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Preventing post-operative 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 compared to mice who 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 MDSC populations were altered in surgically stressed mice. Significantly, perioperative administration of oncolytic 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 demonstrate for the first time that oncolytic vaccinia virus markedly increases NK cell activity in cancer patients. 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.

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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 demonstrated 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,

while 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 perioperative administration. The perioperative administration of recombinant cytokines has been explored in early phase clinical trials (23, 24). These studies have demonstrated 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). OV have several demonstrated mechanisms of action including direct tumour cell cytotoxicity, tumour-specific vascular shutdown, and induction of innate and adaptive immune responses (28). We have also shown that ORFV exerts its anti-tumor effect mainly though NK cell activation (29). The compelling pre-clinical and clinical OV data has led us to hypothesize that perioperative treatment with OV 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 demonstrated. In this study, the function of NK cells in surgically stressed mice was rigorously characterized and the first use of novel OV to recover this defect is provided.

MATERIALS AND METHODS

Mice - C57BL/6 (B6), BALB/c, and IL2γR-KO (NK-deficient) were purchased from The Jackson Laboratory. Animals were housed in pathogen-free conditions and all studies performed were in accordance with institutional guidelines at the Animal Care Veterinary Service facility of the University of Ottawa (Ontario).

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 (0.05mg/kg) administered s.c. the day of surgery and every 8h for 2d postoperatively. Surgical stress was induced in mice by an abdominal laparotomy (3cm midline incision) and left nephrectomy preceded by an i.v. challenge of 3e5 B16lacZ cells to establish pulmonary metastases. Surgery commenced 10 minutes following tumor inoculation. Animals were euthanized at 18h or 3d 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 PFU of ORFV and 1e7 PFU of oncolytic vaccinia virus was injected into mice 4h and 3e5 B16lacZ cell 1h, respectively, before surgery. *Spontaneous metastasis model:* 1e6 4T1 breast tumor cells in 50ul of sterile PBS were injected orthotopically into the fat pad of BALB/c mice at day 0. At 14d post-tumor cell injection, a complete resection of the mammary primary tumor along with abdominal nephrectomy was performed. For groups receiving OV treatment, 1e7

PFU of ORFV was given 1 hour prior to surgery at 14d. At 28d post orthotopic tumor injection, lungs were isolated and photographs taken.

Cell lines and viruses – B16F10LacZ melanoma cell line was obtained from Dr K Graham (LRCP, Ontario) and maintained in cDMEM. Cells were resuspended in DMEM without serum for i.v. injection through the lateral tail vein. 3e5 cells at >95% viability were injected in a 0.1ml volume/mouse. RMA (thymoma) and RMA-S (MHC-deficient variant of RMA) were a kind gift from Dr. Andre Veillette (IRCM, Quebec). 4T1 mammary carcinoma, YAC-1 and K562 leukemic cell lines was purchased from ATCC 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, New Zealand) and was injected and titred 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 RBCs lysed using ACK lysis buffer. The following mAbs were used: anti-TCRβ (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. PI/Annexin V stains and Isotype controls were purchased from BD Biosciences. Spleen NK cell IFNγ secretion was examined following a 5h GolgiPlug (BD Bioscience) incubation using: anti-CD3 (17A2), anti-NK1.1 (PK136) and anti-IFNγ (XMG1.2) all from BD Bioscience. FACS acquisitions were performed on a CyAN-ADP using Summit software (Beckman Coulter). Data was 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 3d post-surgery

Cell transfer experiments – For NK cell transfer experiments, splenocytes were isolated from no surgery control or 18h post-surgery from B6 mice, enriched for NK cells with $DX5^+$ microbeads on an AutomacsPro cell sorter (Miltenyi Biotec). 1e6 $DX5^+$ NK cells as determined by FACS were injected i.v. into NK-deficient mice. For all transfers, 3e5 B16lacZ tumor cells were injected i.v. 1h post immune cell transfer. 3d post immune and tumor cell injection, lungs of NK-deficient mice were isolated and quantified with Xgal. For CFSE labeled whole splenocyte transfers, bulk splenocytes were labeled with 10uM of CFSE (Invitrogen). 10e6 $CFSE^+$ splenocytes were injected i.v. into mice that received surgery 2h prior. 18h post 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 performed as previously described (33). Briefly, splenocytes were isolated from surgically stressed and control mice at 18 h post-surgery. Pooled and sorted NK cells were re-suspended at a concentration of $2.5x10^6$ cells/ml and then mixed with chromium labelled target cells (YAC-1, B16F10LacZ, RMA-S and EL-4), which were re-suspended at a concentration of $3x10^4$ cells/ml at different E:T (50:1, 25:1, 12:1, 6:1). For ADCC, after ^{51}Cr-labelling, target cells were incubated in the presence of 10 μg/ml of anti-Thy1.2 mAb or Isotype control Ab for 30 min and then washed before co-incubation with effector cells. For rescue of NK cell impairment assays, 1e7PFU ORFV or JX-594-GFP^+/βgal^- were injected into mice 4 h before surgery.

***In vivo* NK cell rejection assay -** The *in vivo* rejection assay was performed as previously described (33). Briefly, RMA and RMA-S were differentially labeled with 5 μM and 0.5 μM CFSE, respectively. A mixture of 1e6 cells of each type was injected i.p. into B6 recipient mice treated with surgery (4h prior). After 18h, peritoneal cells were harvested from the peritoneum with PBS-2mM 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 PBMC using Ficoll-Paque (Stemcell). PBMC were resuspended at a concentration of $7.5x10^6$ in freezing media (RPMI, 12.5% Human Serum Albumin and 10% DMSO). K562 were harvested and labelled with ^{51}Cr. Assessment of PBMC killing was determined as above.

Cytokine and chemokine analysis – 26 common cytokines and chemokines were assayed 18h post-surgery or no treatment from the serum of B16lacZ tumor bearing mice using commercial multi-analyte 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 is 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 3d post-abdominal nephrectomy and B16F10lacZ tumor inoculation, lungs were harvested and visualized for metastases. Surgery

clearly increases the amount of pulmonary metastases compared to untreated mice by 2-fold (Figure 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 demonstrated 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 pharmacological 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 (Figure 1A, NK cell depleted). These findings were further confirmed by reproducing the same results in transgenic mice deficient in NK cells (IL2γR-KO, NK-deficient mice) (Figure 1B). Thus, the underlying mechanism of surgical stress resulting in increased metastases appears 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 14d post-orthotopic tumor implantation, a complete resection of the primary mammary tumor along with abdominal nephrectomy (surgical stress) was performed. At 28d post-tumor inoculation, a significant increase in the number of lung tumor nodules was observed in surgically stressed mice compared to untreated controls (Figure 1C, 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 1h after NK cell transfer. At 3d post treatment, we found significantly increased lung tumor burden in NK-deficient mice that received surgically stressed NK cells compared to those that received NK cells from untreated animals (Figure 1E). As an added control, we transfered the negative immune cell population (which consisted of non-NK cell leukocytes) post-NK cell enrichment and observed complete abrogation of the effect of surgery on pulmonary metastases (Figure 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

Since 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 (Figure 2A). Other immune cell populations were also assessed in the spleen and no differences were detected (Supplemental Figure 1A). 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 (Figure 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 demonstrate that the dysfunction in

NK cells observed is not due to cell viability (Figure 2C). Another possibility to explain this discrepancy is the exit of NK cells from the spleen. We assessed various lymph nodes (LN) for accumulation of NK cells and detected no differences between surgery and untreated groups (Supplemental Figure 1B). Since, spleen NK cells did not appear to migrate to LN post-surgery, 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 (Figure 2D). This suggests that following surgery, spleen NK cells migrate preferentially to sites of surgical trauma (abdominal nephrectomy is performed in the peritoneal cavity). A panel of NK cell markers (NKG2D, KLRG1, CD62L and CD11b) were further tested on splenic NK cells (Figure 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 post-surgery. 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 post-surgery. By 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).

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 performing 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 (Figures 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 antibody dependent cellular cytotoxicity (ADCC) assay (Figure 3D). To further support our *ex vivo* killing data, we performed 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%) (Figure 3E). This data strongly suggests 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 MDSC.

Next, we systematically assessed for surgery-induced tissue signals that might be responsible for the suppression of NK cells. Using a multi-analyte protein array, we profiled 14 cytokines and 12 chemokines in the serum of mice taken 18h post surgery. 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 IL5, IL6 and TGFβ in surgically stressed mice (Figure 4A). All three cytokines have documented immune suppressive effects and their increase in serum may contribute to NK cell dysfunction post-surgery (39). In parallel, surgically stressed mice showed an increase in G-CSF (Figure 4A), MCP-1 and Eotaxin levels (Figure 4B). In contrast, SDF-1, IP-10 and 6Ckine levels were reduced following surgery. These proteins are chemo-attractants for monocytes and lymphocytes. Their role in mediating NK cell dysfunction following surgery has not been documented and may represent contributing factors towards the defective NK cell

function/migration observed post-surgery. 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 to no surgery controls (Figure 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 T regulatory (Treg) cell populations following surgery. Treg cells 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 (Figure 4D).

Novel anti-cancer OV as perioperative therapy against immune suppressive effects of surgery

Given the suppressive effects of surgery on NK cell function, we employed perioperative treatments to enhance NK cell function and attenuate the formation of metastatic disease following surgery. Currently, there are a number of novel OV candidates in our lab under assessment, including preclinical ORFV and the clinical candidate JX-594 - an attenuated vaccinia virus strain that contains the immune modulating gene GM-CSF. First, we determined whether perioperative OV can attenuate the formation of metastatic disease following surgery. At 3d post-treatment with therapeutic doses of ORFV or JX-594-GFP^{+}/βgal^{-}, a dramatic attenuation of experimental metastases was observed in surgically stressed mice compared to surgically stressed mice that did not receive OV therapy, suggesting that both viruses mediate

tumor metastases removal (Figure 5A,B). Next, we determined whether the decrease in metastases was a virus-induced NK cell-mediated "tumoricidal" effect or OV-mediated "viral oncolysis" effect. When NK cells were depleted, we observed for both viruses, approximately 1/3 less metastases in the surgery + OV group (Figure 5C,D) compared to the surgery alone group with NK depletion. This data suggests that tumor metastases removal in our surgical stress model is mainly mediated through OV viral activation of NK cells and subsequent NK cell mediated tumor lysis. This does not rule out a partial and important role of OV-mediated killing of tumors. This data is 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 lab. To further characterize NK cell function following perioperative administration of OV, we examined NK cell cytotoxicity and IFNγ 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 OV compared to surgery alone (Figure 5E,F). Similarly, we observed increased IFNγ secretion from surgically stressed mice receiving perioperative OV compared to surgery alone (Figure 5G,H). Lastly, we rescued the formation of lung tumors in the 4T1 mammary tumor model with ORFV given 1h prior to surgery on 14d. At 28d post tumor inoculation, a significant increase in the number of lung tumor nodules was observed in surgically stressed mice compared to untreated controls along with a striking clearance of lung nodules in mice receiving perioperative ORFV (Figure 5I). Taken together, these results demonstrate that we can successfully treat perioperative NK cell suppression with novel OV therapy.

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

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To understand and prevent postoperative NK cell suppression in human patients, we collected blood from cancer patients as part of two different protocols: Ottawa Hospital cancer surgery patients enrolled in the Perioperative Blood Collection Program and in the neoadjuvant JX-594 Phase 2a 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 preOV, postOV D3, postOV D14 and postOV 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 (Figure 6A). In the neoadjuvant JX-594 trial, NK cell cytotoxicity improves in the setting of JX-594 compared to baseline control blood for 2 enrolled patients (Figure 6B).

DISCUSSION

In this study, we have clearly demonstrated that the innate NK cell responses triggered by OV are a vital component of successful perioperative treatment, capable of overcoming immunosuppressive post-surgery microenvironments and clearing metastatic disease. We, therefore, propose the perioperative stimulation of the immune system with OV 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 demonstrating their potential to prevent postoperative NK cell suppression and enhance progression-free survival (23-26, 43-47).

We clearly demonstrate that an analogous mechanism of surgery induced NK cell cyototoxic suppression is occuring in human patients post-surgery. Our human data confirms 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 OV treatment compared to baseline control blood and no adverse events were reported. These results show the feasibility of administering OV perioperatively to cancer patients.

In addition to our research interests in developing OV as direct oncolytic agents, we want to explore the potential for OV to trigger innate and adaptive immune responses. This will maximize the potential clinical impact of this virotherapy**.** Ongoing work in our lab 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 has clearly demonstrated surgery induced suppression of immune cells, in particular we have demonstrated NK cell dysfunction leading to impaired clearing of metastases. There are currently no standard perioperative anti-cancer 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.

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Running Title: Oncolytic virus inhibits surgery induced NK cell dysfunction

FIGURE LEGENDS

Figure 1. Surgical stress increases lung tumor metastases by impairing NK cells. Quantification of lung tumor metastases at 3d from **(a)** NK intact and NK depleted mice subjected to surgical stress and **(b)** NK-deficient mice subjected to surgery. Assessment of 4T1 lung tumor metastases at 28d by **(c)** number of tumor nodules **(d)** photographs of representative lungs. **(e)** Quantification of lung tumor metastases at 3d 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).

Figure 2. Surgically stressed NK cells display abnormal NK cell migration and markers. The mean percentage **(a)** or total number **(b)** of NK cells ($CD122^+$, $TCR\beta^-$) 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 = $PI^-AnnexV^-$, dying = $PI^-AnnexV^+$, dead = $PI^+AnnexV^+$). **(d)** $CFSE^+$ cells from peritoneal lavage, blood and lungs from surgically stressed and untreated mice are shown. **(e)** MFI of the indicated cell surface marker was performed on $CD122^+$, $TCR\beta^-$ gated splenocytes from surgically stressed and untreated mice. Data are representative of 3 similar experiments where 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).

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 **(a)** YAC-1, **(b)** B16F10lacZ, **(c)** RMA-S, **(d)** EL-4 labeled with α-Thy1.2/ isotype control/no Ab. The data are displayed as the mean percent (+/- SD) of chromium release from triplicate wells for the indicated E:T ratios. Data are representative of 3 similar experiments where n=4-6/group

(*, p =0.01;**, p =0.0034; ***, p =0.0001 comparing No surgery to Surgery; ^, p=0.042 comparing No surgery + αThy1.2 to No surgery + Iso; ^^, p=0.02 comparing Surgery + αThy1.2 to 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.

Figure 4. Surgical stress induces altered tissue signals and expansion of myeloid derived suppressor cells. Relative serum cytokine **(a)** and chemokine **(b)** levels 18h post-surgery and untreated from B16lacZ tumor-bearing mice. Data are representative of 2 independent experiments where n =10-20/group. The mean percentage of spleen **(c)** MDSC ($CD11b^+$, $Gr\text{-}1^+$) and **(d)** Treg cells ($CD4^+$, $CD25^+$) is shown from surgically stressed and untreated mice**.** Data is representative of 3 similar experiments where n=4/group (*, p=0.0134; n.s., not significant).

Figure 5. Novel anti-cancer OV as perioperative therapy against immune suppressive effects of surgery. (a,b) Quantification of B16lacZ lung tumor metastases at 3d from the indicated treatment groups (*, p <0.001; **, p =0.0005). **(c,d)** Quantification of lung tumor metastases at 3d from NK intact and NK depleted mice subjected to surgery with and without OV (***, p =0.0001; #, p =0.0034). **(e,f)** *In vitro* killing by indicated treatment groups. **(g,h)** % gated IFNγ expression from NK cells isolated from the spleen of indicated treatment groups. Bar graphs represent data from three similar experiments. N=5/group (+, p =0.04; ++, p =0.012; +++, p= 0.0007). **(i)** Quantification of 4T1 lung tumor metastases at 28d from indicated treatment groups. N=7-10/group. (***, p =0.0001). All data are representative of at least 3 similar experiments.

Figure 6. Defective *ex vivo* killing and OV activation of human NK cells. The ability of human PBMC to kill K562 tumor cells **(a**,**b)** Perioperative blood collection program - preoperative, POD1, POD3, POD28(±14) **(c,d)** Neoadjuvant JX-594 clinical trial – healthy

donor control, PreOV, PostOV D1, PostOV D3, PostOV D14. The data are displayed as the mean percent (+/- SD) of chromium release from triplicate wells for 100:1 (E:T) ratio.

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Figure 1. Surgical stress increases lung tumor metastases by impairing NK cells

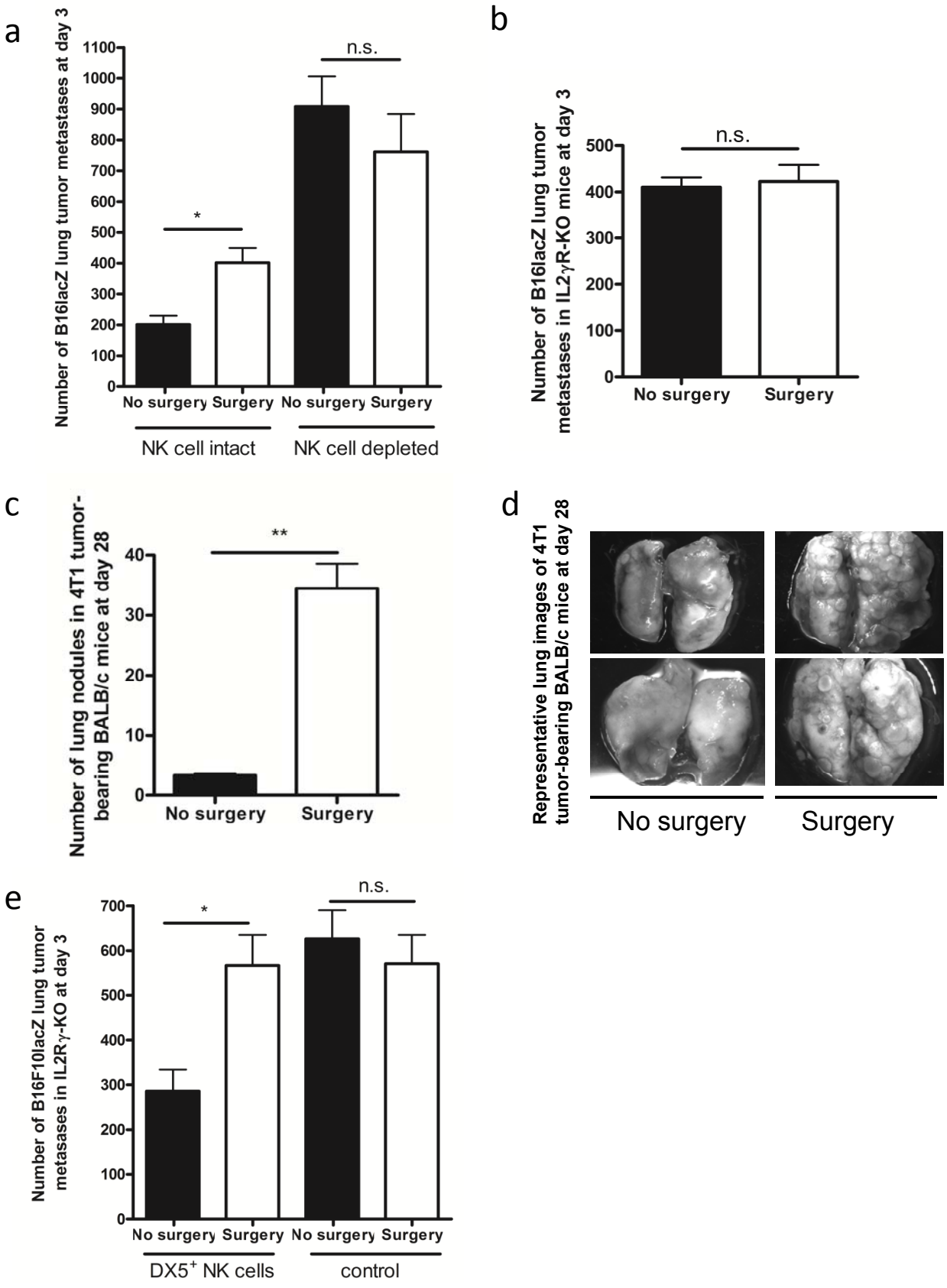

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Figure 2. Surgically stressed NK cells display abnormal NK cell markers and migration

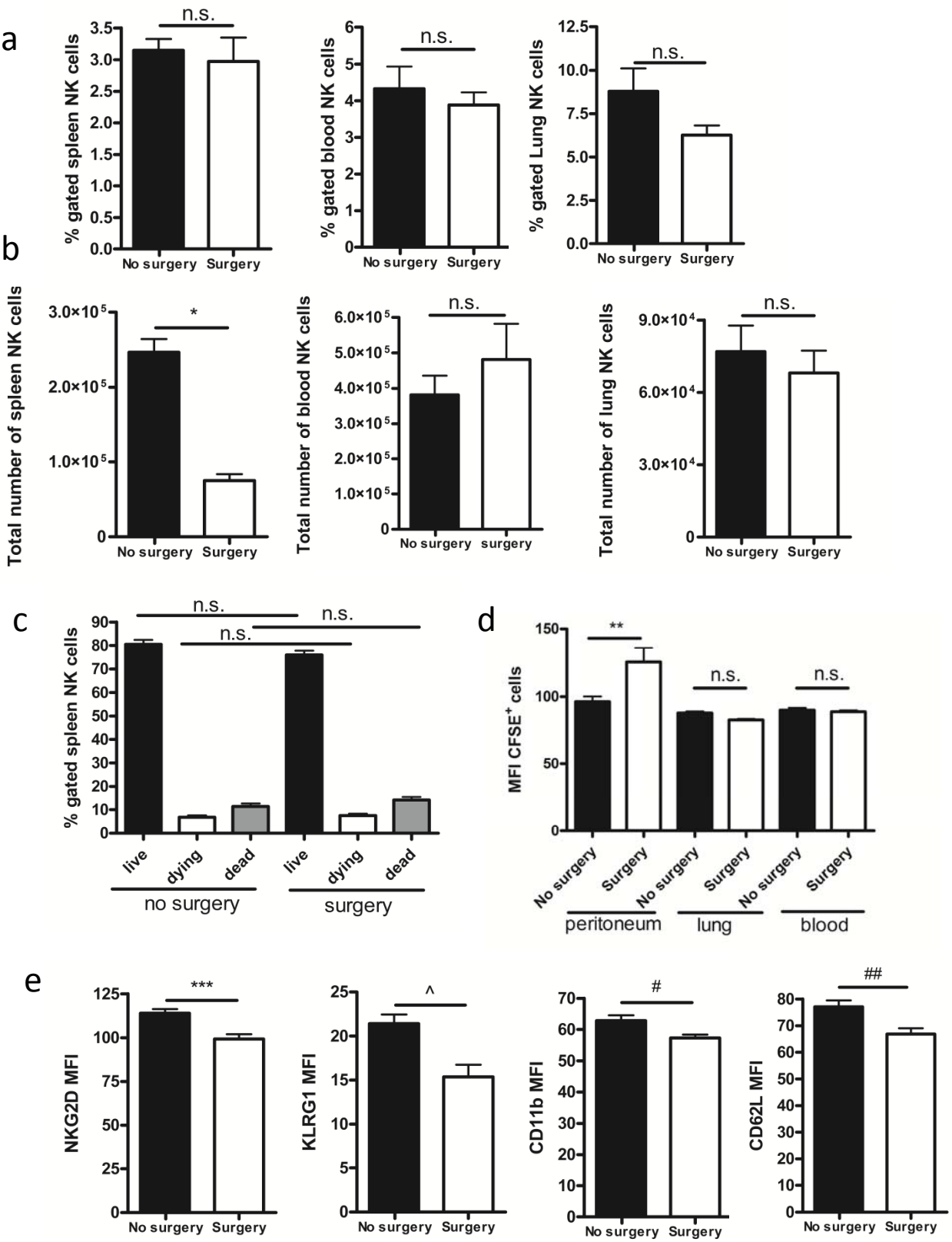

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Figure 3. Defective killing of tumor cells by surgically stressed NK cells

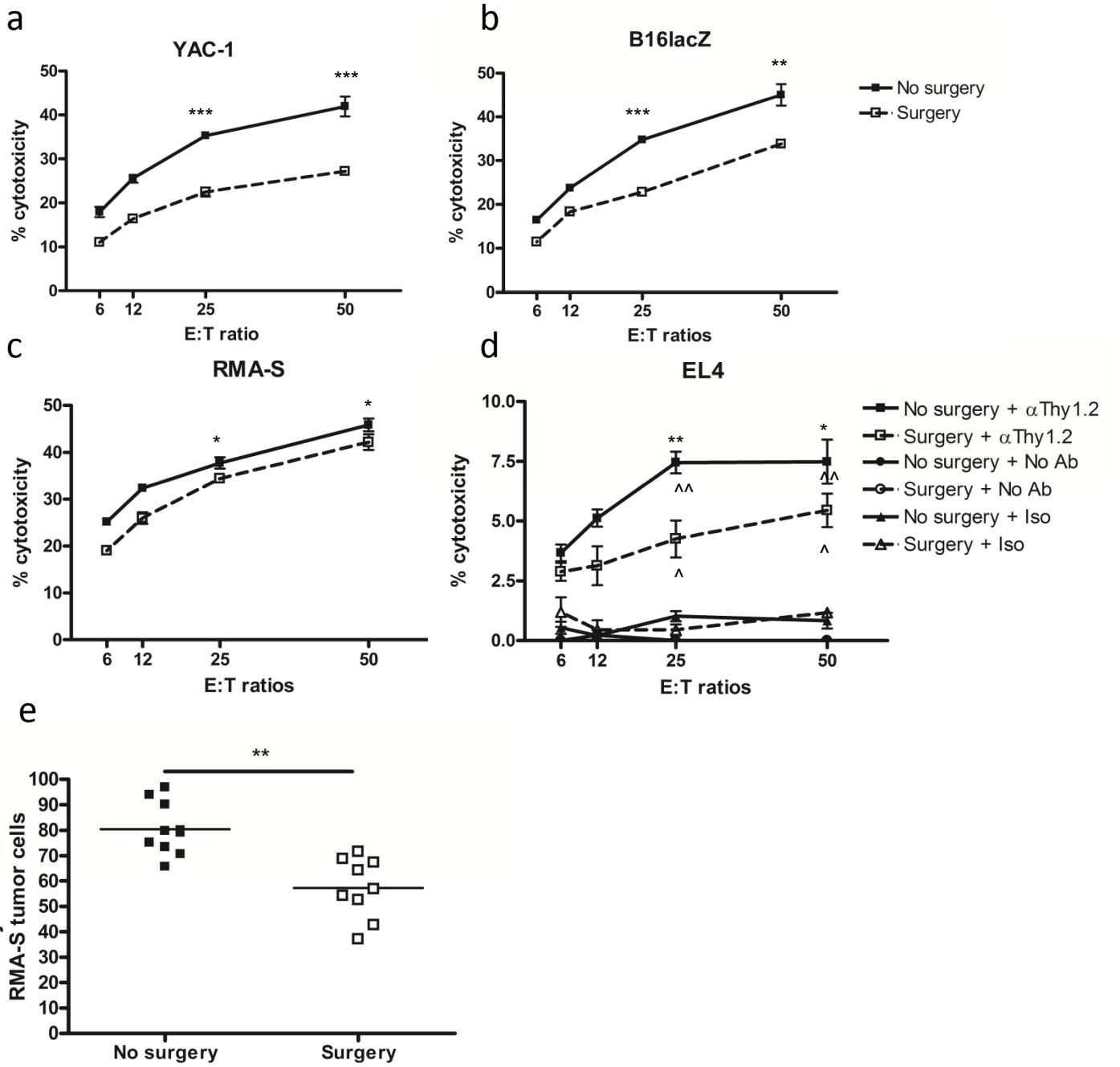

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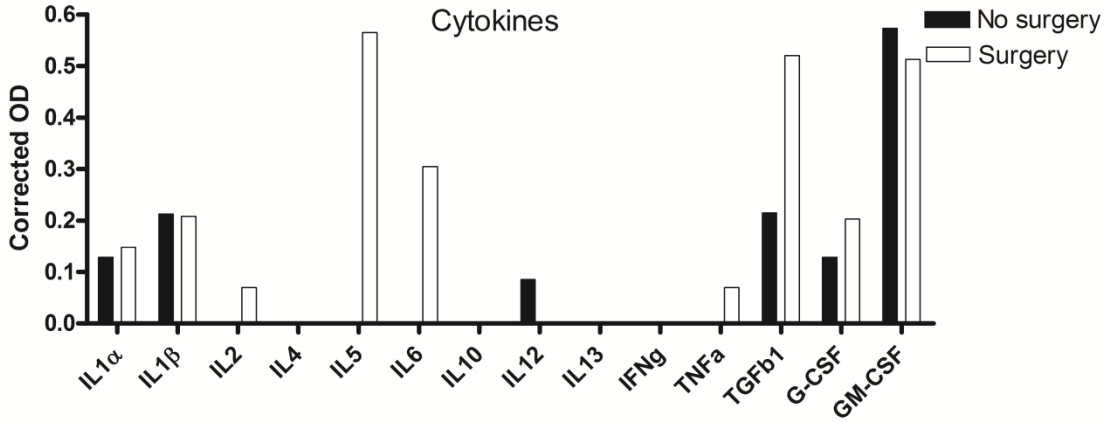
Figure 4. Surgically stressed mice exhibit altered tissue signals and immune suppressive cell populations

a



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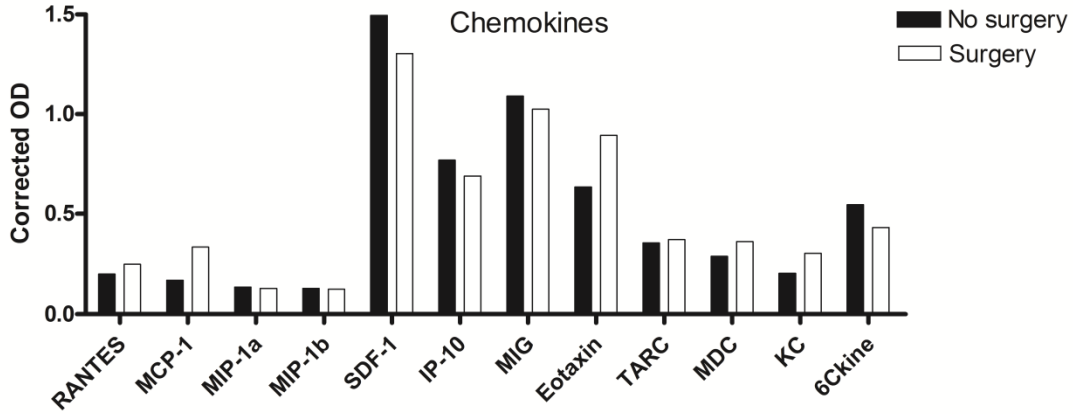
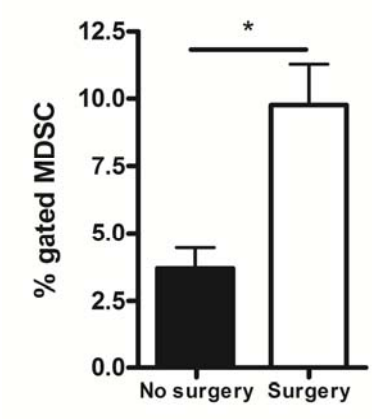


c



d

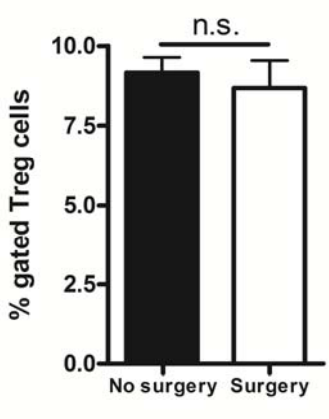


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Figure 5. Novel anti-cancer oncolytic virus as perioperative therapy against immune suppressive effects of surgery

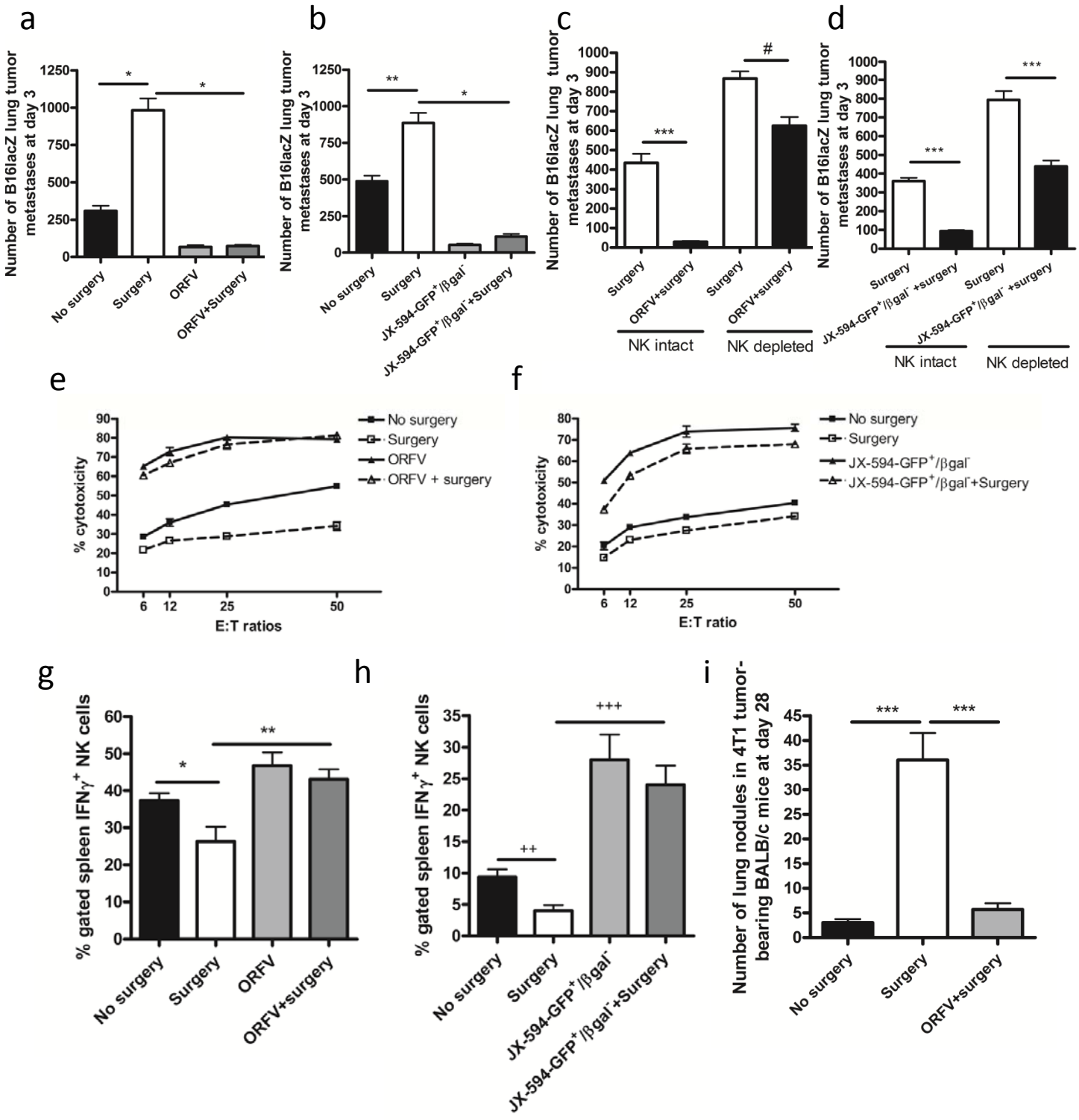
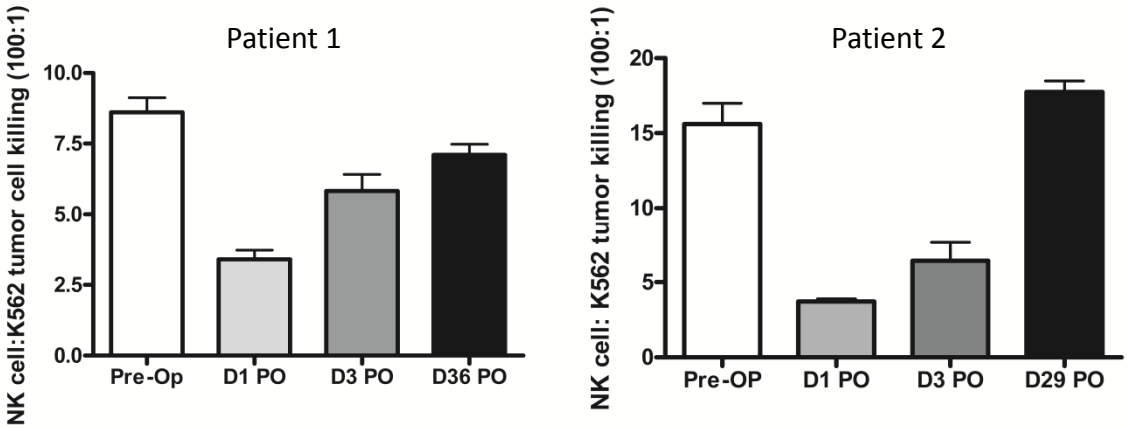


Figure 6. Defective human NK cell killing post-surgery and OV activation of human NK cells

a



b