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Preventing Postoperative Metastatic Disease by Inhibiting Surgery-Induced Dysfunction in Natural Killer Cells

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Abstract

Natural killer (NK) cell clearance of tumor cell emboli following surgery is thought to be vital in preventing postoperative metastases. Using a mouse model of surgical stress, we transferred surgically stressed NK cells into NK-deficient mice and observed enhanced lung metastases in tumor-bearing mice as compared with mice that received untreated NK cells. These results establish that NK cells play a crucial role in mediating tumor clearance following surgery. Surgery markedly reduced NK cell total numbers in the spleen and affected NK cell migration. *Ex vivo* and *in vivo* tumor cell killing by NK cells were significantly reduced in surgically stressed mice. Furthermore, secreted tissue signals and myeloid-derived suppressor cell populations were altered in surgically stressed mice. Significantly, perioperative administration of oncolytic parapoxvirus ovis (ORFV) and vaccinia virus can reverse NK cell suppression, which correlates with a reduction in the postoperative formation of metastases. In human studies, postoperative cancer surgery patients had reduced NK cell cytotoxicity, and we show for the first time that oncolytic vaccinia virus markedly increases NK cell activity in patients with cancer. These data provide direct *in vivo* evidence that surgical stress impairs global NK cell function. Perioperative therapies aimed at enhancing NK cell function will reduce metastatic recurrence and improve survival in surgical cancer patients. *Cancer Res; 73(1); 97–107. ©2012 AACR.*

Introduction

Surgeons have long suspected that surgery, a necessary step in the treatment of solid cancers, facilitates the metastatic process. Despite the initial observation of this phenomenon in 1913 (1), it remains an area of unresolved inquiry. Numerous animal studies using implanted and spontaneous tumor models have clearly shown that surgery promotes the formation of metastatic disease (2, 3), and the number of metastatic deposits that develop is directly proportional to the magnitude of surgical stress (2). In clinical studies, a complicated postoperative course corrresponds to an increase in physiologic surgical stress. This has been shown to correlate with an inferior cancer survival and an increased incidence of metastatic disease (4, 5).

A number of perioperative changes have been proposed to explain the promotion of metastases formation following surgery, including dissemination of tumor cells during the surgical procedure (6, 7), local and systemic release of growth factors (8, 9), and cellular immune suppression. Intraoperative circulating tumor cells have been detected in patients with metastatic cancers and may be a valuable prognostic marker (10, 11). The cellular immune suppression following major surgery peaks at 3 days (12) following surgery but may persist for weeks (12, 13).

Natural killer (NK) cells are cytotoxic lymphocytes that constitute a major component of the innate immune system. Immunosurveillance of the host by NK cells for malignant and virally infected cells results in direct cytotoxicity and the production of cytokines to enhance the immune response. NK cell dysfunction following surgery has been documented in both human patients (13–15) and animal models (16, 17). Postoperative NK cell suppression correlates with increased metastases in animal models of spontaneous (3) and implanted (18, 19) metastases, whereas in human studies low NK activity during the perioperative period is associated with a higher rate of cancer recurrence and mortality in different cancer types (20, 21).

The perioperative period represents not only a window of opportunity for cancer cells to form metastases, but also a therapeutic window in which to intervene in the metastatic process. Traditional cancer therapies, such as chemotherapy, are considered too toxic to be administered to patients recovering from a major surgery as they impair wound healing (22). Alternatively, immune therapies are ideal candidates for

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perioperative administration. The perioperative administration of recombinant cytokines has been explored in early-phase clinical trials (23, 24). These studies have shown that perioperative administration prevent the supression of NK cell activity that occurs following surgery (25, 26).

We recently reported on oncolytic vaccinia virus to selectively infect, replicate, and express a transgene in cancer tissue of human patients following an intravenous infusion (27). Oncolytic virus has shown several mechanisms of action including direct tumor cell cytotoxicity, tumor-specific vascular shutdown, and induction of innate and adaptive immune responses (28). We have also shown that parapoxvirus ovis (ORFV) exerts its antitumor effect mainly through NK cell activation (29). The compelling preclinical and clinical oncolytic vaccinia data have led us to hypothesize that perioperative treatment with oncolytic vaccinia could improve recurrence-free survival following surgical resection.

While the detrimental impact of surgery on NK cell function has clearly been addressed, the direct *in vivo* role of NK cells in clearing tumor metastases following surgery has yet to be shown. In this study, the function of NK cells in surgically stressed mice was rigorously characterized, and the first use of novel oncolytic vaccinia to recover this defect is provided.

Materials and Methods

Mice

C57BL/6 (B6), BALB/c, and IL-2γR-KO (NK-deficient) were purchased from The Jackson Laboratory. Animals were housed in pathogen-free conditions and all studies conducted were in accordance with institutional guidelines at the Animal Care Veterinary Service facility of the University of Ottawa (Ottawa, ON).

Establishment of murine surgical stress model

Experimental metastasis model. Mice were subjected to 2.5% isofluorane (Baxter Corp.) for induction and maintenance of anesthesia. Routine perioperative care for mice was conducted following university protocols including pain control using buprenorphine (0.05 mg/kg) administered subcutaneously the day of surgery and every 8 hours for 2 days postoperatively. Surgical stress was induced in mice by an abdominal laparotomy (3-cm midline incision) and left nephrectomy preceded by an intravenous challenge of 3e5 B16lacZ cells to establish pulmonary metastases. Surgery commenced 10 minutes following tumor inoculation. Animals were euthanized at 18 hours or 3 days following tumor inoculation, and their lungs were stained with X-gal (Bioshop) as described previously (30). Total number of surface visible metastases was determined on the largest lung lobe (left lobe) using a stereomicroscope (Leica Microsystems). This analysis correlates well with the total number of lung metastases on all 5 lobes and was therefore used for the current study. For rescue of tumor cell clearance assays—1e7 plaque forming units (PFU) of ORFV and 1e7 PFU of oncolytic vaccinia virus, were injected into mice 4 hours and 3e5 B16lacZ cell 1 hour, respectively, before surgery.

Spontaneous metastasis model. 1e6 4T1 breast tumor cells in 50 μL of sterile PBS were injected orthotopically into the fat pad of BALB/c mice at day 0. At 14 days posttumor cell injection, a complete resection of the mammary primary tumor along with abdominal nephrectomy was conducted. For groups receiving oncolytic vaccinia treatment, 1e7 PFU of ORFV was given 1 hour before surgery at 14 days. At 28 days postorthotopic tumor injection, lungs were isolated and photographs were taken.

Cell lines and viruses

B16F10LacZ melanoma cell line was obtained from Dr. K Graham (London Regional Cancer Program, Ontario, Canada) and maintained in complete Dulbecco's Modified Eagle's Medium. Cells were resuspended in DMEM without serum for intravenous injection through the lateral tail vein. 3e5 cells at more than 95% viability were injected in a 0.1 mL volume per mouse. RMA (Rauscher murine leukemia virus-induced T cell lymphoma) and RMA-S (MHC-deficient variant of RMA) were a kind gift from Dr. Andre Veillette (Institut de recherches cliniques de Montreal, Quebec). 4T1 mammary carcinoma, YAC-1, and K562 leukemic cell lines were purchased from American Type Culture Collection and maintained in cRPMI. All cell lines were verified to be mycoplasma free and show appropriate microscopic morphology at time of use. Wild-type ORFV (strain NZ2) was obtained from Dr. Andrew Mercer (University of Otago, Dunedin, New Zealand) and was injected and titered as previously described (31). Oncolytic vaccinia virus JX-594-GFP$^+$/βgal$^-$ was prepared as previously described (32).

Antibodies and FACS analysis

To analyze splenic, blood, and lung lymphocyte populations, organs were removed from mice and red blood cells lysed using ammonium chloride-potassium (K) chloride lysis buffer. The following monoclonal antibodies (mAb) were used: anti-TCR-β (H57-597), anti-CD62L (MEL-14), anti-CD11b (M1/70), anti-CD122 (TM-beta1), anti-NKG2D (CX5), anti-KLRG1 (2F1), anti-GR1 (RB6-8C5), anti-CD4 (GK1.5), and anti-CD25 (PC61.5) were purchased from eBiosciences. Propidium iodide (PI)/Annexin V stains and Isotype controls were purchased from BD Biosciences. Spleen NK cell IFN-γ secretion was examined following a 5 hours GolgiPlug (BD Biosciences) incubation using: anti-CD3 (17A2), anti-NK1.1 (PK136), and anti-IFN-γ (XMG1.2), all from BD Biosciences. Fluorescence-activated cell sorting (FACS) acquisitions were conducted on a CyAN-ADP using Summit software (Beckman Coulter). Data were analyzed with Kaluza software.

Evaluation of the role of NK cells in postoperative tumor metastases

NK cells were depleted using α-asialoGM1 antibody (Cedarlane) as previously described (30). The lung tumor burden was quantified at 3 days postsurgery

Cell transfer experiments

For NK cell transfer experiments, splenocytes were isolated from no surgery control or 18 hours postsurgery from B6 mice, enriched for NK cells with DX5$^+$ microbeads on an AutomacsPro cell sorter (Miltenyi Biotec). 1e6 DX5$^+$ NK

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cells as determined by FACS were injected intravenously into NK-deficient mice. For all transfers, 3e5 B16lacZ tumor cells were injected intravenously 1 hour after immune cell transfer. Three days after immune and tumor cell injection, lungs of NK-deficient mice were isolated and quantified with Xgal. For carboxyfluorescein succinimidyl ester (CFSE)-labeled whole splenocyte transfers, bulk splenocytes were labeled with 10 μmol/L of CFSE (Invitrogen). 10e6 $CFSE^+$ splenocytes were injected intravenously into mice that received surgery 2 hours prior. Eighteen hours after splenocyte transfer—lung, peripheral blood, and peritoneal lavage cells were processed and analyzed by flow cytometry for $CFSE^+$ cells.

Ex vivo NK cell cytotoxicity assay

The chromium release assay was conducted as previously described (33). Briefly, splenocytes were isolated from surgically stressed and control mice at 18 hours postsurgery. Pooled and sorted NK cells were resuspended at a concentration of 2.5×10^6 cells/mL and then mixed with chromium-labeled target cells (YAC-1, B16F10LacZ, RMA-S, and EL-4), which were resuspended at a concentration of 3×10^4 cells/mL at different E:T (50:1, 25:1, 12:1, 6:1). For antibody-dependent cell-mediated cytotoxicity (ADCC), after ^{51}Cr-labeling, target cells were incubated in the presence of 10 μg/mL of anti-Thy1.2 mAb or Isotype control Ab for 30 minutes and then washed before coincubation with effector cells. For rescue of NK cell impairment assays, 1e7 PFU ORFV or JX-594-$GFP^+/\beta gal^-$ were injected into mice 4 hours before surgery.

In vivo NK cell rejection assay

The *in vivo* rejection assay was conducted as previously described (33). Briefly, RMA and RMA-S were differentially labeled with 5 and 0.5 μmol/L CFSE, respectively. A mixture of 1e6 cells of each type was injected intraperitoneally into B6 recipient mice treated with surgery (4 hours prior). After 18 hours, peritoneal cells were harvested from the peritoneum with PBS-2 mmol/L EDTA and analyzed for the presence of CFSE-labeled tumor cells by FACS.

Human PBMC cytotoxicity assay

Human whole blood was collected (Perioperative blood collection program = OHREB#2011884, Neoadjuvant JX-594 clinical trial NCT01329809) and processed immediately for peripheral blood mononuclear cell (PBMC) using Ficoll-Paque (Stemcell). PBMC were resuspended at a concentration of 7.5×10^6 in freezing media (RPMI, 12.5% human serum albumin and 10% dimethyl sulfoxide). K562 were harvested and labeled with ^{51}Cr. Assessment of PBMC killing was determined as described earlier.

Cytokine and chemokine analysis

Twenty-six common cytokines and chemokines were assayed 18 hours postsurgery or no treatment from the serum of B16lacZ tumor bearing mice using commercial multianalyte ELISArray kits (SABiosciences) and conducted according to manufacturer's instructions.

Statistical analysis

Statistical significance was determined by Student t test with a cutoff P value of 0.05. Data are presented as ±SD.

Results

Surgical stress increases lung metastases and NK cells are important mediators of this effect

We have developed a reproducible mouse model of surgical stress that results in the dramatic enhancement of pulmonary metastases. At 3 days postabdominal nephrectomy and B16F10lacZ tumor inoculation, lungs were harvested and visualized for metastases. Surgery clearly increases the amount of pulmonary metastases as compared with untreated mice by 2-fold (Fig. 1A, NK cell–intact). In addition, our findings indicate that this prometastatic effect seen following surgery is not mouse strain, cell-type, anesthesia/analgesia, or surgery specific (34).

Previous studies have shown that NK cells play an important role in clearing tumor cells in the vasculature (35, 36). To determine if this mechanism is operating in the postoperative period, the surgical stress experiment was repeated following pharmacologic depletion of NK cells. In mice depleted of NK cells, both surgically stressed and untreated mice developed increased lung tumor burden. More importantly, surgery did not result in an increased number of lung metastases over and above the no surgery controls, suggesting a preventive role for NK cells in the postoperative formation of metastases (Fig. 1A, NK cell depleted). These findings were further confirmed by reproducing the same results in transgenic mice deficient in NK cells (IL-2γR-KO, NK-deficient mice; Fig. 1B). Thus, the underlying mechanism of surgical stress resulting in increased metastases seems to involve NK cells.

To complement our experimental metastasis model, we used a 4T1 murine breast carcinoma model, which is a highly aggressive tumor that spontaneously metastasizes from the primary mammary gland to multiple distant sites including the lungs. At 14 days postorthotopic tumor implantation, a complete resection of the primary mammary tumor along with abdominal nephrectomy (surgical stress) was conducted. At 28 days posttumor inoculation, a significant increase in the number of lung tumor nodules was observed in surgically stressed mice compared with untreated controls (Fig. 1C and D). These results further corroborate the prometastatic effect of surgery in our experimental metastasis model.

Surgery suppresses NK cell function and prevents them from removing experimental lung metastases

To corroborate that NK cells indeed facilitate the removal of lung tumor metastases, we adoptively transfered 1e6-purified splenic $DX5^+$ NK cells from surgically stressed and untreated mice into NK-deficient mice. To establish experimental lung metastases, 3e5 B16lacZ cells were injected into NK-deficient mice 1 hour after NK cell transfer. At 3 days posttreatment, we found significantly increased lung tumor burden in NK-deficient mice that received surgically stressed NK cells compared with those that received NK cells from untreated animals (Fig. 1E). As an added control, we transfered the negative immune cell population (which consisted of non-NK cell leukocytes)

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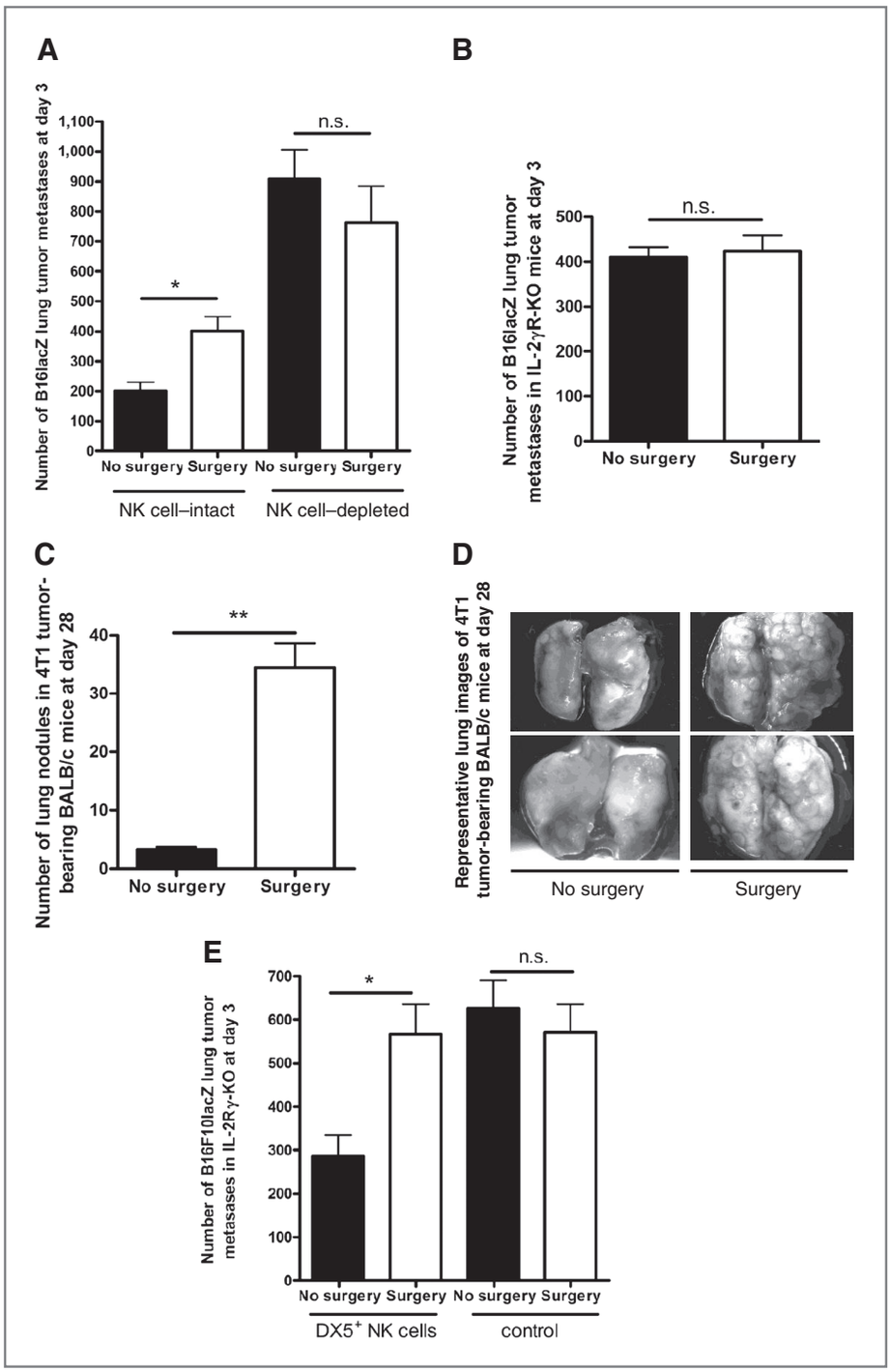

Figure 1. Surgical stress increases lung tumor metastases by impairing NK cells. Quantification of lung tumor metastases at 3 days from NK–intact and NK-depleted mice subjected to surgical stress (A) and NK-deficient mice subjected to surgery (B). Assessment of 4T1 lung tumor metastases at 28 days by number of tumor nodules (C) and photographs of representative lungs (D). E, quantification of lung tumor metastases at 3 days from NK-deficient mice receiving adoptively transferred NK cells or non-NK cells from surgically stressed and control mice. Data are representative of 3 similar experiments with $n = 5$–10/group (*, $P = 0.01$; **, $P < 0.0001$; n.s., not significant).

post-NK cell enrichment and observed complete abrogation of the effect of surgery on pulmonary metastases (Fig. 1E), further eliminating potential roles played by T cells and macrophages in our surgical stress model. By transfering surgically stressed NK cells and recreating the effect of surgery on the formation of metastases, we have definitively established that the prometastatic effect of surgery is mediated by NK cells.

Surgically stressed NK cells display abnormal NK cell migration and markers

Because surgical stress resulted in dysfunctional NK cell clearance of tumor cells *in vivo*, we questioned whether NK cell numbers and cell surface receptor status are affected by surgery. We examined NK cell frequencies in the spleen, blood, and lungs by flow cytometry, and they were found to be similar in surgically stressed and untreated mice (Fig. 2A). Other

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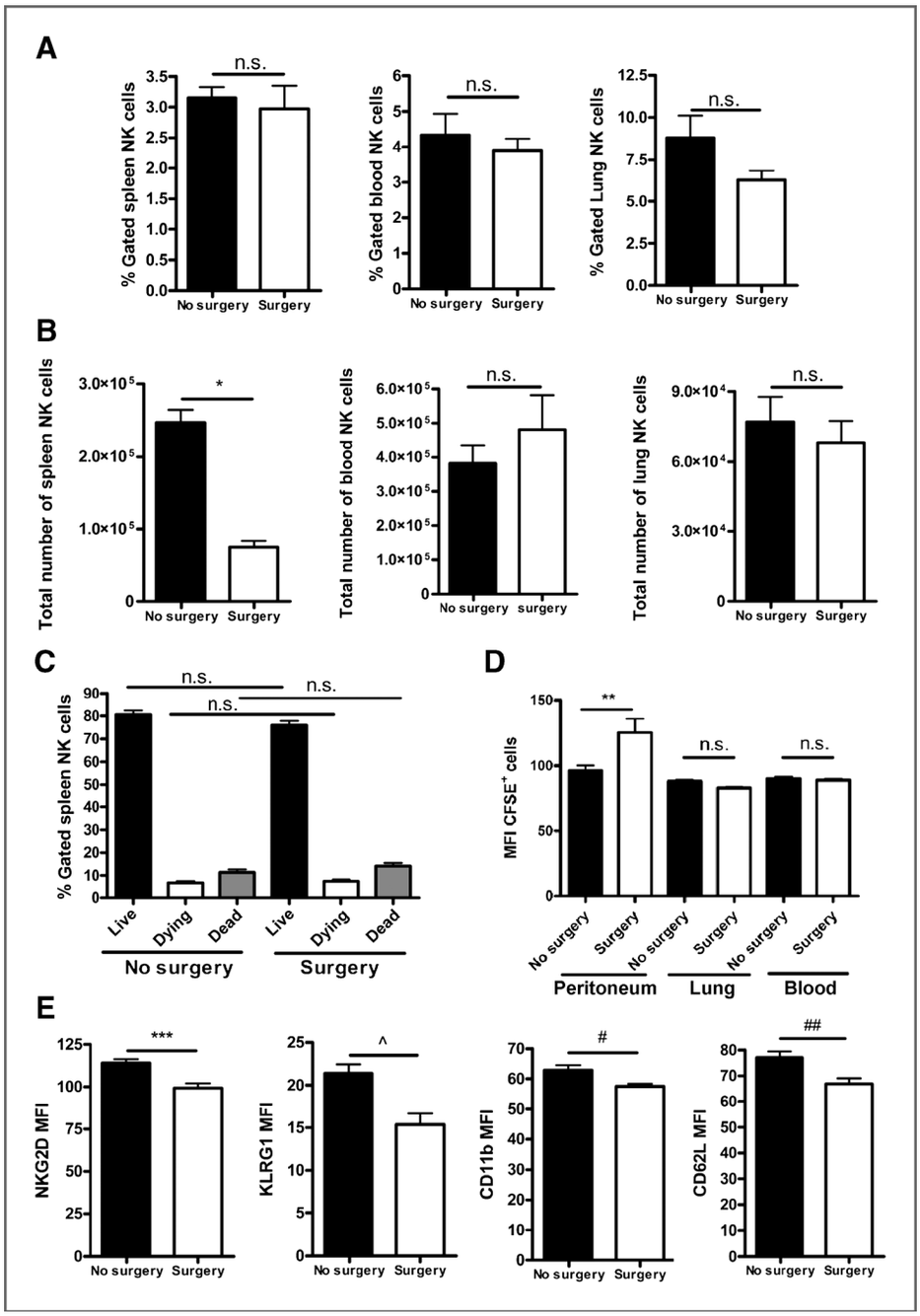

Figure 2. Surgically stressed NK cells display abnormal NK cell migration and markers. The mean percentage (A) or total number (B) of NK cells ($CD122^+$, TCR-β^-) is shown from the spleen, blood, or lung of surgically stressed and untreated mice. C, the mean percentages of splenic NK cells were stained with PI and AnnexV (live, PI^-Annexin V^-; dying, PI^-Annexin V^+; dead, PI^+ Annexin V^+). D, $CFSE^+$ cells from peritoneal lavage, blood, and lungs from surgically stressed and untreated mice are shown. E, mean fluorescence intensity (MFI) of the indicated cell surface marker was conducted on $CD122^+$, TCR-β^- gated splenocytes from surgically stressed and untreated mice. Data are representative of 3 similar experiments in which $n = 5$–6/group (*, $P = 0.005$; **, $P = 0.038$; ***, $P = 0.0008$; ^, $P = 0.00138$; #, $P = 0.0092$; ##, $P = 0.025$; n.s., not significant).

immune cell populations were also assessed in the spleen and no differences were detected (Supplementary Fig. S1A). However, total NK cell numbers in spleen were reduced by half in surgically stressed mice but were found to be similar in blood and lungs (Fig. 2B). Given this discrepancy between spleen NK cell proportions and total numbers, we decided to verify NK cell viability. We observed comparable high percentages of live splenic NK cells (PI^-/Annexin V^-) in both surgically stressed and untreated mice. These results show that the dysfunction in NK cells observed is not due to cell viability (Fig. 2C). Another possibility to explain this discrepancy is the exit of NK cells from the spleen. We assessed various lymph nodes for accumulation of NK cells and detected no differences between surgery and untreated groups (Supplementary Fig. S1B). Because spleen NK cells did not seem to migrate to lymph nodes postsurgery, we assessed for their presence in the peritoneum, blood, and lungs through splenocyte transfer. We observed an increase in $CFSE^+$ cells in the peritoneum of surgically stressed mice but not at other sites (Fig. 2D). This suggests that following surgery, spleen

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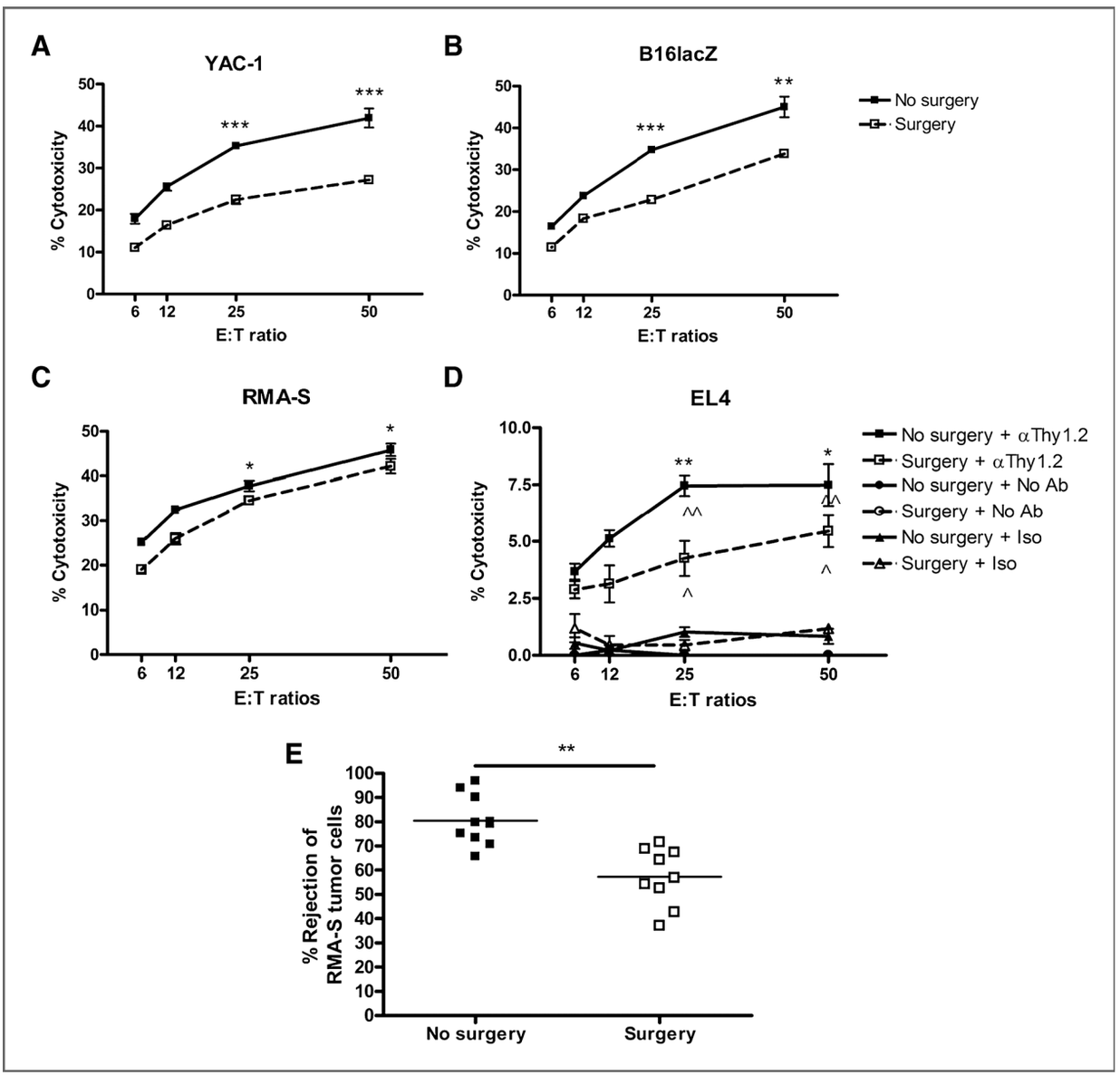

Figure 3. Defective killing of tumor cells by surgically stressed NK cells. The ability of purified $DX5^+$ NK cells from surgically stressed and untreated controls to kill tumor cells YAC-1 (A), B16F10lacZ (B), RMA-S (C), and EL-4 labeled with α-Thy1.2/isotype control/no Ab (D). The data are displayed as the mean percentage (±SD) of chromium release from triplicate wells for the indicated E:T ratios. Data are representative of 3 similar experiments in which $n = 4$–6/group (*, $P = 0.01$; **, $P = 0.0034$; ***, $P = 0.0001$ comparing "No surgery" with "Surgery"; ^, $P = 0.042$ comparing "No surgery + αThy1.2" with "No surgery + Iso"; ^^, $P = 0.02$ comparing "Surgery + αThy1.2" with "Surgery + Iso"). E, the ability of NK cells from surgically stressed and untreated controls to reject RMA-S tumor cells (**, $P = 0.0034$). Data are pooled from 3 independent experiments.

NK cells migrate preferentially to sites of surgical trauma (abdominal nephrectomy is conducted in the peritoneal cavity). A panel of NK cell markers (NKG2D, KLRG1, CD62L, and CD11b) were further tested on splenic NK cells (Fig. 2E) and found to show decreased expression following surgery. The reduction in the expression of NKG2D suggests a probable deficiency in surgically stressed NK cell killing against tumors as most tumors express ligands for the activating NKG2D receptor (37). KLRG1 is a marker of NK cell activation/maturation and its decrease likely reflects impaired NK cell function postsurgery. CD62L is a cell-adhesion molecule that binds to selectins for NK cell migration and CD11b is a complement receptor. Decreased expression of CD62L and CD11b by surgically stressed NK cells also explains the altered splenic NK cell numbers and migration observed postsurgery. In comparison, NK cell surface expression of NK1.1, CD122, DX5, NKp46, NKRP1C, LFA-1, B220, CD27, and Ly49D were assessed and no significant difference was detected between treatment groups (data not shown).

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Defective killing of tumor cells by surgically stressed NK cells

The dysfunctional phenotype and migration of surgically stressed NK cells raises the question of how other facets of NK cell function is affected by surgery. We examined NK cell cytotoxicity by conducting an *ex vivo* NK cell cytotoxicity assay. It was found that against all tumor targets tested (NKG2D ligand bearing YAC-1, MHC-I-deficient B16F10lacZ, and RMA-S), surgically stressed NK cells were significantly inhibited in their ability to kill tumor cells (Figs. 3A–C). Interestingly, the killing of Thy1.2-labeled EL-4 target cells was found to be similarly impaired by surgically stressed NK cells in an ADCC assay (Fig. 3D). To further support our *ex vivo* killing data, we conducted an *in vivo* tumor rejection assay. The rejection of RMA-S cells has also been shown to be due to NK cells by serum-mediated NK cell depletion (38). The rejection of RMA-S by surgically stressed mice (55%) was significantly lower than untreated controls (80%; Fig. 3E). These data strongly suggest that surgical stress impairs various NK cell–activating receptors and causes a generalized NK cell cytotoxicity defect.

Surgical stress induces altered tissue signals and expansion of myeloid-derived suppressor cells

Next, we systematically assessed for surgery-induced tissue signals that might be responsible for the suppression of NK cells. Using a multianalyte protein array, we profiled 14 cytokines and 12 chemokines in the serum of mice taken 18 hours postsurgery. We observed relative changes in protein levels in the serum of surgically stressed mice over untreated controls for numerous cytokines and chemokines. Specifically, we observed an increase in interleukin (IL)-5, IL-6, and TGF-β in surgically stressed mice (Fig. 4A). All 3 cytokines have documented immune-suppressive effects and their increase in serum may contribute to NK cell dysfunction postsurgery (39). In parallel, surgically stressed mice showed an increase in granulocyte colony-stimulating factor (G-CSF; Fig. 4A), MCP-1, and eotaxin levels (Fig. 4B). In contrast, SDF-1, IP-10, and 6Ckine levels were reduced following surgery. These proteins are chemoattractants for monocytes and lymphocytes. Their role in mediating NK cell dysfunction following surgery has not been documented and may represent contributing factors toward the defective NK cell function/migration

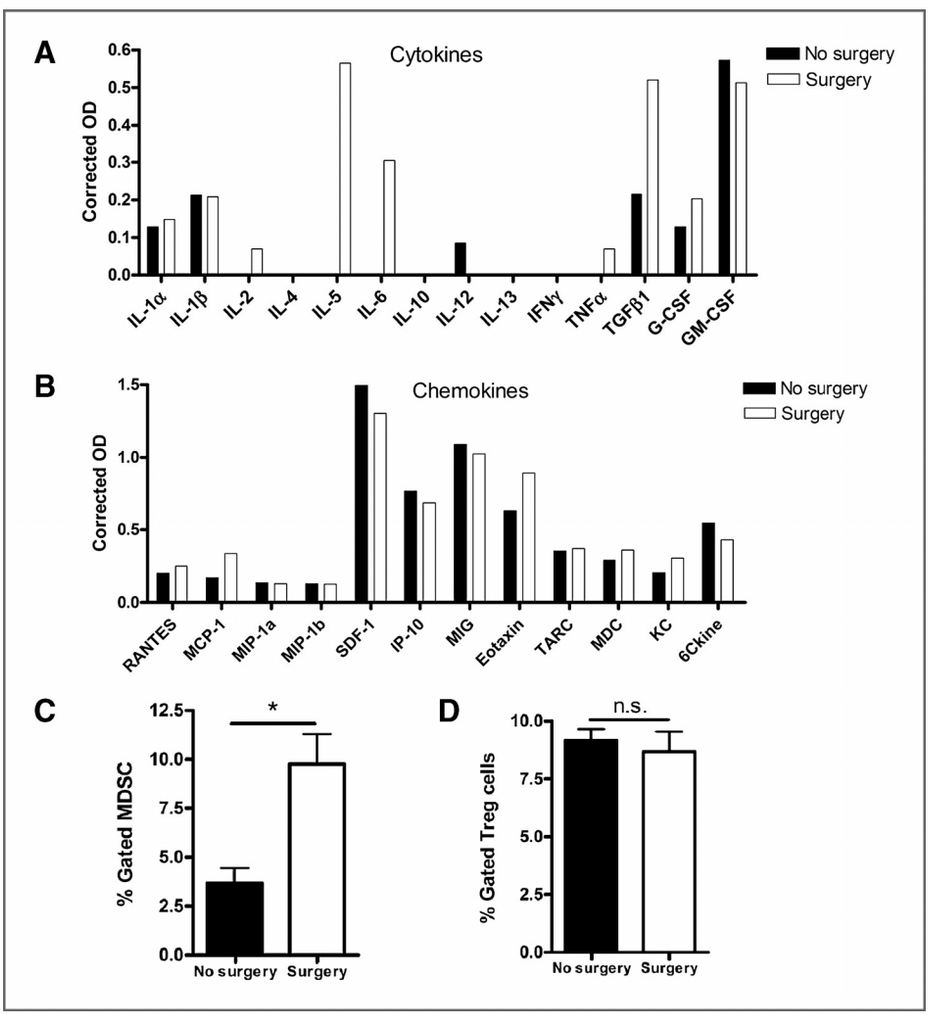

Figure 4. Surgical stress induces altered tissue signals and expansion of myeloid-derived suppressor cells (MDSC). Relative serum cytokine (A) and chemokine (B) levels 18 hours postsurgery and untreated from B16lacZ tumor-bearing mice. Data are representative of 2 independent experiments in which $n = 10–20$/group. The mean percentage of spleen MDSC ($CD11b^+$, $Gr\text{-}1^+$; C) and Tregs ($CD4^+$, $CD25^+$; D) is shown from surgically stressed and untreated mice. Data are representative of 3 similar experiments in which $n = 4$/group (*, $P = 0.0134$; n.s., not significant). OD, optical density.

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observed postsurgery. Further studies are required to characterize each protein and their exact role in perioperative NK cell suppression.

Next, we questioned whether surgery caused alterations in immune-suppressive cell populations. Myeloid-derived suppressor cells (MDSC) represent a population of immature myeloid cells that expand dramatically during tumor progression and impair adaptive immunity (40). In surgically stressed mice, we observed an approximate 2.5-fold increase in the proportion of spleen MDSC compared with no surgery controls (Fig. 4C). However, it remains to be investigated how the expansion of MDSC contribute to perioperative NK cell suppression. In addition, we looked for perturbations in regulatory T cell (Treg) populations following surgery. Tregs have been shown to inhibit the effector functions of immune cells including NK cells (41, 42). We did not observe any differences in spleen Treg populations following surgery (Fig. 4D).

Novel anticancer oncolytic vaccinia as perioperative therapy against immune-suppressive effects of surgery

Given the suppressive effects of surgery on NK cell function, we used perioperative treatments to enhance NK cell function and attenuate the formation of metastatic disease following surgery. Currently, there are a number of novel oncolytic vaccinia candidates in our laboratory under assessment, including preclinical ORFV and the clinical candidate JX-594—an attenuated vaccinia virus strain that contains the immune modulating gene granulocyte macrophage colony-stimulating factor (GM-CSF). First, we determined whether perioperative oncolytic vaccinia can attenuate the formation of metastatic disease following surgery. At 3 days posttreatment with therapeutic doses of ORFV or JX-594-GFP$^+$/βgal$^-$, a dramatic attenuation of experimental metastases was observed in surgically stressed mice compared with surgically stressed mice that did not receive oncolytic vaccinia therapy, suggesting that both viruses mediate tumor metastases removal (Fig. 5A and B). Next, we determined whether the decrease in metastases was a virus-induced NK cell-mediated "tumoricidal" effect or oncolytic vaccinia–mediated "viral oncolysis" effect. When NK cells were depleted, we observed for both viruses, approximately one-third less metastases in the surgery + oncolytic vaccinia group (Fig. 5C and D) compared with the surgery alone group with NK depletion. These data suggest that tumor metastases removal in our surgical stress model is mainly mediated through oncolytic vaccinia viral activation of NK cells and subsequent NK cell mediated tumor lysis. This does not rule out a partial and important role of oncolytic vaccinia–mediated killing of tumors. These data are supported by our previous findings of the strong NK stimulating abilities of ORFV (29). The extent of JX-594-GFP$^+$/βgal$^-$ on NK cell activation is currently being assessed in our laboratory. To further characterize NK cell function following perioperative administration of oncolytic vaccinia, we examined NK cell cytotoxicity and IFN-γ secretion. We observed a significant surgery induced defect in NK cell killing along with a dramatic recovery of NK killing following perioperative administration of both oncolytic vaccinia compared with surgery alone (Fig. 5E and F). Similarly, we observed increased IFN-γ secretion from surgically stressed mice receiving perioperative oncolytic vaccinia compared with surgery alone (Fig. 5G and H). Finally, we rescued the formation of lung tumors in the 4T1 mammary tumor model with ORFV given 1 hour before surgery on 14 days. At 28 days posttumor inoculation, a significant increase in the number of lung tumor nodules was observed in surgically stressed mice compared with untreated controls along with a striking clearance of lung nodules in mice receiving perioperative ORFV (Fig. 5I). Taken together, these results show that we can successfully treat perioperative NK cell suppression with novel oncolytic vaccinia therapy.

Surgery suppresses and intravenous infusion of oncolytic vaccinia virus enhances NK cell function in human cancer patients

To understand and prevent postoperative NK cell suppression in human patients, we collected blood from patients with cancer as part of 2 different protocols: Ottawa Hospital cancer surgery patients enrolled in the Perioperative Blood Collection Program and in the neoadjuvant JX-594 phase IIa clinical trial. Blood is collected at different time points including preoperatively, on postoperative day (POD) 1, POD 3 (±1), and POD 28 (±14) for the first study and at preoncolytic vaccinia, postoncolytic vaccinia D3, postoncolytic vaccinia D14, and postoncolytic vaccinia D49 for the second trial. As part of the first study, we observed a decrease in the NK cell cytotoxicity at POD 1 and 3 and a return to baseline at POD 28(±14) in 2 human patients studied so far (Fig. 6A). In the neoadjuvant JX-594 trial, NK cell cytotoxicity improves in the setting of JX-594 compared with baseline control blood for 2 enrolled patients (Fig. 6B).

Discussion

In this study, we have clearly shown that the innate NK cell responses triggered by oncolytic vaccinia are a vital component of successful perioperative treatment, capable of overcoming immunosuppressive postsurgery microenvironments, and clearing metastatic disease. We, therefore, propose the perioperative stimulation of the immune system with oncolytic vaccinia as a way to avoid the NK suppressive effects of cancer surgery. The early postoperative period is an ideal window for immune-based anticancer therapies because the tumor burden is at its absolute lowest immediately following resection of the primary tumor. There is strong evidence in the animal setting that numerous agents that broadly stimulate the immune system are effective in significantly reducing the incidence of metastatic disease after surgery. Perioperative administration of recombinant IL-2 and IFN-α has been explored in early-phase clinical trials showing their potential to prevent postoperative NK cell suppression and enhance progression-free survival (23–26, 43–47).

We clearly show that an analogous mechanism of surgery-induced NK cell cytotoxic suppression is occuring in human patients postsurgery. Our human data confirm that NK cell activity is indeed suppressed following surgery, but returns to baseline at POD 28(±14). As part of the neoadjuvant JX-594 trial, NK cell cytotoxicity improves in the setting of oncolytic

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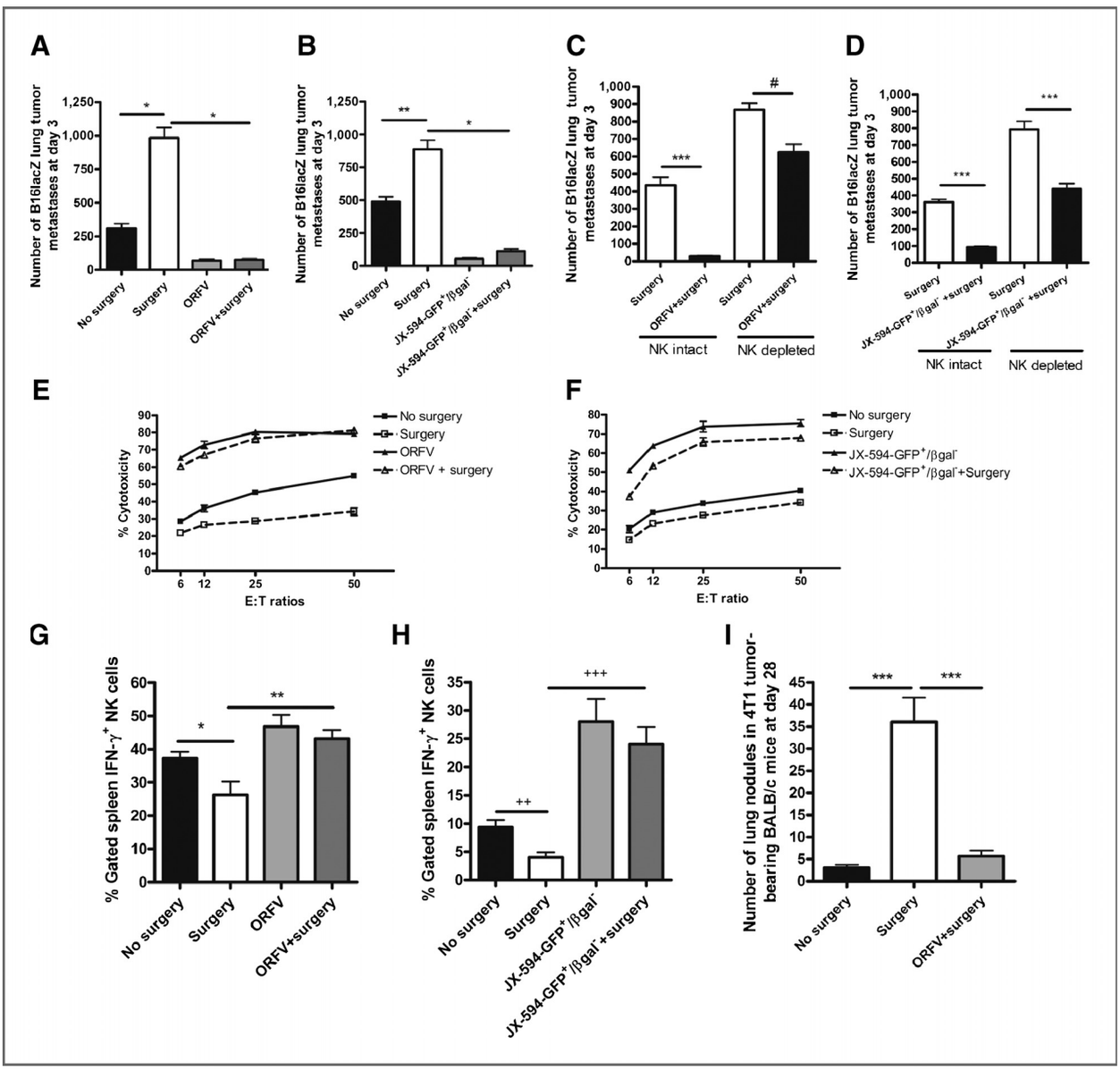

Figure 5. Novel anticancer oncolytic vaccinia as perioperative therapy against immune-suppressive effects of surgery. A and B, quantification of B16lacZ lung tumor metastases at 3 days from the indicated treatment groups (*, $P < 0.001$; **, $P = 0.0005$). C and D, quantification of lung tumor metastases at 3 days from NK-intact and NK-depleted mice subjected to surgery with and without oncolytic vaccinia (***, $P = 0.0001$; #, $P = 0.0034$). E and F, *in vitro* killing by indicated treatment groups. G and H, percentage gated IFN-γ expression from NK cells isolated from the spleen of indicated treatment groups. Bar graphs represent data from 3 similar experiments. $n = 5$/group (+, $P = 0.04$; ++, $P = 0.012$; +++, $P = 0.0007$). I, quantification of 4T1 lung tumor metastases at 28 days from indicated treatment groups. $n = 7$–10/group. (***, $P = 0.0001$). All data are representative of at least 3 similar experiments.

vaccinia treatment compared with baseline control blood and no adverse events were reported. These results show the feasibility of administering oncolytic vaccinia perioperatively to patients with cancer.

In addition to our research interests in developing oncolytic vaccinia as direct oncolytic agents, we want to explore the potential for oncolytic vaccinia to trigger innate and adaptive immune responses. This will maximize the potential clinical impact of this virotherapy. Ongoing work in our laboratory is focused on understanding the molecular mechanism of surgery-mediated NK cell suppression in the preclinical and clinical setting. Specifically, we are further characterizing surgery-induced alterations in cyto/chemokine secretions and MDSC expansion and their role in modulating NK cell dysfunction.

Surgical removal of solid primary tumors is an essential component of cancer treatment. However, we and others have clearly shown surgery-induced suppression of immune cells, in particular, we have shown NK cell dysfunction leading to impaired clearing of metastases. There are currently no

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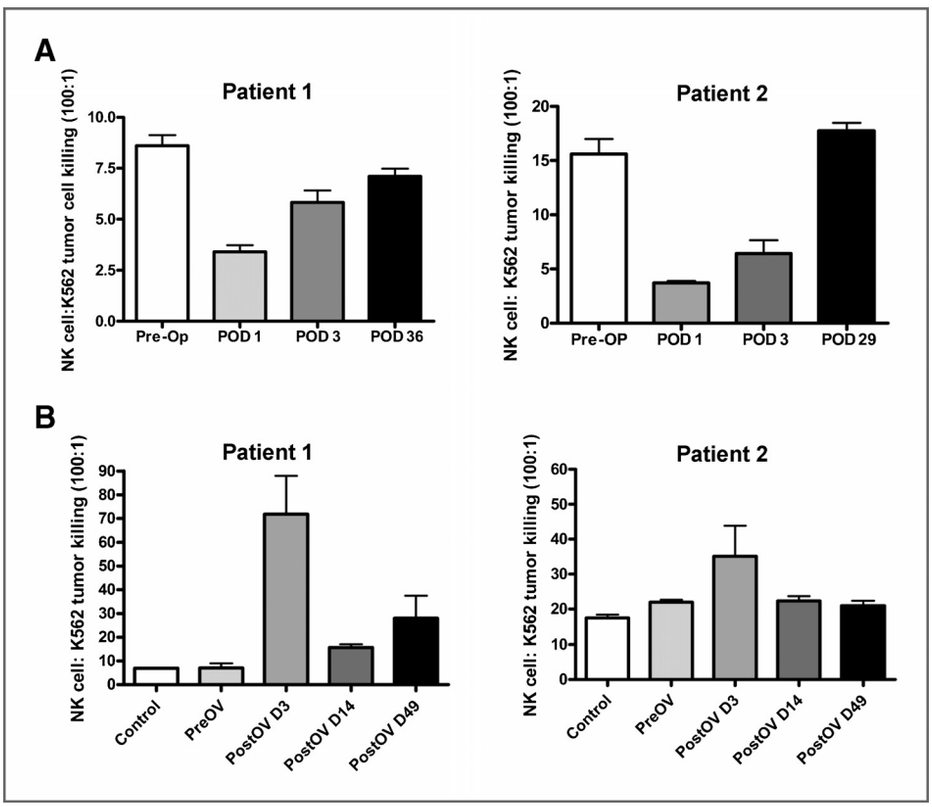

Figure 6. Defective *ex vivo* killing and oncolytic vaccinia activation of human NK cells. A, the ability of human PBMC to kill K562 tumor cells. Perioperative blood collection program: preoperative (Pre-OP), POD 1, POD 3, POD 28 (± 14). B, neoadjuvant JX-594 clinical trial: healthy donor control, preoncolytic vaccinia (PreOV), postoncolytic vaccinia (PostOV) D1, postoncolytic vaccinia D3, and postoncolytic vaccinia D14. The data are displayed as the mean percentage (±SD) of chromium release from triplicate wells for 100:1 (E:T) ratio.

standard perioperative anticancer therapies given to cancer surgery patients. Research into neoadjuvant immune therapy is clearly warranted to prevent immune dysfunction following surgery and to eradicate micrometastatic disease.

Disclosure of Potential Conflicts of Interest

C.J. Breitbach has ownership interest (including patents) in Jennerex, Inc. D.H. Kirn has ownership interest (including patents) in Jennerex, Inc. No potential conflicts of interest were disclosed by the other authors.

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