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Perioperative Influenza Vaccination Reduces Postoperative Metastatic Disease by Reversing Surgery-Induced Dysfunction in Natural Killer Cells

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Abstract

Purpose: Surgical removal of solid primary tumors is an essential component of cancer treatment. Surgery-induced dysfunction in natural killer (NK) cells has been linked to the development of metastases in animal models and patients with cancer. We investigated the activation of NK cells using influenza vaccine in the perioperative period to eradicate micrometastatic disease.

Experimental Design: Both the B16lacZ and 4T1 tumor models in immunocompetent mice were used to assess the *in vivo* efficacy of perioperative influenza vaccine administration. In healthy human donors and cancer surgery patients, we assessed NK cell function pre- and post-influenza vaccination using both *in vivo* and *ex vivo* assays.

Results: Using the TLR3 agonist poly(I:C), we showed as proof-of-principle that perioperative administration of a nonspecific innate immune stimulant can inhibit surgery-induced dysfunction in NK cells and attenuate metastases. Next, we assessed a panel of prophylactic vaccines for NK cell activation and determined that inactivated influenza vaccine was the best candidate for perioperative administration. Perioperative influenza vaccine significantly reduced tumor metastases and improved NK cytotoxicity in preclinical tumor models. Significantly, IFN α is the main cytokine mediator for the therapeutic effect of influenza vaccination. In human studies, influenza vaccine significantly enhanced NK cell activity in healthy human donors and cancer surgery patients.

Conclusion: These results provide the preclinical rationale to pursue future clinical trials of perioperative NK cell activation, using vaccination in cancer surgery patients. Research into perioperative immune therapy is warranted to prevent immune dysfunction following surgery and eradicate metastatic disease. *Clin Cancer Res*; 19(18); 5104–15. ©2013 AACR.

Introduction

Surgical resection is the mainstay of therapy for patients with localized solid malignancies. Even with complete resection, many patients develop a metastatic recurrence and ultimately die of their disease. One of the key mechan-

isms responsible for the prometastatic effects of surgery is postoperative dysfunction of natural killer (NK) cells. NK cells are innate immune cytotoxic lymphocytes and have long been implicated in the control of tumor growth and metastases (1, 2). There are numerous studies documenting NK cell dysfunction post-surgery in animal models (3–8) and human patients (3, 4, 8–10). In animal studies, postoperative NK cell suppression correlates with increased metastases in spontaneous (8, 11) and implanted (8, 12, 13) tumor models. Similarly in human studies, low perioperative NK cell activity is associated with a higher rate of cancer recurrence and mortality in different cancer types (14, 15).

We recently established that NK cells play a crucial *in vivo* role in mediating tumor clearance following surgery (8, 16). In human studies, we established that postoperative cancer surgery patients also had reduced NK cell cytotoxicity (8). Despite the large number of studies that have documented postoperative NK cell dysfunction, very few have attempted to reverse it to improve cancer outcomes (8, 17–22). Current treatment regimens tend to ignore metastatic disease

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Translational Relevance

Surgical resection of solid tumors is a necessary component of cancer therapy. However, there is a large body of evidence to support the concept that the perioperative period is a uniquely susceptible time for the formation of metastases, in large part, due to the suppression of natural killer (NK) cells. Despite this, there are no cancer therapies specifically targeting the perioperative period. In this study, we showed that the perioperative administration of influenza vaccine can enhance NK cell function and eradicate micrometastatic disease. Influenza vaccination can provide a safe, novel way of strengthening the immune system and reducing recurrence of cancer following surgery in patients with cancer. Our data provide the preclinical rationale to propose a preoperative vaccination strategy that, when used in combination with surgery, has the potential to impact countless patients with cancer who undergo surgery to remove their primary tumor every year.

until after the recovery from the stress of surgery. Traditional cancer therapies, such as chemotherapy, are considered too toxic to be administered to patients recovering from a major surgery as they inhibit recovery and impair wound healing (23).

The perioperative administration of cytokine therapy has been explored in early-phase clinical trials showing their potential to prevent postoperative NK cell suppression and enhance progression-free survival (17–22, 24–26). However, further development has been limited due to dose-limiting toxicity (27–29). The perioperative use of oncolytic viruses (OV) are currently in preclinical (8) and early-phase clinical study. We have previously shown that perioperative administration of OV can reverse NK cell suppression which correlates with a reduction in the postoperative formation of metastases (8). In human studies, postoperative cancer surgery patients had reduced NK cell cytotoxicity, and we showed, for the first time, that OV markedly increases NK cell activity in patients with cancer (8). Given these encouraging data, we explored the use of existing prophylactic vaccines as a safe and currently available method of perioperative NK cell stimulation. Prophylactic vaccines contain inactivated pathogens and function by stimulating the innate and adaptive immune system to recognize and destroy the administered foreign agent and thus generate immunologic memory against it. In this way, the immune system can more easily recognize and destroy later encounters of the actual microorganism. Many vaccines have been found to be a source of Toll-like receptor (TLR) ligands that can activate innate immune cells including NK cells (30, 31).

The current project investigates a promising strategy in the emerging field of cancer innate immunotherapy, the nonspecific activation of NK cells using pre-existing, commercially available vaccines in the perioperative period to eradicate micrometastatic disease.

Materials and Methods

Mice

C57BL/6 (B6) and BALB/c were purchased from Charles Rivers Laboratory. IFNAR-deficient mice on a 129/SvEv background were backcrossed to B6 for 14 generations. Animals were housed in pathogen-free conditions, and all studies conducted were in accordance with institutional guidelines at the Animal Care Veterinary Services (University of Ottawa).

Surgery and experimental metastasis model

The experimental metastasis model was carried out as previously described (8). Briefly, an intravenous challenge of 3×10^5 B16lacZ cells was given to establish pulmonary metastases. Surgical stress was induced by an abdominal nephrectomy 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) and quantified. For perioperative rescue experiments, 15 or 150 μ g of poly(I:C) was administered intraperitoneally (i.p.), 1 of 5 of the human dose of influenza vaccine was administered i.v., recombinant IFN α (PBL) at 100 U and 10,000 U was administered i.p. 4 hours and 3×10^5 B16lacZ cells 1 hour, respectively, before surgery.

Surgery and spontaneous metastasis model

The spontaneous metastasis model was conducted as previously described (8). Briefly, 1×10^6 4T1 breast tumor cells were injected orthotopically into the mammary fat pad of BALB/c mice. At 14 days post-tumor cell injection, a complete resection of the primary tumor along with abdominal nephrectomy was conducted. For perioperative rescue experiments, 150 μ g of poly(I:C) was administered i.p.; 1 of 5 of human dose of influenza vaccine was administered i.v. respectively 4 hours before surgery at 14 days. Neoadjuvant influenza vaccine was given i.v. 5 days before surgery. For multidosing, influenza vaccine was administered 4 hours before surgery, at 19 and 23 days. At 28 days post-orthotopic tumor injection, lungs were weighed, photographed, and nodules quantified.

Cell lines, vaccines, and viruses

The B16F10LacZ melanoma cell line was obtained from Dr. K. Graham (London Regional Cancer Program) in 2010 and maintained in complete Dulbeccos' Modified Eagles' Media (DMEM). A total of 3×10^5 cells at more than 95% viability were injected i.v. in 0.1 mL/mouse. Rauscher murine leukemia virus-induced T-cell lymphoma (RMA) and RMA-S (MHC-deficient variant of RMA) were obtained from Dr. A. Veillette (Institut de Recherches Clinique Montreal) in 2011. 4T1, YAC-1, K562, and Daudi cell lines were purchased from American Type Culture Collection in 2010 and maintained in complete RPMI. All cell lines have been tested and authenticated. MHC-I staining and human leukocyte antigen (HLA) typing of all tumors cells are conducted every 6 to 12 months. All cell lines were verified to be mycoplasma free and showed appropriate pathologic morphology during the experiments. The following vaccines

were used: inactivated influenza (Agrimflu, Novartis); BCG (BCG), meningitis (Act-HIB), yellow fever (YF Vax), diphtheria/tetanus/pertussis/polio/pneumonia (Pediace) all from Sanofi-Pasteur; and HPV (Gardasil) and MMR (M-M-RII) from Merck. A 1:5 dose of the human vaccine given i.v. 24 hours before euthanasia was predetermined to maximally stimulate NK cells. Wild-type ORFV (strain NZ2) was obtained from Dr. A. Mercer (University of Otago, North Dunedin, New Zealand) and was injected and titred as previously described (32). Oncolytic vaccinia virus was prepared as previously described (33).

Antibodies and fluorescence-activated cell-sorting analysis

Spleen lymphocyte populations were excluded for RBCs using ammonium chloride–potassium lysis buffer. The following monoclonal antibodies were used: anti-TCR β (H57-597), anti-CD122 (TM-beta1), and anti-CD69 (H1.2F3) from eBiosciences. Anti-NK1.1 (PK136) and human antibodies: anti-CD3 (SK7), anti-CD56 (B159), and anti-CD69 (FN50) from BD Bioscience. Fluorescence-activated cell-sorting (FACS) acquisitions were conducted on a CyAN-ADP using Summit software (Beckman Coulter).

NK cell depletion

NK cells were depleted using an optimized dose and schedule of α -NK1.1 antibody (PK136) or isotype control (BD Bioscience). Two hundred micrograms was injected i.p. on days -4, -1, and +1. The lung tumor burden was quantified at 3 days post-surgery.

Ex vivo NK cell cytotoxicity assay

The ^{51}Cr -release assay was conducted as previously described (34). Briefly, splenocytes were isolated from surgery and control mice at 18 hours post-surgery. DX5 $^{+}$ -sorted NK cells (Miltenyi Biotech) were resuspended at a concentration of 1.5×10^6 cells/mL, mixed with chromium-labeled YAC-1 cells, which were resuspended at a concentration of 3×10^4 cells/mL at different effector-to-target (E:T) ratios. For rescue studies, 15 or 150 μg of poly(I:C) was administered i.p.; 1:5 dose of influenza vaccine was administered i.v., and IFN α (PBL) at 100 U and 10,000 U was administered i.p. 4 hours before surgery.

In vivo NK cell rejection assay

The *in vivo* rejection assay was conducted as previously described (34). Briefly, RMA and RMA-S were differentially labeled with 5 and 0.5 $\mu\text{mol/L}$ carboxyfluorescein succinimidyl ester (CFSE; Biolegend), respectively. A mixture of 1×10^6 cells of each type was injected i.p. into recipient mice treated with surgery (4 hours prior). After 18 hours, peritoneal cells were harvested from the peritoneum with PBS and 2 mmol/L EDTA and analyzed for the presence of CFSE-labeled tumor cells by FACS. For rescue studies, 150 μg of poly(I:C) administered i.p.; 1:5 dose of human influenza vaccine was administered i.v. 4 hours before surgery.

Human peripheral blood mononuclear cell cytotoxicity assay

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation from healthy donors or patients undergoing planned, hepatic resection for colorectal liver metastases. Written, informed consent was obtained from all patients in accordance with local institutional ethics review and approval. Cells were cultured at $3 \times 10^6/\text{mL}$ and treated with 0 or 50 μL influenza vaccine (1:10 dose) for 12 hours (\pm IFN blockade), before being co-cultured with ^{51}Cr -labeled Daudi cells at varying E:T ratios. For *in vivo* influenza vaccination, PMBCs were isolated from vaccinated patients and co-cultured with ^{51}Cr -labeled K562 at varying E:T ratios.

Cytokine analysis

Mouse serum was obtained by cardiac puncture from surgery and control mice at various time points post-surgery. Secretion of IFN α , IFN β , IFN γ , interleukin (IL)2, IL12, and IL15 was detected by FlowCytomix (Ebioscience) kits as per manufacturer's instructions. For human samples, PBMCs were treated with 0 or 50 μL influenza vaccine (\pm IFN blockade) for 12 hours before cell-free supernatants were collected. Secretion of IL2, IL15, IL12p40, IL12p70, IFN γ (BD Pharmingen), IFN α (MabTech AB), IL28 and IL29 (R&D Systems), and IFN- β (PBL) in supernatants was determined using Ab-matched pairs or the IFN- β ELISA Kit as per manufacturer's instructions.

Statistical analysis

Statistical significance was determined by the Student *t* test (2-tailed) with a cutoff $P = 0.05$. Data are presented as \pm SD.

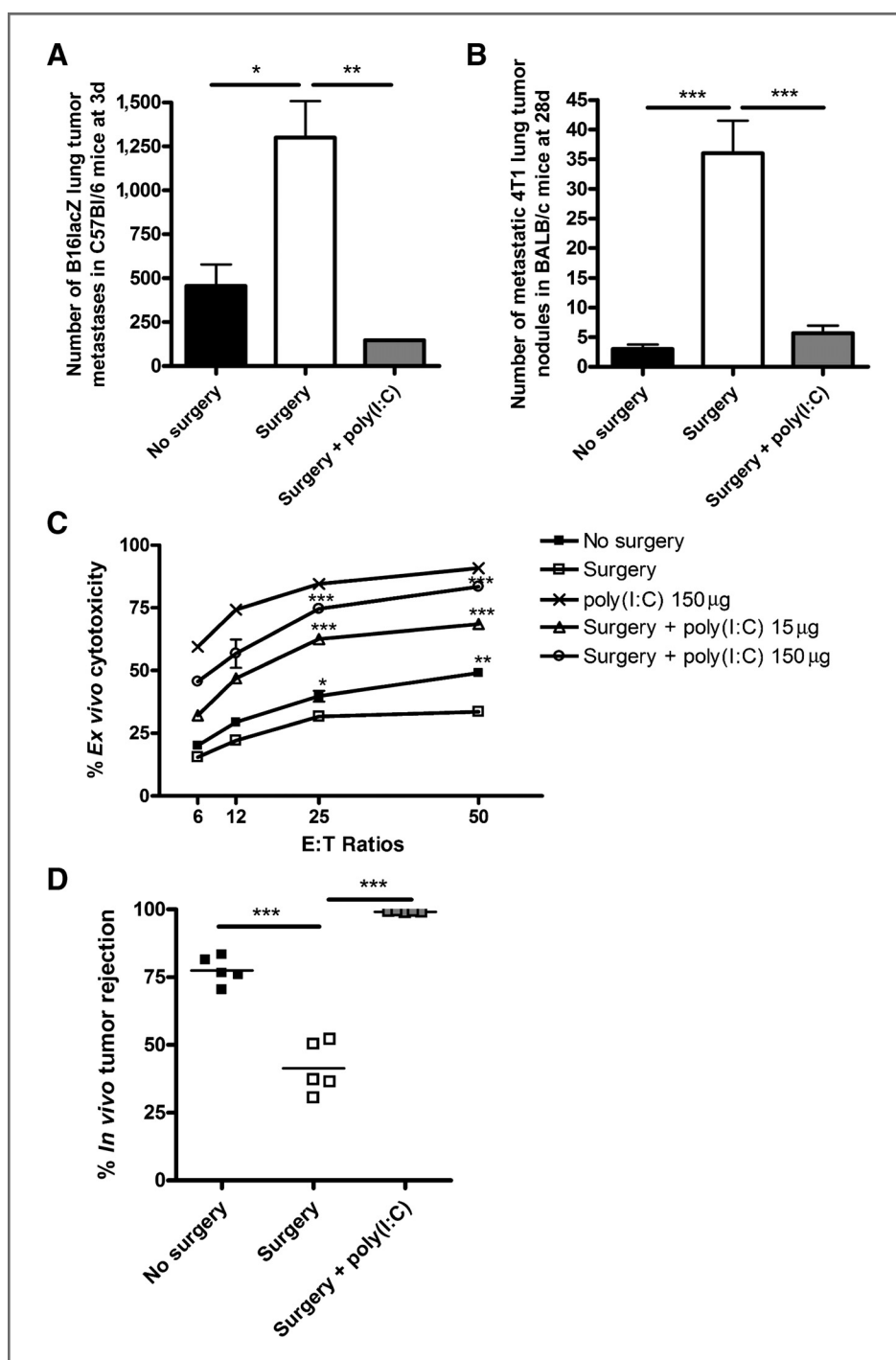
Results

Poly(I:C) as perioperative therapy against immunosuppressive effects of surgery and attenuation of metastatic disease

We have previously shown that a profound dysfunction in NK cells caused by surgical stress can be rescued with perioperative live OV (8). This finding showed the first novel use of OV as perioperative therapy to inhibit metastases after surgical resection of tumors by boosting innate immunity. To determine whether a nonreplicating immune stimulant can accomplish the same result, we used poly(I:C), a synthetic double-stranded (ds)RNA agonist for TLR3 and a well-established activator of NK cells (35). Using both the B16lacZ and 4T1 tumor models, we determined that perioperative poly(I:C) can attenuate the formation of metastatic disease following surgery (Fig. 1A and B).

Next, we assessed NK cell function in the setting of perioperative poly(I:C). A dramatic recovery in *ex vivo* NK cell cytotoxicity against tumor targets was observed following surgery at 2 different doses of poly(I:C) (15 and 150 μg ; Fig. 1C). In parallel, we observed a significant recovery of tumor rejection following perioperative poly(I:C) (Fig. 1D) over and above surgery alone in an *in vivo* rejection assay. These

Figure 1. Poly(I:C) as perioperative therapy against immunosuppressive effects of surgery and attenuation of metastatic disease. Quantification of lung tumor metastases at endpoint in (A) B16lacZ tumor-bearing B6 mice and (B) 4T1 tumor-bearing BALB/c mice from indicated treatment groups. C, the ability of NK cells to kill tumor targets from indicated treatment groups. Data are displayed as the mean percentage (\pm SD) of ^{51}Cr -release from triplicate wells for the indicated E:T ratios (P values are compared with "surgery" group). D, ability of NK cells from indicated treatment groups to reject RMA-S tumor cells. Data are representative of 3 experiments, $n = 4$ –6 per group (*, $P = 0.02$; **, $P = 0.005$; ***, $P = 0.0001$; n.s., not significant).



data establish a proof-of-concept that perioperative administration of a nonreplicating NK cell activator is an effective perioperative therapeutic strategy.

Influenza vaccine is a potent stimulator of NK cell function

Given the ability of poly(I:C) to recover the surgery-induced NK cell functional defect and attenuate the forma-

tion of metastases, we assessed a panel of commercially available prophylactic vaccines to find optimal candidates for perioperative administration: influenza, BCG, meningitis, yellow fever, mumps/measles/rubella, diphtheria/tetanus/pertussis/polio/pneumonia, and human papilloma virus. A 1:5 of the human dose was predetermined to maximally stimulate NK cells without associated toxicity (Supplementary Fig. S1).

Compared with all the vaccines tested, influenza vaccine generated the highest NK cell cytotoxic response and CD69 surface expression (Fig. 2A and B). Next, we compared the ability of influenza vaccine to potentiate NK cell killing against our most promising clinical OV candidates. Not unexpectedly, NK cells from mice treated with live oncolytic ORF and vaccinia viruses induced higher levels of NK cytotoxicity compared with an inactivated influenza vaccine. However, influenza vaccine induced a significant increase in NK cytotoxicity despite being an inactivated vaccine preparation containing only viral proteins (Fig. 2C). In summary, these data provide the first direct comparison of NK cell activation by commercially available prophylactic vaccines and suggest that further investigation

of the best candidate—influenza vaccine in the perioperative context is warranted.

Attenuation of metastatic disease by perioperative NK cell stimulation with inactivated influenza vaccine

The ability of the inactivated influenza vaccine to act as a novel perioperative nonspecific immune stimulator was further characterized. First, we determined whether perioperative influenza vaccine can attenuate the formation of metastatic disease following surgery in the B16lacZ and 4T1 tumor models. At endpoint, a dramatic reduction in lung metastases was observed in surgically stressed mice treated with vaccine compared with surgery alone as evidenced by quantification of lung metastases (Fig. 3A in B16lacZ) and

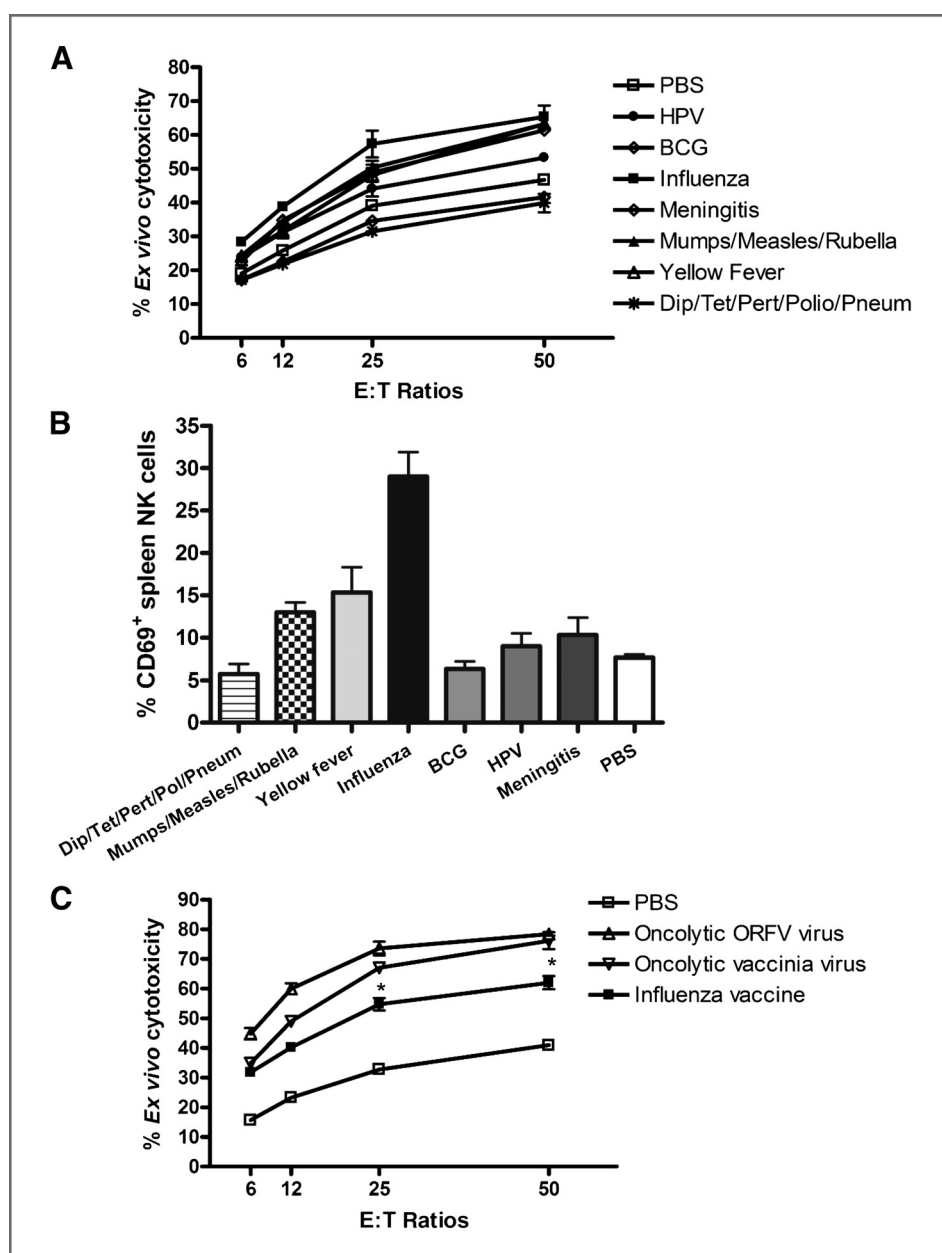


Figure 2. Influenza vaccine is a potent stimulator of NK cell function. A, ability of NK cells to kill tumor targets. B, CD69 activation on NK cells from indicated vaccine treatment groups. C, ability of NK cells to kill tumor targets from indicated treatment groups. Data are representative of 3 experiments, $n = 4-5$ per group. *, $P = 0.005$ comparing "Influenza vaccine" to "PBS" groups.

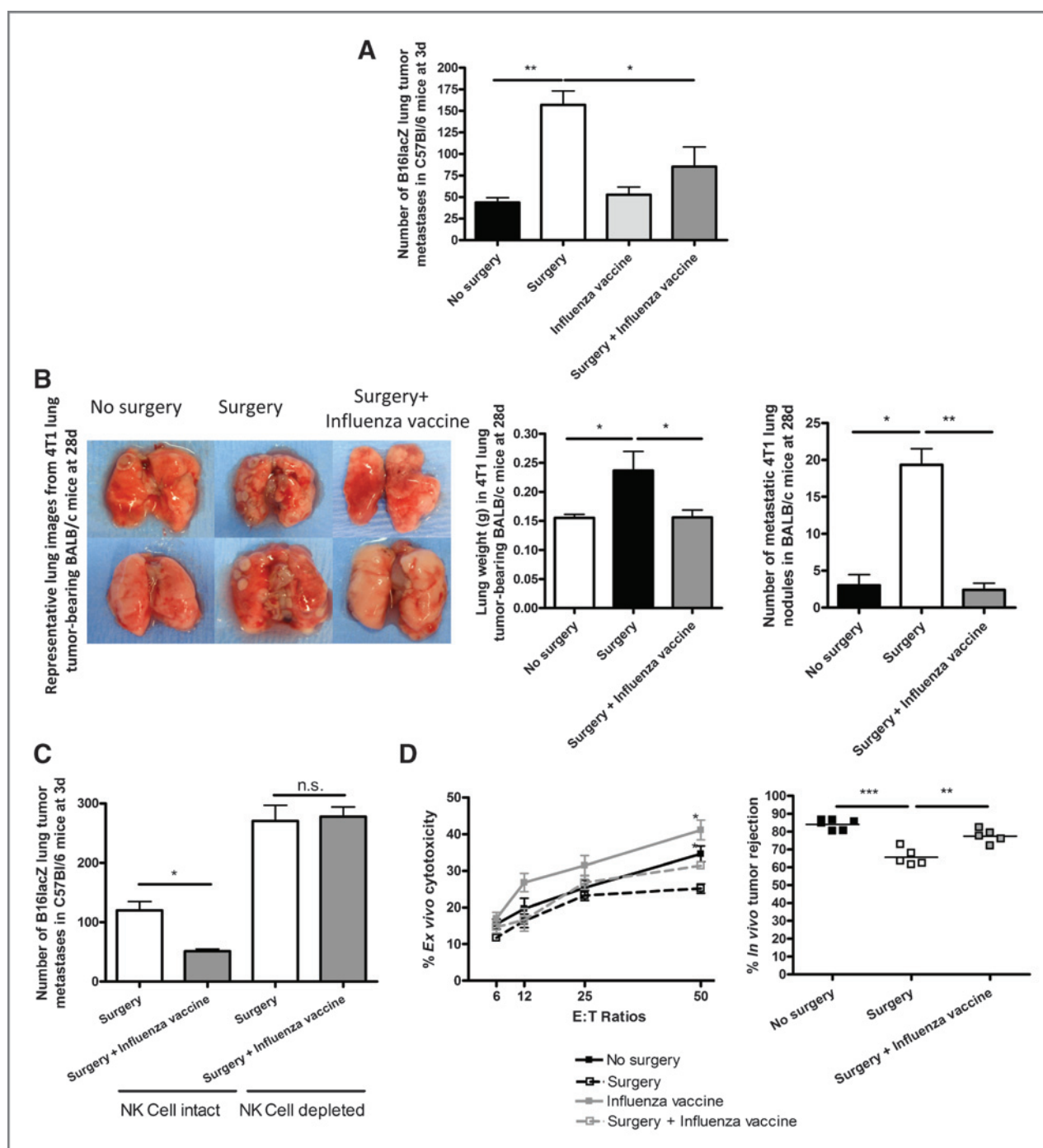


Figure 3. Attenuation of metastatic disease by perioperative NK cell stimulation with influenza vaccine. **A**, quantification of B16lacZ lung tumor metastases in B6 mice from indicated treatments. Assessment of 4T1 lung tumor metastases in BALB/c mice with indicated treatments by **(B)** representative lung photographs, weight, and number of lung nodules. **C**, quantification of B16lacZ lung tumor metastases from NK intact and depleted mice subjected to surgery with and without influenza vaccine. **D**, ability of NK cells to kill tumor targets from indicated treatment groups (*P* value is compared to "surgery" group; left). The ability of NK cells from indicated treatment groups to reject RMA-S tumor cells (right). Data are representative of 3 experiments, *n* = 4–7 per group. *, *P* = 0.05; **, *P* < 0.0042; ***, *P* < 0.0001. n.s., not significant.

representative lung photographs, lung weight, and enumeration of lung nodules (Fig. 3B in 4T1).

To determine whether NK cells play a mediating role in preventing metastases post-vaccine treatment, we pharma-

cologically depleted NK cells using anti-NK1.1 in the B16lacZ metastasis model. In the absence of NK cells, we observed a complete abrogation of the therapeutic effect of influenza vaccine (Fig. 3C). These data suggest that tumor

metastases removal in our surgical stress model is mainly mediated through influenza vaccine activation of NK cells and subsequent NK-mediated tumor lysis.

To further characterize NK cell function following perioperative administration of influenza vaccine, we examined *ex vivo* and *in vivo* NK cell killing. We observed a significant surgery-induced defect in NK killing in both cytotoxicity (Fig. 3D, left) and rejection assays (Fig. 3D, right) along with a significant recovery of NK killing following perioperative administration of influenza vaccine compared with surgery alone. Taken together, these results show that we can successfully treat perioperative NK cell suppression and reduce metastatic disease with influenza vaccination.

Influenza vaccine enhances NK cell function through IFN α

Given the enhanced response of NK cells following influenza vaccine administration, we proceeded to test serum cytokine levels. We chose a panel of cytokines known to directly or indirectly affect NK cells (IFN γ , IFN α , IFN β , IL2, IL12, IL15, IL18) and quantified their serum levels at various time points post-vaccination (6 hours, 18 hours, 24 hours, 3 days, 5 days). We observed an increase in IFN α , IL2, IL12, and IL15 at early time

points (6–24 hours) and a gradual decrease to baseline by 5 days. Notably, the most dramatic increase was seen in IFN α production with a peak production at 18 hours post-influenza vaccine (Fig. 4A). IFN β , IFN γ , and IL18 were not detected.

Next, we injected IFNAR-KO and B6-WT mice with influenza vaccine and observed no increase in NK cytotoxicity in IFNAR-KO mice (Fig. 4B, left). These data suggest that type I IFN is an important mediator of the NK cell activating effect following influenza vaccine. To test the effect of type I IFN administration in our B16lacZ mouse model of surgical stress, we administered recombinant IFN α at a low (100 U) and high dose (10,000 U). We observed a rescue of surgery-induced NK cytotoxicity (Fig. 4B, right) following perioperative treatment with both doses along with a dramatic decrease in the number of lung metastases in IFN α treated surgically stressed mice compared with surgery alone. Significantly, influenza vaccine and low-dose IFN α treatment showed comparable levels of tumor metastases removal (Fig. 4C). Finally, we administered perioperative influenza vaccine into surgically stressed IFNAR-KO mice and observed an abrogation of the NK cell-mediated controlling effect of influenza vaccine on tumor metastases removal (Fig. 4D). Taken together, these data suggest that influenza

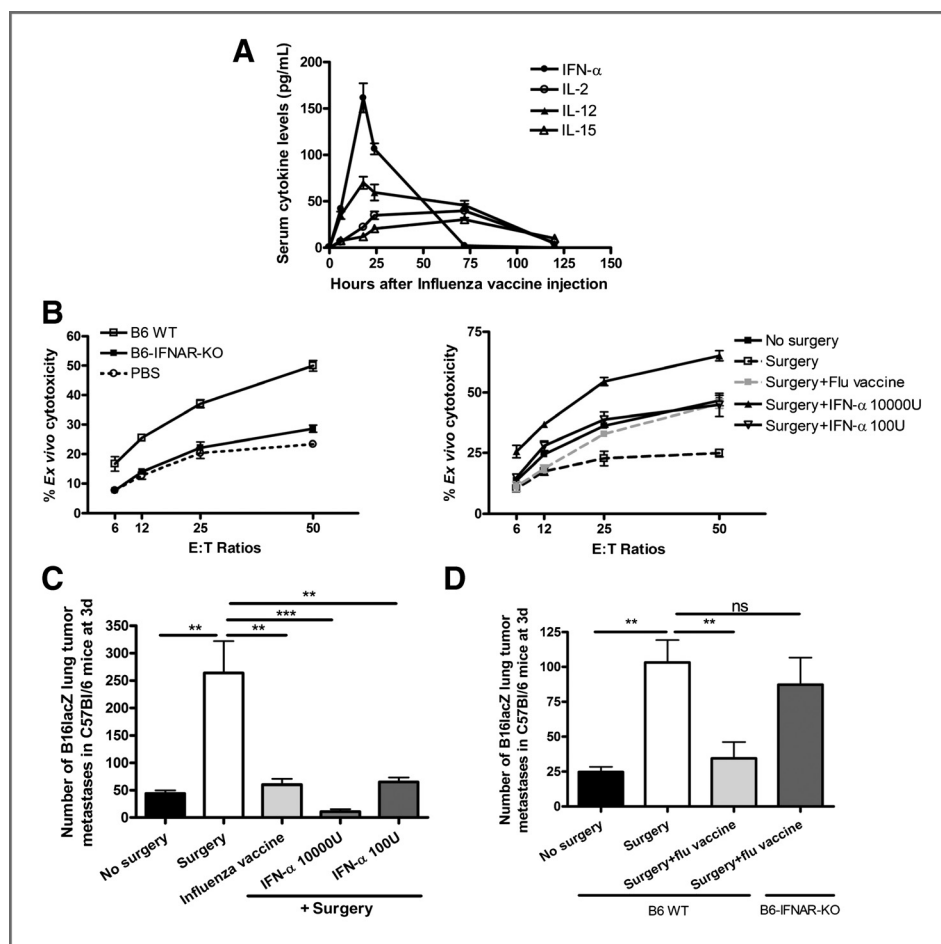


Figure 4. Influenza vaccine enhances NK cell function through IFN- α . A, time course quantification of serum cytokine levels in control B6 mice following influenza vaccine administration. B, ability of NK cells to kill tumor targets from (left) IFNAR-KO and B6 mice post-influenza vaccine and (right) from indicated treatment groups. Quantification of B16lacZ lung tumor metastases in (C) B6 and (D) IFNAR-KO mice following indicated perioperative treatment. $n = 4-5$ per group. **, $P > 0.005$; ***, $P = 0.0001$; n.s., not significant. Data are representative of 3 experiments.

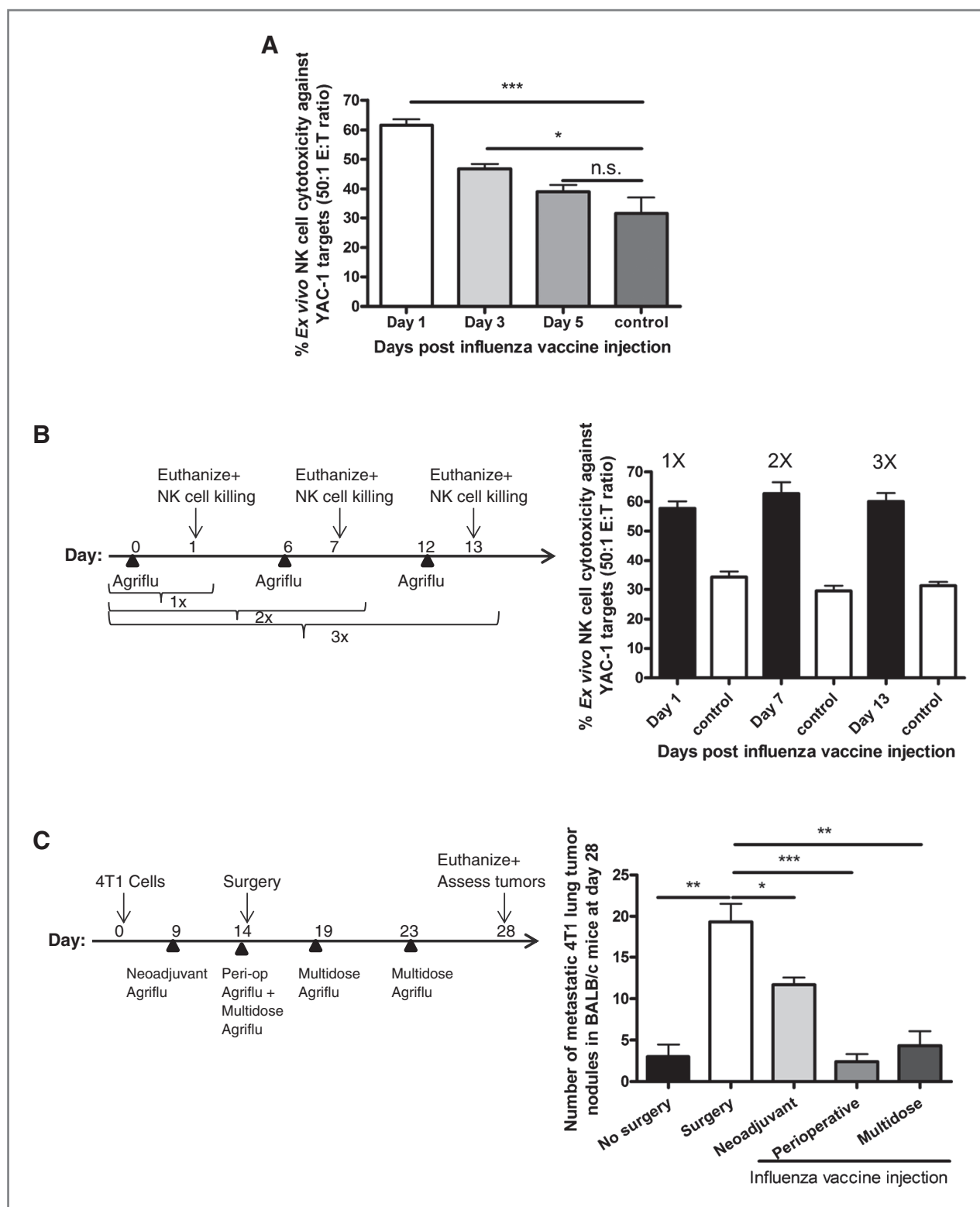
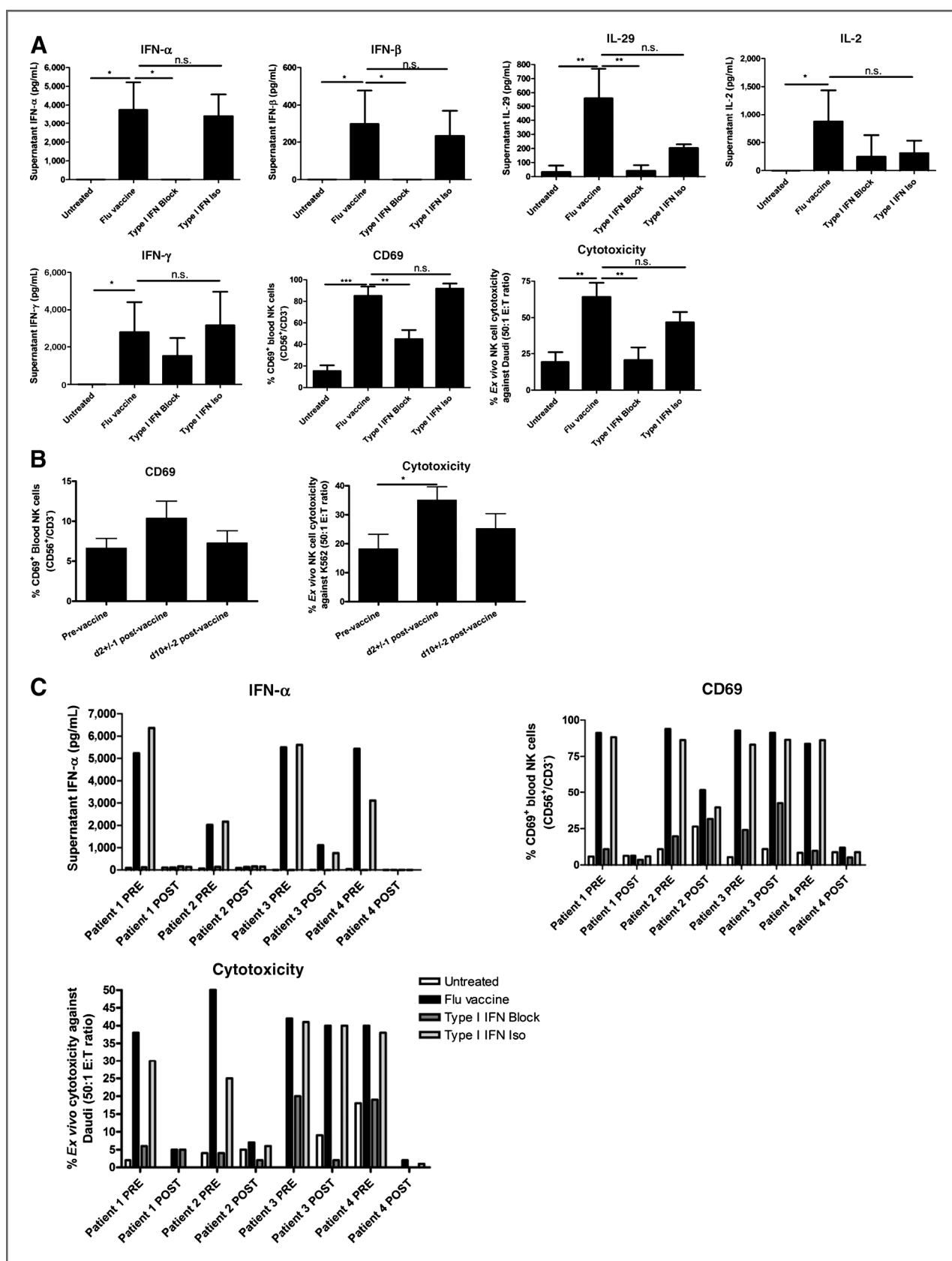


Figure 5. Influenza vaccination in the immediate perioperative period is necessary to achieve maximal efficacy for reducing lung metastases. **A**, ability of NK cells to kill tumor targets from B6 mice at 1, 3, and 5 days post-influenza vaccine along with controls. **B**, ability of NK cells to kill tumor targets from B6 mice treated with 1, 2, or 3 doses of influenza vaccine given 6 days apart along with controls. **C**, quantification of 4T1 tumor metastases in BALB/c mice with the indicated treatment groups. $n = 4-7$ per group. *, $P = 0.01$; **, $P > 0.005$; ***, $P = 0.0001$; n.s., not significant. Data are representative of 3 experiments.



vaccine enhances perioperative NK cell function through modulation of IFN α .

Influenza vaccination in the immediate perioperative period is necessary to achieve maximal efficacy for reducing lung metastases

As influenza vaccine given as a single dose in the perioperative period was able to reduce lung metastases, we next questioned whether other dosing schedules could achieve the same results. First, the duration of NK cell activation by influenza vaccine was evaluated in the *ex vivo* cytotoxicity assay at 1, 3, and 5 days posttreatment. NK cytotoxicity was maximally achieved at 1 day postinjection and was significantly diminished by 5 days (Fig. 5A). We next determined whether we can re-activate NK cells with multiple doses after 5 days. We compared NK cytotoxicity levels in mice that received 1, 2, and 3 doses of influenza vaccine at 5-day intervals and observed re-activation of NK cytotoxicity after each successive round of vaccine treatment (Fig. 5B). Given these results, we treated 4T1 tumor-bearing mice with 3 regimens of influenza vaccine: neoadjuvant (given 5 days before surgery), perioperative (given on the same day of surgery), and perioperative + multidose (given on the day of surgery, followed by 2 additional doses given 5 days apart; Fig. 5C). Remarkably, all 3 modes of vaccine treatment significantly decreased lung metastases. However, influenza vaccine administered perioperatively as a single dose reduced metastases most effectively (Fig. 5C). Collectively, these experiments highlight the importance of the immediate perioperative period as a narrow therapeutic window to intervene in the metastatic process.

Influenza vaccination enhances NK cell function in human donors

It is hoped the current project will provide the preliminary data to support a preoperative vaccination strategy. To validate this goal, human NK cell response to influenza vaccination must be evaluated. We, therefore, first isolated PBMCs from healthy donors and pulsed them *ex vivo* with influenza vaccine. We assessed a panel of pro-inflammatory cytokines 12 hours later and observed a significant production of IL2, IL29, IFN α , IFN β , and IFN γ in 5 of 5 donors. Of these cytokines, IFN α and IFN γ were produced in the highest amounts post-vaccine treatment. Notably, the presence of type I IFN blocking antibody (IFN α/β), while abrogating the production of IFN α and IFN β following vaccine treatment, also caused a significant reduction in IL29 (Fig. 6A). Next we observed significant upregulation of NK cell CD69 expression and cytotoxicity against tumor targets in 5 of 5

donors, which was also inhibited by IFN block but not by IFN isotype control (Fig. 6A). Furthermore, we recapitulated our *ex vivo* vaccine pulsing results with *in vivo* influenza vaccination of healthy donors. Blood was collected at different time points including pre-vaccine, on post-vaccine day 2 (± 1) and post-vaccine day 10 (± 2). We observed an improvement in NK cytotoxicity and CD69 expression at post-vaccine day 2 (± 1) followed by a decrease in both NK cytotoxicity and CD69 expression at day 10 (± 2) for 6 of 6 donors (Fig. 6B). Finally, to address the effects of influenza vaccine in cancer surgery patients, we used the same *ex vivo* vaccine pulsing strategy for PBMCs isolated pre- and post-surgery from patients with cancer undergoing planned resection of colorectal cancer liver metastases. In 4 of 4 patients, there was influenza-induced enhanced NK cell function as evidenced by cytokine (IFN α) secretion, CD69 upregulation, and increased cytotoxicity in the pre-surgery samples. However, in only 1 of the 4 same patients (patient 3) was similar NK activation by influenza seen post-surgery (Fig. 6C), consistent with surgical stress-induced NK cell suppression. In summary, these data suggest that influenza vaccination can stimulate human NK cells systemically and support the rationale that cancer surgery patients can derive an NK cell stimulating benefit from influenza vaccination given in the perioperative setting immediately before surgery.

Discussion

Although the field of antitumor immunotherapy is rapidly advancing, very few studies have been conducted in the perioperative context. We have previously shown that NK cell responses triggered by OV are capable of overcoming immunosuppressive post-surgery microenvironments and significantly reducing metastases in the preclinical setting. In related clinical trials, we have showed that OV markedly increases NK cell activity in patients with cancer (8). While these data are exciting, the perioperative use of OV is in the preclinical and early stages of clinical investigation.

We, therefore, explored the use of existing prophylactic inactivated vaccines, such as influenza vaccine, as a safe and viable method of perioperative NK cell stimulation, improving cancer outcomes by preventing metastatic disease. The most commonly administered influenza vaccine in Canada is the inactivated, protein-based form consisting of viral surface antigens of the seasonal circulating strain. Agriflu is an inactivated influenza vaccine that is immunogenic, associated with minimal side effects, and is recommended for anyone ≥ 6 months of age without contraindications. In humans, there are limited longitudinal

Figure 6. Influenza vaccination enhances NK cell function in human donors. A, quantification of indicated cytokines from cell-free supernatant, CD69 NK (CD56⁺/CD3⁺) expression, and *ex vivo* cytotoxicity with PBMCs obtained from 5 healthy humans pulsed with influenza vaccine in the presence of type I IFN-blocking Ab and isotype control. B, CD69 NK (CD56⁺/CD3⁺) expression and *ex vivo* cytotoxicity with PBMCs obtained from 6 healthy human donors who received *in vivo* influenza vaccination. PBMCs from whole blood were obtained at pre-vaccine, d2 ± 1 post-vaccine, and d10 ± 2 post-vaccine. C, quantification of indicated cytokines from cell-free supernatant, CD69 NK (CD56⁺/CD3⁺) expression, and *ex vivo* cytotoxicity with PBMCs obtained from 4 cancer-surgery (pre and post) patients pulsed with influenza vaccine in the presence of type I IFN-blocking Ab and isotype control. *, $P > 0.05$; **, $P > 0.005$; ***, $P = 0.0005$; n.s., not significant.

studies on the impact of influenza vaccination on innate and NK cell responses (36).

In the present study, we clearly establish that influenza vaccine administered preoperatively reverses surgery-induced NK cell dysfunction and dramatically reduces lung tumor metastases. Furthermore, our data suggest that the perioperative period is the crucial window of opportunity to intervene with an innate immune stimulant (Fig. 5C). Mechanistically, we showed that IFN α is the main mechanism underlying the therapeutic effect of influenza vaccination in both preclinical and clinical samples as the abrogation of IFN α through IFNAR-KO mice, and *ex vivo* IFN block in humans, suppressed NK cell functionality. In healthy donors, NK cell functions are significantly augmented post-influenza vaccine both *in vivo* and *ex vivo*, whereas in cancer surgery patients, our data suggest that preoperative *ex vivo* pulsing of PBMCs with influenza vaccine can enhance NK cell functionality. In contrast, in post-surgery samples, NK cell activation and cytokine secretion was only observed in 1 of 4 patients. However, these *ex vivo* pulsing results do not rule out the possibility that preoperative *in vivo* vaccination of cancer surgery patients can result in clinically beneficial enhanced NK cell functionality in the perioperative setting. Rather, the NK cell impairment in the presence of vaccine pulsing observed in post-surgery PBMC samples argues in favor of a preoperative vaccination strategy to prevent severe immune cell impairment and recurrence of metastatic disease following the stress of cancer surgery, a strategy consistent with the preclinical scheduling data of Fig. 5. We, therefore, propose the preoperative stimulation of the immune system with inactivated influenza vaccine as a way to avoid the NK-suppressive effects of cancer surgery.

Two forms of recombinant IFN α (IFN α 2a, IFN α 2b) have been approved by the U.S. Food and Drug Administration (FDA) for a variety of clinical indications, including hairy cell and chronic myelogenous leukemia and AIDS-related Kaposi sarcoma. However, adverse effects due to IFN α have been described, including hematologic and neurologic toxicities. Many of these side effects are dose-dependent and require dose adjustment or cessation of treatment. In the context of perioperative immune stimulation, previous early-phase studies using low-dose preoperative IFN α showed increased NK activity and acceptable toxicity (17, 18, 25). Furthermore, its preoperative use could provide more precise conditions to achieve a high amount of NK cell stimulation, which may overcome the individual variability that may occur in response to vaccination. The dose and scheduling of preoperative administration of immunomodulatory agents in preclinical models is ongoing work in our laboratory.

Nevertheless, the focal point of this current study is to show that a pre-existing, pre-approved, widely available,

cost-effective, and widely used vaccine can achieve effective results. Influenza vaccine will allow the host to respond by activating physiologic amounts of multiple pro-inflammatory cytokines to enhance the immune system. The impressive safety profile of influenza vaccine is shown by its current indication for the general population ≥ 6 months of age. It is also specifically recommended for vulnerable populations (pregnant women) and safe for administration into immunocompromised individuals. Cancer surgery patients may benefit from immunity against influenza infection, as well as the potential antimetastatic benefits suggested by our study.

Ultimately, our goal is to find a perioperative vaccine that helps to prevent surgery-associated metastatic disease by providing a physiologic amount of NK stimulation without associated toxicities. Ideally, this perioperative treatment modality should be simple ("off the shelf"), safe (FDA-approved), and viable (worthwhile and acceptable to patients). The current project explores the activation of NK cells in the perioperative period to eradicate micrometastatic disease. It will provide the preliminary data to propose a preoperative vaccination clinical trial. When used in combination with surgery, this cancer therapy has the potential to impact countless patients with cancer who undergo surgical resection of their solid tumors every year.

Disclosure of Potential Conflicts of Interest

A.A. Melcher is a consultant/advisory board member of Amgen and Trimod Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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