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Perioperative influenza vaccination reduces post-operative metastatic disease by reversing surgery-induced dysfunction in natural killer cells

Lee-Hwa Tai¹, Jiqing Zhang¹,²,³, Karen J. Scott⁴, Christiano Tanese de Souza¹, Almohanad A. Alkayyal¹,⁵,⁶, Anu Abhirami Ananth¹,⁵, Shalini Sahi¹, Robert A. Adair⁴, Ahmad Bakur Mahmoud⁵,⁷, Subash Sad⁵, John C. Bell¹,⁵, Andrew P. Makrigiannis⁵, Alan A. Melcher⁴ and Rebecca C. Auer¹,⁸

¹Centre for Innovative Cancer Research, Ottawa Hospital Research Institute, Ottawa, Canada
Departments of ²Cellular and Molecular Medicine; ⁵Biochemistry, Microbiology, and Immunology; and ⁸Surgery, University of Ottawa, Ottawa, Canada
³Department of Neurosurgery, The Second Hospital of Shandong University, Shandong, China
⁴Targeted & Biological Therapies Group, Leeds Institute of Cancer Studies and Pathology, Leeds, UK
⁶Department of Medical Laboratory Technology, University of Tabuk, Saudi Arabia
⁷College of Applied Medical Sciences, Taibah University, Saudi Arabia

Corresponding Author: Dr Rebecca Auer, Ottawa General Hospital, 1617 CCW, Box#134, 501 Smyth Road, Ottawa, ON, K1H8L6 Canada, phone: 613-737-8899x79551, fax: 613-739-6646, rauer@ohri.ca

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STATEMENT OF 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 NK cells. Despite this, there are no cancer therapies specifically targeting the perioperative period. In this study, we demonstrated 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 cancer patients. Our data provides the preclinical rationale to propose a preoperative vaccination strategy that, when used in combination with surgery has the potential to impact countless cancer patients who undergo surgery to remove their primary tumor every year.
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ABSTRACT

Purpose: Surgical removal of solid primary tumors is an essential component of cancer treatment. Surgery-induced dysfunction in NK cells has been linked to the development of metastases in animal models and cancer patients. 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 demonstrated as proof-of-principle that perioperative administration of a non-specific 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, IFNα 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.
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 mechanisms 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 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 demonstrating 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
Running Title:  Perioperative influenza vaccine enhances NK cell function 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 demonstrated, for the first time, that OV markedly increases NK cell activity in cancer patients (8). Given this 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 immunological 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 non-specific 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 performed 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 (i.v.) challenge of $3 \times 10^5$ B16lacZ cells was given to establish pulmonary metastases. Surgical stress was induced by an abdominal
Running Title: Perioperative influenza vaccine enhances NK cell function nephrectomy 10mins following tumor inoculation. Animals were euthanized at 18h or 3d following tumor inoculation and their lungs were stained with X-gal (Bioshop) and quantified. For perioperative rescue experiments, 15 or 150µg of poly(I:C) was administered intraperitoneally (i.p.), 1/5 of the human dose of influenza vaccine was administered i.v., recombinant IFNα (PBL) at 100U and 10,000U was administered i.p 4h and 3x10^5 B16lacZ cells 1h, respectively before surgery.

Surgery and spontaneous metastasis model - The spontaneous metastasis model was performed as previously described (8). Briefly, 1x10^6 4T1 breast tumor cells were injected orthotopically into the mammary fat pad of BALB/c mice. At 14d post-tumor cell injection, a complete resection of the primary tumor along with abdominal nephrectomy was performed. For perioperative rescue experiments, 150µg of poly(I:C) was administered i.p.; 1/5 of human dose of influenza vaccine was administered i.v. respectively 4h before surgery at 14d. Neoadjuvant influenza vaccine was given i.v. 5d prior to surgery. For multi-dosing, influenza vaccine was administered 4h before surgery, at 19d and 23d. At 28d 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 DMEM. 3x10^5 cells at >95% viability were injected i.v. in 0.1ml/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 was purchased from ATCC in 2010 and maintained in complete RPMI. All cell lines have been tested and authenticated. MHC-I staining and HLA typing of all tumors cells are performed every 6-12 months. All cell lines were verified to be mycoplasma
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free and showed appropriate pathological morphology during the experiments. The following vaccines were used: Inactivated influenza (Agriflu, Novartis); BCG (BCG), Meningitis (Act-HIB), Yellow fever (YF Vax), Diptheria/Tetanus/Pertussis/Polio/Pneumonia (Pediacl) all from Sanofi-Pasteur; HPV (Gardasil) and MMR (M-M-RII) from Merck. A 1/5 dose of the human vaccine given i.v. 24h prior to euthanasia was predetermined to maximally stimulate NK cells. Wild type ORFV (strain NZ2) was obtained from Dr. A. Mercer (University of Otago, New Zealand) and was injected and titred as previously described (32). Oncolytic vaccinia virus was prepared as previously described (33).

Antibodies and FACS analysis. - Spleen lymphocyte populations were excluded for RBCs using Ammonium-Chloride-Potassium lysis buffer. The following mAbs were used: anti-TCRβ (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. FACS acquisitions were performed 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). 200μg were injected i.p. on days -4, -1 and +1. The lung tumor burden was quantified at 3d post-surgery.

Ex vivo NK cell cytotoxicity assay – The 51Cr-release assay was performed as previously described (34). Briefly, splenocytes were isolated from surgery and control mice at 18h postsurgery. DX5+ sorted NK cells (Miltenyi Biotech) were re-suspended at a concentration of 1.5x10^6 cells/ml, mixed with chromium-labelled YAC-1 cells, which were re-suspended at a concentration of 3x10^4 cells/ml at different E:T ratios. For rescue studies – 15 or 150μg of
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poly(I:C) was administered i.p; 1/5 dose of influenza vaccine was administered i.v., and IFNα (PBL) at 100U and 10,000U was administered i.p 4h before surgery.

In vivo NK cell rejection assay - The in vivo rejection assay was performed as previously described (34). Briefly, RMA and RMA-S were differentially labeled with 5µM and 0.5µM CFSE (Biolegend), respectively. A mixture of 1x10⁶ cells of each type was injected i.p. into 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. For rescue studies – 150µg of poly(I:C) administered i.p; 1/5 dose of human influenza vaccine was administered i.v. 4h before surgery.

Human PBMC cytotoxicity assay - 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 3x10⁶/ml and treated with 0 or 50µl influenza vaccine (1/10 dose) for 12h (+/- IFN blockade), before being co-cultured with ⁵¹Cr–labelled Daudi cells at varying E:T ratios. For in vivo influenza vaccination, PMBC were isolated from vaccinated patients and co-cultured with ⁵¹Cr –labelled 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 IFNα, IFNβ, IFNγ, IL2, IL12, IL15 was detected by FlowCytomix (Ebioscience) kits as per manufacturer’s instructions. For human samples, PBMC were treated with 0 or 50µl influenza vaccine (+/-IFN blockade) for 12h before cell-free supernatants were collected. Secretion of IL2, IL15, IL12p40, IL12p70, IFNγ (BD Pharmingen),
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IFNα (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 student t test (2 tailed) with a cutoff P value of 0.05. Data is presented as +/-SD.

RESULTS

Poly(I:C) as perioperative therapy against immune suppressive 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 non-replicating immune stimulant can accomplish the same result, we used poly(I:C), a synthetic dsRNA 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 (Figure 1A,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 two different doses of poly(I:C) (15ug, 150ug) (Figure 1C). In parallel, we observed a significant recovery of tumor rejection following perioperative poly(I:C) (Figure 1D) over and above surgery alone in an in vivo rejection assay. This data establishes a proof-of-concept that perioperative administration of a non-replicating NK cell activator is an effective perioperative therapeutic strategy.
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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 formation 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 (Supplemental Figure 1).

Compared to all the vaccines tested, influenza vaccine generated the highest NK cell cytotoxic response and CD69 surface expression (Figure 2A, 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 to an inactivated influenza vaccine. However, influenza vaccine induced a significant increase in NK cytotoxicity despite being an inactivated vaccine preparation containing only viral proteins (Figure 2C). In summary, these data provide the first direct comparison of NK cell activation by commercially available prophylactic vaccines and suggests 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 non-specific 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
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metastases was observed in surgically stressed mice treated with vaccine compared to surgery alone as evidenced by quantification of lung metastases (Figure 3A in B16lacZ); and representative lung photographs, lung weight and enumeration of lung nodules (Figure 3B in 4T1).

To determine whether NK cells play a mediating role in preventing metastases post-vaccine treatment, we pharmacologically 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 (Figure 3C). This data suggests 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 (Figure 3D left panel) and rejection assays (Figure 3D right panel) along with a significant recovery of NK killing following perioperative administration of influenza vaccine compared to surgery alone. Taken together, these results demonstrate that we can successfully treat perioperative NK cell suppression and reduce metastatic disease with influenza vaccination.

Influenza vaccine enhances NK cell function through IFNα

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 (IFNγ, IFNα, IFNβ, IL2, IL12, IL15, IL18) and quantified their serum levels at various time points post-vaccination (6h, 18h, 24h, 3d, day 5d). We observed an increase in IFNα, IL2, IL12 and IL15 at early time points (6-24h) and a gradual decrease to
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baseline by 5d. Notably, the most dramatic increase was seen in IFNα production with a peak production at 18h post influenza vaccine (Figure 4A). IFNβ, IFNγ 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 (Figure 4B left panel). This data suggests 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 IFNα at a low (100U) and high dose (10000U). We observed a rescue of surgery-induced NK cytotoxicity (Figure 4B right panel) following perioperative treatment with both doses along with a dramatic decrease in the number of lung metastases in IFNα treated surgically stressed mice compared to surgery alone. Significantly, influenza vaccine and low dose IFNα treatment demonstrated comparable levels of tumor metastases removal (Figure 4C). Lastly, 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 (Figure 4D). Taken together, this data suggests that influenza vaccine enhances perioperative NK cell function through modulation of IFNα.

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

Since 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 1d, 3d and 5d post-treatment. NK cytotoxicity was maximally achieved at 1d post-injection and was significantly diminished by 5d (Figure 5A). We next determined if we can re-activate NK cells with multiple doses after 5d. We compared NK
Perioperative influenza vaccine enhances NK cell function 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 (Figure 5B). Given these results, we treated 4T1 tumor bearing mice with 3 regimens of influenza vaccine: neoadjuvant (given 5 days prior to surgery), perioperative (given on the same day of surgery) and perioperative + multi-dose (given on the day of surgery, followed by 2 additional doses given 5 days apart) (Figure 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 (Figure 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. In order to validate this goal, human NK cell response to influenza vaccination must be evaluated. We, therefore, first isolated PBMC from healthy donors and pulsed them \textit{ex vivo} with influenza vaccine. We assessed a panel of pro-inflammatory cytokines 12h later and observed a significant production of IL2, IL29, IFN\(\alpha\), IFN\(\beta\) and IFN\(\gamma\) in 5/5 donors. Of these cytokines, IFN\(\alpha\) and IFN\(\gamma\) were produced in the highest amounts post-vaccine treatment. Notably, the presence of type I IFN blocking antibody (IFN\(\alpha/\beta\)), while abrogating the production of IFN\(\alpha\) and IFN\(\beta\) following vaccine treatment, also caused a significant reduction in IL29 (Figure 6A). Next we observed significant upregulation of NK cell CD69 expression and cytotoxicity against tumor targets in 5/5 donors, which was also inhibited by IFN block but not by IFN isotype control (Figure 6A). Further, we recapitulated our \textit{ex vivo} vaccine pulsing results with \textit{in vivo} influenza vaccination of healthy donors. Blood was collected at different time
Perioperative influenza vaccine enhances NK cell function points including pre-vaccine, on post-vaccine d2(±1) and post-vaccine d10(±2). We observed an improvement in NK cytotoxicity and CD69 expression at post-vaccine d2(±1) followed by a decrease in both NK cytotoxicity and CD69 expression at d10(±2) for 6/6 donors (Figure 6B). Lastly, to address the effects of influenza vaccine in cancer surgery patients, we employed the same ex vivo vaccine pulsing strategy for PBMC isolated pre- and post- surgery from cancer patients undergoing planned resection of colorectal cancer liver metastases. In 4/4 patients, there was influenza-induced enhanced NK cell function as evidenced by cytokine (IFNα) 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 (Figure 6C), consistent with surgical stress-induced NK cell suppression. In summary, these data suggest that influenza vaccination can stimulate human NK cells systemically and supports the rationale that cancer surgery patients can derive a NK cell stimulating benefit from influenza vaccination given in the perioperative setting immediately prior to 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 demonstrated that OV markedly increases NK cell activity in cancer patients (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 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 suggests that the perioperative period is the crucial window of opportunity to intervene with an innate immune stimulant (Figure 5C). Mechanistically, we demonstrated that IFNα is the main mechanism underlying the therapeutic effect of influenza vaccination in both preclinical and clinical samples as the abrogation of IFNα 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, while in cancer surgery patients, our data suggests that pre-operative ex vivo pulsing of PBMC with influenza vaccine can enhance NK cell functionality. By contrast, in post-surgery samples, NK cell activation and cytokine secretion was only observed in 1/4 patients. However, these ex vivo pulsing results do not rule out the possibility that pre-operative 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 favour of a preoperative
Running Title: Perioperative influenza vaccine enhances NK cell function 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 Figure 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 IFNα (IFNα2a, IFNα2b) have been approved by the FDA for a variety of clinical indications, including hairy cell and chronic myelogenous leukemia and AIDS-related Kaposi’s sarcoma. However, adverse effects due to IFNα have been described, including hematological and neurological 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 IFNα showed increased NK activity and acceptable toxicity (17, 18, 25). Further, 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 lab.

Nevertheless, the focal point of this current study is to demonstrate 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 physiological 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 immune compromised individuals. Cancer surgery patients may
Running Title: Perioperative influenza vaccine enhances NK cell function benefit from immunity against influenza infection, as well as the potential anti-metastatic 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 physiological 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 cancer patients who undergo surgical resection of their solid tumors every year.
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FIGURE LEGENDS

Figure 1. Poly(I:C) as perioperative therapy against immune suppressive 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. The data are displayed as the mean percent (+/-SD) of $^{51}$Cr-release from triplicate wells for the indicated E:T ratios (p values are compared to “surgery” group). (d) The ability of NK cells from indicated treatment groups to reject RMA-S tumor cells. Data are representative of 3 experiments, n=4-6/group (*, p=0.02; **, p=0.005; ***, p=0.0001, n.s. (not significant)).

Figure 2. Influenza vaccine is a potent stimulator of NK cell function.
(a) The ability of NK cells to kill tumor targets and (b) CD69 activation on NK cells from indicated vaccine treatment groups. (c) The ability of NK cells to kill tumor targets from indicated treatment groups. Data are representative of 3 experiments, n=4-5/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) The ability of NK cells to kill tumor targets from indicated treatment groups (p value is compared to “surgery” group) (left panel). The ability of NK cells
Running Title: Perioperative influenza vaccine enhances NK cell function from indicated treatment groups to reject RMA-S tumor cells (right panel). Data are representative of 3 experiments, n=4-7/group (*, p=0.05; **, p<0.0042; *** p<0.0001; n.s.).

Figure 4. Influenza vaccine enhances NK cell function through IFNα.  
(a) Time course quantification of serum cytokine levels in control B6 mice following influenza vaccine administration.  (b) The ability of NK cells to kill tumor targets from (left panel) IFNAR-KO and B6 mice post-influenza vaccine and (right panel) from indicated treatment groups. Quantification of B16lacZ lung tumor metastases in (c) B6, (d) IFNAR-KO mice following indicated perioperative treatment.  N=4-5/group.  (**. p>0.005; ***, p=0.0001; n.s.). 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) The ability of NK cells to kill tumor targets from B6 mice at 1d, 3d and 5d post-influenza vaccine along with controls. (b) The 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/group.  (*, p=0.01; **, p>0.005; ***, p=0.0001; n.s.). Data are representative of 3 experiments.

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 PBMC 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 PBMC obtained from 6 healthy human donors who received in vivo influenza vaccination. PBMC from whole blood was obtained at pre-vaccine, d2±1 post-vaccine, and d10±2 post-vaccine.  (c) Quantification of indicated
Running Title: Perioperative influenza vaccine enhances NK cell function cytokines from cell-free supernatant; CD69 NK (CD56+/CD3−) expression; and ex vivo cytotoxicity with PBMC 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.).
Figure 1: Poly(I:C) as perioperative therapy against immune suppressive effects of surgery and attenuation of metastatic disease

A

Number of B16lac-2 lung tumor metastases in C57Bl/6 mice at 3d

B

Number of metastatic 4T1 lung tumor nodules in BALB/c mice at 28d

C

% ex vivo cytotoxicity

E:T ratios

D

% in vivo tumor rejection
Figure 2. Influenza vaccine is a potent stimulator of NK cell function
Figure 3. Attenuation of metastatic disease by perioperative NK cell stimulation with influenza vaccine

A

Number of B6.11a2 lung tumor metastases in C57Bl6 mice at 3d

No surgery Surgery Surgery+
Influenza vaccine

B

Representative lung images from 4T1 tumor-bearing BALB/c mice at 28d

No surgery Surgery Surgery+
Influenza vaccine

C

Number of B6.11a2 lung tumor metastases in C57Bl6 mice at 3d

Surgery+Influenza vaccine Surgery+Influenza vaccine

NK cell intact NK cell depleted

D

% ex vivo cytotoxicity

% in vivo tumor rejection

E:T ratios

No surgery Surgery Influenza vaccine Surgery + Influenza vaccine
Figure 4. Influenza vaccine enhances NK cell function through IFNα
Figure 5. Influenza vaccination in the immediate perioperative period is necessary to achieve maximal efficacy for reducing lung metastases.

A) Euthanize+ NK cell killing.

B) Agriflu 1x 2x 3x.

C) 4T1 cells surgery Euthanize+ Assess tumors.

Days post influenza vaccine injection.

% ex vivo NK cell cytotoxicity against YAC-1 targets (50:1 E:T ratio).

Days post influenza vaccine injection.

Number of metastatic 4T1 lung tumor nodules in BALB/c mice at day 28.

No surgery Surgery Neoadjuvant Perioperative Multidose Influenza vaccine injection.
Figure 6. Influenza vaccination enhances NK cell function in healthy human donors and cancer surgery patients
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Perioperative influenza vaccination reduces post-operative metastatic disease by reversing surgery-induced dysfunction in natural killer cells

Lee-Hwa Tai, Jiqing Zhang, Karen J Scott, et al.

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