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ORIGINAL RESEARCH

Phosphodiesterase-5 inhibition reduces postoperative metastatic disease by targeting surgery-induced myeloid derived suppressor cell-dependent inhibition of Natural Killer cell cytotoxicity

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ABSTRACT

Cancer surgery while necessary for primary tumor removal, has been shown to induce immune suppression and promote metastases in preclinical models and human cancer surgery patients. Activating the immune system and reversing immunosuppression have emerged as promising ways to treat cancer and they can be safely employed in the perioperative period. In this study, we evaluated the immunotherapeutic potential of phosphodiesterase-5 (PDE-5) inhibitors to target surgery-induced myeloid-derived suppressor cells (MDSC) and restore natural killer (NK) cell function in the clinically relevant perioperative period. Immunocompetent murine tumor models of major surgery were used to characterize the functional suppression of surgery-induced MDSC and to assess the *in vivo* efficacy of perioperative PDE5 inhibition. In cancer surgery patients with abdominal malignancies, we assessed postoperative NK cell function following co-culture with MDSC and PDE5 inhibition. Perioperative PDE5 inhibition reverses surgery-induced immunosuppression. In particular, sildenafil reduces surgery-derived granulocytic-MDSC (gMDSC) function through downregulation of arginase 1 (ARG1), IL4Rα and reactive oxygen species (ROS) expression, enabling NK cell antitumor cytotoxicity and reducing postoperative disease recurrence. By removing surgery-derived immunosuppressive mechanisms of MDSCs, sildenafil can be combined with the administration of perioperative influenza vaccination which targets NK cells to reduce postoperative metastasis. Importantly, sildenafil reverses MDSC suppression in cancer surgery patients. These findings demonstrate that PDE5 inhibitors reduce postoperative metastasis by their ability to inhibit surgery-induced MDSC. Further clinical studies are warranted to investigate the immunotherapeutic role of PDE5 inhibitors in combination with cancer surgery.

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Introduction

Surgery is the only chance for cure for patients with solid tumors. Despite complete resection, many patients die from local or metastatic recurrence because minimal residual disease is present at the time of surgery. Increasingly, it is clear that surgery-induced natural killer (NK) cell immune dysfunction promotes metastatic disease progression. Postoperative NK cell suppression correlates with increased metastases in animal models of spontaneous[1,2] and implanted[2-4] metastases, while in human studies, low NK cell activity is associated with a higher rate of cancer recurrence and mortality.[5,6] Our translational research has shown that postoperative cancer surgery patients have a dramatic reduction in NK cell cytotoxicity.[2] Despite the clear importance of postoperative NK cell dysfunction, very few studies have characterized the mechanism of suppression[1,2,4,7] and even fewer have attempted to reverse it to reduce postoperative metastatic disease.[8-14] There are no therapies administered to prevent surgery-induced immune suppression in the current cancer treatment paradigm.

Myeloid-derived suppressor cells (MDSC) are immature precursor cells of myeloid lineage.[15] They have been most intensively studied in cancer, where they accumulate in the blood, bone marrow and tumour to inhibit innate and adaptive immunity.[15] MDSC display a diverse array of phenotypic markers and are defined by their subtype and associated suppressive activity.[16] Granulocytic MDSC (gMDSC) exert immune suppression through production of reactive oxygen species (ROS) by NAPDH oxidases which catalyzes the nitration of the T cell receptor, while monocytic MDSC (mMDSC) depletes surrounding L-arginine, an essential amino acid for T cell activation, through metabolism by *i*NOS and arginase (ARG-1).[15] There are fewer studies exploring the mechanisms of NK cell suppression by MDSC,[17] but inhibition by production of ROS,[18] ARG-1 activity[19] and by TGFβ[20,21] have been described. Our evolving understanding of MDSC biology suggests that MDSC accumulation represents a programmed response to both chronic and acute inflammatory processes.[22]

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This phenomenon of rapid MDSC accumulation and immune suppression has been demonstrated in the setting of burns,[23] sepsis[24] and trauma.[25] However, studies exploring the role of MDSC in the context of cancer surgery and their mechanism of postoperative immunosuppression is limited.[26-28]

Phosphodiesterase-5 (PDE5) inhibitors are conventionally used as therapies for non-malignant conditions.[29] Sildenafil and tadalafil are clinically approved to treat erectile dysfunction and are currently being tested in pulmonary hypertension and cardiac hypertrophy.[29] Emerging data in the fields of cancer pharmacology and immunotherapy suggest that PDE5 inhibitors are multi-targeting agents that can treat a variety of tumors. PDE5 inhibitors have been shown to exert antitumor activity by increasing intracellular cyclic GMP and its downstream effectors in breast, colon and squamous cell cancer.[30-32] Other reports describe the ability of PDE5 inhibitors to interfere with the efflux functions of the ABC transporters, therefore sensitizing cancer cells toward cytotoxic agents that are substrates of ABC transporters. Thus, these studies suggest the use of PDE5 inhibitors in combination with chemotherapy.[33-35] With the exception of one important study,[36] there are no other mechanistic reports describing PDE5 inhibitors in the modulation of the tumor microenvironment. Serafini et al. used sildenafil to alter the tumor microenvironment by enhancing antitumor immunity in preclinical models and *ex vivo* cancer patient samples by down-regulating MDSC suppressive function.[36]

Since a detailed understanding of PDE5 inhibitors as antitumor therapies is limited, further studies are warranted to establish their role and characterize their mechanism of action. The aim of this work was to characterize the activity of PDE5 inhibitors on the immunosuppressive function of MDSC in clinically relevant mouse tumor models of major surgery and cancer surgery patients with abdominal malignancies. The results of our work present evidence that surgery-derived MDSC mediate NK cell dysfunction and promotes metastatic disease, which can be reversed by perioperative administration of sildenafil.

Materials and methods

Mice – C57BL/6 (B6) and BALB/c mice were purchased from Charles Rivers Laboratory. Animals (6 weeks old females) were housed in pathogen-free conditions and all studies performed were in accordance with institutional guidelines at the Animal Care Veterinary Service facility (University of Ottawa).

Surgery and experimental metastasis model – The experimental metastasis model was carried out as previously described.[2] Briefly, an intravenous (iv) challenge of 3×10^5 B16lacZ cells was given to establish pulmonary metastases which are evident as early as 3 days following tumour cell injection. Surgical stress was induced by an abdominal nephrectomy 10mins following tumor inoculation. Animals were euthanized 3d following tumor inoculation and lungs were stained with X-gal (Bioshop) and quantified. For perioperative treatment, sildenafil (Pfizer) or tadalafil (GlaxoSmithKline) at 20 mg/kg/24h was administered intraperitoneally (ip) starting at 5 days before surgery and twice on the day of surgery (1h before and 4h after surgery); trivalent inactivated influenza vaccine (Agriflu®, Novartis) at 1/5 of the human dose (100 ml of 15 mg / 500 mL) was administered ip 1d before surgery.[8]

Surgery and spontaneous metastasis model – The spontaneous metastasis model was performed as previously described.[2] Briefly, 1×10^6 4T1 breast tumor cells were injected orthotopically into the mammary fat pad of BALB/c mice. At 14d post-tumor cell injection, a complete resection of the primary tumor along with abdominal nephrectomy was performed. For perioperative treatment, 20 mg/kg/24h of sildenafil was administered ip starting at 7 days before surgery and continued for 3 days following surgery. At 28d post-orthotopic tumor injection, visible nodules on the lung surface were visualized with a dissection microscope and quantified.

Cell lines – B16F10LacZ melanoma cell line was obtained from Dr. K Graham (London, Ontario) and maintained in cDMEM. RMA (thymoma) and RMA-S (MHC-I deficient) were from Dr. A Veillette (Montreal, Quebec). 4T1, YAC-1, K562, NK92 cell lines was purchased from ATCC and maintained in cRPMI.

Antibodies and FACS analysis. – Murine spleen and bone marrow lymphocytes were harvested and RBCs lysed. The following mAbs/reagents were used: anti-CD3 (SK7), anti-CD19, anti-NK1.1 (PK136), anti-F4/80 (BM8), anti-CD11c (N418), anti-B220 (RA3-6B2), anti-CD11b (M1/70), anti-GR1 (RB6-8C5), anti-Sca-1 (D7), anti-c-Kit (2B8), anti-CD34 (RAM34), anti-FcgRII/III (3G8) and isotype controls all from eBiosciences; anti-IL4Ra (I01F58) from Biolegend; ARG1 (IC5868F) from R&D Systems; ROS indicator (H_2-DCFDA) from ThermoFisher Scientific; and NOS indicator (DAF-2DA) from Sigma Aldrich. FACS acquisitions were performed on a CyAN-ADP (Beckman Coulter).

***Ex vivo* NK cell cytotoxicity assay** – The ^{51}Cr-release assay was performed as previously described.[2] Briefly, splenocytes were isolated from mice at 18h post-surgery. Purified NK cells ($DX5^+$, Miltenyi) were re-suspended at a concentration of 1.5×10^6 cells/ml and then mixed with ^{51}Cr-labelled target cells, which were re-suspended at a concentration of 3×10^4 cells/ml at different E:T ratios.

***In vivo* NK cell rejection assay** – The *in vivo* rejection assay was performed as previously described.[2,37] Briefly, RMA (wild-type) and RMA-S (MHC class I-deficient) were differentially labeled with 5mM ($CFSE^{high}$) and 0.5mM ($CFSE^{low}$) of CFSE (Biolegend), respectively. A mixture of 3×10^6 cells of each type was injected ip into recipient mice 4h following surgery. After 18h, peritoneal cells were harvested from the peritoneum with PBS-2mM-EDTA and analyzed for CFSE-labeled tumor cells by FACS. The RMA parental cells were not rejected by endogenous NK cells due to missing-self recognition, whereas the RMA-S cells will be rejected due to the absence of MHC class I inhibitory signals. The % rejection of MHC class I-deficient RMA-S is calculated using the following formula and the number of cells in each region:

$$\%\ rejection = \left(1 - \left(CFSE^{low} / CFSE^{high}\right) input \ / \left(CFSE^{low} / CFSE^{high}\right) output\right) \times 100$$

NK cell depletion – NK cells were depleted using an optimized dosing and schedule of α-NK1.1 antibody (PK136) or

isotype (Ebiosciences). 200μg were injected ip on days -4, -1 and +1. The lung tumor burden of the experimental metastasis model was quantified at 3d post-surgery.

MDSC:NK cell co-culture assays – gMDSC were purified using the Mouse MDSC isolation kit (Miltenyi). Purified gMDSC were seeded in triplicates in 96V bottom well plates at 1:1 ratio with purified NK cells with 50 mg/ml of sildenafil. Following 20h of co-culture, ^{51}Cr-labelled YAC-1 cells were added and the killing assay was conducted as described above.

MDSC transfer experiments – Splenocytes were isolated from B6 donor mice according to the treatment schedule in the experimental lung metastasis model at 24h post-surgery and enriched for gMDSC. 5×10^6 gMDSC as determined by FACS were injected ip into recipient B6 mice. For *in vivo* NK cell killing assays, CFSE differentially-labelled RMA and RMA-S cells were injected ip 4h following MDSC transfer. 24h post MDSC and tumor cell injection, peritoneal tumor cells were harvested and analyzed for CFSE expression. For experimental lung metastasis assays, 3×10^5 B16lacZ tumor cells were injected iv 1h post MDSC transfer. 3d post MDSC and tumor cell injection, lungs of recipient mice were isolated and quantified with Xgal.

Human samples for FACS, co-culture and cytotoxicity assays – Human whole blood was collected (Perioperative Blood Collection Program, approved by the Ottawa Health Science Network Research Ethics Board #2011884) preoperatively, postoperative day 1 (POD1), POD3, POD5 and POD28+/-14 from patients that did not have tumor metastasis, surgical complications and did not have chemotherapy prior to or in the relevant postoperative period. The blood samples were processed immediately for PBMC using Ficoll-Paque (Stemcell) and resuspended in freezing media (RPMI, 12.5% Human Serum Albumin, 10% DMSO). For FACS analysis, the following mAb were used: anti-CD11b (ICRF44), anti-CD33 (WM53), anti-HLA-DR (LN3) and anti-CD14 (61D3) (EBiosciences). For human MDSC:NK cell co-culture assays, human MDSC were isolated by slowly thawing frozen aliquots of PBMCs on ice, then at room temperature, followed by washing in complete media and resuspension in 1X PBS prior to magnetic separation using the CD33$^+$ selection kit (Miltenyi). MDSC were seeded in triplicates in 96V bottom well plates at 1:1 ratio with NK92 cells with 50mg/ml of sildenafil. Following 20h of co-culture, ^{51}Cr-labelled K562 cells were added and assessment of target cell killing was determined as above.

Statistical analysis – Statistical significance was determined by one way ANOVA or student t test with a cutoff P value of 0.05. Data is presented as mean +/- SEM.

Results

In vivo *perioperative PDE5 inhibition reduces postoperative metastatic disease*

Since current clinical trials are studying the effect of PDE5 inhibitors on malignant disease, we asked whether the antitumor effects of PDE5 inhibition could be beneficial for the reduction of postoperative metastatic disease. We administered perioperative PDE5 inhibitors in experimental and spontaneous mouse tumor models of major surgery and lung metastasis, including B16F10lacZ (melanoma) (Fig. 1A, B and C) and 4T1 (mammary carcinoma) (Fig. 1D and E). Perioperative sildenafil and tadalafil significantly reduced lung tumor metastasis at day 3 for melanoma-bearing mice (Fig. 1B and C and Supplementary Fig. S1A). Importantly, perioperative sildenafil reduced spontaneous metastatic disease as evident by a reduction in the number of visible nodules on the lung in a 4T1 breast tumor model (Fig. 1E and Supplementary Fig. S1A). To exclude the possibility of direct tumor cell killing by PDE5 inhibitors, we cultured B16F10lacZ and 4T1 tumors with sildenafil and did not observe a direct negative effect of sildenafil on tumor cell viability (Supplementary Fig. S1B and C). We therefore questioned the contribution of the immune system in the antitumor effect of PDE5 inhibitors. As we had previously observed a central role for NK cells in mediating tumor metastasis removal following surgery,[2] the lung metastasis experiments were repeated in the absence of NK cells by targeted depletion (Fig. 1F). The antitumor efficacy of perioperative sildenafil was no longer evident upon NK cell depletion (Fig. 1G). These results suggest that the antitumor activity of PDE5 inhibition in our models is secondary to an innate immune response involving the direct or indirect activity of sildenafil on NK cell function.

In vivo *perioperative PDE5 inhibition recovers natural killer cell function*

To characterize the effects of perioperative PDE5 inhibitor treatment upon NK cell-mediated antitumor activity following surgery, we performed *ex vivo* NK cell cytotoxicity assays using NK cells isolated from animals treated with sildenafil or tadalafil (see Supplementary Fig. S2A for experimental schedule). A significant effect of both PDE5 inhibitors upon NK cell cytotoxicity was observed in the surgery groups (Fig. 2A-D). Notably, sildenafil treatment alone was not found to impact NK cell cytotoxicity in naïve mice suggesting the PDE5 inhibition is not acting to directly enhance NK cell activity (Supplementary Fig. S2B). To confirm these *ex vivo* findings, we performed an *in vivo* NK cell cytotoxicity assay. Similar to our *ex vivo* results, NK cell activity was restored in mice treated with perioperative sildenafil compared to untreated surgery mice (Fig. 2E and F). Taken together, these results demonstrate PDE5 inhibitors indirectly improve perioperative NK cell cytotoxicity, leading to improved cancer target cell killing.

PDE5 inhibition can be combined with perioperative immunostimulation to reduce metastatic disease

Despite significant reductions in lung metastases sildenafil, through its indirect enhancement of NK cell activity, failed to completely eradicate metastatic disease. Therefore we next sought to determine whether a combination of sildenafil with a direct agonist of NK cell activity, type I interferon produced in response to influenza vaccination, could enhance therapeutic efficacy. In the B16F10lacZ model, mice were additionally injected with the influenza vaccine along with perioperative sildenafil (Fig. 3A). Although the PDE5 inhibitor alone significantly reduces lung metastases, combining PDE5 inhibition with influenza vaccination resulted in a greater

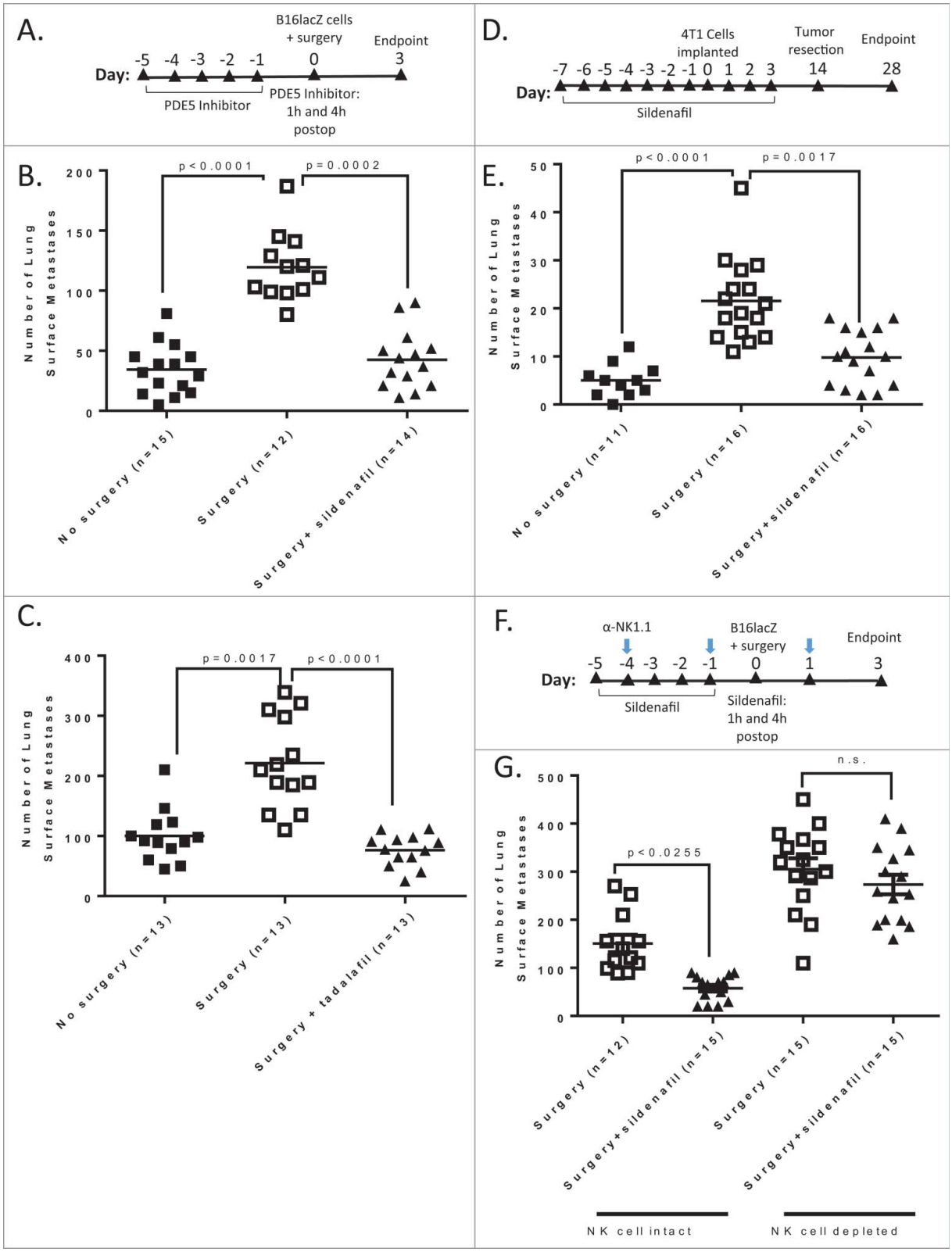

Figure 1. *In vivo* perioperative PDE5 inhibition reduces postoperative metastatic disease. (a) Experimental overview for evaluating the effects of PDE5 inhibtors (b) sildenafil and (c) tadalafil upon B16F10lacz lung metastases when given perioperatively. Animals received 5 daily injections of either drug prior to the surgery and two doses delivered 1h and 4 h postoperatively (postop). Lungs were collected on postop day 3 and metastases quantified as described in the materials and methods. (d) Experimental overview of mice receiving a once daily injection of sildenafil 1 week prior to and for 3 days following tumour implantation. Primary tumours were resected on day 14 and lungs collected on day 28 to (e) quantify the number of 4T1 lung tumor nodules. (f)Experimental overview for determining the effects of depleting NK cells upon the therapeutic efficacy of perioperative sildenafil on reducing (g) B16F10lacZ lung tumor metastases. In addition to receiving 5 daily injections of sildenafil prior to the surgery and two doses delivered 1h and 4 h postoperatively (postop) mice were injected with 200 mg of an anti-NK1.1 depleting antibody on day -4, -1 and +1 as indicated by the arrows. Lungs were collected on postop day 3 at time of endpoint and metastases quantified as described in the materials and methods. Data represent pooled results from 2 or 3 experiments with total number of mice per group indicated (n.s., not significant).

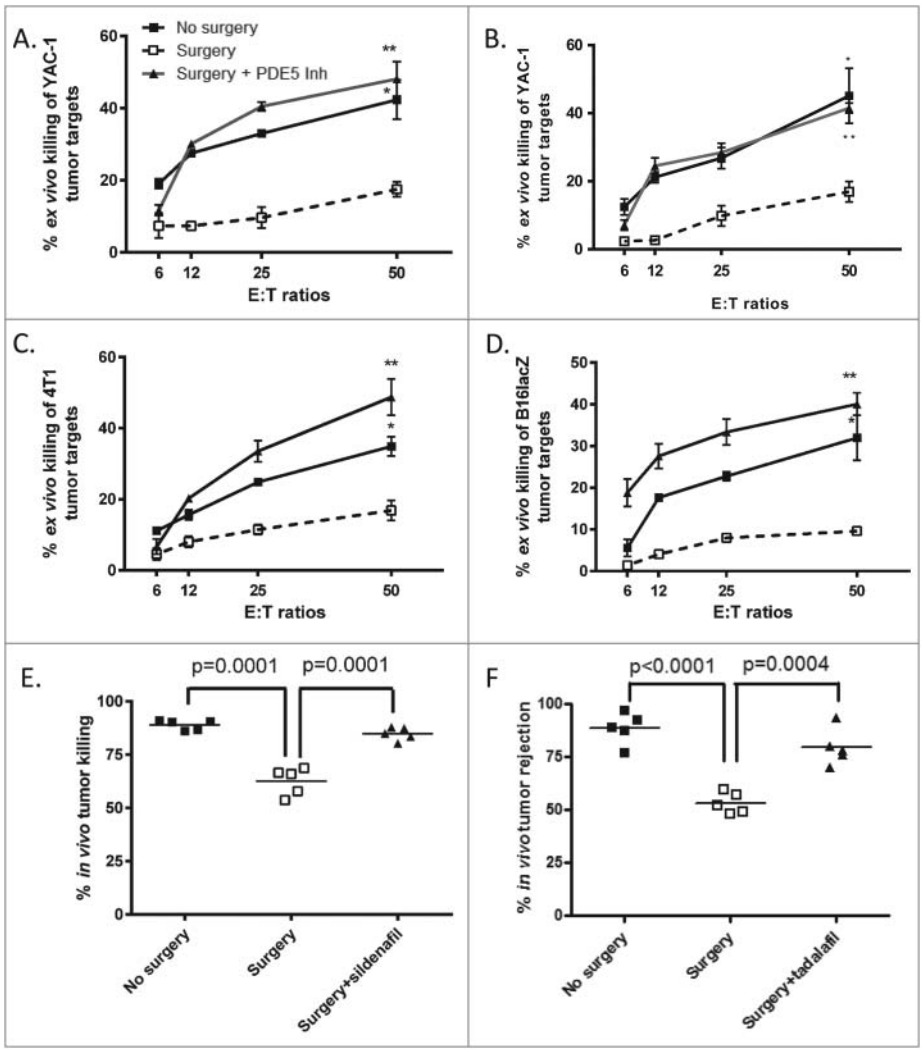

Figure 2. *In vivo* perioperative PDE5 inhibition recovers NK cell function. The ability of purified $DX5^+$ NK cells from animals receiving the PDE5 inhibitor (PDE5 Inh) sildenafil (a, c and d) or tadalafil (b) to kill YAC-1 (a) and (b), B16F10lacZ (c) or 4T1 (d) tumour cells *ex vivo*. The data are displayed as the mean percent (+/- SEM) of chromium release from triplicate wells for the indicated E:T ratios. (*, $p < 0.05$ comparing "No surgery" to "Surgery" groups; **, $p < 0.05$ comparing "Surgery + sildenafil or tadalfil to "Surgery" groups). (e) and (f) The ability of endogenous NK cells from indicated treatments to reject RMA-S tumor cells. Data is representative of 3 independent experiments, where n = 4-6/group.

antitumor effect (24 vs 11 lung metastasis in sildenafil vs sildenafil + flu vaccine) (Fig. 3A). To further investigate a role for NK cells in mediating the additive effects of combining perioperative administration of influenza vaccine and PDE5 inhibitor, we examined *ex vivo* NK cell killing (Fig. 3B). Here, we observed a significant recovery of NK cell killing following perioperative administration of either agent alone compared to surgery treated mice (Fig. 3B). However, the effect on antitumor NK cell cytotoxicity was further improved when the influenza vaccine was used in combination with sildenafil treatment in the perioperative period (Fig. 3B). These results demonstrate a role of sildenafil in modifying the postoperative immunosuppressive environment.

Surgery induces accumulation of granulocytic myeloid derived suppressor cells

The experiments described thus far demonstrate the ability of PDE5 inhibition to augment postoperative antitumor immunity mediated by NK cells. However, the mechanisms resulting in the suppression of NK cell activation and functionality in the postoperative period are unknown. We have previously demonstrated that MDSC and not Treg cells expand following surgery[2] suggesting that myeloid cells may be responsible for the suppression of NK cell activity in the perioperative period. Flow cytometric analysis revealed that surgery was associated with an increased proportion of myeloid precursor cells (common myeloid: $lineage^-$, $Sca1^-$, $ckit^+$, $CD34^{int}$, $FcgRII^{int}/FcgRIII^{int}$ and granulocytic: $lineage^-$, $Sca1^-$, $ckit^+$, $CD34^{int}$, $FcgRII^{high}/FcgRIII^{high}$ progenitor cells) in the bone marrow (BM), (Fig. 4A), but not in the spleen (Fig. 4B). Additionally, our analysis revealed a reduction in the number of $CD11b^+$ $Gr1^+$ cells in the BM which was accompanied by an increase in the spleen suggesting surgery-induced $CD11b^+$ $Gr1^+$ cells are generated in the BM before migrating to the peripheral tissues (Fig 4A – B and Supplementary Fig S3A). Further examination of the population of $CD11b^+$ $Gr1^+$ cells revealed that although there was no change in the number of either the $Gr1^{lo}$ or $Gr1^-$ cells there was a significant accumulation of $CD11b^+$ $Gr1^{hi}$ cells in the spleen during the postoperative period, increasing from 2.6% of all splenocytes at baseline to 13.9% of all cells by 12h and reaching a peak of 15.7% by 24 h

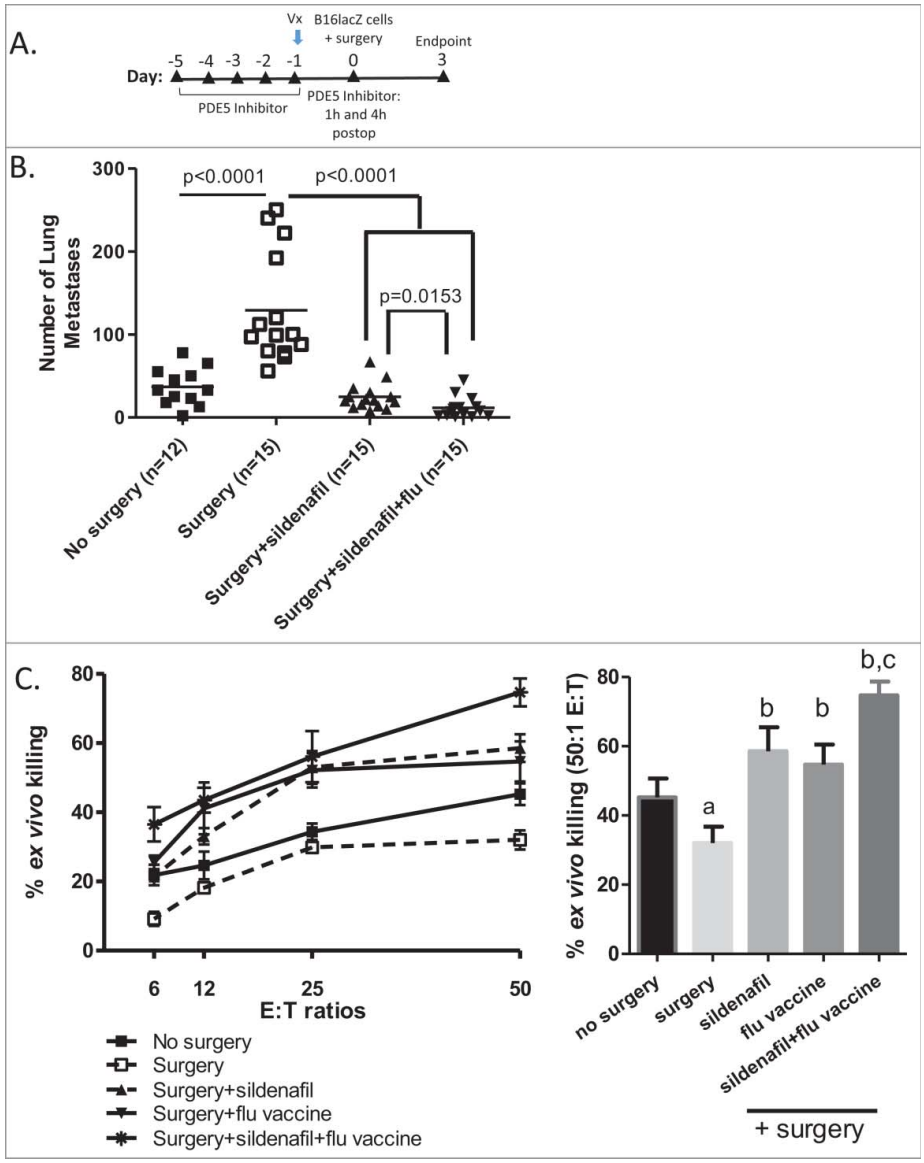

Figure 3. PDE5 inhibition combines with perioperative immunostimulation to improve NK cell function and reduce metastatic disease. (a) Experimental overview for evaluating the combined effects of perioperative PDE5 inhibition and influenza vaccination (Vx). (b) Quantification of B16F10lacZ lung tumor metastases at 3d from indicated treatment groups. (c) The ability of purified $DX5^+$ NK cells from indicated treatment groups to kill YAC-1 tumor cells. The data are displayed as the mean percent (+/- SEM) of chromium release from triplicate wells for the indicated E:T ratios. Data represent the pooled results of 3 independent experiments with total number of mice per group indicated. The right panel is representative of the 50:1 E:T ratio used for assessing statistical significance (a, $p < 0.05$ comparing "No surgery" to "Surgery" groups; b, $p < 0.001$ comparing "Surgery + sildenafil", "Surgery + flu vaccine" or "Surgery + sildenafil + flu vaccine" to "Surgery" groups; c, $p < 0.001$ comparing "Surgery + sildenafil" or "Surgery + flu vaccine" to "Surgery + sildenafil + flu vaccine" groups).

(Fig. 4C, D and Supplementary Fig. S3A). The accumulation of $CD11b^+$ $Gr1^+$cells observed in the spleen was indicative of systemic increases as similar increases were also observed in the lung and blood and was not impacted by sildenafil (Supplementary Fig S3C and D). Similarly, we did not observe any significant effect of sildenafil upon either NK or T cells present in the spleen, blood or lungs in either untreated or surgically stressed groups (Supplementary Fig. S4). Since $CD11b^+$ $Gr1^{hi}$ cells are consistent with the reported phenotype of granulocytic MDSC (gMDSC) we next sought to investigate whether this population expressed known markers associated with immune modulation and determine the effects of sildenafil. An examination of the expression of known mediators of gMDSC immunosuppressive activity including ARG1, ROS, IL-4Ra and NOS revealed an increase in nearly all markers following surgical stress (Fig. 4E-H) Furthermore, with the exception of NOS, which was not altered following surgical stress, sildenafil treatment reduced the significant increases in ARG1, ROS and IL4Ra (Fig. 4E-G) to control levels (Fig. 4H) but did not directly impact the accumulation of $CD11b^+$ $Gr1^{hi}$ population (Supplementary Fig. S3C and D). Considering that ARG1, ROS and IL4Ra are key molecules in MDSC suppressive pathways,[36,38] these results suggest that PDE5 inhibition is a novel perioperative therapy to regulate surgery-derived gMDSC mediated-immunosuppression.

Surgery-induced MDSC are the targets of sildenafil-mediated antitumor activity

The influence of surgery-derived $CD11b^+$ $Gr1^{hi}$ cells on NK cell activity was first assessed by *ex vivo* co-culture assays

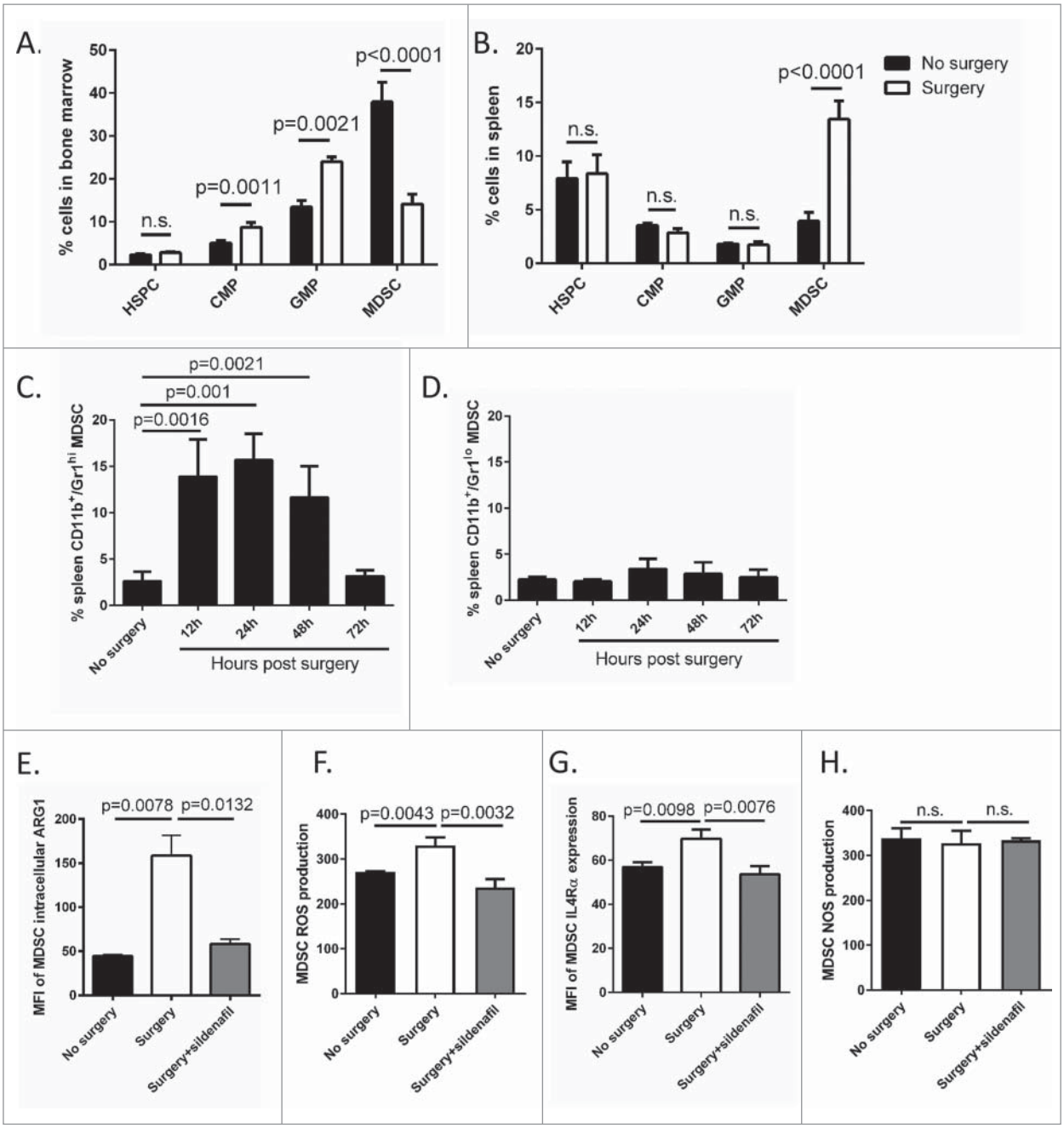

Figure 4. Surgery induces accumulation of granulocytic myeloid derived suppressor cells. The mean percentage of (a) bone marrow and (b) spleen HSPC (lineage$^-$, Sca1$^+$, ckit$^+$), common myeloid progenitor (CMP) (lineage$^-$, Sca1$^-$, ckit$^+$, CD34^{int}, FcgRII^{int}/FcgRIII^{int}), GMP (lineage$^-$, Sca1$^-$, ckit$^+$, CD34^{int}, FcgRII^{high}/FcgRIII^{high}), and MDSC (CD11b$^+$, Gr1$^+$) was evaluated in untreated and surgery treated mice. The mean percentages of splenic (c) gMDSC and (d) mMDSC was evaluated at indicated time points following surgery. MFI of (e) ARG1, (f) ROS (H_2-DCF), (g) IL4Ra and (h) NOS (DAF-2DA) expression on (CD11b$^+$, Gr1^{high}) gated splenocytes was evaluated in indicated treatment groups. Data are representative of 3 similar experiments where n = 4-6/group (n.s., not significant).

using Gr1$^+$ cells isolated from untreated and surgery-treated mice co-cultured with resting (Fig. 5A) and IL2 activated (Fig. 5B) NK cells. In the presence of surgery-derived Gr1$^+$ cells, the cytolytic capacity of resting and IL2 activated NK cells were significantly inhibited compared to untreated mice (Fig. 5A-B). We further supported these findings by demonstrating that surgery-induced gMDSC can functionally inhibit CD3/CD28 stimulated T cells (Supplementary Fig. S5A). Together these data suggest that the myeloid derived CD11b$^+$ Gr1$^+$ cells which accumulate following surgery are suppressive of NK and T cell function and will be referred to as gMDSC as warranted by standardized nomenclature.[39] To verify that surgery-induced gMDSC are indeed the targets of sildenafil-mediated antitumor activity, we adoptively transferred MDSC from surgically stressed animals treated perioperatively with vehicle (PBS) or sildenafil into naïve mice and examined NK cytotoxicity and metastasis formation. As shown in Fig. 5C, the adoptive transfer of MDSC from surgery-treated mice is alone sufficient to significantly impair endogenous NK cell cytotoxicity. In contrast, naïve mice receiving MDSC from surgically stressed animals that had received perioperative sildenafil treatment prior to transfer

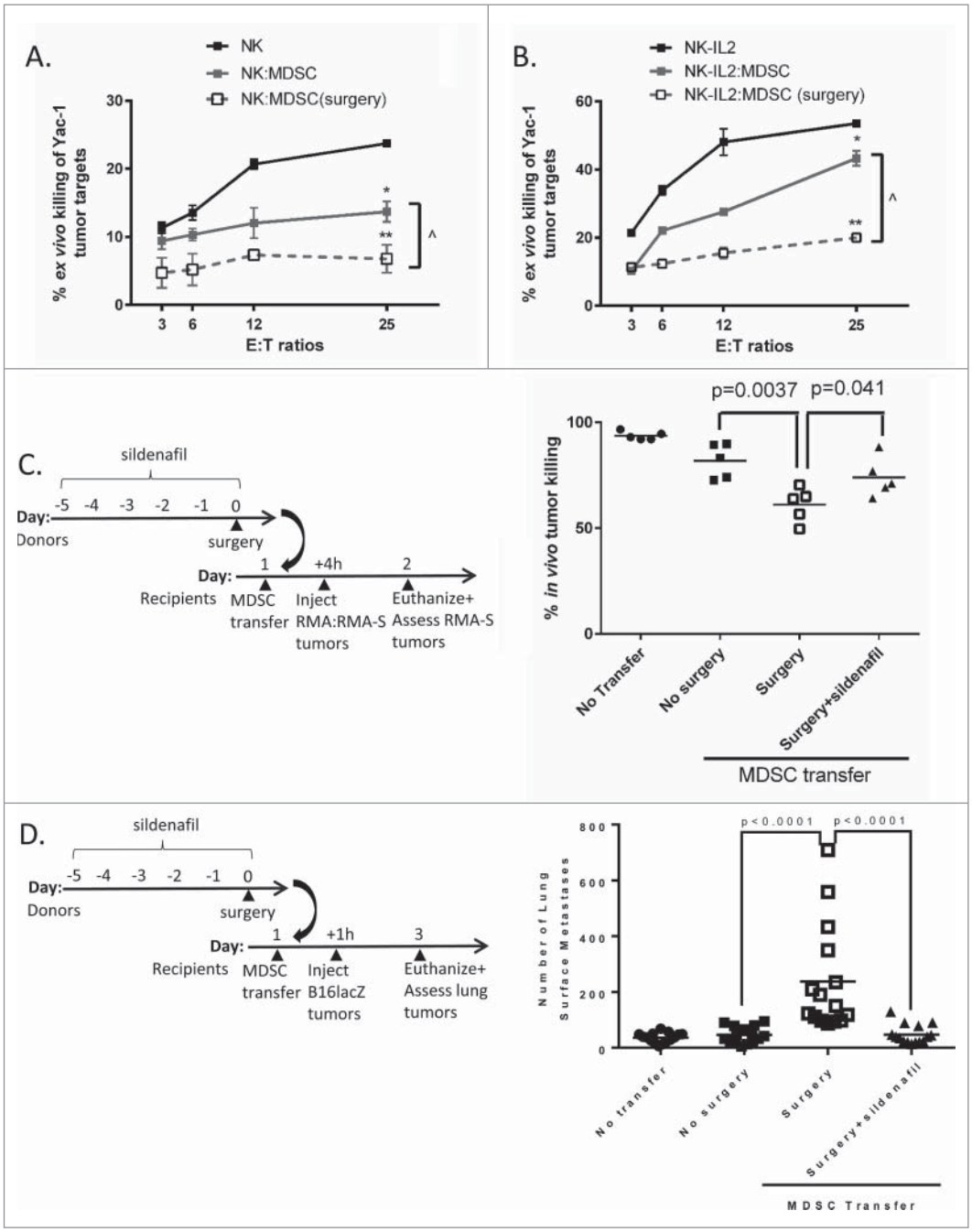

Figure 5. Surgery-induced MDSC are the targets of sildenafil-mediated antitumor activity. The ability of (a) resting NK cells and (b) IL2 activated NK cells to kill YAC-1 targets following 20h of co-culture with MDSC isolated from mice with indicated treatment groups. The data are displayed as the mean percent (+/- SEM) of chromium release from triplicate wells for the indicated E:T ratios. Data represent 3 pooled experiments with total number of mice per group indicated. (*, p = 0.05 comparing "NK:MDSC or NK-IL2:MDSC" to "NK or NK-IL2" alone groups; **, $p < 0.001$ comparing "NK:MDSC(surgery) or NK-IL2:MDSC(surgery)" to "NK or NK-IL2" alone groups; ^, $p < 0.05$ comparing "NK:MDSC(surgery) or NK-IL2:MDSC(surgery)" to NK:MDSC or NK-IL2:MDSC" groups). (c) In vivo tumor rejection at 24h and (d) quantification of lung tumor metastases at 3d from recipient mice receiving adoptively transferred MDSC from indicated treatment groups.

exhibited significantly improved endogenous NK cell cytotoxicity over surgery-treated MDSC. To support these observations, we assessed lung tumor metastases. As shown in Fig. 5D, lung tumor burden was similarly augmented following transfer of surgery-treated MDSC, whereas, it was significantly reduced after transfer of surgery + sildenafil treated MDSC. Furthermore, perioperative sildenafil treatment improved the NK cell cytotoxic activity of NK cells isolated from the lungs and improved IFNγ secretion of NK cells from animals undergoing surgery in comparison to the untreated surgery cohort (Supplementary Fig. S5B and C). In contrast, despite the effects upon on gMDSCs (Fig. 4) and NK function, sildenafil was unable to restore CD8+ T cell IFNγ secretion in response to non-specific stimulation (PMA and Ionomycin) (Supplementary Fig. S5D). Taken together, these findings demonstrate that the immunosuppressive effects of surgery-induced gMDSC upon NK cell activity can be reduced by inhibiting PDE5 activity of gMDSC *in vivo*.

PDE5 inhibition restores NK cell cytotoxicity in colorectal cancer patients

Having demonstrated that PDE5 inhibition can restore MDSC suppressive mechanisms in preclinical models, we sought to determine whether similar effects exist in human cancer patients following tumor resection. Peripheral blood NK cell activity from colorectal patients showed functional impairment following surgery,[2] however, surgery-induced MDSC immunosuppression has not been previously reported in solid cancers. Similar to our preclinical data, we observed a significant accumulation of gMDSC ($CD11b^{+}/CD33^{+}/CD14^{-}/HLA\text{-}DR^{-/low}$) in the peripheral blood of colorectal cancer patients following surgical resection (Fig. 6A). We then examined target cell lysis by co-culturing MDSC from cancer surgery patients with the NK92 tumor cell line. K562 tumor lysis was significantly reduced when NK cells was co-cultured with cancer patient MDSC following surgical resection at POD1 and POD3 (Fig. 6B-C). We next sought to determine whether we could restore NK cytotoxicity when co-cultured with MDSC isolated from cancer patients in the presence of a PDE5 inhibitor. In cancer patients, NK cell cytotoxicity was restored with PDE5 inhibition at POD1 (Fig. 6D). In summary, these results confirm that PDE5 inhibition can augment NK cell immune responsiveness through its effect on surgery-expanded human MDSC.

Discussion

Metastatic recurrence following surgery remains the single biggest cause of mortality in patients with solid tumors.[1-4] We

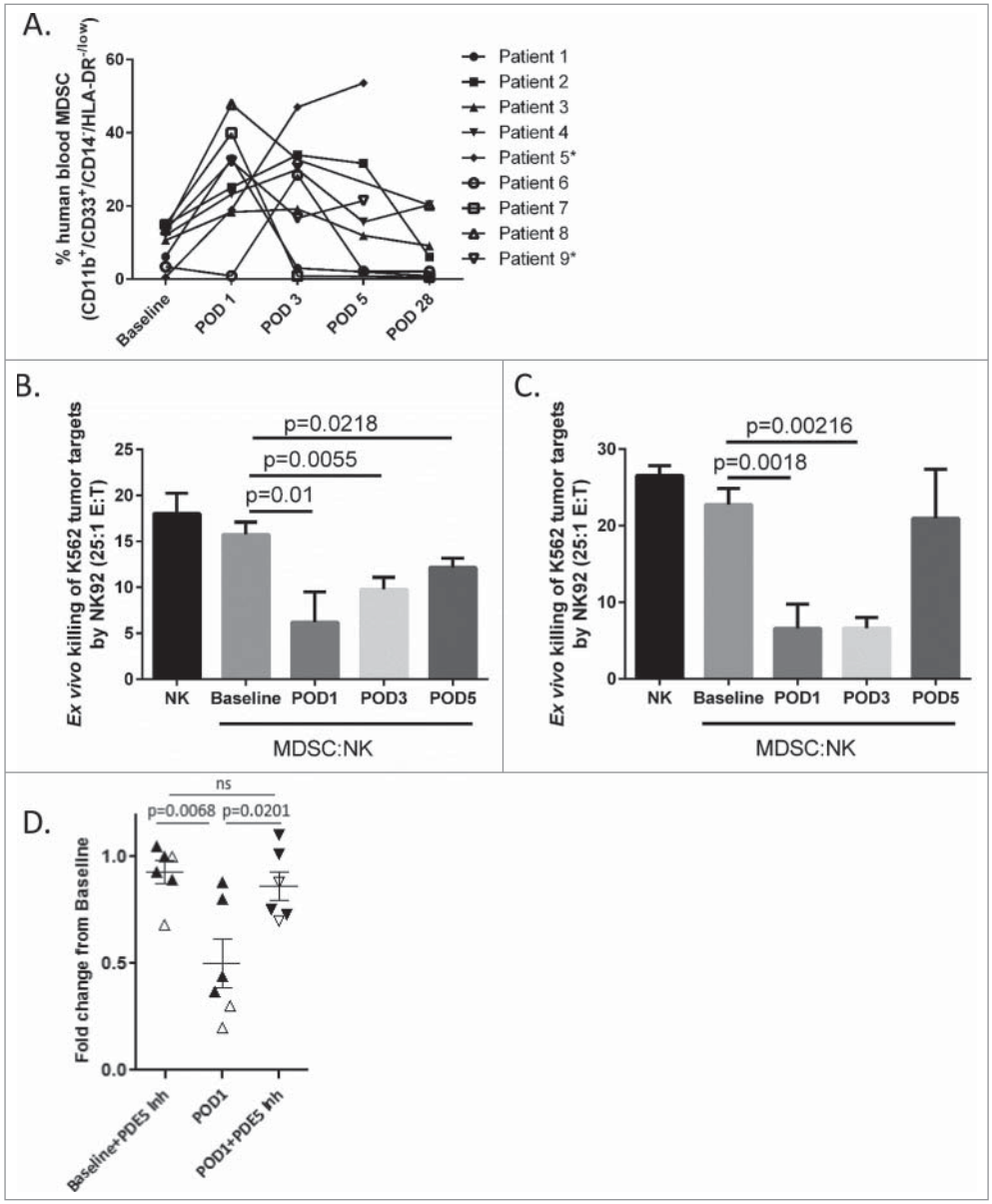

Figure 6. PDE5 inhibition restores NK cell activity in cancer surgical patients with abdominal malignancy. (a) Percentage human MDSC ($CD11b^{+}$, $CD33^{+}$, $CD14^{-}$, $HLA\text{-}DR^{-/low}$) was evaluated in PBMC from patients at baseline (preoperative), POD1, POD3, POD5, and POD28(±14); *patients with POD28 missing. (b and c) The ability of NK92 cells to kill K562 tumor cells after co-culture with purified MDSC from patients at baseline, POD1, POD3, and POD5. (d) The ability of NK92 cells to kill K562 tumor cells after co-culture with purified MDSC from patients at baseline and POD1 in the presence or absence of sildenafil (closed symbols) or tadalafil (open symbols). The data are displayed as the mean percent (+/- SEM) of chromium release from triplicate wells for 50:1 or 25:1 (E:T) ratio.

previously demonstrated that surgery-induced NK cell dysfunction contributes to progression of metastatic disease.[2] However, the underlying mechanisms leading to postoperative NK cell impairment was not fully characterized. In this report, we provide preclinical evidence that gMDSC accumulation following surgery parallels the time line of postoperative NK cell suppression. Given reports on the suppressive function of MDSC on immune effector cell function, we co-cultured resting and IL2-activated NK cells with gMDSC from control and surgery-treated mice and detected functional suppression of NK cells with surgery-treated gMDSC, but not with control gMDSC (Fig 5A and B). Production of ARG1 and ROS have both been described as potential mechanisms of MDSC mediated suppression of NK and T cells. In agreement with Ochoa and colleagues,[25,38] we observed upregulation of ARG1 in gMDSC following surgical injury. However, in contrast, we detected increased levels of intracellular ROS in gMDSC following surgery. In line with tumor-expanded MDSC studies by the Borrello group,[36] we also detected increased IL4Ra and ARG1 expression on surgery-derived gMDSC, but not NOS levels. These phenotypic and biochemical differences could potentially be explained by the heterogeneity of the MDSC populations described by these groups. For instance, we isolated gMDSC populations from tumor-bearing major surgery models involving laparotomy and nephrectomy, whereas total MDSC from non-tumor bearing, laparotomy-treated mice were used by the Ochoa group[38] and tumor bearing (non-surgical resection model) was used by the Borrello group.[36] Thus, because of the multifaceted functions of MDSC, it is reasonable to propose that multiple MDSC suppressive factors/phenotypes exist and in the context of cancer surgery, targeting both ARG1 and ROS may be needed to abolish MDSC-induced immune suppression.

There is accumulating evidence to suggest that PDE5 inhibitors could interfere with the suppressive functions of MDSC.[36,40,41] In this report, we provide evidence that perioperative PDE5 inhibition reverses surgery-induced immunosuppression. This is the first study to link the PDE5 inhibitors sildenafil and tadalafil with MDSC and NK cells in the eradication of postoperative metastases. We show that sildenafil reduces surgery-derived gMDSC function through downregulation of ARG1, IL4Ra and ROS expression, enabling NK cell tumoricidal activity and reducing postoperative disease recurrence. Our results indicate that sildenafil primarily targets $CD11b^{+}/Ly6G^{+}$ gMDSC and enhances NK cell activity *in vitro* (Fig. 5C,D) and *in vivo* (Fig. 5A). Although we have demonstrated that PDE5 inhibition reduces ARG1, IL4Ra and ROS levels, the full mechanisms underlying these effects remains to be characterized. Potential mechanisms include PDE5 inhibition mediated increase in cGMP which leads to destabilization of ARG1 mRNA[42] or reduction of the calcium-dependent protein kinase C activity that in turn prevents the upregulation of IL4Ra and subsequent decrease in ARG1 expression.[36,43]

Notably, our preclinical findings of surgery-induced MDSC-mediated immunosuppressive mechanisms are conserved in cancer surgery patients. Treatment with PDE5 inhibitors *ex vivo* reversed the suppressive functions of postoperative MDSC from patients with colorectal cancer following resection and rescued NK cell responses.

Together our findings support the further investigation of clinical strategies aimed at safely preventing the immunosuppressive environment present in the perioperative period. Interestingly two recently completed clinical trials using the PDE5 inhibitor tadalafil in head and neck squamous cell carcinoma patients, prior to chemotherapy, supports the safety and feasibility of our proposal.[40,41] Both trials reported that tadalafil was well tolerated in patients in addition to the beneficial modulation of the tumor microenvironment, including MDSC, Treg and CD4/CD8 T cell functionality.[40,41] Furthermore, a recently completed Phase II clinical trial reporting that perioperative treatment with a COX-2 inhibitor (etodolac) in combination with a β-adrenergic antagonist (propranolol) could reduce circulating $CD14^{+}$ monocytes and improve NK cell activation[44] supporting the rationale for targeting MDSCs in the perioperative period to improve clinical outcomes.

Based on our preclinical data demonstrating that the perioperative administration of sildenafil in combination with influenza vaccine results in a greater reduction of lung tumor metastasis than either treatment alone and our *ex vivo* sildenafil patient studies, we have proposed a randomized, double-arm phase Ib study in which patients with abdominal malignancies will be treated with tadalafil and influenza vaccine prior to surgical resection of their primary tumor. Blood and tumor MDSC and NK cell functionality will be investigated pre- and postoperatively. Repurposing of the existing drug, tadalafil, for the treatment of postoperative metastases represents a cost and time effective means to deliver a novel and effective immunotherapy to surgical cancer patients.

Abbreviations

ARG1	arginase 1
g/mMDSC	granulocytic/monocytic myeloid derived suppressor cells
IFN	interferon
iNOS	inducible nitric oxide synthase
iv	intravenous
ip	intraperitoneal
NK	natural killer cells
POD	postoperative day
PDE-5	Phosphodiesterase-5
ROS	reactive oxygen species
TGFb	transforming growth factor

Disclosure statement

The authors report no conflict of interest.

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References

1. Glasner A, Avraham R, Rosenne E, Benish M, Zmora O, Shemer S, Meiboom H, Ben-Eliyahu S. Improving survival rates in two models of spontaneous postoperative metastasis in mice by combined administration of a beta-adrenergic antagonist and a cyclooxygenase-2 inhibitor. J Immunol. 2010;184:2449–57. doi:10.4049/jimmunol.0903301.
2. Tai LH, de Souza CT, Belanger S, Ly L, Alkayyal AA, Zhang J, Rintoul JL, Ananth AA, Lam T, Breitbach CJ, et al. Preventing postoperative metastatic disease by inhibiting surgery-induced dysfunction in natural killer cells. Cancer Res. 2013;73:97–107. doi:10.1158/0008-5472.CAN-12-1993.
3. Benish M, Bartal I, Goldfarb Y, Levi B, Avraham R, Raz A, Ben-Eliyahu S. Perioperative use of beta-blockers and COX-2 inhibitors may improve immune competence and reduce the risk of tumor metastasis. Ann Surg Oncol. 2008;15:2042–52. doi:10.1245/s10434-008-9890-5.
4. Goldfarb Y, Sorski L, Benish M, Levi B, Melamed R, Ben-Eliyahu S. Improving postoperative immune status and resistance to cancer metastasis: a combined perioperative approach of immunostimulation and prevention of excessive surgical stress responses. Ann Surg. 2011;253:798–810. doi:10.1097/SLA.0b013e318211d7b5.
5. Tartter PI, Steinberg B, Barron DM, Martinelli G. The prognostic significance of natural killer cytotoxicity in patients with colorectal cancer. Arch Surg. 1987;122:1264–8. doi:10.1001/archsurg.1987.01400230050009.
6. Fujisawa T, Yamaguchi Y. Autologous tumor killing activity as a prognostic factor in primary resected nonsmall cell carcinoma of the lung. Cancer. 1997;79:474–81. doi:10.1002/(SICI)1097-0142(19970201)79:3<474::AID-CNCR8>3.0.CO;2-I.
7. Carter CR, Hogan BV, Cole JY, Shenoy HG, Horgan KM, Hughes TA. The expression of activating natural killer cell receptors in patients with primary breast cancer. Cancer Res. 2009;69(2 Suppl):5047.
8. Tai LH, Zhang J, Auer RC. Preventing surgery-induced NK cell dysfunction and cancer metastases with influenza vaccination. Oncoimmunology. 2013;2:e26618. doi:10.4161/onci.26618.
9. Sedman PC, Ramsden CW, Brennan TG, Giles GR, Guillou PJ. Effects of low dose perioperative interferon on the surgically induced suppression of antitumour immune responses. Br J Surg. 1988;75:976–81. doi:10.1002/bjs.1800751012.
10. Nichols PH, Ramsden CW, Ward U, Sedman PC, Primrose JN. Perioperative immunotherapy with recombinant interleukin 2 in patients undergoing surgery for colorectal cancer. Cancer Res. 1992;52:5765–9.
11. Nichols PH, Ramsden CW, Ward U, Trejdosiewicz LK, Ambrose NS, Primrose JN. Peri-operative modulation of cellular immunity in patients with colorectal cancer. Clin Exp Immunol. 1993;94:4–10. doi:10.1111/j.1365-2249.1993.tb05968.x.
12. Deehan DJ, Heys SD, Ashby J, Eremin O. Interleukin-2 (IL-2) augments host cellular immune reactivity in the perioperative period in patients with malignant disease. Eur J Surg Oncol. 1995;21:16–22. doi:10.1016/S0748-7983(05)80061-7.
13. Houvenaeghel G, Bladou F, Blache JL, Olive D, Monges G, Jacquemier J, Chaudet H, Delpero JR, Guérinel G. Tolerance and feasibility of perioperative treatment with interferon-alpha 2a in advanced cancers. Int Surg. 1997;82:165–9.
14. Elias D, Farace F, Triebel F, Hattchouel JM, Pignon JP, Lecesne A, Rougier P, Lasser P, Duvillard P, Escudier B. Phase I-II randomized study on prehepatectomy recombinant interleukin-2 immunotherapy in patients with metastatic carcinoma of the colon and rectum. J Am Coll Surg. 1995;181:303–10.
15. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol. 2009;9:162–74. doi:10.1038/nri2506.
16. Ostrand-Rosenberg S. Myeloid-derived suppressor cells: more mechanisms for inhibiting antitumor immunity. Cancer Immunol Immunother. 2010;59:1593–600. doi:10.1007/s00262-010-0855-8.
17. Liu C, Yu S, Kappes J, Wang J, Grizzle WE, Zinn KR, Zhang HG. Expansion of spleen myeloid suppressor cells represses NK cell cytotoxicity in tumor-bearing host. Blood. 2007;109:4336–42. doi:10.1182/blood-2006-09-046201.
18. Zhu J, Huang X, Yang Y. Myeloid-derived suppressor cells regulate natural killer cell response to adenovirus-mediated gene transfer. Journal of virology. 2012;86:13689–96. doi:10.1128/JVI.01595-12.
19. Oberlies J, Watzl C, Giese T, Luckner C, Kropf P, Müller I, Ho AD, Munder M. Regulation of NK cell function by human granulocyte arginase. J Immunol. 2009;182:5259–67. doi:10.4049/jimmunol.0803523.
20. Li H, Han Y, Guo Q, Zhang M, Cao X. Cancer-expanded myeloid-derived suppressor cells induce anergy of NK cells through membrane-bound TGF-beta 1. J Immunol. 2009;182:240–9. doi:10.4049/jimmunol.182.1.240.
21. Mao Y, Sarhan D, Steven A, Seliger B, Kiessling R, Lundqvist A. Inhibition of tumor-derived prostaglandin-e2 blocks the induction of myeloid-derived suppressor cells and recovers natural killer cell activity. Clin Cancer Res. 2014;20:4096–106. doi:10.1158/1078-0432.CCR-14-0635.
22. Cuenca AG, Delano MJ, Kelly-Scumpia KM, Moreno C, Scumpia PO, Laface DM, Heyworth PG, Efron PA, Moldawer LL. A paradoxical role for myeloid-derived suppressor cells in sepsis and trauma. Mol Med. 2011;17:281–92. doi:10.2119/molmed.2010.00178.
23. Noel JG, Osterburg A, Wang Q, Guo X, Byrum D, Schwemberger S, Goetzman H, Caldwell CC, Ogle CK. Thermal injury elevates the inflammatory monocyte subpopulation in multiple compartments. Shock. 2007;28:684–93.
24. Delano MJ, Scumpia PO, Weinstein JS, Coco D, Nagaraj S, Kelly-Scumpia KM, O'Malley KA, Wynn JL, Antonenko S, Al-Quran SZ, et al. MyD88-dependent expansion of an immature GR-1(+)CD11b(+) population induces T cell suppression and Th2 polarization in sepsis. J Exp Med. 2007;204:1463–74. doi:10.1084/jem.20062602.
25. Makarenkova VP, Bansal V, Matta BM, Perez LA, Ochoa JB. CD11b+/Gr-1+ myeloid suppressor cells cause T cell dysfunction after traumatic stress. J Immunol. 2006;176:2085–94. doi:10.4049/jimmunol.176.4.2085.
26. Wang J, Su X, Yang L, Qiao F, Fang Y, Yu L, Yang Q, Wang Y, Yin Y, Chen R, et al. The influence of myeloid-derived suppressor cells on angiogenesis and tumor growth after cancer surgery. Int J Cancer. 2016;138:2688–99. doi:10.1002/ijc.29998.
27. Wang J, Yang L, Yu L, Wang YY, Chen R, Qian J, Hong ZP, Su XS. Surgery-induced monocytic myeloid-derived suppressor cells expand regulatory T cells in lung cancer. Oncotarget. 2017;8:17050–8.
28. Yuan L, Xu B, Fan H, Yuan P, Zhao P, Suo Z. Pre- and post-operative evaluation: percentages of circulating myeloid-derived suppressor cells in rectal cancer patients. Neoplasma. 2015;62:239–49. doi:10.4149/neo_2015_029.
29. Sandner P, Hutter J, Tinel H, Ziegelbauer K, Bischoff E. PDE5 inhibitors beyond erectile dysfunction. Int J Impot Res. 2007;19:533–43. doi:10.1038/sj.ijir.3901577.
30. Di X, Gennings C, Bear HD, Graham LJ, Sheth CM, White KL Jr, Gewirtz DA. Influence of the phosphodiesterase-5 inhibitor, sildenafil, on sensitivity to chemotherapy in breast tumor cells. Breast Cancer Res Treat. 2010;124:349–60. doi:10.1007/s10549-010-0765-7.
31. Spoto G, Fioroni M, Rubini C, Contento A, Di Nicola M, Forcella S, Piattelli A. Cyclic guanosine monophosphate phosphodiesterase activity in human gingival carcinoma. J Oral Pathol Med. 2003;32:189–94. doi:10.1034/j.1600-0714.2003.00083.x.
32. Zhu B, Strada SJ. The novel functions of cGMP-specific phosphodiesterase 5 and its inhibitors in carcinoma cells and pulmonary/cardiovascular vessels. Curr Top Med Chem. 2007;7:437–54. doi:10.2174/156802607779941198.
33. Chen JJ, Sun YL, Tiwari AK, Xiao ZJ, Sodani K, Yang DH, Vispute SG, Jiang WQ, Chen SD, Chen ZS. PDE5 inhibitors, sildenafil and vardenafil, reverse multidrug resistance by inhibiting the efflux function of

multidrug resistance protein 7 (ATP-binding Cassette C10) transporter. Cancer Sci. 2012;103:1531–7. doi:10.1111/j.1349-7006.2012.02328.x.
34. Ding PR, Tiwari AK, Ohnuma S, Lee JW, An X, Dai CL, Lu QS, Singh S, Yang DH, Talele TT, et al. The phosphodiesterase-5 inhibitor vardenafil is a potent inhibitor of ABCB1/P-glycoprotein transporter. PLoS One. 2011;6:e19329. doi:10.1371/journal.pone.0019329.
35. Das A, Durrant D, Mitchell C, Mayton E, Hoke NN, Salloum FN, Park MA, Qureshi I, Lee R, Dent P, et al. Sildenafil increases chemotherapeutic efficacy of doxorubicin in prostate cancer and ameliorates cardiac dysfunction. Proc Natl Acad Sci U S A. 2010;107:18202–7. doi:10.1073/pnas.1006965107.
36. Serafini P, Meckel K, Kelso M, Noonan K, Califano J, Koch W, Dolcetti L, Bronte V, Borrello I. Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function. J Exp Med. 2006;203:2691–702. doi:10.1084/jem.20061104.
37. Patel R, Belanger S, Tai LH, Troke AD, Makrigiannis AP. Effect of Ly49 haplotype variance on NK cell function and education. J Immunol. 2010;185:4783–92. doi:10.4049/jimmunol.1001287.
38. Zhu X, Pribis JP, Rodriguez PC, Morris SM Jr, Vodovotz Y, Billiar TR, Ochoa JB. The central role of arginine catabolism in T-cell dysfunction and increased susceptibility to infection after physical injury. Ann Surg. 2014;259:171–8. doi:10.1097/SLA.0b013e31828611f8.
39. Bronte V, Brandau S, Chen SH, Colombo MP, Frey AB, Greten TF, Mandruzzato S, Murray PJ, Ochoa A, Ostrand-Rosenberg S, et al. Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. Nat Commun. 2016;7:12150. doi:10.1038/ncomms12150.
40. Califano JA, Khan Z, Noonan KA, Rudraraju L, Zhang Z, Wang H, Goodman S, Gourin CG, Ha PK, Fakhry C, et al. Tadalafil augments tumor specific immunity in patients with head and neck squamous cell carcinoma. Clin Cancer Res. 2015;21:30–8. doi:10.1158/1078-0432.CCR-14-1716.
41. Weed DT, Vella JL, Reis IM, De la Fuente AC, Gomez C, Sargi Z, Nazarian R, Califano J, Borrello I, Serafini P. Tadalafil reduces myeloid-derived suppressor cells and regulatory T cells and promotes tumor immunity in patients with head and neck squamous cell carcinoma. Clin Cancer Res. 2015;21:39–48. doi:10.1158/1078-0432.CCR-14-1711.
42. Pilz RB, Casteel DE. Regulation of gene expression by cyclic GMP. Circ Res. 2003;93:1034–46. doi:10.1161/01.RES.0000103311.52853.48.
43. Lin CS, Chow S, Lau A, Tu R, Lue TF. Regulation of human PDE5A2 intronic promoter by cAMP and cGMP: identification of a critical Sp1-binding site. Biochem Biophys Res Commun. 2001;280:693–9. doi:10.1006/bbrc.2000.4221.
44. Shaashua L, Shabat-Simon M, Haldar R, Matzner P, Zmora O, Shabtai M, Sharon E, Allweis T, Barshack I, Hayman L, et al. Perioperative COX-2 and beta-Adrenergic Blockade Improves Metastatic Biomarkers in Breast Cancer Patients in a Phase-II Randomized Trial. Clin Cancer Res. 2017;23:4651–61. doi:10.1158/1078-0432.CCR-17-0152.