MSS and MSI tumors by means of single cell RNA sequencing (scRNA-seq), with a focus on the identification of tumor-reactive $CD8^+$ T-cell receptor (TCR) clonotypes.

We applied a gene signature for the prediction of tumor-reactive versus non-reactive (bystander) TCR clonotypes in scRNA-seq data sets, followed by functional testing of TCR reactivity against autologous tumor cells. This pipeline was established in pancreatic cancer and subsequently validated for other human cancer types.

The outcome of these studies differed from our expectations in two aspects. Firstly, we could readily identify multiple tumor-reactive TCR clonotypes in MSS tumor samples that mediated reactivity against autologous tumor cells in vitro. Secondly, our gene signature predicted high numbers of tumor-reactive TCR clonotypes in the MSI samples, but anti-tumor reactivity could not be verified in vitro because the tumor cells had undergone profound immunoediting involving defects in HLA heavy chain, beta-2-microglobulin and/or antigen-processing machinery genes.

The latter finding suggested that the TCR clonotypes predicted to be tumor-reactive in MSI tumors have been instrumental in tumor immunoediting. This was confirmed by the finding that restoration of HLA expression rendered the tumor cells sensitive to recognition by these TCRs.

We are currently diving into the antigen specificity of the tumor-reactive TCRs isolated from the MSS tumors, as this may provide us with clues on how to mobilize an anti-tumor T-cell response in this indication. Using a single-HLA-allele knockout strategy, we determined the HLA-restriction of multiple TCRs, as a first step towards the identification of cognate target antigens by means of mass spectrometry and

immunopeptidomics. A key question in this respect is whether the immunodominant epitopes represent mutanome-encoded neoantigens or tumor-associated antigens that are shared between MSS CRC cases.

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Assessment of an in vitro potency assay for evaluation of immune cell-mediated cytotoxicity using the Omni Pro 12 automated imaging platform.

D. Sullivan[1], S. Meiler[2], L. Stemkens[1], I. Thijssen[1], D. Califano[2], S. Chvatal[2], D. Millard[1]
[1]Axion BioSystems, Applications, Atlanta, United States of America, [2]Axion BioSystems, Product Management, Atlanta, United States of America

The development of immunotherapies relies on the use of in vitro potency assays—which are key for understanding complex interactions between immune cells and cancer cells. Immune effector T-cells are a promising cancer therapy due to their innate cytotoxicity. In particular, CAR T-cell therapy uses genetically engineered T-cells that express a chimeric antigen receptor that binds to a specific antigen on tumor cells. Assessing the efficacy and potency of CAR T-cell therapies in vitro and at high throughputs is vital for the preclinical development of these promising therapies. Here, we describe an in vitro potency assay that uses an automated imaging platform to quantify immune cell-mediated cytotoxicity of cancer target cells by immune effector cells. A GFP fluorescent and HER-2 expressing lung cancer cell line, A549, was seeded into a 96-well cell culture plate. Then, HER-2 CAR T-cells were added at 24 hours post target cell seeding at various E:T ratios (1:10, 1:5, 1:2, 1:1, and 5:1). Fluorescent images of the target cells were captured every 6 hours by the Omni Pro 12, an automated, high-throughput, live-cell analysis platform designed for continuous multi-well imaging inside an incubator. Automated image analysis was performed using the Confluency Module in the Axion Portal to view attachment and proliferation and quantify cytolysis of fluorescent A549 target cells. Percent cytolysis of the target cells was calculated by comparing the green fluorescent confluency of treated wells to no treatment control wells. A549-GFP cells showed a dose-dependent decrease in confluency over time that correlated with increasing amounts of CAR T-cells. At 92 hours post HER-2 CAR T-cell addition, the 5:1 ET group demonstrated approximately 91.8% +/- 2.0% cytolysis of A549-GFP cells, while the 1:10 group demonstrated approximately 69.2% +/- 3.0% cytolysis. Future work will evaluate differences in CAR T-cell potency when co-cultured with target cells that express different levels of HER-2. Overall, the Omni Pro 12 platform enables continuous quantification of the potency and kinetics of immune cell-mediated cytolysis.

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Prediction of tumor-reactive T cell receptors across different tumor entities

C. L. Tan[1,2,3,4], K. Lindner[1,2,3,5], T. Boschert[1,2,3,4,6], Z. Meng[7,8,9], A. Rodriguez Ehrenfried[4,6,8], A. De Roia[4,10], G. Haltenhof[1,3], A. Faenza[11], F. Imperatore[11], L. Bunse[1,2,3], J. M. Lindner[12], R. P. Harbottle[10], M. Ratliff[13], R. Offringa[7,8,9], I. Poschke[1,2,5], M. Platten[1,2,3,5,6,14], E. W. Green[1,2,3]
[1]German Cancer Research Center, CCU Neuroimmunology and Brain Tumor Immunology, Heidelberg, Germany, [2]German Cancer Consortium, Heidelberg, Germany, [3]Medical Faculty Mannheim, Mannheim Center for Translational Neuroscience, Heidelberg University, Department of Neurology, Mannheim, Germany, [4]Heidelberg University, Faculty of Biosciences, Heidelberg, Germany, [5]National Center for Tumor

Diseases, Immune Monitoring Unit, Heidelberg, Germany, [6]Helmholtz Institute for Translational Oncology, Mainz, Germany, [7]University Hospital Heidelberg, Department of General, Visceral and Transplantation Surgery, Heidelberg, Germany, [8]German Cancer Research Center, Division of Molecular Oncology of Gastrointestinal Tumors, Heidelberg, Germany, [9]Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Sino-German Laboratory of Personalized Medicine for Pancreatic Cancer, Wuhan, China, People's Republic of, [10]German Cancer Research Center, DNA Vector Laboratory, Heidelberg, Germany, [11]Cellply Srl, Bologna, Italy, [12]BioMed X GmbH, Heidelberg, Germany, [13]University Hospital Mannheim, Department of Neurosurgery, Mannheim, Germany, [14]German Cancer Research Center–Hector Cancer Institute at the Medical Faculty Mannheim, Mannheim, Germany

Personalized adoptive cell therapies (ACT) employ autologous tumor-reactive T cell receptors (TCRs) to target patient-specific (neo)antigens presented by human leukocyte antigen (HLA) molecules. Conventionally, a suitable tumor (neo)antigen is first identified and used to expand and screen for antigen-specific T cells. However, this current state-of-the-art method is limited to the ex ante definition of the type and immunogenicity of tumor antigens. Furthermore, the process of screening for antigen-specific T cells is time- and cost-intensive, making it difficult to integrate into a clinical workflow.

To circumvent these limitations, we have developed an antigen-agnostic, TCR-centric, and machine learning (ML)-based approach to identify tumor-reactive TCRs from tumor-infiltrating T cells (TILs) using combined single-cell VDJ- and RNA-sequencing. Using the scRNAseq and reactivity validation data, we trained a highly accurate classifier predicTCR to identify tumor-reactive TILs. In contrast to existing gene signature-based algorithms predicTCR does not falsely call TCRs from virus-reactive T cells,which often share transcriptomic signature with tumor-reactive T cells. When validated with four published datasets of various cancer entities, predicTCR also identifies tumor-reactive TCRs more accurately than gene signature-based approaches, increasing specificity and sensitivity (geometric mean) from 0.38 to 0.74.

Taken together, our data demonstrate the potential of predicTCR in identifying tumor-reactive TCRs across different tumor entities and therefore can facilitate and accelerate personalized T cell therapies by prioritizing relevant TCRs.

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Standard of care plus individualized multimodal immunotherapy (IMI) to treat H3.3G34 Diffuse Midline Glioma

S. Van Gool, J. Kosmal, P. Van de Vliet, W. Stücker
IOZK, Translational Oncology, Köln, Germany

The prognosis of patients with the extremely rare H3.3G34 diffuse midline glioma is uniformly dismal with survival of <2 years. Since approval of IO-Vac® as advanced therapy medicinal product (27/05/2015), we treated two young patients with IMI in connection to the standard of care. BN (male 14y, diagnosis 01/2022) and JV (female 21y, diagnosis 12/2021) received partial resection resp. biopsy. Both underwent standard radiochemotherapy. BN received 3/6 TMZ/Lomustin maintenance chemotherapy before start IMI. JV started with 1/12 TMZ courses together with IMI. Five-day immunogenic cell death (ICD) immunotherapy (Newcastle Disease Virus bolus injections and local modulated electrohyperthermia) were

added to each maintenance chemotherapy block. After maintenance chemotherapy plus ICD immunotherapy, both received active-specific immunotherapy with two IO-Vac® vaccinations (autologous mature dendritic cells loaded with ICD-induced antigenic extracellular microvesicles). Both started with modulatory immunotherapy (4x ipilimumab/nivolumab q3w and further 6x nivolumab q4w) in combination with maintenance ICD immunotherapy. BN had a spinal relapse 19 months after initial diagnosis. He restarted with local radiotherapy and thereafter Lomustin plus Nivolumab, ultimately stopped due to arthritis. He died 24 months after diagnosis. Because of the observation of spreading disease, JV is now further treated with maintenance ICD immunotherapy using local modulated electrohyperthermia to the brain and to the upper and lower spine. She shows no evidence of progressive disease after >25 months. The addition of IMI to standard of care for young patients with H3.3G34 diffuse midline glioma might give a glimpse of hope, and further anecdotal experiences should be collected. Additional treatment of the spinal axis with modulated electrohyperthermia might be required for patients with DMG.

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Clinical experience with the concept of multiphase combined treatment for adults with GBM

S. Van Gool, J. Kosmal, P. Van de Vliet, L. Kampers, W. Stücker
IOZK, Translational Oncology, Köln, Germany

We published survival data of 50 adults with GBM treated with individualized multimodal immunotherapy (IMI) as part of a multiphase combined treatment (Van Gool et al, https://doi:org/10.1016/bs.mcb.2023.06.001). We expanded these observation data. Definition of patients for retrospective analysis: start IMI between 27/05/2015 and 31/10/2023, >18y, newly diagnosis, IDH1 wild-type, MGMT promoter-methylation status documented, survival known, no second malignancy. Forty (14 female 26 male) GBM patients were MGMT-promoter-unmethylated (unmeth), while 31 (15 female, 16 male) were MGMT-promoter-methylated (meth). Median age for both groups, 48 years (range 18-65) and 54 years (range 26-72), and Karnofsky performance index, 70 (range 50-100) and 80 (range 60-100), were equal. The distribution of patients (< complete resection, complete resection, not documented) was equal: 17/15/8 (unmeth) versus 17/11/3 (meth). Treatments were not different between both groups: in median 37 (range 0-147) sessions of modulated electrohyperthermia, in median 37 (range 5-147) bolus injections of Newcastle Disease Virus, in median 2 (range 0-6) IO-Vac® vaccines with a total number of DCs in median of 30.7x10e6 DCs (range 0-158x10e6). The median overall survival (mOS) for the total group was 27 months. The 2-year overall survival (OS) was 57.9 % and the 3-year OS 37.1 %. The mOS for unmeth patients was 22.1 month, with a 2-year OS of 42.7%. The mOS for meth patients was 37.7 months, with a 2-year OS of 75.5%. Sampling five control arms of RCTs as external control arm (Liau et al, JAMA Oncol 2023) showed for unmeth and meth patients a mOS of 14.6 resp. 21.3 months with a 2-year OS of 21% resp. 42%, which reflects the current prognosis for these patients. Our analysis confirms that the integration of IMI during and after standard of care improves the OS. Similar multiphase combined treatment strategy, including IMI, should be considered for prospective clinical trials.

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Synergistic multimodal innate and antigen-specific activation mechanisms of hematopoietic stem cell-

derived NK cells potentiate superior and sustained anti-tumor efficacy

A. van Vliet, D. Özkazanc, N. Lamers-Kok, D. de Bruijckere, M. Raimo, A.-M. Georgoudaki, A. Duru, J. Spanholtz
Glycostem Therapeutics, Oss, The Netherlands

Adoptive NK cell therapy is safe and has shown promising efficacy in hematological malignancies, but patients, especially with solid malignancies still pose a great challenge. Thus, there is a need to improve efficacy by exploiting various activation modes, increasing cell persistence and targeting, overcoming tumor immune evasion and functional inactivation, to achieve better anti-tumor responses in more patients in different indications. Using Glycostem's highly functional and versatile hematopoietic stem cell-derived NK cells, we investigated the individual role of multiple innate and antigen-specific tumor surveillance axes and identified the transcriptomic, phenotypic and functional signatures of superior NK cells, which unlike CAR-T cells, can still target and kill tumor cells in the context of antigenic escape.
Combinatorial receptor blockade revealed the contribution of multiple activating (NKG2D, DNAM-1) and natural cytotoxicity receptors, as well as a significant contribution of death receptor TRAIL to cytotoxicity across indications, like melanoma, AML and MM.
To further define the factors differentiating "excellent" and "good" killers we assessed cytotoxicity against solid and hematological tumors (CRC, leukemia, GBM and melanoma) and performed bulk and single-cell RNA-sequencing at different stages of NK differentiation. Distinct transcriptomic features identified excellent donors (4/10), as having a larger population of effector-like NK cells characterized by enrichment in cytotoxicity pathways and depletion of myeloid traits earlier on in differentiation, compared to good donors (6/10). This allowed the definition of a multi-factorial gene expression signature predictive of donor anti-tumor potential.
For further enhancement of anti-tumor responses against more challenging targets we explored antigen (Ag)-specific targeting. Preclinical in vitro explorations of antibody-dependent cellular cytotoxicity (ADCC) against $CD19^+$, $HER2^+$or $EGFR^+$ tumors in combination with monoclonal antibodies (mAb) confirmed efficacy in 2D and 3D tumor settings. Moreover, multiple tumor rechallenges showed sustained cytotoxicity over time, supporting the efficient cytotoxic load replenishment and serial killing. Also, NK cells showed more efficient cytotoxicity in combination with an innate cell engager as compared to the corresponding mAb. Genetic modification for CD19 CAR-expression on NK potentiated efficient Ag-specific cytotoxicity, degranulation, cytokine production, and preserved native cytotoxicity in 2D and 3D assays in vitro. Additionally, in vitro rechallenge assays identified a superior CAR-construct which was further confirmed in a preclinical in vivo model.
In conclusion, we highlight allogeneic NK cells as a powerful and versatile cell therapy across multiple indications due to their multimodal activation mechanisms and enhancement potential using innovative combination therapies or genetic engineering.

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Immune-parameters affecting TIL therapy during chemotherapy in patients with recurrent platinum-sensitive epithelial ovarian cancer

E. Verdegaal*[1], M. Welters[1], M. IJsselsteijn[2], S. Santegoets[1], R. van der Breggen[2], L. de Bruin[1], C. van der Minne[1], I. Roozen[3], D. Ruano[2], P. Meij[4], N. de Miranda[2], J. Kroep*[3], S. van der Burg*[1]

[1]Leiden University Medical Center, Medical Oncology, Oncode Institute, Leiden, The Netherlands, [2]Leiden University Medical Center, Pathology, Leiden, The Netherlands, [3]Leiden University Medical Center, Medical Oncology, Leiden, The Netherlands, [4]Leiden University Medical Center, Center for Cell and Gene Therapy, Leiden, The Netherlands

*Contributed equally

The efficacy of immunotherapy as treatment for epithelial ovarian cancer (EOC) is moderate, possibly due to the tumor's immune-suppressive microenvironment. We hypothesized that adoptive cell therapy with autologous Tumor-Infiltrating Lymphocytes (TIL) could be effective if immunosuppression by myeloid-derived suppressor cells and M2 macrophages is reduced. Platinum-based chemotherapy can mitigate this immunosuppression by normalizing the myeloid cell counts, offering a potential window for T cell-based immunotherapy.
We conducted a phase I/II trial (NCT04072263) in recurrent platinum-sensitive EOC patients. TIL were administered during platinum-based chemotherapy combined with or without interferon-α (IFNa) as conditioning and TIL support. Feasibility, safety, clinical response and immune modulatory effects were assessed.
Sixteen patients were enrolled. Treatment during chemotherapy without IFNa was safe, but the combination with IFNa increased toxicity. Platinum-based chemotherapy reduced myeloid cell numbers and IL-6 plasma levels, confirming its immunosuppression-alleviating effect, while lymphocytes and their function were preserved. Of 14 patients completing treatment, 86% showed objective responses (RECIST 1.1), with two patients showing prolonged progression-free survival exceeding previous platinum-free intervals, indicating sustained immune suppression alleviation.
To unravel the underlying mechanism of action we determine the immune(suppressive) gene-signature in the tumor tissue and evaluated its impact on the TCR-repertoire as well as the phenotype and function of expanded TIL used for infusion. In addition, the spatial interaction of different immune cell populations and mechanisms of immune evasion are studied. Preliminary results will be presented in the context of clinical outcome.
We conclude that the treatment was safe when given without IFNa and that the observed reduction in immunosuppression and prolonged progression-free survival for some patients justify further exploration of timed TIL infusions during platinum-based chemotherapy, possibly with IL-2 support, as a novel treatment option for EOC.

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Tafasitamab-retargeted Cytokine-Induced Killer (CIK) cells as a feasible alternative to CD19-CAR T lymphocytes

E. Vigolo[1], R. Sommaggio[1,2], G. D'Accardio[2], A. Rosato[1,2], E. Cappuzzello[1]
[1]Veneto Institute of Oncology - IOV IRCCS, Padua, Italy, [2]University of Padua, Padua, Italy

Patients affected by aggressive B-cell malignancies who are resistant to chemo-immunotherapy have an extremely poor prognosis and limited therapeutic options. The development of new feasible therapeutic approaches, which also need to be both effective and safe, is an urgent need.
Cytokine Induced Killer (CIK) cells are a very interesting population of effector cells for the development of

new adoptive immunotherapy approaches. In fact, thanks to their expression of the CD16 receptor (FcγRIIIa), they can be made antigen-specific by simple combination with monoclonal antibodies, resulting at the same time in an increase in their intrinsic anti-tumor activity through antibody-dependent cell-mediated cytotoxicity (ADCC).
In this regard, we investigated the combination of CIK cells with the anti-CD19 monoclonal antibody tafasitamab, an antibody approved for the treatment of diffuse large B-cell lymphoma (DLBCL), comparing this approach with lymphocytes transduced with a Chimeric Antigen Receptor (CAR) specific for CD19 (CD19-CAR T cells). CIK cells were expanded from healthy donors and stimulated with rhIFN-γ, and after 24 hours of incubation, with anti-CD3 mAb and rhIL-2. Lymphocytes from the same donors were activated for 48 hours on anti-CD3 and anti-CD28-coated plates and further stimulated by adding hIL-7 and hIL-15, to be subsequently transduced with CD19-CAR viral vectors. The ability of tafa to enhance CIK cell-mediated ADCC was assessed using various CD19+ B-cell lines (Raji, Granta-519, RCK-8, SU-DHL-4, EHEB and Nalm-6 target cells), demonstrating a comparable efficacy to CD19-CAR T cells. Moreover, preliminary data have demonstrated a lower cytokine release from CIK cells compared to CAR T cells, which may be related to a lower risk of developing cytokine release syndrome (CRS) in patients. To assess the in vivo therapeutic efficacy, NSG mice were injected i.v. with luciferase-expressing Raji or Granta-519 cells, were divided into experimental groups according to the treatment (untreated, CIK+tafa, CD19 CAR-T cells) and were monitored for tumor growth and survival. In both models of disseminated disease, CIK+tafa therapy demonstrated a remarkable therapeutic activity, which was more efficient than CD19-CAR T cells in limiting tumor growth and prolonging the survival of treated mice.
Overall, CIK+tafasitamab therapy has proven to be a promising therapeutic approach for the treatment of B-cell malignancies. Furthermore, this strategy would allow obtaining a very high number of cytotoxic cells, without requiring any genetic manipulation, thus reducing costs and risks related to CAR T cell therapy. This approach could be rapidly translated into clinical practice, providing an effective therapeutic alternative for those patients who are not eligible for CAR-T therapy and do not have any further treatment perspective.

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Tumor-specificity validation of T cell receptors identified in single cell interaction experiments utilizing nanofluidics and opto-electropositioning

T. R. Wagner[1,2], B. Schönfelder[1], F. Werner[1], N. Kehl[1,3], S. Steiger[1], T. Boschert[1], M. Kilian[1,3], P. Schmidt[1,4], W. Osen[1], K. Rippe[1], H. Goldschmidt[2,4], M. S. Raab[1,2], M. Platten[1,4,5,6,7], M. J. Friedrich[8], S. B. Eichmüller[1]
[1]German Cancer Research Center, Heidelberg, Germany, [2]Heidelberg University Hospital, Heidelberg, Germany, [3]Mannheim Center for Translational Neuroscience, Mannheim, Germany, [4]National Center for Tumor Diseases (NCT), Heidelberg, Germany, [5]Mannheim Center for Translational Neuroscience, Mannheim, Germany, Mannheim, Germany, [6]Helmholtz Institute of Translational Oncology (HI-TRON), Mainz, Germany, [7]DKFZ Hector Cancer Institute at the University Medical Center Mannheim, Mannheim, Germany, [8]Broad Institute, Cambridge, United States of America

Cancer immunotherapy approaches such as adoptive transfer of chimeric antigen receptor (CAR) T cells or tumor-infiltrating lymphocytes have yielded promising results in hematological malignancies. Despite inducing deep responses in multiple myeloma, both antigen escape and limited persistence lead to CAR-T

cell therapy resistance. Since multiple myeloma remains an incurable malignancy, novel therapeutic approaches are urgently needed. Adoptive transfer of T cells expressing an engineered T cell receptor (TCR-T cells) represent a promising therapeutic alternative. In contrast to CAR-T cells, TCR-T cells can additionally target intracellular antigens, thereby expanding the range of potential immunotherapeutic targets. Furthermore, TCR-T cell therapy may lead to prolonged persistence in vivo and could mediate sustained anti-tumor effects.
In our project, we identified TCRs specifically targeting autologous myeloma cells. Myeloma-reactive T cells were identified applying nanofluidics and opto-electropositioning using the Bruker Cellular Analysis Lightning® platform. This enabled simultaneous functional analysis of up to 1500 individual T cell/target cell interactions on a chip. Myeloma-reactive T cells were discovered based on the secretion of cytokines (IFNγ, IL-2, TNFα) and surface expression of 4-1BB (CD137). Among 15 myeloma patients tested, reactive T cells demonstrating various cytokine secretion patterns and 4-1BB expression were detected (on average 1.2% of T cells screened). Individual myeloma-reactive T cells were isolated and their respective TCR was sequenced. Additionally, single-cell RNA sequencing was conducted for all 15 patients. By mapping TCR sequences of exported T cells to the single-cell RNA sequencing data, a gene expression signature of myeloma-reactive T cells was established. Genomic TCR sequences were in vitro transcribed and electroporated into autologous CD8+ T cells, followed by co-culture with myeloma cells or PBMCs as negative control. Myeloma-reactivity of transgenic T cells was determined for three patients (n = 11 TCRs) by flow cytometric measurement of T cell activation markers CD69 and 4-1BB. Through immuno-precipitation of MHC I molecules and mass spectrometry of the eluted peptides, we identified T cell responses against both shared and private cancer antigens for 12 patients.
To summarize, we established a pipeline for the identification and functional validation of myeloma-reactive T cells. Together with the evidence of expressed shared and private myeloma derived antigens, we envision this approach to facilitate patient-individualized T cell therapy for newly diagnosed multiple myeloma patients.

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Improving efficacy of tumor-agnostic TCRmimic CAR-T cells through drug modulated target presentation

L. Wang[1], M. Zheng[1], B. Zaghla[1], S. Mecklenbräuker[1], O. Popp[2], P. Mertins[2], M. Klatt[1,3,4]
[1]Charite - University Medicine Berlin, Department of Hematology, Oncology and Cancer Immunology, Berlin, Germany, [2]Max Delbrück Center for Molecular Medicine, Proteomics Platform, Berlin, Germany, [3]Berlin Institute of Health, Clinician Scientist Program, Berlin, Germany, [4]German Center for Translational Cancer Research (DKTK), Heidelberg, Germany

TCRmimic CAR-T cells provide the unique opportunity to combine the efficacy of a CAR-T cell with the specificity and wide target selection of TCRmimic antibodies. Recently, we developed a TCRmimic CAR-T cell which targets an HLA-A*02 ligand derived from the protein NDC80, which is essential for mitosis and thus highly expressed in many cancer types. This TCRmimic CAR-T cell demonstrated high efficacy in vitro and in mouse models without toxicities towards healthy resting or proliferating cells. However, given the lower abundance of distinct peptide:HLA complexes compared to conventional CAR-T cell targets their efficacy might still be limited.

Therefore, we wanted to improve the presentation of the targeted peptide:HLA to optimize TCRmimic CAR-T cell killing. To identify drugs that increase the expression or degradation of the peptide target's source protein, we acknowledged the cell cycle dependent expression of NDC80 and that disrupting physical interactions of proteins can increase their degradation. We selected five different drugs which either lead to cell cycle arrest (docetaxel and vincristine) or disrupt the direct interaction of the NDC80 protein and other interaction partners (e.g., a NDC80 inhibitor, a TTK inhibitor and a pan-aurora kinase inhibitor). We determined non-toxic doses of these drugs towards tumor cells and CAR T cells and analyzed the modulation of the peptide:HLA target presentation through isolation of the presented target peptide and subsequent quantitative mass spectrometry using spiked-in external peptide standards.

Docetaxel, vincristine and the TTK inhibitor increased the numbers of presented target peptide:HLA relatively up to 2-fold and to an absolute averaged number of 3,000 molecules per cell. This led to a substantial increase of the CAR T cells' in vitro killing capacity especially for the docetaxel treatment although this did not correlate with the strongest increase in peptide:HLA upregulation. The reason for this discrepancy was revealed through cell cycle dependent analysis of peptide presentation which showed that after M-phase many cells present the target with up to 4,500 molecules per cell and some presented almost no target complexes which might lead to a more robust killing of the target cells in the docetaxel setting. Other quantitative immunopeptidome experiments to investigate the cell cycle dependent presentation of our target and in vivo validation models are still ongoing.

Overall, this study highlighted that even unspecific drugs such as docetaxel can lead to an increase in peptide:HLA presentation of a specific target if the underlying biology of the target protein is taken into account. This approach opens the path to new combination therapies which prime target cells through a low dose, non-toxic pharmacological drug treatment to enhance TCRmimic CAR T cell killing.

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Off-the-Shelf Allogenic T-Cell Combined Therapy for Solid Tumors

M. Warzycha[1,2], O. Suska[1], J. Misiak[1], P. Ciołek[1], T. Kolanowski[1,2], N. Rozwadowska[1,2]
[1]FamiCordTX, Warsaw, Poland, [2]Institute of Human Genetics, Polish Academy of Sciences, Poznan, Poland

Chimeric antigen receptor (CAR) based T cell therapies, which involve autologous T cells as the starting material, have revolutionized clinical practice in the treatment of hematologic cancer. Despite the significant progress achieved with autologous CAR-T cell therapy, this approach faces several challenges. The personalized manufacturing process for each patient makes it expensive and time-consuming. Umbilical cord blood (CB) is an attractive source of cells for CAR-T production since it is readily available from cord blood banks and has lower requirement for Human Leukocyte Antigen (HLA) matching. Besides, the success observed with CAR-T cell treatment of leukemias has not been replicated in solid tumors due to challenges posed by the hostile tumor microenvironment (TME) and tumor heterogeneity. To overcome these obstacles, researchers are exploring strategies to enhance CAR-T effectiveness through combinatorial therapies with immune checkpoint inhibitors, or targeting immunosuppressive cells within TME. The present work proposes combining allogenic CAR-T therapy with strategies tailored for solid tumors. We designed a readily available and "off-the-shelf" source of CAR-T cells from CB targeting solid tumor antigen

coupled with an in situ gene therapy modulating TME. In this study, we have compared the effectiveness of two CAR-T cell products: allogeneic HER2 CAR-T cells produced from CB (CB-CAR) and autologous HER2 CAR-T cells produced from peripheral blood mononuclear cells (PBMC-CAR). The effector functions of both products were investigated on two HER2-positive cancer cell lines: A549 (lung cancer cell line) and HepG2 (liver hepatocellular cancer cells). For a combined approach, CAR-T addition was preceded by the delivery of a recombinant Adeno-Associated Virus (rAAV) vector with a transgene encoding a proinflammatory cytokine interleukin 12 (IL-12), to stimulate CAR-T cell effector function. We employed a two parameter assessment of the initial and post exposure CAR-T cell populations which included cytotoxicity evaluation based on perforin and granzymes levels, and measurement of T cell exhaustion by analysis of expression of immune checkpoint receptors such as PD-1 and LAG-3. The collected data confirmed the functionality of the CB-CAR and PBMC-CAR products. The rAAV-mediated IL-12 transgene expression was measured on mRNA and protein levels. Our findings demonstrate that CB-CAR can effectively target and destroy cancer cells and can be combined with gene therapy designed to reshape tumor microenvironment resulting in improved CAR-T performance.

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HLA-independent T-cell response against PD-L1 in a melanoma patient

C. Wölfel
Universitätsmedizin-Mainz, Onkology-Hematology, Mainz, Germany

Catherine Wölfel[1], Martina Fatho[1], Alice Bury[2], Michael Volkmar[2], Enes Huduti[2], Annette Paschen[3], Matthias Theobald[1], Thomas Wölfel[1]
1 – Internal Medicine III, University Cancer Center (UCT), Research Center for Immunotherapy (FZI), University Medical Center (UMC) of the Johannes Gutenberg University and German Cancer Consortium (DKTK), Partner Site Frankfurt/Mainz, Mainz;
2 – Helmholtz-Institute for Translational Oncology (HI-TRON) Mainz, Mainz;
3 – Dermatology, University Hospital of the Duisburg/Essen University and German Cancer Consortium (DKTK), Partner Site Essen/Düsseldorf, Essen

In melanoma patient Ma-Mel-86 with HLA I-negative disease the anti-tumor T-cell repertoire was over time increasingly dominated by **HLA-independent T cells**. We identified six distinct HLA-independent T-cell clonotypes, with two targeting tyrosinase-related protein 2 (TRP2) and one targeting the GM-CSF receptor-alpha chain (CSF2RA). Notably, two clonotypes, one against CSF2RA and another against an unidentified target antigen, displayed pronounced prevalence within the HLA-independent sub-repertoire in peripheral blood. The target antigens for three clonotypes (CTL clones **11C/25**, **11C/34**, and **5C/169**) were unknown. Their T-cell receptors (TCRs) were cloned, chimerized with murine constant TCR regions (cTCRs) and finally provided in bicistronic retroviral constructs for antigen identification.
Buffy coat-derived T cells were transduced with cTCR**11C/34** after knockout of endogenous TCR genes. These cTCR-T_KO^{TRAC,TRBC} cells were used for cDNA library expression screening, which led to the identification of antigen-encoding cDNA clones. They were found to encode the programmed death-ligand 1 (PD-L1; alias: CD274). Also cTCRs 11C/25 and 5C/169 recognized PD-L1.
In further analyses we found that anti-PD-L1 cTCR-T cells were able to mediate lysis of target cells. Thereby both CD8+ and CD4+ cTCR-T cells were functional. None of the three cTCRs cross-reacted with murine PD-

L1. Recognition by 11C/34 and 11C/25 cTCR-T cells was inhibited by Atezolizumab and Durvalumab, depended on N-glycosylation, and required the presence of both the IgV- and the IgC-like domains of PD-L1. Conversely, 5C/169 cTCR-Tcells were not inhibited by Atezolizumab and Durvalumab, did not depend on N-glycosylation of PD-L1, and only required the IgC-like domain for recognition. From these observations we conclude that the patient's anti-PD-L1 cTCRs were found to recognize at least two distinct PD-L1 epitopes.
These findings shed light on the emergence of an HLA-independent anti-T-cell repertoire in HLA-negative metastatic disease and offer new options for immunotherapy.

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Arming CAR T cells with two immune modulatory molecules to improve therapy of solid tumors

A. Wurzel, S. Stamova, A. Riediger, B. Ott-Rötzer, A. Glander, P. Beckhove
Leibniz-Institute for Immunotherapy, Division of Interventional Immunology, Regensburg, Germany

Chimeric antigen receptor (CAR) T cell therapy has been proven effective in treatment of several hematological malignancies including e.g. lymphoma. Solid tumor entities however still resist this form of adoptive cell therapy by generating a complex immunosuppressive tumor microenvironment (TME), consequently inhibiting T cell infiltration into the tumor tissue and cytotoxic effector functions. Even though therapeutic approaches targeting these issues are used in the clinic, their systemic application often leads to significant adverse effects.
To this end, we developed 4th generation CAR T cells containing an inducible expression cassette (TRUCKs) with transgenes targeting immunosuppression as well as limited immune infiltration. Expression of the transgenes is induced by T cell activation resulting from T cell-tumor cell contact in situ in the TME, ensuring the local application of therapeutic agents. Our system is based on a bicistronic transgene encoding a single-chain variable fragment (scFv) blocking a recently identified immune checkpoint pathway. Simultaneous expression of the chemokine ligand CXCL9 generates a chemotaxis-inducing stimulus in the TME to recruit additional immune cells. High intratumoral concentrations of CXCL9 have been shown to correlate with improved T cell infiltration and prolonged patient survival.
Both molecules were characterized and tested for functionality before incorporation into a TRUCK system. Surface plasmon resonance and FACS data validate binding of the scFv to its target antigen. Sandwich ELISAs show the capability to block interaction of the respective immune checkpoint molecules. In vitro cytotoxicity assays demonstrate improved T cell functionality upon scFv induced immune checkpoint blockade. Functionality of transgenic CXCL9 was validated in Boyden chamber migration experiments. TRUCKs were generated, containing inducible expression cassettes for both molecules. RT-qPCR analyses show that both transgenes are expressed in TRUCKs specifically upon antigen stimulation of the CAR. In addition, ELISA revealed that both molecules are released into the culture supernatant. In vitro co-culture assays of CAR T cells with target cell lines demonstrate increased cytotoxic capacity conveyed by expression of the scFv. In vivo experiments showcase an elevated therapeutic potential. TRUCKs secreting scFv and CXCL9 exhibit improved control of tumor outgrowth in a xenograft mouse model compared to CAR T cells. Tumor progression was inhibited and survival prolonged significantly.
Taken together, our results suggest that inducible secretion of a checkpoint inhibitor and a T cell targeted chemokine contributes to tumor eradication and improved immune control of an established solid tumor

model. This proof-of-principle study provides perspectives for novel immune therapeutics applicable in safer and more efficient solid tumor therapy.

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Abstract has been withdrawn

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Molecular mechanisms underlying the modulation of T-cell proliferation and cytotoxicity by immobilized CCL21 and ICAM1

S. Yado[1], R. Zoabi[1], B. Dassa[2], S. Reich-Zeliger[3], B. Geiger[1]
[1]The Weizmann Institute of Science, Department of Immunology and Regenerative Biology, Rehovot, Israel, [2]The Weizmann Institute of Science, Bioinformatics Unit, Department of Life Sciences Core Facilities, Rehovot, Israel, [3]The Weizmann Institute of Science, Department of Systems Immunology, Rehovot, Israel

Adoptive cancer immunotherapy, using engineered T-cells, expressing chimeric antigen receptor (CARs) or autologous tumor infiltrating lymphocytes (TILs) became, in recent years, a major therapeutic approach for diverse types of cancer. However, despite the transformative potential of adoptive cancer immunotherapy, this field still faces a major challenge manifested by the complex interplay between the proliferation rate and cytotoxic capacity of effector $CD8^+$ T cells. We performed integrated analysis of specific differentiation markers via flow cytometry, together with gene expression profiling to explore the molecular mechanisms through which a “synthetic immune niche” (SIN), composed of immobilized CCL21 and ICAM1, modulates the interplay between the proliferation and cytotoxic potency of effector $CD8^+$ T cells. On day 3, the transcriptomic effect induced by the SIN was largely similar for both DC/OVA and anti CD3/CD28-activated cells. Cell proliferation increased and the cells exhibited high killing capacity. On day 4 and on, the proliferation/cytotoxicity phenotypes were radically “activation-specific”; The DC/OVA-activated cells lost their cytotoxic activity, which, in turn, was rescued by the SIN treatment. Upon longer incubation, the cytotoxic activity further declined, and on day7, could not be rescued by the SIN. SIN stimulation following activation with antiCD3/CD28 beads resulted in a highly proliferative phenotype with low cytotoxicity, yet the cells regained killing activity on day 7. Potential molecular regulations of the SIN effects were identified, based on transcriptomic and multispectral imaging profiling. These data indicate that cell proliferation and cytotoxicity are negatively correlated, and the interplay between them is differentially regulated by the mode of initial activation. The SIN stimulation greatly enhanced the cell expansion, following both activation modes, while maintaining high cytotoxic potency, suggesting that it could reinforce adoptive cancer immunotherapy.

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DCOne-derived dendritic cells promote robust in vitro expansion of memory NK cells with strong tumor cell cytotoxicity and high persistence.

H. Zuo, Z. Wang, J. Naik, J. Kaspers, A. Karlsson-Parra, S. K. Singh
Mendus AB, Stockholm, Sweden

Natural killer (NK) cell therapies have shown promising results in hematological malignancies. Challenges such as poor infiltration into solid tumors and highly restricted persistence in vivo however remain. NKG2C$^+$CD57$^+$ memory NK cells are induced by CMV infection and characterized by improved metabolic fitness and long-term in vivo persistence. Notably, the presence of these memory NK cells is associated with a lower rate of relapse in hematological malignancies, making them attractive candidates for adoptive cell therapy.

In this study we explored the potential of using mature dendritic cells derived from the leukemic cell line DCOne (DCOne mDCs) for the ex vivo expansion of memory NK cells. For this, NK cells isolated from CMV-seropositive donors were co-cultured with irradiated DCOne mDCs in the presence of cytokines for 2 weeks. NK cell expansion, viability and phenotype were monitored on day 14. In vitro tumor cell killing, and NK cell persistence was evaluated in a long-term killing/persistence assay in which expanded NK cells were co-cultured with Raji tumor cells for 3 days.

DCOne mDCs induced selective and potent expansion of NKG2C$^+$CD57$^+$ memory NK cell subpopulation (>100-fold median expansion) compared with NK cells expanded without DCOne mDCs. Subsequent short-term interaction with Raji cells showed that the expanded NK cells were cytotoxic and proinflammatory in nature as assessed by expression of CD107a and IFN-γ, TNF-α, and GM-CSF production, respectively. Additionally, opsonization of tumor target cells with anti-tumor IgG1 antibodies substantially increased IFN-γ production, whereas CD107a expression remained unaffected. In the long-term tumor killing/NK cell persistence assay the NK cells expanded with DCOne mDCs showed high IFN-γ expression, very strong tumor cell cytotoxicity and high persistence/survival that was superior compared to NK cells expanded with the other expansion protocols.

Taken together, the presented data indicate that in vitro expansion of NK cells from CMV$^+$ donors with DCOne mDCs leads to a highly expanded NK cell product with increased frequency of NKG2C$^+$CD57$^+$ memory NK cells and with desirable tumor cell cytotoxicity, IFN-γ production, and persistence in vitro. Such expanded NK cells could be used for adoptive immunotherapy, including combinations with tumor-targeting antibodies or NK cell engagers, in different hematological malignancies.

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Targeting a shared neoepitope derived from non-canonical translation of c-Myc oncogene in cancer cells

E. Baulu[1], A. Bolon[1], A. Merienne[2], F. Raimundo[3], J. Martin[4], T. Richard[5], J. Grandsire[3], L. Tonon[6], C. Dubois[5], N. Gervois[2], N. Labarrière[2], Y. Estornes[5], Q. Wang[7], J. Valladeau-Guilemond[1], V. Marcel[1], N. Chuvin[5], J.-J. Diaz[1], S. Depil[1,3,5,8]

[1]Centre de Recherche en Cancérologie de Lyon (CRCL), UMR INSERM U1052 CNRS 5286, Lyon, France, [2]Nantes Université, Univ Angers, INSERM, CNRS, Immunology and New Concepts in ImmunoTherapy, INCIT, UMR 1302/EMR6001, Nantes, France, [3]Centre Léon Bérard, Lyon, France, [4]Univ Lyon, Université Claude Bernard Lyon 1, CNRS, UMR 5086 MMSB, Lyon, France, [5]Ervimmune, Centre Léon Berard, Lyon, France, [6]Fondation Synergie Lyon Cancer, Plateforme de bio-informatique Gilles Thomas, Centre Léon Bérard, Lyon, France, [7]Complete Omics, Baltimore, United States of America, [8]Université Claude Bernard Lyon 1, Lyon, France

The development of personalized immunotherapeutic approaches based on mutation-associated neoepitopes remains complex and poorly adapted to large numbers of tumors with a low mutational burden. There is therefore a major need to identify new families of neoantigens shared across patients. Cancer cells are exposed to different stresses and rely on alternate modes of translation for protein synthesis and cell growth. In this context, the chemical modifications of tumor ribosomes promote IRES-dependent translation of a subset of mRNA encoding survival factors and oncogenes, while inducing a decrease in translational fidelity. We hypothesized that these translation modifications in tumor cells could generate shared neoepitopes derived from non-canonical translation of IRES-associated oncogenes, such as c-Myc.

We set up a bioinformatic prediction pipeline to identify potential HLA-A2-restricted neoepitopes derived from alternative open-reading frames of c-Myc and selected 2 candidates with evidence of specific translation in tumors in proteomic databases. Targeted immunopeptidomics confirmed the presence of one of these epitopes, PR3, on HLA molecules on the surface of 8/10 tested HLA-A2+ tumor cell lines but not on HLA-A2+ normal primary cells. RNAseq analysis using the nanopore technology did not show any transcript containing PR3 sequence other than c-Myc (out-of-frame), confirming the translational origin of PR3. Using a bicistronic reporter assay, we showed that a +1 ribosomal frameshifting of c-Myc occurring during the oncogenic transformation leads to the generation of PR3 in tumor cells. In addition, PR3-specific T cells were found among tumor infiltrating lymphocytes from 6/22 (27%) colon cancer samples, suggesting that PR3 is presented and immunogenic in cancer patients. PR3-specific CD8+ T cell clones of high functional avidity were generated by in vitro priming of T cells by dendritic cells obtained from HLA-A2+ healthy donors. We showed that PR3-specific CD8+ T cells recognize and kill specifically tumor cells endogenously expressing PR3. To assess the therapeutic potential of targeting the PR3 neoepitope, we genetically engineered T cells to express a PR3-specific TCR (TCR-T cells). We confirmed that these TCR-T cells are cytotoxic against tumor cells while sparing a panel of HLA-A2+ normal primary cells from critical tissues. We also demonstrated the in vivo antitumor activity of these PR3-specific TCR-T cells using the immuno-AVI-cellDX™ model and NSG mice transplanted with tumor cells expressing endogenous levels of PR3.

PR3 is a shared neoepitope derived from a +1 ribosomal frameshift of c-Myc occurring selectively in cancer cells. PR3 is immunogenic and induces specific $CD8^+$ T cells able to kill tumor cells in vitro and in vivo while sparing normal cells. These results provide a proof of concept for developing T-cell based immunotherapies

targeting this new family of shared neoepitopes.

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Direct Detection of Splicing Inhibitor-induced Neoantigens by Quantitative Immunopeptidomics

M. Bernhardt[1], F. Erhard[2], B. Schilling[3], A. Schlosser[4]
[1]Uniklinikum Würzburg, Rudolf-Virchow-Center, Würzburg, Germany, [2]Universität Regensburg, Fakultät für Informatik und Data Science, Regensburg, Germany, [3]Uniklinikum Würzburg, Klinik und Poliklinik für Dermatologie, Venerologie und Allergologie, Würzburg, Germany, [4]Universität Würzburg, Rudolf-Virchow-Center, Würzburg, Germany

Oncogenesis is recurrently accompanied by dysregulated RNA splicing events. Tumor-specific splicing could be of clinical relevance, not only because splicing modulating agents are currently evaluated in clinical trials as promising anti-cancer drugs, but also, because tumor-specific splicing can contribute to the generation of immunogenic T cell epitopes. Accumulating evidence suggest that neoantigens derived from aberrant splicing events, such as intron retentions or generation of new exon-exon-junctions, might represent promising targets for cancer immunotherapy.
So far, the identification of these neoantigens is mainly based on RNA-seq analyses and MHC epitope prediction. However, mass spectrometry-based approaches are more straightforward for target identification purposes. In this study, we used quantitative mass spectrometric analyses to evaluate the generation of neoantigens upon pharmacological splicing modulation.
We therefore combined a quantitative pSILAC immunopeptidomics approach with de novo sequencing-based MHC-peptide identification by Peptide-PRISM. We recently introduced this pSILAC approach for the quantitative analysis of immunopeptidomic changes upon pharmacological intervention.
The manipulation of pre-mRNA splicing was induced in three human cancer cell lines by FR901464, Indisulam and the GSK3β-inhibitor AR-A014418. All experiments were conducted using pSILAC followed by immunoaffinity purification and quantitative mass spectrometry-based analysis of MHC-I peptides.
The formation of dozens of neoantigens was induced upon splicing-inhibitor treatment. Most of these peptides derived from intron retentions, which led to an extension of the coding sequence (CDS) into intronic regions. FR901464, a spliceostatin, induced the highest number of neoantigens. Re-analyzing a large number of tumor and benign immunopeptidomes revealed that some of the splicing inhibitor-induced neoantigens are recurrently presented on tumor, but not on healthy cells.
In summary, our findings confirm that splicing alterations can enhance neoantigen presentation. We identified tumor-exclusive peptide candidates as potential therapeutic targets, that can mechanistically be attributed to altered splicing. Our results can therefore support rational and comprehensive target selection in tumor entities, that predominantly harbor splicing defects, such as hematological malignancies, uveal melanomas and lung adenocarcinomas.

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Human endogenous retrovirus-derived epitopes represent new targets for T-cell based immunotherapies in ovarian cancer

P. Bonaventura[1], F. Raimundo[1,2], Y. Estornes[1], A. Page[2,3], V. Mutez[1], A. Valente[1], Q. Wang[4], J. Valladeau[2,3], S.

Depil[1,2,3,5]

[1]ErVimmune, Lyon, France, [2]Cancer Research Center of Lyon (CRCL), UMR INSERM U1052 CNRS 5286, Lyon, France, [3]Centre Léon Bérard, Lyon, France, [4]Complete Omics, Baltimore, United States of America, [5]Université Claude Bernard Lyon 1, Lyon, France

Ovarian cancer represents the most lethal gynecological cancer with poor results of checkpoint inhibitors. New approaches are thus warranted to induce efficient anti-tumor immune responses. Human endogenous retroviruses (HERVs) are aberrantly expressed by tumor cells and may represent a source of shared T cell epitopes for cancer immunotherapy regardless of the tumor mutational burden.
We developed a transcriptomic analysis based on RNAseq data to quantify the expression of selected HERV-K/HML-2-derived HLA-A2 epitopes. The sum of the epitope-containing HERV transcripts was significantly higher in ovarian cancer compared to normal tissues. We then quantified by immunohistochemistry the presence of HML2 GAG antigen in tissue microarrays of ovarian cancer samples. We observed protein expression in 23 of the 43 tested samples (53%) with high expression (3+) in 7/43 (16 %). No GAG expression was found in normal ovarian tissue samples. Targeted immunopeptidomics analysis showed that these epitopes are presented on HLA molecules on the surface of tumor cell lines but not on normal primary cells from critical tissues.
We next assessed the presence of $CD8^+$ T cells specific for these epitopes in tumor infiltrating lymphocytes (TILs) from ovarian tumors. Epitope-specific T cells were detected in 10 of the 13 (77%) analysed samples, suggesting the immunogenicity of these epitopes in cancer patients. We generated specific $CD8^+$ T cell clones of high functional avidity (IFN-γ produced at low peptide concentration, $<10^{-10}$M) using cells form HLA-$A2^+$healthy donors. In vitro, these HERV-specific T cells specifically killed ovarian cancer cells in an HLA class I-restricted manner while sparing normal HLA-$A2^+$ primary cells derived from critical tissues.
We then evaluated the in vivo anti-tumor efficacy of HERV-specific $CD8^+$ T cells. We used an avian embryo model that allows to establish 3D tumors in a few days in tissues that are homologous to those in which tumors emerge in the patient. Epitope-specific $CD8^+$ T cells co-transplanted with ovarian cancer cells exhibited a strong anti-tumoral activity, inducing a highly significant decrease in tumor volume in comparison with control groups, in which tumor cells were transplanted alone or with unspecific $CD8^+$ T cells (volume reduced of 62%, $P<0.001$).
Overall, these results show that the selected HML2-derived epitopes are specifically expressed in ovarian cancer cells compared to normal cells and that $CD8^+$ T cells specific to these epitopes selectively kill ovarian cancer cells, both in vitro and in vivo, while sparing normal cells. This provides the preclinical rationale for developing T-cell based approaches against these new targets in ovarian cancer.

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Engineering of T-cell retargeting antibodies for intratumoral delivery via oncolytic adenoviruses

M. Boos[1], S. Sawall[1], O. Seifert[2], I. Hofmann[3], R. E. Kontermann[2], G. Ungerechts[1,4], D. M. Nettelbeck[1]

[1]German Cancer Research Center (DKFZ), Clinical Cooperation Unit Virotherapy, Heidelberg, Germany, [2]Institute of Cell Biology and Immunology, University of Stuttgart, Stuttgart, Germany, [3]German Cancer Research Center (DKFZ), Core Facility Antibodies, Heidelberg, Germany, [4]Department of Medical Oncology, National Center for Tumor Diseases (NCT) and Heidelberg University Hospital, Heidelberg, Germany

This study aims to overcome immune escape of tumor cells during immunotherapy due to tumor

heterogeneity by pursuing a multi-targeting approach facilitated by expression of bi- and/or trispecific T-cell engagers encoded by oncolytic Adenoviruses (oAds). Resistance to conventional therapy, toxicity of conventional therapeutic agents and tumor heterogeneity call for novel approaches in cancer therapy. Treatment with oncolytic viruses, viruses replicating specifically or preferentially in tumor cells, leading to tumor cell lysis and release of new infectious particles and optionally encoded biotherapeutics, has proven to be well tolerated, but often lacking efficacy. Here, multiple T-cell engagers, i.e. bispecific T-cell engagers (BiTEs), single-chain Diabodies (scDbs) and Diabody-based trispecifc T-cell engagers (DiTriTEs) were engineered and characterized for insertion into the oAd genome. Initially, recombinant antibody constructs directed at four different targets, i.e. EGFR, HER2, cMET and IL13Rα2, associated with a variety of solid tumor entities, were produced in HEK293 suspension cultures. Qualitative comparison of BiTEs and scDbs was performed for binding analysis via flow cytometry and for antibody-mediated tumor cell killing in co-cultures of PBMCs with target and non-target cancer cells. Performance of scDbs directed against HER2 and cMET was comparable to the corresponding BiTEs, whereas scDbs directed against EGFR and IL13Rα2 were inferior to BiTEs. The results indicated that a fusion of the scDb and BiTE format to the novel DiTriTE format could be promising for selected constructs, on the one hand allowing T-cell retargeting to two distinct tumor-associated antigens with a single molecule and on the other hand considering the limited genomic capacity of oAds for transgene insertion. Based on the bispecific antibody data, DiTriTEs scDb-HER2-scFv-EGFR, scDb-HER2-scFv-IL13Rα2 and scDb-cMET-scFv-EGFR were designed, produced and characterized. Analysis revealed only scDb-cMET-scFv-EGFR exhibiting effective induction of cytotoxicity on single positive cells and superior efficacy on double-positive cells. Pilot experiments have shown its potential for efficacy in mixed cultures of single-target tumor cells. Therefore, scDb-cMET-scFv-EGFR, scDb-cMET and BiTE-EGFR were chosen for insertion into the oAd genome, the combination of the targets EGFR and cMET being relevant for e.g. colorectal cancer, non-small cell lung cancer and glioblastoma. A panel of viruses was cloned to test different expression and multi-targeting strategies. Co-targeting of EGFR and cMET via two viruses expressing one bispecific construct each, one virus co-expressing two bispecifics and one virus expressing the DiTriTE are being investigated and initial results will be presented. Taken together, novel bi- and trispecific T-cell engagers with potent in vitro anti-tumor activity could be identified and adaptation for a viro-antibody therapy approach has been started.

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Abstract has been withdrawn

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Triple immunotherapy for microsatellite-stable metastatic colorectal or pancreatic cancer: updates from the LUMINESCENCE trial

S. Grauling-Halama[1], A. Williams[2], X. Holtkotte[1], S. Roldan Pinzon[3], G. M. Haag[4], N. Hohmann[4], C. Springfeld[4], H. Starke[4], M. Suarez-Carmona[1], A. Ahmed[4], D. Jäger[4], T. Herrmann[5], N. Halama[1]

[1]HI-TRON, Cancer immunology and cancer immunotherapy, Mainz, Germany, [2]Sarah Cannon Research Insitute, London, United Kingdom, [3]National Center for Tumor Diseases Heidelberg, NCT Trial Center, Heidelberg, Germany, [4]National Center for Tumor Diseases Heidelberg, Medical Oncology, Heidelberg, Germany, [5]Westkuestenklinikum Heide, Heide, Germany

Advanced metastatic microsatellite-stable colorectal (CRC) and pancreatic ductal adenocarcinoma (PDAC) represent enormous challenges for medical care. Both entities are regarded as immunologically “cold” tumors and conventional immunotherapy has failed to show clinical benefit, especially in the presence of liver metastases.
The LUMINESCENCE trial (NCT04721301) included 50 patients with advanced metastatic microsatellite-stable colorectal or pancreatic cancer being treated with a triple immunotherapy combination, including anti-PD1 (Nivolumab 240 mg i.v. /q2w), anti-CTLA4 (Ipilimumab 1 mg/kg i.v. q6w) and CCR5-inhibition (Maraviroc 300 mg/bid). Treatment was accompanied by concomitant tissue, blood and stool analyses.
All patients were successfully treated with the triple immunotherapy combination, 25 patients with colorectal and 25 patients with pancreatic cancer. The treatment was very well tolerated, mainly showing expected adverse events typically observed with double anti-PD1 and anti-CTLA4 treatment. Immune-related adverse events (grade ≥3) were noted and included a limited number of cases of hepatitis, pneumonitis, colitis, adrenalitis and constrictive pericarditis. Statistical analyses with Cox models revealed no impact of cancer entity or lactate dehydrogenase (LDH) levels. Objective responses were noted and the resulting final overall survival (OS) and progression free survival (PFS) data is being presented. Median PFS based on RECIST was 2.8 months (95% CI 2.2 to 3.2) for CRC and 2.6 months (95% CI 1.4 to 3.3) for PDAC. Median PFS based on iRECIST was 4.7 months (95% CI 3.3 to 5.2) for CRC and 3.4 months (95% CI 1.9 to 4.7) for PDAC. Median OS is 4.9 months (95 % CI 3.3 to 5.8) for CRC and 4.0 months (95% CI 2.5 to 6.2) for PDAC.
Data from exploratory analyses of the tissue, blood and stool samples are also presented. Nanopore sequencing technology for 16S rRNA analyses of microbiota was established and allows to systematically evaluate cross-entity associations with clinical and laboratory parameters. Serial samples revealing common species and metagenomics of microbiota are currently evaluated.
The triple immunotherapy was very well tolerated and showed clinical effects, with ongoing exploratory analyses allowing for in-depth analyses of associations between different parameters.

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Kinetics of HLA Antigen Presentation in Transition from Latent to Lytic Stage of EBV

N. G.-A. Grinberg, H. Pak, F. Huber, M. Bassani-Sternberg
Centre Hospitalier Universitaire Vaudois, Oncologie, Lausanne, Vaud, Switzerland

Oncoviruses have the capacity to initiate cancer and collectively they are responsible for 12-15% of human cancers. While prophylactic vaccines are effective in reducing the risk of HPV- and HBV-associated cancers by mounting potent neutralizing antibodies, they cannot induce immunity required for prevention or elimination of tumors in previously infected patients. In contrary, T cells can target presented cancer-specific HLA bound peptides derived from viral proteins and eliminate infected cells before and after they transform. Furthermore, T cell epitopes derived from oncoviruses are clonal, driver and immunogenic and are ideal targets for mounting immune responses. However, in order to evade the adaptive host response, viruses employ diverse mechanisms, such as targeting antigen processing and presentation. This results in the downregulation of HLA-I from the cell surface, presenting a significant obstacle for identifying new immunogenic viral epitopes.

In this study we aim to characterize the antigenic landscape of

the Epstein-Barr Virus (EBV), a gamma herpes virus associated with esophageal cancer, nasopharyngeal cancer and lymphomas. In the latent stage, EBV lies dormant in epithelial and B-cells with a handful of viral proteins expressed at low level with anti-apoptotic and anti-inflammatory functions. Due to these anti-inflammatory proteins, it has long been known that few latent proteins are presented as antigens by HLA Class I and II molecules. However, in the lytic stage, virion particles are massively produced exported out of host cell via budding. To our knowledge there hasn't been an effort to map out HLA bound peptides presented in host cells in the various stages of lytic mode. By inducing virus production in EBV positive B-cell lines and performing immunopeptidomics on them, we were able to map more than 28 000 HLA-I and 9 000 HLA-II bound peptides presented at various stages in virus activation. Although viral lytic mode led to a general drop in HLA surface antigen presentation, 18 HLA-I peptides derived from key replicative EBV-proteins were detected throughout the virus activation cycle. Among others, peptides from early and late proteins such as BCRF1 and HEPA were identified. Next, transcriptomics and proteomics assays will be conducted. When combined with immunopeptidomics, these assays will shed light on antigen biogenesis in the induced cells and unveil novel EBV peptides for development of theraeutic cancer vaccines and T cell-based immunotherapies.

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Generation of a novel antibody-drug conjugate, TCX-101, targeting a tumor-associated carbohydrate antigen

F. Gueths Gomes, F. Muraca, G. Marçal Schmidt Garcia Moreira, K. Rösch, M. Bräutigam, P. Sondermann, M. Ocker
Tacalyx GmbH, Berlin, Germany

Tumor-associated carbohydrate antigens (TACAs) are oligosaccharides expressed on the surface of cancer cells, anchored on proteins or lipids, which play key roles in, e.g., cellular communication, immune evasion, apoptosis, and metastasis. Therefore, TACAs are promising targets for antibody-based cancer therapies. We have generated an antibody drug conjugate (ADC), named TCX-101, against a TACA structure. TCX-101 has improved binding affinity compared to the only commercially available reference monoclonal IgG that targets the same antigen, as shown by surface plasmon resonance (~ two orders of magnitude higher target affinity) and flow cytometry (~2-fold lower EC_{50} and ~ 5-fold higher max. median fluorescence intensity) as well as high level of specificity against glycan species related to the target, observed by glycan array. Immunohistochemistry studies on fresh-frozen tissue microarrays showed high expression of the target in various solid tumors (e.g., gastrointestinal, lung, and ovary) and only low expression in normal tissues. In vitro, TCX-101 has a ~ 4-fold faster cellular internalization and induces ~ 2-fold higher antibody-dependent cellular cytotoxicity in target-expressing MCF7 cancer cells, compared to the reference antibody against the same TACA. TCX-101, either naked or coupled with monomethyl auristatin E (MMAE) with a drug-antibody ratio (DAR) of 4, shows a 50% internalization rate of ~1.15 h in MCF7 human breast cancer cells. This ADC shows effective performance in in vitro cytotoxicity assays against murine melanoma B16 cells induced to express the target (half-maximal inhibitory concentration, IC_{50}, of 7.4 nM) with no measurable effect on WT cells, as well as in MCF-7 cells (IC_{50} of 1.97 nM) with ~ 25-fold less activity on target-knockout MCF7 cells. When tested in vivo, TCX-101 showed efficacy in the syngeneic B16 mouse melanoma model and in the cell line-derived xenograft (CDX) model of MCF-7 human breast cancer cells, showing more than 95% tumor

growth inhibition (TGI) compared to vehicle, with no signs of toxicity since no weight loss was observed in the animals, as well as no evidence of organ damage in pathological histology analysis. An ADC with humanized TCX-101, with similar binding properties (EC_{50} 6.27 nM vs. 8.13 nM) was evaluated in vitro, showing cell killing of human lung cancer (NCI-H526) cells as well as a highly resistant human breast cancer cell line, HCC-1428. Tumor-growth inhibition was also observed in a CDX mouse model of NCI-H526 cells treated with the humanized TCX-101, while in vivo studies with mice bearing HCC-1428 cells are currently ongoing. The presented data places TCX-101 as a promising option for the treatment of various solid tumors that express the targeted TACA.

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B7-H3 as a promising target for NK cell-based immunotherapy of ovarian cancer

I. Hagelstein[1,2], K. Wang[1], G. Jung[2,3], H. R. Salih[1,2], M. S. Lutz[1,4]
[1]University Hospital Tuebingen, Clinical Collaboration Unit Translational Immunology, German Cancer Consortium (DKTK), Department of Internal Medicine, Tuebingen, Germany, [2]University of Tuebingen, Cluster of Excellence iFIT (EXC 2180) "Image-Guided and Functionally Instructed Tumor Therapies", Tuebingen, Germany, [3]Eberhard Karls Universitaet Tübingen, Department of Immunology, Tuebingen, Germany, [4]University of Tuebingen, Cluster of Excellence iFIT (EXC 2180), Tuebingen, Germany

To date, ovarian cancer remains the most lethal gynecologic malignancy and therefore represents a major health challenge. The standard of care for ovarian cancer is surgery and chemotherapy, but these treatments often fail, leading to disease recurrence. The introduction of monoclonal antibodies (mAbs) has revolutionized the treatment of many types of cancer, but patients with ovarian cancer have yet not benefitted. Natural killer (NK) cells are cytotoxic lymphocytes and contribute significantly to antibody-dependent cellular cytotoxicity (ADCC), which is a key factor for the success of anti-tumor mAb treatment. Currently, many efforts focus on enhancing this crucial mAb function by modifying the Fc moiety.
B7-H3 (CD276) is highly expressed in various tumor entities, including ovarian cancer, whereas expression on healthy tissue is rather limited. Therefore, B7-H3 is a promising target for immunotherapy. Here we report on an Fc-optimized B7-H3-targeting antibody for treatment of ovarian cancer. We developed a chimeric B7-H3-mAb containing an Fc portion with the amino acid substitutions S239D/I332E to increase affinity to CD16 which is expressed by NK cells (8H8-SDIE). We show that various ovarian cancer cell lines exhibit high B7-H3 expression that is recognized by our 8H8-SDIE.
Functional analyses demonstrated that 8H8-SDIE induced profound NK cell activation, degranulation as well as IFN-γ and TNF release. No effects were observed upon application of a control mAb with unrelated specificity, confirming strictly target antigen-restricted activity of 8H8-SDIE. Therapeutic efficacy of 8H8-SDIE against ovarian cancer cells was documented in various short- and long-term cytotoxicity assays.
In summary, 8H8-SDIE triggers profound NK cell functions resulting in potent tumor cell lysis, which emphasizes its potential as a novel immunotherapeutic option for treatment of ovarian cancer.

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B7-H3 as target for T cell-based therapy of breast cancer

I. Hagelstein[1,2], L. Wessling[1], A. Rochwarger[3], L. Zekri[1,2,4], C. M. Tegeler[1,5,6], G. Jung[2,4], C. M. Schürch[3], H. R. Salih[1,7], M. S. Lutz[1,2]

[1]University Hospital Tuebingen, Clinical Collaboration Unit Translational Immunology, German Cancer Consortium (DKTK), Department of Internal Medicine, Tübingen, Germany, [2]University of Tuebingen, Cluster of Excellence iFIT (EXC 2180) "Image-Guided and Functionally Instructed Tumor Therapies", Tübingen, Germany, [3]University Hospital and Comprehensive Cancer Center Tübingen, Department of Pathology and Neuropathology, Tübingen, Germany, [4]University of Tuebingen, Department for Immunology, Tübingen, Germany, [5]University Hospital Tuebingen, Department of Obstetrics and Gynecology, Tübingen, Germany, [6]University and University Hospital Tuebingen, Department of Peptide-based Immunotherapy, Tübingen, Germany, [7]University of Tuebingen, Cluster of Excellence iFIT (EXC 2180), Tübingen, Germany

Invasive breast cancer is the most prevalent cancer in women globally. The recent years have seen significant improvements in the treatment landscape like the implementation of checkpoint inhibitor-based immunotherapy in routine treatment. However, many patients are not eligible for this T cell-based therapy, and others do not respond to treatment or for limited time only. Thus, there remains a high clinical need for new therapies, in particular for triple-negative breast cancer, where treatment options are still very limited. B7-H3 is overexpressed in various cancers on both tumor cells and tumor vessels. Besides directing T cells against the malignant cells themselves, targeting B7-H3 may enhance infiltration of immune effector cells into the tumor site, making B7-H3 a promising target for immunotherapy. Here we explored the potential of B7-H3 as a target for a bispecific antibody (bsAb) for treatment of breast cancer. We analyzed tumor samples of n=25 patients using immunohistochemistry to assess B7-H3 expression levels. We found substantial expression of B7-H3 with a minimum staining intensity of 2+ (clear positive). Notably, none of the cases was B7-H3 negative. In addition, various breast cancer cell lines showed high B7-H3 expression regardless of the molecular subtype. We evaluated the effectiveness of CC-3, a novel B7-H3xCD3 bsAb, for inducing T cell immunity against breast cancer cells. Functional analysis using breast cancer cell lines revealed that CC-3 induces profound T cell activation and secretion of immunostimulatory cytokines like IL-2, IFN-γ, and TNF. Importantly, CC-3 also induced potent tumor cell lysis, significant T cell proliferation, and T cell memory subset formation.
In summary, our findings reveal B7-H3 as promising target in breast cancer and provide evidence that CC-3 has therapeutic potential for this malignancy. Based on these findings, a clinical trial to evaluate CC-3 in patients with breast cancer is presently in preparation.

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IOMX-0675, a LILRB1 and LILRB2 cross-specific antibody, effectively repolarizes immunosuppressive myeloid cells and activates T cells leading to potent tumor cell killing

C. Hartl, J. Zantow, K. Heinig, I.-P. Maser, M. Stebegg-Wagner, S. Friedrich, J. Schilz, S. Kaden, R. Milde, E. Korotkova, M. Maraslis, B. Langer, M. Swiat, C. Ginzel, C. Ammerhauser, L. Albert, L. Majunke, A. Huth, K. Sudan-Grimpe, C. Strobl, T. Michels, M. Yule, C. Rothe, N. Khandelwal, H. Loferer, M. Aigner, A. N. Marziale,

S. Bissinger
iOmx Therapeutics AG, Planegg/Martinsried, Germany

In the context of tumor immune evasion, myeloid checkpoints in the tumor microenvironment have gained increased attention. LILRB1 (ILT2) and LILRB2 (ILT4) are immunosuppressive receptors of the leukocyte immunoglobulin-like receptor (LILR) family that recognize both classical and non-classical MHC-I molecules (e.g., HLA-G). While tumor-infiltrating myeloid cells express both LILRB1 and LILRB2, lymphoid cells are restricted to LILRB1 expression. LILRB1 and LILRB2 are frequently co-expressed with the immune-activating LILR family members LILRA1 and LILRA3 in several tumor indications and are upregulated in patients non-responsive to T cell checkpoint blockade. Furthermore, the non-classical MHC-I molecule HLA-G, a major ligand of LILRB1 and LILRB2 in the tumor environment, is overexpressed and associated with poor prognosis in multiple solid cancer types.
IOMX-0675 is a fully human, Fc-silenced monoclonal antibody, identified from iOmx' proprietary phage display library. It displays a highly differentiated binding profile, with selective binding to the inhibitory receptors LILRB1 and LILRB2 with high affinity, while binding to the closely related immune-activating LILR family members LILRA1 and LILRA3 with only low affinity. A high-resolution Fab-based Octet assay was established to monitor IOMX-0675-mediated blocking of LILRB1 or LILRB2 binding to HLA-G. The LILRB1/2 cross-specific antibody promotes phagocytic and pro-inflammatory activity of various macrophage subtypes, which was assessed by flow cytometry and cytokine profiling. In co-cultures of monocyte-derived macrophages with autologous T cells, IOMX-0675 shows high potential to dose-dependently reprogram immunosuppressive macrophages and to further activate pro-inflammatory macrophage subtypes, even under conditions dominated by the presence of LILRA1/A3. Finally, in a $CD34^+$ stem cell-engrafted humanized mouse xenograft tumor model, IOMX-0675 demonstrates potent anti-tumor activity and pharmacodynamic modulation of tumor-associated macrophages.
In summary, we discovered IOMX-0675, a cross-specific antibody antagonizing both LILRB1 and LILRB2 with high selectivity and negligible binding to the immune-activating LILR family members LILRA1 and LILRA3. IOMX-0675 exhibits potent reprogramming capacity of the immunosuppressive myeloid compartment and restores cytotoxic T cell activity in the tumor microenvironment. The unique binding profile of IOMX-0675 provides best-in-class potential for a dual-targeting myeloid checkpoint inhibitor that may maximize anti-tumor efficacy in patients across a variety of solid cancer indications.

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Distinct immune resistance profiles of malignant subpopulations contribute to intra-tumoral heterogeneity of pancreatic cancer

A. Hussein[1], A. N. Menevse[1], J. Sax[1], L. Bellersheim[1], A. Sorrentino[1], M. El-Tager[2], D. Ferrari[3], A. Markus[3], A. Mahfouz[2], M. J. T. Reinders[2], F. Alves[3], P. Beckhove[1,4]
[1]Leibniz Institute for Immunotherapy (LIT), Division of Interventional Immunology, Regensburg, Germany, [2]Delft Bioinformatics Lab, Delft University of Technology, Delft, The Netherlands, [3]Translational Molecular Imaging, Max-Planck-Institute for Multidisciplinary Sciences, Göttingen, Germany, [4]University Hospital Regensburg, Department of Internal Medicine III, Regensburg, Germany

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive and prevalent exocrine tumor that associates

with low survival rates due to a desmoplastic tumor microenvironment critical for its progression, metastasis, and drug resistance. Advances in cancer treatment have been achieved with the development of immunotherapies that exploit the immune system's ability to recognize aberrant cells. Certain immune checkpoint molecules, that are upregulated by tumor cells resulting in the evasion of anti-tumor immunity, are therapeutically targeted to restore the immune response. Although nanovaccines show great potential for the improvement of cancer vaccine efficacy, long-term immunity to PDAC has proved elusive. By broadening the potentials of immunotherapy to PDAC, we hypothesize that this elusiveness is due to the presence of hitherto unknown immune resistance genes (IRGs) expressed by PDAC. To systematically identify IRGs, we performed high-throughput RNA interference screens of tumor-T cell co-cultures on human primary PDAC cells beside five other tumor entities. We explored the expression profiles of 235 validated IRGs by analyzing transcriptome data from primary PDAC patients, investigating the degree of heterogeneity between tumor subjects and in comparison to control pancreases based on bulk expression levels. By merging different single-cell RNA-seq datasets, we obtained subpopulations of malignant ductal cells reflecting distinct biological states and characterized by a differential co-expression of IRGs and upstream transcription factors driving their co-expression. Ten candidates confirmed to be significantly upregulated in PDACs and associated to worse survival rates. These were selected for deeper functional analyses, all of which proved to protect against the immune rejection mediated by tumor-specific cytotoxic T lymphocytes. The tested IRGs were shown to mediate tumor-intrinsic resistance to T cell-derived cytotoxic molecules such as TRAIL, FasL and TNFa with differential impacts on distinct downstream death-receptor signaling pathways. Downregulation of some IRGs resulted in elevated death receptor expression on tumor cells sensitizing them towards immune rejection. Murine PDAC cells were transduced with viral-based shRNA for the generation of cell lines with the stable knockdown of three IRGs and were further tested in a KPC orthotopic mouse model. In vivo experiments proved that IRG expression promotes tumor progression in immune-competent mice. Combination of IRG inhibition with peptide-based PDAC nanovaccine improved the therapeutic efficacy over monotherapies. The tumor microenvironment of mice that received the combination therapy showed an induced T cell activation as well as cytokine secretion. In this work, we have identified and validated novel therapeutic targets for inhibition in PDAC for an enhanced tumor-sensitivity towards effector immune responses.

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Targeting HHLA2-mediated immunosuppression with NPX267 and NPX887, two fully human monoclonal antibodies that target KIR3DL3 and HHLA2 respectively

E. Loeuillard, M. Hayes, R. O'Connor, Y. Hashambhoy-Ramsay, J. Strand, E. Wang, M. Lamb, A. Dulak, K. Kis-Toth, L. Gandhi, B. Etemad-Gilbertson, M. Rausch, T. Novobrantsava
Nextpoint Therapeutics, Cambridge, United States of America

Human endogenous retrovirus H long terminal repeat associating protein 2 (HHLA2), also called B7-H7, a member of the B7 family, exhibits both immuno-suppressive and stimulatory functions through its interactions with the co-inhibitory receptor killer cell immunoglobulin-like receptor, three immunoglobulin domains and long cytoplasmic tail 3 (KIR3DL3) and the co-stimulatory receptor transmembrane and immunoglobulin domain containing 2 (TMIGD2) expressed on T and NK cells. While HHLA2 expression is restricted in most normal tissues, its expression in cancer is associated with adverse patient outcomes,

highlighting HHLA2 as a promising immune checkpoint target. In this context, NextPoint Therapeutics has developed two human antagonistic monoclonal antibodies targeting the HHLA2 axis: NPX267 binds to KIR3DL3 and blocks the interaction with HHLA2 and NPX887 targets HHLA2 and blocks the KIR3DL3 inhibitory signal but spares TMIGD2-mediated co-stimulation.

NPX267 is a first in class, fully human antibody that binds to KIR3DL3 on the surface of primary human T and NK cells. NPX267 blocks the binding of recombinant HHLA2 protein to KIR3DL3+ cells and inhibits KIR3DL3-mediated suppression in a T cell reporter assay. NPX267 treatment enhances the cytotoxicity of human NK cell lines and primary NK cells against HHLA2+ tumor cells in vitro. Finally, anti-KIR3DL3 treatment enhances NK cell-mediated killing of HHLA2+ tumors in vivo in a humanized mouse model.

NPX887 is a fully human, Fc-enhanced monoclonal antibody that binds to HHLA2 on the surface of human tumor cell lines and tumor cells from dissociated tumors. NPX887 blocks the on-cell binding of recombinant KIR3DL3 to HHLA2+ cells, but spares the binding of recombinant TMIGD2 protein. NPX887 binding blocks KIR3DL3-mediated suppressive signaling, but spares TMIGD2-mediated co-stimulation in T cell reporter assays. In addition, NPX887 treatment augments NK cell-mediated cytotoxicity and ADCC activity of HHLA2+ tumor cells in vitro. Finally, NPX887 treatment demonstrates anti-tumor efficacy against HHLA2+ tumors in a humanized mouse model.

Collectively, our findings demonstrated that the HHLA2 axis represents a novel immune checkpoint that mediates tumor immune evasion by suppressing both NK and T cell activity. NPX267, a first-in-class KIR3DL3 blocking antibody, and NPX887, a novel fully human, monoclonal antibody targeting HHLA2 represent attractive approaches to treat certain cancers by potentiating anti-tumor immune responses. Additionally, HHLA2 represents a novel tumor target with limited normal tissue expression. NPX267 and NPX887 are currently being evaluated in Phase I clinical trials for patients with solid tumors known to express HHLA2 / B7-H7 [NCT05958199] and [NCT06240728] respectively.

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The monoclonal antibody FC-4A7G8 defines MUC4 as a viable target against pancreatic cancerThe monoclonal antibody FCCTA1 defines MUC4 as a viable target against pancreatic cancer

L. Lordello[1], J. R. Lérias[1], M. Sedas[1], C. M. Gorgulho[1], J. Kamiki[1], P. A. António[1], S. Cascais[1], R. Eduardo[1], M. El Alaoui[2], S. El Alaoui[2], E. de Sousa[1], V. Balan[1], M. Maeurer[1,3]
[1]Champalimaud Foundation, Immunotherapy/Immunosurgery, Lisbon, Portugal, [2]Covalab, Bron, France, [3]Johannes Gutenberg University Mainz, I Medizinische Klinik,, Mainz, Germany

MUC4 is a membrane-anchored glycoprotein. Transcription, splicing and differential MUC4 glycosylation is associated with aging and cell division; some MUC4 isoforms are associated with malignant transformation and metastasis. MUC4-expressing tumors are associated with a worse clinical course in patients with pancreatic adenocarcinoma (PA). MUC4 shows stronger expression in malignant cells as compared to non-transformed cells in the pancreas. It represents a viable theranostic target for the development of a monoclonal antibody (mAb), either for diagnosis or treatment for patients with MUC4+ malignancies. We manufactured a set of mAbs, among them FCCTA1, targeting a viable epitope, being analyzed in greater

detail in a cohort of 27 patients with PA: A) 6 wild-type and 6 mutant epitopes, which included the FCCTA1 epitope, were tested for recognition by tumor-infiltrating lymphocytes (TIL), which showed reactivity against the FCCTA1 target epitope, defined by exclusive production of IFNγ in 27% (n=6/22), IL-17 in 23% (n=5/22) and simultaneous production of IFNγ and IL-17 in 32% (n=7/22) TIL lines. B) MUC4 expression, based on FCCTA1 staining of PA tissue samples, was positive in 74% (Hscore>0, n=14/19) and scored as low in 42% (Hscore 1-99, n=8/19), moderate in 10.5% (Hscore 100-199, n=2/19) or high in 21% (Hscore 200-300, n=4/19). C) Serum IgG against the FCCTA1 epitope was detected in 70% of patients with PA (n=19/27). Samples were considered positive if the IgG target equivalent was above 10 ng IgG/mL suggesting that this epitope was accessible in vivo. In summary, we report the development of anti-MUC4 mAbs, among them the FCCTA1 mAb that reacts against a unique MUC4 epitope. The mAb reacts specifically with about 75% of PA and shows negative or weak reactivity to adjacent non-malignant pancreas tissue. T- and B-cell responses were detected in patients with PA suggesting that this epitope is accessible in vivo.
We are currently testing i) functional mAb FCCTA1 reactivity against PA and ii) $2^{nd}/3^{rd}$ generation CAR-constructs against the FCCTA1 epitope alone or in combination with anti-HER2. The FCCTA1 defined epitope holds promise to serve as a viable target for anti-MUC4+ PA directed therapies using either a mAb or CAR-based cellular immunotherapeutic strategies.

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CAMYO-01: an off-the-shelf MSS-CRC vaccine targeting camyotopes, a novel and unique class of shared antigen targets derived from the dark genome

L. Lybaert, G. Boons, M. Montyne, C. Bogaert, B. Fant, L. Van Oudenhove
myNEO BV, Ghent, Belgium

We present camyotopes, a novel class of dark genome antigens with unprecedented immunogenicity and sharedness, introducing a promising avenue for the development of shared therapeutic cancer vaccines.

Leveraging vast cancer omics data, we developed a pipeline to screen the 'dark genome' —the non-coding part of the genome— for camyoRNAs, tumor-specific lncRNAs that are consistently producing micropeptides. The CamyoRNA Discovery Pipeline comprises differential expression analysis to identify tumor-specific camyoRNAs, in silico prediction of smORF (small open reading frame) translation, and peptide identification in MS (mass spectrometry) data to validate translation. Our proprietary, first-in-class presentation and immunogenicity prediction algorithms, neoMS and neoIM, were employed to identify immunogenic epitopes derived from these camyoRNAs combined with in-vitro immunogenicity screening.

The CamyoRNA Discovery Pipeline screened 240 transcriptomics and 95 proteomics CRC (colorectal cancer) samples, identifying 15 camyoRNAs. From these, 79 camyopeptides were identified as being translated, with in silico presentation and immunogenicity screening leading to the selection of 73 camyotopes. These camyotopes are distinguished by their high population coverage (>50% of patients express at least 6 camyoRNAs), high tumor-specific abundance, favorable translation and presentation profiles. Furthermore, we found that camyotopes have unprecedented immunogenicity. ELISpot readouts revealed that a strong majority of the selected camyotopes are significantly more immunogenic than shared antigens (e.g. MAGE-A3) and neoantigens. These results enabled the selection of 50 camyotopes which are now being brought

to the clinic as an mRNA-LNP vaccine product. A unique strength of this vaccine formulation lies in the multiplicity of T-cell responses it will elicit, guaranteeing that immune responses will be elicited towards several epitopes in any patient, which minimizes the chances of immune escape while enhancing the clinical efficacy of the therapy.

To conclude, camyotopes represent a novel and uniquely interesting target for immunotherapy. Their combined broad population coverage, high abundance, and strong immunogenicity make camyotopes an attractive off-the-shelf solution for cancer patients. Given that any patient within the target population is likely to be affected by a large fraction of the vaccine formulation, this approach minimizes the risks of immune escape and the introduction of ineffective epitopes. Indeed, the coverage achieved by camyoRNAs significantly surpasses that of other antigen-based off-the-shelf approaches. We are now initiating our lead program CAMYO-01, a camyotope-encoded mRNA-LNP vaccine, which will enter the clinic in 2025 as a novel therapeutic strategy for CRC patients.

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Identification of a T cell receptor recognizing shared HLA-A*02:01 presented epitope from TP53 frameshift

M. Martin[1], S. Santegoets[2], J. van Langelaar[1], W. van Esch[1], H. Olsman[1], A. Stolk[2], W. Kloosterman[1], S. van der Burg[2], K. Franke[1]
[1]CureVac Netherlands, Amsterdam, The Netherlands, [2]Leiden University Medical Center, Leiden, The Netherlands

Background: The recognition of epitopes derived from tumor antigens via T cell receptors (TCR) of antigen specific $CD8^+$ T cells is prerequisite for efficient tumor cell destruction. One of the limiting factors in T cell-mediated immunotherapy is the availability of relevant targets highly expressed on tumor cells and absent from healthy tissue, ideally shared across patients and/or cancer types. Neo-open reading frame peptides (NOPs) arising from insertions or deletions in tumor DNA are a class of antigens, providing opportunities for designing T cell receptor therapies with high tumor specificity.
Methods:
NOPs were predicted for all patients in The Cancer Genome Atlas. Epitopes from shared NOPs were analyzed for their predicted presentation on the HLA-A*02:01 allele. The top 30 epitopes prioritized based on presentation prediction and level of sharedness were used to make HLA-A*02:01 fluorescently labeled tetramers. Buffy coats of at least three healthy individuals were used to isolate peripheral blood mononuclear cells and incubated with pooled peptide loaded tetramers. Tetramer positive cells were further expanded and enriched for antigen specific $CD8^+$ T cells which were further used to sort single cells. The alpha and beta chains of TCR were identified and cloned into naïve $CD8^+$ T cells of another HLA-A*02:01 donor. Both in vitro expanded and TCR engineered $CD8^+$ T cells were used in co-culture assays with either HEK293 Free Style cells overexpressing selected frameshifts of interest or with cell lines naturally expressing the same frameshift mutation. The activation of antigen specific $CD8^+$ T cells was detected by increased CD137 and CD107a expression. The engineered T cells were tested in a dose titration experiment to assess their affinity for pHLA-A*02:01 complex.
Results: The in vitro expansion of antigen specific CD8 T cells recognizing HLA-A*02:01 bound peptides

derived from frameshifts of multiple genes was successful for 15 out of 30 screened peptides. The relevance of such TCRs for the peptide recognition was tested first in a cell line model where individual mutated proteins were overexpressed, and peptide presentation was tested in a T cell co-culture assay. Out of all in vitro expanded $CD8^+$ T cell populations, only 2 recognized their cognate peptide presented in the context of HLA-A*02:01 allele. One of the TCRs recognized an epitope derived from TP53 NOP. This TCR was successfully cloned into naïve $CD8^+$ T cells of a healthy donor which recognized naturally expressing and presented TP53 NOP peptide as shown by increased expression of CD137 and CD107a. No $CD8^+$ T cell activation was observed upon co-culture with an irrelevant cell line.
Conclusions: We identified an HLA-A*02:01 presented epitope specific for TP53 NOP and its cognate TCR, a target expressed in 1-3% of patients across multiple cancer types. This study shows promise of NOP-derived peptides as off-the-shelf TCR T cell therapy targets.

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GD2 is a relevant target for cellular and antibody-based therapies in the treatment of paediatric brain tumours.

C. Paret[1], A. Wingerter[1], S. Wagner[1], K. El Malki[1], F. Alt[1], A. Russo[1], O. Beck[1], K. Frauenknecht[2], R. Sandhoff[3], J. Faber[1]
[1]Children's Hospital Mainz, Pediatric oncology, Mainz, Germany, [2]Laboratoire National de Santé, Luxembourg Center of Neuropathology (LCNP), Luxembourg, Luxembourg, [3]German Cancer Research Center, Lipid Pathobiochemistry, Heidelberg, Germany

While immunotherapy has revolutionized cancer treatment for adult patients, its application in childhood cancer remains limited. This restriction arises not only from the imperative to safeguard paediatric patients from poorly defined risks and toxicities but also from our constrained understanding of target expression in paediatric tumour and normal tissues. The gangliosides GD2, an oncofetal plasma membrane glycolipid currently used as target in neuroblastoma (NBL), and N-glycolyl GM3, uniquely expressed on tumor cells, are currently being explored as promising therapeutic targets for monoclonal antibodies and CAR-T cells across various tumour types. Gangliosides elude detection through pipelines reliant on genomic and transcriptome analyses. Dedicated technologies are thus indispensable for the identification and characterization of such targets. Nonetheless, given the significant role of gangliosides in the developing brain, it is reasonable to anticipate heightened expression of such targets in paediatric brain tumours (BT).

Here we analysed 40 BT clinical samples, including medulloblastomas (MB), pilocytic astrocytomas (PA), ependymomas (EP), H3K27M mutant diffuse midline gliomas (DMG) and CNS tumors with BCOR internal tandem duplication (HGNET-BCOR) by mass spectrometry and/or flow cytometry.

High expression of GD2 was found in Posteriora Fossa EP (PFA) and some PA. In MB, GD2 expression was depending from the subtype, with the highest expression found in Group 4 and the lowest in WNT. In HGNET-BCOR the expression was depending on the localisation, with the highest expression found at diagnosis. All H3K27M mutant diffuse midline gliomas expressed GD2 but the level of expression was lower than in PFA. Positive samples exhibited a uniform expression pattern of GD2. N-glycolyl GM3 expression was not detected in any of the samples.

In conclusion, the expression of GD2 displays notable heterogeneity within the same tumor type, underscoring the importance of precise quantification methods to pinpoint patients with heightened GD2 levels. Interestingly, while GD2 expression appears uniform in positive BT samples, it frequently exhibits heterogeneity in other solid tumors like Ewing sarcoma and osteosarcoma, potentially facilitating tumor evasion mechanisms. The identification of surrogate biomarkers such as H3K27 alterations in DMG and PFA is pivotal, particularly in cases where biopsy for direct GD2 expression measurement isn't feasible. These biomarkers offer a promising avenue for identifying positive patients, contributing significantly to treatment decisions. These findings hold substantial implications for the treatment of pediatric brain tumor patients, advocating for the inclusion of anti-GD2 therapies in basket clinical study programs.

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Expression of Target-independent T-cell engagers (MATEs) by oncolytic adenoviruses facilitates intratumoral T-cell activation and broad range applicability

M. Peter[1], B. Mundt[1], N. Woller[1], V. Volk[2], H. Wedemeyer[1], C. Falk[3], F. Feuerhake[2], T. Wirth[1], F. Kühnel[1]
[1]Hannover Medical School, Dept. Gastroenterology, Hepatology, Infectious Diseases and Endocrinology, Hannover, Germany, [2]Hannover Medical School, Dept. of Pathology, Hannover, Germany, [3]Hannover Medical School, Inst. of Transplantation Immunology, Hannover, Germany

Oncolytic viruses (OV) expressing bispecific T-cell engagers (BiTEs) are promising tools for tumor immunotherapy but their application range is limited to tumors which express a specific target antigen. To equip oncolytic viruses with direct and potent T-cell stimulation capabilities with broad-range applicability, we established soluble, non-selective, membrane-associated T-cell engagers (MATEs). MATEs contain an αCD3 single-chain antibody fragment linked to the protein transduction domain of the HIV-Tat protein to achieve efficient, but non-selective binding of the T-cell engager to cellular membranes. In vitro, MATEs effectively activated murine T-cells in splenocytes as shown by the activations markers CD25 and CD69, enhanced proliferation, and IFNγ-production. MATEs improved direct killing of murine MC38 colon carcinoma cells. Similarly, humanized MATEs were capable of activating T-cells in PBMCs from human donors. In vivo, a chimeric oncolytic adenovirus (Ad5/11) was used for intratumoral delivery of MATEs. After i.t. injection of MATE-expressing Ad5/11 in MC38 tumors in C57BL/6 mice, intratumoral T cells were significantly activated. In contrast, no T-cell activation was detectable in tumor draining lymph nodes at early time points suggesting that MATEs were sufficiently retained by the infected tumor tissue thus demonstrating the safety of this target-independent approach. Antitumoral immunity reduced tumor growth and prolonged survival accompanied by infiltration of tumor-directed CD8 T-cells and improved CD8/CD4 T cell ratio. Consistent with the strong intratumoral immune activation, MATE-expression by Ad5/11 was capable of breaking tumor resistance to αPD-1 checkpoint blockade. Multiplex IHC analyses suggested that MATE-expression during virotherapy resulted in improved T-cell/tumor cell proximity and also in improved clustering of CD8 and CD4 T cells. In summary, non-target selective MATEs for T-cell activation are a powerful tool for arming OVs for local immunotherapy of a broad range of tumors.

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Generation of an NG2 targeting bispecific antibody for induction of T cell immunity against Melanoma

N. Prakash[1,2,3], S. Hörner[1,2,3], G. Jung[2,3], H. R. Salih[1,2]

[1]Clinical Collaboration Unit Translational Immunology, German Cancer Consortium (DKTK), Department of Internal Medicine, University Hospital Tübingen, Tübingen, Germany, [2]Cluster of Excellence iFIT (EXC 2180) "Image-Guided and Functionally Instructed Tumor Therapies", University of Tübingen, Tübingen, Germany, [3]Department for Immunology and German Cancer Consortium (DKTK), Eberhard Karls University, Tübingen, Germany

Incidence of melanoma has continued to rise, making it the fifth most frequently occurring cancer in Europe, with a high risk of metastasis making it one of the 20 most frequent causes of cancer death. Although the introduction of immune checkpoint inhibitors (ICI) has revolutionized treatment options for melanoma patients, these monoclonal antibodies (mAbs) neither possess tumor-specific selectivity nor the ability to directly recruit T cells to the tumor site. As a consequence, many patients do not respond to ICI or for limited time only, and treatment is associated with substantial side effects. This calls for the development of novel, targeted immunotherapies. Here we report on bispecific antibodies (bsAbs) that stimulate T cells via the TCR/CD3 complex and bind simultaneously to the Tumor Associated Antigen (TAA) NG2 (also known as Melanoma-associated Chondroitin Sulfate Proteoglycan) that is overexpressed in 90% of melanoma cases.

Three NG2 bsAbs with different T cell binders including a TCR binding bsAb (NG2xBMA) and two CD3 binding bsAbs (NG2xUCHT-1 and NG2xM18) were generated. Dose titration of the different bsAbs on several melanoma cell lines and T cells documented dose dependent binding with saturation for all constructs at ~5nM and ~25nM, respectively. As expected, the constructs exhibited highly differing affinity to T cells. The TCR-directed NG2xBMA bsAb and the CD3-directed NG2xUCHT-1 bsAb bound T cells with high affinity, in contrast to NG2xM18 that was constructed using a UCHT-1 sequence mutated to reduce CD3 affinity about 100-fold. In in-vitro assays measuring T cell proliferation, the two CD3-constructs displayed superior ability to induce T cell proliferation, whereas the TCR-directed NG2xBMA bsAb induced only marginal T cell proliferation. Superiority of the CD3-binding constructs was further confirmed using flow cytometry to measure T cell activation and target cell lysis. Among the CD3-directed constructs, NG2xUCHT-1 induced higher T cell activity than NG2xM18.

Our results indicate that NG2xCD3 bsAbs may be promising compounds for treatment of melanoma. Careful analysis of cytokine release in the context of desired activity with regard to the respective CD3/TCR binder is presently ongoing to select an optimal construct that maintains desired T cell activity with a favourable cytokine release profile to allow for effective clinical dosing.

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Molecular mimicry of SARS-COV-2 antigens as a possible natural anti-cancer preventive immunization

C. Ragone[1], A. Mauriello[1], B. Cavalluzzo[1], E. Cavalcanti[2], C. Manolio[3], S. Mangano[1], B. Cembrola[1], M. Tagliamonte[1], L. Buonaguro[1]

[1]Istituto Nazionale Tumori - IRCCS Pascale, Experimental Oncology, NAPOLI, Italy, [2]Istituto Nazionale Tumori - IRCCS Pascale, Clinical Pathology, Naples, Italy, [3]University of Padua, Padua, Italy

In the present study we investigated whether peptides derived from the entire SARS-CoV-2 proteome share homology to TAAs and cross-reactive CD8+ T cell can be elicited by the BNT162b2 preventive vaccine or the SARS-CoV-2 natural infection.
Viral epitopes with high affinity (<100nM) to the HLA-A*02:01 allele were predicted. Shared and variant-specific epitopes were identified. Significant homologies in amino acidic sequence have been found between SARS-CoV-2 peptides and multiple TAAs, mainly associated with breast, liver, melanoma and colon cancers. The molecular mimicry of the viral epitopes and the TAAs was found in all viral proteins, mostly the Orf 1ab and the Spike, which is included in the BNT162b2 vaccine. Predicted structural similarities confirmed the sequence homology and comparable patterns of contact with both HLA and TCR α and β chains were observed. CD8+ T cell clones cross-reactive with the paired peptides have been found by MHC class l-dextramer staining.
Our results show for the first time that several SARS-COV-2 antigens are highly homologous to TAAs and cross-reactive T cells are identified in infected and BNT162b2 preventive vaccinated individuals. The implication would be that the SARS-Cov-2 pandemic could represent a natural preventive immunization for breast, liver, melanoma and colon cancers. In the coming years, real-world evidences will provide the final proof for such immunological experimental evidence. Moreover, such SARS-CoV-2 epitopes can be used to develop “multi-cancer” off-the-shelf preventive/therapeutic vaccine formulations, with higher antigenicity and immunogenicity than over-expressed tumor self-antigens, for the potential valuable benefit of thousands of cancer patients around the World.

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Link between bovine meat and milk factor protein expression and inflammation as possible driver of colorectal cancer – new options for prevention and therapy?

G. Shukla[1], E. Nikitina[1], A. Burk-Körner[1], M. Wiesenfarth[1], P. Schrotz-King[1,2], H. Brenner[1,2,3], M. Schneider[4], M. Heikenwälder[1,5], M. Hoffmeister[1], A. Pöchmann[1,6], N. Halama[1,6], E.-M. de Villiers[1], H. zur Hausen[1], T. Bund[1]

[1]German Cancer Research Center (DKFZ), Heidelberg, Germany, [2]National Center for Tumor Diseases (NCT), Heidelberg, Germany, [3]German Cancer Consortium, Heidelberg, Germany, [4]Thorax Clinics, Heidelberg, Germany, [5]Eberhard Karls University, M3 Research Center, Tübingen, Germany, [6]Helmholtz Institute for Translational Oncology (HI-TRON), Mainz, Germany

Bovine Meat and Milk Factors (BMMFs) are plasmid-like DNA molecules isolated from bovine serum and milk. BMMFs have been described to contribute to the development of different cancers including colorectal and breast cancer after decades-long latency based on indirect carcinogenesis outlined by H zur Hausen. In tissues of colorectal cancer (CRC) patients, BMMF DNA and high levels of expression of a

conserved BMMF protein (Rep) have been identified by antibody staining, the latter also in tissues of lung and pancreatic cancer patients. Rep was expressed in the tumor-adjacent mucosa of 99% of CRC patients (TMA, n = 246) and was histologically associated with CD68+CD163+ macrophages. Macrophage-linked Rep expression was increased in CRC patients when compared to age-matched healthy controls supporting a causative contribution to CRC. Rep was also expressed in low grade dysplasia and LGD/HGD-adjacent tissues indicating a role as early risk factor.

This supported the previously proposed model of BMMF-induced chronic inflammation resulting in increased levels of diffusion radicals, which in turn induce random DNA mutations in neighboring replicating crypt cells as precursors of dysplasia and tumors. Incidence curves for CRC-specific death were increased for higher Rep expression supporting also a prognostic relevance of BMMF and a basis to test therapy.

If the BMMF cancer model holds true, we expect that exposure of BMMF to specific immune cells, tissue explants or to mice results in phenotypic changes fueling chronic inflammation, faster progression of bigger tumors or a general increase in proliferation or a M2-like immune signature as observed in clinical tissues. The same setup would allow to test preventive and therapeutic measures to restrict BMMF activity and to revert any possible immune blockade.

Therefore, we currently test exposure of mice, patient explants and cultured cells to different BMMF formulations (e.g. BMMF DNA, protein, milk). Monitoring efficient uptake, BMMF replication, expression and immune regulation represent the first goals and basis to understand BMMF pathology. It allows to subsequently test possible interventions such as application of specific inhibitors e.g. Fucosyllactose, NSAIDs, and treatments against specific macrophages and to test the function of the sialic acid receptor Neu5Gc as putative receptor for BMMF. Identification of BMMF-induced regulatory patterns might help to deepen our pathological understanding and add additional inhibitory candidates. In addition, we test a possible neutralizing and/or anti-tumorigenic effect of the application of human chimeric anti-Rep antibodies, which might help to overcome any (M2-type) BMMF- and tumor-induced regulatory immune blockade.

Efficient targeting and modulation of BMMF-linked immune cell populations, occasionally as high as 40% of the entire peritumour macrophage population, might help to finally address the 40-60% of cancers linked with diffuse chronic inflammation.

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Link between bovine meat and milk factor protein expression and inflammation as possible driver of colorectal cancer – new options for prevention and therapy?

G. Shukla[1], E. Nikitina[1], A. Burk-Körner[1], M. Wiesenfarth[1], P. Shrotz-King[1,2], H. Brenner[1,2,3], M. Schneider[4], M. Haikenwälder[1,5], M. Hoffmeister[1], A. Pöchmann[1,6], N. Halama[1,6], E.-M. d. V. de Villiers[1], H. zur Hausen[1], T. Bund[1]

[1]Desutches Krebs Forschung Zentrum, Episomal-Persistent DNA in Cancer- and Chronic Diseases, Heidelberg, Germany, [2]National Center for Tumor Diseases (NCT), Heidelberg, Georgia, [3]German Cancer

consortium, Heidelberg, Germany, [4]ThoraxKlinik Germany, Heidelberg, Germany, [5]Eberhard Karls University, M3 Research Center,, Tübingen, Germany, [6]Helmholtz Institute for Translational Oncology, Mainz, Germany

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Efficient targeting and modulation of BMMF-linked immune cell populations, occasionally as high as 40% of the entire peritumour macrophage population, might help to finally address the 40-60% of cancers linked with diffuse chronic inflammation.

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Immunomodulation induced by the drug conjugates 5-Fluorodeoxyuridine-alendronate and 5-Fluorodeoxyuridine-C-ethynylcytidine in human epithelial ovarian carcinoma

M. Suarez-Carmona[1], S. Schott[2], N. Halama[1,3,4]
[1]Helmholtz-Institute for Translational Oncology Mainz, Translational Immunotherapy, Mainz, Germany, [2]Heidelberg University Hospital, department of Obstetrics and Gynecology, Heidelberg, Germany, [3]National Center for Tumour Diseases (NCT), University Hospital Heidelberg, Medical Oncology, Heidelberg, Germany, [4]University medical Center, University Cancer Center (UCT), Mainz, Germany

Patients with epithelial ovarian cancer (EOC) facing chemotherapy resistance are in dire need of new therapeutic options. An optimal therapy should be effective in chemotherapy-naïve and in pretreated patients at relapse, support the anti-tumor immune response and be well tolerated without heavy side effects. Fully human explants from EOC patients were cultured in the presence of innovative duplex drugs, in which the antimetabolite 5-Fluorodeoxyuridine (5-FdU) is linked to alendronate (5-FdU-ale) or to C-ethynylcytidine (5-FdU-ECyd or 5-FdU-lipid-ECyd). The effects on the tumor immune microenvironment (TIME) were analyzed by Luminex-based multiplex measurement of 50 cytokines, chemokines and growth factors. The density of immune cells was studied by automated immunostaining and virtual pathology. 5-FdU-ale reshapes the TIME into a landscape supportive of the anti-tumor immune response and of T cell survival, selectively in tissues from chemotherapy-treated patients – precisely where chemotherapy resistance dictates the need for new treatment options. Moreover, 5-FdU-ale treatment increases the density of $CD8^+$ cytotoxic T cells in the tumor synergistically with immune checkpoint blockade (nivolumab). Indeed, in 7 patient-derived explants, only the 5-FdU-ale and nivolumab combined treatment leads to expansion of tumor-infiltrating lymphocytes, whilst either drug alone fails. The treatment with 5-FdU-ECyd leads to the expansion of $CD8^+$ cytotoxic T cells in most treated tissues, relying on the presence of tertiary lymphoid structures (TLS) in the tissue before treatment. 5-FdU-ECyd additionally mediates a decrease in the concentration of immunosuppressive factors in the tissue. Based on our dataset in tissue explants, both drugs are excellent candidates for the treatment of EOC and definitely warrant further translational and clinical studies.

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Gene network- and artificial intelligence-based selection of self-tolerant tumor-associated antigens for targeted immunotherapy

J. Vera[1], C. Lischer[1], M. Eberhardt[1], J. Dörrie[1], N. Schaft[1], J. J. Freen-van Heeren[2], B. Hohberger[3], G. Schuler[1], C. Berking[1], S. Gupta[4], H. Bruns[5]
[1]Uniklinikum Erlangen, Dermatology, Erlangen, Germany, [2]Sanquin Diagnostic Services, Amsterdam, The Netherlands, [3]Uniklinikum Erlangen, Department of Systems Biology and Bioinformatics, Rostock, Germany, [4]Universität Rostock, Systems Biology and Bioinformatics, Rostock, Germany, [5]Uniklinikum Erlangen, Medicine 5, Erlangen, Germany

Tumor-associated antigens (TAAs) and their derived peptides constitute an opportunity to design off-the-shelf mainline or adjuvant anti-cancer immunotherapies for a broad array of patients. Here, we present an experimentally validated, data-driven computational pipeline that selects and ranks antigens in a multi-

pronged approach. In addition to minimizing the risk of immune-related adverse events (irAE) by selecting antigens based on their expression in tumor biopsies and healthy tissues, we incorporated a network analysis-derived antigen indispensability index based on computational modeling results, and candidate immunogenicity predictions from a machine learning ensemble model relying on peptide physicochemical characteristics. In a model study of uveal melanoma, HLA docking simulations and experimental quantification of the peptide MHC binding affinities confirmed that our approach discriminates between high- and low-binding affinity peptides with a performance similar to that of gold-standard methodologies. Blinded validation experiments with autologous T-cells yielded stimulation-induced IFN-γ secretion despite high inter-donor variability. Upon translation to the clinic, our pipeline supports fast turn-around validation, e.g., for adoptive T-cell transfer preparations, in both generalized and personalized settings.

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The intra-tumoral spatial heterogeneity of T cell antigens in glioblastoma: An integrated multi-omics approach

M. Wacker[1,2], G. Medici[3,4], M. L. Dubbelaar[1,2,5], J. Bauer[1,2], A. Nelde[1,2], F. Hanssen[5], C. Schwitalla[5], L. Regli[3], M. Weller[4], H.-G. Rammensee[2,6], M. C. Neidert[3,7,8], J. S. Walz[1,2,9,10]

[1]University and University Hospital Tübingen, Department of Peptide-based Immunotherapy, Tübingen, Germany, [2]University of Tübingen, Cluster of Excellence iFIT (EXC2180) "Image-Guided and Functionally Instructed Tumor Therapies", Tübingen, Germany, [3]University Hospital Zurich, University of Zurich, Clinical Neuroscience Center and Department of Neurosurgery, Zurich, Switzerland, [4]University Hospital Zurich, University of Zurich, Laboratory of Molecular Neuro-Oncology, Department of Neurology & Brain Tumor Center, Zurich, Switzerland, [5]University of Tübingen, Quantitative Biology Center (QBiC), Tübingen, Germany, [6]University of Tübingen, Institute of Immunology, Tübingen, Germany, [7]Cantonal Hospital St. Gallen, Department of Neurosurgery, St. Gallen, Switzerland, [8]University of Zurich and ETH Zurich, Neuroscience Center Zurich (ZNZ), Zurich, Switzerland, [9]University Hospital Tübingen, Clinical Collaboration Unit Translational Immunology, Department of Internal Medicine, Tübingen, Germany, [10]German Cancer Consortium (DKTK) and German Cancer Research Center (DKFZ), partner site Tübingen, Tübingen, Germany

Glioblastoma is the most common primary malignant neoplasm of the central nervous system in adults. Surgical resection followed by radiation and/or chemotherapy, with temozolomide, represents the current standard of treatment option. However, residual tumor cells lead to frequent recurrence, accounting for the poor prognosis of the disease. Glioblastoma is a highly infiltrative tumor with recurrence originating from the unresectable peritumoral infiltration zone (INF). Therefore, novel treatment options that specifically target tumor cells in the INF zone are needed to prevent relapse and achieve durable remissions. Here, we performed a multi-omics spatial analysis of the necrotic center (Core), the gadolinium contrast-enhancing central tumor region (CER), and the INF zone in primary glioblastoma (n = 15) integrating mass spectrometry-based immunopeptidome analysis with next-generation sequencing methods (whole exome and RNA sequencing) to assess immunologically relevant aspects of tumor heterogeneity.

In total 31,227 unique HLA class I and 22,340 unique HLA class II peptides from all three zones were identified in 12 of 15 glioblastoma patients. Overlap analysis of glioblastoma-derived peptides and a benign

tissue immunopeptidome database (HLA ligand atlas (https://hla-ligand-atlas.org)) identified 44% (13,741/31,227) of HLA class I and 44% (9,871/22,340) of HLA class II peptides as tumor-exclusive. Among these exclusive HLA class I ligands, 16%, 17%, and 15% and of the HLA class II tumor-associated antigens (TAAs) 16%, 19%, and 42%, were exclusively presented in the INF, CER, or Core zones, respectively. 6% of HLA class I and 1% of HLA class II peptides were presented with high frequency among all three zones. Notably, five INF-associated HLA class I ligands and nine INF-associated HLA class II ligands showed frequent representation in 42% and 62% of the glioblastoma cohort, respectively. Among these INF-associated ligands, peptides derived from the glioblastoma-associated proteins BAALC (overexpression), NCAN (central role in glioma progression), and SLC20A1A (overexpression in glioblastoma with a poor prognosis), were identified. Integrated RNA/DNA sequencing enabled a greater understanding of spatial tumor antigen presentation, identifying 7,072 and 4,448 in silico predicted HLA class I and HLA class II neoepitopes. 38% of these HLA class I and 36% of HLA class II neoepitopes were predicted in at least two of the three zones, underlining the comparable mutation profile of the three zones. 27 HLA class I and 2 HLA class II neoepitopes derived from highly expressed, mutated proteins (TPM ≥ 50). The immunogenicity of these peptides will be further validated in upcoming T cell assays of tumor-infiltrating lymphocytes.

In summary, this multi-omics approach identified intra-tumoral regional heterogeneity of tumor antigens, which could be used for specific immunotherapy approaches targeting the INF zone in glioblastoma.

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A Novel Avidity Optimized Tri-specific T-cell Engager Co-targeting Trop-2 and Cadherin-17 to Achieve Selective Lysis of GI Tumor Cells

D. Weismann[1], S. Kasturirangan[2], S. Kende[1], Y. Karabacak[1], J. Weinzierl[1], N. Sabarth[1], F. Trapani[1], P. Adam[1]
[1]Boehringer Ingelheim RCV GmbH & Co KG, Wien, Austria, [2]Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, United States of America

T-cell engagers (TcEs) are immunotherapeutic multi-specific antibodies designed to potently redirect the cytolytic activity of T cells selectively to cancer cells. A key factor to maximize the therapeutic window of TcEs is the discovery of tumor-specific surface antigens, which are very rare. This study describes the discovery of a novel tri-specific TcE that achieves tumor specificity by avid binding to both Trop-2 and cadherin-17 (CDH17), two proteins that are co-expressed in various solid tumors, but mutually exclusively expressed in normal tissues. The 'Avidity Optimized' TcE (AOTcE) was designed using a heterodimeric IgG-like scaffold with low affinity monovalent binding to Trop-2, CDH17, and CD3. We demonstrated that the AOTcE can bind to Trop-2/CDH17 co-expressing cell lines and CD3-positive purified human T cells, but not to Trop-2/CDH17 and CD3-negative cells. The TcE induced T-cell redirected lysis selectively of Trop2/CDH17 co-expressing cancer cells in a dose-dependent manner. Consistent with the mode-of-action AOTcE induced upregulation of CD69 and CD25 on both CD4+ and CD8+ T cells, as well as release of IFN-γ by human T cells, in a time- and dose-dependent manner. In vivo activity of AOTcE was demonstrated in a model consisting of an implanted pancreatic cancer cell line expressing both Trop2 and CDH17 and re-constituted with human T cells. This novel targeting concept offers the opportunity to utilize the TcE mode-of-action in tumors for which no selective cell surface proteins are known.

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Target cell-restricted CD28 bispecific antibodies that sustain antitumor activity of CD3-bispecific antibodies and prevent T cell exhaustion

L. Zekri[1,2], N. Prakash[1,2], M. Engel[1], T. Manz[3], S. Müller[1], J. Heitmann[1,2], A. Henrich[1], I. Hagelstein[1], S. Hörner[1,4], M. Pflügler[1,4,5], G. Jung[3,4,5], H. R. Salih[1,2,5]

[1]CCU Translational Immunology - DKTK - DKFZ, Department of Internal Medicine, UKT, Tuebingen, Germany, [2]Cluster of Excellence iFIT (EXC 2180) "Image-Guided and Functionally Instructed Tumor Therapies", University of Tübingen, Tuebingen, Germany, [3]Institut of Immunology, University Hospital Tübingen, Tuebingen, Germany, [4]BiconY Therapeutics GmbH, Leipzig, Germany, [5]TWYCE GmbH, Tuebingen, Germany

Bispecific antibodies engaging CD3 (bsAbs) emerged as a promising class of therapeutics that effectively link tumor cells to T cells and trigger T cell activation through the so-called "T cell-signal 1" resulting in tumor cell destruction. Their therapeutic success is limited by T cell exhaustion induced by excessive TCR/CD3 receptor signaling, and lack of the costimulatory "signal 2" to sustain T cell function. Recently, we introduced a PSMAxCD3 bsAb termed CC-1 that was evaluated as monotherapy in patients with metastatic castration resistant prostate carcinoma (NCT04104607). Whereas CC-1 treatment substantially reduced PSA levels in all the heavily pretreated patients, the induced T cell activity was rather short-lived. Following an approach that has been introduced by our group already 30 years ago[1], we here report on a combinatorial approach using novel bispecific costimulators for activation of CD28 (BiCos) to sustain the antitumor reactivity of CD3-directed bsAbs including CC-1. Various bispecific formats containing different proprietary target binders were comparatively evaluated, and an optimal molecule showing superior affinity, good producibility, low aggregation tendency and, most importantly, exclusive target cell-restricted CD28 activity was selected and a lead construct directed to Endoglin was designated BiCo-1.

In line with the fact that physiologically CD28 stimulation alone does not induce T cell activation but is strictly dependent on the presence of T cell-signal 1, BiCo-1 induced neither therapeutic activity nor toxicity as single agent. In combination with CC-1, the antibody allowed for (i) increased selectivity of the induced T cell response for tumor cells due to targeting two different antigens with non-overlapping expression on healthy tissues, (ii) increased and sustained T cell proliferation, even at low CC-1 doses, (iii) long-lasting T cell memory formation, and (iv) significantly improved antitumor activity as documented in multiple in vitro assays as well as xenograft mouse models evaluating prevention of metastasis and eradication of large established tumors. GMP compliant production of BiCo-1 has meanwhile been completed, and a combinatorial clinical "first in man" study with CC-1 in prostate cancer patients will start recruitment in 2024. In addition, the preclinical development of two additional BiCo molecules targeting B7H3/CD276 and Fibroblast Activation Protein (FAP) and their functional characterization with bsAb directed to CD3 and various target antigens has been completed. We envisage that, in general, the combinatorial approach based on our BiCo format will allow for reduction of side effects while at the same time improving efficacy of T cell-based immunotherapy for solid tumors.

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Discovery of Highly Potent and Selective HPK1 Inhibitors for the Treatment of Cancer

Z. Zhang[1], L. Guo[1], T. Chen[1], H. Pan[1], Z. Zhang[1], L. Wang[1], M. Huang[2], J. Ma[2], D. Liu[1]
[1]Huadong Medicine Co., Ltd., Hangzhou, China, People's Republic of, [2]XtalPi Inc., Shanghai, China, People's Republic of

Background：
Despite great successes of immune checkpoint inhibitors, innate and therapy-induced resistance often limit the response rate and durability in many cancers. Therefore, new therapeutic strategies are urgently needed to overcome the limitations. Hematopoietic progenitor kinase 1 (HPK1), a negative feedback regulated kinase of the TCR, can overcome some problems in tumor immunotherapy, such as tumor heterogeneity or suppressive factors in the tumor microenvironment (TME) that weaken T cell effects. Thus, pharmacological inhibition of HPK1 has the potential to enhance effector T cell function and antitumor activity. Herein, our effort of identifying HPK1 inhibitors exhibiting excellent anti-tumor response is disclosed.

Methods：
In our study, small molecule inhibitors targeting HPK1 have been designed based on an artificial intelligence (AI) and structure-based drug design (SBDD) approach. Our small molecule inhibitor series based on a novel chemical core. HPK1 kinase activity was measured by the ADP-Glo assay. Pharmacokinetic studies using mice and rats were conducted at Huadong Meicine. Serial blood samples were analyzed by liquid chromatography−mass spectrometry (LC−MS)/MS. Pharmacokinetic parameters were calculated by noncompartmental analysis. The female BALB/c mice used for CT26 or MC38 syngeneic tumor models.

Results：
Utilizing AIDD and SBDD, a series of potent inhibitors of HPK1 was identified. Eventually, we identified a novel compound as an optimal HPK1 inhibitor for further studies. In Jurkat cells, our compound potently inhibited the phosphorylation of SLP76 at a concentration as low as 0.1 μM. The compound can enhance both T cell activation, reinvigorate exhausted T cells, and inhibit the suppressive capacity of T regulatory cells. Furthermore, our compound exhibited >30-fold selectivity against GLK in the MAP4K family, and weak inhibitory activity against kinases in other families at 1000 nM. It showed excellent PK properties with high oral bioavailability (F = 83.1% on female rats, 78% on female cynomolgus). In an in vivo pharmacodynamic evaluation in a tumor-bearing mouse model (CT-26 xenograft mouse model), the compound was shown to induce robust and statistically significant tumor growth inhibition with a TGI value >70% in combination with anti-PD-L1.

Conclusion
Herein we have reported drug discovery efforts resulting in the identification of a potent and selective HPK1 inhibitor with strong immune cell activation and robust anti-tumor activity in a mouse syngeneic tumor model, as a single agent and in combination with anti-PD-L1. In addition to excellent in vivo tumor activity, an acceptable non-clinical safety profile supported selection as a pre-clinical candidate. This compound is about to undergo IND-enabling study.

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Tetraspanin CD81 as a therapeutic target for treatment of Mozambican children diagnosed with B cell acute lymphoblastic leukemia

T. Babetine[1], A. Sigauque[2], E. Mongo[3], V. Maphossa[3], C. Mudenyanga[4], R. Matavele Chissumba[5]
[1]Instituto Nacional de Saude, Investigator, maputo city, Mozambique, [2]Instituto Nacional de Saúde, Investigação, Maputo cidade, Mozambique, [3]Instituto Nacional de Saude, Investigador, Maputo city, Mozambique, [4]Clinton health acess initiative, Maputo city, Mozambique, [5]Instituto Nacional de Saude, Investigadora, Maputo city, Mozambique

Background and aims:CD81 belongs to the tetraspanin family, is considered a marker of poor prognosis in patients with myeloid acute leukemia, associated with low response after chemotherapy and high mortality. Knockout of CD81 was associated to increased chemosensitivity in acute lymphoblastic leukemia. This study aims to assess the potential of CD81 as a therapeutic target for treatment of B cell acute lymphoblastic leukemia (B-ALL) in Mozambican children. **Methods:** The study included 36 patients from the Pediatric Hemato-oncology Service (SHOP) of Hospital Central de Maputo, aged between 0 and 14 years. Immunophenotyping analysis was performed on bone marrow samples at Instituto Nacional de Saúde using the EuroFlow complementary panel. Descriptive statistical analysis was performed to determine the frequency of expression of CD81. Median comparisons were performed using the Mann-Whitney test. **Results:** Of the 36 patients diagnosed with B-ALL, 17 (44%) were classified as a high-risk group and 22 (56%) were classified as a low-risk group. Expression of CD81 marker was observed in 92% of the B-ALL cases. We also observed that the proportion of CD81 expression on leukemic clone was higher in those from the standard-risk group compared to those from the high-risk group (p=0.003). Furthermore, that proportion was also high in those who died compared to those alive, at time of analysis (p=0.0077). **Conclusion**: We observed high rate of expression of CD81 marker on children diagnosed with B-ALL in Mozambique. Furthermore, in our cohort higher expression of CD81 was correlated to poor prognosis in B-ALL. These results might have implications on therapeutic strategies aiming to target CD81 for treatment of B-ALL in underrepresented African children.

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VEGF-Specific cellular response associated to a cancer therapeutics vaccine administration: From bench to bedside.

M. Bequet-Romero[1], Y. Morera[1], J. Sanchez[1], I. González[1], C. Canaan-Haden[1], F. Hernández[2], M. Ayala[1]
[1]Center for Genetic Engineering and Biotechnology, Biomedical Research / Pharmaceutics, Havana, Cuba, [2]Center for Genetic Engineering and Biotechnology, Clinical Research, Havana, Cuba

The Vascular Endothelial Growth Factor (VEGF) is a key player in tumor survival given its known functions in the induction of new vascular networks. HEBERSaVax is a vaccine based on a mutated variant of VEGF isoform 121 unable to bind VEGFR2. Immunization schedules based on this recombinant protein pursue the establishment of a cellular response that generates a T-cell mediated cytotoxicity that accompanies the B cell's secretion of VEGF-specific neutralizing antibodies. In the last ten years, the vaccine has been administered in the context of phase I/II clinical trials in two main formulations: (1) aqueous VSSP adjuvant

(Very Small Size Proteoliposomes from Neisseria meningitides membrane) in weekly schedules for up to 8 weeks (induction), and (2) adsorbed in a suspension of 0.7 mg of Al+ in the form of Alum Phosphate in bi-weekly schedules for the same induction period followed in both cases by monthly immunization. Herein we summarize the cellular response follow-up for the vaccine development from the preclinical to the clinical setting and offer a meta-analysis of the pool results of 4 clinical trials including 138 patients. The enrichment of the cellular arm of the immune response was characterized using Flow cytometry-based phenotyping of leucocytes and ELISPOT and FLUOROSPOT assays whenever possible for mice, Non-Human Primates, and Humans. Earlier results in mice indicate that a direct cytolytic response specific for syngeneic tumor cells can be observed a week after the induction cycle in splenocytes of 75-100% of CIGB-247 treated inbred mice. In a small cohort of non-human primates (n=9), cytolytic responses to autologous “charged” cells, were detected in 66% after the induction, and in all the animals after the booster. In the first four clinical trials for HEBERSaVax (n=138 total patients for advanced malignancies), the cellular response was assessed after week 13 (the first booster, n=73) and also after a year of treatment (n=26). Results indicate a 33% positive outcome for the patients with pair samples for the induction phase, while 50% of the pair samples analyzed after one year of treatment showed a significant increase in VEGF-specific IFN gamma response in PBMCs. So far HEBERSaVax is the only active immunotherapeutic intervention using VEGF as a target that has managed to induce VEGF-specific cellular response. Of higher importance, such response was associated in terms of its presence and intensity with the increment in overall survival of the patients within the clinical studies.

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Systemic markers for predicting and monitoring response to immune checkpoint blockade therapy in patients with advanced microsatellite-unstable gastrointestinal cancers

L. Bohaumilitzky[1,2], A. Ahadova[1,2], T. Walle[3], J. Kraske[3], M. Tariverdian[3], K. Heiss[4], D. Hildebrand[4], V. Stadler[4], U. Ganserer[5], I. Hausser[5], S. Haupt[6,7], V. Heuveline[6,7], J. Gebert[1,2], A. Stenzinger[8], D. Jäger[3,9], M. von Knebel Doeberitz[1,2], G. M. Haag[3,9], M. Kloor[1,2], E. Busch[3]

[1]Heidelberg University Hospital, Applied Tumor Biology, Heidelberg, Germany, [2]German Cancer Research Center (DKFZ), Clinical Cooperation Unit Applied Tumor Biology, Heidelberg, Germany, [3]National Center for Tumor Diseases Heidelberg, Heidelberg University Hospital, Medical Oncology, Heidelberg, Germany, [4]PEPperPRINT GmbH, Heidelberg, Germany, [5]Heidelberg University Hospital, EM Laboratory, Institute of Pathology, Heidelberg, Germany, [6]Interdisciplinary Center for Scientific Computing (IWR), Heidelberg University, Engineering Mathematics and Computing Lab (EMCL), Heidelberg, Germany, [7]Heidelberg Institute for Theoretical Studies (HITS), Data Mining and Uncertainty Quantification (DMQ), Heidelberg, Germany, [8]Institute of Pathology, Heidelberg University Hospital; German Cancer Consortium (DKTK) and German Cancer Research Center (DKFZ), Heidelberg, Germany, [9]German Cancer Research Center (DKFZ), Clinical Cooperation Unit Applied Tumor-Immunity, Heidelberg, Germany

Cancers presenting with microsatellite instability (MSI), which is associated with high immunogenicity mediated by frameshift peptide (FSP) neoantigens, are susceptible to immune checkpoint blockade (ICB) therapy. However, a relevant proportion of MSI cancer patients do not durably respond to ICB and reliable biomarkers for therapy response prediction as well as therapy monitoring are still lacking. In this study we analyzed systemic FSP-specific immune responses and MSI status of extracellular vesicle (EV) DNA for

predicting and monitoring treatment response in ICB-treated MSI cancer patients.

IFN-γ ELISpot and PEPperCHIP® peptide microarray were used to analyze systemic T cell and antibody responses against FSPs in 52 peripheral blood samples from 19 patients with advanced MSI gastrointestinal adenocarcinomas before and during ICB therapy. The applied FSP panel consisted of up to 40 recurrent FSPs (rFSPs) originating from putative driver mutations and with widespread occurrence in MSI tumors. Plasma samples from 18 patients and 30 healthy controls were utilized for precipitation-based EV isolation and EV DNA was analyzed using four standard diagnostic microsatellite markers.

Future ICB responders displayed significantly higher rFSP-specific T cell responses at baseline compared to non-responders. However, immune responses during therapy were not correlated with the patients' clinical course. In 7/10 patients the MSI phenotype was successfully identified in EV DNA obtained prior to therapy start. In six of those patients EV-associated MSI signals were lost during treatment, typically before the first staging at three months, which was generally associated with a favorable treatment response and an objective response in three patients.

Among patients with advanced MSI adenocarcinoma, future ICB therapy response was associated with higher rFSP-specific T cell responses before treatment initiation. Favorable response to ICB therapy was further associated with a switch from the EV-associated MSI status from MSI to non-MSI during treatment, warranting further exploration of EV DNA for liquid biopsy-based therapy monitoring.

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Personalized Viral Based Cancer Vaccine (NOUS-PEV) elicits potent neoantigen specific T cells response infiltrating the tumor

A. M. D'Alise[1], G. Leoni[1], G. Cotugno[1], L. Siani[1], R. Vitale[1], V. Ruzza[1], I. Garzia[1], L. Antonucci[1], E. Micarelli[1], V. Venafra[2], S. Gogov[3], A. Capone[1], S. Runswick[3], J. Martin-Liberal[4], E. Calvo[5], V. Moreno[6], S. N. Symeonides[7], E. Scarselli[1], O. Bechter[8]
[1]Nouscom Srl, Rome, Italy, [2]University of Rome "Tor Vergata", Department of Biology, Rome, Italy, [3]Nouscom AG, Basel, Switzerland, [4]Catalan Institute of Oncology (ICO), Barcelona, Spain, [5]START Madrid-CIOCC, Centro Integral Oncológico Clara Campal, Madrid, Spain, [6]START Madrid-FJD, Hospital Fundacion Jimenez Díaz, Madrid, Spain, [7]Edinburgh Experimental Cancer Medicine Centre, University of Edinburgh, Edinburgh, United Kingdom, [8]Leuven Cancer Institute, University Hospitals, Department of General Medical Oncology, Leuven, Belgium

Personalized cancer vaccines hold promise as immunotherapeutic approach based on their capability to elicit potent and durable tumor-specific immune response. Multi-neoepitope vaccines are often pursued as strategy to induce a more diversified antitumor T cell response and to overcome the issue of tumor heterogeneity. NOUS-PEV is a personalized viral based cancer vaccine targeting 60 patient specific neoantigens identified by next generation sequencing (NGS) and selected with a proprietary algorithm VENUS. Vaccine is administered intramuscularly, with an injection of the first vector (Great Ape Adenovirus, GAd20) as prime, followed by Modified Vaccinia Ankara (MVA) "boosts" vaccination, in combination with anti PD-1 Pembrolizumab. In this study (NCT04990479), we evaluated the feasibility, safety,

immunogenicity and anti-tumor activity of NOUS-PEV in combination with Pembrolizumab in 6 vaccinated patients with unresectable stage III/IV cutaneous melanoma. Vaccine manufacturing was feasible, with release and administration to six patients of 11 out of 12 vaccines within 8 weeks from biopsy collection to GAd20 administration, and safe. Immunogenicity was evaluated on peripheral blood mononuclear cells (PBMC) before and after vaccination by ex-vivo IFNγ ELISpot assay to detect neoantigen specific immune response. High magnitude vaccine-induced immune responses were elicited in all evaluable patients receiving the prime/boost regimen (n=4), with polytope responses measured against multiple peptides comprising of both CD4 and CD8 T cells. Expansion and diversification of vaccine-induced TCR clonotypes was observed in the post-treatment biopsies of patients with clinical response providing demonstration of tumor infiltration by neoantigen-specific T cell clones following vaccination. Moreover, gene signatures analysis of transcriptomic data obtained from pre-treatment biopsies were evaluated to identify biomarkers potentially associated with resistance to the combination treatment. Overall, this study demonstrated the feasibility of a novel personalized neoantigen-based vaccine that in combination with pembrolizumab was shown to be safe and able to activate tumor specific T cells trafficking to the tumor, supporting the use of this vaccine platform as a valuable therapeutic option.

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Prediction and identification of MHC class I presented T cell epitopes of the oncolytic virus VSV-GP in BALB/c mice

S. Danklmaier[1,2,3], S. V. Vijver[1,2], L. Pipperger[1,3,4], G. Floriani[5], R. Gronauer[5], H. Hackl[5], K. Das[6], G. Wollmann[1,2,3,4]

[1]Institute of Virology, Department for Hygiene, Microbiology and Public Health, Medical University of Innsbruck, Innsbruck, Austria, [2]Christian Doppler Laboratory for Viral Immunotherapy of Cancer, Medical University of Innsbruck, Innsbruck, Austria, [3]Tyrolean Cancer Research Institute (TKFI), Innsbruck, Austria, [4]Department of Internal Medicine V, Haematology & Oncology, Medical University of Innsbruck, Innsbruck, Austria, [5]Institute of Bioinformatics, Medical University of Innsbruck, Innsbruck, Austria, [6]ViraTherapeutics GmbH, Innsbruck, Austria

VSV-GP is a novel oncolytic virus (OV) platform that recently entered clinical phase I trials. The virus induced strong immune responses in preclinical studies. However, upon OV treatment not only antitumor but also antiviral T cells are activated. To be able to evaluate OV therapies, it is critical to distinguish antiviral and antitumor components of the immune responses. Therefore, we aimed to identify the antiviral $CD8^+$ T cells upon VSV-GP treatment in the widely utilized BALB/c mouse model using a multilevel adapted bioinformatic viral epitope prediction approach. Viral epitopes presented on mouse MHC-I alleles H2-Kd, H2-Dd and H2-Ld were identified using the ELISpot assay, where the IFN-γ secretion upon T cell activation is a measure. To validate the identified VSV-GP epitopes, $CD8^+$ T cells were further analyzed by using intracellular cytokine staining. In total eleven candidates significantly activated $CD8^+$ T cells in the BALB/c mouse model. Custom peptide-MHC-I multimers using the newly identified epitopes allow the direct detection of virus-specific T cells and therefore provide an additional tool to measure anti-VSV-GP T cell responses. Additionally, the identified epitopes were used to compare the antiviral T cell dynamic between intravenous and intratumoral treatment routes in the tumor and the periphery of BALB/c mice. Taken together, the identified epitopes enable monitoring of the full repertoire of antiviral T cells upon VSV-GP

oncolytic virotherapy in BALB/c mice. These findings contribute to preclinical development of novel VSV-GP variants by improving assessment of antiviral T cell immunity.

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Florescent Split-TCR reporter: A novel platform for high throughput investigation of T cell receptor function and dynamics

E. Fakhr[1], M. Volkmar[1], M. L. Wiegand[1], A. L. Gerstner[1], S. Klag[1], N. Halama[2], M. Platten[2,3], E. W. Green[3]
[1]TCR Discovery Platform, Helmholtz Institute for Translational Oncology (HI-TRON), Mainz, Germany, [2]Helmholtz Institute for Translational Oncology (HI-TRON), Mainz, Germany, [3]German Cancer Research Center,DKFZ, Heideberg, Germany

Adoptive T cell therapy with transgenic T cell receptors (TCRs) holds great promise for cancer therapy. Yet, while TCR discovery has seen quick progress in the past decade, e.g. by single-cell sequencing, functional TCR characterization is lagging behind and still experiences basic problems such as mispairing of exogenously expressed with endogenous TCR chains, high variability in functional assay results and high manual workload. To facilitate TCR functional characterization, we are developing a Jurkat-based reporter cell line and a novel split fluorescent protein-based assay system.
Jurkat E6.1 was used as the parental cell line and modified by CRISPR/Cas9. Following each modification, the knock-out/-in was confirmed by flow cytometry and PCR- and Sanger sequencing-based genotyping. We knocked out TRAC, TRBC1/2 and CD4, and introduced CD8A+B into the AAVS1 genomic safe harbor. The edited cell lines went through single cell expansion monitored by live cell imaging and displayed unchanged growth rate and medium requirements compared to the parental line.
In the next step, we designed a set of MART-1 and NY-ESO1 TCRs where the TCRa chain was joined to 1st-10th strand of beta barrel of mNeonGreen2 while TCRb chain carried the 11th beta sheet. Upon pairing of the TCR chains, the complex becomes fluorescent due to reconstitution of the beta barrel structure. We call this system ‘Split-TCR’. We first demonstrated that Split-TCRs become fluorescent only upon correct pairing of the TCR chains. Importantly, Split-TCRs are functional in that they are complexed with CD3 and able to trigger TCR signaling. We further used Split-TCR for live cell imaging and monitored the gradual reduction of mNeonGreen2 fluorescence upon contact with target. Moreover, we were able to visualize trogocytosis between the Split-TCR reporter cell line and antigen-presenting cells.
In this study, we have developed a novel engineered TCR coupled to a split-fluorescent protein and confirmed its functionality in a reporter cell line. By combining these two elements, we achieved a robust platform for the detection and monitoring of TCR activation and internalization dynamics which could be used for evaluating the interaction of transgenic T cells with their cognate antigens. Our findings highlight the synergistic potential of integrating reporter cell lines with novel TCR engineering to facilitate functional TCR characterization.

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Unraveling the dynamics of immunosurveillance: T cell responses and dysfunction in tumor development.

M. Faro Craveiro Nogueira[1], I. Martinez-Reyes[1], K. Hönig[1], A. Milojkovic[1], K. Anders[1], B. Obermayer[2], E. Belbaraka[1], M. T. Norcia[1], A. Dhamodaran[1], D. Beule[2], T. Blankenstein[1]
[1]Max Delbrück Center for Molecular Medicine, Berlin, Germany, [2]Berlin Institute of Health at Charité - Universitätsmedizin Berlin, Core Unit Bioinformatics, Berlin, Germany

Immunosurveillance, the ability of the immune system to detect and destroy malignant cells, is well-established for virus-associated tumors. However, immunosurveillance of spontaneous tumors is still controversial. A limitation to address this question has been the lack of cancer mouse models that resemble the sporadic nature of human cancer. To fill this gap, we developed a model that involves inoculating cancer cells in which antigenic oncogene expression and proliferation are triggered once the artificial transplantation-induced inflammation subsides. We used cancer cells with doxycycline (dox)-inducible expression of the tumor antigen and tumor driver gene SV40 large T antigen (Tag) fused to luciferase (TagLuc). Dox deprivation leads to growth arrest and loss of TagLuc expression in vitro. To investigate T cell responses against de novo-expressed Tag under non-acute inflammatory conditions, cells were deprived of dox and subsequently inoculated into mice subcutaneously as well as in visceral organs. After a resting period, mice were administered dox to induce TagLuc expression and proliferation. As control, cells growing on dox where inoculated into mice that received dox immediately after tumor injections (acute inflammatory conditions). In vivo imaging revealed that, while in immunodeficient mice tumors progressively grew, in young T cell competent mice, tumors were rejected in both acute and non-acute inflammatory conditions. Interestingly, old mice were able to reject tumors albeit with a significant delay. Tag expressing tumors were also rejected in mice lacking MHC class II which suggest that CD4 help is not necessary for mounting an anti-Tag immune response. Moreover, our data indicate direct priming of Tag-specific CD8 T cells by the cancer cells. Single-cell analysis of tumors at various stages revealed expanded TCR clonotypes with different phenotypes in rejected tumors. These results demonstrate immunosurveillance under resting conditions using a potent antigen with multiple MHC-I epitopes. However, most neoantigens result from a single amino acid exchange. Cancer cells expressing the neoantigen mutant p68 showed rejection if dox was never withdrawn (inflammatory conditions), but in sharp contrast to TagLuc, not anymore under resting conditions. These results suggest that CD8 T cells might have encountered mut-p68 but become dysfunctional. Overall, our model resembles pathophysiologic conditions allowing us to study the immune response to clinically relevant antigens. Our results provide a better understanding of the mechanisms underlying immunosurveillance or the inability of the immune system to control nascent tumors.

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Modelling human tumor antigen specific T cell responses in humanized mice for preclinical assessment of cancer immunotherapies

S. Gebhardt[1,2], M. Le Clech[1], A. Varol[1], S. Lang[1], C. Münz[2], J. Sam[1]

[1]Roche Innovation Center Zürich, Schlieren, Switzerland, [2]University of Zürich, Institute of Experimental Immunology, Zürich, Switzerland

Humanized immune system (HIS) mice reconstituted with human CD34+ hematopoietic stem cells (HSCs) provide a valuable platform for bridging preclinical findings to clinical applications in cancer immunotherapy research. However, a significant limitation of this model is the incomplete understanding of the T cell selection process. Human T cells are believed to be mainly selected on murine major histocompatibility complex (MHC) within the murine thymus. This species-specific education process leads to a fundamental mismatch when evaluating T cell responses against human tumor antigens, as the T cells are not optimally educated to recognize human-specific MHC-peptide complexes. This gap not only hinders the tracking of antigen-specific T cell responses but also precludes the evaluation of cancer immunotherapies that depend on such responses. To circumvent this challenge, we adopted and further optimized an innovative approach that enables the generation of T cells with human-specific antigen recognition capabilities within HIS mice. By transducing a subset of human CD34+ HSCs with a lentiviral vector encoding a T cell receptor (TCR) specific for the NY-ESO-1 antigen presented on HLA-A2.1, we successfully directed the development of T cells that are both naïve and capable of expressing the transgenic TCR. These T cells demonstrate the ability to recognize their cognate antigen ex vivo and proliferate in response to antigenic stimulation, thus confirming the functionality of the introduced TCR. Upon challenge with various HLA-A2.1-positive human tumor cell lines expressing NY-ESO-1, these HIS mice were able to convert lymphocyte excluded tumors into inflamed tumors, evidenced by either complete rejection or significant delay in tumor growth and an increase in infiltrating T cells. Interestingly, some tumor models continued to grow despite ongoing presence of tumor-reactive T cells and target antigen expression, offering the possibility to test novel cancer immunotherapies. Phenotypic analysis of the tumor-infiltrating lymphocytes (TILs) following tumor outgrowth provided insights into the effector states of the TCR-transgenic human T cells within the tumor microenvironment. The TILs comprised a diverse spectrum of differentiation and exhaustion states, including TCF-1+/PD-1+ precursor exhausted T cells (Tpex), which represent a key target population for cancer immunotherapies. As such, our model serves as a robust platform for testing cancer immunotherapeutic strategies that aim to mount tumor antigen-specific T cell responses in a human relevant environment. Consequently, our approach has the potential to refine therapeutic strategies, enhance the translational relevance of preclinical findings, and inform the design of clinical trials with improved outcomes in cancer immunotherapy.

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Immunomonitoring of adaptive NK cell and antigen-specific T cell dynamics in blood cancer patients post-hematopoietic stem cell transplantation

A.-E. Häkkinen[1,2,3], J. Huuhtanen[1,2,3,4], S. Lundgren[1,2,3], R. Niittyvuopio[5], H. Lähdesmäki[4], M. Keränen[1,2,3], M. Myllymäki[1,2,3], S. Mustjoki[1,2,3]

[1]University of Helsinki, Translational Immunology Research Program and Department of Clinical Chemistry and Hematology, Helsinki, Finland, [2]Helsinki University Hospital Comprehensive Cancer Center, Hematology Research Unit Helsinki, Helsinki, Finland, [3]iCAN Digital Precision Cancer Medicine Flagship, Helsinki, Finland, [4]Aalto University, Department of Computer Science, Espoo, Finland, [5]Helsinki University Hospital Comprehensive Cancer Center, Stem Cell Transplantation Unit, Helsinki, Finland

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) remains the only curative therapy for various blood cancers, but impaired immune cell reconstitution poses risks for complications, such as cytomegalovirus (CMV) reactivation. If left untreated, CMV infection can be lethal, but even less severe viremia profoundly shapes the immune system. The development of CMV-associated adaptive NK cells and anti-CMV T cells in vivo is still largely undescribed. Importantly, both NK and T cells are also known to partake in the graft-versus-leukemia effect. To characterize immune cell dynamics after allo-HSCT, we sequenced 32 longitudinal CD45+ sorted peripheral bloodsamples with single-cell RNA and T cell receptor sequencing (scRNA+TCRαβ-seq) from 5 allo-HSCT donor-recipient pairs (pre-HSCT sample from donor and recipient and follow-up samples over 3 months) and 6 healthy controls. All recipients underwent myeloablative conditioning and were CMV+, while 2 and 3 donors were CMV+ and CMV-, respectively. CMV viremia occurred in 4 patients, on average 32 days post-HSCT. Our analysis yielded a total of 197,887 immune cells. With Souporcell, we detected an average of 3,6% patient-derived cells persisting two weeks after transplant, with the share declining to only 0,5% on average at three months. Persisting patient-derived cells comprised mostly CD4+ T cell subsets, including cytotoxic CD4+ T cells. After transplant, adaptive NK cell expansion was observed in all patients, fueled by CMV reactivation. The proportion of adaptive NK cells out of CD45+ cells was higher post-viremia in 2 patients with CMV+ donors (18.9% and 7.4%), compared to 2 patients with CMV- donors (3.1% and 1.7%). All adaptive NK cells after viremia were donor-derived, and adaptive NK cells from CMV+ donors expressed higher levels of KLRG1, CD3E and FCGR3A/CD16, compared to those from CMV- donors. To determine most important viral epitopes, we screened 32 common viral epitopes from 3 patients post-transplant with scRNA+TCRαβ+pMHC-seq. $pp65_{NLV}$ was found to be the immunodominant CMV epitope; thus, we profiled T cells specific to $pp65_{NLV}$-multimer with TCRβ-seq (donor-recipient pairs n=5, healthy n=4), and matched CMV-epitope-recognizing TCRs in the scRNA+TCRαβ-seq data. We identified $pp65_{NLV}$-specific TCRs in all patients, with marked heterogeneity in clonotype abundance between patients. Phenotypically these cells were CD8+ $T_{EM/EMRA}$ cells. One patient was noted to have a 14-fold expansion of a donor-derived CMV-specific clone, which made up 64,0% of the patient's CD8+ TCR repertoire at the onset of CMV viremia. This manyfold clonal expansion was accompanied by upregulation of STAT1 and cytotoxicity markers PRF1 and GZMB compared to the same clone in the donor. In summary, our study improves understanding of NK cell and antigen-specific T cell dynamics in the unique setting of allo-HSCT and CMV reactivation, implicating donor CMV serostatus as a key factor shaping immune reconstitution.

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Characterization of the immunogenicity of human neoantigens in vivo

T.-J. Kuo[1], E. Belbaraka[1], I. Gavvovidis[1], A. Ben Hamza[2], N. Fellmer[3], M. Heichenko[3], L. Hansmann[2], T. Blankenstein[1]
[1]Max Delbrück center for Molecular Medicine in the Helmholtz Association, Berlin, Germany, [2]Charité – Universitätsmedizin Berlin, Department of Hematology, Oncology, and Tumor Immunology, Berlin, Germany, [3]T-knife Therapeutics, Berlin, Germany

Neoantigens have been implicated as target during effective cancer immunotherapy, e.g. by immune checkpoint inhibitors or adoptive T cell therapy. Additionally, vaccine trials started where patients are treated with a multitude of predicted neoantigens. Neoantigens, mostly single non-synonymous point mutations, are often defined by a high predicted peptide-MHC (pMHC) affinity, yet only a low percentage of predicted neoantigens are in fact immunogenic, e.g. processed and presented. Relative to tumor mutational load, tumor-infiltrating lymphocytes against only few neoantigens are typically detected. Since somatic mutations creating a neoantigen occur sequentially over an extended period of time, the question arises, whether immunodominance obscures neoantigen-specific T cell responses. As neoantigens are difficult to define, we argue that immunogenicity in the strict sense can only be analyzed in vivo. Here, we analyzed the immunogenicity of naturally occurring patient-individual cancer neoantigens, which had elicited specific CD8 T cell responses in the respective patients. Simultaneous expression of 16 neoantigens in a spontaneous, low-immunogenic tumor in mice with a diverse human T cell receptor (TCR) repertoire restricted to HLA-A*02:01 resulted in cancer cell rejection or selection of antigen-negative variants and effective T cell activation was shown in all mice. The CD8 T cell response was directed against one dominant and occasionally low responses to 2-3 other neoantigens. TCRs specific for the dominant neoantigen used a restricted set of Vb/Va-Ja gene segments, contained almost no nucleotide insertions, had short CDR3 and shuffling Vb/Va chains between individual T cell clones retained specificity as long as the Vb CDR3 contained a certain germline-encoded D-segment motif. Thus, T cells against the dominant neoantigen occurred at a high precursor frequency in the naïve repertoire, which may explain the immunodominance. The dominant neoantigen prevented T cell responses against inferior neoantigens. Further analysis of neoantigen-specific TCRs is ongoing.

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Adaptive immune response in primary uveal melanoma: tumor-specific T-cell infiltration and pattern of recirculation in the blood

A. I. Lalanne, F. Lucibello, A.-L. Le Gac, A. Soumare, S. Aflaki, C. Ekwegbara, C. Jamet, S. Gardrat, N. Cassoux, A. Matet, M.-H. Stern, M. Rodrigues, O. Lantz
Institut Curie, Paris, France

Uveal melanoma (UM), the most common cancer of the eye in adults, is characterized by recurrent chromosomal abnormalities. The loss of one copy of chromosome 3 (M3) together with the inactivation of the BRAC1-associated (BAP1) protein in the remaining chr3 is the hallmark of the most aggressive and rapidly metastatic tumors. As compared with low-risk disomic 3 tumors (D3), M3 tumors are more infiltrated by T lymphocytes and myeloid cells. This observation appears contradictory with other tumor

types where T cell infiltrate correlates with good prognosis. It is not known whether these infiltrating T cells represent an anti-tumor Antigen (Ag) response. As the eye is an immune privileged organ the way and site of T lymphocyte priming is unknown.

Herein, we characterized the T cells infiltrating primary UM tumors using multiparametric flow cytometry, tumor Ag tetramers, V(D)J-scRNA-seq and TCR repertoire analysis. We evidenced differences in the number and phenotype of the T cells according to the M3 vs D3 status of the tumor. CD8+ and Treg T cells were more abundant in M3 tumors than in D3 tumors and juxta-tumoral healthy tissues. $CD39^+PD\text{-}1^+CD8^+$ T cells were enriched in M3 tumors suggesting a tumor Ag specific T cell response, which was confirmed using HLA-A2 tetramer detecting the Melan-A tumor Ag. V(D)J-scRNA-seq analysis of 3 primary UM tumors evidenced a large number of TCR clonal expansions in proliferating $CD39^+PD\text{-}1^+CD8^+$ T cells suggesting in situ anti-tumor Ag responses. The number of Melan-A specific clones in the tumor bed varied a lot among the different patients: in all cases, large clones were present but smaller clones were also observed in some patients. Still, a more diversified anti-tumor response towards other tumor Ags is very likely as most expanded clonotypes belonging to cluster of proliferating $CD39^+PD\text{-}1^+CD8^+$ T cells were not labelled by the Melan-A tetramer. We also characterized the systemic T cell phenotype and looked for tumor Ag specific immune response in the blood. We characterized the recirculation pattern in the blood of the anti-tumor responses in both M3 and D3 tumors. By flow cytometry we observed an early tumor Ag-specific systemic response at the time of primary treatment in some patients. Using TCRseq, we found that $CD39^+PD\text{-}1^+CD8^+$ T cells were among the TILs with less counterparts in the blood, a pattern of tissue residency.

Altogether, our results suggest that a tumor Ag response occurs in UM tumors since their early stages of development raising the question of the priming mechanisms in the absence of known lymphatic drainage.

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Large scale identification, tracking and characterization of vaccine-induced neoepitope-specific T cell receptors in patients with IDH1-mutant glioma

K. Lindner[1,2], C. L. Tan[1,3], L. Bunse[1,4,5], E. W. Green[1,4], G. Haltenhof[1,4], A. Kurtz[1,2], I. Mildenberger[1,4], G. Tabatabai[6], W. Wick[7,8], I. Poschke[1,2], M. Platten[1,2,4,5,9]

[1]DKFZ Heidelberg, DKTK Clinical Cooperation Unit (CCU) Neuroimmunology and Brain Tumor Immunology, Heidelberg, Germany, [2]National Center for Tumor Diseases (NCT), Immune Monitoring Unit, Heidelberg, Germany, [3]MCTN Medical Faculty, Heidelberg University, Department of Neurology, Mannheim, Germany, [4]MCTN, Medical Faculty Mannheim, Heidelberg University, Department of Neurology, Mannheim, Germany, [5]DKFZ Hector Cancer Institute at the University Medical Center Mannheim, Mannheim, Germany, [6]University Hospital Tübingen, Department of Neurology and Interdisciplinary Neuro-Oncology, Tübingen, Germany, [7]University Hospital Heidelberg and National Center for Tumor Diseases (NCT), Neurology Clinic, Heidelberg, Germany, [8]DKFZ Heidelberg, DKTK Clinical Cooperation Unit (CCU) Neurooncology, Heidelberg, Germany, [9]Helmholtz Institute of Translational Oncology (HI-TRON), Mainz, Germany

The R132H mutation in isocitrate dehydrogenase type 1 (IDH1) is found in ~70% of WHO grade 2-3 gliomas, a class of brain tumors with limited therapeutic options. The mutation is an oncogenic driver, making it an attractive therapeutic target. Ten years ago, our lab first described a long peptide vaccine targeting

IDH1R132H, that was shown to be presented on MHC II molecules and which moreover induced a robust T helper cell response. On the basis of these findings, two clinical trials (NOA16 and NOA21) were initiated, with NOA16 demonstrating both safety and immunogenicity of the vaccine.

The ongoing phase 1 “window-of-opportunity” clinical trial NOA21 assesses safety, tolerability and immunogenicity of the long IDH1R132H peptide vaccine alone and in combination with Avelumab. It allows for in-depth characterization of the vaccine-induced intratumoral immune response: since only patients with clinically warranted re-resection of a recurrent IDH1R132H-mutated glioma are recruited, single cell analysis of immune infiltrates from on-treatment tumor tissues is feasible.

Using peptide-based expansion cultures and the blood of 17 vaccinated patients, we classified 1423 T cell receptors (TCRs) as candidate IDH1R132H-reactive TCRs. A representative subset of 120 TCRs from seven patients was selected for in vitro validation, of which reactivity of 104 TCRs (87%) was confirmed. TCR tracking suggests that all TCRs were induced by vaccination and showed IDH1R132H reactive TCRs to be enriched in post-vaccination tumor tissue as compared to peripheral blood. The kinetics of the cumulative frequencies of IDH1R132H-reactive T cells over time matched those observed in ELISpot assays using PBMCs, indicating that the peptide-based expansion assay allowed to select the most relevant T cell populations. IDH1R132H-reactive T cells isolated from on-trial tumor tissue showed a distinct gene expression profile compared to bystander CD4+ T cells, characterized by expression of activation markers such as CD40 ligand.

Analysis of the HLA-restriction of all 93 IDH1R132H-reactive TCRs using autologous immortalized B-lymphoblastoid cell lines revealed that 72% were restricted to HLA-DR alleles, 28% to HLA-DQ alleles, but none to HLA-DP. These findings suggest promiscuous peptide binding and explain T cell responses across a wide range of HLA alleles in both NOA16 and NOA21 clinical trials. Affinity titration of 21 selected TCRs from two patients revealed that vaccine-induced IDH1R132H-reactive T cells show a broad range of affinities. A subset of these CD4(+)-derived TCRs shows very strong affinity to pMHC, enabling them to work in a co-receptor independent fashion in CD8+ TCR-transduced T cells in vitro.

We were able to rapidly identify and validate reactivity of 104 TCRs, improving our understanding of the mechanism of action of the IDH1R132H long peptide vaccine and supporting design of future vaccine or cell-based therapeutic approaches for patients with IDH1-mutant gliomas.

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EO2401, a novel immunotherapy, plus nivolumab induces a strong and robust immune response in patients with recurrent glioblastoma

A. Maia dos Santos Leite[1], H. Toussaint[2], J. Gamelas[2], A. Idbaih[3], M. Vieito[4], G. Tabatabai[5], A. Stradella[6], F. Ghiringhelli[7], M. Burger[8], I. Mildenberger[9], U. Herrlinger[10], M. González[4], A. Hervieu[7], M. GilMartin[6], M. Renovanz[5], M. Touat[3], P. Wen[11], A. Wick[12], C. Bonny[2], J.-M. Paillarse[2], D. Reardon[11], W. Wick[12], C. Gouttefangeas[1]

[1]Institute for Immunology, University of Tübingen, Tübingen, Germany, [2]Enterome, Paris, France, [3]Hôpital Universitaire La Pitié-Salpêtrière, Paris, France, [4]Hospital Universitari Vall d'Hebron, Barcelona, Spain,

[5]Universitätsklinikum Tübingen, Tübingen, Germany, [6]Institut Catala D'Oncologia, Hospital Duran i Reynals, Barcelona, Spain, [7]Centre Georges-François Leclerc, Dijon, France, [8]Universitätsklinikum Frankfurt Goethe-Universität, Frankfurt, Germany, [9]Medizinische Fakultät Mannheim, Mannheim, Germany, [10]Universitätsklinikum Bonn, Bonn, Germany, [11]Dana-Farber Cancer Institute, Boston, United States of America, [12]Universitätsklinikum Heidelberg, Heidelberg, Germany

Introduction

EO2401 is a novel generation of peptide cancer immunotherapies designed to activate memory commensal-specific T cells that cross-react with tumor-associated antigens (TAAs). Three synthetically-produced HLA-A2 peptides with molecular mimicry to commonly upregulated TAAs in glioblastoma (GBM) (IL13RA2, BIRC5 and FOMX1) are included in EO2401, as well as a CD4 helper peptide (UCP2). The study (NCT04116658) was approved by all participating institution's Ethics Boards.

Methods

Patients with GBM at first progression after standard treatment received EO2401 with nivolumab ± bevacizumab. Blood was collected at baseline, every two weeks in the first 5 months, then every month until disease progression. Immune response was evaluated ex vivo and after in vitro stimulation (IVS) in cryopreserved PBMCs using tetramer staining, IFNγ ELISpot and intracellular cytokine staining (ICS).

Results

T cells against the commensal peptides were observed in 93% of tested patients (n=55) ex vivo after EO2401 administration based on tetramer staining. Commensal-specific T cells could be readily detected after a single dose of EO2401 in 42% of evaluated patients (n=24), showing an early response by EO2401. Out of the three HLA-A2 peptides, two were highly immunogenic – EO2317 and EO2318 – with 90% (maximal response: 15.4% of $CD8^+$ T cells in tetramer staining) and 85% (maximal response: 3.7%) of responding patients, respectively. Importantly, cross-reactivity against homologous-TAA (BIRC5 for EO2317, FOXM1 for EO2318) was detected in 100% and 48% of tested patients, respectively. Ex-vivo IFNγ ELISpot validated the functionality of these cells with 90% of tested patients (n=21) showing IFNγ-producing T cells against the pool of commensal-derived peptides, and 56% and 22% reacting against two or three of these peptides, respectively. 76% of the patients also showed ex vivo responses against UCP2. Additional flow cytometry-based analyses demonstrated an effector memory phenotype (T_{EM} and T_{EMRA}) of the antigen-specific $CD8^+$ T cells (>90%). Also, expression of the late activation marker CD57 increased over time. However, no increase in PD-1 or LAG-3 was observed, hinting that these cells are not exhausted. Supporting this observation is the fact that vaccine-specific T cells were strongly expanded and produced high levels of cytokines after IVS. Finally, long-term analyses revealed a sustained T cell response of almost one year after the first vaccination.

Conclusion

Together, our data shows that EO2401 with the support of nivolumab can induce fast, strong and sustained T cell responses in patients. Commensal-derived peptides with molecular mimicry to TAAs lead to the expansion of commensal-specific $CD8^+$ T cells which cross-react with homologous TAAs. This is a promising approach to generate broad immune responses in tumors with low neoantigen expression and poor T cell priming, as is the case of GBM.

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Phase I trial of personalized multi-peptide vaccines combined with the TLR1/2 ligand XS15 under Bruton-Tyrosine-Kinase inhibitor (BTKi) treatment in CLL patients

Y. Maringer[1,2], M. Wacker[1,2], J. S. Heitmann[2,3], A. Nelde[1,2], S. Jung[1,2,3,4], C. Hackenbruch[3,4], L. Zieschang[1,2,4], C. Kammer[1,2,4], M. T. Özbek[1,2], M. Richter[1,2,4], M. Denk[1,2,4], H. R. Salih[3,4], J. S. Walz[1,2,3,4]

[1]University Hospital Tübingen, Department of Peptide-based Immunotherapy, Tübingen, Germany, [2]Cluster of Excellence iFIT (EXC2180) "Image-Guided and Functionally Instructed Tumor Therapies", Tübingen, Germany, [3]University Hospital Tübingen, Clinical Collaboration Unit Translational Immunology, Department of Internal Medicine, Tübingen, Germany, [4]German Cancer Consortium (DKTK) and German Cancer Research Center (DKFZ), partner site Tübingen, Germany

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults. Treatment of CLL has greatly improved since the introduction of targeted substances like the Bruton-Tyrosine-Kinase inhibitors (BTKi). Nevertheless, the survival of residual CLL cells frequently causes disease relapse, calling for novel therapeutic options to eliminate minimal residual disease. Here, we report on the preliminary results of a Phase I trial evaluating a warehouse-based personalized multi-peptide vaccine adjuvanted with the toll-like receptor (TLR) 1/2 agonist XS15 emulsified in Montanide ISA51 VG in CLL patients under BTKi treatment (NCT04688385, iVAC-XS15-CLL01).

The peptide warehouse comprising a total of 12 high-frequent CLL-associated antigens (9 HLA class I epitopes, restricted to the common allotypes HLA-A*02, HLA-A*24 and HLA-B*07, and 3 HLA class II epitopes) was designed using mass spectrometry-based immunopeptidome analyses of a large cohort of primary CLL samples. In the trial, personalized vaccine cocktails are selected from this warehouse based on the allotype and, if available, on the immunopeptidome of the individual patients. Twenty CLL patients who have achieved at least partial remission upon BTKi treatment are administered three personalized vaccine doses in a monthly interval.

All twenty patients (median age 59 years, male:female ratio: 2.3 to 1) have been recruited and fully vaccinated. At least one warehouse peptide could be identified as naturally presented in the individual immunopeptidome of the 16 analyzable study patients (median 3, range 1-5). The expected local granuloma formation at the vaccination site was observed in 100% of patients, which enables continuous local stimulation of CLL-specific T cells without relevant systemic inflammation. Immunogenicity analyses revealed T-cell responses in all patients (15/15) analyzed to date after the third vaccination, as well as in 13/15 already after the second vaccination. T cell responses were directed against multiple vaccine peptides (47% CLL-associated HLA-class I epitopes, 94% HLA-class II epitopes) and persisted in all patients until the six-month follow up. The vaccine induced responses were mediated by both $CD4^+$ and $CD8^+$ T cells that produced multiple cytokines, including IFN-g, TNF and IL-2, as well as upregulated the degranulation marker CD107a.

In conclusion, the preliminary results of this Phase I trial in CLL patients show a beneficial safety profile and induction of profound and long-lasting $CD4^+$ and $CD8^+$ T-cell responses targeting multiple CLL-associated epitopes following vaccination with our personalized peptide vaccine adjuvanted with XS15.

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GLP-compliant FluoroSpot validation to monitor antigen vaccinations in preclinical mouse models

A. Mauthe, R. Villar-Hernández, M. Springer, R. Preyer, M. Gutekunst
Genome Identification Diagnostics GmbH, Immune Analytics, Strassberg, Germany

Introduction:
While tumor vaccines increasingly target personalized neoantigens, the need for preclinical testing with regard to neoantigen selection, optimization of vaccine formulations or adjuvant development to obtain optimized individual immune responses also increases. The FluoroSpot assay enables the quantification of antigen-specific immunity on a single-cell level. Due to the biologically dynamic setup of using living immune cells, a standardization is necessary to abide by the international regulatory requirements for GLP-compliant (pre-)clinical sample analysis.
In this context, we performed a generic mouse FluoroSpot validation with IFN-γ as model cytokine for potential multiplexing using splenocytes derived from naïve C57BL/6 mice vaccinated with an immunogenic peptide (SIINFEKL/OVA257-264) as a potent model antigen.

Methods:
Spleens came from a preclinical adjuvant development project conducted by Synovo GmbH (Tübingen, Germany). Naïve C57BL/6 mice were vaccinated with OVA257-264 peptide (± adjuvant) with boost vaccinations on day 7 and 14 and termination on day 15 or 21, respectively. Control animals received adjuvant only.
After splenocyte isolation, FluoroSpot analysis was done to quantify antigen-specific IFN-γ secreting cells. Splenocytes were plated in triplicates including negative control (NC, medium), mock control (AP, actin peptide pool), positive control (PC, Pokeweed) and OVA257-264 peptide to assess inter- and intra-assay precision in three runs performed by three operators. Moreover, assay linearity, specificity, minimum PC response, limit of detection (LOD), lower and upper limit of quantification (LLOQ, ULOQ) were determined.

Results:
The following assay parameters were determined: a) a LOD of 6 spots per well (NC) or 7 spots per well (AP), respectively, b) a LLOQ of 7 spots per well, c) an ULOQ of 384 spots per well, d) a minimum PC response of 50 spots per well.
All predefined acceptance criteria were met. Regarding splenocyte viability, all samples were ≥ 50% upon overnight resting. For intra-assay and inter-assay precision, 5 of 5 samples ≥ LLOQ and 6 of 6 samples ≥ LLOQ showed a CV ≤ 30%, respectively. In terms of linearity, the analyzed samples showed $R^2 \geq 0.9$ for cell counts between 100,000 and 300,000 per well (spot counts ≥ LLOQ). Regarding specificity, all PC were > LOD and 17 of 18 AP were ≤ LOD. Moreover, in the OVA257-264 + adjuvant-treated group, all samples were > LLOQ and the stimulation index was ≥ 3 whereas in the control group, all samples were < LOD.

Conclusion:
The validated IFN-γ FluoroSpot is suitable for the detection of antigen-specific immune responses in a preclinical mouse model fulfilling regulatory requirements for bioanalytical assays and method validation. A validation study with a neoantigen peptide pool for an established mouse tumor cell line (CT26) is currently under way.

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Mapping T cell signatures associated to response to neoadjuvant chemotherapy in colorectal liver metastasis.

M. Mayoux[1], F. Wiesmann[2], G. Litscher[1], M. Vermeer[1], S. Dergun[1], K. Long[1], A. Karimi[1], S. Burr[1], N. Nunez[3], T. Wertheimer[1], E. Friebel[4], C. Sparano[1], F. Mair[5], B. Becher[1], B. Sobottka-Brillout[2], S. Tugues[1]
[1]University of Zurich, Institute of experimental Immunology, Zurich, Switzerland, [2]University Hospital Zurich, Institute for Pathology and Molecular Pathology, Zurich, Switzerland, [3]Universidad Nacional de Córdoba, Department of Clinical Biochemistry, Córdoba, Argentina, [4]Charite universitatsmedizin Berlin, Institute of Neuropathology, Berlin, Germany, [5]ETH Zurich, Institute of Molecular Health Sciences, Zurich, Switzerland

Colorectal cancer ranks among the most prevalent malignancies worldwide, and frequently progress into colorectal cancer liver metastasis (CRLM), resulting in a 5-year survival rate ranging from 30 to 50%. For those patients, partial hepatectomy is the therapeutic approach with the highest success rate, but the eligibility for surgery is restricted to merely 20% of patients. Neoadjuvant chemotherapy (NAC) is used as standard of care to facilitate the feasibility of curative surgery, as it can convert up to 30% of initially unresectable CRLMs into resectable metastases. However, the underlying reasons of why some patients fail to benefit from neoadjuvant treatment are currently unknown. The success of immunotherapeutic interventions hinges upon the presence and functional activity of infiltrating lymphocytes, which is tightly governed by the metastatic microenvironment. In this study, we collected CRLM samples from patients treated with NAC and studied the unicity of three spatially distinct patient-matched regions: the metastatic nodule, the invasive margin, and the distal liver tissue. Using high dimensional flow cytometry, we revealed distinct immune landscapes characteristic of each region. As such, we observed a scarcity of cytotoxic lymphocytes in the metastatic nodule, concomitant with an enrichment of exhausted, regulatory and memory T cells. Subsequent correlations of these immune features with NAC response revealed that patients with a high tumor burden and low fibrosis exhibited a more suppressive/exhausted lymphocytic landscape. Conversely, metastases that regressed in response to NAC were highly infiltrated by specific subsets of memory T cells. The integration of whole transcriptome analysis with proteomics revealed distinct immune signatures for these memory T cell subsets, emphasizing mechanisms for enhanced anti-metastatic activity. Our findings provide valuable insights into the biology of CRLM and unveil immune signatures associated with response to NAC. Based on this study, we suggest new therapeutic strategies to enhance NAC efficacy in CRLM management.

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Evaluation of the immune response using TCR repertoire analysis in patients with non-small cell lung cancer treated with a PDC*line cell-based cancer vaccine in combination or not with an anti-PD-1

S. Michel[1], A. Sibille[2], E. Pons-Tostivint[3], I. Demedts[4], S. Derijcke[5], K. Cuppens[6,7], C. Duchayne[1], H. Gazon[1], P. Alves[1], M. Devos[8], C. Debruyne[1], B. De Vos[1], J. Vansteenkiste[9], J. Carrasco[8,10], J. Plumas[11]
[1]PDC*Line Pharma, Liege, Belgium, [2]University Hospital of Liège, Department of Pulmonology and Thoracic oncology, Liege, Belgium, [3]University of Nantes, Medical Oncology, Nantes, France, [4]AZ Delta, Department

of Pulmonary Diseases, Roeselare, Belgium, [5]AZ Groeninge, Department of Pulmonology and Thoracic oncology, Kortrijk, Belgium, [6]Jessa Hospital, Department of Pulmonology and Thoracic oncology and Jessa and Science, Hasselt, Belgium, [7]UHasselt, Faculty of Medicine and Life Sciences, Diepenbeek, Belgium, [8]Seqalis, Gosselies, Belgium, [9]University Hospitals KU, Department of Respiratory Oncology, Leuven, Belgium, [10]Grand Hopital de Charleroi, Department of Pulmonology and Thoracic oncology, Charleroi, Belgium, [11]PDC*line Pharma, R&D, La Tronche / Grenoble, France

In the ongoing Phase I/II NCT03970746 clinical trial, the therapeutic cancer vaccine PDC*lung01 (based on an irradiated plasmacytoid dendritic cell line loaded with 7 HLA-A*02:01-restricted tumor peptides) is being evaluated in the treatment of HLA-A*02:01+ lung cancer patients in monotherapy or in combination with an anti-PD1 immune checkpoint inhibitor (pembrolizumab). PDC*lung01 is injected intravenously and subcutaneously for 6 times at two dose levels, 14 million, or 140 million cells in resected stage II/IIIA (cohorts A1 & A2; monotherapy) or stage IV NSCLC patients (cohorts B1 & B2; in combination with pembrolizumab). Blood samples were obtained before the treatment and one week, 4 weeks and 10 weeks after the last injection. Beside the assessment of antigen-specific T-cells among CD8+ population, we sequenced the CDR3 region of the TCRβ gene of CD8+ populations purified at different timepoints. In addition, as a control, T cells specific against influenza or EBV were sorted and subjected to TCRβ sequencing. TCRβ sequencing is based on NGS libraries incorporating unique molecular barcodes (UMIs) before targeted amplification, allowing correction for technical bias and absolute quantification of each TCRβ rearrangement transcripts sequenced and therefore of the abundance of each T cell clonotype present in the population. Generally, at least 200,000 CD8+ T-cells were used in that study. We present here, the strategy and results of the TCRβ repertoire immunomonitoring of patients treated in A1 and B1 cohorts representing 12 patients and 46 timepoints. To characterize the different repertoires obtained, we first compared their diversity based on Hill diversity measures (richness, Shannon and Simpson diversity). We also compared their evenness using the Pielou's index. These assessments did not reveal any clear trends in the evolution of the T cell repertoire over time. However, more detailed analyses allowing individual monitoring of each T cell clonotype, such as pairwise comparison of T cell repertoires after standardization of the number of TCRβ transcripts compared, or monitoring of the Top 100 clonotypes, revealed the expansion of some T cell clonotypes during treatment. To focus on most significant changes, we monitored those for which the expansion was at least 10x relative to the baseline with a significance level of $p<10^{-6}$. This strategy enabled us to clearly identify some CD8+ T cells clonotypes presenting significant variations over time in the patients assessed. We will present this strategy and the characterization of this evolution in patients receiving PDC*lung01 in combination or not with pembrolizumab.

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Immunophenotypic diversity and prognostic significance of CD3 and CD117 expressions in pediatric acute myeloid leukemia

E. Mongo[1,2], F. Amod[3], A. Sigaúque[1], V. Maphossa[1], O. Guiliche[1], V. Monteiro[1], T. Babetine[1], J. Mutchamua[3], F. Matola[3], A. Dias[3], E. Granado[4], D. Mutsaka[5], C. Mudenyanga[5], R. Matavele Chissumba[1]
[1]Instituto Nacional de Saúde, CISPOC, Maputo, Mozambique, [2]Universidade Católica de Moçambique, Public Health, Maputo, Mozambique, [3]Hospital Central de Maputo, Hematoncology, Maputo, Mozambique,

[4]Instituto Nacional de Cancer, Brasil, Brazil, [5]Clinton Health Access Initiative, Maputo, Mozambique

Introduction: Acute Myeloid Leukemia (AML) presents a complex landscape characterized by diverse immunophenotypic profiles. Among these, the expressions of CD3 and CD117 have garnered particular interest due to their potential prognostic significance and therapeutic implications.
Objective: This study aimed to analyze the immunophenotypic diversity of AML, focusing specifically on the expressions of CD3 and CD117, and to assess their correlation with patient prognosis.
Methodology: We conducted a retrospective analysis of 106 pediatric acute leukemia cases, specifically focusing on 30 AML cases. Immunophenotypic profiling was performed using flow cytometry to assess the expression of key biomarkers Patient survival data were correlated with marker expression using statistical analysis.
Results: Notably, 85% of the AML cases expressed Myeloperoxidase (MPO). The immunophenotypic diversity observed underscored the complexity of AML, with 15% of cases expressing CD64, 37% displaying CD33, 26% exhibiting CD123, and 33% showing positivity for CD38. We found that 70% of patients succumb to the disease. We observed a significant correlation between lifetime and the expression of one of the markers mCD3 (p=0.01, r=0.66) or CD117 (p=0.03, r=0.59). Patients expressing CD3 or CD117 markers exhibit a median lifespan of 528 days, compared to those who do not present this profile, whose median lifespan is around 36 days (p=0.009).
Conclusion: This study sheds light on the immunophenotypic intricacies of pediatric AML in Mozambique, emphasizing the diverse diagnostic significance of MPO and unveiling potential prognostic markers. The observed associations between the combination expression of mCD3 or CD117 and extended survival open avenues for further research and may pave the way for personalized therapeutic strategies, ultimately improving outcomes for Mozambican children battling AML.

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Serum NY-ESO-1 and XAGE1 antibodies are potentially predictive and immunomonitoring markers of response to immune checkpoint therapy in non-small cell lung cancer

M. Oka[1], K. Kurose[2], H. Taniguchi[3], S. Takemoto[3], M. Fukuda[4], N. Sugasaki[4], M. Fukuda[5], T. Masuda[6], N. Hattori[6], K. Shimizu[7], M. Nakata[7], H. Mukae[3], T. Oga[2]
[1]Kawasaki Medical School, Immuno-Oncology, Kurashiki, Japan, [2]Kawasaki Medical School, Respiratory Medicine, Kurashiki, Japan, [3]Nagasaki University Hospital, Respiratory Medicine, Nagasaki, Japan, [4]Nagasaki Prefecture Shimabara Hospital, Respiratory Medicine, Shimabara, Japan, [5]The Japanese Red Cross Nagasaki Genbaku Hospital, Respiratory Medicine, Nagasaki, Japan, [6]Hiroshima University Hospital, Respiratory Internal Medicine, Hiroshima, Japan, [7]Kawasaki Medical School, General Thoracic Surgery, Kurashiki, Japan

Introduction: Immune checkpoint inhibitor (ICI) therapy is the standard care of non-small cell lung cancer (NSCLC). Although tumor PD-L1 levels are used as predictive markers of ICI therapy response, tumor PD-L1 assays require sufficient tumor tissues and are rarely repeatable. On the other hand, TLS with T and B cells increase ICI responsiveness in various cancers. Since B cells activated within TLS produce antibodies (Abs) against cancer antigens, NY-ESO-1/XAGE1 Abs are B-cell activation markers. We previously reported that NY-ESO-1/XAGE1 Abs predicted clinical benefit from ICI monotherapy, and the Abs were detected in about 25% of NSCLC. Thereafter, we developed an Automated Immunoassay System HISCL measuring NY-ESO-

1/XAGE1 Abs. Here, we applied the Abs using our immunoassay for predicting the benefit. Additionally, we serially monitored NY-ESO-1/XAGE1 Abs during ICI therapy.
Patients and Methods: Cohort 1) The cutoff value (10 units/mL) of NY-ESO-1 and XAGE1 Ab in our immunoassay was determined. The Abs were measured before nivolumab monotherapy in 99 previously treated NSCLC patients, including 21 with EGFR, ALK, or KRAS alterations. Differences in PFS and OS between the Ab-positive and -negative groups were retrospectively analyzed using Cox regression analysis after applying inverse probability of treatment weighting (IPTW). **Cohort 2)** NY-ESO-1 and XAGE1 Abs of IgG and IgA isotype were measured by ELISA during ICI therapy. XAGE1 protein in tumors and NSCLC cell lines with driver genes was detected by IHC and immunoblot, respectively.
Results: Result 1) NY-ESO-1/XAGE1 Abs were positive in 28 NSCLC, who responded more highly to nivolumab than the Ab-negatives (ORR 50.0% vs. 15.5%, $p < 0.0007$). The Ab-positives showed significantly longer IPTW-adjusted PFS (HR = 0.59, 95% CI: 0.39-0.90, p = 0.014) and IPTW-adjusted OS (HR = 0.51, 95% CI: 0.32-0.81, p = 0.004) than the Ab-negatives. Among NSCLC with driver genes, the Ab-positives (n = 10) showed significantly longer PFS (HR = 0.34, 95% CI: 0.13-0.89, p = 0.029) and OS (HR = 0.27, 95% CI: 0.098-0.75, p = 0.012) than the Ab-negatives (n = 11). **Result 2)** NY-ESO-1 and XAGE1 IgG-Abs were decreased and increased corresponding to clinical responses during ICI therapy, and the IgA-Abs were increased with disease progression. Five patients (3 EGFR, 2 ALK) of 12 with EGFR/ALK alterations had XAGE1 Ab with XAGE1 expression among available specimens. These five patients showed two CR, one PR, one SD, and one PD in ICI therapy after targeted therapy. Interestingly, XAGE1 protein was expressed in 8 of 9 cell lines with driver genes (EGFR/ALK/ROS1) and increased in targeted-drug resistant cells.
Conclusions: Our immunoassay for NY-ESO-1/XAGE1 Abs is probably useful for predicting the clinical benefit from nivolumab in NSCLC, including those with driver genes, and it maybe also in first-line ICI monotherapy. Additionally, NY-ESO-1/XAGE1 Ab levels are probably immunomonitoring markers of ICI therapy response.

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Characterization of neoantigen-specific T cells induced by personalized peptide vaccination within the clinical trial IVAC-ALL-1 in pediatric patients with relapsed acute lymphoblastic leukemia

A. Rabsteyn[1,2], C. Mohr[3], J. Matthes[4], O. Witt[5], R. Meisel[6], C. Chen-Santel[7], T. Feuchtinger[8], C. Schröder[4], N. Casadei[4], M. Denk[2,9], C. Seitz[1], A. von Stackelberg[7], T. Milde[5], S. Stevanovic[2,9], O. Rieß[4], O. Kohlbacher[3], H.-G. Rammensee[2,9], R. Handgretinger[1], S. Pfister[5], J. Schulte[1], P. Lang[1,2]
[1]University Children's Hospital Tübingen, General Paediatrics, Oncology/Haematology, Tübingen, Germany, [2]German Cancer Consortium (DKTK) and German Cancer Research Center (DKFZ), Partner Site Tübingen, Tübingen, Germany, [3]University of Tübingen, Institute for Applied Bioinformatics, Tübingen, Germany, [4]University of Tübingen, Institute of Medical Genetics and Applied Genomics, Tübingen, Germany, [5]Heidelberg University Hospital, Pediatric Hematology and Oncology, Heidelberg, Germany, [6]Clinic for Pediatric Oncology, Hematology and Clinical Immunology, Heinrich-Heine-University Düsseldorf, Division of Pediatric Stem Cell Therapy, Düsseldorf, Germany, [7]Charite University Medicine Berlin, Pediatric Oncology and Hematology, Berlin, Germany, [8]Dr. von Hauner'sches Children's Hospital, LMU Munich, Pediatrics, München, Germany, [9]University of Tübingen, Institute for Cell Biology, Department of Immunology, Tübingen, Germany

Acute lymphoblastic leukemia (ALL) is the most common pediatric malignancy. Although standard treatment cures most patients, about 20% of patients develop a disease relapse. Patients with recurrent disease have a dismal prognosis and limited treatment options. Prevention of relapse after first-line chemotherapy or stem cell transplantation is therefore an urgent clinical need. We established a platform for the design of patient-individual peptide vaccination cocktails by combination of whole exome sequencing of tumor and normal tissue within silico epitope prediction algorithms. We started clinical translation of this approach in a phase I/II clinical trial in 2016 (NCT03559413). Besides feasibility and toxicity assessments, we aimed to assess the capability of the peptide vaccination to induce neoantigen-specific T cell responses. Vaccine cocktails consisting of 3-5 individual peptides and a control wildtype peptide derived from the antigen Survivin were produced and formulated under GMP conditions. The vaccination schedule was 16 vaccinations over 33 weeks using GM-CSF and Imiquimod as adjuvants. Response to the vaccination was monitored by detection of vaccine-induced neoantigen-specific T cells in peripheral blood of patients. The rationale for this approach was targeting of residual tumor cells after salvage therapy by tumor-specific T cells to prevent leukemic relapses.
In total, 28 patients were recruited to the trial, whole exome sequencing was completed for 24 patients. 15 patients were vaccinated, as planned in the trial design. The vaccine was generally well tolerated and only mild side effects were observed. Immune monitoring was performed by prestimulation with vaccine peptides and subsequent intracellular cytokine staining and assessment of polyfunctional T cells. Immunomonitoring revealed sustained T cell responses against vaccinated peptides in 14/15 vaccinated patients. Peptide-specific T cell responses were of CD4+ and CD8+ subtype. 14/15 patients of the vaccination cohort are still alive and only three of these suffered a disease relapse.
Concluding, this individualized peptide vaccination approach is feasible in pediatric ALL. Despite low mutational profile, there are sufficient tumor-specific mutations to predict HLA-binding peptides which induce de novo T cell responses against neoantigens by peptide vaccination. With the favorable safety profile, remarkably good immunogenicity as well as good clinical outcome of patients, this approach warrants further evaluation in a larger phase II clinical trial.

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A sidekick's Immuno-superhero story: The role of CD226 on CD4 T cells in cancer

N. Salim[1,2], S. Eickhoff[2], L. Doll[2], F. AbdelAziz[2], R. Joshi[2], A. Lapp[2], D. Rommel[2], F. Schneppenheim[2], S. Ng[3], D. Baumjohann[4], M. Hölzel[2], T. Bald[2]
[1]Uniklinikum Bonn, University of Bonn, Institute of Experimental Oncology, Bonn, Germany, [2]Uniklinikum Bonn, University of Bonn, Bonn, Germany, [3]Uniklinikum Bonn, Institute of Clinical Chemistry and Clinical Pharmacology, Bonn, Germany, [4]Uniklinikum Bonn, Medical Clinic III for Oncology, Hematology, Immuno-Oncology and Rheumatology, Bonn, Germany

Activating receptors on T cells, such as CD226 (DNAM-1), play an important role for anti-cancer responses. We have previously shown that tumour cells can downregulate CD226 on CD8 T cells in mouse and human tumours and that the success of immune checkpoint blockade in melanoma patients correlates with the presence of CD226 positive CD8 T cells. Although anti-cancer roles of CD4 T cells have been demonstrated, most immunotherapy studies mainly focus on CD8 cytotoxic T cells. Understanding of the role of CD226 for CD4 T cell subsets within the tumour microenvironment (TME) remains limited. In mice, CD226 expression

is upregulated during activation and correlates with the functionality of CD4 T cells. Furthermore, a rapid downregulation of CD226 is observed upon ligation with its ligand, CD155. The downregulation of surface CD226 was also observed in tumour-bearing mice. A mutation at the CD226 tyrosine 319 (Y319) residue showed resistance to CD155-driven downregulation. Mechanistically, CD155 induces Y319 phosphorylation, which leads to CBL-B mediated ubiquitination, internalisation, and ultimately proteasomal degradation of CD226. Our preliminary human data suggests varying CD226 surface expression across various CD4 memory subsets, and that naïve CD4 T cells upregulate CD226 upon stimulation. Thus, suggesting a role for $CD226^{+}$ CD4 T cells in anti-tumour responses. To further curate the role of CD226 in CD4 T cell functionality, we have optimised a CRISPR-Cas9 system to obtain CD226KO primary human CD4 T cells – a valuable toolbox for downstream analysis.Data yielded from this project will provide further understanding on the mechanisms of CD226-expressing CD4 T cells and possibly unmask other kinetics of immune invasion.

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Addition of Durvalumab to neoadjuvant chemoradiotherapy (CROSS) in esophageal adenocarcinoma is feasible and safe - first results from the prospective Phase-II RICE trial.

H. A. Schlößer[1,2], A. Lohneis[3], M. A. Garcia Marquez[1], M. Thelen[1], P. Schiller[4], K. Rosenberger[4], N. M. Wirsik[2], L. Lukomski[2], A. M. Hillmer[5], M. Hellmich[4], M. Hallek[3], R. Büttner[5], W. Schröder[2], M. S. von Bergwelt-Baildon[6], A. Quaas[5], C. J. Bruns[2], T. Zander[3]
[1]University of Cologne, Center for Molecular Medicin, Cologne, Germany, [2]University of Cologne, Department of General, Visceral, Cancer and Transplantation Surgery, Cologne, Germany, [3]University of Cologne, Department of Internal Medicine I, Cologne, Germany, [4]University of Cologne, Department of Medical Statistics and Biometry, Cologne, Germany, [5]University of Cologne, Institute of Pathology, Cologne, Germany, [6]Ludwig Maximilian University Munich, Department of Internal Medicine III, Cologne, Germany

Anti-PD-(L)1 is effective in esophago-gastric adenocarcinoma (EGA) and appears synergistic with chemoradiotherapy (CRT). The RICE trial aims to evaluate safety, feasibility and efficacy of addition of neoadjuvant durvalumab and adjuvant durvalumab ± tremelimumab to standard CRT. In this open-label, randomized phase-2 trial, patients with locally advanced (≥uT3/Nx or uT2/N+) non-metastatic EGA (including EGJ-I and EGJ-II) receive two doses of neoadjuvant durvalumab (1500mg) in addition to CRT according to the CROSS protocol. After a safety-run in of 6 patients, adjuvant therapy was randomized 1:1 to 12 doses of durvalumab monotherapy or 12 doses of durvalumab (1500mg/4w) plus a single dose of adjuvant tremelimumab (300mg d1 of adjuvant treatment). Here we report the results from the neoadjuvant part of the trial including the primary endpoints for safety and efficacy and extensive translational immune monitoring. In the modified intention-to treat analyses 95% (53/56) of patients completed neoadjuvant therapy and 93% (52/56) underwent resection (3 excluded due to peritoneal carcinomatosis, 1 to intraoperatively identified cirrhosis; R0 in all patients), which was not lower than 90.4% as predefined benchmark for feasibility. Regarding the primary endpoints for safety, the rate of anastomotic leakage was 6% (3/52) and significantly lower than the predefined threshold of 22% ($p<0.05$), while the rate of grade 3/4 toxicity exceeded the predefined threshold of 13% (25%, 13/56 G3, 1/56 G4). Clinicopathological evaluation of response revealed 5% (3/56) peritoneal carcinomatosis, 39% (22/56) minor response (>10% vital tumor), 30% (17/56) major response (<10%), 23% (13/56) complete response and 2% not evaluable (1/56, cirrhosis). The primary endpoint for efficacy (35% CR) was not reached, but at

least <10% residual tumor was achieved in 53%, which was higher than in patients treated with CROSS during the same period at the same center. Patterns of response and resistance from translational analyses will be presented as well. The first analyses of the RICE trial demonstrate safety and feasibility of addition of neoadjuvant immunotherapy with durvalumab to CROSS. Despite not meeting the primary endpoint, comparison to CROSS and FLOT standard patients suggests efficacy and the trial provides further evidence supporting evaluation of RICE in a randomized controlled trial.

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Exploring the potential of urine-based immune profiling: evaluating sample quality and feasibility

A. Sevko[1], C. Prevosto[1], D. Ambrose[2], N. Carroll[2], S. Hailey[2], S. Domingos-Pereira[3], V. Cesson[3], L. Derre[3], L. Deban[1]

[1]Prokarium, London, United Kingdom, [2]KCASBio, Lower Gwynedd, United States of America, [3]Lausanne University Hospital, Urology Research Unit and Urology Biobank, Department of Urology, Lausanne, Switzerland

Urine biomarkers are crucial for monitoring patient responses and understanding the mechanism of action when treating urological pathologies, including Non-Muscle Invasive Bladder Cancer (NMIBC). Yet, analysing urine biomarkers poses challenges regarding specimen stability and analytical processing. This study investigates the stability of urinary leukocytes and proteins to optimize urine specimen logistics in clinical trials.

We demonstrate, by flow cytometry analysis, the stability of healthy donor peripheral blood leukocytes spiked into cell-free urine supernatants from healthy donors or untreated NMIBC patients over 72 hours under refrigeration without viability loss or changes in subsets distribution. Additionally, urine samples from five NMIBC patients receiving Bacillus Calmette-Guérin (BCG) therapy were split in two and processed immediately or after overnight (ON) refrigeration. ON refrigeration of BCG-treated patients' urine samples did not affect viability, absolute number and frequency of total leukocytes ($CD45^+$ cells), T cells ($CD3^+$ cells), granulocytes ($CD15^+$ cells) and monocytes ($CD14^+$ cells).

In addition to urine leukocytes, we also addressed the stability of urinary inflammatory proteome and normalization requirements. Multiple proteins were successfully quantified in urine supernatants using 51-plex Luminex^{tm} technology and the data demonstrates stability in total protein, creatinine levels, and 51 protein analytes in overnight refrigerated and frozen urine supernatants from BCG-treated NMIBC patients, providing a flexible and practical non-invasive approach for immune biomarker analysis.

This study affirms the feasibility of immunophenotyping refrigerated urine samples within a 72-hour sample quality window. The custom-designed 18-parameter flow cytometry panel holds applicability for monitoring cellular phenotypes in human urine, facilitating the longitudinal evaluation of phenotypic changes in cellular composition and phenotype during therapeutic intervention.

Taken together these findings suggest that urine biomarker analysis is feasible even in decentralized or multicentred clinical trials, addressing logistical challenges in transporting samples from trial sites to analytical laboratories, thus supporting broader implementation in clinical research. This biomarker strategy is implemented in the ongoing PARADIGM-1 clinical trial (NCT06181266) investigating ZH9, a novel live attenuated bacterial immunotherapy, aiming to redesign the treatment paradigm in NMIBC with a single induction dose therapy.

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Weak potential of using CD66c and CD123 markers in risk group classification among pediatric patients diagnosed with B ALL in Mozambique

A. Sigauque[1], E. Mongo[1], T. Babetine[1], V. Mapossa[1], F. Matola[2], F. Amodo[2], A. Dias[2], C. Mudenyanga[3], D. Mutsaka[3], R. Matavele[4]

[1]Instituto Nacional de Saude, CISPOC, Mozambique, Mozambique, [2]Hospital Central de Maputo, Hematooncologia Pediatrica, Maputo, Mozambique, [3]Clinton Health Access Initiative, Maputo, Mozambique, [4]Instituto Nacional de Saude, Maputo, Mozambique

Background and aims

The markers CD66c and CD123 have been mentioned in some studies as possible markers of good prognosis in pediatric patients with acute lymphoblastic leukemia (ALL). That information is crucial for risk group classification, thus allowing for appropriate clinical management and consequent reduction of mortality. The present study aims primarily to analyze the frequency of aberrant markers related to good prognosis (CD66c+ and CD123+) in pediatric patients diagnosed with B-cell ALL in Mozambique.

Methods

Retrospectively, immunophenotypic, demographic, and clinical data were collected from pediatric patients diagnosed at the Hemato-Oncology Service of the Hospital Central de Maputo and at Instituto Nacional de Saúde, Mozambique, between January 2020 and December 2023. The criteria used for risk group classification were age >1 and <10 years, leukocyte count above 50x103, and the type of leukemia. CD Markers were selected based on convenience, considering their frequency of citation in studies highlighting significant markers for prognosis assessment in acute leukemia (ALL B). The chi-square test was employed for frequency comparisons ($P<0.05$).

Results

Out of the total of 39 children diagnosed as ALL-B, 32 (82%) were in the age range of 1-10, and 23 (58.0%) were female. Regarding the risk group, 24 (61.5%) were classified as in the standard risk group, and 15 (38.5%) in the high-risk group. Overall, 14 (36.0%) and 20 (51.3%) had expression of CD66c+ and CD123+ respectively, and only 5 (12.8%) were negative for these markers. Among those classified as in the standard risk group, 11 (78.5%) and 13 (65.0%) showed expression of CD66c+ and CD123+, respectively. On the other hand, in the high-risk group, only 3 (21.4%) and 7 (35.0%) showed expression of CD66c and CD123, respectively ($P=0.3927$).

Conclusion

No statistically significant difference was observed in the comparison of CD66c+ and CD123+ marker expression between individuals classified into high-risk and standard-risk groups according to the number of leukocytes, age, and type of leukemia. This indicates a weak potential of mentioned markers in risk group classification among pediatric patients in Mozambique

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High-dose IL-2 dynamically reduces IL-2 receptor signaling especially in CD4+ T cells

C. Sommer[1,2,3], S. Jacob[1,2,3], T. Bargmann[1,2,3], S. Dehmel[1,2,3], V. Neuhaus[1,2,3], A. Braun[1,2,3], K. Sewald[1,2,3], imSAVAR Consortium

[1]Fraunhofer Institute for Toxicology and Experimental Medicine, Hannover, Germany, [2]Member of the German Center for Lung Research (DZL), Biomedical Research in Endstage and Obstructive Lung Disease Hannover (BREATH) research network, Hannover, Germany, [3]Member of the Fraunhofer Cluster of Excellence Immune-Mediated Diseases CIMD, Hannover, Germany

More than 40 interleukin-2 (IL-2)-based compounds are currently being tested in clinical trials to treat inflammatory diseases or cancer. However, IL-2 therapy can induce severe side effects including systemic inflammation, affecting multiple organs. Similar autoimmune-like symptoms are observed in patients with mutations in the IL-2 receptor β subunit (IL-2Rβ). In these patients, T cells show highly reduced IL-2Rβ surface expression, leading to impaired IL-2R signaling while signaling in natural killer (NK) cells is relatively unaffected. We hypothesize that high-dose IL-2 (hdIL-2) – used for anti-cancer treatment – decreases IL-2Rβ surface expression and reduces IL-2R signaling capacity especially in T cells but not NK cells, similar to patients with IL2RB mutations. Therefore, human peripheral blood mononuclear cells (PBMCs) were continuously stimulated with 1-10,000 IU/mL IL-2 (aldesleukin) for up to 7 days. Receptor surface expression and IL-2R signaling (pSTAT5) of cell subsets were measured using flow cytometry.
While IL-2Rβ surface expression remained high on CD8+ T cells and NK cells after 15 min of hdIL-2 stimulation, we observed significant decreases on CD4+ T cell subsets to levels below 0.4% (vs. 2.5% for unstimulated CD4+ T effector cells [Teffs] and 26.1% for regulatory T cells [Tregs]). Prolonged IL-2 exposure (up to 7 days) reduced IL-2Rβ expression on CD8+ T cells to 0.2%. Expression on NK cells also was reduced but 55.8% of cells remained IL 2Rβ+. CD4+ and CD8+ T cells stimulated with hdIL-2 for 7 days showed lower pSTAT5 signal when re-stimulated with IL-2 than unstimulated cells (reduction in mean fluorescence intensity of 1.9-fold, 1.8-fold, and 1.7-fold for CD4+ Teffs, Tregs, and CD8+ T cells, respectively). In contrast, hdIL-2 did not impact IL-2 signaling in NK cells as pSTAT5 signaling remained unaltered upon re-stimulation with IL-2. Similarly, re-stimulation with IL-15 – also signaling through IL-2Rβ – induced significantly reduced pSTAT5 in T cells after high-dose IL-2 stimulation, while pSTAT5 in NK cells was unaltered. Similar to IL-2Rβ expression, impaired IL-2R signaling was more pronounced at earlier time points for CD4+ T cell subsets compared to CD8+ T cells.
Conclusively, IL-2Rβ is basically absent on hdIL-2-stimulated T cells, leading to concomitant decreases in IL-2R signaling. NK cells retained relatively high IL-2Rβ expression and IL-2R signaling. CD4+ T cell subsets seem to be more broadly affected than CD8+ T cells, underlining lineage-specific differences in IL-2 responsiveness. Given the resemblance of cellular characteristics of hdIL-2-stimulated cells and cells from patients with defective IL-2Rβ, impact of continuous IL-2 stimulation on IL-2R signaling should be considered in the onset of adverse events during IL-2 therapy.

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A multipeptide vaccine targeting individual somatic mutations induces tumor infiltration of neoantigen-specific T cells in a patient with metastatic colorectal cancer

H. Zelba[1], A. Rabsteyn[1], B. Shao[1], A. Reinhardt[1], C. Kyzirakos[1], S. Kayser[1], A. Golf[2], S. Biskup[1]
[1]Zentrum für Humangenetik Tübingen, Tübingen, Germany, [2]MVZ Zentrum für ambulante Onkologie, Tübingen, Germany

When first diagnosed, 25% of all colorectal tumors are metastasized. Although treatment strategies, such as immunotherapy, chemotherapy or targeted therapy, were developed rapidly in recent years, the prognosis of colorectal cancer (CRC) is still unsatisfying. The median survival for metastatic CRC is reported to be about 19 months with a 5-year overall survival rate of 15%. Here we present a 50-year-old patient who was first diagnosed with stage IV colorectal cancer. After the tumor progressed several times under guideline therapies, the patient received individualized neoantigen-derived peptide vaccination in the setting of an individual treatment attempt.

The tumor was analyzed for somatic mutations by whole exome sequencing and potential neoepitopes were vaccinated over a period of 13 months. For each vaccination, 0.5 ml multipeptide solution was injected intracutaneously in the lower abdomen followed by subcutaneous injection of sargramostim and superficial application of imiquimod. The vaccine was well tolerated with no severe side effects.

Intracellular cytokine staining after 12 day in-vitro expansion measuring four T-cell activation markers (IFN-γ, TNF, IL-2, CD154) was used to determine vaccine-induced T cell responses.

Immune monitoring performed three months after the first vaccination revealed strong and polyfunctional CD4+ and/or CD8+ T cell responses against 10 of 20 vaccinated neoantigens, including those against relevant driver mutations. During the course of treatment, vaccine-induced responses remained robust, durable, and polyfunctional.

A liver metastasis that was surgically removed five months after the first vaccination, revealed neoantigen-specific tumor-infiltrating lymphocytes against at least five vaccinated neoantigens. This was confirmed by T cell receptor sequencing. Two of those five targeted mutations were no longer detectable in the recurrent tumor. Afterwards, the patient was stable and survived more than 40 months.

We show that peripheral vaccine-induced neoantigen-specific T cells are able to infiltrate tumors and specifically kill mutation-bearing tumor cells. The results presented here implicate the necessity of broad neoantigen-targeting vaccines for successful cancer immunotherapies.

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Treatment of colon cancer cells with Lacticaseibacillus casei: induction of apoptosis and release of DAMPs associated with immunogenic cell death

G. Aindelis[1], V. Glaros[2,3], K. Fragoulis[4], A. Mouchtari[1], K. Spyridopoulou[1], K. Chlichlia[1]

[1]Democritus University of Thrace, Department of Molecular Biology and Genetics, Alexandroupolis, Greece, [2]Karolinska Institutet, Department of Medicine, Division of Immunology and Allergy, Stockholm, Sweden, [3]Karolinska Institutet, Center for Molecular Medicine, Stockholm, Sweden, [4]Karolinska Institutet, Department of Microbiology, Tumor and Cell Biology, Stockholm, Sweden

Lactic acid bacteria have long been known to exert anti-proliferative and pro-apoptotic effects against cancer cells, among other health-beneficial properties. Emergence of immunogenic cell death (ICD) has opened new possibilities on the functionality of these probiotic bacteria. Herein, we investigated whether Lacticaseibacillus casei, previously reported to induce apoptosis in colon cancer cells, could also result in the release of damage-associated molecular patterns (DAMPs) indicative of ICD. Treatment of murine and human cancer cells, with various concentrations of L. casei initiated apoptosis, mediated by the extrinsic pathway, as evident by the upregulation of death receptors, increased caspase 8 activity and cleavage of caspase 3. Along that, a distinct signaling equilibrium in favor of apoptosis, including modulation of Bax, Puma, survivin, and phosphorylation of p-38, Chk2, Akt and HSP27, was evident. In addition, we detected the presence of DAMPs in response to exposure to the probiotic. Presence of calreticulin on the cytoplasmic membrane was evaluated with flow cytometry and translocation was noticeable, acting as a potent inducer of engulfment of dying cells from antigen-presenting cells. Moreover, the localization of HMGB1 protein in the nucleus and cytoplasm of cancer cells and their ATP content was investigated with the CellInsight High-Content platform. In tumor cells incubated with L. casei, nuclear HMGB1 was found to be decreased, with the protein accumulating in the cytoplasm and its subsequent release from the dying cells induces dendritic cell (DC)-mediated immune responses. Similarly, the ATP content of cancer cells was reduced and secretion of the metabolite acts as a chemo-attractant and immuno-stimulatory signal of DC-associated adaptive immune responses. Finally, expression of type I interferons was upregulated, with the production of such molecules facilitating immune cell cascades, enhancing CD8 T cell-induced cytotoxicity. To our knowledge, this is one of very few instances of probiotic treatment potentially inducing ICD in cancer cells. Interestingly, we have also previously found L. casei to confer antitumor immunomodulatory effects in preclinical colon cancer models. We aspire to possibly combine these diverse anticancer activities, with novel bioengineering tools to enhance and modify the innate properties of the probiotic, to use it as a platform for immunotherapeutic approaches. This research was co-financed by Greece and the European Union in the context of the project “Strengthening Human Resources Research Potential via Doctorate Research” (MIS-5000432) and was supported from the BioActiveScreen Center, funded by the Hellenic Foundation for Research and Innovation (HFRI) and the General Sekretariat for Research and Technology (GSRT), under grant agreement HFRI-FM17C3-2007.

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Development of a heterologous prime-boost vaccine in a cold and very low T-cell infiltrated mouse tumor model.

F. Alfei, G. Philippin, L. Devy-Dimanche, M.-L. Santiago-Raber
AMAL Therapeutics, Boehringer Ingelheim, Geneva, Switzerland

Cancer is the second leading cause of death worldwide and immunotherapy represents one of the most promising strategies in cancer treatment. However, a substantial number of patients do not respond to the treatments and have a poor clinical outcome. The low response rate can be due to the low number of tumor infiltrating T-cells and the presence in the tumor of exhausted T-cells and other immune suppressive cells. Among all immunotherapy strategies, therapeutic cancer vaccines have the potential to generate and to amplify immune activation against cancer and increase response rate of patients. At AMAL Therapeutics, a vaccine regimen composed of a prime with the therapeutic vaccine KISIMA™ and the boost with an oncolytic vesicular stomatitis virus variant (VSV-GP) has been developed and showed great efficacy due to the extensive reshaping of the tumor microenvironment and the significant increase of T-cell functionality.
The therapeutic vaccine KISIMA is made as a single chimeric fusion protein, containing a proprietary cell-penetrating peptide (CPP) for antigen delivery, a proprietary Toll-like receptor (TLR)- peptide agonist with self-adjuvant properties and a modulable multi-antigenic domain (Mad), which include antigens expressed by cancer cells. VSV-GP is chimeric viral vector where neurotoxicity is abrogated by replacing the VSV glycoprotein with the LCMV glycoprotein (GP). VSV-GP carries the same antigens included in the Mad domain, VSV-GP-Antigens (VSV-GP-Ags), able to boost antigen specific T-cells primed by the KISIMA vaccine.
The KISIMA/VSV-GP-Ags vaccination has been tested in hot tumor model such as MC-38 and in cold tumor model such as TC-1, showing the ability to induce a strong antigen specific immune response and efficacy in tumor control. To investigate the action of KISIMA/VSV-GP-Ags vaccination in a very cold and poor infiltrated tumor, the LLC1 mouse tumor model has been chosen.
For the design of KISIMA and VSV-GP-Ags construct against mouse tumor antigens expressed by LLC1, published data have been analyzed and antigen expression confirmed in the LLC1 cells. Two peptides resulted to be mutated and immunogenic, therefore a KISIMA and VSV-GP-Ags constructs targeting those two mouse tumor antigens have been developed.
The treatment of LLC1-tumor-bearing mice with the novel KISIMA/VSV-GP-Ags regimen significantly increases the number and functionality of antigen-specific CD8 and CD4 T-cells in both tumor and peripheral compartments compared to control group.
The significant increase in immunogenicity confirms the ability of the KISIMA/VSV-GP-Ags heterologous prime boost to generate a specific immune response against mouse tumor antigens in the very poorly infiltrated LLC1 tumor model.

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Novel vaccination strategies based on optimal stimulation of CD4 + T helper cells for the treatment of Oral Squamous Cell Carcinoma

L. Azzi[1,2], F. Celesti[3], A. M. Chiaravalli[2], A. K. Shaik[1], M. Shallak[1], A. Gatta[1], P. Battaglia[4,5], S. La Rosa[1,2], A. Tagliabue[2,6], R. S. Accolla[6], G. Forlani[1]

[1]University of Insubria, Department of Medicine and Technological Innovation, Varese, Italy, [2]ASST sette laghi, Varese, Italy, [3]University of Siena, Department of Medicine, Surgery and Neuroscience, Siena, Italy, [4]University of Insubria, Department of Biotechnology and Life Sciences, Varese, Italy, [5]ASST Lariana, Varese, Italy, [6]University of Insubria, Department of Medicine and Surgery, Varese, Italy

Oral Squamous Cell Carcinoma (OSCC) is the most common malignant tumor of the oral cavity. Despite recent advances in the field of oral cancer therapy, including the introduction of immunotherapeutic approaches, the 5-year survival rate remains steadily assessed around 50%. Thus, there is an urgent need for new therapeutic strategies. After the characterization of the immune phenotype of three human OSCC cell lines (CAL-27, SCC-25, and SCC-4) and one mouse OSCC cell line (MOC2) showing their similarities to resected patient tumors, we explored for the first time an experimental preclinical model of therapeutic vaccination with mouse OSCC MOC2 cell line stably expressing MHC class II antigens after CIITA gene transfection (MOC2 CIITA).[1] [2] Mice injected with MOC2-CIITA reject or strongly retard tumor growth; more importantly, vaccinated animals that fully reject MOC2-CIITA tumors display anti-tumor immunological memory protective against challenge with parental MOC2 tumor cells. Further experiments of adoptive cell transfer or in vivo cell depletion show that both CD4 + and CD8 + T lymphocytes prove fundamental in tumor rejection. This unprecedented approach for oral cancer opens the way for possible future translation of novel immunotherapeutic strategies to the human setting for the treatment of this tumor.[3] [4]

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Detection and validation of gene fusion-induced neoepitopes and cognate T cell receptors in dedifferentiated liposarcoma

J. P. Becker[1,2], S. Blobner[3,4], D. Weber[5], S. Uhrig[6], J. D. Förster[1,2], A. Baude[3,6], J. Wagner[3,6], I. Poschke[7,8,9], M. Volkmar[10], M. Platten[7,8,9,11], S. Fröhling[3,6,8,12], P. Horak[3,6], A. B. Riemer[1,2]

[1]German Cancer Research Center (DKFZ), Division of Immunotherapy and Immunoprevention, Heidelberg, Germany, [2]German Center for Infection Research (DZIF), Molecular Vaccine Design, Heidelberg, Germany, [3]German Cancer Research Center (DKFZ), Division of Translational Medical Oncology, Heidelberg, Germany, [4]National Center for Tumor Diseases (NCT); NCT Heidelberg, a partnership between DKFZ and Heidelberg University Hospital, Division of Translational Medical Oncology, Heidelberg, Germany, [5]TRON gGmbH, Expression & Cell Biology, Mainz, Germany, [6]National Center for Tumor Diseases (NCT); NCT Heidelberg, a partnership between DKFZ and Heidelberg University Hospital, Molecular Precision Oncology Program, Heidelberg, Germany, [7]National Center for Tumor Diseases (NCT); NCT Heidelberg, a partnership between DKFZ and Heidelberg University Hospital, Immune Monitoring Unit, Heidelberg, Germany, [8]German Cancer Consortium (DKTK), Heidelberg, Germany, [9]German Cancer Research Center (DKFZ), Clinical Cooperation Unit Neuroimmunology and Brain Tumor Immunology, Heidelberg, Germany, [10]Helmholtz Institute for Translational Oncology Mainz (HI-TRON Mainz), TCR Discovery Platform, Mainz, Germany, [11]Heidelberg University Mannheim Center for Translational Neuroscience, Department of Neurology, Heidelberg, Germany, [12]Heidelberg University, Institute of Human Genetics, Heidelberg, Germany

Liposarcomas are malignant soft-tissue tumors of which only a subset responds to conventional cytostatic drugs and there is currently no approved targeted therapy available. One of the most common histologic subtypes, dedifferentiated liposarcoma (DDLS), is characterized by focal amplification of CDK4 and MDM2 on chromosome 12. Using WES/WGS and RNA sequencing, we previously found that these structural changes generate open reading frames and thus presumably transcribed and translated chimeric genes. The neoepitopes encoded in these genes are promising targets for epitope-centric individualized therapies if they are presented by HLA class I molecules.

Using two different variant calling pipelines – Arriba and EasyFuse – we identify gene fusions from whole genome/exome and RNA sequencing data of eight DDLS patients. Next, we performed a comprehensive mass spectrometry (MS) analysis of the patients' tumor immunopeptidome by combining both untargeted and targeted methods in order to identify fusion-derived neoepitopes. For the untargeted approach, we used data-independent acquisition with ion mobility separation by high-field asymmetric waveform ion mobility spectrometry (FAIMS-DIA) identifying up to 16.500 unique peptides per patient, including several neoepitope candidates. For the targeted analysis, we utilized our recently established optiPRM workflow. Here, candidate neoepitopes identified by FAIMS-DIA as well as additional candidates from variant calling with excellent predicted binding properties were synthesized as stable isotope-labeled (SIL) variant and assay-relevant parameters such as normalized collision energy (NCE) and exact retention times were determined relative to iRT peptides. Targeted analysis not only confirmed several of the FAIMS-DIA candidates but also yielded additional detections in 5 out of 6 analyzed patients.

Using our complimentary FAIMS-DIA and PRM immunopeptidomics workflows, we have identified fusion-derived neoepitopes in 5 out of 8 DDLS patient samples. Importantly, the identifications contained both neoepitopes which would have been missed by the less sensitive FAIMS-DIA approach or which would have been excluded from the targeted analysis due to their poor predicted binding properties, highlighting the

advantage of the combined approach.
Currently, we are testing all identified fusion-derived neoepitopes in epitope-specific expansion cultures (ESPEC) from the respective patients for reactive T cell populations. Additionally, we plan to perform TCR repertoire analysis by deep sequencing and single cell VDJ sequencing to identify fusion neoepitope-reactive T cell receptors. Both detected neoepitopes and cognate T cell receptors identified will provide starting points for the clinical development of personalized treatment options in liposarcoma such as cancer vaccines or TCR-based therapies.

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Functional characterization of targeted polymeric nanoparticles for personalized mRNA-based immunotherapy of non-small cell lung cancer (NSCLC)

A. K. Binder[1], I. Espinós i Longa[2], A. Torres Coll[2], R. E. Voll[3], C. Berking[1], S. Borrós[2], C. Fornaguera[2], N. Schaft[1], J. Dörrie[1]
[1]University Hospital Erlangen, Department of Dermatology, CCC Erlangen-EMN, German Centre of Immunotherapy, Friedrlich-Alexander Universität Erlangen-Nürnberg (FAU), Erlangen, Germany, [2]Universitat Ramon Llull, Grup d'Enginyeria de Materials (Gemat), Institut Químic de Sarrià (IQS), Barcelona, Spain, [3]University of Freiburg, Faculty of Medicine, Department of Rheumatology and Clinical Immunology, Freiburg, Germany

This project aims to establish a nanoparticle-based mRNA immunotherapy involving therapeutic vaccination and in situ reprogramming of immune cells as a new treatment option for NSCLC. Up to now, no therapeutic tumor-associated antigen (TAA)-based vaccine showed survival benefits in clinical phase III. An approach to enhance the immune response is to use targeted nanoparticles (tNPs) shuttling mRNA directly to antigen presenting cells (APCs). Thereby the translated protein, as intracellular antigen, is mainly presented on MHC-I of APCs and induces $CD8^+$ cytotoxic anti-tumoral T-cells. Furthermore, co-shuttled mRNA can be used to reprogram immune cells in situ to overcome immune suppression and induce a fulminant immune activation.
In this project, the TAA MAGE-A3 and tNPs equipped with targeting moieties specifically binding to CLEC9A and CD206 were used. These phagocytosis receptors are present on several APCs including M1($CD206^{low}$) and M2($CD206^{high}$) macrophages, monocyte-derived dendritic cells ($CD206^{high}$) and classical dendritic cells type I ($CLEC9A^+$). To evaluate the functionality of in-house produced TAA-encoding tNPs, transfection assays were conducted with the above-mentioned APCs. These transfected APCs were used in priming experiments with 2- and 3-partner cell systems containing $CD8^+$ or $CD4^+$ T-cells. As readout, the surface expression of T-cell-activation markers (via flow cytometry) and the cytokine release in cytometric beady assays (CBA) were evaluated. For reprogramming APCs, we used mRNA encoding functional proteins e.g. constitutively active IKKβ mRNA. To evaluate this effect, either M2 macrophages or $CD14^+$ cells were cultured in the supernatant of the NSCLC cell line A549 and the tested mRNA was either delivered via electroporation or tNPs. Subsequently, the cell phenotypes were characterized by surface marker expression and cytokine release. Furthermore, we aimed to reprogram tumor cells to be more immunogenic. Therefore, tNPs were used to shuttle mRNA encoding co-stimulatory molecules (e.g. CD80) to the tumor cells. After co-culture of these tumor cells with $CD8^+$ T cells, the activation status of the latter was evaluated as described above.

Our results showed that the tNP-transfected APCs were able to present the TAA MAGE-A3 and induce a MAGE-A3-specific $CD8^+$ T-cell-mediated immune response. Further, M2 as well as $CD14^+$ cells cultured in A549 supernatant were reprogrammed by IKKβ RNA which was shown by the phenotypical shift towards M1 macrophages in marker expression and cytokine release. The cells upregulated the surface markers CD86 and CD40 as well as the cytokines TNF and IL-6. Lastly, the reprogrammed cancer cells enhanced $CD8^+$ T cell activation.
We conclude that the tNPs can be used to transfect APCs for efficiently presenting TAAs as well as to reprogram the APCs and tumor cells.

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Abstract has been withdrawn

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$CD8^+$ T cell responses to homologous tumor-associated antigens (TAAs) and microbiota-derived antigens (MoAs)

B. Cavalluzzo[1], M. C. Viuff[2], S. A. Tvingsholm[2], C. Ragone[1], C. Manolio[3], A. Mauriello[1], F. Izzo[4], A. Morabito[1], M. Tagliamonte[1], L. Buonaguro[1]
[1]Istituto Nazionale Tumori - IRCCS Pascale, Experimental Oncology, NAPOLI, Italy, [2]Technical University of Denmark, Department of Health Technology, Section of Experimental and Translational Immunology,, Kongens Lyngby, Denmark, [3]University of Padua, Padua, Italy, [4]Istituto Nazionale Tumori - IRCCS Pascale, Gastrointestinal Dept, NAPOLI, Italy

Background: We have recently shown extensive sequence and conformational homology between tumor-associated antigens (TAAs) and antigens derived from microorganisms (MoAs). The present study aimed to assess the breadth of T-cell recognition specific to MoAs and the corresponding TAAs in healthy subjects (HS) and patients with cancer (CP).
Method: A library of >100 peptide-MHC (pMHC) combinations was used to generate DNA-barcode labelled multimers. Homologous peptides were selected from the Cancer Antigenic Peptide Database, as well as Bacteroidetes/Firmicutes-derived peptides. They were incubated with CD8+ T cells from the peripheral blood of HLA-A*02:01 healthy individuals (n=10) and cancer patients (n=16). T cell recognition was identified using tetramer-staining analysis. Cytotoxicity assay was performed using as target cells TAP-deficient T2 cells loaded with MoA or the paired TuA.
Results: A total of 66 unique pMHC recognized by CD8+ T cells across all groups were identified. Of these, 21 epitopes from microbiota were identified as novel immunological targets. Reactivity against selected TAAs was observed for both HS and CP. pMHC tetramer staining confirmed CD8+ T cell populations cross-reacting with CTA SSX2 and paired microbiota epitopes. Moreover, PBMCs activated with the MoA where shown to release IFNγ as well as to exert cytotoxic activity against cells presenting the paired TuA.
Conclusions: Several predicted microbiota-derived MoAs are recognized by T cells in HS and CP. Reactivity against TAAs was observed also in HS, primed by the homologous bacterial antigens. CD8+ T cells cross-reacting with MAGE-A1 and paired microbiota epitopes were identified in three subjects. Therefore, the microbiota can elicit an extensive repertoire of natural memory T cells to TAAs, possibly able to control

tumor growth ("natural anti-cancer vaccination"). In addition, non-self MoAs can be included in preventive/therapeutic off-the-shelf cancer vaccines with more potent anti-tumor efficacy than those based on TAAs.

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DUSP6-deficient CD4$^+$ T cells exhibits a superior tumor killing abiltiy via escaping the immunosuppression from the bystander B cells.

M.-Y. Chen[1], Y.-W. Su[2]

[1]National Health Research Insitutes, Immunology Research Center, Miaoli County, Taiwan, [2]National Health Research Institutes, Immunology Research Center, Miaoli County, Taiwan

Dual specificity phosphatase 6 (DUSP6) is a protein phosphatase that negatively regulates the mitogen-activated protein (MAP) kinase. Our previous study has demonstrated that deficiency of DUSP6 in T cells leads to a stronger T cell receptor (TCR)-mediated ERK- and JNK-signaling, which promotes T cell differentiation to Th1 and follicular helper T (Tfh) cells[1]. In the present study, we attempt to address whether DUSP6 deficiency in CD4$^+$ T cells bearing the Th1- and Tfh-features ameliorates the immune surveillance against tumors. We utilized OTII-transgenic mice, by which TCR expressed on all CD4$^+$ T cells from the mice are OVA-specific, as the syngeneic tumor model to address the question. We implanted ovalbumin overexpressing MC38 (MC38-OVA) tumor cells into OTII-transgenic mice, and observed that OTII-DUSP6$^{-/-}$ mice were resistant to tumor growth. The dissection of immune cell profiles in MC38-OVA tumor cells revealed a significant CD4$^+$ T cells-infiltration in tumor bearing OTII-DUSP6$^{-/-}$ mice. To confirm a role of DUSP6$^{-/-}$ CD4$^+$ T cells in the immune surveillance, we implanted MC38-OVA tumor cells into RAG2$^{-/-}$ mice, which were adoptively transferred with OTII-CD4$^+$ T cells with or w/o DUSP6 on one day before tumor transplantation. The transfer of OTII-DUSP6$^{-/-}$ CD4$^+$ T cells were able to lower 50% of tumor incidence and reduce the tumor size. The superior tumor killing effect of DUSP6$^{-/-}$ CD4$^+$ T cells was verified by in vitro killing experiment by co-culture of MC38-OVA with OTII-DUSP6$^{-/-}$ CD4$^+$ T cells. The tumor killing ability of DUSP6$^{-/-}$ CD4$^+$ T cells was not enhanced by the co-transfer of antigen non-specific CD8$^+$ T cells, indicating the tumor killing effect was DUSP6$^{-/-}$ CD4$^+$ T cells-intrinsic. Meanwhile, immunohistochemical staining in tumors section in OTII-DUSP6$^{-/-}$ mice revealed a significant reduction of tumor-infiltrated B cells. In MC38-OVA tumor-bearing TCRb knockout mice, by which abT cell subsets were absent but B cell subset was normally developed, the transferred OTII-DUSP6$^{-/-}$ CD4$^+$ T cells exhibited a similar anti-tumor effect compared to DUSP6 expressing-OTII CD4$^+$T cells, indicating that B cells interfered the tumor killing ability of DUSP6$^{-/-}$ CD4$^+$ T cells. To test a role of tumor-infiltrated B cells on CD4$^+$ T cell's activity, we co-transferred OTII-DUSP6$^{-/-}$ CD4$^+$ T cells with wild type splenic B cells prior to tumor inoculation in RAG2$^{-/-}$mice. The anti-tumor effect of DUSP6$^{-/-}$ CD4$^+$ T cells was blocked by the co-transfer of wild type B cells, suggesting that tumor-infiltrated B cells acted to repress the anti-tumor ability of CD4$^+$ T cells. Taken together, our finding uncovers that DUSP6 deficiency strengthens the anti-tumor ability of antigen-specific CD4$^+$ T cells by escaping from the immunosuppressive bystander B cells.

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Differential regulation of Calcium-NFAT signaling pathway by Akt isoforms: unraveling effector dynamics and exhaustion in tumor microenvironment

W.-L. Chen[1], Y.-L. Chang[1], H.-C. Yang[2,3,4], M. Isogawa[5], L.-R. Huang[1]
[1]National Health Research Institutes, Institute of Molecular and Genomic Medicine, Miaoli County, Taiwan, [2]National Taiwan University Hospital, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Taipei, Taiwan, [3]National Taiwan University, College of Medicine, Graduate Institute of Clinical Medicine, Taipei, Taiwan, [4]National Taiwan University, College of Medicine, Graduate Institute of Microbiology, Taipei, Taiwan, [5]National Institute of Infectious Diseases, Research Center for Drug and Vaccine Development, Tokyo, Japan

Impairment of Akt signaling has been observed in antigen-specific cytotoxic T lymphocytes (CTLs) during chronic viral infections and within the tumor microenvironment. Despite numerous studies emphasizing Akt's role in driving CTL effector functions, there is limited exploration of utilizing Akt molecules in T-cell engineering to enhance their anti-viral or anti-tumor capabilities for therapeutic purposes. Some researchers even propose that inhibiting Akt activation during the in vitro expansion process can prevent T-cell exhaustion and boost the anti-tumor effector functions of chimeric antigen receptor (CAR)-T cells in vivo. Given the unique expression patterns and functions of the three Akt isoforms in immune cells, our hypothesis suggests that Akt isoforms in CTLs may regulate effector functions and T-cell exhaustion distinctly. In this study, we analyzed tumor-Ag-specific TCR tg CTLs ectopically expressing Akt isoforms for their Akt expression, in vivo anti-tumor efficacies, functionalities, and transcriptome. We found that both Akt1 and Akt2 overexpression enhanced the cytotoxic capabilities of CTLs, albeit with different dynamics. Specifically, Akt2 signaling in CTLs accelerated effector functions, leading to a swift attack on tumor cells. Conversely, Akt1 signaling triggered calcium influx and subsequent NFAT activation, while Akt2 signaling suppressed calcium influx, preventing excessive NFAT expression and nuclear translocation. This repression of NFAT transcriptional activity by Akt2 signaling during prolonged antigen stimulation subsequently led to reduced expression of transcription factors associated with T-cell exhaustion, such as Egr2, Nr4a, Tox, and immune checkpoints. Consequently, Akt2-OE CTLs experienced reduced T-cell exhaustion within the tumor microenvironment and efficiently eradicated tumors. These findings highlight the essential role of Akt signaling in enabling tumor-specific CTLs to eliminate cancer cells in the solid tumor microenvironment, with Akt isoforms differentially regulating the Calcium–Calcineurin–NFAT Signaling Pathway. This discovery suggests the potential of AKT2 in T-cell engineering technology to enhance the survival and effector functions of adoptively transferred T cells for treating liver malignancies or chronic viral infections.

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Analysis of mechanisms promoting T cell tolerance against autochthonous cancer

L. Dyck*[1], K. Anders*[1,2], M. Milek[3], E. Belbaraka[1], M. T. Norcia[1], D. Beule[1,3], I. Martinez-Reyes[1], T. Blankenstein[1]

[1]Max Delbrück Center for Molecular Medicine (MDC), Berlin, Germany, [2]Department of Pediatric Oncology and Hematology, Charité, Berlin, Germany, [3]Core Unit Bioinformatics Berlin Institute of Health, Charité, Berlin, Germany

*Contributed equally

Chronic antigen exposure in solid tumors leads to T cell dysfunction and cancer progression. Cancer immunotherapies have demonstrated the induction of functional T cell responses in specific tumor models and human cancer types. However, the efficacy observed in mouse models with transplanted tumors often does not translate to the same degree in humans. To address this disparity, we have developed a transgenic, autochthonous tumor model called TTC, in which the oncogene and T cell antigen SV40 large T antigen (Tag) is induced by doxycycline (dox) from birth in cells with (a history of) tyrosinase expression. This model allows us to study the interplay between tumor-reactive T cells and tumor development over time.

After 4 months on dox, TTC mice failed to mount an immune response against transplanted Tag-expressing cancer cells, indicating the onset of T cell dysfunction. Between 6-12 months on dox, TTC mice developed singular tumors. Transfer of dysfunctional TTC T cells into Rag-ko mice, followed by immunization, induced polyclonal expansion of Tag-reactive T cells. However, these cells failed to reject Tag-expressing tumor cells in vivo. Single cell TCR sequencing revealed a notable proportion of Tag-specific T cells in tumor-bearing TTC donor mice that were undetectable by Tag-directed tetramer staining. This suggests that tumor-specific dysfunctional T cells downregulate their TCRs. Consistent with this, the expression of several genes associated with the TCR complex (Cd3, Zap70, Cd8) was significantly downregulated in tumor-reactive CD8 T cells in TTC mice. This downregulation was reversed upon transfer into Rag-ko mice. Tumor-reactive CD8 T cells in autochthonous cancer-bearing mice predominantly exhibited an exhausted or progenitor-exhausted state, a phenotype they maintained after transfer and immunization. These findings indicate that, in addition to upregulation of inhibitory receptors, tumor-directed T cells downregulate TCR expression, which hampers T cell activation and potentially undermines the efficacy of immunotherapy.

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Antigen specific immune responses and mechanisms of impaired antigen presentation in HPV positive and negative head and neck cancer

H. Eckel, M. Thelen, H. Schlößer

Center for Molecular Medicine Cologne, Faculty of Medicine and University Hospital of Cologne, Cologne, Germany

Antigen-specific immune response is a hallmark of cancer immunotherapy. Potential antigens in head and neck squamous cell carcinoma (HNSCC) include tumor-associated antigens (TAAs), mutation-associated neoantigens (MANAs) and viral proteins, including high-risk human papillomavirus (HPV) proteins. Studies combining multiple antigens for cellular therapy renewed interest in TAAs. While antigen expression in tumor cells is frequent, immunotherapy does not induce durable tumor regression in the majority of HNSCC patients. Impaired HLA class I antigen processing and antigen loss as key factors of immune evasion in HNSCC have been recognized previously, but the precise mechanisms remain elusive. HPV status was determined by overexpression of p16 INK4a (p16) and positive HPV DNA testing. Endogenous antigen specific T cell and humoral responses against HPV viral proteins and TAAs were determined by FluoroSpot and protein-bound bead assays. Expression of components of the HLA class I antigen processing pathway was analyzed by immunohistochemistry (IHC) in a large cohort of HPV-positive and HPV-negative HNSCC patients and correlated to intratumoral immune cell abundance. Using previously published sequencing data, we identified shared antigens specific to HPV positive and negative HNSCC. Antigen specific T-cell responses directed against HPV E6 and E7 and TAAs FK506 binding protein 6 (FKBP6) and Zona Pellucida Binding Protein 2 (ZPBP2) were frequent in peripheral blood mononuclear cells (PBMCs) and lymph nodes (LNs) from patients with HPV positive HNSCC. In HPV negative HNSCC, some T-cell responses to Melanoma-Associated Antigen 3 (MAGE A3), PRAME and Survivin were detectable in PBMCs while responses from lymph node derived T cells were sparse. Corresponding data describing humoral responses and deficient antigen presentation will be presented.

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DuoBody-EpCAMx4-1BB mediates conditional T-cell co-stimulation and promotes antitumor activity in preclinical models

S. Fellermeier-Kopf*[1], A. Ioan-Facsinay*[2], A. Imle[1], A. M. Diks[2], K. B. Nürmberger[1], L. Guelen[2], M. Köhne[1], M. Houtkamp[2], A. Toker[1], S. M. Burm[2], J. Gamse[3], C. Janaitis[1], D. Satijn[2], A. Muik[1], T. Ahmadi[3], Ö. Türeci[1], K. Kemper[2], U. Sahin*[1], E. Breij*[2]

[1]BioNTech SE, Mainz, Germany, [2]Genmab BV, Utrecht, The Netherlands, [3]Genmab US Inc., Princeton, United States of America

*Contributed equally

While 4-1BB has shown preclinical promise as an immuno-oncology target, classical 4-1BB agonists have demonstrated limited success in clinical trials due to modest efficacy or severe hepatotoxicity. DuoBody-EpCAMx4-1BB (BNT314/GEN1059) is an Fc-inert immunomodulatory bispecific antibody (bsAb) designed to boost antitumor immune responses through EpCAM-dependent 4-1BB agonist activity. EpCAM is highly and homogeneously expressed on the surface of cancer cells in many solid tumor indications. Here we report preclinical studies characterizing the mechanism-of-action of DuoBody-EpCAMx4-1BB in vitro and ex vivo, a proof-of-principle in vivo study in mice, and non-clinical safety assessment in cynomolgus monkeys [1]. DuoBody-EpCAMx4-1BB bound to EpCAM on tumor cells and 4-1BB on activated T cells, as shown by flow cytometry analysis. By cross-linking target-expressing cells, DuoBody-EpCAMx4-1BB induced 4-1BB agonist activity in a cell-based reporter assay. Agonist activity of DuoBody-EpCAMx4-1BB was conditional, i.e. dependent on binding to EpCAM, due to its inert IgG1 Fc domain. DuoBody-EpCAMx4-1BB dose-dependently enhanced activation of anti-CD3 stimulated $CD4^+$ and $CD8^+$ T cells in co-cultures of $EpCAM^+$

tumor cells and PBMCs from both healthy donors and cancer patients. Furthermore, DuoBody-EpCAMx4-1BB enhanced proliferation and cytokine secretion of activated healthy donor T cells and increased $CD8^+$ T-cell mediated killing of $EpCAM^+$ tumor cells in vitro. In ex vivo assays using tissue samples of patient-derived lymphocyte-infiltrated $EpCAM^+$ tumors, DuoBody-EpCAMx4-1BB enhanced the expansion of $CD8^+$ T-cells and NK-cells. An Fc-inert EpCAMx4-1BB bsAb targeting human EpCAM and mouse 4-1BB exhibited antitumor activity in human EpCAM-transgenic mice bearing subcutaneous human EpCAM-expressing MC38 tumors. DuoBody-EpCAMx4-1BB was well-tolerated in cynomolgus monkeys at doses up to 50 mg/kg (QWx5). In conclusion, through conditional 4-1BB agonist activity, DuoBody-EpCAMx4-1BB enhanced T-cell activation, proliferation, and effector functions in vitro and ex vivo. In a murine model, an Fc-inert EpCAMx4-1BB bsAb promoted antitumor activity in vivo. The clinical safety and preliminary antitumor activity of DuoBody-EpCAMx4-1BB are being investigated in patients with solid tumors in a first-in-human trial (NCT06150183).

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[1] Previously presented at the ESMO Congress 2023, Final Publication Number: 1072P, Sina Fellermeier-Kopf et al. Reused with permission.

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Protective anti-tumor vaccination against glioblastoma expressing the MHC class II transactivator CIITA

A. Gatta*[1], F. Celesti*[2], M. Shallak[1], A. M. Chiaravalli[3], M. Cerati[3], R. Accolla[1], F. Sessa[1,3], G. Forlani[1]
[1]University of Insubria, Department of Medicine and Technological Innovation, Varese, Italy, [2]University of Siena, Dipartimento di Scienze Mediche, Chirurgiche e Neuroscienze, Siena, Italy, [3]ASST Sette-Laghi, Unit of Pathology, Varese, Italy

*Contributed equally

Glioblastoma is the most malignant tumor of the central nervous system. Current treatments based on surgery, chemotherapy, and radiotherapy, and more recently on selected immunological approaches, unfortunately produce dismal outcomes, and less than 2% of patients survive after 5 years. Thus, there is an urgent need for new therapeutic strategies. Here, we report unprecedented positive results in terms of protection from glioblastoma growth in an animal experimental system after vaccination with glioblastoma GL261 cells stably expressing the MHC class II transactivator CIITA. Mice injected with GL261- CIITA express de novo MHC class II molecules and reject or strongly retard tumor growth as a consequence of rapid infiltration with CD4+ and CD8+ T cells. Importantly, mice vaccinated with GL261-CIITA cells by injection in the right brain hemisphere strongly reject parental GL261 tumors injected in the opposite brain hemisphere, indicating not only the acquisition of anti-tumor immune memory but also the capacity of immune T cells to migrate within the brain, overcoming the blood-brain barrier. GL261-CIITA cells are a potent anti- glioblastoma vaccine, stimulating a protective adaptive anti-tumor immune response in vivo as a consequence of CIITA-driven MHC class II expression and consequent acquisition of surrogate antigen-presenting function toward tumor-specific CD4+ Th cells [1]. This unprecedented approach for glioblastoma demonstrates the feasibility of novel immunotherapeutic strategies for potential application in the clinical setting [2][3].

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Pattern of tumor regression of recurrent glioblastoma after intratumoral virus injection of oncolytic parvovirus H-1 strongly supports virus contribution in effective viro-immunotherapy

K. Geletneky[1], L. Zeller[1], C. Weiss[2], A. Bartsch[3], H. Bernhard[4]
[1]Klinikum Darmstadt, Neurosurgery, Darmstadt, Germany, [2]Klinikum Darmstadt, Radiation Oncology, Darmstadt, Germany, [3]Radiologie Bamberg, Radiology, Bamberg, Germany, [4]Klinikum Darmstadt, Oncology, Darmstadt, Germany

Glioblastoma (GBM) remains a tumor entity with dismal prognosis. Efforts to use immunotherapy have generally shown little effect due to -among others- strong immunosuppression by the tumor, low mutational burden and the blood-brain/tumor barrier. Parvovirus H-1 (H-1PV) is an oncolytic virus with anti-tumor activity against glioblastoma that was already tested in a phase I/II clinical trial (Parvoryx-study). Upon request and based on a compassionate use agreement, a group of unselected patients with recurrent GBM were treated with H-1PV in combination with immunotherapy using Bevacizumab and PD-1 blockade with Nivolumab or Pembrolizumab. The majority of patients (9/11) showed an objective tumor response (ORR), thus exceeding the expected ORR for bevacizumab and PD-1 blockade alone which is around 30%. As tissue sampling for analysis of therapeutic effects is obviously very limited in GBM the contribution of the virus remained likely from the data but unproven. Here we report on two of the patients with recurrent GBM who had first responded to H-1PV based viro-immunotherapy but eventually showed tumor progression while under continuous treatment. Both patients demanded re-treatment with H-1PV that was administered intravenously and locally into the tumor at a combined individual dose of 2E9 pfu. Afterwards, patients continued with bevacizumab and PD-1 blockade. Due to the pharmaceutical formulation of the virus preparation which contains iodixanol, an x-ray contrast agent, it was possible to show the location of the intratumoral virus distribution by computer tomography (CT) immediately after surgery. In both patients, subsequent MRI at 6 and 12 weeks after local virus injection showed tumor regression with tumor necrosis in the exact areas where the virus inoculum had been demonstrated by CT. Interestingly, in patient 2 adjacent tumor areas not covered by local injection had no morphological changes on MRI and were slowly progressing. In conclusion, while clinical data with a high ORR under triple H-1PV based viro-immunotherapy indicated a strong additional effect of the virus, the co-localization of

intratumoral virus inoculum and tissue destruction adds further compelling evidence.

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GDF-15 neutralization overcomes treatment resistance to platinum-based chemoimmunotherapy

S. Genßler, D. Schätzlein, M. Haake, T. Ross, E. Leo, C. Schuberth-Wagner
CatalYm GmbH, Planegg-Martinsried, Germany

The combination of platinum-based chemotherapies with anti-PD-(L)1 immune checkpoint inhibitors as first-line treatment has led to significant improvements in overall survival in metastatic non-small-cell lung cancer (NSCLC). However, a significant proportion of tumors is directly refractory to frontline treatment or in most other cases experiences relapse post initial remission. Growth Differentiation Factor 15 (GDF-15), a member of the TGF-beta superfamily, is a stress-induced cytokine secreted by tumors. Elevated serum levels of GDF-15 correlate with a reduced response of cancer patients to immune checkpoint therapy. Mechanistically, GDF-15 inhibits the extravasation of T cells and thereby T cell infiltration into the tumor. Furthermore, GDF-15 prevents the activation and maturation of antigen-presenting cells and thus has a negative impact on priming of adaptive immune responses. In a recent clinical trial (NCT04725474), we demonstrated that GDF-15 neutralization in combination with anti-PD-1 can restore response to PD-(L)1 checkpoint therapy in patients with advanced or metastatic NSCLC. In addition to the role of GDF-15 as an immunosuppressive factor in immune checkpoint therapy, it has also been shown that platinum-based chemotherapies increase serum levels of GDF-15, thus potentially limiting effectiveness and tolerability of platinum-based chemoimmunotherapy. To test this hypothesis, we investigated the impact of a combination treatment of cisplatin, PD-1 blockade and GDF-15 neutralization on tumor growth and GDF-15 serum levels in preclinical tumor models. Our data showed that cisplatin monotherapy significantly increased GDF-15 serum levels in tumor-bearing mice. While GDF-15 neutralization, PD-1 blockade and cisplatin alone or a dual combination had no effect on tumor volumes in vivo, the triple combination of GDF-15 neutralization with anti-PD-1 and cisplatin reduced tumor growth and led to prolonged survival of mice. Analysis of PBMCs during treatment further shows that GDF-15 blockade alone already increased the expression of activation and proliferation markers on circulating CD4+ and CD8+ T cells. These data reveal that GDF-15 blockade positively influences T cells and acts independently from PD-1 blockade. Our data demonstrate that GDF-15 inhibition acts synergistically with chemoimmunotherapy and leads to improved antitumoral effects. This reinforces the evidence of GDF-15 as a central protein in immune evasion in advanced solid cancers. GDF-15 blockade in combination with chemoimmunotherapy is therefore a potential new strategy in the treatment of metastatic NSCLC.

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Epigenetic therapies induced antigens – a novel class of immunotherapy targets

A. Goyal*[1], J. Bauer*[2,3], J. Hey*[1], J. Walz[2,3,4,5], C. Plass[1]
[1]DKFZ, Cancer Epigenomics, Heidelberg, Germany, [2]Institute of Immunology, University and University Hospital Tübingen, Department of Peptide-based Immunotherapy, Tübingen, Germany, [3]University of Tübingen, Cluster of Excellence iFIT (EXC2180) “Image-Guided and Functionally Instructed Tumor

Therapies", Tübingen, Germany, [4]University Hospital Tübingen, Clinical Collaboration Unit Translational Immunology, Department of Internal Medicine, Tübingen, Germany, [5]German Cancer Consortium (DKTK) and German Cancer Research Center (DKFZ), partner site Tübingen, Tübingen, Germany

*Contributed equally

Immunotherapies targeting cancer-specific neoantigens have revolutionized the treatment of cancer patients. Recent evidence from our lab suggests that epigenetic therapies synergize with immunotherapies, mediated by the de-repression of endogenous retroviral element (ERV) - encoded promoters (Brocks et al, Nature Genetics, 2017). Now, using deep RNA sequencing from cancer cell lines treated with DNA methyltransferase inhibitor (DNMTi) and/or Histone deacetylase inhibitor (HDACi), we assemble a de novo transcriptome and identify several thousand ERV-derived, treatment-induced novel polyadenylated transcripts (TINPATs). Using immunopeptidomics, we demonstrate the human leukocyte antigen (HLA) presentation of several treatment-induced neopeptides (t-neopeptides) arising from TINPATs. We illustrate the potential of the identified t-neopeptides to elicit a T-cell response to effectively target cancer cells. We further verify the presence of t-neopeptides in AML patient samples after in vivo treatment with the DNMT inhibitor Decitabine. Our findings highlight the potential of ERV-derived antigens in epigenetic and immune therapies directed against cancer (Goyal et al, Nature communications, 2023). These antigens hold the promise to treat to any type of cancer and offer the possibility to generate off the shelf immunotherapeutic approaches.

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From DC activation to T-cell infiltration – Tracking the adaptive immune response to oncolytic virotherapy

L. Hanesch[1], S. Glauß[1], N. Hartmann[2], T. Feng[2], J. Altomonte[1]
[1]Klinikum rechts der Isar Technische Universität München, Klinik und Poliklinik für Innere Medizin II, Munich, Germany, [2]Ludwig-Maximilians-Universität München, Munich, Germany

rVSV-NDV is a chimeric oncolytic virus, which consists of the backbone of the vesicular stomatitis virus (VSV), with its targeting glycoprotein having been replaced by the envelope proteins of the Newcastle disease virus (NDV) consisting of the HN protein and the fusion (F) protein, which has been further modified to mediate potent syncytial reactions within the tumor. We have previously demonstrated that tumor lysis by this virus leads to a highly immunogenic cell death (ICD), characterized by the release of danger associated molecular patterns (DAMPs) and the antiviral response to the virus itself. These signals attract immune cells to the tumor microenvironment (TME) and activate them, which can lead to a transition from an immunologically "cold" to an immunologically "hot" TME. We hypothesized that this transition plays a key role in the initiation of an acquired anti-tumoral immune response by stimulating DCs, specifically the DC subtype cDC1. cDC1 cells are capable of cross-presenting antigens on MHC-I to $CD8^+$ T-cells, making them responsible for the most direct cytotoxic anti-tumor response. Testing this hypothesis, we demonstrated that in vivo treatment of subcutaneous B16 melanoma lesions with rVSV-NDV leads to increased migration of DCs, including cDC1, into the tumor draining lymph node, where we found them to be positive for the migration marker, CCR7, and co-stimulatory factors like CD80 and CD86. We also observed that these cDC1 cells cross-present tumor antigen-specific peptides, which corresponds to a

subsequent increase in tumor antigen-specific CD8⁺ T cell numbers and activation in the tumor and tumor-draining lymph nodes and a delay in tumor growth, both in directly injected and in contralateral lesions. In an in vitro setup we were able show that cDC1 cells co-cultured with VSV-NDV-lysed B16 cells display a comparable activation signature to that observed in vivo, and that these cells similarly prompt antigen-specific T-cell proliferation and activation. Together, these data indicate a correlation between the initial DC activation and therapeutic success, which is now being tested in a study utilizing cDC1 knock-out mice. This study investigates the immunogenic chain of events kickstarted by oncolytic rVSV-NDV therapy, from DC activation to T-cell infiltration using in vivo and in vitro methods. We believe that studies like this are crucial to understanding the recent successes in the field of oncolytic virotherapy and to finding avenues for improved virus vectors or combined therapy approaches.

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Combination of HexaBody-CD27 with PD-(L)1 blockade potentiates single-agent activity leading to enhanced human T-cell effector functions in vitro

A. Imle[1], I. Altıntaş[2], K. B. Nürmberger[1], J. Blum[3], A.-L. Krause[1], A. Toker[1], L. Daszkiewicz[2], A. Muik[1], F. Gieseke[1], T. Ahmadi[3], S. Fellermeier-Kopf[1], K. Kemper[2], Ö. Türeci[1], E. Breij[2], U. Sahin[1]
[1]BioNTech SE, Mainz, Germany, [2]Genmab BV, Utrecht, The Netherlands, [3]Genmab US Inc., Princeton, United States of America

Activation of the T-cell co-stimulatory receptor CD27 and blockade of the PD-1:PD-L1 axis can augment antitumor immune responses through distinct mechanisms. HexaBody®-CD27 (GEN1053/BNT313) is a novel CD27 human monoclonal antibody with a functionally inert IgG1 Fc domain harboring a hexamerization-enhancing mutation to induce CD27 activation independently of Fc gamma receptor crosslinking, while also avoiding T-cell depletion. This unique mechanism of action distinguishes HexaBody-CD27 from other CD27-targeting monoclonal antibodies. Previously presented preclinical data show that HexaBody-CD27 enhanced proliferation and effector functions of activated T cells. Here we present preclinical studies investigating the effect of combining HexaBody-CD27 with anti-PD(L)1 antibodies (pembrolizumab, nivolumab, and atezolizumab) on human T cells [1]. In co-cultures of human CD8⁺ T cells specific for the model antigen claudin-6 with cognate antigen-expressing autologous dendritic cells, combination treatment with HexaBody-CD27 and all tested anti-PD-(L)1 antibodies enhanced T-cell proliferation and proinflammatory cytokine secretion, compared to the respective single-agent treatments. Furthermore, combination treatment potentiated single-agent effects on the expression of the cytotoxic effector molecules granzyme B and CD107a by antigen-specific CD8⁺ T-cells in co-culture with cognate antigen-expressing MDA-MB-231 tumor cells. Real-time analysis of adherent tumor cells showed that combination treatment also enhanced CD8⁺ T-cell-mediated cytotoxic activity towards cognate antigen-expressing MDA-MB-231 cells. In mixed lymphocyte reaction assays of human CD8⁺ T cells and allogeneic dendritic cells, the combination of HexaBody-CD27 and pembrolizumab synergistically enhanced IFN-γ secretion, as determined by synergy analysis using the Highest Single Agent model. In conclusion, the combination of HexaBody-CD27 with anti-PD-(L)1 antibodies potentiates the effects of each single agent on effector functions of antigen-specific T cells in vitro. These findings provide preclinical rationale for investigation of this combination in clinical trials. The clinical safety and preliminary antitumor activity of HexaBody-CD27 is currently being evaluated in patients with advanced solid tumors (NCT05435339).

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The clinical-stage bispecific B7H4x4-1BB agonist, CLN-418, elicits robust B7H4-dependent immune activation and potent single-agent anti-tumor efficacy

E. Ioffe[1], K. Meetze[1], I. Shapiro[1], S. Chaudhry[1], Y. Zhang[1], M. Sardinha[1], K. Sauer[1], B. Huang[2], Y. He[2], P. A. Baeuerle[1], J. S. Michaelson[1]

[1]Cullinan Oncology, Inc., Cambridge, United States of America, [2]Harbour BioMed, Shanghai, China, People's Republic of

4-1BB is a key immune receptor that mediates robust and durable tumor control by providing a costimulatory signal to antigen-experienced T cells. First-generation 4-1BB agonistic antibodies required an Fc receptor for 4-1BB engagement and showed signs of efficacy but triggered hepatotoxicity in cancer patients. The bispecific antibody CLN-418 was designed to direct 4-1BB agonism to tumors for improved safety. CLN-418 binds both 4-1BB on T cells and B7H4, a highly prevalent solid tumor antigen with minimal expression in normal tissues. B7H4 also acts as an immunosuppressive checkpoint often uncorrelated with tumor PD-L1 expression. CLN-418 agonism of the 4-1BB pathway is strictly dependent on its cross-linking by B7H4. Moreover, targeting B7H4 may potentially overcome resistance to PD-1 blockade in difficult-to-treat indications.

To further elucidate the B7H4-dependent mechanism of action of CLN-418, B7H4 and 4-1BB expression and CLN-418 binding to B7H4 or 4-1BB positive cells were evaluated by flow cytometry. The B7H4-dependence of CLN-418 activity was studied via co-incubation of pan T cells with B7H4-expressing cancer cells. CLN-418 efficacy was assessed in 4-1BB-humanized mice bearing MC38-hB7H4 tumors (human B7H4 overexpressing MC38 tumor cell line), and in a B7H4-positive human carcinoma model in human PBMC-engrafted mice. The prevalence of B7H4 and PD-L1 expression in human tumors was examined using immunohistochemistry (IHC).

A novel B7H4 IHC assay revealed broad B7H4 expression across ovarian, endometrial and other solid cancers. CLN-418 showed high-affinity binding to cells expressing either B7H4 or 4-1BB, as well as dual cell binding to form a trimeric complex. 4-1BB expression was minimal on resting PBMC, but strongly induced on several immune cell subsets upon persistent TCR stimulation. Despite strong binding to activated T cells, CLN-418 did not increase 4-1BB expression or soluble 4-1BB in the absence of tumor cells. In contrast, CLN-418 mediated potent dose-dependent increases in 4-1BB expression, T cell activation and cytokine production, associated with release of soluble 4-1BB, in the presence of B7H4-positive, but not B7H4-negative, cancer cells. CLN-418 exhibited robust single-agent anti-tumor efficacy in mice bearing B7H4-positive human ovarian carcinoma or syngeneic MC38-hB7H4 tumors.

Collectively, enriched expression of B7H4 and 4-1BB in tumors, together with robust B7H4-dependent single-agent efficacy of CLN-418 in preclinical tumor models, support the development of CLN-418 in patients with difficult-to-treat solid cancers. CLN-418-001 (NCT05306444) is a phase 1 open-label, multicenter study to evaluate the safety, tolerability, pharmacokinetics, and anti-tumor activity of CLN-418 in subjects with advanced solid tumors.

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HexaBody-OX40, a novel Fcγ receptor crosslinking-independent OX40-targeting antibody, exhibits agonistic activity in vitro and antitumor activity in vivo

K. Kemper*[1], M. Köhne*[2], M. van der Kroef[1], K. B. Nürmberger[2], M. Russier[1], G. Zom[1], A.-L. Krause[2], P. de Goeje[1], F. Gieseke[2], A. Gorlani[1], A. Muik[2], L. Guelen[1], D. Satijn[1], S. Fellermeier-Kopf[2], T. Ahmadi[3], Ö. Türeci[2], E. Breij*[1], U. Sahin*[2]

[1]Genmab BV, Utrecht, The Netherlands, [2]BioNTech SE, Mainz, Germany, [3]Genmab US Inc., Princeton, United States of America

*Contributed equally

Clustering of the costimulatory tumor necrosis factor receptor superfamily member OX40 on activated T cells activates signaling pathways that enhance T-cell activation, survival, and proliferation. OX40 agonists in development, which require FcγR-mediated crosslinking to induce OX40 agonism, have demonstrated limited clinical activity. We present the preclinical characterization of HexaBody-OX40 (GEN1055/BNT315), a novel OX40 agonist antibody designed to induce OX40 clustering independently of FcγR-mediated crosslinking to enhance antitumor T-cell responses [1]. HexaBody-OX40 bound to activated OX40-expressing T cells, as shown by flow cytometry analysis. In a T-cell based reporter assay, HexaBody-OX40 induced FcγR-independent OX40 agonist activity, whereas the activity of conventional OX40 agonists depended on the presence of FcγR-bearing cells. HexaBody-OX40 enhanced proliferation and activation of human $CD4^+$ and $CD8^+$ T cells as well as cytokine secretion in an in vitro assay using polyclonally stimulated human PBMCs. In this assay, OX40 agonist activity in $CD8^+$ T cells depended on the presence of $CD4^+$ T cells. In an antigen-specific in vitro assay using OX40-overexpressing $CD8^+$ T-cells, HexaBody-OX40 enhanced $CD8^+$ T-cell proliferation independently of $CD4^+$ T cells. HexaBody-OX40 exhibited antitumor activity in vivo in human OX40 knock-in mice bearing subcutaneous syngeneic MC38 tumors, which was associated with peripheral T-cell proliferation and activation, increased percentages of tumor-specific $CD8^+$ T cells, and an intratumoral increase in $CD4^+$ T cells and granzyme B^+ cells. In conclusion, HexaBody-OX40 exhibited FcγR-crosslinking-independent OX40 agonist activity in preclinical studies, a unique mechanism of action that is distinct from conventional OX40 agonists. HexaBody-OX40 enhanced T-cell activation and proliferation in vitro and showed antitumor activity in vivo. The clinical safety and preliminary efficacy of HexaBody-OX40 will be investigated in a first-in-human clinical trial in patients with advanced solid tumors.

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Towards Effective CAIX-targeted Radionuclide and Immune Checkpoint Inhibition Combination Therapy

for Advanced Clear Cell Renal Cell Carcinoma

S. Kleinendorst[1], E. Oosterwijk[2], J. Molkenboer-Kuenen[1], M. Konijnenberg[1,3], M. Wheatcroft[4], K. Twumasi-Boateng[4], S. Heskamp[1]

[1]Radboud University Medical Center, Medical Imaging, Nuclear Medicine, Nijmegen, The Netherlands, [2]Radboud University Medical Center, Urology, Nijmegen, The Netherlands, [3]Erasmus MC Cancer Institute, Radiology and Nuclear Medicine, Rotterdam, The Netherlands, [4]Telix Pharmaceuticals Limited, Melbourne, Australia

Background. The treatment of advanced clear cell renal cell carcinoma (ccRCC) has evolved with the implementation of immune checkpoint inhibitors (ICIs) as standard of care. However, a substantial group of patients does respond to this treatment strategy. A promising approach to increase ICI response rate is radiation since it can generate anti-tumor immunity. Targeted radionuclide therapy (TRT), a systemic radiation treatment, targeting carbonic anhydrase IX (CAIX), which is overexpressed in ccRCC, holds great potential. Therefore, this study aims to explore the therapeutic efficacy of combined TRT/ICI and characterize changes in the tumor microenvironment (TME).

Methods. Renca-CAIX tumor-bearing mice were treated with control (vehicle), ICI (aPD-1/aCTLA4), TRT ([^{177}Lu]Lu-DOTA-hG250), or combined TRT/ICI. Tumor growth and survival were monitored for 6 weeks, and mice with complete tumor regressions were re-challenged with tumor cells. In a follow-up experiment, mice were sacrificed before or 5 or 8 days after treatment, and changes in the TME were characterized by flow cytometry, immunohistochemistry, and transcriptional profiling.

Results. Combined TRT/ICI demonstrated significant tumor growth inhibition and improved survival compared to control at a dose at which TRT or ICI monotherapies were not effective. Complete responses were observed in 80% of mice treated with combination treatment, and 94-100% of these mice rejected tumor cell re-challenge, suggesting tumor-specific immune responses. Ex vivo analyses of treated tumors revealed DNA damage, T-cell infiltration, and modulated immune signaling pathways in the TME after combination treatment.

Conclusion. Subtherapeutic TRT combined with ICI showed superior therapeutic outcome and significantly altered the TME.

Disclosures. Telix Pharmaceuticals provided funding and CAIX-specific antibody for this study.

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Knockdown of NRP1 by third generation antisense oligonucleotides combined with immune checkpoint inhibition as tumor therapy

A. Maaske[1], N. Kirchhammer[2], J. Festag[1], M. Buchi[2], M. Schell[1], S. Raith[1], S. Michel[1], A. Zippelius[2,3], R. Klar[1], F. Jaschinski[1]

[1]secarna pharmaceuticals GmbH & Co. KG, Immune oncology, Planegg, Germany, [2]University Hospital Basel, Cancer Immunology, Department of Biomedicine, Basel, Switzerland, [3]University Hospital Basel, Medical Oncology, Basel, Switzerland

The multi-domain surface receptor NRP1 has been shown to be of importance in several tumorigenic pathways and processes, including exhaustion of effector T-cells, phenotypic stability of regulatory T-cells, migration and polarization of macrophages, and angiogenesis. Since these functions are mediated by different domains of NRP1 and can involve several co-receptors and ligands, knockdown of the entire protein, induced by locked nucleic acid (LNA) antisense oligonucleotides (ASOs), seems intrinsically advantageous over functional blocking of single domains. Immune checkpoint proteins with less diverse functionality, like PD-1 or PD-L1, on the other hand, are likely to be blocked adequately by antibody-based regimens and show efficacy in some cancer patients. However, the majority of patients is refractory to anti-PD-1 treatment, demonstrating a clear medical need for additional therapy approaches and combination regimens.
We designed LNA ASOs with specificity for human and mouse NRP1, which achieve robust knockdown in vitro in several cell lines and in vivo in different organs and immune cell types, including cells both of myeloid and lymphoid origin.
We employed different syngeneic mouse tumor models. After establishment of a palpable tumor, the animals were treated with NRP1-specific ASOs alone, anti-PD-1 or anti-PD-L1 alone, or a combination of NRP1-ASO with either anti PD-L1 or PD-1. Tumors were either excised for analysis or monitored regarding growth and subsequently survival. Knockdown of cellular and soluble NRP1 is shown in tumor and serum, respectively.
Systemic application of NRP1-specific LNA-ASOs, has led to a preferential knockdown in immune cells, which was already observable three days after start of the treatment. ASO treatment as monotherapy suppressed tumor growth and could even induce tumor eradication in some animals resulting in a profound survival benefit. Moreover the combination of both treatments could achieve complete tumor regression in the majority of mice resulting in long-term survival.
Here, we demonstrate the compelling potential of LNA-ASOs to reversibly affect molecules and pathways that are difficult to target with antibodies or small molecules. In this regard, we believe NRP1 is a particularly suitable target for this drug modality, since knockdown of the entire protein leads to simultaneous reduction of all functions of NRP1 in the affected cells. Furthermore, we show that this broad knockdown has promising anti-tumor effects in mouse models, which can be further increased by combination with antibodies targeting other immune checkpoints. Taken together, LNA-modified ASOs targeting NRP1 can be considered as a novel approach for treatment of cancer and the translation towards the clinic of this approach has already started.

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Generation of recombinant antibody-based fusion proteins for radioimmunodiagnosis and -therapy of brain cancer

S. Malindi, S. Barth
University of Cape Town, Integrative Biomedical Science, Cape Town, South Africa

Brain cancer stands as a prominent contributor to morbidity worldwide. In 2020, more than 300,000 individuals globally were diagnosed with brain cancer, leading to 251,329 deaths. Among all human malignant brain tumors, glioblastoma (GBM) is the deadliest and most aggressive, with an infiltrative nature. Even with the current treatments, which include surgery followed by radiation and chemotherapy,

the prognosis is dismal. It remains incurable and has a median survival of about 15 months. A further difficulty is early detection and imaging of brain cancer cells, especially in African countries where resources are limited. Thus, the development of targeted diagnostic modalities and treatments for brain cancers is crucial. Immunotherapy, at the forefront of cancer treatment, relies on targeted antibody approaches, selecting biomarkers exclusively present on tumor cell surfaces. Although antibody-based therapies targeting these receptors have made significant progress, they face limitations like poor tumor tissue penetration and limited persistence. To address these issues, this study aims to use a unique SNAP tag-based antibody format to selectively deliver radionuclides comprising substrates by targeting overexpressed biomarkers on the surface of brain cancer cells. In this study, SNAP-tag, a modified DNA repair enzyme, is combined with a scFv antibody to target specific tumor receptors. The SNAP-tag antibody and the BG chemical have a 1:1 ratio, allowing for complete saturation of the fusion protein. In prior studies, BG-modified SNAP-tag fusion proteins have shown potent cytotoxicity and preclinical effectiveness in immunodiagnostic imaging. The SNAP-tag-based fusion proteins are transiently expressed in mammalian cells, followed by purification with immobilized metal ion affinity chromatography (IMAC). The enzymatic activity of the SNAP-tag is assessed using BG-modified fluorophores. SNAP-tagged fusion proteins are then conjugated to BG-Alexa fluorophores to provide highly specific labeling in vitro. Subsequently, cell-surface binding is confirmed by confocal microscopy. Additionally, for cytotoxic proof-of-concept studies, fusion proteins are conjugated with a small molecule of BG-toxin to selectively kill tumor cell lines in vitro. Advanced studies utilizing radiolabeled isotopes in xenografted human brain cancer-bearing mice will further validate the recombinant protein's applicability for immunodiagnostic and targeted therapy for brain cancers. Following this, preclinical investigations will be conducted, paving the way for precision therapy in brain cancer patients. Thus far, this study has successfully demonstrated optimal conjugation efficiencies and validated the site-specific targeted activity of the SNAP-tag-based fusion protein. This work represents a proof-of-concept for exploring the use of radio-labeled SNAP-tag-based antibody fusion proteins in brain cancer detection and patient stratification, offering personalized therapeutics.

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Genetically armed macrophages eliminate liver metastases by expanding and reprogramming tumor-reactive T cells

M. Notaro[1], M. Borghetti[1], C. Bresesti[1], G. Giacca[1,2], T. Kerzel[1], C. M. Mercado[1], S. Breggion[1,2], S. Beretta[3], M. Monti[3], I. Merelii[3], S. Iaia[4], A. Annoni[4], S. Gregori[4], L. Naldini[1,2], M. L. Squadrito[1,2]
[1]San Raffaele Scientific Institute, SR TIGET, Cancer targeted gene therapy, Milano, Italy, [2]Vita-salute San Raffaele University, Milano, Italy, [3]San Raffaele Scientific Institute, SR TIGET, BioInformatics Core, Milano, Italy, [4]San Raffaele Scientific Institute, SR TIGET, Immune Core, Milano, Italy

Liver metastases (LM) are associated with unfavorable prognosis and are often the cause of death in cancer patients. The immune-suppressive environment of the liver promotes metastatic seeding and proliferation of tumor cells, concurrently reducing the efficacy of cancer therapies, including immunotherapy. Therefore, it is of pivotal importance to develop innovative therapeutic interventions aimed at unleashing immune responses within the liver in presence of LM.
We designed a new type of lentiviral vector (LV)-based immunotherapy, which allows simultaneous delivery

of tumor antigens (TA) and immune-activating cytokines selectively to liver macrophages (hereon TA.Combo). The therapeutic efficacy of TA.Combo was investigated in distinct mouse models of liver metastases obtained by intrahepatic or intrasplenic injection of cancer cells. We used MC38-OVA cells, a colorectal cancer (CRC) cell line engineered to express the surrogate TA ovalbumin (OVA); B16 cells, a melanoma cell line expressing the tumor-associated antigen Trp-2; and AKTPF cells, a cell line of CRC in which we identified tumor neoantigens exploiting whole exome sequencing, RNA sequencing and bioinformatic predictions. To characterize the molecular determinants of immune activation after treatment we used flow cytometry, single cell, and spatial transcriptomics.
We found that prophylactic vaccination with the TA protected mice from developing tumors only when low doses of LV driving the expression of TA containing both MHC-I and MHC-II immunogenic epitopes were employed. However, in the presence of established LM, TA LV delivery failed to control LM growth. Conversely, simultaneous delivery of liver specific LVs carrying the TA, IL-12 and IFNa (TA.Combo) reduced the growth of established LM up to complete eradication. While IFNa alone promoted MHC-I presentation in cancer cells, macrophages and dendritic cells; IL-12 ameliorated the phenotype of tumor-reactive T cell while boosting both MHC-I and MHC-II presentation on liver and tumor antigen presenting cells. TA.Combo reshaped the genetic program and effector functions of TA-specific T cells, which displayed enhanced markers of immune activation and reduced expression of markers associated with T cell exhaustion. TCR clonotype tracking revealed that CD4 and CD8 T cell clones expanding in the liver upon treatment efficiently reached LM in presence of TA.Combo , but not when TA was delivered alone. Of note, TA.Combo promoted acquisition of effector function in T cell independently of MHC-II-restricted presentation of the delivered TA.
In summary, we found that simultaneous gene-based delivery of tumor antigens and cytokines to liver macrophages, in mice hosting established liver metastasis, enables the generation and reprogramming of tumor-specific CD8+ T cells leading to tumor eradication. TA.Combo holds promise for advancing LM treatment strategies in cancer patients.

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Recombinant listeria monocytogenes vaccine induces antigen-specific anti-tumor immune response in pyroptosis and necroptosis-deficient mice

A. Olagunju, A. Sardinha, G. Amarante-Mendes
Institute of Biomedical Sciences, Immunology, Sao Paulo, Brazil

Effective induction of an in vivo CD8+ T cell-mediated anti-tumor immune response is one of the major focuses of developing anti-cancer vaccines. Here, we present studies with a live recombinant vector, Listeria monocytogenes (LM), and the effect of individual deficiencies in regulatory or effector molecules associated with necroptosis or pyroptosis. Recombinant LM has been shown to elicit a robust in vivo CD8+ T cell response in preclinical settings. Significantly, LM has been demonstrated to induce inflammatory/immunogenic cell death mechanisms such as pyroptosis and necroptosis. Therefore, we hypothesized that the host's response to LM and the consequent induction of CD8+ T cell-mediated immunity would be compromised by a lack of regulatory or effector molecules involved in these cell death pathways. We used recombinant LM carrying the ovalbumin gene (LM.OVA) to vaccinate C57Bl/6 wild-type (WT), caspase-1/11-/-, gsdmd-/-, ripk3-/-, and mlkl-/- mice to test our hypothesis. An in vivo cytotoxicity

experiment was performed to assess the effectiveness of OVA-specific CD8+ T lymphocytes in eliminating target cells. Furthermore, B16 and B16.OVA melanoma cell line growth in vivo was assessed in vaccinated and control mice. Our findings demonstrated that while caspase 1/11 and GSDMD deficiency contribute to the regulation of LM.OVA infection, the antigen-specific anti-tumor response elicited by LM-OVA, was not compromised by a single deficiency of these key molecules in vaccine optimized settings.

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A DNA plasmid melanoma cancer vaccine, SCIB1, combined with nivolumab + ipilimumab in patients with advanced unresectable melanoma

S. Paston[1], H. Shaw[2,3], P. Patel[4], M. Payne[5], S. Kumar[6], S. Danson[7], M. Highley[8], C. Barlow[9], R. Miller[1], G. Cane[1], J. Chadwick[1], S. Shah[10], V. Brentville[10], R. Metheringham[10], G. Goodhew[1], F. Master[1], L. Durrant[1,10]
[1]Scancell Ltd, Oxford, United Kingdom, [2]Mount Vernon Cancer Centre, Northwood, United Kingdom, [3]University College London Hospital, London, United Kingdom, [4]Nottingham Hospitals University Trust, Nottingham, United Kingdom, [5]Churchill Hospital, Oxford University Hospitals, Oxford, United Kingdom, [6]Velindre Cancer Centre,, Cardiff, United Kingdom, [7]Weston Park Cancer Centre, Sheffield, United Kingdom, [8]Derriford Hospital, University Hospitals NHS Trust, Plymouth, United Kingdom, [9]Musgrove Park Hospital, Somerset NHS Foundation Trust, Taunton, United Kingdom, [10]Scancell Ltd, Nottingham, United Kingdom

Targeting melanoma by T cells drives anti-tumour responses. Previously in a phase 1/2 we successfully evaluated a DNA vaccine, SCIB1, incorporating T cell epitopes from TRP-2/gp100 into an antibody framework to allow Fc targeting of activated dendritic cells. In stage 3/4 unresectable melanoma patients receiving SCIB1 as a monotherapy we showed a 60% DCR and 88% of patients treated with SCIB1 post tumour resection remained disease free for 5 years.

Checkpoint inhibitor (CPI) therapies are now standard of care for melanoma, with nivolumab and ipilimumab being the most used combination. The current Phase 2 SCOPE trial tests the hypothesis that unresectable patients may have an improved response when SCIB1 is combined with CPI. Patients were treated with nivolumab with ipilimumab and SCIB1 (8mg) i.m. using needle-free injection at a fixed dosing schedule for a total of 10 doses over 24 months.

To date, 19 patients received the combination of SCIB1 with nivolumab and ipilimumab. At study entry, all patients were stage IV. Eleven patients had reached the first imaging timepoint at 13 weeks, and the objective response rate is 82% with only one PD. Eight responses were confirmed in a subsequent scan, one is pending. Patients showed a 40-95% reduction in tumour volume between 13 and 25 weeks. Functional T cell responses to the TRP2 and gp100 peptides can be detected in patients post vaccination.

The limitation of SCIB1 is the targeting of select HLA haplotypes which has restricted its use to 40% of patients. To overcome the limitation of select HLA haplotypes of SCIB1 we have developed iSCIB1+ that covers the most common HLA alleles and incorporates modifications in its Fc region to enhance the potency of the vaccine. Data from preclinical murine models show that iSCIB1+ is more potent than SCIB1 and has broader HLA allele coverage, iSCIB1+ has been included as a separate cohort in the current SCOPE trial.

SCIB1 in combination with nivolumab and ipilimumab as first line treatment for unresectable melanoma resulted in an ORR of 82% without an increase in clinically meaningful adverse events. The addition of the iSCIB1+ vaccine cohort should increase the number of patients that can benefit from this vaccine.

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GEN1042-mIgG2a, an Fc-inert mouse-human chimeric variant of GEN1042 (DuoBody®-CD40x4-1BB), exhibits in vivo antitumor activity and peripheral immune modulation

K. Pilones[1], A. Sette[2], K. B. Nürmberger[3], M. Luijten[2], M. Köhne[3], J. Wolthoorn[2], A. Muik[3], S. Fellermeier-Kopf[3], T. Ahmadi[1], O. Türeci[3], U. Sahin[3], M. Jure-Kunkel[1], H. Adams III[1]
[1]Genmab, Plainsboro, United States of America, [2]Genmab B.V., Utrecht, The Netherlands, [3]BioNTech SE, Mainz, Germany

GEN1042 (DuoBody®-CD40x4-1BB) is an investigational, novel, bispecific antibody combining targeting and conditional activation of CD40 and 4-1BB on immune cells. Preclinical characterization and encouraging clinical activity of GEN1042 in solid tumors were recently reported. We investigated the in vivo biologic activity and mechanism of action of GEN1042 using the mouse-human chimeric, Fc-inert, surrogate antibody GEN1042-mIgG2a in immunocompetent human CD40/human 4-1BB double knock-in (hCD40/h4-1BB dKI) mice. Biologic activity of GEN1042-mIgG2a, compared with GEN1042, was evaluated in vitro using cell-based reporter and human T-cell proliferation assays. hCD40/h4-1BB dKI mice subcutaneously implanted with syngeneic colorectal MC38 tumors were treated systemically with three biweekly doses of GEN1042-mIgG2a or isotype control after tumor establishment; pharmacokinetics, tumor growth and survival were then examined. A parallel study investigated dose-dependent effects on circulating immune cells and plasma cytokines. Pilot studies investigated the combination of GEN1042-mIgG2a with PD-1 blockade and a platinum-based chemotherapy doublet. Comparable biologic activity of GEN1042 and GEN1042-mIgG2a was confirmed in vitro. GEN1042-mIgG2a at 1 and 10 mg/kg biweekly largely maintained plasma concentrations within predicted levels in tumor-bearing dKI mice. Treatment with GEN1042-mIgG2a 1 and 10 mg/kg delayed tumor growth with observed significance on Day 12 after treatment initiation (P=0.0015 and P=0.0232, respectively). GEN1042-mIgG2a 1 mg/kg significantly improved progression-free survival versus control (P=0.001). Antitumor activity at GEN1042-mIgG2a 1 mg/kg was associated with favorable peripheral immune modulation, including an increased pool of memory T cells, upregulation of T-cell activation markers, induction of 4-1BB and CD86 on B cells, and transiently increased IFN-γ concentrations. Improved survival was observed with a combination of GEN1042-mIgG2a 1 mg/kg, PD-1 blockade, and a platinum-based chemotherapy doublet compared with GEN1042-mIgG2a alone in preliminary data with this model, resulting in complete tumor regressions in 3/10 (30%) mice. In summary, GEN1042-mIgG2a showed dose-dependent in vivo antitumor activity in immunocompetent MC38 tumor-bearing hCD40/h4-1BB dKI mice and generated a peripheral immune profile consistent with its hypothesized mechanism of action. This model has enabled ongoing preclinical exploration of GEN1042 in combination with PD-1 blockade and a platinum-based chemotherapy doublet, which is hypothesized to potentiate antitumor activity through complementary immune modulatory effects. These data support ongoing clinical studies evaluating combination therapy in patients with advanced solid tumors (NCT04083599, NCT05491317).

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An oncolytic HSV-1 vector induces a therapeutic adaptive immune response against glioblastoma

A. Reale*[1], A. Gatta*[2], A. K. B. Shaik[2], M. Shallak[2], A. M. Chiaravalli[3], M. Cerati[3], A. Calistri[1], R. Accolla[2], G. Forlani[2]

[1]University of Padua, Department of Molecular Medicine, Padua, Italy, [2]University of Insubria, Department of Medicine and Technological Innovation, Varese, Italy, [3]ASST Sette-Laghi, Unit of Pathology, Varese, Italy

*Contributed equally

Glioblastoma (GBM) is the most frequent and aggressive brain tumor in adults, and it carries one of the lowest survival rates five years post-diagnosis. Oncolytic viruses (OVs) selectively target and damage cancer cells and, for this reason, they are being actively investigated as new therapeutic tools also against GBM. Here we report that an oncolytic herpes simplex virus type 1 (oHSV1) expressing the reporter gene enhanced green fluorescent protein and with deletions in the γ34.5 neurovirulence gene and the Us12 gene (EGFP-oHSV1), replicates and induces cell death in various human and murine GBM cell lines [1]. Using an orthotopic, syngeneic mouse model of GBM (GL261) we compared the efficacy of EGFP-oHSV1 treatment with vaccination with GL261 cells expressing MHC class II molecules after transfection with a construct encoding CIITA (GL261-CIITA) [2][3]. Our results show that a single intracranial injection of EGFP-oHSV1 in established GL261 tumors significantly prolongs survival in all treated mice compared to placebo treatment. Notably, 45% of treated mice became long-term survivors, and all rejected GL261 cells upon rechallenge in the contralateral brain hemisphere**,** indicating an anamnestic antitumoral immune response. Overall survival rate of EGFP-oHSV1-treated animals was lower than that obtained following GL261-CIITA vaccination, which also results in complete rejection of GL261 parental tumors after challenge. Post-mortem analysis revealed increased infiltration by CD4+ and CD8+ T lymphocytes and intratumoral vascular collapse in OV-treated tumors, along with a profound modification of the tumor microenvironment witnessed by the activation and redistribution of macrophage, microglia and astroglia cells in the tumor area, with the formation of intense fibrotic tissue, suggestive of complete rejection in long-term survivor mice. Thus, EGFP-oHSV1 demonstrates potent antitumoral activity in an immunocompetent GBM model as a monotherapy, as a result of direct cell killing combined with the stimulation of a protective adaptive immune response. The similar picture observed in GL261-CIITA vaccinated mice strongly suggests that two distinct treatments, oncolytic HSV1 and CIITA-driven MHC class II expression in tumor cells, could be combined to reach complete regression of established GBM [4].

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Abstract has been withdrawn

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Enhanced anti-tumor effects in a preclinical model for colon carcinoma by combining carbon ion irradiation with neoantigen RNA-LPX vaccines? A proof of principle.

N. Salomon[1], A. Helm[2], C. Totis[2], A. Selmi[1], C. Fournier[2], M. Diken[1], B. Schrörs[1], M. Scholz[2], S. Kreiter[1], M. Durante[2], F. Vascotto[1]
[1]TRON- University Medical Center, Johannes Gutenberg University gGmbH, Mainz, Germany, [2]GSI Helmholtz Center for Heavy Ion Research GmbH, Biophysics, Darmstadt, Germany

Conventional radiotherapy uses sparsely ionizing X-rays, whereas densely ionizing radiation, i.e. carbon ions are increasingly applied. Their specific physical characteristics, i.e. the inverted depth dose profile allows to escalate the tumor dose and/ or to spare the normal tissue. In addition, the DNA damage induced at the tumor site is clustered and difficult to repair.
For a growing number of tumor types, preclinical and clinical studies showed the expected enhanced efficacy of carbon ions compared to X-rays in substantially shrinking or even killing the primary tumors. Based on the induction of different DNA damage and cell death patterns, first preclinical studies show an enhanced immunogenic effect of carbon ion compared to X-ray. However, further fostering the immune response in a combination of radio and immunotherapy bears the potential for a long lasting anti-tumor effect. Along this line, we set out to investigate in an adenocarcinoma model the combination of carbon ion irradiation with a treatment using a tumor specific neoantigen (neoAg) LPX vaccine, which has already shown promising results in combination with XRT
We assessed in vitro in several murine tumor cells (adenocarcinoma cell lines MC38 and CT26, the lung cell line TC-1 and the breast carcinoma cell line 4T1) the potential immunogenic effects of irradiation alone, comparing cell death induction and immunogenic markers (HMGB1, Calreticulin) for carbon ions with X-ray up to a dose of 8 Gy.
The in vivo part of our study was carried out in a colon adenocarcinoma mouse model (MC38 cells, injected in the flank of C57BL/6 mice). To study the potential to enhance anti-tumor effects, we used densely

ionizing carbon ions under therapy relevant conditions (CIRT) and for comparison isoeffective doses of XRT, in combination with RNA-LPX vaccines, encoding for MHC class I- and class II- tumor-specific neoantigens. We assessed tumor growth inhibition and characterized tumor immune cell infiltrates and antigen-specific T cell responses.
We observed in vitro that cell death induction, expression of calreticulin and release of HMGB1 is more efficient after carbon ion compared to X-ray irradiation. The same accounts for tumor growth inhibition in vivo and combined treatment with NeoAg RNA-LPX reduces growth even further. The main driver for NeoAg-specific T cell responses, and infiltration and cytotoxicity of neoAg-specific T cells are the RNA-LPX vaccines. Compared to XRT, the combination of CIRT with RNA-LPX vaccines shows a comparable therapeutic efficiency, rendering CIRT especially for radioresistant tumors in combination with RNA-LPX vaccines a promising strategy.
The carbon ion experiments (SBIO_08_Salomon) were performed at the SIS18 accelerator (GSI Helmholtz Center for Heavy Ion Research, FAIR Phase-0).

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CD8+ T cell-derived CD40L mediates non-canonical cytotoxicity crucial for tumor immunity and tumor progression in renal cell carcinoma patients

P. Schiele[1], A. Sada Japp[1], R. Stark[1], J. J. Listopad[2], C. Nikolaou[1], T. Blankenstein[2], I.-K. Na[1], A. Thiel[1], M. Frentsch[1]
[1]BIH Center for Regenerative Therapies (BCRT), Berlin Institute of Health at Charité - Universitätsmedizin Berlin, Berlin, Germany, [2]Max-Delbrück-Center for Molecular Medicine & Institute for Immunology, Charité - Universitätsmedizin Berlin, Berlin, Germany

T cells and their effector functions play a crucial role in tumor immunity. Cytotoxicity is considered the canonical effector function of CD8+ T cells, where perforin, granzymes, and death receptors are acknowledged as the cytotoxic factors. We demonstrate that tumor-specific CD8+ T cells can induce cytotoxicity in cancer cells directly through CD40L, thereby controlling tumor progression. This effect cannot be compensated by other canonical cytotoxic factors. Non-canonical cytotoxic signaling of CD40 is primarily delivered by CD8+ T cells, as many tumors do not express MHC-II, limiting CD40L derived from CD4+ T cells. In murine models, up to 50% of tumor-specific CD8+ T cells can express CD40L, and conditional CD40L gene ablation in CD8+ T cells resulted in tumor formation. CD4+ T cells as well as CD40L-/- but not CD40L competent CD8+ T cells failed to prevent tumor growth in lymphocyte-deficient mice. Further in vivo experiments showed that the prevention of tumorigenesis by CD40L+ CD8+ T cells strictly depended on CD40 expression by cancer cells. We also demonstrated that CD40L-CD40 signaling in CD40+ cancer cells initiates caspase 8 activation, resulting in increased cell death. Consistent with these observations, gene expression signatures associated with resistance to CD40 signaling-induced cell death correlated with survival in different RCC patient cohorts as well as the presence of CD40L expression on tumor-infiltrating CD8+ T cells. Our results show that CD40L expressed by tumor-reactive CD8+ T cells is an unknown antitumor function that exploits the largely overlooked susceptibility of various cancers to CD40-mediated apoptosis. These findings will unveil new treatment and stratification options for patients with malignant diseases.

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Induction of anti-HPV16 cellular immune responses by a silica nanoparticle vaccination platform in MHC-humanized mice

A.-K. Schlosser[1,2], L. T. Roth[1,3,4], S. Kruse[1], P. Uhl[5], A. K. Grabowska[5], A. Kübelbeck[5], A. B. Riemer[1,3]
[1]German Cancer Research Center (DKFZ), Immunotherapy & Immunoprevention, Heidelberg, Germany, [2]Heidelberg University, Faculty of Biosciences, Heidelberg, Germany, [3]German Center for Infection Research (DZIF), partner site Heidelberg, Molecular Vaccine Design, Heidelberg, Germany, [4]Heidelberg University, Faculty of Medicine, Heidelberg, Germany, [5]Heidelberg University, Institute of Pharmacy and Molecular Biotechnology, Heidelberg, Germany

Persistent infection with high-risk types of the human papillomavirus (HPV) causes cancer in both women and men and accounts for approximately 5% of cancer cases worldwide. HPV16 is by far the most important cancer-causing high-risk type. Many therapeutic vaccines targeting HPV16-associated malignancies have already been tested and shown to be highly effective in preclinical studies but lacked effectiveness when tested in human patients. Here, we present a novel vaccine platform consisting of silica nanoparticles (SiNP) in combination with the HPV16-derived HLA-A2-restricted epitope E7/11-19 and the adjuvant poly(I:C) for application against HPV16-derived malignancies. SiNP consist of an amorphous silica core with a functionalized surface that allows for adsorption of various epitopes to its surface. Using fluorescence microscopic and flow cytometric analyses, we confirmed the uptake of the SiNP vaccine construct by antigen-presenting cells (APCs) and the presentation of the epitope on the APCs' cell surface in vitro. Biodistribution studies in rats showed that the epitope remained at the injection site longer when coupled to SiNP compared to free epitope. For evaluation of the ability of SiNP to induce epitope-specific systemic and mucosal immune responses in vivo, MHC-humanized mice were vaccinated three times subcutaneously, followed by flow cytometric analysis of splenocytes and immune cells isolated from the female genital tract. Vaccination with SiNP loaded with the CD8 T cell epitope HPV16 E7/11-19 resulted in a successful induction of CD8 T cells which could be significantly enhanced by additionally including the CD4 T cell epitope PADRE (pan-DR epitope) in the vaccine formulation. Furthermore, we assessed the effect of vaccinating with a larger pool of epitopes. Additional SiNP vaccination studies aiming at inducing a strong mucosal immune response are currently under investigation. These include the evaluation of different administration routes and prime-pull approaches in order to pull systemically induced T cells into the vaginal mucosa. The most promising vaccination strategies, as determined by these analyses, will be tested for efficacy by assessing orthotopic HPV16$^+$ tumor shrinkage/elimination. Taken together, these experiments will be a crucial step in the preclinical assessment of SiNP as a new therapeutic HPV vaccine platform. The obtained results will provide important insights for the translation to clinical trials for therapeutic HPV vaccination.

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Deletetious knockouts in the HLA class I antigen processing and presentation machinery induce distinct changes in the immunopeptidome

I. Shapiro[1,2], C. Maschke[1], A. de Waard[3,4], T. Verkerk[3,4], R. Spaapen[3,4], M. Bassani-Sternberg[1,2]

[1]CHUV, Unil, Department of Oncology, Lausanne, Switzerland, [2]Ludwig Institute for Cancer research, Lausanne, Switzerland, [3]University of Amsterdam, Amsterdam, The Netherlands, [4]Sanquin Research, Department of Immunopathology, Amsterdam, The Netherlands

Antigen presentation is an indispensable trigger for T-cell-based immune responses where T-cell receptors recognize HLA-peptide complexes on the cell surface. In the case of HLA class I, cells constantly sample the cytoplasmic proteome for short peptides to present them as antigens. For antigen presentation, an entire network of proteins acts in concert to uphold a molecular assembly line that delivers a flow of HLA-peptide complexes to the cell surface to potentially bind T-cell receptors. Functionally impairing mutations of the antigen processing and presentation machinery (APPM) are a frequently observed immune evasion mechanism in cancer. Our aim is to understand how deleterious knock-outs of key players in the APPM affect the antigenic landscape. Here we show that impairments of the APPM result in discernable characteristics of the antigenic landscape for a human cancer-derived cell line.
In this study we leveraged a series of previously generated 11 isogenic HAP1 cell lines bearing single knock-outs (IRF2, B2M, GANAB, CALR, CALX, TAPBP, PDIA3, ERAP1, TAP1, TAP2, or SPPL3), including genes involved if modifying, chaperoning, translocating, and loading the HLA I-peptide complex. HAP1 is a near-haploid leukemia-derived cell line model with highly frequent HLA class I alleles among the European population, and, as such, a suitable model to generate and study the immunopeptidome of APPM knock-outs. We applied mass spectrometry-based proteomics and immunopeptidomics methods to understand the effects on the proteome and antigen landscape. We collected data in discovery- and quantitation-based acquisition modes to achieve a depth that exceeds 22’000 quantified unique peptides originating from more than 8000 unique proteins. We notice varying levels of impact on the overall ability to present antigens, ranging from complete elimination of antigen presentation capability such in the case of B2M to sampling depths resembling those of the wildtype controls as in CALR. We also determined knock-out specific effects that are directly linked to the biological function of knocked-out genes, e.g., knocking-out ERAP shifted the peptide length distribution to longer peptides. Thirdly, we observe effects that are not explained by and are poised to expand current models, such as TAP2 knock-outs showing a significant relative decrease in peptides presented by HLA-B specifically.
This work improves our understanding of the role of different APPM actors in how they shape the immunopeptidome and bears implications regarding the prioritization of T-cell targets for personalized cancer immunotherapy interventions.

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Improving personalized cancer vaccines by encapsulating MHC class I and class II neoepitope peptides in cationic liposomes

F. S. Spitzer[1], J. Heuts[1], M. Camps[1], S. Romeijn[2], F. Ossendorp[1], K. van der Maaden[1]
[1]Leiden University Medical Center, Department of Immunology, Leiden, The Netherlands, [2]Leiden University, Leiden Academic Centre for Drug Research, Division of BioTherapeutics, Leiden, The Netherlands

Personalized cancer therapy is the future of oncology, as it offers the potential to improve clinical outcomes with less side effects. Tailoring the treatment to the tumor avoids the use of expensive drugs that may be

ineffective and can cause side effects that severely decrease the patient's quality of life. Novel approaches, such as patient-specific vaccination, aim to improve anti-tumor immunity and minimize adverse effects. Underlying this approach is the identification of neoepitopes, which derive from patient-specific mutations. Neoepitopes can be presented to T cells in the form of peptides bound to MHC class I and class II molecules, leading to highly specific anti-tumor immune responses. Neoepitope peptides for personalized vaccines can be produced easily under GMP conditions at low cost, but are poorly immunogenic by themselves. One approach to boost the immunogenicity of peptides is by encapsulating them in cationic liposomes.
In this study, we introduce a novel method for the efficient encapsulation of peptides with diverse physicochemical properties into cationic liposomes. Liposome-encapsulated peptide neoepitopes are capable of activating neoepitope-specific CD8 T cells in vitro upon uptake by dendritic cells. Vaccination with liposomal MHC class I and class II neoepitope peptides increased the magnitude of vaccine-induced CD8 and CD4 T cell responses compared to vaccination with the same free neoepitope peptides. Furthermore, combining several liposome-encapsulated MHC class I and class II neoepitope peptides into one vaccine strongly enhanced tumor control and survival in a murine colorectal cancer model.
In conclusion, formulating neoepitope peptides in cationic liposomes is a potent strategy to improve personalized cancer vaccination. Liposomal neoepitope peptide vaccines induce strong and robust tumor-specific T cell responses and significantly improve tumor control, highlighting the huge potential of liposomal peptide delivery in the field of personalized cancer therapy.

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Combination of artLCMV and ANV419 strongly potentiates the tumor-specific T cell response resulting in complete tumor clearance in a colorectal cancer model

J. Strauss[1], J. Raguz[1], K. Richter[2], N. Rammer[1], K. Pojar[1], D. Reckendorfer[1], C. Huber[2], K. K. Orlinger[1], H. Lauterbach[1]
[1]Hookipa Pharma Inc., Vienna, Austria, [2]Anaveon AG, Basel, Switzerland

CD8+ T cells play a critical role in controlling tumor growth and are essential for clinical responses to immune checkpoint inhibitors. Yet, the induction and maintenance of functional tumor antigen-specific T-cell responses still pose a great challenge in cancer immunotherapy. Replicating arenavirus vectors are currently evaluated in multiple clinical trials in infectious diseases and oncology and have demonstrated potent induction of target antigen-specific CD8+ T cells, reaching up to almost 50% of the total CD8+ T cell compartment in peripheral blood of patients with human papillomavirus (HPV) 16+ cancer. ANV419 is a selective IL-2Rβγ targeted anti-IL-2 antibody/IL-2 fusion protein that preferentially stimulates cytotoxic CD8+ T and natural killer (NK) cells over immunosuppressive regulatory T cells, with a significantly longer half-life than that of conventional IL-2. We, therefore, hypothesized that ANV419 could even further enhance and maintain arenavirus vector-induced tumor antigen-specific T cell responses and enhance antitumor efficacy. The combination of replicating lymphocytic choriomeningitis virus vectors (artLCMV) encoding the tumor-associated antigen GP70 with ANV419 led to a strong increase of peripheral tumor antigen- specific CD8+ T cells and NK cells compared to vector treatment alone. Notably, the CD8+ T cell/regulatory T cell ratio also increased substantially. The increase of tumor antigen-specific CD8+ T cells was associated with enhanced anti-tumor efficacy in the syngeneic MC38 colorectal cancer model, resulting

in a tumor cure rate of 100%. Furthermore, complete tumor clearance resulted in immunological memory as demonstrated by protection against tumor re-challenge. These preclinical data highlight the great potential of the combination of arenavirus vectors and ANV419 as a next generation cancer immunotherapy.

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MAdCAM-1+ tumor-associated high endothelial venules are key for LIGHT-mediated anti-glioma immunity

T. van de Walle, S. Franke, A. Vaccaro, M. Melssen, M. Hedlund, A. Shanti, M. Ulvmar, M. Ramachandran, M. Essand, A. Dimberg
Uppsala University, Department of Immunology, Genetics and Pathology, Uppsala, Sweden

High endothelial venules (HEVs) are specialized blood vessels that express various molecules involved in the recruitment of specific immune cell subtypes to the tissue. Peripheral node addressin (PNAd) is the standard marker used to identify these vessels in the lymph nodes, while mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) is not typically used since its expression is confined to the HEVs of gut-associated lymphoid tissues in adults. Furthermore, PNAd+ HEVs play a role in the formation of cancer-associated tertiary lymphoid structures (TLS), which are ectopic clusters of lymphoid cells that are associated with positive prognosis, as well as in the recruitment and maintenance of the intratumoral stem-like T cell population, which is responsible for tumor control following checkpoint blockade. Our group has previously demonstrated that treatment of murine glioma with the lymphoneogenic cytokine LIGHT results in prolonged survival in association with the development of functional MAdCAM-1+ tumor-associated HEVs (TA-HEVs), formation of T cell-rich TLS, and a boost in the population of TCF1+CD8+ stem-like T cells. However, the key driver in this system or potential interplay between these effects is yet to be clarified. In the current study, we aimed to elucidate the role of MAdCAM-1+ TA-HEVs in the LIGHT-mediated anti-tumor response by using a MAdCAM-1–/– mouse line to abolish their formation. Strikingly, while LIGHT overexpression still prolonged survival in the absence of MAdCAM-1, the survival benefit in MAdCAM-1–/– mice was reduced compared to their MAdCAM-1+/+ counterparts, suggesting that MAdCAM-1+ TA-HEVs have an important role in LIGHT-mediated tumor clearance but are not the only mechanism at play. Interestingly, although PNAd+ TA-HEVs were still observed in the tumors, knockout of MAdCAM-1 completely prevented the LIGHT-induced boost in stem-like T cells, indicating that the recruitment of these cells is specifically driven by MAdCAM-1. Furthermore, TLS formation was unaffected by the absence of MAdCAM-1, suggesting that these structures may still contribute to the survival benefit provided by LIGHT. As such, this study demonstrates that the roles of TA-HEVs can differ depending on their expression patterns, with MAdCAM-1 playing a central role in LIGHT-mediated anti-tumor immunity.

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Optimization of HPV16 E6/E7-positive orthotopic tumor models in MHC-humanized mice for the development of therapeutic HPV vaccines

K. van der Gouw[1,2], A.-K. Schlosser[1,3], S. Zottnick[1,4], C. Schifflers[1,5], J. D. Förster[1,4], A. B. Riemer[1,4]

[1]German Cancer Research Center (DKFZ), Immunotherapy & Immunoprevention, Heidelberg, Germany, [2]Leiden University, Faculty of Medicine/Leiden University Medical Center (LUMC), Leiden, The Netherlands, [3]Heidelberg University, Faculty of Biosciences, Heidelberg, Germany, [4]German Center for Infection Research (DZIF), partner site Heidelberg, Molecular Vaccine Design, Heidelberg, Germany, [5]University of Namur, Cell Biology Research Unit (URBC)-Namur Research Institute for Life Sciences (NARILIS), 5000 Namur, Belgium

Therapeutic vaccines are a promising approach to treat human papillomavirus (HPV)-induced malignancies. Despite promising results in preclinical studies, therapeutic HPV vaccines have only achieved modest efficacy in clinical trials. This might be partly due to the use of suboptimal preclinical tumor models. Many vaccine candidates are tested in wildtype mice, which express murine major histocompatibility complexes (MHC), and in subcutaneous tumor models. However, murine MHC molecules present different peptides than human MHC molecules, so antigens that are immunogenic in mice might not be in humans. Furthermore, the effect of a vaccine-induced immune response on a tumor strongly depends on the tumor's localization. To address these problems, we established syngeneic and orthotopic HPV16 E6- and E7-dependent tumor models in the vagina and base of the tongue of MHC-humanized A2.DR1 mice. For this, lung cells from A2.DR1 mice were isolated, immortalized with HPV16 E6 and E7, made tumorigenic with constitutively active H-Ras^{G12V} and transfected with firefly luciferase to allow monitoring of tumor growth in live animals. We confirmed that the resulting tumor cell line, which is called E6/7-lucA2, expresses HPV16 E6 and E7, H-Ras^{G12V}, luciferase and sufficient levels of the humanized MHC molecule HLA-A2, using western blots, bioluminescent imaging and flow cytometry. The cell line could also be killed by antigen-specific CD8$^+$ T cells in a cytotoxicity assay. However, further analysis revealed that E6/7-lucA2 is of mesenchymal origin, whereas HPV naturally only infects epithelial cells. Therefore, we now aimed to develop an optimized tumor model for HPV-driven cancer that is based on epithelial cells. For the generation of the tumor cell line, we single-cell sorted HPV16 E6- and E7-positive, tumorigenic cells from an early stage of the development of E6/7-lucA2 for the epithelial cell adhesion molecule EpCAM. We confirmed the expression of cytokeratin (to prove the cells' epithelial origin), HPV16 E6 and E7, H-Ras^{G12V}, luciferase and humanized HLA-A2 as well as recognition by antigen-specific CD8$^+$ T cells before passaging the cells subcutaneously in vivo. Tumors were isolated and resulting tumor cell lines were again analyzed for these parameters. As a next step, cells that still fulfill all requirements will be passaged and then titrated intravaginally in A2.DR1 mice. In parallel, cells will be injected and titrated at the base of the tongue. These two orthotopic tumor models will resemble the clinical situation of cervical and oropharyngeal carcinoma more closely than the currently available tumor models for HPV-induced malignancies. In doing so, they will provide more translationally relevant data and can contribute to the development of an efficacious therapeutic vaccine against HPV-induced cancer.

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Vaccination with a leukemic-cell derived cancer vaccine (vididencel) improves anti tumor immune competency in AML patients correlating with improved survival

H. J. van Zeeburg[1], E. Wagner-Drouet[2], U. Platzbecker[3], C. Van Elssen[4], T. A. Holderried[5], A. Giagounidis[6], B. T. Gjertsen[7], A. A. Van de Loosdrecht[8], J. Rovers[9]

[1]Mendus AB, Stockholm, The Netherlands, [2]Universitätsmedizin der Johannes Gutenberg-Universität, Mainz, Germany, [3]Universitätsklinikum Leipzig, Leipzig, Germany, [4]Maastricht University Medical Centre,

Maastricht, The Netherlands, [5]Universitaetsklinikum Bonn (UKB), Bonn, Germany, [6]Marien Hospital Düsseldorf, Dusseldorf, Germany, [7]Haukeland Universitetssykehus,, Bergen, Norway, [8]Amsterdam UMC location VUmc, Amsterdam, The Netherlands, [9]Mendus AB, Stockholm, Sweden

Use of active immunotherapy in AML was long thought not to be possible due to compromised immune responses after initial therapy with high doses of chemotherapy. In this phase 2 study (ADVANCE-II, Clintrials.gov: NCT03697707), AML patients in CR1, but with residual disease (MRD positive) were given vididencel, an intradermal leukemic-cell derived vaccine as active immunotherapy. Vididencel expresses tumor associated antigens (TAA) frequently upregulated in AML like WT1, PRAME and RHAMM. Immune profiling of peripheral blood mononuclear cells (PBMC) was done before and during treatment to evaluate changes in immune cell composition.
Methods. Twenty evaluable AML-patients received four biweekly intradermal injections of vididencel followed by two booster doses at week 14 and 18. PBMC were cryopreserved on day 1, 3, 42, 44, week 11, 18, 20 and 32. IFNy ELISPOT was performed with WT1, PRAME and RHAMM peptide pools to evaluate T-cell responses toward these antigens. Multiparametric flow cytometry (40 marker panel; Cytek 5 laser Aurora) was performed, standard gating on immune cell subsets was applied, for for T, B, NK cells,and dendritic cellss. Hierarchial clustering was applied to evaluate the most dominant cell subsets predicting relapse free and overall survival (cut off date 24th November 2023) using JMP17.
Results

Baseline analysis of immune cell composition based on > 100 predefined immune cell subsets in peripheral blood showed an immune compromised profile in relapsed patients. This immune compromised profile was characterized by low levels of B-cells, high numbers of $CD8^+$ CM and $CD8^+$ $LAG3^+$ and lower level of dendritic cells (cDC1 and cDC2).
During treatment changes in immune cell composition were observed towards a more immune competent profile, shown by increases in dendritic cells, B-cells and decreases in $CD8^+$ $LAG3^+$ cells. Heatmap analysis per patient showed clear shifts in immune cell compositions over time.
Effective T-cell responses to TAAs were induced by vididencel in 85% of patients, which showed a clear correlation with clinical responses. Patients with more and durable immune responses had improved relapse free and overall survival.
In conclusion, active immunotherapy in AML patients using vididencel resulted in an improvement in immune competency and induction of effective T-cell responses. This analysis showed that an immune competent profile and higher numbers of T-cell responses correlate strongly with longer relapse free and overall survival.

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Pharmacologic inhibition of nonsense-mediated decay induces anti-tumour immunogenicity in ex vivo patient tumours

R. Vendramin[1,2,3], H. Fu[1,3], Y. Zhao[1,3,4], S. Fernandez Patel[1,3], D. Qian[1,3], L. Ligammari[1,3], R. Levy[5], P. Greenberg[5], A. Castro[1,3], K. Thakkar[1,3], G. Beattie[1], J. Murai[1], D. Karagianni[1,3], M. Shah[1,3], P. Vlkova[1], M. Rodriguez-Justo[1], C. C. Sng[1,3], B. S. Simpson[1,3], K. K. Dijkstra[6,7], O. Bartok[5], F. Galvez-Cancino[1,3], E. Grönroos[2,3], S. Quezada[1,3], J. L. Reading[1,3], Y. Samuels[5], C. Swanton[2,3], K. Litchfield[1,3]

[1]UCL Cancer Institute, London, United Kingdom, [2]The Francis Crick Institute, London, United Kingdom, [3]CRUK Lung Cancer Centre of Excellence, London, United Kingdom, [4]Fudan University Shanghai Cancer Center, Shangai, China, People's Republic of, [5]Weizmann Institute of Science, Rehovot, Israel, [6]Netherlands Cancer Institute, Amsterdam, The Netherlands, [7]Oncode Institute, Amsterdam, The Netherlands

Frameshift insertion/deletions are a key source of neoantigens. Frameshifted transcripts often contain premature termination codons and are degraded by the nonsense-mediated mRNA decay (NMD) pathway. Leveraging exome, transcriptome, and checkpoint inhibition response data from over 1,000 patients we identify an association between genetic loss or lower expression of the NMD mediator SMG1 with improved responses to immunotherapy. Pharmacological targeting of SMG1 in patient-derived tumour fragments results in activation and clonal expansion of tumour-reactive tissue-resident $CD8^+$ lymphocytes. Mechanistically, SMG1 inhibition increases the abundance of frameshifted transcripts and their HLA presentation, converting the neoepitope count from a low- to a high-TMB-like state without inducing mutations. Co-culture of $CD8^+$ lymphocytes with patient-derived tumour organoids or tumour cells upon SMG1 inhibition induces NMD- and antigen-dependent T cell activation and tumour cell killing. Our findings, in a clinically relevant platform, highlight SMG1 inhibition as a novel immune-oncology approach to exploit an untapped source of highly immunogenic peptides.

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Effect of chemoradiotherapy on the immunopeptidome of HPV16-positive cervical and head and neck cancer cells

N. Vuckovic[1,2,3], J. Förster[1,2,3], C. Schifflers[1,2,4], J. Becker[1,2], A. Riemer[1,2]
[1]German Cancer Research Center (DKFZ), Immunotherapy and Immunoprevention, Heidelberg, Germany, [2]German Center for Infection Research (DZIF), Molecular Vaccine Design, Heidelberg, Germany, [3]Heidelberg University, Faculty of Biosciences, Heidelberg, Germany, [4]University of Namur, Cellular Biology Research Unit (URBC), Namur Research Institute for Life Sciences (NARILIS), Namur, Belgium

Cervical cancer (CxCa) and oropharyngeal cancer, a subset of head and neck squamous cell carcinoma (HNSCC), are largely associated with a persistent Human Papillomavirus (HPV) infection, the majority of which are caused by high-risk type HPV16. First-line standard-of-care for both advanced CxCa and HNSCC patients, regardless of HPV status, is cisplatin treatment concurrent with radiotherapy; however, patients with HPV-induced disease have a more favourable prognosis than patients with HPV-independent cancer. HPV oncoproteins E6 and E7 are critical for the transformation and immortalization of infected cells by disrupting the normal cell cycle and preventing apoptosis and are always present in HPV-driven cancer cells. Peptides derived from E6 and E7 comprise the cancer cells´ immunopeptidome, i.e. the repertoire of HLA-presented peptides. Immunopeptidomics is a technique that allows identification of HLA-presented epitopes using liquid-chromatography mass-spectrometry (LC/MS). A targeted LC/MS method was established in the group that allows detection of low-abundance epitopes, such as HPV-derived viral ones, and several HLA-presented HPV16-derived epitopes were identified. On the other hand, it has been reported that chemoradiotherapy has an immunomodulatory effect. By in vitro mimicking the treatment regimens applied in the clinic, we aim to assess chemoradiotherapy effects on the HLA-presentation of HPV16 epitopes. We have adjusted clinically relevant combinations of cisplatin and irradiation dose to

expand a surviving fraction of CxCa and HNSCC cell lines, compared HLA surface levels and E6 and E7 expression as well as LC/MS peptide identifications across four condition groups. Gained knowledge on the effect of chemoradiation on the immunopeptidome of treated CxCa and HNSCC compared to untreated respective cell lines will be a step forward towards the design and implementation strategy of an effective therapeutic HPV vaccine.

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Increasing the immunogenicity of neuroblastoma by modulating ganglioside expression

S. Wagner[1], C. Paret[1,2,3], A. Wingerter[1,2], K. El Malki[1,2], R. Sandhoff[4], J. Faber[1,2,3]
[1]University Medical Center of the Johannes Gutenberg-University Mainz, Pediatric Hematology/Oncology, Center for Pediatric and Adolescent Medicine, Mainz, Germany, [2]University Cancer Center (UCT), University Medical Center of the Johannes Gutenberg-University Mainz, Mainz, Germany, [3]German Cancer Consortium (DKTK), site Frankfurt/Mainz, Germany, German Cancer Research Center (DKFZ), Heidelberg, Germany, [4]Lipid Pathobiochemistry, German Cancer Research Center, Heidelberg, Germany

Neuroblastoma (NBL) is one of the most common extracranial solid tumours in children. NBL is poorly immunogenic, due to a low mutational burden as well as low expression of MHC-I. NBL express high amount of simple, such as GD2, and/or complex gangliosides, with a high variability in the gangliosides composition within patients. Gangliosides are glycosphingolipids, consisting of a ceramide backbone connected to different carbohydrate and neuraminic acid residues, and are important immunomodulators. Complex gangliosides are found in the mature brain, while simple gangliosides are found at high concentration particularly in the developing brain. Their expression can be regulated by glucosylceramide synthase inhibitors such as eliglustat, which are used for treating patients with Gaucher's disease.

Here we analysed by flow cytometry in NBL cell lines the effect of eliglustat and of a purified solution of complex gangliosides on the expression of the MHC complexes MHC-I and MHC-II and the non-classical MHC protein CD1d. Interferon-gamma was used to induce the expression of MHC complexes. The expression of GD2 was used to monitor the efficacy of eliglustat in reducing the synthesis of gangliosides. The effect of gangliosides modulation on NBL proliferation was evaluated.

Eliglustat exhibited a dose- and time-dependent effect on GD2 expression and cell proliferation. A significant decrease in CD1d and an increase in MHC-1 were observed upon treatment with eliglustat, while other surface proteins remained unchanged. Interferon induced the expression of MHC-1 and/or MHC-2, but not CD1d, depending on the cell line. The lipid solution significantly inhibited the interferon-mediated induction of MHC-2.

In conclusion, gangliosides, and particularly complex gangliosides, have an important impact on the expression of MHC complexes in NBL. The MHC expression in NBL has been shown to impact the immune response, the density of tumour-infiltrating CD3+ T cells and the survival. Thus, eliglustat can be potentially used to increase the immunogenicity of NBL. As the drug is already used in paediatric patients with Gaucher syndrome, it could be easily tested whether it is also effective in the treatment of tumours. Moreover, the effect of gangliosides expression on the MHC expression could be relevant also for adult tumour entities

with a neuroendocrine origin such as small cell lung cancer (SCLC), which has an altered ganglioside expression and a low intrinsic expression of MHC-1.

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Characterization of HLA-restricted HPV16 E6/E7-derived T cell epitopes for their suitability as immunotherapy targets

K. Wellach[1,2,3], K. van der Gouw[1], M. Kleinert[1], N. Asadi[1], J. Tiebel[1], H. Maier[1], J. D. Förster[1,2], M. Bonsack[1,2], A. B. Riemer[1,2]

[1]German Cancer Research Center (DKFZ), Immunotherapy & Immunoprevention, Heidelberg, Germany, [2]German Center for Infection Research (DZIF), partner site Heidelberg, Molecular Vaccine Design, Heidelberg, Germany, [3]Heidelberg University, Faculty of Biosciences, Heidelberg, Germany

Persistent infections with high-risk human papillomaviruses (HPVs) cause several anogenital (mainly cervical) and oropharyngeal cancers. The malignant transformation of infected cells is driven by the viral oncoproteins E6 and E7, which are consistently expressed during disease progression. The presence of these non-self proteins makes HPV-driven malignances the ideal model systems for therapeutic vaccination and peptides derived from the viral proteins E6 and E7 optimal vaccine candidates. However, the epitopes presented are specific to the individual human leukocyte antigen (HLA) molecules, requiring short peptide vaccines to match a patient's HLA type. This can be overcome by combining epitopes binding to HLA supertypes, which are groups of HLA molecules presenting the same peptides. A combination of epitopes binding to the six HLA-supertypes A1, A2, A3/A11, A24, B7 and B15 is computed to result in a world population coverage of >99%. In previous work, ligands to these HLA supertypes were identified.
In the present study, HPV16 E6- and E7-derived HLA-ligands are functionally characterized to enable the selection of promising epitopes for the design of immunotherapeutic treatments against HPV16-mediated malignancies, such as therapeutic vaccination. The immunogenicity of the HLA-ligands is first analyzed in interferon-γ ELISpot assays. For this, PBMCs of HLA-matched heathy female donors above 40 years of age are tested for T cell memory responses. Additionally, a flow cytometry-based phenotyping for CD3, CD4 and CD8 and an intracellular cytokine staining (ICS) for IFNγ and TNFα are performed to further characterize the responsive immune cell population. At least ten donors per HLA-supertype were analyzed. This resulted in the identification of 42 HLA-A2-, 8 HLA-A1-, 18 HLA-A3- , 12 HLA-A11, 16 HLA-A24, 11 HLA-B7 and 29 HLA-B15-restricted epitopes in ELISpot assays. Moreover, the ICS data proved that at least two of the identified epitopes for each HLA supertype specifically induced CD8 T cell activation. In parallel, a highly sensitive image-based cytotoxicity assay was established, allowing the time-course analysis of T cell cytotoxicity. The cytotoxicity assay was found to be suited for the detection of specific killing executed by rare T cell populations. Up to know, 11 HLA-A2-binding epitopes were validated to mediate epitope-specific killing by $CD8^+$ T cells in at least one donor. The analysis of additional donors and epitopes also in the context of other HLA-supertypes is ongoing.
Together with a parallel immunopeptidomics project that identifies peptides that are truly HLA-presented on HPV16-transformed tumor cells, the immunogenicity data will provide an HPV16 E6/E7 epitope

repertoire map, which can serve as a rational basis for the development of epitope-centric HPV16 immunotherapies.

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Single-cell and spatial dissection of the effect of combined PD-L1 and TGFβ blockade (bintrafusp alfa) in head and neck squamous cell carcinoma

J.-T. Werle[1], C. Hoffmann[1,2], J. Gal[3], P. Sirven[1], B. Demaille[1], A. Abbas[1], J. Mesple[1], J. Klijanienko[1], S. Lameiras[1], N. Badois[1], O. Choussy[1], L. Geoffrois[4,5], G. Dolivet[4,5], G. Marret[1], I. Bieche[1], C. Simon[6], W. Zeitouni[7], N. Robil[7], A. Dubray-Vautrin[1], A. Zhubanchaliyev[1], J. Merleverde[1], K. Borgman[1], D. Louis[1], R. Leclere[1], I. Puisieux[1], K. Maud[1], L. Monard[6], C. Le Tourneau[1], H. Salmon[1]
[1]Institut Curie, Paris, France, [2]Owkin, Paris, France, [3]Unicancer, Nice, France, [4]Institut de Cancérologie de Lorraine, Nancy, France, [5]Unicancer, Nancy, France, [6]Unicancer, Paris, France, [7]Genosplice, Paris, France

The efficacy of immune checkpoint inhibitors in head and neck squamous cell carcinoma (HNSCC) is limited, underscoring the need for novel therapeutic targets. TGFβ signaling has been linked to T cell exclusion as well as attenuated response to immune checkpoint blockade, supporting a rationale of co-targeting TGFβ for potentially synergistic effects. Here, we investigate efficacy of, and changes induced by, combined PD-L1 and TGFβ blockade in a phase II window-of-opportunity clinical trial of bintrafusp alfa in patients with untreated, resectable stage III/IV HNSCC (NCT04428047). For this purpose, tumor biopsies were taken before treatment and at the time of surgical resection in a similar location, and subjected to single-cell RNA sequencing and multiplexed imaging. Resections were utilized to assess treatment efficacy via the pathological response (PathR). Seven patients were included, with six receiving the full treatment scheme (2 doses à 1200 mg on D1 and D15). Four patients experienced a partial response (PathR > 10%), including one major partial response (MPR) with a PathR > 50%. Independent of treatment response, we report a remodeling of the stromal architecture together with a phenotypic shift in cancer-associated fibroblasts (CAF) reducing activated LRRC15+ CAF and an increase in CD10+IL24+ inflammatory CAF. This was accompanied by a broad reduction of TGFβ-induced genes in both cancer and stromal cells, but most strongly impacting CAF and endothelial cells. Furthermore, we observed IFNγ-mediated activation of macrophages and dendritic cells increasing the expression of CXCL9 and CXCL10, and a reduction in both conventional and regulatory CD4+ T cells. In three patients whose pre-treatment biopsies were marked by an architecture of LRRC15+ CAF lining tumor cell islets, we observed a disappearance of this structure after treatment associated with an increase of CD8+ T cell infiltration into the tumor. The MPR's tumor microenvironment was distinguished by the presence of p16+ (confirmed HPV-negative) tumor cells with higher genomic alterations, and a higher presence of immune cell aggregates composed of T cells, dendritic cells, B cells and Plasma cells, suggesting a pre-existing immune reaction against the tumor. Finally, MERSCOPE and XENIUM spatial transcriptomics analysis of the MPR's tumor resection highlighted clear spatial heterogeneity of the treatment response, with a significant resistant region characterized by less differentiated tumor cells with stemness phenotype surrounded by an intact fibrotic stroma. Collectively, our study deciphers the effects of bintrafusp alfa both molecularly and spatially at unprecedented

granularity. This study was financially supported and bintrafusp alfa provided by Merck (CrossRef Funder ID: 10.13039/100009945).

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Ubi-tagging: a novel ubiquitination-based conjugation technique to enhance dendritic cell-targeted antigen delivery

Z. Wijfjes*[1,2], A. el Hebieshy*[3,4], C. Le Gall[1], I. Derksen[4], C. Talavera[3], F. Scheeren[3,4], M. Verdoes[1,2]
[1]Radboud University Medical Center, Department of Medical BioSciences, Nijmegen, The Netherlands, [2]Institute for Chemical Immunology, Nijmegen, The Netherlands, [3]Leiden University Medical Center, Department of Cell and Chemical Biology, Leiden, The Netherlands, [4]Leiden University Medical Center, Department of Dermatology, Leiden, The Netherlands

*Contributed equally

Targeting peptide-based cancer vaccines to dendritic cells is well-established to enhance antigen (cross-)presentation. Induction of a cytotoxic immune response is crucial for the efficacy of such vaccines. However, CD8-epitopes are notorious for their hydrophobic character complicating conjugation to dendritic cell-targeting moieties. Here, we developed a novel conjugation technique utilizing the ubiquitination machinery in vitro to conjugate ubiquitin-tagged antibody fragments (Fabs), nanobodies or peptides. Using this method, which we named “ubi-tagging”, we generated fusions of antigenic peptides and dendritic cell-targeting Fabs and nanobodies, including a set of conjugates challenging to produce using conventional techniques. Our ubi-tagged vaccine conjugates induce potent T cell activation in vitro outperforming comparable conjugates obtained with traditional ligation techniques. In vivo we observe a strong T cell proliferation highlighting the potency as well as the stability of our ubi-tagged conjugates. We hypothesize the ubiquitin-linkage in the conjugates contributes to the potent responses observed in vitro and in vivo. Our current research focuses on elucidating the observed benefit in efficacy of ubi-tagged conjugates. We expect “ubi-tagging” to contribute to the field of therapeutic peptide-based vaccines by offering a novel approach to conjugate hydrophobic entities. Moreover, we are excited to further explore the platform in the context of immunotherapeutic applications.

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Abstract has been withdrawn

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Dual targeting of DNA and histone methylation suppresses AML and improves response to T Cell-based Immunotherapy

A. Ziegfeld[1,2], U. R. Schmits[1], C. Zanetti[2,3], E. Schmitt[1,2], K. Woods[1,2], S. Kelekci[4], C. Plass[4], M. Theobald[1,2,5], H.

Echchannaoui[1,2], B. Guezguez[1,2,5]

[1]University Medical Center Mainz (UMC), III. Med (Hematology/Oncology), Mainz, Germany, [2]German Cancer Consortium (DKTK), Partner site Frankfurt/Mainz, Mainz, Germany, [3]HI-TRON, Helmholtz Institute for Translational Oncology Mainz - A Helmholtz Institute of the DKFZ, Mainz, Germany, [4]Division of Cancer Epigenomics, German Cancer Research Center (DKFZ), Heidelberg, Germany, [5]Research Center for Immunotherapy (FZI), University Medical Center Mainz (UMC), Mainz, Germany

Acute myeloid leukemia (AML) is an aggressive form of hematological cancer that is associated with poor prognosis and low survival rates. Elderly patients are often ineligible for intensive chemotherapy, so novel, tolerable, and specific therapies are needed. Epigenetic dysregulation is a hallmark of AML disease progression and resistance. Epigenetic modifiers, in particular hypomethylation drugs that target DNA methyltransferase (DNMT), have emerged as promising therapeutics, and many received FDA approval. Nevertheless the efficacy of these first generation DNMT therapies remains limited in improving overall survival or preventing disease relapse. Recently, it has been demonstrated that DNMTs interact with Histone Methyltransferases (HMTs) like G9a in distinct epigenetic complexes, enabling AML oncogenic activity and gene silencing. However, the potential role of this DNMT/HMT complex towards tumor outgrowth and immune-evasion across the AML mutational spectrum remain to be fully explored.

Our study aims is to decipher the anti-tumorgenic role of dual DNMT/HMT-G9a inhibitor (CM-272) in targeting AML and to investigate the synergistic potential of CM-272 with adoptive T cell immunotherapy in overcoming AML immune resistance.

Functional in vitro screen of AML cell lines with CM-272 allowed to pinpoint a specific vulnerability of cancer cells harboring DNMT3A/NPM1 mutations (OCI-AML3 and OCI-AML2) with potent anti-tumor effect and restoration of myelomonocytic differentiation. CM-272 in vivo monotherapy demonstrate similar cytoreductive effect by reduction of tumor burden in OCI-AML3-PDX mouse model. At the molecular level, Gene expression (RNA-seq) and methylome profiling (EPIC array) demonstrated that CM-272 revert HOXA9/MEIS1 oncogenic program and induce a high expression of core gene-sets related to “viral mimicry” Type I interferon signaling pathway which is characterized by the transcriptional reactivation of specific set of hypermethylated repeat elements (LTR, ERV). Furthermore, the combination of CM-272 and p53-antigen-TCR-specific T cells showed an improved eradication of leukemic cells compared to CM-272 or T cells alone. CM-272 treatment induced an upregulation of the co-stimulatory molecule CD86 and the antigen-presenting ligand MHC-I on the AML cells without impacting T-cell intrinsic properties.

In conclusion, the combinatorial epigenetic/TCR data presented here provides the basis for further investigation as a potential approach to circumvent immune resistance in AML therapy. As a next step, we will explore the therapeutic efficacy of this approach in the Luciferase-OCI-AML3 xenograft NSG model and further characterize the influence of CM-272 on the T cells using single-cell RNA sequencing. Our research will provide a better understanding of the effect of epigenetic drugs on T cell effector functions and a proof of principle demonstration for this novel combinatorial treatment.

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High-density peptide microarrays for MHC I- immunopeptidome profiling in cancer

E. Aydin Kurtulmus, D. Hildebrand, B. Gonzalez, V. Stadler
PEPperPRINT GmbH, Heidelberg, Germany

Identification of peptides presented by major histocompatibility complex (MHC) is important for multiple applications in immunology. One especially promising area is the discovery of reactive T-cells against neoantigens to develop personalized therapies centered around adoptive T-cells and therapeutic vaccines. Despite numerous efforts to profile MHC-I immunopeptidome, several challenges have constrained their success, including limitations in instrument sensitivity, labor-intensive processes, and the requirement for substantial amounts of patient material, in addition to dependence on predictions and complex computational methodologies. Here, we present a novel, chip-based methodology that overcomes these limitations, allowing for the discovery of epitope-specific T-cell responses in a remarkably short timeframe of a few weeks. Given the devastating rate of disease progression, implementation of our method can contribute significantly to the advancement of personalized immunotherapies in cancer and form the basis of a life-saving strategy.
In this study, we employed high-density peptide microarrays, a robust method capable of simultaneously screening thousands of peptides against specific binders in a high-throughput manner. The amino acid sequences of melanoma patient-specific somatic mutations (neoantigens) were printed in a peptide library format. Literature-confirmed HLA-A2-specific melanoma epitopes were incorporated into the same array as positive controls. The resulting custom melanoma neoepitope microarray contained 5400 peptides printed in duplicate, framed by hemagglutinin quality control peptides and screened for recombinant HLA-A2 protein binding. Data was analyzed by PepSlide® Analyzer software and and the immunogenicity of the identified T-cell epitopes was validated through an ELISpot assay.
Incubating the melanoma antigen microarray with recombinant HLA-A2 construct resulted in validation of 94 out of 100 positive control peptides, alongside new epitope discoveries. The approach can be easily transferred to any other immunogenicity screening of custom antigens against specific MHC-I constructs. In conclusion, our study shows the potential of PEPperCHIP® Peptide Microarrays in advancing our understanding of peptide-MHC interactions.

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Visualizing antibody-receptor interactions at single protein resolution

I. Baudrexel[1,2], L. A. Masullo[1,2], S. C. M. Reinhardt[1,2], P. R. Steen[1,2], C. Klein[2,3], R. Jungmann[1,2]
[1]Max Planck Institute of Biochemistry, Planegg, Germany, [2]Ludwig Maximilian University, Munich, Germany, [3]Roche Innovation Center Zurich, Schlieren, Switzerland

The B cell marker CD20 is a primary target for immunotherapy in non-Hodgkin's lymphoma. CD20-therapeutic antibodies (Abs) induce cancer cell depletion by multiple mechanisms, including cellular effects via Fc receptor recognition, complement-dependent cytotoxicity, and direct binding effects. These processes are intricately regulated by the nanoscale arrangement of CD20 and its cognate Abs on the cell

surface. In the specific case of CD20, two distinct types of therapeutic Abs with different depletion mechanisms have been previously identified. However, the structural basis for this difference in the cellular context remains elusive.

To address and resolve this structural biology challenge, we applied the molecular specificity of super-resolution fluorescence imaging. Traditionally, super-resolution microscopy approaches can achieve 15-20 nm resolution in cells, which prevents the detection of oligomer-spacings of less than 10 nm. Therefore, we have recently developed the DNA-barcoding method Resolution Enhancement by Sequential Imaging (RESI), which improves the resolution of fluorescence microscopy down to the Ångström scale in whole intact cells. By performing 2-color, 3D RESI in a monomeric enhanced green fluorescent protein (mEGFP)-tagged CD20 cell line after treatment with either the type I mAb rituximab (RTX) or the type II mAb obinutuzumab (OBZ), we were able to visualize the molecular arrangement of CD20 and bound therapeutic Abs at 1-nm resolution.

Strikingly, by performing cluster analysis and structural modeling, we reveal distinct differences in CD20 arrangement after RTX versus OBZ treatment. On the one hand, OBZ binding induces a shift from pure monomers and dimers to trimers and tetramers. Further analysis shows that there is a potential to increase the oligomerization capacity of OBZ in cells. RTX treatment, on the other hand, results in a flexible chain with periodic CD20-RTX arrangements. Further analysis of the stoichiometry and substructure of RTX-mediated clusters allows us to propose a novel binding model for C1q recognition.

These observations suggest potential strategies for antibody engineering based on determined binding angles and Fab to Fab distances, thereby facilitating informed decisions for modulating mechanisms of cancer cell depletion. Importantly, the modular nature of our technology can be used to investigate other mAb-receptor interactions where receptor clustering is linked to activation of downstream signals or immune effector mechanisms. Overall, our study provides insight into the structure-function relationship of cancer immunotherapy targets, bridging the gap between in vitro structural biology and functional studies in living systems.

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In-depth phenotyping of CAR T cells in suspension and tissue samples by highly multiplexed microscopic analysis and super resolution imaging

A. Brauchle, P. McCormick, S. Halbfeld, R. Diekmann, D. Missing, M. Winkels, A. Freitag, R. Rosner, S. Rüberg, A. Appelshoffer, F. El Yassouri, S. Wilmes, M. Schütte, H. Spiecker, J. Fauerbach, A. Bosio, C. Dose, A. Richter, M. Mues
Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany

Multicolor flow cytometry is commonly used to characterize CAR T cells for the treatment of hematological malignancies. However, spatial information, which is crucial for CAR T cell treatment of solid tumors and autoimmune disorders, cannot be obtained using flow cytometry. This gap clearly indicates the need for novel analytical tools to assess CAR T cell distribution within tissue samples. The MACSima™ Imaging Cyclic Staining (MICS) technology is an automated imaging platform that allows detection of potentially hundreds

of cellular markers using a cyclic staining and imaging approach, thus enabling in-depth phenotyping of suspension cells and tissue samples. On CD2019 CAR T cell suspensions, we were able to unambiguously detect CAR-expressing cells by using CAR detection reagents such as an CD19 CAR FMC63 idiotype antibody. In addition, a variety of cellular markers and their subcellular localization could be analyzed. With the help of spatial segmentation algorithms, various cell populations could be distinguished according to their unique marker expression pattern. This enabled a hierarchical gating strategy similar to multicolor flow cytometry data analysis, while additionally preserving important spatial information. This is particularly crucial when studying entire tissue slices. We managed to successfully apply this imaging workflow to CD2019 CAR T cells infiltrated in xenograft tissues, where we identified and characterized these cells. Thereby we were able to quantify the amount of tissue-infiltrating CAR T cells, along with other phenotyping markers, in good agreement with corresponding flow cytometric data. While MICS imaging enables high-content phenotyping of a multitude of proteins, super resolution imaging provides a powerful tool to analyze proteins below the diffraction limit with single-molecule sensitivity. This is particularly relevant for the assessment of low-abundance tumor antigens in CAR T cell therapy, and thus for the optimization of cell surface CAR expression. The BMBF-funded IMAGINE project addresses this challenge using super resolution imaging approaches, such as dSTORM and DNA-PAINT. We are currently investigating corresponding technical solutions and reagents with a dedicated use for research and clinical applications. Here, we aim to showcase the outstanding sensitivity and localization precision of super resolution imaging approaches and demonstrate its potential benefit for patients receiving CAR T cell treatment. Taken together, the MICS technology along with super resolution microscopy are versatile and complementary tools to analyze samples in a CAR T cell context and provide powerful new perspectives for CAR T cell research and therapy.

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T-FINDER: delivering targets and ammunition in the battle against cancer and beyond

M. Cetin[1], T. Boschert[2,3,4], V. Pinamonti[1,2], E. W. Green[3,5], M. Platten[3,4,5], J. M. Lindner[1]
[1]BioMed X Institute, Heidelberg, Germany, [2]Heidelberg University, Faculty of Biosciences, Heidelberg, Germany, [3]German Cancer Research Center (DKFZ), DKTK CCU Neuroimmunology and Brain Tumor Immunology, Heidelberg, Germany, [4]Helmholtz Institute for Translational Oncology (HI-TRON), Heidelberg, Germany, [5]Medical Faculty Mannheim, MCTN Heidelberg University, Department of Neurology, Mannheim, Germany

Accumulating evidence for the safety and efficacy of T cell therapies, such as CAR-T, TCR-T, and TILs, strongly support future investment into cellular and genetic precision medicine. However, the re-introduction of TILs and/or unscreened TIL-derived TCRs is frequently complicated by frequent bystander receptors with no anti-tumor reactivity, leading to wasted resources and potentially impacting safety profiles. Therefore, systems to rapidly validate functional T cell reactivity and identify cognate TCR epitopes are crucial for the development of new and improved cellular therapies. Notably, while class I HLA-restricted TCRs from CD8 T cells have been widely applied due to their cytotoxic role and relative ease of target identification, class II HLA-presented epitopes and the CD4 TCRs that bind them have been historically undervalued. While the role of CD4 T cells in orchestrating anti-tumor responses across the immune system has now been more clearly elucidated, strategies for the functional validation and

deconvolution of these TCRs has remained a challenge. Our recently published T-FINDER (T cell Functional Identification and (Neo)-antigen Discovery of Epitopes and Receptors) platform accomplishes these goals with several key features: a highly sensitive and functionally quantitative cellular reporter system of T cell activation, the use of physiological TCR:peptide:HLA complexes recapitulating the interaction of T cells with antigen-presenting cells in vivo, and a novel strategy for achieving high levels of antigen presentation from genetically encoded putative target libraries. T-FINDER can be used independently from, but is also compatible with, predictive methodologies based on computational algorithms and/or mass spectrometry, loaded peptide ligands, and tetramer-labeled TCR discovery. We describe each element of the system which can be used individually for a range of applications (e.g. CAR evaluation) or start-to-finish as a complete pipeline for, e.g., de novo target discovery, functional validation of predicted interactions, and safety profiling for off-target effects of tgTCRs. T-FINDER was designed with class II HLA-presented ligands in mind, which we have demonstrated in-depth by identifying more than 30 H3K27M-specific TCRs from a single diffuse midline glioma patient treated with the H3-vac peptide vaccine. We characterized in detail the ability of this vaccine to drive CD4 T cell (and corresponding B cell) responses in the central nervous system, highlighting the importance of targeting this arm of the immune response in anti-tumor therapies. This and further applications (SARS-CoV-2, rheumatoid arthritis, and celiac disease) effectively showcase T-FINDER's potency, flexibility, and ability to provide unique biological insights.

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Identifying clinically relevant metabolic cell states in metastatic breast cancer using multiplexed imaging

M. Cetin[1,2], C. Fremd[3], F. Hartmann[1,2]
[1]German Cancer Research Centre (DKFZ), Heidelberg, Germany, [2]Germany, German Cancer Consortium (DKTK), Heidelberg, Germany, [3]National Center for Tumor Diseases (NCT), Heidelberg, Germany

Breast cancer is a major cause of cancer-related deaths in women, as it is often diagnosed after metastasis has occurred, leading to a poor prognosis. It is a complex disease with different molecular subtypes, each exhibiting distinct metabolic and immune characteristics within the tumor microenvironment. Unlike many other tumor types, immunotherapy in breast cancer has not been very effective. We hypothesize that metabolic suppression within the tumor microenvironment is a major mechanism of tumor immune evasion. We know that the metabolic environment influences immune function, resulting in T cell exhaustion and polarization of myeloid cells towards immune suppressive phenotypes. However, we currently do not know the metabolic states of tumor and immune cells in human breast cancer.
In this study, we are constructing a spatially-resolved, single-cell landscape of the tumor microenvironment and its metabolic state across various types of metastatic breast cancer. To do so, we are combining the recently established single-cell metabolic profiling (scMEP) approach with spatial proteomic imaging by multiplexed ion beam imaging (MIBI) which enables high-dimensional proteomic imaging of clinical FFPE tissues.
First, we have established and validated a 40-plex antibody panel spanning 13 lineage markers, 10 metabolic markers, 4 transcription factors, 9 immune state markers, and segmentation markers, together allowing us to identify all cellular components of the TME and reveal their immunological and metabolic state. Next, we made use of this panel to stain a cohort of N=60 metastatic breast cancer tissue samples enrolled in the CATCH running register and diagnostic study (NCT04272970). We have already imaged 161

FOVs across 19 sections, which we are currently complementing by imaging additional 376 FOVs across 47 sections.
To analyze this data, we are employing neural network and machine-learning approaches to automatically identify cells, cluster them into established cell lineages, and reveal their metabolic state. In addition, we will employ recently-developed spatial neighbourhood identification approaches to comprehensively map spatially-defined metabolic niches in these tissues.
Overall, we anticipate that the data generated from this study will provide valuable insights into the metabolic and immune landscape of breast cancer subtypes, potentially leading to the identification of novel therapeutic targets and strategies for personalized cancer treatment.

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Multi-Effector Cell Targeting using Bispecific Single-Chain Variable Fragments for B Cell Lymphoma

M. Freihammer*[1], S. Heidenreich[1], P. Reichelt[1], P. Jayalath[1], Y. Yang[1], M. Nill[1], D. Stahl[1], O. Käsgen[1], S. Moskopp[1], M. Babaki[2], C. Jungst[3], J. Schleifenbaum[1,4], M. Heger[1], T. Braun[1], R. Ullrich[1], S. Borchmann*[1,4]
[1]Uniklinik Köln, Department I of Internal Medicine, Center for Integrated Oncology Aachen Bonn Cologne Duesseldorf, University of Cologne, Cologne, Germany, Koeln, Germany, [2]Uniklinik Köln, Center for Molecular Medicine Cologne (CMMC), University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne, Germany, Koeln, Germany, [3]Uniklinik Köln, University of Cologne, Faculty of Mathematics and Natural Sciences, Cluster of Excellence Cellular Stress Responses in Aging-associated Diseases (CECAD), Koeln, Germany, [4]German Hodgkin Study Group, Cologne, Germany, Koeln, Germany

*Contributed equally

Bispecific antibodies (BsAbs) with various designs have been developed to treat patients with aggressive B cell lymphomas such as diffuse large B cell lymphoma (DLBCL) or classic Hodgkin lymphoma (cHL). Despite effective first-line treatments, the prognosis for these patients remains relatively poor in the relapsed/refractory (r/r) situation. BsAbs that recruit immune effector (IE) cells to the malignant cell population, have improved the treatment of DLBCL in particular and might be an alternative to other immunotherapeutic approaches such as CAR-T cells that can be associated with a long and expensive manufacturing process.
Here, we introduce our platform for the design and production of various bispecific single-chain variable fragments (bi-scFvs) targeting CD19 and CD30 positive hematological malignancies by combined targeting of multiple IE cell antigens on T cells (CD3 and CD28) and NK cells (CD16). The two antigen binding regions targeting the tumor and IE cells are linked together via a well characterized linker with low immunogenicity. Protein production is performed using a mammalian protein expression system. The bi-scFvs are subsequently recovered through an optimized purification process, the size and purity of which is verified using Western Blot and Coomasie Blue Staining. Flow cytometry is used to show specific binding to tumor and IE cells. In vitro cytotoxicity (TOX) assays are then conducted to investigate the potential of the bi-scFvs in our library to induce tumor cell killing in a dose-dependent manner. In addition, confocal microscopy is performed to detect tumor-IE cell colocalization caused by the bi-scFvs and studies analyzing cytokine release from IE cells after bi-scFv-mediated target cell engagement have been performed as well.
We were able to show target-specific binding for all tested bi-scFv constructs. Additionally, the CD3- and

CD16- bi-scFvs showed a dose-dependent effect in their ability to induce IE cell-mediated tumor cell killing of Raji (CD19+) or Hodgkin (CD30+) cell lines. In each case, the cytotoxicity observed was specific to the tumor cell of interest. Additionally, the CD3- and CD16- bi-scFvs, when combined, showed a synergistic effect, indicating that the idea of targeting multiple IE cells might indeed be beneficial. The increased cytotoxicity caused by the bi-scFvs correlated with the release of pro-inflammatory cytokines and increased cell-cell colocalization.
In conclusion, we have established a platform for the production and purification of a library of bi-scFvs and are able to show dose-dependent binding specificity, cytotoxicity and expected in vitro functionality for bi-scFvs targeting CD19 and CD30. Using combinations of bi-scFvs targeting CD3 and CD16, we were able to achieve a synergistic effect by targeting both T and NK cells. In vivo studies are currently in progress for further preclinical validation of our approach.

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B4GALNT1 amplification as a promising surrogate biomarker indicating the expression of the immunotherapy target GD2 in dedifferentiated liposarcoma

M. Fried[1], M. Gaida[2], A. Ustjanzew[3], C. Ritzel[4,5], A. Desuki[4], J. Faber[6], T. Kindler[4], R. Sandhoff[7], C. Paret[6]
[1]University Hospital Mainz, University Cancer Center/ Dept. of Internal Medicine III, Mainz, Germany, [2]University Hospital Mainz, Institute of Pathology, Mainz, Germany, [3]University Hospital Mainz, Institute for Medical Biometry, Epidemiology and Informatics, Mainz, Germany, [4]University Hospital Mainz, University Cancer Center, Mainz, Germany, [5]University Hospital Mainz, TRON-Translational Oncology, Mainz, Germany, [6]University Hospital Mainz, Clinic and polyclinic for pediatric and adolescent medicine, Mainz, Germany, [7]German Cancer Research Center (DKFZ), Lipid Pathobiochemistry, Heidelberg, Germany

Dedifferentiated liposarcoma (DDLS) is a high-grade and aggressive disease with high local recurrence and metastatic rate. DDLS is characterized by amplified sequences of 12q13-15 including murine double minute 2 (MDM2) and cyclin-dependent kinase 4 (CDK4) cell cycle oncogenes. The ganglioside GD2, an oncofetal plasma membrane glycolipid originally identified in Neuroblastoma (NBL), has been suggested as a therapeutic target for monoclonal antibodies and CAR-T cells in sarcoma. However, the assessment of GD2 expression in tissues samples, and particularly in FFPE, is challenging and surrogate biomarkers to identify patients with high GD2 expression are necessary.

Here we analyzed by Mass Spectrometry the GD2 concentration in ten DDLS native clinical samples. The expression and amplification of genes required for the synthesis of GD2 (B4GALNT1 and ST8SIA1) was analysed by qRT-PCR and using the cBioPortal software application.

The median expression of GD2 was of 0.035 nmol GD2/mg protein. Only one sample showed very high expression of GD2, comparable to the expression described in NBL (1.2 nmol GD2/mg protein). This sample was characterized by a high expression and amplification of B4GALNT1 which is found on chr. 12q13.3. According to cBioPortal, B4GALNT1 amplification is found in circa 50% of DDLS and is associated with high expression of B4GALNT1. Interestingly, also ST8SIA1 is found on chr.12 (p12) and is amplified in 5% of DDLS samples.

Our results indicate that the expression of GD2 is heterogeneous within DDLS and the measurement of GD2 is mandatory to select patients for basket studies with anti-GD2 based immunotherapies. DDLS are defined by amplification of several regions within 12q13-15. Co-amplification of MDM2 on 12q15 and CDK4 on 12q14 is a common feature in DDLS. However, several samples also have amplification of 12q13.3, which carries B4GALNT1. B4GALNT1, together with ST8SIA1, is required for the synthesis of GD2 and its amplification could be the main reason of GD2 expression in DDLS. The analysis of gene amplification on FFPE samples is a standard method used in pathology and could therefore in the future be used to identify GD2-positive DDLS patients.

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Original TCR sequencing and cloning methods for repertoire analysis and isolation of tumor-reactive TCRs.

R. Genolet, S. Bobisse, J. Chiffelle, M. Arnaud, R. Petremand, L. Queiroz, A. Michel, P. Reichenbach, J. Cesbron, A. Auger, P. Baumgaertner, P. Guillaume, J. Schmidt, M. Irving, L. E. Kandalaft, D. Speiser, G. Coukos, A. Harari
Ludwig Institue for Cancer Research, Lausanne University Hospital, Lausanne, Switzerland

T-Cell Receptor (TCR) technologies are increasingly important in the clinical management of cellular immunity in cancer, transplantation and other immune diseases. Successful applications of these TCR analyses will certainly rely on our ability to faithfully capture the complexity of repertoires to avoid misleading conclusions and unappropriated treatments and to provide time- and cost-effective tools to manipulate and engineer T cells. Yet, sensitive and reliable methods for repertoire analyses and TCR cloning are still lacking. The wildly used multiplex PCR is known to introduce biases in clonotype quantification related to differences in primer efficiency. While the 5'RACE solved the multiplex issue, efficiency of the template-switch is quite poor, resulting in a lower sensitivity. Here we report on SEQTR, a novel high-throughput approach to analyze T-cell repertoires that circumvents the aforementioned biases. We demonstrated that RNA-based assays remain suitable for clonotypes quantification and that variations in TCR expression do not significantly affect clonotype quantification, in disagreement with the consensual hypothesis. Benchmarking SEQTR against ImmunoSEQ, highlighted a better reproducibility and a clear reduction of the bias induced by the amplification (mean R^2 of 0.786 vs mean R^2 of 0.278). Similarly, comparison with SMARTer (5'RACE assay) demonstrated the better sensitivity with twice more detected clonotypes having an average frequency of 0.0004%. Therefore, SEQTR preserves repertoire integrity and provides data of greater accuracy and sensitivity than the current assays. While sensitivity is an important parameter enabled by RNA-based assays, the quantitative aspect of the method is even more fundamental. Indeed, while sensitivity allows analysis of samples of limited size and the detection of larger amounts of low-frequency TCRs, biases in quantification likely affect the entire repertoire analyses, including dominant clonotypes. It may thus lead to misrepresented repertoire profiles and distort their interpretations. The development of scTCRseq has opened opportunities to obtain more information on individual clones, including TCR identification, yet the number of T cells analyzed is limited such that single-cell technologies are not appropriate to reach the deepness of analyses required for some clinical monitoring. Furthermore, cloning and screening strategies remain mandatory steps to validate specificities of TCR identified with scTCRseq. Therefore, we also describe a cloning strategy to reduce timelines and resources needed to

isolate TCR of interest. Used separately or in combination our cloning strategy, we illustrate how SEQTR could improve patient stratification using TCR metrics and describe a cost- and time-effective pipeline to identify, clone and validate tumor-specific TCRs in only few days.

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High-dimensional fluorescence imaging for characterization of the tumor microenvironment with a focus on unconventional T cells

N. Herold[1], J. Spreuer[1], M. Claassen[1,2], K. Wistuba-Hamprecht[1,2,3,4]
[1]University Hospital Tübingen, Section for Clinical Bioinformatics, Department for Internal Medicine I, Tübingen, Germany, [2]Faculty of Medicine, University Tübingen, M3 Research Center for Malignome, Metabolome and Microbiome,, Tübingen, Germany, [3]German Cancer Research Center (DKFZ), Skin Cancer Unit, Heidelberg, Germany, [4]University of Heidelberg, Department of Dermatology, Venereology and Allergology, University Medical Center Mannheim, Mannheim, Germany

High-dimensional tissue imaging has emerged as a powerful tool for the in-depth investigation of the tumor microenvironment, providing a better understanding of the role, interplay, and spatial organization of infiltrating immune cells. Based on an automated iterative cyclic staining system we developed a comprehensive antibody panel designed for fresh-frozen paraformaldehyde-fixed tissue and encompassing more than 60 markers. This approach enables the concurrent assessment of a diverse array of immune cell populations, including unconventional γδ T cells and MAIT cells, coupled with a detailed phenotypic investigation of the differentiation signature, activation markers, and immune checkpoints. In addition to that, our panel also covers the extracellular matrix, as well as epithelial and endothelial cells.
We emphasize the importance for utilizing B and T cell-specific nucleic markers to unequivocally identify cells within each lineage, since membrane signals can be inconclusive in dense areas or due to the transfer of plasma-membrane fragments via trogocytosis. Furthermore, we elaborate on the staining order, and the combination of antibodies and fluorophore conjugates in a single cycle, along with the potential of incorporating an indirect staining step enabling a prolonged incubation time outside the automated system.
Integration of this high-dimensional fluorescence imaging panel with bioinformatics analysis and clinical data offers great potential for translational research and the immuno-oncology domain. This lays the foundation for future studies that may guide the development of immunotherapies, for example by identifying novel targets of solid tumors.

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Thorough syngeneic mouse model characterization to enable rational, biomarker-based model selection for experimental immunotherapy

A.-M. Herzner[1], P. Slos[1], H. J. Huber[2], A. Kowalczyk[1], M. Renn[1], C. Ogris[1], M. van Attekum[1], A. Dick[1], C. Viollet[1], F. Heinemann[1], G. Peter[1], B. Stierstorfer[1], T. Schönberger[1], C. Lempp[1], J. F. Rippmann[1]
[1]Boehringer Ingelheim, Biberach an der Riss, Germany, [2]Boehringer Ingelheim, Vienna, Austria

Mouse model selection is a fundamental challenge for proof of principle studies in experimental cancer immunotherapy. Of the most frequently employed mouse models – transplantation of syngeneic tumor cell

lines - a multitude of models are available. While these models may not perfectly reflect human tumor biology, they can reflect certain aspects, such as the expression of a certain immune suppressor, infiltration with an immune cell type, or expression of cytokines. In absence of baseline characterization data, rational model selection based on biomarkers expected to be the basis for a successful therapy is challenging. Therefore, frequently, multiple models have to be screened to show biomarker modulation by or efficacy of experimental immunotherapy.

We set out to characterize fundamentally and thoroughly the most frequently utilized mouse models at different stages of tumor growth. We analyzed four tissues, tumor, tumor-draining lymph node, spleen as well as blood, employing bulk RNAseq, flow cytometry, immunohistochemistry, and cytokine arrays.

We have now characterized eleven mouse models, with and without anti-PD-1/anti-LAG-3 antibodies as combination therapy, at three different time points, each.

This rigorous characterization enables informed model selection based on critical biomarkers, ultimately reducing the need for animal model screening in pharmacological research.

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3D Bioprinting: new models in cancer immunotherapy

X. Holtkotte, N. Halama
DKFZ HI-TRON, Translational Immunotherapy, Mainz, Germany

In vitro testing of therapeutics with human cells has great potential to accelerate discovery. It can help identify whether the knowledge we gain from interspecies models is suitable for translating to humans, thereby, possibly avoiding late-stage clinical failure. Three-dimensional (3D) bioprinting allows us to better recapitulate tissue morphology, physiology, and the complex microenvironment in vitro, thereby providing more realistic measurements of drug efficacy and toxicity. In this proof-of-concept study, we 3D bioprinted cancer cells in alginate-based hydrogel and showed that tumor cells form tumor-like structures. Furthermore, we observed the migration of T cells toward cancer cells when printing T cells as a layer beside the tumor cell layer. Also, we were able to study modulatory effects and identify the mode of action in various established 3D bioprinting models by adding a neutralizing antibody targeting NIM16. Specific effector cytokine changes (TNF alpha, IFN alpha) and effects on chemokines (CXCL10 and CCL5) were noted.

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Exploring Functional Heterogeneity of Immune Cells in Adoptive Cell Therapy

T. M. Hulen*[1], A.-C. K. Rasmussen*[1], D. L. Petersen[2], M. J. Jacobsen[2], Ö. Met[1,3], M. Donia[1], C. A. Chamberlain[1,4], P. Mouritzen[2]
[1]National Center for Cancer Immune Therapy (CCIT-DK), Department of Oncology, Copenhagen University Hospital, Herlev, Denmark, [2]Samplix ApS, Birkerød, Denmark, [3]Technical University of Denmark, Department of Health Technology, Lyngby, Denmark, [4]University College London Cancer Institute, Cancer Immunology Unit, Research Department of Haematology, London, United Kingdom

*Contributed equally

Assessing individual cells within bulk cell populations is essential for understanding the functional heterogeneity within immune cell populations. Yet, exploring this diversity while preserving cell viability has been hindered by technological complexity and associated costs. Here, we present an easily accessible droplet technology that enables accurate evaluation of live cell populations at the individual cell level. This technology seamlessly integrates with standard cell laboratory equipment for handling, culture, analysis, and sorting of cells.

Rapid single-cell secretion assays can readily be performed on living cells. We demonstrate this capability by measuring the secretion of the cytokines IFNγ and TNFα as well as granzyme B. By performing cytokine assays in a single-cell format on peripheral blood mononuclear cells (PBMCs), it was possible to identify a subset of cells exhibiting high levels of TNFα secretion, highlighting population heterogeneity within the PBMC bulk population. Granzyme B secretion was detected with single-cell resolution within a 30-minute assay timeframe.

This technology enables cell-cell interaction assays in flow cytometry, demonstrated by encapsulating single cell-pairs of target and effector cells within droplets. Using flow cytometry, we monitor immune cell mediated cytotoxicity over time by co-encapsulating natural killer (NK) cells, tumour-infiltrating lymphocytes (TIL), or chimeric antigen receptor (CAR) T cells with their respective target cells.

Furthermore, the immune cell mediated cytotoxicity assay can be combined with the measurement of other functionalities, such as granzyme B secretion. Applying this approach, we investigated the sequential events in cell mediated cytotoxicity at the single-cell level using NK and CAR-T cells. Our findings confirm that granzyme B secretion follows effector cell recognition of target cells, preceding cell death.

In summary, the droplet platform, combined with flow cytometry, provides a versatile approach for exploring functional heterogeneity within immune cell populations, unlocking new insights essential for advancing immune cell therapy.

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Deep Learning Powered Imaging of Nanocarriers Across Entire Mouse Bodies at Single-Cell Resolution

J. Luo[1,2,3], Y. Chen[1,2,4], I. Horvath[1,5], K. Kadletz[1,3], R. Al-Maskari[1,5], M. Ali[1,6,7], J. Paetzold[1,5], F. Hellal[1], M. Elsner[1], A. Erturk[1,2,3,6,8]

[1]Helmholtz Munich, Institute for Tissue Engineering and Regenerative Medicine, Munich, Germany, [2]University Hospital, LMU Munich, Institute for Stroke and Dementia Research, Munich, Germany, [3]Deep Piction GmbH, Munich, Germany, [4]Ludwig-Maximilians University Munich, Faculty of Medicine, Munich, Germany, [5]Technical University of Munich, TUM School of Computation, Information and Technology, Munich, Germany, [6]Graduate School of Systemic Neurosciences, Munich, Germany, [7]Helmholtz Munich, Institute of Computational Biology, Munich, Germany, [8]Munich Cluster for Systems Neurology, Munich, Germany

We introduce SCP-Nano (Single Cell Precision Nanocarrier Identification Pipeline), an advanced imaging and deep learning pipeline for cell-level analysis of nanocarrier biodistribution across intact mouse bodies. SCP-Nano accurately detects and quantifies the targeting of tens of millions of cells by various nanocarriers at extremely low, clinically relevant doses (0.0005 mg/kg). Application of SCP-Nano elucidated the tropism of lipid nanoparticles (LNPs), highlighting differences in biodistribution among various injection routes. Furthermore, SCP-Nano enables the identification of all cells targeted by adeno-associated viruses (AAVs) for gene therapy and antibody-targeted DNA origami structures for cancer treatment. Notably, off-target accumulation revealed by SCP-Nano can be further investigated with proteomics to reveal potential side effects and toxicity of the delivered therapeutics. In summary, SCP-Nano provides two to three orders of magnitude greater sensitivity for visualizing nanocarrier distribution in mouse bodies compared to existing approaches. This approach will significantly accelerate the development of precise and safe nanocarrier-based therapeutics.

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Breast cancer-on-chip model for efficacy and safety testing of CAR-T cells

T. I. Maulana[1], C. Teufel[1], M. Cipriano[1,2], J. Roosz[3], L. Lazarevski[1], F. E. van den Hil[4], L. Scheller[5], V. Orlova[4], A. Koch[6], M. Hudecek[5], M. Alb[5], P. Loskill[1,2,3]

[1]University of Tübingen, Department of Microphysiological Systems, Institute of Biomedical Engineering, Tübingen, Germany, [2]3R Center Tübingen for In Vitro Models and Alternatives to Animal Testing, Tübingen, Germany, [3]NMI Natural and Medical Sciences Institute at the University of Tübingen, Reutlingen, Germany, [4]Leiden University Medical Center, Department of Anatomy and Embryology, Leiden, The Netherlands, [5]Universitätsklinikum Würzburg, Medizinische Klinik und Poliklinik II, Lehrstuhl für Zelluläre Immuntherapie, Würzburg, Germany, [6]University of Tübingen, Department of Women's Health, Tübingen, Germany

Physiologically relevant human in vitro models that recapitulate the challenges of solid tumors and the tumor microenvironment (TME) are highly desired in the field of chimeric antigen receptor (CAR)-T cell therapy. To evaluate CAR-T cell efficacy and safety, we developed a microphysiological breast cancer model on a chip that incorporates relevant TME components and allows constant perfusion of CAR-T cells.

The chip is designed to compartmentalize tumor aggregates, exemplified here with aggregates from a triple negative ROR1+ breast cancer cell line MDA-MB-231 or patient-derived tumor organoids (PDOs) obtained from metastatic breast cancers with varying ROR1 expression levels. They were embedded together with primary macrophages in a dextran-based hydrogel, and loaded into microfluidic tumor chambers located underneath a medium channel that is lined with primary microvascular endothelial cells mimicking the vasculature. ROR1- or CD19-specific CAR-T cells or control untransduced T cells from the same donor with an equal ratio of CD4+ and CD8+ T cells were constantly perfused (20 µL/h) through the medium channel, where the (CAR-)T cells were able to extravasate from the circulation across the endothelium and infiltrate the tumor. The platform was utilized to assess CAR-T cell cytotoxicity via quantitative imaging analysis (CAR-T cell recruitment, tumor infiltration, tumor growth), monitor "real-time" cytokine release kinetics (IL-2, IL-6, IL-10, IFN-γ, TNF-α, and granzyme B), and safety intervention by controlling the immune reaction with a pharmacological on/off switch using dasatinib.

Throughout the culture period of 8 days, we found that CAR-T cell infiltration into the tumor aggregates, their persistence, and ability to control tumor growth depended on the ROR1 expression of the tumor. Although it did not hamper CAR-T cell efficacy, the presence of endothelial cells reduced the number of recruited (CAR-)T cells and the resulting infiltration density into the tumor. Additionally, high levels and release kinetics of inflammatory cytokines typically observed in the clinic to monitor the development of cytokine release syndrome were observed in the CAR-T cell condition, which were significantly higher than in the control T cell condition at almost all measurement time points. Applying dasatinib during the chip culture enabled an on/off switch of CAR-T cells and could temporally control CAR-T cell response. Lastly, the integration of PDOs with varying ROR1 expression levels enabled the demonstration of a patient-specific response.

The ability to investigate the efficacy and safety of CAR-T cell therapy and recapitulate patient heterogeneity in this model may provide new insights into the mechanisms of CAR-T cell-mediated cytotoxicity in a physiologically relevant TME. This, therefore, provides the opportunity for a broad application in the bench-to-bedside translation and acceleration of the preclinical development of novel CAR-T cell products.

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Achieving flexibility and scalability in cell therapy manufacturing through optimized cell isolation and activation

H. Meås, D. Tollefsrud Gjølberg, M. Tellez Gabriel, R. Hartberg, I. Nyhus Moen, J. Malm Rasmussen, N. Nejati Moharrami, S. Mevatne, H. Vebø, T. Holt Hereng
Thermo Fisher Scientific, Bio Production Group, OSLO, Norway

Gibco™ CTS™ Dynabeads™ CD3/CD28 are regarded as the gold standard in clinical CAR-T cell manufacturing for simultaneous isolation, activation, and expansion of T cells. They enable research, development and manufacturing of commercial CAR-T cell drugs, including the first FDA approved CAR-T immunotherapy Kymriah™.
Dynabeads CD3/CD28 selectively target and bind cells co-expressing CD3 and CD28 in heterogenous cell populations. In addition to the antibody-coated Dynabeads providing the activation and co-stimulatory signals required for T cell activation and expansion, their paramagnetic property allows the Dynabead-bound cells to be captured on a magnet while unwanted cells are removed. The isolated Dynabead-bound cells have then traditionally been placed in culture for a minimum of 5 days to allow for the passive dissociation of the cells from the Dynabeads before their subsequent magnetic removal. Removing the Dynabeads at earlier time points is possible but can result in reduced cell yields as the Dynabeads are not fully dissociated from the cells. This presents a challenge for manufacturing processes that require downstream applications to be carried out shortly after isolation. Building upon our well established Dynabeads and CaptureSelect™ offerings, our new CTS Detachable Dynabeads CD3/CD28 can similarly provide simultaneous isolation and activation of T cells while also allowing full flexibility over the timing of bead removal.
The new Detachable Dynabeads technology platform employs an innovative and highly effective active release mechanism. The Detachable Dynabeads are coated with streptavidin and conjugated with the

variable domain of camelid heavy-chain only antibodies (VHH ligands) that target and efficiently bind to specific surface markers. The active release mechanism is subsequently based on the VHH ligands being conjugated to a low-affinity biotin derivative, which allows the beads to be actively detached from target cells at any desired timepoint through competition with a biotin buffer.
Detachable Dynabeads CD3/CD28 are optimized for use with Gibco™ CTS™ DynaCellect™ Magnetic Separation System, thus allowing for full automation and scalability of the protocol in process development and clinical manufacturing.

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Preclinical testing of therapeutic transgenes for locoregional immunotherapy

A. Menze, D. Ostroumov, F. Kühnel, H. Wedemeyer, T. Wirth
Hannover Medical School, Department of Gastroenterology, Hepatology, Infectious Diseases and Endocrinology, Hannover, Germany

The efficacy of systemic immunotherapies is limited for poorly immunogenic tumors which suppress T cell priming and tumor infiltration. Modern antigen carriers including lipid nanoparticles/mRNA complexes have renewed the interest in local immunotherapy due to their ability to express multiple transgene constructs simultaneously. The identification of therapeutically active combinations, however, is hampered by the lack of preclinical models to rapidly express and evaluate transgenes combinations in vivo. To enable empirical testing of immunogenic transgenes we have combined a doxycycline-inducible expression system with flow cytometry and multiplex immunohistochemistry imaging. In animal models of liver and colon cancer we demonstrate that the impact of a single transgene on the immune milieu is limited and heavily dependent on the studied tumor entity. Compared to single transgenes, transgene combinations induced more complex and only partially predictable alterations in the tumor micromilieu but strongly enhanced therapeutic efficacy. By combining expression of transgenes with synergistic impact on antigen-presenting cells and T cells, we identified the combination of IL-12, FLT3L and CXCL9 as the most promising combinatorial approach, resulting in complete tumor remission in mice. Taken together we show the feasibility of preclinial testing and identification of therapeutic candidate genes for more efficacious locoregional immunotherapy of solid tumors.

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Non-invasive monitoring of melanoma heterogeneity dynamics using substrate-specific NanoLuciferase variants

J. Messmer, J.-Y. Huang, M. Effern, S. Vadder, M. Yong, N. Glodde, M. Hölzel
Institute of Experimental Oncology, University Hospital Bonn, Bonn, Germany

Malignant melanoma is a rare but very aggressive form of skin cancer. Despite tremendous successes of immunotherapeutic approaches treatment of melanoma patients remains challenging. Tumor plasticity such as shifting between differentiated and undifferentiated cellular states altering the expression of tumor-antigens contributes to the development of therapy resistance. The cellular and molecular mechanisms of tumor plasticity allowing certain melanoma subpopulations to escape from therapy are

currently not well understood. Secreted NanoLuciferase (sNanoLuc) is a small enzyme that can undergo renal excretion in mice and can therefore be used to monitor tumor growth non-invasively by measuring its activity in mouse urine. sNanoLuc can act promiscuously with a variety of substrates. We identified a novel point mutation in sNanoLuc which reduces luciferase activity with its substrate coelenterazine ca. 1000-fold compared to its activity using furimazine as a substrate. In combination with the reverse mutant sNanoLuc^{18Q27L} variant which has enhanced activity with coelenterazine, we can tag two distinct tumor subpopulations and quantify their frequency within a heterogenous mixture of tumor cells. We have established this assay for robust, non-invasive tracking of tumor subpopulations by measuring substrate-specific sNanoLuc activity in cell culture supernatants in vitro and are currently developing its in vivo application. This method will allow highly time-resolved longitudinal non-invasive monitoring of the kinetics of frequencies of tumor subpopulations in mouse models of melanoma. It is a novel approach to refine and reduce animal experiments and has a lot of potential for studying tumor plasticity and resistance to immunotherapy in mouse tumor models.

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Efficacy evaluation of an IgG therapeutic in a humanized immune system mouse model lacking murine Fc gamma receptors

M. Oliverio[1], N. Smith[2], M. Buczek[2], M. MacBride[2], E. Andino[2], E. Sack[2], D. Freer[2], M. Vedder[2], K. Bott[2], L. Baskin[2], J. Richardson[2]

[1]Taconic Biosciences GmbH, Leverkusen, Germany, [2]Taconic Biosciences, Inc, Rensselaer, NY, United States of America

Traditional humanized immune system (HIS) mice retain murine immune cells such as neutrophils and macrophages, and Fc gamma receptors (FcγRs) on these residual murine immune cells can interact with human IgG-based therapeutics to confound preclinical results. To determine whether knockout of murine Fc gamma receptors (FcγRs) in a super-immunodeficient mouse model would alter anti-PD1 efficacy compared to the parent strain, we studied tumor growth kinetics, human reconstitution, and tumor-infiltrating leukocytes (TILs) in each strain engrafted with HCC827 lung adenocarcinoma tumor cells treated with pembrolizumab or vehicle. We also present humanization results for the newly generated FcγR knockout NOG-EXL.

Methods: HIS NOG (huNOG) or HIS FcγR knockout NOG mice (FcResolv™ huNOG) were made using identical protocols with CD34$^+$ cells (3 shared donors). Reconstitution was evaluated in naïve animals, and HCC827 cells were inoculated in the remaining animals. Following randomization on D7, mice were dosed twice weekly for 4 weeks and then euthanized for blood, spleen, and tumor analysis. Bodyweight, clinical observations, and tumor growth were measured.

Results: For a given donor, humanization was equivalent between huNOG and FcResolv huNOG. Pembro treatment showed significant tumor growth inhibition in 1 donor in FcResolv huNOG, but not in donor-matched huNOG. Human TILs in pembro-treated mice were significantly different between the strains for all donors, with more CD8$^+$ T cells and fewer TAMs in FcResolv huNOG compared to vehicle-treated mice, and no significant differences in huNOG. Evaluation of murine TILs revealed differences in murine

macrophage populations, regardless of treatment, with Ly6C^{lo} dominant in FcResolv huNOG and Ly6C^{hi} dominant in huNOG.

Conclusions: Our study demonstrates when treated with anti-PD1, FcResolv huNOG mice show expected pharmacodynamic changes and donor-dependent efficacy whereas, despite identical donors and creation protocols, pembrolizumab-treated huNOG mice showed neither. These differences are due to the presence or absence of murine FcγRs and their impact on antibody IgG-based therapeutics.

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Efficient Computational Docking of TCR:pMHC-I Complexes Using Restricted Rotation Matrices and Distance Restraints

F. Parizi*, J. Aarts*, G. Ramakrishnan, L. Xue
RadboudUMC, Medical BioSciences, Nijmegen, The Netherlands

*Contributed equally

The T cell's ability to discern self and non-self depends on its T-cell receptor (TCR), which recognizes tumor-specific peptides presented by MHC molecules. Understanding this TCR-peptide-MHC (TCRpMHC) interaction is important for cancer immunotherapy design, pathogen identification, and autoimmune disease treatments. Despite its potential, understanding the intricacies of TCR recognition, encapsulated in TCRpMHC structures, remains challenging due to the immense diversity of TCRs (>10^8/individual), rendering experimental determination and general-purpose computational docking impractical.
Addressing this gap, we've developed a rapid integrative 3D modeling protocol leveraging unique docking patterns in TCRpMHC complexes. Built upon PIPER (1) software, our pipeline significantly cuts down docking rotation sets, exploiting the consistent polarized docking angle of TCRs at pMHC. Additionally, our ultra-fast structure superimposition tool, GradPose (2), accelerates clustering of modeled conformations to select models closely resembling experimental structures. On a benchmark set of 36 TCRpMHC-I complexes, our protocol completes a case in just ~1 min, outperforming state-of-the-art Physics-based dockings (3,4) by 30 times in docking speed, while improving modeling quality.
This protocol promises to offer a valuable structural landscape over TCR repertoires targeting specific peptides, along with associated energies. Its computational efficiency can also enrich existing pMHC-specific single-cell sequencing TCR data, facilitating the development of structure-based deep learning algorithms. These insights are essential for understanding T-cell recognition and specificity, advancing the development of therapeutic interventions.

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Combinatorial in silico algorithm to identify clinically-relevant TCRs for personalized T-cell therapy

R. Pétremand[1,2,3,4], J. Chiffelle[1,2,3,4], S. Bobisse[1,2,3,4], M. A. S. Perez[5], J. Schmidt[1,2,3,4], M. Arnaud[1,2,3,4], D. Barras[1,2,3,4], M. Lozano-Rabella[1,2,3,4], R. Genolet[1,2,3,4], C. Sauvage[1,2,3,4], D. Saugy[1,2,3,4], A. Michel[1,2,3,4], A.-L. Huguenin-Bergenat[1,2,3,4], C. Capt[1,2,3,4], J. S. Moore[6], C. De Vito[7], S. I. Labidi-Galy[6,8], L. E. Kandalaft[1,2,3,4,9], D. Laniti Dangaj[1,2,3,4], M. Bassani-Sternberg[1,2,3,4,9], G. Oliveira[10,11,12], C. J. Wu[10,11,12], G. Coukos[1,2,3,4,13], V. Zoete[1,2,3,5], A. Harari[1,2,3,4]

[1]Lausanne University Hospital (CHUV), Oncology, Lausanne, Switzerland, [2]University of Lausanne (UNIL), Biology and Medicine, Lausanne, Switzerland, [3]Ludwig Institute for Cancer Research, Lausanne, Switzerland, [4]Center for Cell Therapy, CHUV-Ludwig Institute, Lausanne, Switzerland, [5]Molecular Modelling Group, Swiss Institute of Bioinformatics (SIB), Lausanne, Switzerland, [6]Faculty of Medicine, University of Geneva, Swiss Cancer Center Leman, Department of Medicine and Center of Translational Research in Onco-Hematology, Geneva, Switzerland, [7]Division of Clinical Pathology, Hôpitaux Universitaires de Genève, Diagnostics, Geneva, Switzerland, [8]Department of Oncology, Hôpitaux Universitaires de Genève, Oncology, Geneva, Switzerland, [9]Center of Experimental Therapeutics, Lausanne University Hospital (CHUV), Lausanne, Switzerland, [10]Dana-Farber Cancer Institute, Boston, MA, United States of America, [11]Harvard Medical School, Boston, MA, United States of America, [12]Broad Institute of MIT and Harvard, Cambridge, MA, United States of America, [13]Immuno-oncology Service, Lausanne University Hospital, Oncology, Lausanne, Switzerland

The adoptive transfer of genetically engineered cells expressing specific T-cell receptors (TCRs) represents a promising personalized cellular therapy. Given the requirement to match both tumor antigens and HLA restriction, only few patients are eligible to these therapies. By exploiting the distinct transcriptomic profile of bone fide tumor-reactive T cells (TRT) relative to bystander cells, we built a personalized in-silico predictor of orphan tumor-reactive TCRs. The predictor, named TRTpred, was benchmarked against several published signatures on 42 tumors, which included 19 melanomas, 17 gastrointestinal cancers, 4 lung cancers, and 2 breast cancers. Using the predictor, we also revealed that the TRT repertoire is consistently richer and more clonal than its bystander counterpart in all tumor indications investigated. Furthermore, in melanoma, we observed that TRTs were predominantly located within tumor islets, while bystanders T-cell clones accumulated in the stroma. Beyond its discovery applications, TRTpred was utilized to identify clinically relevant clones for personalized T-cell therapy. To this end, we developed MixTRTpred by integrating TRTpred with a TCR avidity predictor and a TCR clustering tool (to select panels of relevant TCRs inferred to target different antigens) and benchmarked it in vitro and in vivo. Taking advantage of recent advances in the field of T cell engineering, the accuracy of MixTRTpred indicates that personalized TCR-based therapy is now achievable for most patients with solid tumors.

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The FcResolv™ hIL-15 NOG mouse model facilitates the investigation of therapeutic antibody efficacy by mitigating interference from murine Fc receptors and to explore antibody-dependent cellular cytotoxicity mediated by NK cells.

S. Rhein[1], M. Stecklum[1], M. Buczek[2], J. Richardson[2], J. Hoffmann[1]
[1]EPO GmbH, Berlin, Germany, [2]Taconic Biosciences, Inc., New York, United States of America

A novel mouse model deficient in Fc receptors and expressing human IL-15 (FcResolv™ hIL-15 NOG) was employed in anti-cancer therapy of a patient-derived xenograft (PDX) tumor models. Treatments involved antibodies alone or in combination with human NK cells to explore antibody-dependent cellular cytotoxicity (ADCC).
Targeted antibody therapy operates through direct binding to tumor antigens or indirect interactions via the constant region (Fc) of the antibody, which can enhance anti-tumor efficacy. Indirect mechanisms engage the innate immune system, mediated by both the complement system (complement-dependent cytotoxicity (CDC)) and immune cells (antibody-dependent cellular phagocytosis (ADCP) and antibody-dependent cellular cytotoxicity (ADCC)). In immune-deficient mouse strains (e.g. NOG), false positives and/or negatives may occur due to interactions with murine Fc receptors. These can either result in anti-tumor responses via activation of the murine innate immune system or can interfere with the human-targeted therapy's primary mode of action.
PDX tumor models were transplanted into hIL-15 NOG and FcResolv™ hIL-15 NOG mice. Cetuximab was used to treat a human head and neck squamous cell carcinoma and a lung adenocarcinoma PDX model, while pertuzumab and trastuzumab were applied in a breast ductal carcinoma PDX model. There was no difference in percent tumor growth inhibition between the FcResolv™ hIL-15 NOG and hIL-15 NOG mice with regards to cetuximab treatment in the lung and head and neck cancer or for trastuzumab treatment of breast ductal carcinoma. However, pertuzumab treatment revealed a false positive efficacy, with the false positive effect more pronounced in hIL-15 NOG mice than in FcResolv™ hIL-15 NOG mice. Rituximab treatment was evaluated in two diffuse large B cell lymphoma PDX models. In one of the lymphoma models, a false negative result was observed. Rituximab showed minimal tumor inhibition in hIL-15 NOG mice but was effective in FcResolv™ hIL-15 NOG mice. Based on growth kinetics, the lung cancer PDX model was selected for further testing of ADCC in the NK cell-humanized FcResolv™ hIL-15 NOG or hIL-15 NOG mouse. NK cells were transferred after irradiation of mice and followed by transplantation of the PDX tumor. NK cells engrafted successfully without influencing tumor growth before treatment initiation. Cetuximab treatment strongly delayed tumor growth irrespective of NK cell transfer in this tumor model. FcResolv™ hIL-15 NOG enables the detection of false-positive or false-negative results of antibody efficacy in anti-cancer treatment and can be utilized to investigate NK cell-mediated effects on antibody treatment.

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Microfluidic Technologies for Tumor Cell Isolation in Immunotherapy: Parallel Multi-analyte Pipeline using CTCelect to isolate Circulating Tumor Cells and other solid and liquid Tumor Markers

J. Stiefel
Fraunhofer IMM, Diagnostics, Mainz, Germany

Immunotherapy has revolutionized cancer treatment by harnessing the body's immune system to target and eliminate malignant cells. However, the success of immunotherapy relies heavily on the isolation of tumor cells from complex biological samples with precision and efficiency. Microfluidic technologies have emerged as promising tools for this purpose, offering advantages such as high throughput, sensitivity, and compatibility with small sample volumes. For example, microfluidic devices incorporating antibody-coated surfaces can selectively capture CTCs based on specific antigen expression, enabling precise molecular profiling and personalized treatment strategies. Among these technologies, CTCelect stands out as a novel microfluidic platform designed specifically for the isolation of circulating tumor cells (CTCs). This microfluidic device was developed to capture and isolate CTCs from peripheral blood, providing a minimally invasive method for monitoring disease progression and guiding treatment decisions in cancer patients. By integrating microscale features and functionalized surfaces, CTCelect enables the enrichment of rare CTCs while minimizing contamination from normal blood cells. Moreover, the platform facilitates downstream analysis of isolated CTCs, allowing for molecular characterization and potentially assessment of immunotherapy response. Commercial technologies are often price-intensive and limit user-friendliness due to tight protocols, while a comprehensive combined multi-analyte process chain together with tissue biopsy and cfDNA analysis is usually not pursued at all. Here we present a platform technology that can pave the way for personalized diagnostics for potential immunotherapies through automation. We have recently investigated the feasibility of multi-analyte assays from cell phase, plasma and tissue section using CTCelect and gained first promising handling insights. For example, we can demonstrate a diagnostic procedure for the examination of whole blood and tissue biopsy on the same day of a tumor resection in the operating room. We can detect immune checkpoint-relevant targets at the qPCR level, analyze cfDNA levels, and provide nucleic acid from the samples to sequencing service providers. Despite the significant progress in microfluidic technologies for tumor cell isolation, there is a need for standardized protocols and validation studies to ensure the reliability and reproducibility of microfluidic-based assays in clinical settings. In conclusion, microfluidic technologies, including platforms like CTCelect, hold great promise for advancing tumor cell isolation in the context of immunotherapy. Continued research and innovation in microfluidics are essential for overcoming current limitations and translating these technologies into clinical applications that improve patient outcomes in cancer immunotherapy.

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Artificial Targets: a versatile cell-free platform to characterize CAR T cell function in vitro

N. Tokarew[1], X. Wang[1], N. Borgelt[1], R. Siemer[1], C. Casonato Melo[1], I. Kasampalidis[1], I. Ogusuku[1], T. Cathomen[2], I. Gessner[1], C. Dose[1], J. Fauerbach[1], A. Richter[1], C. Evaristo[1]
[1]miltenyibiotec, Bergisch Gladbach, Germany, [2]University Hospital Freiburg (Universitätsklinikum Freiburg), Freiburg, Germany

Chimeric antigen receptor (CAR) T cells have proven efficacy against a number of cancers, however, further research is needed to extend their therapeutic potential. Functional characterization of CAR T cells plays is central across fundamental research and therapeutic development, with increasing needs for standardization. Typically, effector functions are assessed following co-culture with target-expressing cell lines. This poses several limitations, including high variability, allogeneic-induced background, and work and time intensive workflows. To overcome these limitations, we developed a synthetic bead-based platform

("Artificial Targets",or "AT") to characterize CAR T cell function in vitro. AT could specifically induce CAR T cellactivation, and multiple effector functions of CAR T cells, mimicking the response triggered by target cell lines. These include cytotoxic activity, expression and secretion of cytokines, and cell proliferation. Finally, AT demonstrated flexibility to engage multiple co-stimulatory molecules to enhance CAR T cell function, representing a powerful tool for modulating CAR T cell responses. Collectively, our results show that ATs can specifically activate CAR T cells for essential effector functions that could significantly advance standardization of functional assessment of CAR T cells, from early development to clinical applications.

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The MHC MACSimer technology combines flexibility, quality and releasabilty for the detection of antigen specific T cells

N. Tokarew[1], L. Henschel[2], L. Geiger[2], L. Wessels[2], G. Günther[2], M. Schuster[2], J. Jaufmann[2], M. Wieczorek[2]
[1]Miltenyi Biotec B.V. & Co. KG, R&D, Bergisch Gladbach, Germany, [2]Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany

Antigen (Ag)-specific T cell responses play an essential role in monitoring and combating cancer, infectious diseases and autoimmunity and the precise analysis, isolation and characterization of rare Ag-specific T cells is crucial for the development of cellular therapies and cancer- or virus-specific vaccinations. Given the strong personal component of Ag-specific T cell responses ("MHC/HLA" restriction), innovative tools must provide researchers with full flexibility and high quality enabling integration into translational workflows. We have therefore designed a new reagent, the MHC MACSimer. The innovative construct design of MHC MACSimers is overcoming major challenges of antigen-specific T cell analytics. MHC MACSimers feature superior specificity and fluorescence brightness through an optimally balanced amount and ratio of MHC molecules and fluorophores, enabling the detection of even very rare T cell subsets with highest reliability, stability and robustness.
To further satisfy the demand for flexibility and compatibility and potential integration of our MHC MACSimer with translation workflows, we have additionally integrated
A peptide-loading technology into our peptide-loadable **MHC MACSimer FleX Kits** allowing for extensive T cell epitope screening.
The REAlease® technology into our MHC MACSimer FLeX Kits and **peptide-loaded MHC MACSimers** for label-free T cells after sorting, e.g. for multiple sequential sorting steps or functional downstream assays.
A broad collection of **MACSpep** single peptides covering a broad variety of T cell epitopes related to human diseases, such as cancer, infection, and autoimmunity and the ideal comparability with our MHC MACSimer FleX kits.
We show that both, our MHC MACSimer FleX kits as well as our peptide-loaded MHC MACSimers feature superior performance when staining and analysing shared cancer antigens, Neo-Ags or virus-specific T cells for both, CD4 and CD8 T cells of different origins (PBMCs, whole bloods samples or dissociated organs) and comparing them to other state-of-the art MHC multimer reagents.
They perfectly integrate into secondary antibody staining panels with multimer staining approved Abs, e.g. T cell exhaustion or differentiation panels, allowing to get the most information out of the rarest T cells.
Combining them with fluorochrome-specific magnetic beads using MACS technology leads to highly pure (>>90%) and high-yield-enriched T cell populations, displaying the "expected" T cell phenotype. The

MACSpep peptide come with MHC loading protocol and can be, aside from FleX kit loading, applied for the Epitope-specific activation and expansion of T cells ex vivo.
In conclusion , our three reagent classes improve Ag-specific T cell analysis, enumeration and isolation and cover the need for flexibility, stability and standardization.

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Targeting the untargetable: inducing anti-tumor antibodies with an innovative immunization platform based on the African trypanosome

K. Urban[1], A. Gkeka[1], M. Chandra[1], S. Kruse[1], E. Stebbins[2], F. N. Papavasiliou[3], J. P. Verdi[1]
[1]Panosome GmbH, Heidelberg, Germany, [2]German Cancer Research Centre (DKFZ), Structural Biology of Infection and Immunity, Heidelberg, Germany, [3]German Cancer Research Centre (DKFZ), Division of Immune Diversity, Heidelberg, Germany

Therapeutic antibodies have shown immense potential in cancer treatment, but certain tumor antigens remain challenging to target effectively because of their small size or specific conformation. We address this hurdle by introducing a novel immunization platform derived from the antigenic surface coat of the African trypanosome, enhancing the antigenicity of small molecules like sugars conjugated to it.

We developed the VAST (VSG-immunogen Array by Sortase Tagging) technology, which involves sortase-mediated conjugation of haptens to the trypanosome surface coat protein VSG. This approach significantly boosts the antigenicity of small molecules, enabling efficient antibody generation.

Using our VAST technology in combination with a single-cell RNAseq strategy, we achieved remarkable results against the small molecule opioid fentanyl as a proof of concept, producing femtomolar affinity fentanyl-binding antibodies without the need for extensive candidate screening.
More recently, our VAST technology demonstrated promising outcomes in generating specific antibody responses against tumor-associated MUC1, particularly targeting the sialylated Tn antigen.
Additionally, we are leveraging VAST to target the H3K27M mutation in diffuse midline glioma, aiming to produce TCR-like antibodies that therefore bind to neoepitopes presented on HLA.

Our VAST technology presents an innovative approach for generating therapeutic antibodies against challenging tumor antigens, offering new avenues for effective cancer treatment. We are interested in developing our own antibody assets, but also in deploying the VAST to target a wide array of additional “difficult antigens” in collaboration with interested parties.

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High-throughput single-cell whole transciptome analysis of various blood cell types including neutrophils using a microwell-based system

R. van Haaren
Becton Dickinson GmbH, Biosciences, Heidelberg, Germany

Microfluidic technologies have been developed and used for single-cell analysis over the past decade, and devices have been developed to support higher thoughput single-cell multiomics analysis. A microwell-based system that relies on gentle settling of cells in microwells was used for this study, which made it flexible to capture a wide range of cell sized ranging from 5 µm to 20 µm as well as a magnitude of different cell types including fragile cells such as neutrophils. In this system, the microwell cartridge has eight lanes instead of one to increase the maximum thoughput of cell capture. In this study, we tested the feasibility of the eight-lane cartridge for analysis of various cell types and compared the performance with the current single-lane cartridge. In brief, neutrophils, NK cells and T cells were isolated and loaded in two separate lanes of the eight-lane cartridge to show reproducibility of data from multiple lanes. Cartridge metrics showed a capture rate of >60% from viable cells loaded in the cartridge, which showed comparability to the current single-lane cartridge. In addition, whole transcriptome analysis of the same samples loaded in separate lanes showed high correlation ($R^2 > 0.95$) of gene expression detection. This study demonstrated the flexible application of the eight-lane cartridge in terms of capture of various cell types. We also demonstrated feasibility of use for specific research designs based on individual researcher needs while keeping similar performance with the single lane. The consistency of results between multiple lanes also supports high-throughput single-cell analysis using the eight-lane cartridge without batch effect.

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An imaging-based 3D organoid-myeloid co-culture drug screening assay advances myeloid targeting-drug efficacy testing

T. Veenendaal, S. de Man, S. Chavez Abiega, M. Kop, R. Heeren, D. Okkes, A. Weterings, G. Goverse, L. Price
Crown Biosciences, Immuno-oncology, Leiden, The Netherlands

Within the tumor microenvironment (TME), the myeloid compartment plays an important role in drug responses and tumor survival and represents a heterogenous population that exerts either tumor- or immune-suppressive responses. Because of its role in tumor progression, the myeloid compartment is increasingly recognized as a druggable target. While several immuno-oncology (IO) drugs targeting different effector cells demonstrated great efficacy in a clinical setting, the potential of the myeloid compartment remain largely unrevealed. To advance the field of IO therapeutics specifically for the myeloid compartment, we have developed complex 3D myeloid-tumor cell co-cultures to allow high throughput myeloid-targeting drug testing.
$CD14^+$ monocytes were isolated from healthy PBMC donors, seeded in hydrogels in 384-well format and polarized into M1 and M2 macrophages by activation with granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) respectively. in a 6-day period or remained unpolarized (M0) and matured with LPS. We employed 3D high content imaging and our

proprietary image-based analysis to generate an automated image analysis pipeline to identify the various myeloid subsets based on their distinct morphological characteristics. This methodology provided data on macrophage polarization and proliferation. We next subjected M2 macrophages to macrophage-targeting drugs CSF1Ri, STING agonist and IFN-alpha 2b (n=4), known for their ability to repolarize macrophages towards a tumor suppressive phenotype (M1), and found that treatment of M2 macrophages with these targeted drugs indeed shifted their phenotype from an M2-type towards a M1-type of macrophages. These profiles were subsequently validated by cytokine analysis.
Next, non-repolarized and repolarized macrophages were co-cultured with patient-derived organoids (PDOs) and tumor spheroids (colorectal, breast and lung), to assess their functional tumor killing capacity. PDOs and tumoroids exhibited different sensitivity for macrophage-mediated cytotoxicity with M1 macrophages showing a greater degree of cytotoxicity than M2 macrophages which could be altered upon drug treatment, showing repolarization resulted in functional reprogramming. Our 3D cell culture platform presented here enables screening of IO drug candidates that target myeloid cells with simultaneous assessment of both the myeloid and tumor compartment. Combined, these data provide insight into the drug mode of action and allows fast-tracking the advancement of the field of IO therapeutics.

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Adaptation of automated cell segmentation approaches for highly multiplexed tissue images

M. Wirth*[1,2], N. Herold[1,2], K. Wistuba-Hamprecht*[1,2,3,4,5], S. Babaei*[1,2], M. Claassen*[1,2,3,6,7]
[1]University Hospital Tübingen, Department of Internal Medicine I - Section for Clinical Bioinformatics, Tübingen, Germany, [2]University of Tübingen, M3 Research Center for Malignome, Metabolome and Microbiome, Tübingen, Germany, [3]Cluster of Excellence iFIT (EXC 2180) "Image-Guided and Functionally Instructed Tumor Therapies, Tübingen, Germany, [4]German Cancer Research Center (DKFZ), Skin Cancer Unit, Heidelberg, Germany, [5]University of Heidelberg, University Medical Center Mannheim, Department of Dermatology, Venereology and Allergology, Mannheim, Germany, [6]University of Tübingen, Department of Computer Science, Tübingen, Germany, [7]University of Tübingen, Institute for Bioinformatics and Medical Informatics, Tübingen, Germany

*Contributed equally

Highly multiplexed tissue imaging as implemented on the CODEX (PhenoCycler-Fusion) or MACSima platforms is increasingly used to study the mechanisms conveying the association of tissue microenvironment and macroscopic phenotypes. These images give insights into tissue samples, particularly with multiplexed imaging allowing for the simultaneous recording of multiple markers. Cell segmentation, the step of delineating individual cell shapes, constitutes crucially in making this data amenable to interpretation and ultimately mechanistic hypothesis generation. Automated segmentation, though challenging, offers scalability and reproducibility advantages over manual methods, especially with the advent of deep learning approaches. However, many of the currently used approaches are based on the watershed algorithm expanding from a nuclear segmentation, only few apply deep learning models, and there are very little efforts in trying to utilize the information of more than two channels for segmentation. The introduction of Cellpose, a deep learning model trained on a diverse dataset of over 70,000 segmented objects, addressed some of the issues like generalization. Cellpose employs a U-net deep learning architecture to predict masks of individual cells. It's convenient graphical user interface enables manual

refinement of segmentations, enhancing usability. Additionally, Cellpose offers multiple prediction models trained on different datasets, like the Tissuenet and LiveCell dataset, broadening its applicability.
In this study, we leverage Cellpose for the analysis of highly multiplexed images, with the aim of tailoring it to this specific data type. For that, we have implemented modifications to streamline manual annotation by domain experts. Notably, our enhancements enable the visualization of an arbitrary number and combination of marker channels, empowering annotators to seamlessly switch between different markers expressed by specific cells to achieve comprehensive cell segmentation. Since there is not one marker expressed reliably on every cell membrane, this can significantly improve the segmentation for various tissues, as the annotator can now use a suitable marker for each cell type. The manual annotations can then be used to finetune the automated segmentation model.
Multiplexed tissue imaging holds promise for revealing insights into the mechanisms of cancer immunotherapy in situ. Our approach aims to facilitate this by simplifying the challenging task of cell segmentation in multiplexed tissue imaging data, allowing for interactive analysis. Moreover, our method is versatile and can be utilized beyond proprietary software solutions.

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Improving generalizability for MHC-I binding peptide predictions through structure-based deep learning

L. Xue[1], D. Marzella[1], G. Crocioni[2], T. Radusinović[3], S. Georgievska[2], A. Kessler[4], S. Buschow[4]
[1]Radboudumc, Nijmegen, The Netherlands, [2]NLeSC, Amsterdam, The Netherlands, [3]University of Amsterdam, Amsterdam, The Netherlands, [4]Erasmus MC, Rotterdam, The Netherlands

Recent advances in cancer immunotherapies demand for more accurate computational prediction of MHC-bound peptides. While existing MHC-bound peptide predictors have made important contributions to clinical studies, they tend to work less well on HLA alleles with less experimental data. This is mainly because they only rely on protein sequences to make the predictions, thus is heavily data dependent. Here we address the generalizability challenge of MHC-bound peptide predictions, by boosting the peptide-MHC (pMHC) binding data with three-dimensional (3D) structures, which can provide much richer information than sequence alone. 3D structures also allow us to exploit the power of geometric deep learning, a booming branch of deep learning especially designed for handling 3D objects.

On a dataset of ~100,000 pMHC binding affinity data spanning 114 HLA-I alleles, we demonstrate that our structure-based predictive approaches are more robust and more data-efficient on unseen MHC alleles than sequence-based approaches. Further, inspired by the success of chatGPT, we tackle data efficiency by introducing a similar approach on structures, 3D-GPT. Without being exposed to any binding affinity data, our 3D-GPT outperforms sequence-based methods trained on ~90 times more data points. Finally, we demonstrate the resilience of structure-based deep learning methods to biases in binding data on an Hepatitis B virus vaccine immunopeptidomics case study. Besides improving MHC-binding peptide identification, this proof-of-concept study also paves the way for data-intensive fields like T-cell receptor specificity predictions.

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Polymer-based antibody mimetics (iBodies) target human PD-L1 and function as a potent immune checkpoint blocker

M. R. Zamani[1,2], M. Hadzima[1,3], K. Blazkova[1], V. Šubr[4], T. Ormsby[1], J. Celis-Gutierrez[5], B. Malissen[5], L. Kostka[4], T. Etrych[4], P. Šácha[1], J. Konvalinka[1]

[1]Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, Prague, Czech Republic, [2]Department of Cell Biology, Faculty of Science, Charles University, Prague, Czech Republic, [3]Department of Organic Chemistry, Faculty of Science, Charles University, Prague, Czech Republic, [4]Department of Biomedical polymers, Institute of Macromolecular Chemistry, Czech Academy of Sciences, Prague, Czech Republic, [5]Centre d'Immunologie de Marseille-Luminy, Aix-Marseille Université, INSERM, CNRS, Marseille, France

Immune checkpoint blockade (ICB) using monoclonal antibodies (mAbs) against programmed cell death protein 1 (PD-1) or programmed death-ligand 1 (PD-L1) have demonstrated therapeutic efficacy in a broad range of cancer types. However, limitations with antibody-based ICB such as low tissue permeability, inherent or acquired resistance, immunogenicity, and high cost could be possibly improved using innovative approaches. On the other hand, other alternatives like synthetic low-molecular-weight (LMW) PD-1/PD-L1 blockers suffer from lower solubility, stability, inadequate pharmacokinetics, and efficacy as compared to antibodies. We have developed polymer-based anti-human PD-L1 antibody mimetics (α-hPD-L1 iBodies). The approach is based on attaching a potent PD-L1-targeting LMW ligand (giving it a specificity), a fluorophore (for visualization) and an affinity anchor to N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer. This copolymer is biocompatible, non-immunogenic, non-toxic, and very versatile. Moreover, it improves the solubility, stability, and functional affinity (avidity) of LMW ligand.
We were able to rescue activation of T cells comparably to therapeutic antibodies like avelumab, atezolizumab and durvalumab. Our findings suggest that iBodies can be used as experimental tools to target hPD-L1 and could serve as a platform to potentiate the therapeutic effect of hPD-L1-targeting small molecules.

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Insights from rapid bench-to-bedside development and manufacturing of individualized peptide-based vaccines for clinical trials

L. Zieschang[1,2,3], C. Kammer[1,2], M. Denk[1,2], M. T. Özbek[1,2], H.-G. Rammensee[4], J. S. Walz[1,2,3,5]

[1]University and University Hospital Tübingen, Institute of Immunology, Department of Peptide-based Immunotherapy, Tübingen, Germany, [2]University of Tübingen, Cluster of Excellence iFIT (EXC2180) "Image-Guided and Functionally Instructed Tumor Therapies", Tübingen, Germany, [3]German Cancer Consortium (DKTK) and German Cancer Research Center (DKFZ), partner site Tübingen, Germany, [4]University of Tübingen, Institute of Immunology, Tübingen, Germany, [5]University Hospital Tübingen, Clinical Collaboration Unit Translational Immunology, Department of Internal Medicine, Tübingen, Germany

The production of peptide-based cancer vaccines (pVACs) for use in clinical trials (CT) requires a GMP-certified facility, holding a valid manufacturing license. However, for smaller GMP facilities, the production of CT supplies has been increasingly difficult and costly due to recent changes in regulatory requirements. One approach to overcome this hurdle is the close collaboration between the research and GMP

departments to facilitate the translation of the latest research findings into CT. We here present our strategy for accelerated formulation development and manufacturing of pVACs for CT.
pVACs can be used to serve different therapeutic strategies, comprising off-the shelf single agent vaccines (FusionVAC22_01; EU-CT Nr. 2022-502869-17-01) as well as warehouse-based personalized vaccine composition (iVAC-XS15-CLL01; EudraCT Nr. 2020-002367-65) and individualized de novo vaccine design (GAPVAC-101; EudraCT Nr. 2013-002801-71). The latter represents the most complex, as peptide sequences for patient-specific pVACs will only be made available in the course of the study. To fulfill regulatory requirements for the different study designs we have implemented a comprehensive and reliable vaccine production process using thoroughly developed and validated manufacturing and analytical procedures. Our production capabilities include peptide synthesis with up to 22 amino acids followed by aseptic filling to produce vaccines containing up to 10 peptides. Peptides are manufactured by solid-phase synthesis using Fmoc-chemistry under GMP conditions (yield of 50 mg and up to 200 mg depending on the peptides' physiochemical properties). To enable feasibility and compatibility studies within personalized pVACs, so far unknown peptides are produced under non-GMP conditions prior to the production of GMP-peptides. These preliminary experiments are critical to ensure the final peptide vaccine formulation and aseptic filling during GMP pVAC production. Both, peptides and pVAC are subject of stability studies as part of lifecycle management and are continuously monitored from manufacturing to delivery to the CT site. If a depot-effect is desired after vaccination, a mixing kit containing Montanide ISA™ 51 VG and the vaccine is supplied. Prior to application, the vaccine is mixed with Montanide ISA™ 51 VG to produce a water-in-oil emulsion to protect the peptides from elimination after vaccination. So far nine finished and six ongoing clinical trials were successfully supplied by us with pVACs for more than 210 patients.
In conclusion, the modular design for the fast-track development and production of pVACs has been successfully established to serve clinical vaccine trials to combat cancer.

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Characterization of the tumor microenvironment in breast cancers derived from patient xenografts using humanized mice

S. Al-Haidar, J. Hald, J. S. Sommer, T. B. Engel, C. H. Nielsen, E. Christensen
Minerva Imaging, Ølstykke, Denmark

Preclinical evaluation is critical for developing safer and more efficacious anti-cancer therapies. These studies have mostly been performed using mice with a murine immune system and murine cancers (syngeneic models) or using immunodeficient mice with xenografted human tumors. However, the lack of an intact human immune system in these preclinical models makes them less clinically relevant. To evaluate cancer in a more clinically relevant setting, we characterized patient derived breast cancer xenografts (PDXs) in mice engrafted with a human immune system (humanized mice).
Female NSG mice were either engrafted intravenously with human peripheral blood mononuclear cells (PBMCs humanized) or human CD34+ umbilical cord hematopoietic stem cells (CD34+ humanized) after whole body irradiation. After evaluating engraftment kinetics in both PBMCs and CD34+ humanized mice in peripheral blood, we established luminal B (ST1099, XenoSTART) and basal-like (ST2789, XenoSTART) breast cancers subcutaneously in CD34+ stem cell humanized NSG mice. Ex vivo evaluation was performed for ST1099 tumors, ST2789 tumors, spleens and lungs by multicolor flow cytometry and multiplex immunohistochemistry.
The engraftment level in PBMC humanized mice gradually increased until reaching ≈55% human CD45+ (hCD45+) six weeks after PBMC humanization. Here mice exhibited symptoms of graft versus host disease. Furthermore, the PBMCs humanized model showed reconstitution of predominantly T cells (>99% of hCD45+ of both CD4+ and CD8+ T cells). In contrast, the blood level of monocytes and B cells were much higher in CD34+ humanized mice. In the CD34+ humanized setting, the levels of circulating CD4+ T cells and CD8+ T cells started to increase after week 12, while monocytes started to develop after week 20. From week 6 to week 46, the humane immune cells constituted 30 to 60% in CD34+ humanized mice.
In line with literature showing that basal-like breast cancers have poorer prognosis than luminal B breast cancer, tumor growth was only slowed by humanization for luminal B (ST1099) PDXs. Furthermore, the ST1099 PDX was immunologically colder (<2% hCD45+ of all viable cells) compared with ST2789 PDX (<8% hCD45+ of all viable cells). Together, this confirms the PDXs capability to maintain properties of the breast cancer subtypes in CD34+ humanized mice.
Our data indicates that humanized mice with PDXs can serve as clinically relevant tumor models to assess in vivo preclinical therapeutic effects with a human immune system and human tumor model present.

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Prognostic Utility of Epithelial Mesenchymal Transition-Related Transcription Factors in Papillary Renal Cell Carcinoma Prognosis and Immune Microenvironment

W. Ali[1], V. Cali[1], D. Jacobs[1], A. Kajdacsy-Balla[2]
[1]Albert Einstein College Of Medicine, The Bronx, United States of America, [2]University of Illinois at Chicago, Chicago, United States of America

Background: The transformation of cells from polarized epithelial to mobile mesenchymal forms, known as the epithelial mesenchymal transition (EMT), represents a significant shift in our understanding of cancer progression. It is a process tightly coordinated by transcription factors such as Snail (SNAI1), Slug (SNAI2), Smuc (SNAI3), Twist1/2, and Zeb1/2. While there have been other studies examining their expression in cancers such as breast and colon, comparatively less has been examined in papillary renal cell carcinoma (pRCC), the second most common type of kidney cancer. Through in-silico methods, this study examined these transcription factors and their expression in regards to survival, and also immune cell infiltrate and immunomodulator expression in pRCC.
Methods: Survival analysis was conducted with Kaplan-Meier plots generated for each of the aforementioned transcription factors using the TCGA "Kidney Renal Papillary Cell Carcinoma" dataset (n=288). Bonferroni correction was applied for multiple hypothesis testing (adjusted p-value cutoff: 0.0022). Additionally, deconvolution methods were used to estimate immune cell infiltration of CD8+ T cells, CD4+ T cells, Tregs, neutrophils, and macrophages and examine correlation to EMT transcription factor expression through the TIMER2.0 platform. Further correlation analysis was conducted between the expression levels of a panel of 24 common immunoinhibitor-related genes and the aforementioned EMT transcription factors in pRCC. For both correlation analyses rho of >.4 and above were considered moderate correlations ($p<0.001$ being significant).
Results: Out of the 7 EMT-related transcription factors examined, 5 showed differential expression in regards to survival. SNAI1($p<8.20e-05$), SNAI2 ($p<0.0014$), TWIST1($p<4.20e-07$), TWIST2($p<0.00013$), and ZEB1 ($p<0.0022$) all showed higher expression in cases with worse outcomes. All genes showed at least some level of significant positive correlation with immune cell infiltration, with 4/7 of the genes (SNAI1, SNAI3, ZEB1/2) showing moderate correlation with neutrophil infiltration ($p<0.0001$). Several of the immunoinhibitory genes were also highlighted by the correlation analysis such as ADORA2A and TGFB1 among others.
Conclusions: While the EMT is relatively-well established in other cancers, the same isn't as true for pRCC, especially in regards to how it can relate to the tumor immune microenvironment. This study highlights the prognostic utility of the aforementioned genes, warranting further wet-lab analysis to better understand the interplay between the EMT and immune microenvironment.

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Melanoma stem cells: orchestrating macrophages polarization into a mixed M1/M2 phenotype

M. Anselmi[1], P. Limonta[1], E. Montanari[2], G. Bernardo[2], V. M. Le Noci[2], T. Cesetti[3], R. Rudolf[3], L. Sfondrini[2,4], M. Sommariva[2,4]
[1]Università degli Studi di Milano, Dipartimento di Scienze Farmacologiche e Biomolecolari, Milano, Italy, [2]Università degli Studi di Milano, Dipartimento di Scienze Biomediche per la Salute, Milano, Italy, [3]Hochschule Mannheim, Mannheim, Germany, [4]Fondazione IRCCS Istituto Nazionale dei Tumori di Milano, Microambiente e Biomarcatori dei Tumori solidi, Milano, Italy

Cancer stem cells (CSCs) play an important role in driving the aggressiveness of melanoma and can impact on immune cell phenotype and function, like inducing the polarization of macrophages towards a pro-tumor phenotype. Nonetheless, the precise influence of melanoma stem cells (SCs) on macrophage biology remains to be fully understood. Our study aimed to investigate whether melanoma SCs have the capability

to alter the phenotype of macrophages. To achieve this goal, it was exploited an in vitro model of human macrophages utilizing the THP-1 monocyte cell line, which was differentiated into macrophages (M0) using phorbol 12-myristate 13-acetate (PMA). Macrophages were exposed to the conditioned medium (SCs-CM) derived from A375 and WM115 melanoma SCs, and macrophage phenotype was assessed using flow cytometry, real-time PCR, and ELISA assays. We observed that melanoma SCs can influence macrophage polarization by increasing the expression of Arginase 1 while decreasing the levels of CD163, two well-known M2 markers associated with cancer immunosuppression and progression. Surprisingly, RT-PCR analysis revealed an elevated expression of both M1 and M2 markers, such as IL-6, IL-12B, STAT1, IL-10, VEGF, and MARCO, in macrophages upon exposure to melanoma SCs-CM compared to untreated controls. Conversely, treated macrophages demonstrated reduced levels of M1 markers like IL-1β and TNF-α, as well as the M2 marker TGF-β, compared to control macrophages. Accordingly, ELISA data showed a higher secretion of IL-6, IL-10, and MMP-2 compared the control counterpart, reinforcing the idea of the acquisition of an M1/M2 mixed phenotype in macrophages treated with melanoma SCs-CM. To delineate the functional phenotype of melanoma SCs-activated macrophages, we assessed their impact on NK cell cytotoxicity in vitro. Surprisingly, SCs-treated macrophages failed to influence NK cell cytotoxic activity compared to controls, indicating that despite expressing pro-inflammatory mediators, they lack the capacity to mount an effective immune response. This study enhances our comprehension of how melanoma SCs can modulate macrophage biology, highlighting their potential to induce a mixed M1/M2 phenotype.

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A human immune organoid model of CRISPR-induced hematological malignancies

T. Bilich[1], V. Shankar[1], A. Long[1], C. Beppler[1], E. Sola[1], L. Kamalyan[1], M. M. Davis[1,2,3]
[1]Stanford - School of Medicine, Institute for Immunology, Transplantation, and Infection, Palo Alto, United States of America, [2]Howard Hughes Medical Institute, Stanford, United States of America, [3]Stanford - School of Medicine, Department of Microbiology and Immunology, Palo Alto, United States of America

In the past, studies on cancer immunology have benefited from technological advancements. In particular, organoids created vast opportunities to study the human immune system. Although animal models and cell culture techniques produced many important insights, they have not shed much light on what happens at the very moment when malignancies arise. In contrast, the organoid technology has the potential to fill this gap by providing a robust system resembling true-to-life interactions of the human immune system with malignant cells in tissues.

Here, we aim to explore the cancer immunosurveillance and immunoediting hypotheses using the CRISPR/Cas9 system to induce malignant transformation and thereby mimic hematological malignancies in human tonsil and spleen organoids. CRISPR/Cas9 ribonucleoproteins were delivered into primary lymphocytes by electroporation, introducing genetic alterations such as knockout of the tumor suppressor P53 or CALR5ins mutation. To benefit from the genetically and phenotypically robust organoid system, we co-cultured the transformed cells with their respective autologous origin in organoid format.

To assess whether the transformed cells resemble known cancer biology, we analyzed metabolic features

described for the respective malignancies within the altered cells compared to scramble control treated cells of the same donor. Comparing T and B cells with induced CARL5ins mutation versus scramble control CRISPR, we assessed proliferation by EdU click-it assay, glucose uptake (2-NBDG), reactive oxygen species (ROS), and STAT phosphorylation. We observed an overall higher proliferation capacity in CALR5ins cells, with $CD8^+$ T cells showing the strongest tendency for increased proliferation. $CD4^+$ CALR5ins T cells had a significantly lower uptake of glucose, whereas B cells tended to have increased 2-NBDG uptake. A trend towards higher ROS production in CALR5ins B and T cells was observed, however not reaching significance. Finally, CALR5ins T cells showed a significantly reduced STAT3(pS727) activation. After showing the feasibility of using CRISPR editing to induce malignant transformation in primary human cells, we investigated the biology of induced BCR-ABL and IgH-Myc translocations in primary B cells. For BCR-ABL however, the transformed B cells did not have any proliferative advantage over control B cells, which is likely due to the translocation affecting pre-B cells instead of mature B cells as used in our experiments. Thus, currently ongoing experiments are focused on mimicking Burkitt Lymphoma in mature B cells.

Together, combining organoids with the CRISPR technology allows us to study how malignant transformation affects anti-cancer immune responses at the very time point of alteration. In combination, these technologies are a powerful tool with the potential to advance our knowledge of cancer immunology, thus providing valuable insights for the improvement of cancer immunotherapy and ultimately patient outcomes.

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HLA binding capacity of human papillomavirus-derived peptides is dependent on the pH in the tumor microenvironment

M. Bonsack[1,2], A. Kummer[1,2,3], M. R. Schrimpf[1,3], S. Diehl[1,3], R. Köhler[1,2], J. P. Becker[1,2], A. B. Riemer[1,2]
[1]German Cancer Research Center (DKFZ), Immunotherapy & Immunoprevention, Heidelberg, Germany, [2]German Center for Infection Research (DZIF), partner site Heidelberg, Molecular Vaccine Design, Heidelberg, Germany, [3]Heidelberg University, Faculty of Biosciences, Heidelberg, Germany

Therapeutic human papillomavirus (HPV) vaccines in clinical trials have not yet entered the market because of limited clinical efficacy. One direction for improvement is to study the impact of an immunosuppressive tumor microenvironment (TME). The TME acidifies upon increased tumor cell glycolysis. The pH can drop below 6.0, impairing T cell activation and functionality. Effector T cells are normally activated by recognition of a cognate HLA-presented peptide on the tumor cell surface. However, acidic pH influences the stability of peptide:HLA class I complexes allowing peptide dissociation and exchange which could reduce the repertoire of targetable epitopes.
We investigate the influence of acidification on HLA-presentation of HPV16-derived peptides and resulting implications for immunotherapies against HPV16-associated cancers. Using in vitro cellular competitive binding assays, the HLA binding affinity of known HPV16 E6-/E7-derived HLA-A*02:01 ligands is systematically assessed under a pH gradient from 7.5 to 5. This allows to classify subsets of acid-labile and acid-stable peptide:HLA complexes based on reduced and robust binding affinity, respectively. Both subsets are analyzed for sequence motifs and peptide properties predictive for pH-dependent HLA binding affinity. So far, a set of 35 HPV16 E6-/E7-derived peptides with characterized HLA-A2 affinity has been investigated

at a pH of 7.5, 7.0, 6.5, 6.0, 5.5 and 5.0 in two independent experiments. Eight previously characterized nonbinders retained their inability to bind HLA-A2. Eleven peptides showed stable HLA binding affinity, among them were 8 of 10 strong binders (IC_{50}: <5µM) and 2 of 3 intermediate binders (IC_{50}: 5-15µM). For ten peptides, the binding affinity decreased upon acidification and half of them completely lost HLA binding at pH 5.0. Five peptides displayed no linear binding affinity changes. The binding affinity of a single peptide increased from weak (IC_{50}: 43µM) to intermediate (IC_{50}: 12µM) upon pH lowering. Comparison of the peptide sequences revealed that acid-stable peptides overall contained less charged residues than acid-sensitive peptides, specifically in the auxiliary anchor positions. Using immunopeptidomics, this observation was confirmed in the endogenous peptide repertoire of a HLA-$A2^+$ lymphoblastic B cell line.
Upcoming experiments will investigate the pH-dependent peptide binding of other major HLA types. Further, functional T cell assays will compare the presentation on target cells, and the T cell recognition of HPV-derived epitopes in differentially acidified milieu.
The identification of pH-stable HLA ligands improves the selection of potent epitopes for effective targeted immunotherapies. Furthermore, this approach could reveal a general peptide sequence motif of acid-stability that aids predicting robust therapeutic targets in other indications.

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Immune-targeting of quiescent cancer stem cells

M. S. Brennan[1], M. Lehander[1], Z. Földvári[2,3], N. Chandler[2,3], T. Tran[4], A. Titov[2,3], T. Sylaidi[1], K. Högstrand[1], R. Chand Bollineni[2,3], F. Lund-Johansen[4,5], R. Braathen[4], J. Olweus[2,3], P. Woll[1], S. E. Jacobsen[1,6]
[1]Center for Hematology and Regenerative Medicine, Department of Medicine Huddinge, Karolinska Institutet, Stockholm, Sweden, [2]Department of Cancer Immunology, Institute for Cancer Research, Oslo University Hospital Radiumhospitalet, Oslo, Norway, [3]K.G. Jebsen Center for Cancer Immunotherapy, Institute of Clinical Medicine, University of Oslo, Olso, Norway, [4]Department of Immunology, Institute of Clinical Medicine, Oslo University Hospital Rikshospitalet, Oslo, Norway, [5]PRIMA Centre of Excellence, University of Oslo, Oslo, Norway, [6]MRC Weatherall Institute of Molecular Medicine, MRC Molecular Haematology Unit, University of Oxford, Oxford, United Kingdom

The cancer stem cell (CSC) model suggests that cancers depend on continuous replenishment from rare and distinct CSCs. These cells largely lay dormant, dividing only when needed to replenish malignant cells. Since most conventional cancer treatments preferentially target rapidly dividing cells, CSCs escape conventional treatments and are thought to be a source of relapse. Elimination of CSCs is therefore necessary and potentially sufficient for cure. CSCs have been best studied in chronic blood cancers. However, the cellular identity of leukemic-SCs (LSCs) remains a challenge. Thus, these cells are studied using transplantation assays (often in immune-compromised mice), disrupting their cell cycle. We aim to study LSCs in vivo, in unperturbed conditions to preserve their quiescent and low immunogenic state thereby better mimicking patient conditions. To this end we are engineering CD8 TCR-T cells that target cells expressing mouse MPL. MPL is the receptor for thrombopoietin, and its expression is predictive of LSC capabilities in the BCR-ABL1 mouse model of chronic myeloid leukaemia (CML). Additionally, it is a very attractive therapeutic target as it is only expressed in few blood cell populations. To generate MPL-specific mouse CD8 TCR-T cells we will vaccinate Mpl knock-out mice with MPL Vaccibody adenovirus and boost with MPL lentivirus. The Vaccibody construct has been designed with the Mpl-sequence tethered to the MIP1a chemokine to

enhance CD8 response, and we have shown that it is successfully secreted after expression in HEK293E cells. To isolate candidate T cells we will generate tetramers using MPL peptides presented on C57BL/6 MHC-I that we have identified by immunoprecipitation of MHC-I molecules from EL4 cells overexpressing MPL and mass spectrometry. Tetramer-stained T cells from lymphoid organs from vaccinated mice will be sorted and sequenced using the 10x genomics sequencing platform. MPL-specific TCR-T cells will then be used to target MPL expressing LSCs in the BCR-ABL1 driven CML mouse model to target and eliminate quiescent LSCs in their native environment. This model will allow us, for the first time, to ask if BCR-ABL1 driven CML is strictly dependent on distinct LSCs by monitoring and characterising remaining cells after TCR-therapy. Furthermore, if LSCs remain after treatment we aim to explore if they are the cellular source of relapse and what cellular processes we can exploit to enhance targeting of LSCs by MPL-TCR-T cells. These studies seek to provide novel insights to fundamental cell biology and clinically important questions about the importance of LSCs in CML. Immune-targeting of otherwise therapy-resistant and dormant CSCs could reduce relapses and lead to higher cure rates, in CML as well as other haematological malignancies and solid tumours.

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Human 3D in vitro models for the assessment of Cancer Immunotherapy Mode of Action

F. Cremasco[1], K. Apostolopoulou[2], H. Gomez[2], L. Kunz[2], M. Kiratzis[2], S. Colombetti[2]
[1]Roche Glycart AG, Pharmacology, Schlieren, Switzerland, [2]Roche Glycart AG, Oncology Discovery Pharmacology, Schlieren, Switzerland

Cancer Immunotherapy (CIT) strategies have enabled significant breakthroughs in cancer treatment. However, their development remains extremely challenging, also due to intrinsic limitations of currently available preclinical experimental approaches in vivo and specifically in vitro. The next generation of CIT-drugs will require a more dynamic and content-rich analysis allowing for mid-throughput readouts, in the most physiological human tumor microenvironment (TME) possible in vitro.
To this end we utilized the microfluidics OrganoPlate 3-lane 40 system by MIMETAS, to establish two distinct, fully human, imaging approaches for the screening of next generation CIT-drug candidates.
The first system is a 3D in vitro imaging model which enables the dynamic visualization and characterization of interactions between cancer cells, immune cells, stromal cells, and extracellular matrix (ECM), recapitulating the human TME organization and immune cell dynamics. After carefully cross-validating our platform against in vivo and 2D in vitro analyses, we now employ this new system to shed light on the Mode of Action (MoA) of immune cell engagers, such as Glofitamab (CD20-TCB).
The second system is a fully human mid-throughput 3D in vitro screening system for imaging and quantifying immune cell trafficking and infiltration to tumors in response to CIT-drugs. It combines endothelial tubules, peripheral immune cells, solid or liquid tumors, ECM and tumor-resident immune cells. This model provides insights on the impact on immune cell infiltration of immunomodulation and T cell redirection CIT drug candidates.
While using those systems at this moment, as a next step towards more physiological 3D in vitro models, we are implementing the use of Patient-derived Tumor Organoids (PDTOs) co-cultures with stromal components (Cancer associated fibroblasts, immune cells) derived from the same patient.
In summary, our next generation in vitro models allow for mid-throughput high-content screening of drug

candidates, in vitro evaluation of combination strategies and a mechanistic understanding of their MoA in a fully human 3D in vitro system and are a valuable addition to existing preclinical models with their ability to mimic physiological immune cell dynamics, such as immunological synapse formation and trafficking. Consequently, these platforms support now, and will support even better next generation CIT-drug development.

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Significant involvement of mast cells in the bladder cancer development

M. Degoricija[1], L. Franković[2], I. Gabela[2], K. Vilović[3,4], T. Feyerabend[5], H.-R. Rodewald[5], J. Korać Prlić[2]
[1]University of Split, School of Medicine, Department of Medical Chemistry and Biochemistry, Split, Croatia, [2]University of Split School of Medicine, Laboratory for Cancer Reaserch, Department of Immunology and Medical Genetics, Split, Croatia, [3]University of Split School of Medicine, Split, Croatia, [4]University Hospital Split; School of Medicine, Department of Pathology, Split, Croatia, [5]German Cancer Research Center, Division of Cellular Immunology, Heidelberg, Germany

Bladder cancer stands as the fourth most prevalent cancer among men in Europe, with the tumor microenvironment playing a crucial role in its development and progression. Immunotherapy emerges as a promising avenue for bladder cancer treatment, as evidenced by the successful application of immune checkpoint inhibitors in cases of muscle-invasive bladder cancer. Mast cells, integral to the tumor immune milieu, exhibit a dualistic influence on cancer, their impact being both cancer- and stage-specific. Despite their significance, the mechanisms underlying how mast cells shape bladder cancer formation and progression remain unclear. To explore the role of mast cells in bladder cancer, we utilized a prevalent preclinical mouse model induced by the carcinogen N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN). This model closely mimics the human basal-like bladder cancer subtype, sharing significant similarities in histopathology and a notable mutational burden, particularly the Trp53 mutation. In our study, we delved into the modulation of the bladder cancer microenvironment by analyzing mast cell-deficient mice ($Cpa3^{Cre/+}$), conducting pathohistological examinations and gene expression profiling on BBN-treated bladder specimens. Our findings unveil a protective role played by mast cells in the development of bladder cancer through their modulation of the tumor microenvironment.

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Impact of eosinophils on bladder cancer development

M. Degoricija[1], I. Gabela[2], L. Franković[2], K. Vilović[3,4], J. Terzić[2], J. Korać Prlić[2]
[1]University of Split School of Medicine, Department of Medical Chemistry and Biochemistry, Split, Croatia, [2]University of Split School of Medicine, Laboratory for Cancer Reaserch, Department of Immunology and Medical Genetics, Split, Croatia, [3]School of Medicine, University of Sp, Split, Croatia, [4]University Hospital Split, Department of Pathology, Split, Croatia

Urothelial carcinoma stands as the predominant histological variant of bladder cancer and ranks as the ninth most prevalent malignancy globally. The immunogenic nature of muscle-invasive urothelial carcinoma renders it responsive to immune checkpoint inhibitors. Tumor-infiltrating immune cells play pivotal roles in the tumor microenvironment, serving both prognostic and predictive functions in cancer. The potential for

pharmacological modulation of the tumor microenvironment holds considerable promise in cancer therapy. Eosinophils, recognized for their contributions to tissue repair and normalization of tumor vasculature, play a significant role in shaping the tumor microenvironment. However, their specific impact on bladder cancer remains enigmatic. The administration of N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) to mice induces tumor growth resembling the characteristics of the human basal-like subtype of urothelial carcinoma. This mouse model is widely acknowledged for its histological resemblance and high mutational burden, making it representative of human bladder cancer. To investigate the role of eosinophils in the bladder cancer microenvironment, we employed eosinophil-deficient male mice (ΔdblGATA1). Carcinogenic changes in bladder tissue were elucidated through pathohistological analysis, complemented by gene expression profiling of BBN-treated bladder specimens. Our findings unveil a protective role of eosinophils in the development of bladder cancer, emphasizing their significance as integral constituents of the tumor microenvironment with a positive impact on bladder cancer progression.

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Anti-CD39 (TTX-30) treatment activates CD39$^+$/CD103$^+$/CD8$^+$ tissue resident T cells in lung tumor tissue

S. Dehmel[1], T. Bargmann[1], L. Stowasser[1], S. Konzok[1], C. Werlein[2], P. Zardo[3], D. Jonigk[2], H.-G. Fieguth[4], K. Sewald[1], A. Braun[1]
[1]Fraunhofer Institute for Toxicology and Experimental Medicine ITEM, Preclinical Pharmacology and Toxicology, Hannover, Germany, [2]Hannover Medical School, Institute of Pathology, Hannover, Germany, [3]Hannover Medical School, Cardiac, Thoracic, Transplantation and Vascular Surgery, Hannover, Germany, [4]Klinikum Siloah and Klinikum Nordstadt, Pathology, Hannover, Germany

A high infiltration of CD39$^+$/CD103$^+$/CD8$^+$ resident T cells in solid tumors is associated with improved response to immunotherapies and overall survival rates. CD39 is an Ecto-ATPase which contributes to an enzymatic cascade converting ATP into adenosine, thus shaping an immunosuppressive tumor microenvironment (TME). To counteract this, the therapeutic anti-CD39 antibody TTX-30 was developed to restore the TME back to an inflammatory state. Here, we aim to evaluate the effectiveness and potential side effects of TTX-30 on lung cancer by analyzing patient-specific immune responses using precision-cut lung tissue slices.

Primary tumor and tumor far tissue was prepared from human lung resections. Tumor lung tissue was distinguished from tumor far tissue by macroscopic and microscopic analysis. Tumor far lung tissue viability was assessed by metabolic activity using WST-1 assay and plasma membrane integrity by Lactate Dehydrogenase Release Assay (LDH) five days after stimulation with 0.5-5 µg/mL TTX-30. After treatment with TTX-30 (0.5 µg/mL) or anti-PD1 antibody Nivolumab (50µM), we leveraged histological profiling, flow cytometry of T cell subsets, eATP levels and cytokine analysis in the culture supernatant to understand how TTX-30 impacts immune regulation within TME after 30h ex vivo.

Tumor slices contained significantly higher frequencies of CD8$^+$/PD-1$^+$ (1.6-fold) and CD8$^+$/CD39$^+$/CD103$^+$ (2.2-fold) T cells. At a concentration of 0.5 µg/mL, TTX-30 effectively inhibited CD39 activity, evidenced by a 4.3-fold increase in eATP levels in the culture supernatant of tumor-far tissue. A non-cytotoxic dose of 5µg/mL TTX-30 was defined in tumor far tissue by maximum reduction of 15% of metabolic activity and

maximum increase of 10 % LDH release in comparison to the medium control. In tumor tissue, 0.5 µg/mL TTX-30 significantly increased activation of $CD8^+/CD103^+/CD39^+$ cells, denoted by an increase $CD107a^+$ expression (1.3-fold change in frequency). Additionally, $CD3^+$ T cells upregulated the early activation marker CD137 after treatment (MFI 1.4-fold change in frequency) in two of four donors. As treatment reference, nivolumab treated tumor tissue induced an increase in the activation marker CD107a on $CD8^+$ T cells (1.4-fold change in frequency) in one of four donors. However, all donors displayed significantly higher levels of IFN-gamma (12-fold) and lowered levels of TGFβ2 (1.5-fold) compared to the medium control. Taken together, specific targeting of anti-tumor reactive $CD39^+/CD103^+/CD8^+$ with therapeutic anti-CD39 in lung tumor tissue may induce T cell activation at an effective and non-cytotoxic concentration of 0.5 µg/ml. In addition, we demonstrated the added value of viable patient-derived lung tumor tissue, providing data on the toxicity and efficacy of immunotherapies in context of the TME.

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Unveiling the Intricacies of PD-1/PD-L1 Interaction: Insights into Intercellular Exchanges in Tumor-Immune Cell Crosstalk

P.-H. Desimpel, B. Van den Eynde, J. Zhu
De Duve Insititute, Ludwig Institute for Cancer Research, Brussels, Belgium

In the tumor microenvironment, the expression of Programmed Death-Ligand 1 (PD-L1) by cancer cells is a critical indicator of immunosuppression. Its interaction with PD-1 on activated Cytotoxic T Lymphocytes (CTLs) adversely regulates the latter, leading to T cell exhaustion and compromising the anti-tumor immune response. Targeting the PD-1/PD-L1 immune checkpoints has become a focal point for numerous FDA-approved cancer immunotherapies. Our research digs into a comprehensive exploration of the dynamics governing PD-1/PD-L1 interactions and the behaviour of these proteins during co-culture assays involving T cells and tumor cells.

In our experiments, co-cultures between a human melanoma tumor cell line (Dauv MEL.A-1) and $CD8^+$ T cells recognizing the MHC-I-restricted MUM3 antigen presented by Dauv MEL.A-1 cells (anti-MUM3 CTLs) surprisingly revealed the appearance (by flow cytometry) of PD-L1 on the surface of anti-MUM3 CTLs, only when their target tumor cells expressed PD-L1.

Similar intriguing findings emerged from co-culture assays involving Dauv Mel.A-1 tumor cells and 2D3 Jurkat cells ($CD8^+$). Our investigations illustrated that the detection of PD-L1 on T cells required PD-1/PD-L1 interaction, necessitated close cell-to-cell contact, and was independent of TCR/MHC-I interaction. Importantly, our data established that the detection of PD-L1 on T cells arise from an intercellular transfer of PD-L1 from tumor cells through a phenomenon known as trogocytosis.

Notably, our study uncovered not only the presence of PD-L1 from tumor cells on the surface of T cells but also the occurrence of PD-1, originating from T cells, on the surface of tumor cells after co-culture assays.

These data unveil, for the first time, the existence of an intercellular protein exchange involving PD-1 and PD-L1 between T cells and tumor cells, potentially influencing our comprehension of anti-tumor immunity

and the mechanisms underpinning PD-1/PD-L1-targeting immunotherapies. Our forthcoming investigations aim to unravel the functional implications of this protein exchange for both T cells and tumor cells.

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Direct detection of the Zap70/LAT complex in the tumor microenvironment with in situ proximity ligation technology

H. Elofsson, D. Raykova, F. Oliveira, A. Zieba Wicher
Navinci Diagnostics, Uppsala, Sweden

Immunotherapy has emerged as a revolutionary approach in cancer treatment, harnessing the immune system's ability to target and eliminate malignant cells. Pivotal to the success of immunotherapeutic strategies is the activation and proliferation of T cells, a process involving a complex network of signaling molecules. Among these, the interaction between the Zap70 and LAT proteins stands out as an important regulator of T lymphocyte function. Upon T cell receptor (TCR) activation, Zap70 is recruited to the TCR-associated CD3 complex and phosphorylated. Activated Zap70 phosphorylates LAT, leading to the formation of a Zap70/LAT complex. The complex coordinates the activation of various signaling pathways, culminating in T cell activation, proliferation, and effector functions. To go beyond observing the expression patterns of Zap70 and LAT separately, which does not convey information about their functional states, it is desirable to detect the interaction directly. To this end, we employed the Naveni® in situ proximity ligation technology (isPLA), which allows the detection of protein interplay based on proximal Navenibody™ binding to antibodies against Zap70 and LAT. As long as the detected epitopes are within interaction range (10-40 nm), signal is generated and amplified. Signal is then detectable by either brightfield or fluorescent microscopy. We performed a proof-of-principle stain of the Zap70/LAT interaction in tonsil FFPE tissue with both chromogenic and fluorescent readout. We then detected the complex in breast cancer FFPE tissue and in parallel performed cytokeratin and CD3 co-staining by immunofluorescence, to visualize the tumor tissue and its infiltration by T cells, respectively. In consecutive breast cancer tissue sections, we used isPLA to detect the PD1/PDL1 interaction, demonstrating the activity of this inhibitory pathway with high relevance in immune checkpoint inhibition therapy. Activated PD1/PDL1 and the Zap70/LAT complexes were observed in mostly overlapping tissue regions. The possibility to directly observe the Zap70/LAT complex in cancer tissues along with other relevant markers of the tumor microenvironment contributes to better understanding of the functional roles of these proteins and makes them interesting as a panel of biomarkers that may comprise a specific immune signature in cancer. Such signatures may be used for patient stratification and treatment selection.

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ZBTB18 hinders cytokine expression to impair microglia and macrophage recruitment and the establishment of a tumor-supportive microenvironment in glioblastoma

R. Ferrarese[1], K. Joseph[1], G. Andrieux[2], I. V. Haase[1], F. Zanon[1], E. Kling[1], A. Izzo[3], E. Corrales[2], M. Schwabenland[4], M. Prinz[4], V. Ravi[3], M. Börries[2], D. H. Heiland[3], M. S. Carro[1]
[1]Freiburg University Medical Center, Neurosurgery, Freiburg, Germany, [2]Freiburg University Medical Center, Institute of Medical Bioinformatics and Systems Medicine, Freiburg, Germany, [3]Freiburg University

Medical Center, Freiburg, Germany, [4]Freiburg University Medical Center, Neuropathology, Freiburg, Germany

Glioma associated macrophages/microglia (GAMs) are massively recruited to the tumor site where they commit to a tumor promoting phenotype, driving glioblastoma (GBM) progression. GAMs secrete several factors that facilitate GBM proliferation and invasion, and prevent an effective immune response against the tumor. Here, we investigate how the GBM tumor suppressor ZBTB18 affects GAMs and ultimately shapes the tumor microenvironment.
We used gene expression analyses of primary stem-like GBM cell lines (GSC) and immortalized microglia, immunolocalization of GAM markers in GSC-derived mouse xenografts, and in vitro functional assays of microglia, to demonstrate that ZBTB18, a transcriptional repressor with tumor suppressive function in GBM, impairs the production of key cytokines, which function as chemoattractant for GAMs. Consistently, we observed a reduced migration of GAMs when ZBTB18 is expressed by GBM cells, both in cell culture and in in vivo experiments. Moreover, RNA sequencing analysis delineated how the presence of ZBTB18 in GBM cells alters the commitment of conditioned microglia, suggesting the loss of the immune-suppressive phenotype and the acquisition of pro-inflammatory features.
Overall, we show that ZBTB18 expression in GBM cells affects the tumor microenvironment by halting GAM recruitment and their tumor supportive commitment. Additionally, GAMs exposed to ZBTB18-expressing GBM cells acquire a more pro-inflammatory phenotype. Thus, therapeutic approaches to increase ZBTB18 expression in tumor cells could represent a promising adjuvant to immune therapy in GBM.

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PD1-targeted delivery of an IL2 variant boosts effector responses and antitumor immunity in ex vivo models of human lung cancer

I. Fusi*[1], C. Serger*[1], P. Herzig[1], L. Codarri Deak[2], C. Klein[2], P. Umaña[2], M. Natoli[1], A. Zippelius[1]
[1]University Hospital Basel and University of Basel, Department of Biomedicine, Basel, Switzerland, [2]Roche Pharma Research & Early Development, Cancer Immunotherapy Discovery, Oncology Discovery & Translational Area, Zurich, Switzerland

*Contributed equally

Novel antibody-cytokine fusion proteins are being developed as next-generation cancer immunotherapeutics, aiming to deliver activation signals to targeted immune populations. Among these, PD1-IL2v - an engineered IL2 variant lacking CD25 binding, fused to a high affinity PD-1 blocking antibody - has shown concrete promise in mouse tumor models, by delivering an IL2 signal in cis to $PD1^+$ cells while concomitantly blocking the PD1-PDL1 axis. In this work, we aim to elucidate the mechanism of action of PD1-IL2v in human tumors. Utilising in vitro model systems including PBMCs, in vitro generated exhausted T cells and patient-derived tumor infiltrating immune cells, we observed that PD1-IL2v significantly increased tumor cell killing, T cell activation and interferon-γ (IFN-γ) secretion, compared to anti-PD1 alone or controls. We next dissected PD1-IL2v mediated responses by performing high-parameter spectral flow cytometry and scRNAseq on a patient-derived tumor fragment (PDTF) platform, which is predictive of clinical responses to anti-PD1. Our analyses on PDTFs show that PD1-IL2v leads to the expansion of proliferating T cells, cytotoxic $GZMB^+PRF1^+CD8^+$ T cells and $GZMB^+CXCL13^+$ $CD4^+$ T follicular helper (Tfh)

cells. Furthermore, PD1-IL2v treatment significantly upregulated CXCR6 on PD1$^+$ (GranzymeB$^+$) intratumoral CD8$^+$ T cells and endowed CD8$^+$ T cells with increased migration properties towards CXCL16, in vitro. On the other hand, PD1-IL2v promoted the expression of CXCL13 on Tfh cells. In conclusion, PD1-targeted cis-delivery of an IL2 variant can induce a potent T cell antitumor response mediated by IFN-γ and GranzymeB, while at the same time triggering the expression of chemotactic receptors on both CD8$^+$ and CD4$^+$ T cells, which downstream may facilitate their interaction with other immune cell populations, including dendritic cells and B cells, towards an effective anti-tumor immune response.

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Tumoral interferon beta induces an immune-stimulatory phenotype in tumor-associated macrophages in melanoma brain metastases

J. Gellert[1,2,3], D. A. Agardy[1,2,3,4], S. Kumar[5], K. Jähne[1,2,3], L. Bunse[1,2,3,6], M. A. Davies[5], M. Platten[1,2,3,6,7,8], T. Bunse[1,2,3]

[1]German Cancer Research Center (DKFZ), Clinical Cooperation Unit (CCU) Neuroimmunology and Brain Tumor Immunology, Heidelberg, Germany, [2]German Cancer Consortium (DKTK), DKFZ, Core Center Heidelberg, Heidelberg, Germany, [3]Department of Neurology, Medical Faculty Mannheim, Mannheim Center for Translational Neuroscience (MCTN), Heidelberg University, Mannheim, Germany, [4]Faculty of Bioscience, Heidelberg University, Heidelberg, Germany, [5]Department of Melanoma Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, United States of America, [6]DKFZ Hector Cancer Institute at the University Medical Center Mannheim, Mannheim, Germany, [7]Immune Monitoring Unit, National Center for Tumor Diseases (NCT), NCT Heidelberg, a partnership between DKFZ and University Hospital Heidelberg, Heidelberg, Germany, [8]Helmholtz Institute for Translational Oncology Mainz (HI-TRON Mainz) – A Helmholtz Institute of the DKFZ, Mainz, Germany

Type I interferons (IFN) such as IFNβ and IFNα are pro-inflammatory cytokines involved in antiviral and antitumor immune responses. Increasing evidence indicates that the success of several anticancer therapies, including radio- and chemotherapy, relies on the immune-stimulatory effects of type I IFNs on innate and adaptive immune cells in the tumor microenvironment (TME). Tumor-associated macrophages (TAMs) are one of the most abundant innate immune cells in the TME of melanoma brain metastases (MBMs) and can exert potent immune-suppressive functions compromising effective antitumor immune responses.

Here, we investigate the potential of tumoral type I IFNs to re-educate TAMs towards an antitumor M1-like polarization state to enhance the efficacy of immunogenic anticancer therapies in MBMs. In order to minimize confounding effects of tumor-cell death-associated antigen release and other immune-modulatory cytokines associated with type I IFN-dependent anticancer therapies, we make use of an inducible IFNβ-overexpressing melanoma mouse model. In this murine MBM model, tumoral IFNβ markedly impaired tumor growth and TAMs from IFNβ-expressing tumors exhibited a decreased expression of CD206, a widely used marker for immune-regulatory M2-like macrophages. Functional and transcriptomic analyses of tumoral IFNβ-treated bone marrow-derived macrophages revealed an immune-stimulatory M1-like phenotype characterized by expression of a set of anti-tumor immunity genes. We next sought to validate this myeloid type I IFN-response signature gene set in a recently published bulk RNA

sequencing dataset of treatment-naïve and previously irradiated MBM patient samples. Following radiotherapy, over 40 percent of MBMs showed an upregulation of the myeloid type I IFN-response signature which associated with a higher M1/M2-like TAM ratio and increased overall survival.

Taken together, our work demonstrates the immune-stimulatory capacity of tumoral IFNβ in the context of type I IFN-inducing anticancer therapies in MBM by re-polarizing TAMs towards a pro-inflammatory M1-like phenotype.

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CD109 as a target for lung cancer and tumor-associated macrophages

T. Haesen[1,2], M. Schinke[1,3], V. Neuhaus[1], S. Dehmel[1], M. Ackermann[1,3], P. Laakkonen[4,5], A. Braun[1], K. Sewald[1]
[1]Fraunhofer Institute for Toxicology and Experimental Medicine ITEM, Hannover, Germany, [2]University of Bielefeld, Bielefeld, Germany, [3]Hannover Medical School, Hannover, Germany, [4]Translational Cancer Medicine Research Program, Faculty of Medicine, University of Helsinki, Helsinki, Finland, [5]Laboratory Animal Centre, Helsinki Institute of Life Science (HiLIFE), University of Helsinki, Helsinki, Finland

Lung cancer remains a leading cause for cancer-related deaths worldwide, and the need for novel targeted therapies persists. The glycoprotein CD109 has been shown to contribute to tumorigenesis and presents an appealing target for anti-cancer therapy. We hypothesize that CD109 supports the M2 macrophage phenotype, and thereby promotes a pro-tumorous micro-environment. Herein, we apply a primary human ex vivo setting to reveal the expression of CD109 on tumor-associated macrophages (TAM) and explore the use of RNA therapeutics in vitro. Using confocal microscopy, the expression of CD109 could be visualized in ex vivo primary human lung tissue (i.e. precision-cut (tumor) lung slices, PCLS/PCTLS). Utilizing flow cytometry (FC), CD109 expression was also determined on macrophages isolated from donor-paired tumor and non-tumorous tissue. Pro (M1)- and anti (M2)-inflammatory macrophages were differentiated from either monocytes or induced pluripotent stem cells (iPSC), and CD109 expression was confirmed by FC or whole transcriptome analysis. Comparison of tumor PCTLS and non-tumorous PCLS confirmed overexpression of CD109 in the tumor tissue, mainly showing surface expression on clusters of small cells as well as on larger single cell types. Additionally, a higher expression of CD109 on TAMs compared to non-tumoral macrophages (55.6% ± 0.1 vs. 16.4% ± 0.9 $CD45^{+}CD109^{+}$ cells, n = 2) was revealed. Moreover, CD109 was shown to be differentially expressed on in vitro differentiated macrophages, displaying a higher protein expression on monocyte-derived M2-like macrophages (median fluorescence intensity (MFI) fold change 2,8 ± 1.1, n = 4) and iPSC-derived M2a macrophages, compared to their M1-like phenotypes. Furthermore, effective CD109 downregulation was achieved using small interfering RNA (siRNA) in A549 cells (MFI fold change 0.21 ± 0.07, n = 5 and 96% gene knockdown, n = 1), in monocyte-derived M1 and M2 macrophages (MFI fold change 0.35 and 0.30 respectively, n =1) and in iPSC-derived macrophages with a M0, M1and M2a phenotype (MFI fold change 0.39 ± 0.02, 0.41 ± 0.05 and 0.50 ± 0.06 respectively, n = 2). Moreover, preliminary results showed an increased expression of co-stimulatory T-cell activator CD86 upon downregulation of CD109 in iPSC-derived macrophages with a M0, M1 and M2a phenotype (MFI fold change 1.32 ± 0.11, 1.39 ± 0.21 and 1.29 ± 0.04 respectively, n = 2). Our results confirm the overexpression of CD109 in tumor tissue and reveal an increased expression on ex vivo TAMs and in vitro M2-like macrophages. Moreover, a decrease in CD109 expression was achieved in vitro via siRNA-targeted

knockdown. Henceforth, the immunomodulatory effects of CD109 downregulation on cancer and macrophage functionality will be further investigated.

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Organization of virus-specific immune responses within the microenvironment of Merkel cell carcinoma determines patient response and survival upon checkpoint-inhibitor therapy - a spatially resolved single-cell analysis

M. Haist[1,2,3], Y. Tan[1,2], M. Matusiak[2], J. W. Hickey[1,2,4], S. Zimmer[5], H. Stege[3], S. Mitschke[5], P. Chu[2], B. Weidenthaler-Barth[3], F. Rogall[3], A. Delgado Gonzalez[1,2], M. A. Baertsch[1,6,7], S. Grabbe[3], G. Nolan[2]
[1]Stanford University School of Medicine, Department of Microbiology and Immunology, Stanford, United States of America, [2]Stanford University School of Medicine, Department of Pathology, Stanford, United States of America, [3]University Medical Center of the Johannes Gutenberg University Mainz, Department of Dermatology, Mainz, Germany, [4]Duke University, Department of Biomedical Engineering, Durham, United States of America, [5]University Medical Center of the Johannes Gutenberg University Mainz, Institute of Pathology, Mainz, Germany, [6]Department of Medicine V, Hematology, Oncology and Rheumatology, University of Heidelberg, Heidelberg, Germany, [7]German Cancer Research Center, Clinical Cooperation Unit Molecular Hematology/Oncology,, Heidelberg, Germany

Introduction
Merkel cell carcinoma (MCC) is a rare but aggressive neuroendocrine skin cancer, caused by the integration of Merkel cell polyomavirus (MCPyV), or chronic UV exposure. Immune-checkpoint blockade (ICB) is effective in advanced MCC, but 60% of patients show primary or acquired resistance to ICB. Our current understanding of anti-tumor immune responses in MCC is largely based on flow-cytometry, TCR-sequencing and single-cell transcriptomics that lack spatial information. Hence, critical aspects of the in-situ tumor ecosystem can only be inferred.
In order to understand how the spatial cellular organization within the TME is modulated by MCPyV and how it impacts responses to ICB, we conducted the first comprehensive multiplex protein imaging analysis of MCC which we integrated with matched RNAseq data.

Methods
We characterized the TME of 60 MCC patients with longitudinal follow-up using a 56-marker Co-Detection by Indexing (CODEX) panel on 6 tissue microarrays (TMA). After quality control (<1% unclassifiable cells) we retained data from 356 cores representing 7.9M single cells. Single-cell profiling and spatial analysis was performed using custom python and R scripts. Matched bulk RNAseq data were collected from consecutive TMA sections using laser-capture microdissection.

Results
We identified 29 major cell types (CT) within the MCC TME, encompassing cell lineages, functional states (i.e. T cell exhaustion and dysfunction) and receptor-ligand networks. While at the compositional level, we did not detect major changes in CT abundance between virus-positive (VP) and virus-negative (VN) MCC, we found that disease outcomes were differentially regulated depending on MCPyV status. In particular, VP patients with dense infiltrates of T cells, cDC1 and plasma cells were less likely to progress during ICB,

whereas survival outcomes in VN-patients were driven by the presence of macrophages and fibroblasts. Given the similarity in CT composition between VP and VN patients, we reasoned that the spatial organization of CT within the TME might drive differential roles in tumor control. Hence, we leveraged the spatial resolution of CODEX to identify 11 distinct functional units within which CTs are organized, termed cellular neighborhoods (CN). Using this approach, we found that cells within the TME of VN-patients were organized in fibroblast-enriched CN whereas a plasma-cell enriched CN was characteristic of VP-patients. Also, we identified a Follicle-CN and B cell priming zones as prerequisites for ICB response, whereas a vessel-enriched CN was associated with ICB failure.

Conclusions

In this study we show that high dimensional imaging of the MCC TME provides a framework to interrogate cellular interactions and tissue architecture in-situ at single-cell resolution. This framework allowed us to identify cellular and molecular predictors of ICB response, unique functional units organizing the TME and track changes occurring in the TME during tumor progression.

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Development of tissue resident memory T cells in pancreatic cancer

H. Heimans[1,2], T. Arakelian[1,2], L. Griffioen[1,2], N. Kragten[3], S. H. Van der Burg[1,2], K. Van Gisbergen[3,4], T. Van Hall[1,2]

[1]Leiden University Medical Center, Medical Oncology, Leiden, The Netherlands, [2]Oncode Institute, Leiden, The Netherlands, [3]Sanquin Research and Landsteiner Laboratory, Hematopoiesis, Amsterdam, The Netherlands, [4]Champalimaud Foundation, Cancer and Physiology, Lisbon, Portugal

Tissue resident memory (T_{rm}) T cells are a subset of memory $CD8^+$ T cells that have the capability to stably reside in tissues for prolonged periods of time. When settled in tissues, T_{rm} provide immediate protection against localized pathogens or cancer cells. The presence of T_{rm} in human tumors, characterized by CD103 and CD69 expression, correlates with good prognoses and therapy responses in various cancers, including pancreatic cancer. Despite the positive correlation, it is not yet understood how T_{rm} develop in cancers where the presence of chronic antigen stimulation can also induce T cell exhaustion. We aim to understand the relationship between T_{rm} and exhausted T cell populations.
Previously, we identified Hobit as a master regulator for T_{rm} development, distinguishing them from circulating memory T cell lineages. To investigate T_{rm} in in vivo tumor models of pancreatic cancer, we have created a Hobit-reporter mouse which allows specific tracing and manipulation of T_{rm}. Crossing the Hobit-reporter mice with OT-I mice enables us to track $CD8^+$ T cells specific for the model antigen ovalbumin (OVA) and use this in adoptive transfer settings. As a tumor model we used the pancreatic ductal adenocarcinoma (PDAC) cell line KPC3 that mimics the human PDAC architecture.
Ex vivo analysis of tumor infiltrating lymphocytes (TILs) from subcutaneous KPC3-OVA tumors showed strong OT-I cell infiltration of which a large proportion expressed Hobit. Residency marker CD69 was co-expressed with Hobit, whereas CD103 only partially overlapped. However, CD69 and CD103 expression was also found on non-Hobit-expressing cells. In concordance, in vitro culture experiments indicated that environmental factors such as TGF-β signaling can induce retention integrins, including CD103, independent from Hobit expression. Moreover, we found that clearance of antigen improved $Hobit^+$ T_{rm} formation,

without losing the residency markers CD69 and CD103. Adoptively transferred T cells that upregulated Hobit did express PD-1 but not the terminal exhaustion regulator TOX, while endogenously activated T cells expressed TOX. This implies that T_{rm} display an activated but not fully exhausted phenotype in tumors. These data suggest that Hobit expression imprints tissue residency upon clearance of antigen in the memory phase, but that the induction of functional retention integrins is induced by local environmental factors in the tissues.
Future experiments with our Hobit-reporter system can further unravel T_{rm}- versus exhaustion-developmental pathways.

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Characterization of the IL-33/ST2 axis between cancer cells, fibroblasts and immune cells

F. Hoffmann[1], L. Schmidleithner[1], N. Beumer[2], S. Materna-Reichelt[3], T. Hehlgans[1], M. Feuerer[1]
[1]Leibniz Institute for Immunotherapy, Immunology, Regensburg, Germany, [2]German Cancer Research Center (DKFZ), Division of Applied Bioinformatics, Heidelberg, Germany, [3]Fraunhofer Institute for Toxicology and Experimental Medicine (ITEM-R), Regensburg, Germany

'Interleukin-33' (IL-33), is a member of the IL-1 superfamily and plays a critical role in tissue homeostasis and in the tumor microenvironment. Under homeostatic conditions, IL-33 can be produced by fibroblasts. During inflammation, stress, and tissue damage, IL-33 is released by epithelial cells, endothelial cells, and fibroblastic cells. IL-33 serves as an alarmin, indicating damage and eliciting immune responses by activating various immune cells, such as Th2, ILC2, and tissue Treg cells which constitutively express the IL-33 receptor. The IL-33 receptor consists of the ubiquitously expressed co-receptor IL-1 receptor accessory protein (IL-1RAcP) and the unique 'suppression of tumorigenicity 2' domain (ST2), which is why the IL-33 receptor is widely known and referred to as ST2. Even though the IL-33/ST2 axis has been described to create a pro-metastatic microenvironment and to contribute to tumor progression, the role of IL-33 signaling in the tumor microenvironment is not yet fully understood. We aim to gain a better understanding of this crucial signaling pathway to target the IL-33/ST2 axis.

To this end, we investigated the interplay of the IL-33/ST2 axis in fibroblasts, T cells, and tumor cells by performing cell culture stimulation assays and bulk RNAseq. In this context, we screened compounds of the Selleck bioactive library and identified 5 compounds capable of inhibiting the IL-33/ST2 signaling pathway, providing a potential path forward to address the axis in cancer therapy. Interestingly, we observed that a functional ST2 receptor can be expressed by tumor cells themselves and that tumor cells can use IL-33 signaling to promote tumor cell proliferation and the release of certain chemokines.

Our data indicate that the IL-33/ST2 signaling cascade is far more complex than initially anticipated since tumor cells can both produce and respond to IL-33, fibroblasts can produce IL-33 and different immune cells express the ST2 receptor in a constitutive or inducible manner. This highlights the importance of better understanding the role of IL-33/ST2 signaling in the complex tumor microenvironment.

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Heterotypic CD8 T cell clusters isolated from clinical samples are distinct and enriched for antitumor activity

S. Ibáñez-Molero*[1], J. Veldman*[1], J. J. H. Traets[2], A. George[1], K. Hoefakker[1], S. M. Pack[1], L. Tas[1], P. Alóndiga-Mérida[1], B. van den Broek[3], R. Harkes[3], M. Nieuwland[4], M. van Baalen[5], E. Mul[6], S. Tol[6], J. B. A. G. Haanen[1], W. J. van Houdt[7], D. S. Peeper[1]

[1]Netherlands Cancer Institute, Oncode Institute, Division of Molecular Oncology and Immunology, Amsterdam, The Netherlands, [2]Netherlands Cancer Institute, Division of Tumor biology and Immunology, Amsterdam, The Netherlands, [3]Netherlands Cancer Institute, BioImaging Facility, Amsterdam, The Netherlands, [4]Netherlands Cancer Institute, Genomic Core Facility, Amsterdam, The Netherlands, [5]Netherlands Cancer Institute, Flow Cytometry Facility, Amsterdam, The Netherlands, [6]Sanquin, Flow Cytometry Facility, Amsterdam, The Netherlands, [7]Netherlands Cancer Institute, Department of Medical Oncology, Amsterdam, The Netherlands

*Contributed equally

Functional tumor-T cell interactions are dynamic and complex, underlying both cancer progression and therapy response. By genome-scale CRISPR-Cas9 screens we previously identified several targets in tumor cells, inhibition of which increases their susceptibility to T cell attack in vitro and in vivo. Similar screens in T cells identified genes whose inactivation decreased dysfunction while boosting tumor-killing potential. More recently, we set out to study functional tumor-T cell interactions in clinical cancer samples as emerging evidence suggests a correlation between CD8+ T cell-tumor cell proximity and immunotherapy response. For example, favorable responses to immune checkpoint inhibitors in melanoma are associated with higher densities of CD8+ tumor-infiltrating lymphocytes (TILs) close to tumor cells. In addition, T cell receptor (TCR) structural and functional avidity of CD8+ TILs correlates strongly with their activity against cancer cells. Together, these observations imply that direct interactions between cytotoxic T cells and tumor cells in the tumor microenvironment determine clinical outcome. However, it is unknown whether in clinical samples these interacting cells can also be directly captured together as functional clusters. To study this, we made use of several assay and sample types: (1) in vitro tumor-T cell co-cultures; (2) patient samples and (3) ex vivo assays. We found that in defined co-cultures, tumor antigen-recognizing T cells were commonly enriched over non-specific T cells in heterotypic clusters with tumor cells, prompting us to investigate whether such specific clusters could be isolated also from cancer specimens. From almost all human melanoma metastases analyzed, we were able to isolate heterotypic clusters, comprising CD8+ T cells interacting with one or more tumor cells and/or antigen presenting cells (APCs). These observations were validated by imaging flow cytometry. Upon ex vivo rapid expansion, CD8+ T cells from tumor cell clusters and APC clusters exerted 8-fold increased melanoma-killing activity over T cell singlets, which was associated with enhanced cytokine production. Using single cell sequencing, we observed that CD8+ T cells from clusters were enriched for tumor-reactive and exhausted gene signatures. Integration with TCR-sequencing showed increased clonality of clustered T cells, indicative of expansion upon antigen recognition. Together, these results demonstrate that tumor-reactive CD8+ T cells are enriched in functional clusters with tumor cells and/or APCs, and that they can be isolated and expanded from clinical samples. Being often excluded in cell sorting procedures, these distinct heterotypic CD8+ T cell clusters serve as valuable source amenable to deciphering functional tumor-immune cell interactions, while they

may also be therapeutically explored.

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SARS-CoV-2 specific T-cells in tumor infiltrating lymphocytes (TIL) from patients with epithelial cancer show MHC – restricted cross-reactivity against tissue antigens

J. Kamiki*[1], P. A. António*[1], S. Cascais[1], R. Eduardo[1], B. Marinheiro[1], A. Maia[2], E. de Sousa[1], L. Lordello[1], J. R. Lérias[1], C. M. Gorgulho[1], D. Ligeiro[1], M. Castillo-Martin[2], L. Zitvogel[3,4,5,6], M. Maeurer[1,7]
[1]Champalimaud Foundation, ImmunoTherapy/ImmunoSurgery, Lisboa, Portugal, [2]Champalimaud Foundation, Molecular and Experimental Pathology, Lisboa, Portugal, [3]Gustave Roussy Cancer Campus (GRCC), Clinicobiome, Villejuif Cedex, France, [4]Institut National de la Santé Et de la Recherche Médicale (INSERM) U1015, Equipe Labellisée - Ligue Nationale contre le Cancer, U1015, Villejuif, France, [5]Faculté de Médecine, Université Paris-Saclay, Gustave Roussy, Villejuif, France, [6]Center of Clinical Investigations in Biotherapies of Cancer (BIOTHERIS), Villejuif, France, [7]I Medizinische Klinik, Johannes Gutenberg University Mainz, Mainz, Germany

*Contributed equally

T-cells reacting to SARS-CoV-2 epitopes (not shared with SARS-CoV-1 epitopes) have been described in healthy individuals' blood prior to the SARS-CoV-2 pandemic and prior to vaccination. These T-cells have been reported to react to commensal antigens. In order to effectively contain viral infection, efficient tissue homing and T-cell fitness are important factors to facilitate viral clearance. Specific SARS-CoV-2 epitope reactivity directed against defined epitopes has not been reported in TIL from patients with cancer or in tissue-resident cells. We took advantage of TIL harvested from patients with epithelial malignancies (n =4) prior to summer of 2019, TIL obtained during the pandemic, yet prior to the SARS-CoV-2 vaccine rollout (n=3) which started in the end of 2020 in Portugal and TIL obtained posterior to the vaccination (n=3). 15-mer peptides from the Spike, Membrane, Nucleocapsid, ORF8, ORF10 and ORF3a from SARS-CoV-2 were screened for IFN-γ and IL-17 production. Peptide-specific production of both cytokines were identified prior to the pandemic with 177/187 epitopes recognized by IFN-γ production and 139/187 epitopes defined by IL-17 production, in at least one patient. In order to define potential mimicry candidates for epitopes recognized in the pre-pandemic period, we searched the Swiss-Prot database for protein homologies with the peptides. Among 25 mimicry epitopes, 3 candidates, restricted by HLA-A*0101, HLA-B*3501 and HLA-C*0701 were chosen for further analysis. Peptide specific oligoclonal TIL were established either with peptides provided from the spike protein or with the corresponding mimicry epitopes provided from WARS2, SNRNP35 and TNS1. Cross-reactivity was defined by IL-17 production against the SARS-CoV-2 epitopes and the non-mutant human protein. IHC was performed to select for recognition of autologous tissue cells expressing the potential cross-reactive tissue antigen. Th1 and Th17 cytokine production was detected in either TIL reactive to SARS-CoV-2 peptides or against the corresponding mimicry peptide. T-cell recognition was associated with protein expression, tested by IHC in the target cells and MHC restriction was confirmed by blocking mAbs. Deep TCR sequencing of SARS-CoV-2 or mimicry peptide specific TIL lines showed either shared or different TCRs, which were under the detection level or at very low frequency in the tumor tissue. These results show that cross-reactive T-cells are not only present in PBMCs in the pre-pandemic period, yet also in tissue/tumor- resident cells. The data show that molecular mimicry is an integral part of the cellular immune response and that the specific SARS-CoV-2 precursor T-cell frequency in

tissue is low based on TCR sequencing. Precursor T-cells reacting to SARS-CoV-2 in tissue from patients with cancer may aid to expand protective immune responses upon exposure to the pathogen.

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Loss of CD20 epitope through splicing and NONO regulation in Childhood B-acute lymphoblastic leukemia

M. Kermani[1], N. Ziegler[1], S. Maxeiner[1], F. Alt[1], C. Paret[1,2,3], J. Faber[1,2,3]
[1]University Medical Center of the Johannes Gutenberg-University Mainz, Department of Pediatric Hematology/Oncology, Center for Pediatric and Adolescent Medicine, Mainz, Germany, [2]University Cancer Center (UCT), University Medical Center of the Johannes Gutenberg-University Mainz, Mainz, Germany, [3]German Cancer Consortium (DKTK), site Frankfurt/Mainz, Germany, German Cancer Research Center (DKFZ), Heidelberg, Germany

CD20 is a B cell lineage–specific marker expressed by normal and leukemic B cells and targeted by several antibody immunotherapies. Nevertheless, the loss or absence of CD20 expression on B-ALL blasts presents a clinical challenge, reducing the effectiveness of treatments and driving resistance to anti-CD20 monoclonal antibodies (mAbs) and CAR-T cell immunotherapies. Antigen loss can derive from alternative splicing or downregulation of CD20, two processes that are regulated by RNA-binding proteins (RBPs). The RBP NONO, a non-POU domain-containing octamer-binding protein implicated in several cancers, has been shown to regulate the surface expression of CD20.

To analyze mechanisms leading to CD20 antigen loss, we analyzed the expression of the CD20 gene (MS4A1), MS4A1 isoforms, and NONO in pediatric B-ALL clinical samples. CRISPR/Cas9-mediated Gene knockout (KO) was employed to investigate the potential role of NONO in the regulation of CD20 expression.

In CD20-positive B-ALL blasts, our qRT-PCR results revealed a significant negative correlation between the mRNA expression of NONO and MS4A1. KO of NONO significantly increased the mRNA expression of CD20, but not of CD19. At the time of diagnosis, CD20 positive B-ALL blast exhibited two isoforms: the wild-type variant and a shorter isoform lacking the CD20 epitope recognized by the monoclonal antibodies. The isoforms were not affected by NONO KO.

The advent of anti-CD20 monoclonal antibodies, exemplified by rituximab, and CAR-T cells, has significantly advanced the treatment landscape for B-cell malignancies, particularly when combined with chemotherapy. In this study, we demonstrated that inhibiting the RNA-binding protein NONO, significantly increased the abundance of CD20 levels by increasing its RNA level but not by affecting the isoforms composition. It is conceivable that the deregulation of NONO in B-ALL significantly impacts the abundance of B cell markers that are immunotherapeutic targets. Moreover, CD20 splice variants that are already present at diagnosis can further impair the recognition of the blasts by mAbs.

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Histone modification for the epigenetic interference at co-stimulatory and co-inhibitory factors in immunological synapse between T cells and glioblastoma cells

H. G. Kim, Y. Z. Kim
Sungkyunkwan University Samsung Changwon Hospital, Neuro Oncology / Neurosurgery, Changwon, Republic of Korea (South Korea)

BACKGROUND: This study aimed to identify the specific T cell co-stimulatory and co-inhibitory factors that play prognostic roles in patients with glioblastoma. Additionally, the unique histone H3 modification enzymes that regulate the expression levels of these specific co-stimulatory and co-inhibitory factors were investigated.
MATERIALS AND METHODS: The medical records of 84 patients newly diagnosed with glioblastoma at our institution from January 2006 to December 2020 were retrospectively reviewed. Immunohistochemical (IHC) staining for T cell co-stimulatory factors (CD27, CD28, CD137, OX40, and ICOS), T cell co-inhibitory factors (CTLA4, PD1, PD-L1, TIM3, and CD200R), and histone H3 lysine modification enzymes (MLL4, RIZ, EZH1, NSD2, KDM5c, JMJD1a, UTX, and JMJD5) was performed on archived paraffin-embedded tissues obtained by biopsy or resection. Quantitative real time-polymerase chain reaction (qRT-PCR) was performed for specific factors, which demonstrated causal relationships, in order to validate the findings of the IHC examinations.
RESULTS: The mean follow-up duration was 27.5 months (range, 4.1–43.5 months). During this period, 76 patients (90.5%) died, and the mean OS was 19.4 months (95% confidence interval, 16.3–20.9 months). Linear positive correlations were observed between the expression levels of CD28 and JMJD1a (R2 linear = 0.982) and those of CD137 and UTX (R2 linear = 1.528). Alternatively, significant negative correlations were observed between the expression levels of CTLA4 and RIZ (R2 linear = −1.746) and those of PD-L1 and EZH1 (R2 linear = −2.118); these relationships were confirmed by qRT-PCR. In the multivariate analysis, increased expression levels of CD28 (P = 0.042), and CD137 (P = 0.009), and decreased expression levels of CTLA4 (P = 0.003), PD-L1 (P = 0.020), and EZH1 (P = 0.040) were significantly associated with longer survival.
CONCLUSION: These findings suggest that the expression of certain T cell co-stimulatory factors, such as CD28 and CD 137, and co-inhibitory factors, such as CTLA4 and PD-L1 are associated with prognosis of glioblastoma patients.

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Identification of synergistic drug combinations including MEK inhibitor and agents modulating the tumor microenvironment in an electroporation-induced pancreatic cancer model

A. Konrad[1], T. Hägele[1], S. Zens[1], C. Vent[1], S. Gutierrez-Minguez[1], M. Hamberger[1], S. Baiker[1], A. Maes[1], C.-S. Leonhardt[2], O. Strobel[2], R.-F. Jackstadt[3], O. Politz[4], D. Zopf[4], D. Baumann[1], J. Mochayedi[1], R. Offringa[1]
[1]German Cancer Research Center, Molecular Oncology of Gastrointestinal Tumors, Heidelberg, Germany, [2]Heidelberg University Hospital, Department of General, Visceral and Transplantation Surgery, Heidelberg, Germany, [3]German Cancer Research Center, Cancer Progression and Metastasis, Heidelberg, Germany, [4]Bayer AG, Berlin, Germany

Pancreatic ductal adenocarcinoma (PDAC) is refractory to cytostatic and immuno-oncology therapeutic

strategies, leaving surgical resection of the tumor as the only treatment that can significantly prolong survival. We are therefore exploring optimized treatment strategies targeting cancer cells as well as infiltrating immune cells and the tumor microenvironment (TME) in an autochthonous mouse model for PDAC. Single focal tumors are induced through in vivo electroporation of gene constructs into the pancreas which encode mutated Kras and facilitate knock out of the tumor suppressor genes P53, Cdkn2a and/or Smad4 via CRISPR/Cas9. The resulting tumors recapitulate the human disease with respect to genetics, histology and TME composition. Furthermore, this model allows the generation of tumors reflecting different PDAC subtypes, as well as the resection of primary tumors in combination with the (neo-)adjuvant regimens.

Like the human disease, these tumors are refractory to chemotherapy and immune checkpoint blockade, whereas targeted therapy by means of MEK inhibitors merely leads to temporary delay of tumor outgrowth. We therefore tested the combination of MEK inhibitor treatment with different, potentially complementary modalities. We found the combination with agonist anti-CD40 antibodies to be highly effective. Prominent regressions are observed, and in combination with primary tumor resection long-term complete responses can be achieved even in a metastatic variant of the model. Apart from the direct cytostatic impact of MEKi on the Kras-transformed tumor cells, the mechanism of action of this synergistic drug combination involves enhancement of the anti-tumor T-cell response, suppression of CD4+ Treg cells, and reduction of MDSCs and M2-type macrophages in favor of M1-type pro-inflammatory macrophages.

A second highly effective regimen as identified in our experiments involves the combination of MEK inhibitors with the multi-kinase inhibitor regorafenib. In contrast to the aforementioned regimen, the complementary action of these drugs involves modulation of the TME by regorafenib. While the clinical translation of the MEKi/CD40 Ab regimen is hampered by toxicity issues encountered with agonist anti-CD40 Ab, the synergy as observed in the MEKi/regorafenib regiment provides a tangible path towards clinical testing.

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PTDSS1–dependent triggering of macrophage-driven tumor immune tolerance in breast cancer

I. M. Kur[1], A. Weigert[1,2,3]
[1]Institute of Biochemistry I, Faculty of Medicine, Goethe-University Frankfurt, Frankfurt am Main, Germany, [2]Frankfurt Cancer Institute, Goethe-University Frankfurt, Frankfurt am Main, Germany, [3]German Cancer Consortium (DKTK), Frankfurt am Main, Germany

Breast cancer is the leading cause of cancer-related mortality in women worldwide. Onset and progression of cancer malignancies is directly associated with the dynamic adaptations that occur in the tumor microenvironment. Hereby, the interaction between the immune system and the tumor is crucial and is influenced by factors such as cytokines, metabolites and tumor cells undergoing apoptotic cell death. Macrophages, the apoptotic-cell-phagocytes, can induce self-tolerance and anti-inflammatory responses through the recognition of phosphatidylserine (PS) exposed at the outer leaflet of the plasma membrane by Mer tyrosine kinase (MerTK) receptors. PS is a phospholipid that is produced by two phosphatidylserine synthase (PTDSS) isoforms (PTDSS1/2). Overexpression of PTDSS1 was detected in human tumors, and

increased PTDSS1 levels in mammary carcinoma patients predicted poor survival. We observed increased levels of PS in mammary tumors of mice. Tumor-associated macrophages from both PTDSS1-knockdown tumors and tumors of macrophage-specific MerTK KO mice revealed a common gene signature indicating reduced proliferation and increased inflammation, which was accompanied by reduced macrophage numbers in tumors and smaller tumor mass. Importantly PTDSS1-knockdown cells exposed less PS at their surface during apoptosis, which prevented anti-inflammatory macrophage activation and proliferation in vitro. These data support the view that interfering with apoptotic cell-derived signals may serve to restrict tumor-promoting and to enhance protective inflammation. Selective and specific pharmacological PTDSS1 inhibitors were identified and are being validated using an established model of murine breast cancer organoids. A co-culture system of organoids and primary monocytes to study monocyte-differentiation- and PTDSS-1-dependent macrophage-polarization after treatment with PTDSS1 inhibitors, combined with future use of PTDSS1 inhibitors in therapeutic tumor mouse models will provide insights on the translational potential of PTDSS1 inhibition in breast cancer.

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Functional role of tumor-specific B cells and tertiary-lymphoid structures in preclinical models of immune checkpoint therapy

J. Lehmann[1], M. Thelen[1], M. Garcia-Marquez[1], P. Volkmar[1], K. Siepmann[1], S. Schran[1], T. Seidlitz[2], D. Stange[2], P. Fervers[3], T. Persigehl[3], C. J. Bruns[4], M. von Bergwelt-Baildon[5,6], K. Wennhold[1], H. Schlößer[1,4]
[1]Center for Molecular Medicine Cologne (CMMC), Cologne, Germany, [2]Universitätsklinikum Carl Gustav Carus Dresden, Dresden, Germany, [3]University Hospital of Cologne, Radiology, Cologne, Germany, [4]University Hospital of Cologne, Department of General, Visceral, Cancer and Transplantation Surgery, Cologne, Germany, [5]LMU Munich, Department of Internal Medicine III, Cologne, Germany, [6]German Cancer Consortium (DKTK), Heidelberg, Germany

Immune checkpoint inhibition (CKI) demonstrated breakthrough therapeutic efficacy in a variety of cancer types. Recent data suggest an important role of B cells and tertiary-lymphoid structures for susceptibility of cancer patients to CKI.
To advance our understanding of B cell specific mechanisms in the setting of CKI, we used two genetically modified mouse models, which express a tamoxifen-inducible Cre-recombinase in the stomach-specific Anxa10 locus. The genomically stable (GS) cancer model ($Cdh1^{fl/fl}$, $Kras^{G12D/+}$ and $Smad4^{fl/fl}$) demonstrates a slow progressing tumor growth as observed by magnetic resonance imaging (MRI). The chromosomally instable (CIN) model ($Kras^{G12D/+}$, $Tp53^{R172H/+}$ and $Smad4^{fl/f}$) shows a more rapid, intestinal tumor growth. For study of antigen-specific immune responses, we included the subcutaneous PancO2-OVA tumor models. All three mouse models received single or combination CKI with anti-PD-1 and anti-4-1BB.
CKI in the PancO2-OVA model resulted in a significant increase of OVA-specific B cells and secretion of OVA-specific antibodies. This was accompanied by an increase of T follicular helper cells and activated $CD86^+$ B cells. In the genetically modified cancer models, tumor growth was accompanied by development of metastases in liver, lymph nodes and lungs at later stages. We detected B-cell clusters in the tumor microenvironment indicating the presence of tertiary-lymphoid structures. Combination therapy with anti-PD1 and anti-4-1BB in the orthotopic models did not result in a significant reduction of tumor growth. However, we observed increased percentages of memory B cells, germinal center B cells, activated $CD86^+$ B

cells and activated T-cell subpopulations. We demonstrated resemblance of the genetically modified mouse models with human gastric cancer by comparing infiltrating immune cells in the tumor microenvironment. TLS are usually not observed in cancer mouse models, thus the here described gastric cancer models offer a unique opportunity to study TLS formation. CKI seems to increase the anti-tumor B-cell response by enhanced antigen presentation and humoral-immune response and thus our results underline the role of B cells in CKI

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The tumor microenvironment of epithelial ovarian cancer: exhausted and immunosuppressive

A. Leutbecher[1,2], S. Geweniger[3], G. Hänel[1,2], A.-S. Neumann[1,2], B. Czogalla[3], F. Trillsch[3], A. Burges[3], M. von Bergwelt[1,4], M. Subklewe[1,2,4], A. Reischer[1,2,4]
[1]University Hospital, LMU Munich, Department of Medicine III, Munich, Germany, [2]LMU Gene Center, Laboratory for Translational Cancer Immunology, Munich, Germany, [3]University Hospital, LMU Munich, Department of Obstetrics and Gynecology, Munich, Germany, [4]German Cancer Consortium (DKTK) and German Cancer Research Center (DKFZ), Heidelberg, Germany

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy. 75% of the patients are diagnosed in advanced stage with an overall survival rate of about 30%. Immunotherapeutic approaches with checkpoint inhibitors have not had a prognostic impact so far. Accordingly, for the development of highly needed novel therapeutic strategies, investigation of the tumor microenvironment (TME) and tumor-infiltrating lymphocytes (TILs), as well as identification of immune checkpoints (ICPs) and tumor-associated antigens (TAAs), appears indispensable.
Hence, we analyzed primary EOC tumor tissue of 45 patients. The tumor tissue was mechanically and enzymatically dissociated into a single-cell suspension. $CD45^+$ TILs were magnetically isolated. Multiplex flow cytometry (MPFC) was used to characterize the composition of the TME and to determine ICP expression (PD-1, TIGIT, TIM3) on T cells. In addition, the T-cell function of TILs was compared to healthy donor (HD)-derived T cells in an established cytotoxicity assay system using OCI-AML3 cells and a T-cell bispecific antibody (TCB) in a co-culture setup for 7 days. To overcome the dysfunction of TILs, a combination of the TCB and an anti-CD28 monoclonal antibody (mAb) or IL-2 was performed. Furthermore, T-cell metabolism was evaluated using the Seahorse XF analyzer. Moreover, potential TAAs (mesothelin (MSLN), mucin 1 (MUC1), EpCAM), and ICPs (B7H3 and CD47) were assessed on OC cells.
The $CD45^+$ TILs consisted mainly of T cells (67.7%), followed by a monocyte/macrophage population (15.6%) and B cells (10.3%) as well as NK cells (5.9%) and pDCs (1%). The CD4:CD8 T-cell ratio varied widely among patients. Within the monocyte/macrophage population, we predominantly detected M2-like polarized cells ($CD163^+$ and $CD206^+$). There was a high (co)-expression of PD-1, TIGIT, and TIM3 on T cells. In co-culture with OCI-AML3 cells and a TCB, TIL-derived T cells showed an exhausted phenotype with reduced proliferation, cytokine secretion, and killing capacity compared to HD-derived T cells (94.7% vs. 55.06% specific lysis). The cytotoxic capacity was rescued in combination with anti-CD28 mAb (16.4% vs 37.7% specific lysis) and IL-2 (16.4% vs 85.0% specific lysis). The metabolic capacity was also diminished in comparison to HD-derived T cells. The TAAs MSLN (median MFI ratio 2.2), MUC1 (median MFI ratio 22.9), and EpCAM (median MFI ratio 51.2), and the ICPs B7H3 (median MFI ratio 2.5) and CD47 (median MFI ratio 49) were expressed on primary EOC cells.

In summary, the immune contexture of EOC samples was dominated by immunosuppressive type 2 macrophages and TILs with an exhausted phenotype, as detected by surface expression but also functional data. Albeit TAAs could be identified, the data highlights that immunotherapeutic strategies must integrate immunomodulatory agents, to counteract the immunosuppressive TME of EOC.

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The therapeutic benefits of type I interferons in polycythemia vera involve NK cells

J. Lossa*[1], T. Schnöder[2], F. Marini[3,4], F. Ries[5], N. Lindhauer[5], A. Henrich[6], M. Hoehne[6], M. Klein[1,4], T. Bopp[1,4], H. Schild[1,4], F. Heidel[2,7], H. C. Probst[1,4], M. Radsak*[4,5,8], S. Muth*[1,4]
[1]Institute of Immunology, University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany, [2]Innere Medizin C, Hämatologie, Onkologie, Stammzelltransplantation und Palliativmedizin, Universitätsmedizin Greifswald, Greifswald, Germany, [3]Institute of Medical Biostatistics, Epidemiology and Informatics (IMBEI), Mainz, Germany, [4]Research Center for Immunotherapy (FZI), University Medical Center of the Johannes Gutenberg University, Mainz, Germany, [5]IIIrd Department of Medicine - Hematology & Oncology, University Medical Center of the Johannes Gutenberg-University, Mainz, Germany, [6]TRON – Translational Oncology at the University Medical Center of the Johannes Gutenberg University Mainz GmbH, Mainz, Germany, [7]Leibniz Institute on Aging, Fritz-Lipmann Institute (FLI), Jena, Germany, [8]Department of Medicine VII - Hematology & Oncology, Donau-Isar-Klinikum, Deggendorf, Germany

*Contributed equally

Myeloproliferative neoplasms (MPN) comprise clonal hematopoietic stem cell (HSC) disorders of the myeloid cell lineage and are classified by somatic driver mutations. Polycythemia vera (PV) is based on a gain of function mutation in the tyrosine kinase JAK2 (Janus kinase 2), which leads to its overactivation and ultimately to an increased production of erythrocytes. This JAK2V617F mutation is highly abundant in PV and has triggered the development of JAK inhibitors relieving PV induced symptoms. However, JAK inhibitors do not affect the allelic burden of mutated JAK2 and thus cannot alter the natural course of the disease. In contrast, Interferon α (IFNα) is widely recognized for its efficacy in treating all MPN and has the potential to induce long-term remissions in PV. Despite its effectiveness, the precise mechanism of action remains inadequately understood. As immunostimulants, type I interferons drive the activation of immune cells in general. Thus, it is reasonable to assume that indirect effects of IFNα may contribute to therapeutic effects. To investigate the immunological mechanisms by which IFNα attack malignant HSCs in MPN, we use a mouse model for PV, in which mice suffer from increased hematocrit and enlarged spleens and in which we can reduce disease symptoms by the injection of IFNα. Performing high-dimensional flow cytometry we analysed the contribution of immune cells to therapeutic effects of IFNα in PV mice. We identified NK cell frequencies and numbers to be increased in the bone marrow of PV mice upon IFNα therapy. In the absence of NK cells after antibody mediated depletion the reduced disease symptoms such as decreased hematocrit and smaller spleen sizes in IFNα treated PV mice were completely reversed, indicating that NK cells play a critical role in the beneficial effects of IFNα treatment of PV mice. In addition, we observed changes in NK cell phenotypes in response to IFNα treatment as well as changes in NK cell receptor ligands expressed on HSCs in mice following IFNα therapy, which may imply enhanced potential interactions between NK cells and malignant stem cell clones in PV mice following IFNα therapy. Thus, NK cells may serve as a novel target in the development of future treatments for PV.

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IL-8 and association with acute myeloid Leukemia (AML) in a pediatric Mozambican oncology ward

V. Maphossa[1,2], E. Mongo[1], A. Sigaúque[1], V. Monteiro[1], O. Guiliche[1], T. Babetine[1], C. Castiano[1], L. Manhique[1], C. Mudenyanga[3], J. Sacarlal[2], E. Granado[4], F. Amodo[5], R. Matavele[1,6]
[1]Instituto Nacional de Saúde (INS), Maputo, Mozambique, [2]Univerisdade Eduardo Mondlane, Faculdade de Medicina, Maputo, Mozambique, [3]Clinton Health Acess Iniciative, Maputo, Mozambique, [4]Instituto Nacional de Câncer (INCA), Bahia, Brazil, [5]Hospital Central de Maputo, Serviço de Hemato-Oncologia, Maputo, Mozambique, [6]Centro de Investigação e Desenvolvimento em Etnobotânica, Namahacha, Mozambique

Background
IL-8 has been described as a prognostic biomarker of acute myeloid leukemia (AML). In AML disease, IL-8 creates a microenvironment that supports the survival and growth of AML cells, therefore being proposed as an immunotherapy target. We aim to evaluate the levels of bone marrow (BM) cytokines, including IL-8, in an Mozambican cohort of children diagnosed with AML.

Methods
Bone marrow (BM) samples of diagnosis of 42 patients from Haemato-Oncology Pediatric Service at Maputo Central Hospital, were used in the analysis of the IL-8/CXCL8 and additional pro-inflammatory cytokines such as: IL-6, IL-7, IL-18, CCL2, (MIP-1α), CCL4 (MIP-1β), CXCL10 (IP-10) and CCL28 by Luminex assay as mean florescence intensity (MFI). Patients were aged below 14 and grouped as negative (n=16), B-cell acute lymphoblastic leukemia (B-ALL, n=13), T-cell acute lymphoblastic leukemia (T-ALL, n=3), AML cases (n=10). Association and correlation between variables were assessed using Man Whitney and Spearman Rank, tests respectively. Receiver operating characteristic (ROC) curve analysis evaluated the ability of cytokines in classification of AML cases. All tests were done with $\alpha=0.05$.

Results
IL-8 levels were not significantly different between AML, B-ALL and T-ALL cases ($p>0.05$). AML cases showed IL-8 levels significantly higher than negative cases ($p=0.02$), with a median three times higher. IL-8 efficiently distinguishes positive cases from negatives with an AUC of 0.77 ($p=0.021$) and was found to be strongly associated to IL-6 ($r=0.86$, $p=0.002$), IL-7 ($r=0.89$, $p=0.001$), IL-15 ($r=0.75$, $p=0.016$) and CCL4 ($r=0.92$, $p=0.001$). No differences were found between AML positive and negative cases regarding the cytokines IL-6, IL-7, IL-15 and CCL4.

Conclusions
Study findings corroborate with previously reports of association of IL-8 with AML condition. Despite the absence of differences between AML positive and negative cases regarding the levels of IL-6, IL-7, IL-5 and CCL4, those were found to be strongly associated to IL-8 in this cohort. Further studies are needed to determine the potential role of the referred cytokines on the immune microenvironment pathway of AML.

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Tumor cell-intrinsic factors induce development of myeloid subpopulations in PDAC

C. Marx[1], V. Kolarik[1], C. Hörth[1], L. Delius[1], C. Rambuscheck[1], P. Metzger[1], N. Röhrle[1], D. Böhmer[1], G. Beyer[2], J. Mayerle[2], S. Endres[1], M. Schnurr[1], L. M. König[1]
[1]LMU Munich, Clinical Pharmacology, Munich, Germany, [2]LMU Munich, Medical Department II, Munich, Germany

In recent years, immune therapies have significantly improved treatment options for cancer patients. However, these therapies often fail to enhance outcomes in solid tumors due to the development of an immune-suppressive tumor microenvironment (TME). Pancreatic adenocarcinomas (PDAC) are known for their highly complex TME, driven largely by tumor-infiltrating neutrophils. While neutrophils initially possess anti-tumor properties in early stages of malignant diseases, under pathological inflammatory conditions, they can transform into immune-suppressive myeloid-derived suppressor cells (PMN-MDSCs). Despite their critical role, it remains unknown which tumoral factors trigger the development of PMN-MDSCs in the TME. Additionally, there is a lack of sufficient surface markers to distinguish between pro-tumoral and anti-tumoral neutrophils, making targeting PMN-MDSCs challenging without harming essential physiological myeloid cells.

We are investigating the influence of the PDAC cell biology on the formation of myeloid subpopulation based on surface markers in the murine model as well as in patient samples. To investigate the immune cell composition in the tumor, we facilitate an orthotopic PDAC model. Immune cell composition in various tissue is analyzed using full spectrum flow cytometry. Marker expression on myeloid cells in blood samples from PDAC patients is investigated by an explorative flow cytometry-based screen which allows to examine 360 different surface markers in one sample. High-dimensional cluster analysis is performed using various R packages (e.g. Infinity Flow, CATALYST, Diffcyt).

We found that peripheral neutrophils from PDAC patients form at least three subclusters that can be distinguished based on surface marker expression. The cells in the subclusters express surface markers associated with immune suppression, e.g. CD14, Lox-1 or PD-L1. Subpopulations with a similar marker expression can be found in neutrophils derived from murine PDAC and these cells have increased immunosuppressive capacities. A neutrophil subpopulation with a comparable phenotype can be induced by stimulating neutrophils from healthy mice with PDAC cell conditioned medium. Additionally, this subpopulation shows significantly enhanced survival in vitro compared to neutrophils which were not stimulated with PDAC secreted factors.

Our findings suggest that tumor cell intrinsic factors influence the neutrophil compartment in the TME. The PDAC secretome induces a subpopulation that phenotypically differs from healthy neutrophils and expresses markers that are associated with immune suppression. Future work will identify the relevant factors in the secretome which induces this subpopulation and in addition, will functionally characterize the tumor associated subpopulation. Eventually, our work will contribute to identify immunosuppressive neutrophil subpopulations which might provide a new therapeutic target in cancer immunology.

Type I interferon signaling is downregulated in monocytes during their crosstalk with glioblastoma cells

S. Meyer[1], R. Bauer[1], M. Weidmann[1], R. Raue[1], S. Kuntschar[1], V. M. Guerrero Ruiz[1], B. Aliraj[1], G. Cardamone[1], I. Bode[1], B. Brüne[1,2,3], T. Schmid[1,2]
[1]Institute of Biochemistry I, Faculty of Medicine, Goethe University Frankfurt, Frankfurt, Germany, [2]German Cancer Consortium (DKTK), Partner Site Frankfurt, Frankfurt, Germany, [3]Frankfurt Cancer Institute, Goethe University Frankfurt, Frankfurt, Germany

Glioblastoma is the most aggressive type of brain cancer with an average survival of less than 15 months. The glioblastoma microenvironment is characterized by a massive infiltration with monocyte-derived macrophages which take on an immunosuppressive phenotype. Therefore, we aimed to determine if immune responses are altered in myeloid cells and glioblastoma cells during their crosstalk, and if so, how this impacts immune cell functions. To this end, we co-cultured primary human monocytes isolated from buffy coats with various glioblastoma cell lines and followed changes in mRNA and protein expression. RNA-sequencing analyses revealed substantially decreased expression of type I interferon-stimulated genes in monocytes upon co-culture with glioblastoma cells, while the same genes were not affected by co-culture in glioblastoma cells. Interestingly, interferon-stimulated gene expression was not only reduced in monocytes upon direct cell-cell contact with glioblastoma cells, but also decreased in an indirect transwell assay, indicating that soluble mediators facilitate the alterations in type I interferon signaling. The downregulation of interferon responses was further accompanied by decreased expression and activation of mediators of the type I IFN signaling pathway. Functionally, co-cultured monocytes with reduced interferon responses displayed enhanced surface expression of markers related to immunosuppressive M2-like macrophage phenotypes. The attenuated interferon responses did not result from a general immunosuppressive effect of glioblastoma cells on monocytes though, as the secretion of classical pro-inflammatory cytokines, i.e., interleukins 6 and 1β, was increased in monocytes upon co-culture with glioblastoma cells. Thus, we propose, that type I interferon signaling might be selectively inhibited in myeloid cells infiltrating the glioblastoma microenvironment, which could affect anti-tumor immune responses and consequently the success of immunotherapies.

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Combating glioblastoma: the potential of Vγ9Vδ2 TILs as novel therapeutic

M. Nicolasen[1], L. Gatti[1], M. Botas Miret[1], D. Zawal[1], Z. Sebestyen[1], P. Robe[2], D. Beringer[1], J. Kuball[3]
[1]UMC Utrecht, Centre for Translational Immunology, Utrecht, The Netherlands, [2]UMC Utrecht, Department of Neurology and Neurosurgery, Utrecht, The Netherlands, [3]UMC Utrecht, Department of Hematology, Utrecht, The Netherlands

Glioblastoma (GBM) remains the most aggressive and prevalent primary brain tumor with an extremely poor patient prognosis. Immunotherapeutic strategies are emerging as interesting option for GBM patients, however the effectivity of αβ T cell therapies remains low, possibly due to tumor escape mechanisms. An unconventional T lymphocyte population, γδ T cells, has been identified as interesting effector cells for immunotherapeutic strategies as they possess anti-tumor characteristics against a broad spectrum of cancers. However, phenotypic characterization of γδ tumor-infiltrating lymphocytes in the context of GBM and their cytotoxic capacity remains elusive. In order to assess the role of different T cell subsets in GBM,

we analyzed αβ and γδ T cells from peripheral blood and tumor of 40 GBM patients and 15 HD PBMCs. The T cells were phenotypically characterized using flow cytometry and matched T cells, PBMCs and TILS, from 9 patients were expanded in vitro to assess fitness and anti-GBM reactivity. Phenotypic analyses revealed a relative decrease in γδ tumor-infiltrating lymphocytes (TILs) present in tumor tissue compared to γδ T cells found in peripheral blood, as well as an altered phenotype of both the αβ and γδ TILs. Vδ1+ and vδ1-δ2 TILs as well as CD8 and CD4 αβ TILs were significantly less naïve than the PBMC derived T cells and exhibited more of an effector-memory phenotype. We also observed an increase in expression of cytotoxic markers in vδ2+ TILs, activating and inhibitory NK receptors in vδ1+ TILs and NK receptors and exhaustion in CD8+ αβ TILs. Additionally, various expanded patient-derived γδ T cells from the peripheral blood and TILs displayed anti-GBM reactivity in an autologous setting for all 9 patients, especially vγ9^{+}vδ2^{+} T cells, while αβ T cells from both tumor and peripheral blood did not show reactivity against GBM tumors. However reactivity varied between patients in terms of potency of the γδ T cells as well as susceptibility of the tumor. Collectively, our results suggest an important role part but also varying role of γδ T cells in GBM and highlight the therapeutic potential of tumor-residing γδ T cells as promising target for future immunotherapeutic strategies, which needs to be tailored to individual patients.

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Spatio-functional Characterization of Tumor-infiltrating B cells in the Tumor Microenvironment of Cutaneous T cell Lymphoma

S. Oganesian[1,2,3], G. Piontek[4], M. Heide[1,3,5], M. Kirmaier[1,2,3], A. Hadzic[1,2,3], S. Krebs[6], J. Watter[7], H. Blum[6], S. Mages[7], J. Klughammer[7], C. Posch[8], O. Weigert[1,3,5], M. Rudelius[4], M. Bergwelt-Baildon[1,3], S. Theurich[1,2,3]
[1]University Hospital LMU, MED3, Munich, Germany, [2]LMU Genecenter, Cancer and Immunometabolism Laboratory, Munich, Germany, [3]German Cancer Consortium (DKTK), Partnersite Munich, Munich, Germany, [4]Universitiy Hospital LMU, Department of Pathology, Munich, Germany, [5]Experimental Leukemia and Lymphoma Research, ELLF, Munich, Germany, [6]LMU Gencenter, Laboratory for Functional Genome Analysis, Munich, Germany, [7]LMU Gencenter, Systems Immunology, Munich, Germany, [8]University Hospital TUM Rechts der Isar, Department of Pathology Campus Biederstein, Munich, Germany

Introduction

The majority cutaneous T cell lymphoma (CTCL) run an indolent clinical course. However, advanced stages (>=IIB) or certain CTCL subtypes such as folliculotropic Mycosis Fungoides (FMF) and Sézary Syndrome (SS) are associated with significantly worse prognosis and treatment options are limited. In previous studies, our group
has described increased numbers of CTCL tissue infiltrating B cells as an adverse prognostic factor. However, the underlying mechanisms are still poorly understood.
This project therefore aims to characterize the role of B cells within the CTCL Tumor Microenvironment with a special focus on spatial distribution and immunometabolic and cellular interaction patterns.

Methods

An independent cohort of clinically annotated, Formalin fixed and Paraffin embedded (FFPE) clinical CTCL probes were analyzed by multiplex Immunohistochemistry (IHC) to characterize the entire tumor and immune cell infiltrate and to determine spatial distribution and interaction patterns. Gene expression within these probes was quantified from consecutive tissue sections by Nanostring© and spatial

transcriptomic analyses via the 10x Genomics© and compared with multiplex IHC results. In vitro functional and metabolic characterization of CTCL cells (co-culture experiment, migration experiments, extracellular flux analysis) and the consequences of distinct B cell subtype interaction were addressed.

Results

In a total of 29 CTCL cases, B cells were enriched in aggressive CTCL subtypes in this independent cohort and formed a complex network, which differed from patterns found in indolent CTCL cases. Panel gene expression analysis confirmed B cell enrichment seen IHC and furthermore showed distinct gene expression patterns in aggressive CTCL cases associated with the B cell infiltrate, showing also a strong correlation of tumor associated and immunosuppressive inhibitory M2 Macrophages (TAMs). Metabolic pathways were also found to be significantly altered depending on the clinical course. Characterization of cellular metabolic programs of CTCL cells compared to activated non-malignant $CD4^+$ T cells showed that cell lines HuT78 and HH (aggressive types of CTCL) have distinct glycolytic and mitochondrial capacities compared to CTCL lines representing more indolent subtypes, $CD30^+$ ALCL and normal $CD4^+$ T cells. Notably, B cell conditioned media exhibited higher metabolic capacities in SS cell line SeAx compared to non-conditioned media after 24h cultivation.

Conclusion

Our preliminary data further support the observation that tumor infiltrating B cells and TAMs play a functional role in CTCL and are associated with more aggressive clinical courses of the disease. The ongoing experiments aim to provide a detailed characterization of spatio-temporal distribution, cellular interaction patterns, and immunometabolic factors within these networks.

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Liposomal formulations enable potent co-stimulation assisted anti-cancer vaccinations

D. Ostroumov[1], N. Benne[2], F. L. Vigario[3], S. Knocke[1], H. Wedemeyer[1], F. Kühnel[1], B. Slütter[3], T. C. Wirth[1]
[1]Hannover Medical School, Department of Gastroenterology, Hepatology, Infectious Diseases and Endocrinology, Hannover, Germany, [2]Utrecht University, Department of Infectious Diseases and Immunology, Utrecht, The Netherlands, [3]Leiden University, Division of BioTherapeutics, Leiden, The Netherlands

Since cancer vaccination efficacy in clinical practice is limited by low frequency of induced T cell responses and mostly require repetitive immunizations, there is a clear need for further development of T cell vaccines. We utilized liposomal formulations to deliver peptide antigens in vivo and encapsulated them with different adjuvants in order to enhance dendritic cell activation, measured by CD80/CD86 expression by flow cytometry. The generation of specific T cell responses was investigated by intracellular staining for interferon gamma in samples from peripheral blood and different tissues of immunized mice. Analysis of lymph nodes and spleen from mice immunized with liposomes containing different adjuvants revealed that the stimulator of interferon genes (STING) agonist c-di-GMP resulted in the highest upregulation of CD80/CD86 and the strongest immune response in peripheral blood after priming. One week after liposomal priming the tumor neoepitope-specific CD8 T cell responses were amplified by systemic administration of synthetic peptide, costimulatory CD40 antibody and a toll-like-receptor 3 agonist. This heterologous T cell vaccination regimen induced a high frequency of tumor-specific CD8 T cells within two weeks after priming. Comparison of different application routes for liposomal immunization showed a

similar strength of the resulting immune response in peripheral blood after boosting. The versatility of liposomal formulations allowed not only the encapsulation of short CD8 T cell peptides, but also longer polypeptides that require cross-presentation by dendritic cells for the generation of CD8 T cell responses. Addition of CD4 T cell peptides into the same liposomes not only induced a high frequency of specific CD4 T cells but also further enhanced the specific CD8 T cell response. Treatment of subcutaneous MC38 tumor-bearing mice with the established prime-boost regimen resulted in complete regression of large solid tumors and the survival of all immunized animals. Our data demonstrate the feasibility and efficacy of a fully synthetic anti-cancer T cell vaccine.

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S100A8/9 regulates MDSC-mediated immunosuppression and predicts poor response to immune checkpoint inhibitors in melanoma

F. G. Ozbay Kurt[1,2], B. A. Cicortas[1,2], B. Balzasch[3], C. De La Torre[4], V. Ast[4], E. Tavukcuoglu[1,2], C. Ak[1,2], S. Wohlfeil[1,2], A. Cerwenka[3], J. Utikal[1,2], V. Umansky[1,2]
[1]German Cancer Research Center, Skin Cancer Unit, Heidelberg, Germany, [2]University Medical Center Mannheim, Department of Dermatology, Venereology and Allergology, Mannheim, Germany, [3]Mannheim Institute for Innate Immunoscience (MI3), Medical Faculty Mannheim, Department of Immunobiochemistry, Mannheim, Germany, [4]Institute for Clinical Chemistry, Medical Faculty Mannheim, NGS Core Facility, Mannheim, Germany

Immunotherapeutic strategies in malignant melanoma face challenges due to the resistance developed by a considerable number of patients. Myeloid-derived suppressor cells (MDSC), a heterogeneous population of myeloid cells, accumulate in the melanoma microenvironment. With the ability to inhibit anti-tumor T cell responses, MDSC were shown to promote immunosuppression, enhancing tumor progression and tumor cell resistance to the immunotherapy. TLR4 signaling was reported to be involved in promoting the immunosuppressive properties of human $CD14^+$ monocytes. Damage-associated molecular patterns S100A8/9, TLR4 and RAGE ligands, drive MDSC activation and have been shown to be highly expressed in the tumor microenvironment of solid tumors. Nevertheless, the role of TLR4 and RAGE signaling in the acquisition of MDSC immunosuppressive properties remains to be better defined. This study investigates how TLR4 and RAGE signalling, together with their ligand S100A8/9, shape MDSC-mediated immunosuppression in melanoma. $CD14^+$ monocytes were purified from the peripheral blood of late-stage melanoma patients and healthy donors. Monocytes were treated with S100A9 in the presence of GM-CSF for 72 hours. The immunosuppressive capacity of stimulated monocytes was assessed in the functional assays with T cells with or without TLR4 inhibitor (Resatorvid) and RAGE inhibitor (FPS-ZM1). Expression of markers and pathways involved in the stimulation of MDSC were assessed by flow cytometry, western blotting, and gene expression profiling. S100A8/9 levels were measured in the plasma of melanoma patients by ELISA. TCGA data analysis was performed to evaluate the association between the expression of immunosuppressive markers and S100A9 in melanoma TME. In addition, comparative analysis of monocyte clusters was performed using single-cell RNA sequencing between cohorts of melanoma patients and healthy donors. Elevated plasma levels of S100A8/9 were found to correlate with poor response to immune checkpoint inhibitors in melanoma patients. Analysis of single-cell RNA sequencing (scRNA-seq) data revealed an increase in inflammatory gene expression, including NF-κB and TLR4 signalling, and S100 gene

expression, alongside a decrease in major histocompatibility complex II (MHC II) gene expression in patient monocytes. S100A9 stimulated monocytes acquired suppressive activity against T cells with increased ROS production, PD-L1 and IDO expression. Blockade of TLR4 and, to a lesser extent, RAGE signaling, attenuated the suppression of T cell proliferation. In conclusion, these results highlight the prognostic importance of S100A8/9 and suggest the potential of targeting TLR4 signaling as a strategy to counteract MDSC-mediated immunosuppression in melanoma.

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Exploring the role of therapeutic targets alongside intermediate filament proteins in UTUC immune evasion

J.-M. Peng, J.-W. Luo, Y.-T. Weng, P.-C. Wang
Institute for Translational Research in Biomedicine, Kaohsiung Chang Gung Memorial Hospital, Kaohsiung, Taiwan

Background:
Immunotherapy targeting immune checkpoint proteins has become a key strategy in cancer treatment. However, research on how immune-tolerant tumors promote the expression of these checkpoint proteins remains limited. Understanding the mechanisms by which cancer cells activate immune checkpoints, and enhancing the efficacy of tumor immunotherapy, is critical. Our study focuses on the progression of urothelial carcinoma and its interaction with immune cells.

Materials and Methods:
Our research involved examining upper urinary tract urothelial carcinoma (UTUC) during its progression, with a particular focus on the tumor islands infiltrated by T lymphocytes. We observed the cancer cells surrounding these areas expressing an abundance of fibrous reticulated vimentin. We conducted experiments to assess the impact of increasing or decreasing these intermediate filament proteins on the expression of the immune checkpoint protein PD-L1. We also extended our investigation to other significant checkpoint proteins like CD155, SIRPalpha, and TSP1 to support our findings.

Results:
The results showed that increasing vimentin protein levels promoted PD-L1 expression, while its depletion significantly reduced PD-L1 levels. Additionally, the removal of these intermediate filament proteins affected the expression of other key immune checkpoint proteins. We further discovered that T lymphocyte attacks on cancer cells triggered an increase in the expression and fibrotic netting of vimentin in the resistant cancer cells. A correlation between the expression of a group of immune checkpoint proteins and immune evasion was also noted.

Conclusions:
Our study highlights the role of intermediate filament proteins in the immune response within UTUC, particularly their influence on immune checkpoint protein expression. The detailed investigation into the signaling mechanisms behind this interaction holds promise for uncovering translational clinical applications, potentially leading to more effective immunotherapies in treating immune-tolerant tumors.

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Role of ICAM-1 in mediating actin cytoskeleton remodeling-dependent tumor immune evasion

D. Pereira Fernandes[1,2], L. Filali[1], C. Hoffmann[1], C. Thomas[1]
[1]Luxembourg Institute of Health, Department of Cancer Research, Luxembourg, Luxembourg, [2]University of Luxembourg, Faculty of Science, Technology and Medecine, Esch-sur-Alzette, Luxembourg

Natural killer (NK) cells are essential anti-tumor immune effector cells. They interact with potential target cells by assembling a specialized cell-cell interface termed immunological synapse, which is necessary to recognize and kill cancer cells. Relatively little attention has been given to the postsynaptic (cancer cell) side of the lytic immunological synapse, which has been regarded as a rather passive area. Our recent findings on actin dynamics in cancer cells challenge this view and shed light on a new fundamental layer of tumor immune evasion. We have established that a subset of cancer cells respond to the attack of NK cells by exhibiting a rapid and massive accumulation of filamentous actin within the synaptic region, which is associated with cancer cell resistance to NK cell-mediated cytotoxicity. Our ongoing research focuses on elucidating the signaling pathways responsible for inducing actin cytoskeleton remodeling-dependent tumor immune evasion. Here, we explore the provocative idea that ICAM-1, while being essential for NK cell adhesion and activation, may also mediate outside-in signaling leading to these processes and immune evasion. To investigate this, we generated CRISPR/Cas9-mediated ICAM-1 knockout breast cancer cell lines in which we reintroduced variants of ICAM-1 with modifications in their intracellular domain. This approach enables us to investigate the role of ICAM-1 in mediating synaptic actin cytoskeleton remodeling in cancer cells upon their interaction with NK cells without compromising conjugate formation dependent on the extracellular domain of ICAM-1. The modified cell lines are currently under investigation. Elucidating the associated pathways could uncover novel markers of tumor immune resistance and lead to the development of innovative anti-tumor therapies.

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Cross-disease integration of single-cell RNA sequencing data from lung myeloid cells reveals TAM signature in in vitro model

C. Pinto[1], J. Widawski[2], S. Lukowski[1], F. Ramírez[2], I. Tirapu[1]
[1]Boehringer Ingelheim RCV GmbH & Co KG, Cancer Immunology and Immune Modulation, Vienna, Austria, [2]Boehringer Ingelheim Pharma GmbH & Co. KG, Global Computational Biology and Digital Sciences, Biberach an der Riß, Germany

The innate immune system is required for the development of durable antitumor responses. Macrophages, with several tumor-promoting functions, emerged as promising therapeutic targets. Concurrently, advances in single-cell RNA sequencing (scRNA-seq) highlighted their phenotypic and functional diversity and uncovered specific populations with direct impact in the antitumor response. To build on these advances, we need preclinical models that enable the study of immunotherapies targeting such macrophages, which often lack counterparts in mouse models. Thus, understanding the macrophage diversity present in human cell-based in vitro coculture models is crucial.
Here we applied scRNA-seq to a 3D human cell-based model comprising tumor cell line-derived spheroids,

cancer-associated fibroblasts and primary monocytes, a setup widely used in immuno-oncology research. Integration of our in vitro data with publicly available patient-derived datasets showed that the macrophages in this model share phenotypic characteristics with the pro-angiogenic and pro-fibrotic $SPP1^{+}$ tumor-associated macrophage (TAM) population recently found across multiple cancer types and inflammatory lung diseases. This population was linked to aspects of disease progression and associated with poor prognosis in several tumor indications, highlighting the need for relevant models enabling its study as an immunotherapy target.
Overall, we show that integration of scRNA-seq datasets across diseases and from different sources can be a valuable approach in finding conserved populations of interest and in helping the design of relevant preclinical models. This opens new avenues for the use human cell-based coculture models in preclinical research, possibly contributing to reduced friction in the translation of preclinical findings to the clinic.

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Immune modulatory drug profiling using perfused, microfluidic on-chip co-cultures of patient-derived ovarian cancer microtumors and autologous immune cells

S. Plöger[1], E. Wegner[1], T. Schmidt[1], J. Roosz[1], N. Anderle[1], C. Teufel[1,2], T. I. Maulana[2], L. Christ[1], H. Graf[1], J. Alber[1,3], S. Williamson[1], A. Koch[4], S. Brucker[4], K. Schenke-Layland[1,3], J. Marzi[1,3], P. Loskill[1,2], C. Schmees[1]
[1]NMI Natural and Medical Sciences Institute at the University of Tübingen, Reutlingen, Germany, [2]Institute for Biomedical Engineering, Department for Microphysiological Systems, Eberhard Karls University Tübingen, Tübingen, Germany, [3]Institute for Biomedical Engineering, Department for Medical Technologies and Regenerative Medicine, Eberhard Karls University Tübingen, Tübingen, Germany, [4]Department of Women's Health, University Women's Hospital, Eberhard Karls University Tübingen, Tübingen, Germany

Ovarian cancer (OvCa) is one of the deadliest cancer types in women with 5-year survival rates of approximately 40% [1]. The disease is characterized by a lack of early diagnostic markers and non-specific symptoms. Additionally, OvCa is characterized by a high degree of intra- and intertumoral heterogeneity, which significantly impacts individualized treatment response. Since OvCa is usually detected when it has already metastasized and recurs after standard of care (SoC) therapy, there is a need to gain insight into novel therapeutic approaches and individual patient treatment responses. Supported by Wellcome LEAP as part of the HOPE program, a novel ex vivo platform combining OvCa patient-derived microtumors (PDM) and tailored organ-on-chip technology is developed to achieve this goal.
Established protocols allow for perfused on-chip OvCa PDM culture for 14 days with stable viability. The current focus of the project is the profiling of immunomodulatory drug treatment using on-chip co-cultures of OvCa PDM and autologous immune cells. For this aim, OvCa PDM isolated from residual fresh tumor tissue specimen of n = 7 different patients were pre-treated on-chip with or without taxane-based chemotherapy, followed by addition of autologous tumor-infiltrating lymphocytes (TILs), and anti-PDL1 immunotherapy at different concentrations. Tumor-cell killing in response to different treatments was monitored from flow-through samples collected daily over a 14-day period, using caspase-cleaved cytokeratin 18 ELISA. Moreover, parallel, multiplexed bead-based immunoassay analysis of respective flow-through samples enabled the quantification of pro-inflammatory and cytotoxic immune cell responses. Immunofluorescence staining of on-chip co-cultures for pan-cytokeratin (labeling tumor cells) and CD3 (labeling T cells) provided insights into the TIL migration and recruitment in response to applied treatments.

The combination of patient-derived tumor models and autologous immune cells with a customized microfluidic chip platform enables the parallel monitoring of different aspects of patient-individual treatment response towards different treatment modalities over extended periods of time. At present, we are combining this approach with a toolbox of additional analytical methods including label-free Raman spectroscopy to evaluate preclinical treatment efficacy in even greater detail.

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Immunomodulation induced by Interleukin-1 inhibition activates T cells and dendritic cells in colorectal cancer

A. Pöchmann[1,2], M. Suarez-Carmona[1], X.-W. Zhang[1,3], A. Ahmed[1,3], U. Prüfer[3], A. Höflich[1], J. Wolf[1], X. Holtkotte[1], T. Bund[4], D. Tschaharganeh[5], E. Voronov[6], M. Gaida[7], N. Halama[1,3,8]

[1]DKFZ / HI-TRON, Translational Immunotherapy, Mainz, Germany, [2]Ruprecht Karl University, Faculty of Biosciences, Heidelberg, Germany, [3]University Hospital Heidelberg, National Center for Tumor Diseases, Heidelberg, Germany, [4]DKFZ, Episomal-Persistent DNA in Cancer- and Chronic Diseases, Heidelberg, Germany, [5]DKFZ, Cell Plasticity and Epigenetic Remodeling, Heidelberg, Germany, [6]Ben-Gurion University of the Negev, Beersheba, Israel, [7]University Medical Center, Institute of Pathology, Mainz, Germany, [8]University Medical Center, University Cancer Center, Mainz, Germany

Colorectal cancer is a public health challenge being the third most common and the second most lethal cancer globally. Despite novel and innovative treatment strategies having improved patients' life, there is still a high unmet medical need, particularly for advanced metastatic patients who typically experience recurrence and drug resistance. Since response to therapy is highly dependent on the state of inflammation within the tumor microenvironment, its modulation represents a reasonable treatment approach. In light of this, we examine the classical pro-inflammatory cytokine Interleukin-1 (IL-1) as a possible target for advanced metastatic colorectal cancer patients. In order to maximize the translational power of this work, we decided for a patient-derived explant model system. Besides from being human, this model system has two big advantages. On the one hand, it reflects patient heterogeneity, and on the other hand, it allows to test treatment options for a patient, because it retains the microenvironment of a tumor. Here, we inhibit IL-1 signaling by the clinically approved IL-1 receptor antagonist Anakinra (r-metHuIL-1ra, Kineret, Swedish Orphan Biovitrum) and evaluate its effect on the tumor microenvironment by immunohistochemistry and multiplex cytokine profiling. In our explant model system, we observed a striking induction of IFN-gamma upon IL-1 inhibition. Immunohistochemistry revealed that both T cells and dendritic cells produced IFN-gamma and simultaneously, these cells increased in number in tumor regions, but not in adjacent tissue. Based on beneficial explant data, an incurable end-stage colorectal cancer patient received IL-1 inhibition as a monotherapy followed by one cycle FOLFIRI plus Bevacizumab, the latter being a treatment the patient previously did not respond to. With IL-1 inhibition however, two liver lesions shrank. In summary, our data shows that IL-1 inhibition is promising, feasible and safe for advanced metastatic colorectal cancer patients. It activates both T cells and dendritic cells with a distinct effect on tumor tissue. Hereby, it is able to reinvigorate antitumor immunity in human patients as demonstrated by the responding lesions in a prospective advanced metastatic case.

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Integrative analysis of immunopeptidomics, proteomics, transcriptomics and TCR profiling defining epitope specificities in colorectal cancer

T. Pu[1], K. Boonen[2], I. Bergiers[3], R. Vandoren[4,5], B. Fant[6], C. Bogaert[6], Y. Hong[7], E. Pepermans[2], A. Peddle[7], Z. D. Hu[7], K. Laukens[4,5], P. Meysman[4,5], J. Reumers[3], G. Baggerman[2], S. Verbandt[7], S. Tejpar[7]
[1]KU Leuven, Digestive Oncology, Leuven, Belgium, [2]University of Antwerp, Centre for Proteomics, Antwerp, Belgium, [3]Pharmaceutical Companies of Johnson & Johnson, Discovery Technology and Molecular Pharmacology, Janssen Research & Development, Beerse, Belgium, [4]University of Antwerp, Adrem Data Lab, Antwerp, Belgium, [5]University of Antwerp, Antwerp Unit for Data Analysis and Computation in Immunology and Sequencing, Antwerp, Belgium, [6]myNEO NV, Ghent, Belgium, [7]KU Leuven, Digestive oncology unit, Leuven, Belgium

Colorectal cancer (CRC) displays highly diverse immune responses, with only the minority of patients with Microsatellite instable (MSI) tumors responding to immune checkpoint inhibitors. While the majority of patients fail to respond to immunotherapy in late-stage disease, recent neoadjuvant studies pinpointed high heterogeneity and immunogenic potential also in microsatellite stable (MSS) tumors. Our previous research showed that specifically tumors arising through metaplasia display increased T cell infiltration indicative of active immunity. However, the specificity of the immune infiltrate is still unknown, requiring further studies to identify its therapeutic potential. Therefore, we analyzed the immunopeptidome and TCR landscape in 25 primary CRC spanning all subgroups, using transcriptomics, proteomics, immunopeptidomics and single cell immune repertoire profiling.

By Mass spectrometry (MS) analysis of HLA-I- and HLA-II-bound peptides from 25 tumor and adjacent normal tissue, and identification using custom genome- and transcriptome-based databases, we identified a total of 317355 different immunopeptides, comprising 156,872 HLA-I peptides with an average of 16,578 peptides per patient, and 172,936 HLA-II peptides, averaging 17,083 peptides per patient. Seven neo-epitopes originating from three tumor mutations were identified, a low yield for the expected neoepitopes predicted. Furthermore, we identified 199 epitopes (80 HLA-I, 119 HLA-II) from 41 cancer-testis antigens.

Differential analysis at the source protein level was performed followed by the Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses to identify signalling cascades with potential antigenicity. The analysis revealed distinct CRC-associated pathways such as Wnt, TGFb, PI3K, p53, and RTK-RAS. Proteomic data indicates discernible differences between tumor and healthy samples, with a notable upregulation in functional pathways and biological processes linked to tumorigenesis, inflammation and CRC. Enrichment analyses aligned with disease phenotypes: tumors developing through metaplasia were enriched in proteins associated with epithelial-mesenchymal transition, infection, and immunological processes.

Analysis of the T cell states by scRNAseq alludes to a varied spectrum of immunogenicity, with reactive or bystander orientation, in metaplasia derived CRC which contrasts to the classical adenomatous tumour pathway. Further annotating the specificity of the TCR to viral and self-antigens reveals that metaplasitic CRC, encompassing a subset of MSS as well as MSI CRC, have a large infiltration of T cells that cannot be

mapped to known epitopes, suggesting an exploitable source of antigens that needs further investigation.

In conclusion, we have generated an extensive dataset suitable for initiating predictions and matching of the tumor ligandome to T cell responses in CRC. Further validation of immunogenicity could enhance its potential to expand CRC treatment options.

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Delineating the role of tumor-intrinsic WNT signaling on immune cell function using CRC organoids

S. Reuter[1], K. E. Boonekamp[1], A. von Kries[2], A. Cerwenka[2,3], J. Betge[4,5,6,7], M. P. Ebert[5,6,7], M. Boutros[1,8,9]
[1]German Cancer Research Center (DKFZ), Division Signaling and Functional Genomics and Department of Cell and Molecular Biology, Heidelberg, Germany, [2]University Medical Center and Medical Faculty Mannheim, Department of Immunobiochemistry, Mannheim, Germany, [3]University Medical Center and Medical Faculty Mannheim, European Center for Angioscience, Mannheim, Germany, [4]German Cancer Research Center (DKFZ), Junior Clinical Cooperation Unit Translational Gastrointestinal Oncology and Preclinical Models, Heidelberg, Germany, [5]Heidelberg University, Department of Medicine II, Mannheim, Germany, [6]DKFZ-Hector Cancer Institute at University Medical Center Mannheim, Mannheim, Germany, [7]Mannheim Cancer Center, Mannheim, Germany, [8]Heidelberg University, Institute for Human Genetics, Heidelberg, Germany, [9]Faculty of Medicine Mannheim, Heidelberg University, Heidelberg, Germany

Colorectal cancer (CRC) poses a global health burden due to its high prevalence and the limited successful treatment options especially for metastatic disease (Piawah et al, 2019). WNT signaling is one of the main drivers of CRC with mutations in the pathway found in over 90 % of CRC cases (Cancer Genome Atlas Network, 2012). Dysregulation of the pathway results in stabilization and nuclear translocation of the core canonical pathway component β-catenin, acting as a transcriptional coactivator to initiate target gene expression (Korinek et al., 1997, Clevers and Nusse, 2012). Aberrant Wnt/β-catenin signaling is involved in several oncogenic processes in CRC such as cancer cell proliferation, apoptosis, therapy resistance and metastasis (Zhao et al., 2022). Of note, high Wnt signaling/β-catenin has been correlated with reduced immune cell infiltration in multiple cancers, including CRC (Luke et al., 2019). However, appropriate tools to study Wnt signaling in CRC and the underlying molecular mechanisms of tumor-immune cell interactions are missing. Patient-derived organoids constitute a suitable human model system since they recapitulate the in vivo tissue architecture allowing for more physiological interaction of immune cells and cancer cells. In this project, we aim to generate CRC organoids with an endogenously tagged version of CTNNB1/β-catenin which allows for targeted and reversible degradation using the dTAG system (Nabet et al., 2018 and Nabet et al., 2020). Additionally, we will incorporate a fluorophore to study the levels and localization of β-catenin at physiological levels. Subsequently, co-cultures of CRC organoids and immune cells such as NK cells will be performed with manipulation of WNT/β-catenin signaling using engineered organoids or by drug treatment. Cell viability assays and flow cytometry analysis serve as readout to quantify the degree of cancer cell killing and the activation status of immune cells upon co-cultivation. Eventually, we aim to disentangle the potential role of WNT/β-catenin signaling on the interaction of tumor cells and immune cells in CRC.

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Neoantigen architectures define immunogenicity and drive immune evasion of sub-clonal tumors

M. Roerden[1], A. B. Castro[2], Y. Cui[1,3], N. Harake[1], B. Kim[1], J. Dye[1], L. Maiorino[1], F. M. White[1,3], D. J. Irvine[1,3,4,5], K. Litchfield[2], S. Spranger[1,4,6]

[1]Koch Institute for Integrative Cancer Research, Massachusetts Institute for Technology, Cambridge, MA, United States of America, [2]Cancer Research UK Lung Cancer Centre of Excellence, University College London Cancer Institute, London, United Kingdom, [3]Department of Biological Engineering, Massachusetts Institute for Technology, Cambridge, MA, United States of America, [4]Ragon Institute at MGH, MIT and Harvard, Cambridge, MA, United States of America, [5]Howard Hughes Medical Institute, Chevy Chase, MD, United States of America, [6]Department of Biology, Massachusetts Institute for Technology, Cambridge, MA, United States of America

Sub-clonal neoantigen (NeoAg) presentation in intra-tumorally heterogenous tumors effectively blunts anti-tumor T cell responses and is associated with poor responses to immune-checkpoint blockade therapy (ICB). Yet, the mechanisms by which sub-clonal NeoAg presentation facilitates immune evasion thus far remained elusive, impeding the rational design of NeoAg-based immunotherapies. We therefore modelled clonal and sub-clonal NeoAg architectures to analyze their respective impact on NeoAg-specific T cell responses using a transplantable, syngeneic mouse model of lung adenocarcinoma with engineered NeoAg expression. Using pre-clinical models as well as cancer patient data we demonstrate that NeoAg architectures, defined by the abundance and clonality of NeoAgs, determine the immunogenicity of individual NeoAgs in a tumor. Mechanistically, we identified concerted interplays between concurrent T cell responses induced by cross-presenting dendritic cells (cDC1) mirroring the tumor NeoAg architecture during T cell priming in the lymph node. Depending on MHC binding characteristics of respective NeoAgs, this interplay was either beneficial for both T cell responses (NeoAg synergy) or led to an establishment of immunodominance hierarchies with suppression of sub-dominant responses (NeoAg competition). NeoAg synergy was dependent on clonal NeoAg presentation in the tumor and was induced through differential T cell priming by cDC1, as cDC1 concurrently presenting multiple NeoAgs showed a more stimulatory phenotype. In sub-clonal tumors, synergistic effects were reduced, impairing immune-mediated tumor control. In immunodominance hierarchies, clonal expression of high-affinity NeoAgs restricted to the same MHC allele led to competition between NeoAg peptides for MHC binding on DCs. Sub-dominant T cell responses were characterized by impaired expansion, limited tumor infiltration and diminished target-cell killing in vivo, driving immune evasion in sub-clonal tumors. In line with clinical data, ICB alone failed to overcome the immunological challenges induced by sub-clonal NeoAg presentation. Therapeutic RNA-based vaccination targeting suppressed T cell responses however synergized with ICB to enable control of sub-clonal tumors. Analysis of a large pan-cancer patient cohort suggested that sub-clonal NeoAg presentation impedes NeoAg synergy, hindering immune responses to low-affinity NeoAgs. Further, clonality of high-affinity NeoAgs was associated with response to ICB, suggesting a role for immundominance hierarchies in defining ICB responses. Our study provides novel mechanistic insights to explain the poor immunogenicity of tumors with sub-clonal tumor antigen presentation. These findings have strong implications for the design of immunotherapy approaches, including but not limited to cancer vaccines. Our study demonstrates that combining ICB with targeted vaccination could represent a potent treatment approach for intra-tumorally heterogenous tumors.

Activin Receptor-like Kinase 1 governs monocytic lineage differentiation and mobilization to shape a tumor-promoting immune landscape in breast cancer

M. Safaee Talkhoncheh
Lund University, Translational Cancer Research, Lund, Sweden

Lack of decisive clinical benefit halted the development of drugs targeting the endothelial activin receptor-like kinase 1 (ALK1, encoded by ACVRL1). Here, in efforts to better define the therapeutic context in which to use ALK1 inhibitors, we uncover a population of tumor-associated macrophages (TAMs) that, by virtue of their unanticipated Acvrl1expression, were effector targets for adjuvant anti-angiogenic immunotherapy in mouse models of metastatic breast cancer. The combinatorial benefit depended on ALK1-mediated modulation of the differentiation potential of bone marrow-derived granulocyte-macrophage progenitors, the release of monocytes into circulation, and their eventual extravasation. Notably, $ACVRL1^{+}$ TAMs coincided with an immunosuppressive phenotype, and were over-represented in human cancers progressing on therapy. Accordingly, breast cancer patients with a prominent $ACVRL1^{hi}$ TAM signature exhibited a significantly shorter survival. In conclusion, we shed light on an unexpected multimodal regulation of tumorigenic phenotypes by ALK1, and demonstrate its utility as a target for anti-angiogenic immunotherapy.

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Vaccination escaped tumors in mice are re-sensitized to therapeutic vaccination with cisplatin-induced CD8 T cell infiltration

P. Schol, C. Labrie, M. van Elsas, S. van der Burg
Leiden University Medical Center, Medical Oncology, Leiden, The Netherlands

Immunotherapy for cancer leads to durable tumor regression in just a fraction of patients. Some tumors are completely unresponsive (primary resistance), and others initially respond, only to relapse at a later stage (secondary resistance). Therapeutic vaccination against tumor antigens can effectively induce a strong CD8 T cell response, but often fails to effectively treat tumors due to primary or secondary resistance. Mouse TC-1 tumors, which express HPV16 E7, can be effectively treated with a therapeutic vaccination of E7 synthetic long peptide (SLP) and the TLR9 agonist CpG in IFA. When this vaccine was administered in the flank contralateral to the tumor, tumors initially regressed but relapsed after several weeks, modeling secondary resistance. Immune escaped TC-1 tumors displayed significantly lower infiltration of E7-reactive CD8 T cells, as measured by flow cytometry, indicating that their resistance mechanism could rely on T cell exclusion. When immune escaped TC-1 tumor cells were isolated and reinjected into naïve mice, the resulting tumors were completely resistant to therapeutic vaccination. However, these reinjected immune escaped tumors were sensitive to a combination of SLP vaccination and cisplatin, a widely-used chemotherapeutic with immunogenic effect. The immune composition of blood, spleen, tumor draining lymph node and tumors was interrogated at two different timepoints during combination treatment with cisplatin and SLP vaccination. SLP vaccination alone induced an E7-reactive CD8 response in the blood and spleen, but the infiltration of E7-reactive, GZMB+, Ki67+, CD8+ T cells was significantly higher in tumors treated with SLP vaccination and cisplatin. Surprisingly, we found that tumors treated with SLP alone had a significant influx of iNOS+ M1-like macrophages, while the level of iNOS+ M1-like macrophages was low in

tumors treated with SLP vaccination and cisplatin, and did not differ significantly from untreated tumors. In addition, treatment with SLP vaccination and cisplatin resulted in a significantly higher infiltration of NK cells, when compared to tumors treated with control or only SLP vaccination. Our results indicate that the synergistic effect between cisplatin and SLP vaccination in immune escaped TC-1 tumors relies mainly on the induction of an intratumoral lymphoid response, particularly on E7-reactive CD8 T cells. Current efforts focus on the mechanisms underlying how cisplatin attracts and/or activates these T cells and how it influences macrophages and other myeloid populations in the TME.

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Subtype-specific immune interactions in small cell lung cancer indicate distinct vulnerabilities to immunotherapeutic approaches

M. Schork[1], K. Khinvasara[1], E. Diken[1], L. Kolb[1], O. Akilli-Oeztuerk[1], F. Vascotto[1], F. Beleggia[2], F. Lang[1], B. Schrörs[1], C. Reinhardt[3], U. Sahin[1,4,5], M. Diken[1,5], A. Kuebler[1]
[1]TRON - Translational Oncology at the University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany, [2]University Hospital Cologne, Department of Translational Genomics, Cologne, Germany, [3]Essen University Hospital, Clinic for Hematology and Stem Cell Transplantation, Essen, Germany, [4]University Medical Center Mainz, Institute for Immunology, Mainz, Germany, [5]BioNTech SE, Mainz, Germany

Lung cancer is one of the leading causes of cancer-related deaths with small cell lung cancer (SCLC) being an exceptionally aggressive form. SCLC is a tumor type of neuroendocrine origin with a high intra- and intertumoral heterogeneity and extremely poor response to checkpoint inhibition, despite harboring a high mutational burden. Four molecular subtypes of SCLC can be defined by expression of master regulators - Ascl1, NeuroD1, POU2F3 and Yap1 that have neuroendocrine (NE) or non-neuroendocrine (non-NE) features, reflecting different degrees of cellular differentiation. Hereby, Ascl1-high tumors (SCLC-A) show the highest level of neuroendocrine (NE) gene expression, while Yap1-high tumors (SCLC-Y) are considered non-NE. Despite the high level of heterogeneity, SCLC in clinic is treated as a single disease, leading to a survival-rate of lower than 5 % and new therapeutic approaches are desperately needed. Our aim is to study subtype-related differences of immune interaction and mRNA-based vaccination approaches to establish next generation immunotherapeutic strategies for SCLC. For this purpose, we established and characterized cell-line based multimetastatic murine SCLC models reflecting different subtypes of SCLC. Interestingly, cell lines reflecting different subtypes spread into different organs upon intravenous injection. Using histological analyses, we detected a pronounced subtype-specific difference in the spatial distribution of immune cells. $CD11b^+$ macrophages and dendritic cells can be identified as most prominent cell types in the TME, while T cells are nearly absent in neuroendocrine SCLC-A tumors. In contrast, SCLC-Y tumors are readily infiltrated by $CD4^+$ and $CD8^+$ T cells. High throughput qPCR analysis of tumors enabled us to identify differences in cytokine levels encoding for pro-survival signaling in T cells, such as IL15 or IL18, as well as cytokines supporting T cell infiltration and M0 to M2 differentiation such as members of the CXCL9/10/11 pathway. Subtypes furthermore show differences in T cell recognition in vitro, as shown by IFN-g production of CD8+ T, likely caused by low MHC-I expression of SCLC-A tumor cells. Using RNA-Seq, we identified low expression of Tap1/2 in SCLC-A cell lines that is in line with previous reports in literature. We are therefore currently investigating the effect of epigenetic modulators targeting histone-methylation

pathways to modulate subtype identity as well as MHC-I expression in order to create synergy with mRNA vaccination. In summary, our model confirms clinical observations of differential immune infiltration and T cell escape in different subtypes of SCLC enabling us to study mRNA vaccination efficacy and immune interaction in a subtype-specific model. Future experiments will include combinatory treatments of RNA vaccination and epigenetic drugs in order to improve therapeutic options in SCLC.

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DC-targeted nanoparticles to evoke anti-tumor immune responses

K. Schouren[1], C. Le Gall[2], B. van der Schoot[3], M. Verdoes[3], J. de Vries[3]
[1]Radboudumc, Medical Biosciences (previously Tumor Immunology), Nijmegen, The Netherlands, [2]Boston Children's Hospital, Boston, United States of America, [3]Radboudumc, Nijmegen, The Netherlands

Dendritic cells (DCs) orchestrate the immune system and thereby harness the potential to elicit potent anti-tumor immune responses. Therapeutic vaccination with ex vivo matured and tumor antigen-loaded DCs can induce durable T cell responses without severe side effects. However, DC vaccination is costly, labor intensive, relies on sophisticated infrastructures and suffers from low response rates. To address these issues, we aim to set up an off-the-shelf DC-targeted nanoparticle platform which activates and loads DCs with tumor antigens in vivo. The nanoparticles consist of the biocompatible and biodegradable polymer PLGA, and the block-co-polymer PLGA-PEG which is equipped with an azide moiety for click chemistry. To enhance DC-specific uptake of nanoparticles, the nanoparticle surface is engrafted with DC-targeting molecules. Site-specific nanoparticle functionalization is established via strain-promoted alkyne azide click chemistry and enzymatic peptide ligation. Within this project we seek to find the optimal combination of a DC-specific target and a targeting molecule, for effective DC activation and subsequent tumor-specific T cell responses.

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Endogenous Glucocorticoids Immunomodulatory Role of in Ovarian Cancer

A. Seida
Cairo University, Immunology and Microbiology Department, Cairo, Egypt

Background: Tumour-infiltrating myeloid-derived suppressor cells (MDSC) or tumour-associated macrophages (TAM) which are abundant in ovarian cancer show a high expression of the enzyme 11Beta-Hydroxysteroid dehydrogenase I (11b-HSD1). This enzyme is essential for the conversion of biologically inactive cortisone into active cortisol which has been detected in ascitic fluid and tumour exudates from ovarian cancer patients. Considering that cortisol has strong suppressive effects on all kinds of immune cells, we hypothesize that the activation of endogenous glucocorticoids by MDSC or TAM may contribute to the immune escape of ovarian cancer. Material and methods: Using immunohistochemistry, real-time PCR, luminescent immunoassays (LIA), immunofluorescent double staining and adoptive transfer of glucocorticoid receptor knock out immune cells into immune deficient mouse model for ovarian cancer. Results: We found that 11b-HSD1 enzyme is highly expressed in human and murine ovarian cancer tissue. Luminescent immunoassays (LIA) showed elevated cortisol levels in serum, ascites and tissue exudates from ovarian cancer patients as compared to healthy controls. Immunofluorescent double staining revealed

a co-localization of 11b-HSD1 with CD14, CD68, and CD85, but not with EpCAM. Expression of 11b-HSD1 can thus be attributed to tumour associated macrophages (TAM) or myeloid derived suppressor cells (MDSC).To test our hypothesis about activation of endogenous glucocorticoids by immune cells may contribute to the immune escape of ovarian cancer is now being tested in PTEN$^{loxP/loxP}$; loxP-Stop-loxP-kras^{G12D} mice which spontaneously develop ovarian cancer after intra-bursal injection of adenoviral Cre recombinase. The ongoing experiments involve adoptive transfer of glucocorticoid receptor knock out immune cells as well as pharmacological inhibition of 11b-HSD1 which shall be combined with various immune stimuli. In a first functional in vivo assay, the adoptive transfer of glucocorticoid receptor-deficient T cells led to increased immune cell infiltration of the tumour tissue - which did not translate into prolonged survival. Instead, infiltrating T cells assumed mostly a Foxp3^{+} (regulatory) phenotype and survival was even shortened. Conclusion: We thus propose that endogenous glucocorticoids exert immunomodulatory functions in ovarian cancer. Their putative role in tumour immune escape, however, needs to be assessed in context of further tolerogenic mechanisms that may be simultaneously present.

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Visceral adipose tissue proximity as a biomarker of response to immunotherapy in epithelial ovarian cancer

M. Suarez-Carmona[1], A. Pöchmann[1,2], X.-W. Zhang[1,3], S. Grauling-Halama[1], S. Schott[4], N. Halama[1,3,5]
[1]Helmholtz-Institute for Translational Oncology Mainz, Translational Immunotherapy, Mainz, Germany, [2]Ruprecht Karl University Heidelberg, Faculty of Biosciences, Heidelberg, Germany, [3]National Center for Tumour Diseases (NCT), University Hospital Heidelberg, Medical Oncology, Heidelberg, Germany, [4]Heidelberg University Hospital, department of Obstetrics and Gynecology, Heidelberg, Germany, [5]University medical Center, University Cancer Center (UCT), Mainz, Germany

While durable responses to immune checkpoint blockade occur in epithelial ovarian carcinoma (EOC) patients, the low response rates preclude routine implementation of immunotherapy. We report that omental metastases are required for response to immunotherapy in EOC in a retrospective trial. To achieve patient stratification using non-invasive methods, we apply machine learning to radiomics to assess omental involvement. Omental metastases harbor a specific immuno-metabolic landscape, including lymphocyte exclusion maintained by a CCL5-generated chemokine trap at the tumour-omentum interface and lymphocyte fitness. Tumor-associated macrophage (TAM) dysfunction is also observed in omental metastases and is related to cellular lipid content and processing. In patient-derived tumor explants, maraviroc-mediated CCR5 inhibition or CD36 blockade repolarize TAMs, allowing the re-allocation of tumor infiltrating lymphocytes (TILs) towards the tumor core and supporting the anti-tumor immune response in omental metastases. Biologically, TAMs exposed to lipids are burdened with oxidative distress and activate response pathways including HDAC3-dependent stress response and ferroptosis. Maraviroc or CD36 blockade restore oxidative eustress, reestablishing their proper response to danger signals. Collectively, our findings illustrate a metabolic-inflammatory equilibrium that can be modulated to increase response rates to immunotherapy in EOC.

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Development of a model to analyze the relevance of major histocompatibility complex class II epitopes for hepatocellular carcinoma immunity

L. L. Suhl, D. Ostroumov, A. Menze, S. Sauer, A. Kumthekar, H. Wedemeyer, F. Kühnel, T. Wirth
Hannover Medical School, Department of Gastroenterology, Hepatology, Infectious Diseases and Endocrinology, Hannover, Germany

The role of CD4 T cells in tumor immunity is not yet understood, as CD4 T cells recognize major histocompatibility complex (MHC) class II epitopes expressed on antigen-presenting cells but not on tumor cells. Nevertheless, they send critical signals to dendritic cells, which then upregulate the expression of MHC class I molecules, costimulatory molecules, and secretion of cytokines (signal III). The overall goal of our study is to determine the importance of helping CD4 T cells in the form of MHC class II epitopes in adaptive immune responses and immunotherapy of hepatocellular carcinoma (HCC). Mice underwent implantation with HCC cells with different expression of MHC class II epitopes, the resulting tumor growth was monitored and endogenous specific CD8 and CD4 T cell immune responses in peripheral blood were investigated by intracellular staining for interferon gamma and analyzed by flow cytometry. Using a heterologous T cell vaccination that allows for rapid and strong T cell responses, we generated CD8 T cell responses specific for a MHC class I neoepitope, as well as CD4 T cell responses specific for a MHC class II neoepitope. Boosters were performed with or without concurrent administration of the MHC class II epitope. The T cell responses were analyzed in peripheral blood and spleen by flow cytometry. CD8 specific responses were higher in mice inoculated with tumors expressing both MHC class I and MHC class II epitopes compared to tumors only expressing MHC class I epitopes. The specific CD8 T cell response was even higher with tumors containing the class II, major histocompatibility complex, transactivator (CIITA). Additional experiments will involve investigation of cancer-directed T cell responses using immune checkpoint blockade. The best vaccination regimen and booster peptides will again be tested to identify the best vaccine/booster combination. The therapeutic efficacy of this combination against different HCC cell lines will then be investigated in a subcutaneous cancer model where tumor growth, overall survival and specific immune response towards the MHC class I and class II neoepitopes in the peripheral blood and organs will be analyzed. Our study will bring us closer to understanding the importance of CD4 T cell help in HCC-directed CD8 T cell immune responses.

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Overcoming Challenges in Neoantigen-Based Cancer Vaccines: Harnessing mRNA vaccination against Intratumoral Heterogeneity

S. Thevissen[1], F. Lang[1], B. Schrörs[1], N. Salomon[1], F. Vascotto[1], Y. Saumels[2], M. Diken[1]
[1]TRON, Mainz, Germany, [2]Weizmann Institute, Rehovot, Israel

Neoantigen-based cancer vaccines offer a promising treatment strategy in personalized immunotherapy. However, their efficacy is hampered by limitations such as poor immunogenicity and standardized target identification especially in heterogenous tumors. Moreover, a major limitation for the clinical efficacy of tumor vaccine approaches can be mainly attributed to tumor heterogeneity. Emerging evidence suggests that tumors are not composed of a single tumor clone, but are rather polyclonal, which is often a reason for treatment failure due to secondary resistance. In essence, high intratumoral heterogeneity is associated

with poor immune response and is thus predictive for worse survival in patients.
We aim to elucidate the impact of the immune system on tumor subclones in a heterogenous murine tumor model and how heterogenous tumors can be targeted by personalized neoantigen-specific mRNA vaccines. Therefore, our team established a heterogenous tumor model based on the B2905 murine melanoma cell line with different degrees of heterogeneity. Through multiomics analysis encompassing genomic, transcriptomic, and immunopeptidomic profiling, we identified B2905-specific neoantigens as potential targets for cancer vaccines. We conducted a comprehensive immunogenicity screen of 130 candidates and immunogenic neoantigen candidates were selected for vaccine studies in heterogeneous tumors. Yet, we specifically restricted our study to neoantigens arising from non-synonymous somatic single nucleotide variants (SNVs). However, to enlarge the scope of tumor antigens, we currently investigate self-antigens as well as cryptic peptides as potential targets for mRNA vaccines. Central to our approach is the observation of neutral tumor clonal evolution as well as neoantigen repertoire dynamics and how both changes during antigen-based mRNA vaccination. We and others found that the immune system shapes intratumoral heterogeneity and clonal composition. A deeper understanding, especially in the interaction between the immune system and tumor subclones, can guide the rational selection of tumor antigens and inform the design of a vaccine tailored to target heterogeneous tumor. We plan iterative refinements for different vaccine approaches as well as combinational therapies to reduce the risk of clonal selection and therapeutic resistance. In line with that, we aim to identify a therapeutic setting that fosters epitope spreading after antigen-specific vaccination. Epitope spreading is a process in which anti-tumoral T cell responses are expanded against secondary epitopes that were not part of the initial therapeutic vaccine, which can facilitate complete tumor clearance of a heterogenous tumor. By addressing the complexity of intratumoral heterogeneity and expanding the repertoire of therapy options, we strive enhance the transformative potential of mRNA-based cancer vaccines in heterogeneous tumors.

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Advanced spatial transcriptomics identifies stages of tertiary lymphoid structure formation in glioblastoma

A. Vaccaro[1], F. Yang[1], T. van de Walle[1], S. Franke[1], R. Lugano[1], A. Denés[2,3], A. Magoulopoulou[4], I. Cid[5], A. Smits[2,3], S. Libard[1,6], M. Nilsson[4], L. He[1], T. Olsson Bontell[7,8], A. S. Jakola[2,9], M. Ramachandran[1], A. Dimberg[1]
[1]Uppsala University, Department of Immunology, Genetics and Pathology, Uppsala, Sweden, [2]University of Gothenburg, Department of Clinical Neuroscience, Institute of Neuroscience and Physiology, Gothenburg, Sweden, [3]Sahlgrenska University Hospital, Department of Neurology, Gothenburg, Sweden, [4]Stockholm University, Department of Biochemistry and Biophysics, Science for Life Laboratory, Solna, Sweden, [5]University of Magallanes, Laboratorio de Medicina Molecular CADI-UMAG, Punta Arenas, Chile, [6]Uppsala University, Department of Pathology, Uppsala, Sweden, [7]University of Gothenburg, Department of Physiology, Institute of Neuroscience and Physiology, Gothenburg, Sweden, [8]Sahlgrenska University Hospital, Department of Clinical Pathology, Gothenburg, Sweden, [9]Sahlgrenska University Hospital, Department of Neurosurgery, Gothenburg, Sweden

Tertiary lymphoid structures (TLS) are ectopic lymphoid aggregates that form in inflamed tissues, which correlate with increased T cell infiltration and improved prognosis in numerous solid tumors. To date, the steps underlying the process of TLS formation are unknown, and their prognostic value in central nervous

system cancers remains to be elucidated. In this study, we found that the presence of TLS in surgically-resected tissues correlates with improved survival in a large cohort of glioblastoma (GBM) patients. Coupling advanced spatial transcriptomics of human glioma tissues with studies in murine glioma models, we demonstrated that TLS formation starts with the assembly of T cell-rich clusters harboring a large proportion of $CD4^+$ helper T cells, which were encapsulated by collagen IV fibers into tightly packed perivascular niches. The presence of $MMP2^+$ fibroblasts correlated with a loss of collagen IV encapsulation and a TLS-like organization of the lymphoid structures. TLS T cells expressed lymphoid tissue inducer (LTi) molecules, and depletion of $CD4^+$ T cells prevented TLS formation, suggesting an important role of these cells as LTis. Finally, B cell recruitment in the TLS was preceded by that of T cells, resulting in the assembly of T cell-rich TLS first, followed by the development of canonical B cell-rich TLS. In summary, our work defines TLS as a positive prognostic factor in GBM and identifies critical stages of TLS assembly. These findings have broader implications for TLS research and the immuno-oncology field.

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High infiltration of virtual memory T cells is unique to untreated liver metastases but OX-40 agonism in conjunction with PD-L1 targeting uniformly stimulates tumoricidal CD8 T cell infiltration across all metastases.

T. van der Sluis[1], S. van Duikeren[1], S. Cai[1], S. Keizer[1], D. Veerkamp[1], S. Mostert[1], N. Mülling[1], M. Beijnes[1], S. van der Burg[2], E. Kapiteijn[2], R. Arens[1]
[1]Leiden University Medical Center, Immunology, Leiden, The Netherlands, [2]Leiden University Medical Center, Medical Oncology, Leiden, The Netherlands

Cancer immunotherapy has strongly improved the prognosis of a subset of cancer patients, but the results are disappointing in patients with metastatic disease. It has become clear that synchronous metastases (i.e. metastasis isolated from anatomically distinct locations in one patient on the same timepoint) can differ in immune composition, which can impact the responsiveness to (immune-) therapy. Furthermore, immunotherapy with PD-1/PD-L1 targeting therapy is less efficient in metastatic patients with tumor lesions in the liver. These studies highlight that a better understanding of intralesional heterogeneity is important as specific targeting of these differences may directly impact patient survival. Here we hypothesized that lesion specific immune infiltration is not only shaped by the tumor but also by the target organ. Therefore, we set out to analyze the immune infiltrate in tumors that were located in different organs. First, we injected melanoma cells intravenously in mice, resulting in tumor growth in different tissues. Interestingly, we noticed that the immune infiltrate of liver tumors was clearly distinct from the infiltrates in tumors located in other tissues. Unexpectedly, these difference did not relate to tissue resident myeloid and T cell subsets. In fact, we noticed a strong increase in virtual memory T cells (Tvm). Analysis of multiple organs from naïve mice showed that Tvm were more abundantly present in the liver than in other organs, indicating that the liver could shape the local tumor immune infiltrate. Further characterization of these Tvm showed that their proliferation and metabolic profile was similar to Tvm in different tumor locations and different memory $CD8^+$ T cell populations. PD-L1 blockade increased the number of $CD8^+$ T cells in liver with an effector-memory phenotype ($CD44^+/CD62L^-$), while Tvm cells were barely affected. Furthermore, the cytolytic activity $CD8^+$ T cells was not impacted. Next, we aimed to further improve therapy responses by using a combination of αPD-L1 and OX-40 ligation (PDOX), which

jointly increases the effector potential. Using this therapy combination, we noticed that in contrast to anti-PD-L1 monotherapy, PDOX combination therapy increased the abundance of $CD8^+$ T cells across tumors located in different tissues, including the liver. Further characterization of these cells showed that while the abundance of Tvm in the liver remained stable, a strong infiltration of $CD8^+$ T cells with an cytolytic effector phenotype was observed. In conclusion, our data shows that the liver has an imprint on the tumor immune infiltrate, specifically on Tvm. PDOX therapy does not affect Tvm cells but uniformly increases tumoricidal $CD8^+$ T cell infiltration, showing that PDOX therapy may be effective in patients with metastatic disease, including those with liver metastasis.

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Complement activation induces immunogenic cell death in human melanoma cell lines

S. Vanbellingen, M. Gonçalo, S. Nedyalkova, J. L. Aerts
Vrije Universiteit Brussel, Neuro-Aging and Viro-Immunotherapy, Jette, Belgium

Despite being one of the oldest and most extensively characterized immunologic pathways in health and disease, the significance of the complement system in tumor immunology is often overlooked. Overall, its role within the tumor microenvironment remains controversial, since it can exhibit both tumor-promoting and tumor-suppressing properties, depending on the tumor type, stage and specific complement factor(s) involved. To safeguard host cells against complement-mediated lysis, the complement system is tightly regulated at different stages by a series of complement regulatory proteins (CRPs), such as CD46, CD55 and CD59. However, several reports have indicated that overexpression of CRPs on cancer cells is associated with a poorer prognosis. While some studies have explored the impact of blocking or downregulating CRPs on tumor growth and survival, particularly in B-cell lymphoma cell lines, the functional role of CRPs in melanoma remains largely unexplored. CD59 is a membrane-bound CRP that inhibits the very last step in the formation of the membrane attack complex, which has been shown to be rate-limiting. Therefore, we aim to investigate the effect of knocking-out (KO) CD59 in melanoma cell lines on complement mediated tumor cell death and subsequent anti-tumor immune responses.
The expression of CD46, CD55 and CD59 on human melanoma cell lines and the Raji B-cell lymphoma cell line was analyzed by flow cytometry. CD59 was successfully knocked-out in the 624-mel and 1087-mel cell lines by using the CRISPR-Cas9 technology. To assess complement-dependent cell lysis (CDC), both knockout (KO) and wildtype (WT) variants of the 624-mel and 1087-mel cell lines were treated with normal human serum (NHS). Eculizumab, an anti-C5 monoclonal antibody (mAb) served as a negative control. The effect of the complement system on cell death was monitored using the IncuCyte Live Cell Analysis system. Annexin-V staining was employed to measure the phosphatidylserine (PS) flip flop indicating regulated cell death. Additionally, calreticulin (CRT) exposure, indicative of immunogenic cell death, was assessed using an anti-CRT mAb, and vesicular ATP release was detected with quinacrine via flow cytometry. While all tested melanoma cell lines exhibited positivity for CD46, CD55 and CD59, their expression levels varied on a per cell basis. Our findings demonstrated that even low concentrations of NHS induced cell death in CD59 KO cell lines, contrasting with their WT counterparts. Moreover, we observed PS flip-flop, CRT exposure and ATP release preceding cell membrane disruption, suggesting that melanoma cells undergo complement induced immunogenic cell death. Overall, these findings underscore the critical role of CRPs, particularly CD59, in shielding tumor cells from complement-mediated lysis and emphasize the potential of harnessing

the complement system by targeting CD59 as a therapeutic strategy in the treatment of melanoma and potentially other cancers.

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Targeting and Activation of NK cells using Cytokine-functionalized Immunofilaments

L. Weiss[1,2,3], L. Verhoeven[1], P. K. J. D. de Jonge[4], D. Zaharoff[5], H. Dolstra[4], K.-J. Malmberg[6,7,8], C. G. Figdor[1,2,3], E. Sohlberg[6], R. Hammink[1,2]
[1]Radboudumc, Department of Medical BioSciences, Nijmegen, The Netherlands, [2]Oncode Institute, Division of Immunotherapy, Nijmegen, The Netherlands, [3]Institute for Chemical Immunology, Nijmegen, The Netherlands, [4]Radboudumc, Department of Hematology, Nijmegen, The Netherlands, [5]North Carolina State University, Raleigh, United States of America, [6]Karolinska Institutet, Department of Medicine Huddinge, Stockholm, Sweden, [7]Institute for Cancer Research, Oslo University Hospital, Department of Cancer Immunology, Oslo, Norway, [8]The University of Oslo, The Precision Immunotherapy Alliance, Oslo, The Netherlands

Natural Killer (NK) cells are cytotoxic lymphocytes highly proficient in cancer cell elimination. Cancer cell elimination by NK cells can be directed using cancer cell-targeting antibodies. These antibodies bind to specific cancer cell antigens and mark the cancer cell for NK cells. NK cells expressing the CD16 receptor, which binds to the fragment crystallizable region of the antibody, are triggered to release a cytotoxic cocktail to kill the antibody-opsonized cancer cell. This mechanism – called antibody-dependent cellular cytotoxicity – is thought to play a key role in monoclonal antibody therapies currently used in the clinic as cancer therapies. Pre-clinical data demonstrated that cytokines can greatly benefit monoclonal antibody therapies. Particularly, IL-12 is a potent cytokine which enhances NK cell cytotoxicity and IFNγ secretion which can further support anti-tumor immune responses. Yet, administering cytokines, like IL-12, systemically to patients is often associated with severe adverse side effects. To overcome this limitation, innovative tools like synthetic biomaterials can be engineered which can deliver therapeutically effective doses of cytokines and other immunomodulatory molecules in a precisely defined and highly controllable manner. To this end, we developed a nanosized filamentous synthetic polymer to which we covalently attach different biomolecules with high control over their multivalent presentation by using straightforward click-chemistry. The so-called immunofilaments for NK cell stimulation were decorated with varying amounts of IL-12 and CD16-stimulating and/or tumor-targeting antibodies, Importantly, the backbone of the polymer is based on polyisocyanopeptides, endowing it with a semiflexible character, which can potentially support receptor rearrangements on the NK cell membrane to lead to improved signal integration. Here, we evaluate immunofilaments for their ability to stimulate NK cells and induce tumor cell killing. Immunofilaments functionalized with both IL-12 and CD16-targeting moieties outperformed immunofilaments functionalized with one type of biomolecule in binding efficiency to NK cells, thereby increasing the pool of potential effector cells. In addition, dual delivery through immunofilaments enhances NK cell activation, degranulation and production and secretion of cytokines, as well as cytotoxicity synergistically. Taken together, immunofilaments are a novel and versatile biomaterial-based platform to effectively activate NK cells and boost cytotoxicity towards cancer cells.

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Dissecting the single-cell landscape of leukemic mantle cell lymphoma

T. Wertheimer[1,2], H. Baniadam[3], S. Reisenauer[4], M. Pohly[5], J. Gawron[5], L. Rindlisbacher[2], M. Lin[2], S. Kummer[4], S. Kreutmair[2,4], S. Unger[2], J. Kivioja[4], N. Beerenwinkel[5], A. Moor[5], W. Huber[3], B. Becher[2], T. Zenz[4,6]
[1]University Medical Center Freiburg, Internal Medicine I, Freiburg, Germany, [2]University of Zurich, Institute of Experimental Immunology, Zurich, Switzerland, [3]European Molecular Biology Laboratory, Heidelberg, Germany, [4]University Hospital Zurich, Medical Oncology and Hematology, Zurich, Switzerland, [5]ETH Zurich, Dept. of Biosystems Science and Engineering, Basel, Switzerland, [6]The LOOP Zurich Institute, Zurich, Switzerland

Background:
Resistance to therapy remains a major challenge for Mantle Cell Lymphoma (MCL) patients, despite a diverse and expanding treatment landscape. There is thus a crucial need to better understand the mechanistic underpinnings of therapy resistance to optimize treatment selection. Given the advent of immunotherapies in recent years, there has been increasing evidence that not only tumor-intrinsic factors affect therapy resistance, but that tumor-immune cell interactions are an additional important contributor. In the context of MCL, such interactions have been poorly characterized, but hold the promise of identifying novel treatment approaches and better understanding resistance mechanisms to commonly applied therapies such as BTK inhibition.

Aims and methods
This study aimed to investigate the single-cell landscape of leukemic MCL using single-cell profiling (high-dimensional cytometry and CITE-seq) of 24 patients and 10 healthy donors. In addition, we performed in vitro drug testing on a subset of patient samples (n=12; 6 treatments) to understand the effects of drugs on both tumor and immune cells in MCL. To assess the disease specificity of the immune landscape, we generated single-cell data from other B-NHL (n=60).

Results:
Our data indicate profound changes in the T cell compartment of the peripheral blood in leukemic MCL patients. More specifically, we observed an expansion of highly activated regulatory T cells (~5% of T cells) along with a high fraction of several dysfunctional CD8+ T cells subsets. We further sought to link tumor cell signatures with the healthy immune cell signatures to assess potential interactions and mechanisms of immune escape. The malignant B cells displayed high expression of inhibitory immune checkpoint ligands such as PVR, HLA-G, CD70 and PD-L1 and antiphagocytic proteins such as CD47 and CD24, which may contribute to the dysfunctional T cell compartment. In line with this, we discovered that high expression of the immune checkpoint ligand CD70 on malignant B cells was inversely associated with an activation module in CD4+ T cells. Through cell-cell communication analysis, we identified that the CD70 signaling pathway was most active between B cells and regulatory T cells. Finally, in patients undergoing in vivo ibrutinib treatment we found a less pronounced suppressive phenotype in the effector regulatory T cells and concomitantly observed an increase of proteins crucial for cytotoxic function such as Granzyme B and Interferon-γ in effector T cells. Interestingly, we detected a significant reduction of CD70 expression in malignant B cells after in vitro drug treatment with ibrutinib.

Summary/Conclusion:
This study provides insights into the single-cell landscape of leukemic MCL and how tumor-immune cell interactions may contribute to disease progression and treatment resistance. In particular, we suggest

CD70 signaling pathway as a potential axis of immune escape in MCL that could be targeted via ibrutinib.

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Phenotypic diversity of T cells in primary and metastatic brain tumors

V. Wischnewski[1,2,3,4], R. R. Maas[2,3,4,5], K. Soukup[2,3], G. Galletti[6], M. Kornete[2,3], P. Guerrero Aruffo[2,3], N. Fournier[7], P. Wirapati[7], J. Lourenco[7], A. Scarpa[6], R. T. Daniel[5], A. F. Hottinger[2,8], J.-P. Brouland[9], A. Losurdo[10], E. Voulaz[11,12], M. Alloisio[11,12], M. E. Hegi[4,5], J. A. Joyce[2,3,4]

[1]TRON - Translational Oncology at the University Medical Center of the JGU Mainz, Mainz, Germany, [2]Department of Oncology, University of Lausanne, Lausanne, Switzerland, [3]Ludwig Institute for Cancer Research, University of Lausanne, Lausanne, Switzerland, [4]Lundin and Family Brain Tumor Research Center, Lausanne, Switzerland, [5]Neuroscience Research Center, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland, [6]Laboratory of Translational Immunology, IRCCS Humanitas Research Hospital, Milan, Italy, [7]Translational Data Science, Swiss Institute of Bioinformatics, Lausanne, Switzerland, [8]Department of Oncology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland, [9]Department of Pathology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland, [10]Oncology Department, IRCCS Humanitas Research Hospital, Milan, Italy, [11]Department of Biomedical Sciences, Humanitas University, Milan, Italy, [12]Division of Thoracic Surgery, IRCCS Humanitas Research Hospital, Milan, Italy

Despite improvements in tumor detection and local treatment, as well as the introduction of novel therapies including molecular-targeted and immune-based approaches, the prognosis for patients diagnosed with aggressive brain cancer remains poor. The estimated 2-year survival rates are < 20%, underscoring the need to better understand this disease as a means to develop effective therapies.

Although cytotoxic T cells are generally excluded from healthy brain parenchyma to prevent excessive neuro-inflammation under homeostatic conditions, the impairment of the blood-brain barrier (BBB) in advanced brain malignancies can facilitate the infiltration of peripheral immune cells, including T cells. To interrogate potential roles of T cells in brain tumors, we profiled these cells from patients with primary or metastatic brain cancers, via integrated analyses on the single-cell and bulk population levels. We identified both interpatient similarity and variability in T cell biology, with phenotypical differences being most pronounced in a subgroup of brain metastasis (BrM) patients, characterized by accumulation of CXCL13-expressing CD39+ potentially tumor-reactive T cells (pTRT). In this subgroup, high pTRT abundance was comparable to that in primary lung cancer, whereas all other brain tumors had low levels, similar to primary breast cancer. These findings indicate that T cell-mediated tumor reactivity can occur in specific BrM, thereby providing a strategy for future patient stratification for treatment with immunotherapy.

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An inflammatory microenvironment state remodels the Bone Marrow and T-cell landscape in Human Clonal Hematopoiesis and Myelodysplastic Syndromes

K. Woods*[1,2], K. Prummel*[3], M. Kholmatov*[3], G. Poschmann[4], K. Stühler[4], R. Wehner[5], M. Schmitz[5], M. Theobald[1,2,6], J. Zaugg[3], B. Guezguez[1,2,6]

[1]University Medical Center Mainz (UMC), III. Med (Hematology/Oncology), Mainz, Germany, [2]German

Cancer Consortium (DKTK), Partner site Frankfurt/Mainz, Mainz, Germany, [3]European Molecular Biology Laboratory (EMBL), Heidelberg, Germany, [4]Heinrich-Heine University Düsseldorf, Düsseldorf, Germany, [5]Faculty of Medicine Carl Gustav Carus, TU Dresden, Dresden, Germany, [6]Research Center for Immunotherapy (FZI), University Medical Center Mainz (UMC), Mainz, Germany

*Contributed equally

Hematopoiesis is an essential process for blood development that is tightly regulated by the intrinsic properties of hematopoietic stem/progenitor cells (HSPC). In turn, HSPC are extrinsically maintained in the bone marrow (BM) microenvironment by complex networks of stromal cells, including mesenchymal stromal cells (MSCs) and their derived progeny (osteoblasts, adipocytes), as well as immune cells (T cells). Upon aging, HSPCs acquire somatic mutations in epigenetic regulators (DNMT3A, TET2, SF3B1,…) that can lead to the development of Clonal Hematopoiesis of Indeterminate Potential (CHIP). In some individuals, CHIP progresses towards stem cell-driven myeloid neoplasms, most commonly myelodysplastic syndromes (MDS). Recent evidence suggests that BM niche remodeling contributes to disease progression in hematological malignancies by generating a pro-inflammatory and tumor-supportive microenvironment. However, the specific cellular interactions leading to this remodeling in CHIP and associated MDS, as well as its consequences on T cell functions, remain to be fully explored.

Our study aims to resolve the extent of BM niche reshape and to identify the major cell subsets responsible for instigating the inflammatory state across the CHIP/MDS disease spectrum. Using FACS-based enrichment strategy for single-cell RNA-seq (10X Genomics, SORT-Seq) combined with high-resolution multispectral tissue imaging, we analyzed the pathological interplay between $CD34^+$ HSPC, $CD271^+$ MSC, and $CD3^+$ T cell populations from a curated cohort of matching BM liquid and trephine biopsies from a prospective longitudinal CHIP/MDS study (NCT02867085). Moreover, we used single-cell long-read sequencing (RaCH-seq) to identify mutated HSPCs, allowing us to computationally unveil their specific cross-talk with inflammatory stromal cells and T cells in CHIP/MDS.

Bone imaging revealed significant BM remodeling in CHIP-carriers (n=9) compared to healthy donors (n=10) and associated with emergence of inflammatory MSCs and expansion of sinusoidal endothelium. These stromal changes were exacerbated in MDS patients (n=16) by a buildup of exhausted regulatory T cells. These findings were supported by scRNA-seq (n=10) with the identification of stress-induced MSC subsets in BM-CHIP and enrichment of inflammatory IFN-responsive cytotoxic T cell subsets in BM-MDS, especially those harboring SF3B1-Mutation. Lastly, cytokine profiling assessed a specific IL1-β/TNF inflammatory signature while proteomic analysis from BM-MDS co-culture model replicated these inflammatory and pro-angiogenic processes, emphasizing the importance of these cues for stroma remodeling.

In conclusion, our study revealed novel insights into BM niche adaptations under inflammatory stress with specific reshape of stromal and immune landscapes. Taken together, our findings pave the way for further research into this inflammatory BM phenotype as a possible risk stratification tool for disease progression in CHIP/MDS.

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Circulating tumor antigen reactive effector T cells contain precursors of intratumoral regulatory T cells in breast cancer patients

M. Xydia[1], S. Laidlaw[2], M. Tarabichi[2], R. Lohmayer[1], I. Macaulay[2], T. Voet[2,3], R. Rahbari[2], P. Beckhove[1]
[1]LIT - Leibniz Institute for Immunotherapy (former RCI), Regensburg, Germany, [2]Wellcome Sanger Institute, Hinxton, United Kingdom, [3]KU Leuven, Leuven, Belgium

Cancer patients are enriched with Tumor Antigen (TA)-specific conventional T cells (Tconv), including TA-reactive effector T cells (Teff), that can recognize and kill cancer cells. Thus, TA-Teff are a great tool of modern cancer immunotherapies that aim to enhance their numbers and cytotoxic function in tumors. However, a major obstacle of cancer immunotherapy are regulatory T cells (Treg) that produce cancer-promoting factors and suppress TA-Teff, inhibiting tumor eradication. Thus, efficient anti-tumor immunity depends on mechanisms that drive Treg and TA-Teff repertoire formation. Although many studies have focused on intratumoral T cells, little is known about the T Cell Receptor (TCR) and transcriptomic repertoire of circulating TA-Teff in cancer patients. Here we analyzed single-cell transcriptome and TCR sequencing data of $CD4^+$ TA-Teff in blood of breast cancer patients compared to $CD4^+$ T cells infiltrating the respective primary breast tumors. Despite their homogenous capacity to secrete IFNγ after TA-specific stimulation, circulating TA-Teff separated into two clusters with distinct gene expression profiles and functions. In the first cluster, marker and differential gene expression analysis revealed the upregulation of cytotoxic genes, while Gene enrichment analysis showed its high similarity with intratumoral Th1 Teff, confirming its tumor-killing capacity (Killers). However, the second cluster exerted enhanced transcription of genes associated with immune-suppressive Treg and Treg induction from Tconv. Its transcriptome resembled intratumoral Treg, suggesting a Treg-like phenotype. While Killers lacked CCR7 and CD62L, supporting their effector state, Treg-like Teff expressed both genes, indicating a naive or central memory profile. Moreover, CellTypist analysis identified Killers as cytotoxic Teff but Treg-like Teff as naive or central memory Treg. Treg-like Teff, Killers and intratumoral Early activated Tconv shared identical TCRs, proving their common clonal origin. Indeed, based on Slingshot trajectory analysis, Early activated Tconv are not only progenitors of circulating Killers and Treg-like Teff, but also differentiate within the tumor towards either cytotoxic Th1 Teff or into immune-suppressive and pro-tumorigenic Treg. Most importantly, Treg-like Teff constitute an intermediate step in the developmental trajectory of Early Activated Tconv conversion into Treg. In conclusion, we reveal the functional division of circulating TA-Teff into cytotoxic Killers and Treg precursors that can convert into fully functional effector Treg after their intratumoral activation. As TA-Teff are a major source of cell therapy products and a key target of modern therapies for cancer patients, this finding has important implications for anti-tumor immunity and the design of efficient immunotherapies against cancer.

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Synergistic systematic analyses of therapeutic response mechanisms in renal cell carcinoma with fully human tissue models and in silico modelling of “copycats”

X.-W. Zhang[1,2], A. Pöchmann[1,3], X. Holtkotte[1], M. Suarez-Carmona[1], A. Höflich[1], J. Wolf[1], U. Prüfer[1], S. Duensing[4], S. Zschäbitz[2], D. Jäger[2], M. Gaida[5], N. Halama[1,2,6]

[1]German Cancer Research Center, Helmholtz Center for Translational Oncology (HI-TRON), Division of Translational Immunotherapy, Mainz, Germany, [2]National Center for Tumor Diseases, Department of Medical Oncology, Heidelberg, Germany, [3]Ruprecht Karls University, Faculty of Biosciences, Heidelberg, Germany, [4]University Hospital Heidelberg, Department of Urology, Heidelberg, Germany, [5]University Medical Center Mainz, Institute for Pathology, Mainz, Germany, [6]University Medical Center Mainz, University Cancer Center (UCT), Mainz, Germany

Immune checkpoint inhibitors have become part of the standard therapy for advanced clear cell renal cell carcinoma (ccRCC). However, response to immunotherapy is heterogeneous and many patients are resistant or eventually develop resistance against immunotherapies. As the tumor microenvironment of ccRCC is distinct from other solid tumors, a better understanding of functional mechanisms governing immune responses in ccRCC is needed to identify biomarkers for therapeutic responses and to unravel novel therapeutic agents to overcome primary or acquired resistance to therapies. To address this need, we developed a fully human explant model derived from freshly resected ccRCC tissue. Our tissue explant model preserves all components of the tumor microenvironment including not only tumor cells, but also the immune cell compartment as well as tumor stroma and vasculature in culture. Following treatment with combined immune checkpoint inhibition (ipilimumab plus nivolumab), the tissue explants are analyzed by immunohistochemistry staining and multiplex cytokine profiling of 50 cytokines. Spatial and functional insights are incorporated in an agent-based simulation, generating in silico models of ccRCC tumors for unlimited exploration of functional cellular dynamics in the tumor microenvironment. Spatial analyses of immune cell populations revealed clusters of $CD8^+$ and $CD4^+$ T cells and $CD163^+$ macrophages accumulating in the perivascular area in a highly vascularized tumor microenvironment. Further immunohistochemical characterization revealed PD-1 expressing lymphocytes in the perivascular area as well as CCR5 expression on endothelial cells. Cytokine profiling of untreated and treated tissue explants uncovered distinct clustering of T cell recruiting chemokines (CXCL9, CXCL10) and cytotoxic cytokines (TNFalpha, IFNgamma, TRAIL) under treatment, indicating a disconnection between immune cell recruitment and activation in the ccRCC tumor microenvironment. In silico models, derived based on the spatial and functional information from tissue explants, elucidated the combined effects of immunosuppressive macrophages and endothelial cells leading to T cell exhaustion. Our findings provide evidence for immunosuppression in the perivascular area mediated by macrophages and endothelial cells. Further characterization of immune cell compositions and functional mechanisms in the perivascular area is imperative to identify therapeutic targets for enhancing therapeutic responses to immunotherapies for ccRCC.