Restoring Postoperative Natural Killer Cell Function by Targeting the Immunosuppressive Machinery of Surgery-Induced Myeloid Derived Suppressor Cells

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ABSTRACT

In the aftermath of cancer surgery, Natural killer (NK) cells are severely suppressed. NK cells are critical for anti-tumour surveillance and their postoperative dysfunction creates an opportunity for metastases. *I hypothesized that NK cell suppression is mediated by multiple suppressive mechanisms of surgery-induced Myeloid Derived Suppressor Cells* (Sx-MDSCs). In this thesis, I first show that NK cell dysfunction is far worse than previously described. In a cohort of colorectal cancer (CRC) surgery patients (n=42), the ability of NK cells to secrete IFN-gamma in response to stimulation was suppressed for up to 2 months after surgery. Secondly, since Sx-MDSCs have been poorly characterized in humans, I thoroughly phenotyped Sx-MDSCs from cancer surgery patients using flow cytometry (n=32 patient samples) and single-cell RNA sequencing (n=6 patient samples). Additionally, upon screening a library of 150 compounds, I showed that Sx-MDSC rely on PI3K signaling for their suppression of NK cells in *ex vivo* NK cell suppression assays. The third part of this thesis explores the contribution of Sx-MDSCs to the rapid reduction in postoperative arginine, the perioperative importance of arginine for NK cells, and the therapeutic effects of a perioperative arginine enriched supplement (AES) on metastases in murine models of surgical stress. Here, I showed that perioperative AES attenuates postoperative metastases by accelerating NK cell recovery after surgery. These promising preclinical data combined with evidence from the scientific literature led us to initiate a Phase II randomized-controlled clinical trial assessing the ability of perioperative AES to improve NK cell function after surgery in CRC patients (n=12/arm). In the last part of this thesis, I present the results from our clinical trial, which showed only a transient and, at best, modest improvement in NK cell function. Importantly, this may have been heavily influenced by poor postoperative patient compliance in taking the AES. In conclusion, this body of work describes the multifactorial role that Sx-MDSCs play in mediating postoperative NK cell suppression, and that safe, effective, and targeted perioperative interventions should be further investigated as a strategy to attenuate metastatic disease recurrence after surgery.

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LIST OF ABBREVIATIONS

ACK - Ammonia Chloride Potassium
ADC - Antibody drug conjugate
ADCC - Antibody dependent cellular cytotoxicity
AED - Arginine enriched diet
AES - Arginine Enriched Supplement
ALL - Acute lymphoblastic leukemias
Arg1 - Arginase-1
BL - Baseline
CAT - Cationic amino acid transport
CI - Confidence interval
COX2 - Cyclooxygenase-2
CRC - Colorectal cancer
CRP - C-reactive protein
CTC - Circulating tumour cells
DAMPs - Damage-associated molecular patterns
DFS - Disease Free Survival
DNAM1 - DNAX accessory molecule 1
E-MDSC - Early/immature-MDSCs
eIF2α - eukaryotic initiation factor 2 alpha
EMN - Enhanced Medical Nutrition
EMT - Epithelial to mesenchymal transition
EtHD - Ethidium homodimer
G-MDSC - Granulocytic MDSC
GCN2 - General control non-depressible 2
GM-CSF - Granulocyte-macrophage colony-stimulating factor
GSEA - Gene set enrichment analysis
HD - Healthy Donor
HDN - High density neutrophil
i.p. - Intraperitoneal injection
i.v. - Intravenous injection
IFN - Interferon
IL - Interleukin
iNOS - Inducer of Nitric Oxide Synthase
IQR - Interquartile range
ITAM - Immunoreceptor tyrosine-based activation motifs
KIRs - Killer Ig-like receptors
LDN - Low density neutrophil
LOS - Length of stay
LPS - Lipopolysaccharide

LRFS - Local recurrence free survival
M-MDSC - Monocytic MDSC
MDSC - Myeloid derived suppressor cell
MHC - Major histocompatibility
MIC - MHC-class I polypeptide-related sequence
MSigDB - Molecular Signatures Database
mTOR - Mammalian target of rapamycin
NCR - natural cytotoxicity receptors
NES - Normalized enrichment scores
NK - Natural Killer
NKA - NK cell activity (IFN-gamma following stim)
NKC - NK cell cytotoxicity
NMF - Non-negative matrix factorization
NO - Nitric Oxide
NSAIDs - Non-steroidal anti-inflammatory drugs
PBL - Peripheral blood lymphocytes
PBMCs - Peripheral Blood Mononuclear Cells
PDE5 - Phosphodiesterase-5
PFS - Progression free survival
PGE2 - Prostaglandin E2
PMN-MDSC - Polymorphonuclear MDSC
POD - Postoperative Day
PRR - Pattern recognition receptors
RBC - red blood cell
ROS - Reactive oxygen species
s.d. - standard deviation
scRNA-seq - single cell RNA sequencing
SD - Surgery day
SNPs - Single nucleotide polymorphisms
Sx-MDSC - Surgery induced MDSC
TAMs - Tumour associated macrophages
TANs - Tumour associated neutrophils
TGF-β - Transforming growth factor beta
Th1 - T helper 1
Th2 - T-helper 2
TID - “Ter in die” or, 3 times a day
TNF-α - Tumor necrosis factor alpha
ULB - UL16-binding proteins
ΔΨm - Mitochondrial membrane potential

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Chapter 1

General Introduction

1.0 PREFACE

In this Chapter, I will give a general introduction for the main pillars of my project which are: *i)* Cancer metastases, *ii)* Natural killer cells, *iii)* Myeloid derived suppressor cells, and *iv)* Perioperative immunotherapies. Each section will end with how they are affected by surgery.

Parts of this chapter have been taken and edited from my review manuscript [1]:

Angka L, Khan ST, Kilgour MK, Xu R, Kennedy MA, Auer RC. Dysfunctional Natural Killer Cells in the Aftermath of Cancer Surgery. International Journal of Molecular Sciences. 18(8):1787 (2017).

Author contributions for this work are as follows:

Angka L: Worked together with Auer RC to conceive the outline and focus. Coordinated the efforts of the co-authors. Wrote the manuscript in its entirety, with input and background research from co-authors. Edited and created the figures and tables.

Khan ST: Contributed to "4. Surgical Stress, Inflammation and NK cell Dysfunction", "4.5. Correlating Post-Op NK cell Suppression to the Degree of Surgical Stress" and "5. Post-Op MDSC and NK cell Dysfunction".

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The sections of this review manuscript that have been deliberately excluded from this thesis for brevity and focus, are: *4.2. Analgesics, Anaesthetics, Blood Transfusions and Their Effect on Post-Operative NK Cell Function, 4.3 The Post-Operative Hypercoagulable State Shields Cancer Cells and Blocks NK cell Cytotoxicity*, *4.4 Decreased Mitochondrial Membrane Potential Increases NK Cell Susceptibility to Apoptosis,* and *4.5. Correlating Post-Operative NK Cell Suppression to the Degree of Surgical Stress [1]*.

The title page and permissions of use for this work can be found in Appendix A.

1.1 CANCER AND METASTASES

Cancer is a disease characterized by the uncontrolled proliferation of abnormal cells which can acquire traits that enable them to metastasize to distant organ sites, disrupting vital physiological processes along the way. Although cancer is the second-leading cause of death worldwide, advances in our understanding of the disease coupled with the application of new technologies and therapies is making progress in reducing the overall death toll from cancer. Cancer derives from a stepwise transformation of normal cells into malignant cells that can occur at any time in one's lifespan. These transformations have been classified into six original "hallmarks of cancer" with each hallmark representing a physiological change that budding cancer cells acquire to overcome specific anti-cancer mechanisms [2]. These hallmarks include *i)* self-sufficiency in growth signals, *ii)* insensitivity to growth-inhibitory signals, *iii)* programmed cell death evasion, *iv)* limitless replicative potential, *v)* sustained angiogenesis, and *vi)* tissue invasion and metastasis [2]. Genetic mutations were regarded as the enabling characteristic that allow abnormal cells to acquire the original six hallmarks. However, it is now appreciated that tumour-promoting inflammation from the cells of the immune system is a second enabling characteristic that allows abnormal cells to avoid immune destruction and acquire hallmarks [3]. Therefore, while genetic predispositions may make one susceptible to cancer, external factors that directly impact host inflammation and immunity are also critical driving events in the initiation and the metastatic progression of cancer.

The metastatic cascade is first initiated within the primary tumour where it is thought that a subset of malignant cells are enabled, either by genetic mutations or the state of inflammation, to invade tissues and metastasize [2,4]. These cells will ultimately undergo an epithelial to mesenchymal transition (EMT) from integrating cytokine signals from cells within the tumour microenvironment (i.e., fibroblasts, endothelial, myeloid, and lymphoid

cells). An important EMT signal comes from stromal cell derived transforming growth factor beta (TGF-β) [4]. Once those migrating cancer cells from the primary tumour reach circulation, they must be able to survive as circulating tumour cells (CTCs) until they are able to extravasate into a distant tissue. From there, the CTCs can form *micrometastatic* niches where they can persist for very long periods in a dormant state until receiving cellular queues to proliferate into secondary *macrometastases* [5].

Metastasis accounts for ~90% of cancer-related deaths, making it the leading cause of death for cancer patients [4]. Across a number of cancer types, 5-year survival rates plummet once metastases develop [6,7]. Defined as secondary outgrowths of tumour cells at distant anatomical sites, metastases are most dangerous when they develop undetected. Importantly, metastasis is not a late stage event that occurs only after the primary tumour has proliferated to a sizable mass [5]. Accumulating evidence shows that metastasis begins even before the initial symptoms of a primary tumour are detected [8]. Furthermore, metastases can remain dormant in distant secondary sites for more than a decade after primary tumour resection [8]. These features of metastatic disease highlight its insidious nature and research targeting the prevention of metastasis is warranted.

1.1.1 Cancer Surgery

Surgery is the oldest treatment modality for cancer with records of tumour resections dating back to ancient Egypt [9]. The basic principles of cancer surgery were simple, remove the malignancy before it has a chance to spread. However, early documentation of cancer surgeries would often mention the return of a more aggressive cancer at auxiliary sites [10]. This reality of cancer surgery ushered in an era of radical tumour resections pioneered by Dr. William Halsted and colleagues in the 1890s, and was further utilized with the advent of anesthetics. His radical mastectomies involved the removal of the entire breast, in addition to the underlying pectoral muscle and regional lymph nodes to ensure that the source of the

cancer would be removed [11]. To their credit, radical mastectomies were able to reduce the incidence of local recurrences down to ~6%, a marked improvement from non-radical mastectomies at the time which had a local recurrence rate of 51-82% after surgery. It was only until the 1940's that the use of radical surgeries was challenged, and the whole paradigm had shifted by the 1970's towards breast-conserving surgeries since they were shown to have comparable efficacy without compromising the patient's quality of life after surgery [11].

The field of cancer surgery has since undergone even more changes in large part due to technological advancements such as improved tools of detection, precision instruments, and surgical techniques. The thirty-day mortality rate after cancer surgery has dropped from 17% in the 1960's, to now below 1% for similar procedures [9]. However, postoperative recurrence with aggressive metastatic disease is still an issue that looms over cancer surgeries today [12], as it did over a century ago [13].

1.1.2 Cancer Surgery and Metastases

Despite the indisputable benefits of tumour resections, the pronounced aftereffects of surgery may be detrimental to overall patient survival. Almost a third of all colorectal cancer (CRC) patients who have no signs of metastases before undergoing primary tumour resection will develop metastases within 5 years after surgery [10]. Furthermore, Baum et al. found that there was an increase in the hazard rate for relapse or death within the first 3 years after surgery in breast cancer patients [14]. Numerous aspects of cancer surgery have been postulated to contribute to metastatic relapse including the use of anaesthetics, a hypercoagulable state, tumour cell dissemination during resection, and inflammation leading to immune suppression [12,15,16].

Ceelen et al. describes how wound healing processes and the components of "wound fluid" can stimulate tumour growth [17]. The upregulation of pro-inflammatory cytokines interleukin (IL) -1 and tumor necrosis factor alpha (TNF-α) can enhance the adhesion of CTC to the endothelial walls of blood vessels to facilitate tumour cell extravasation [18]. Additionally, these cytokines result in the recruitment and expansion of neutrophils that can release neutrophil-extracellular traps after surgery which can promote metastases by ensnaring CTCs [18]. As mentioned above (Section 1.1), the inflammatory tumour microenvironment is an enabling feature of tumorigenesis [3]. Therefore, the act of surgery, which disrupts endothelial barriers and inflicts tremendous amounts of pain and stress, initiates the surgical stress response and facilitates tumorigenesis and metastases through inflammation and immune suppression.

1.1.3 Cancer Surgery and the Surgical Stress Response

Likening the preoperative state to an unassuming body of water, whereby all of the unseen components thrive together seamlessly underneath the surface, the surgeon's scalpel would then be the cannonball that results in a flurry of concentric waves, emanating from the site of incision. This analogy describes the response to surgical stress as an "Ebb and Flow" whereby the *Ebb* represents the outgoing response to surgery and the *Flow* represents the incoming attempt to return to homeostasis.

Postoperative Ebb and Flow

The inflammatory response to surgery is made up of an acute pro-inflammatory "Ebb" phase followed by a prolonged anti-inflammatory "Flow" phase [19]. Surgical stress starts at the breaking of endothelial cell barriers which lead to excessive inflammation at the wound site. Neutrophils are first recruited to the site of inflammation and the ratio of neutrophil to lymphocyte count by complete blood cell counts has been used as a measure of postoperative inflammation [20,21]. Additionally, the release of stress signals known as

"damage-associated molecular patterns" (DAMPs) immediately after surgery can be sensed by pattern recognition receptors (PRRs) on monocytes. Monocyte PRRs (e.g., toll-like receptors, C-type lectin receptors) bind to DAMPs (e.g., HMGB1, S100 proteins, actin, HSP60 and HSP70) which leads to the large production of pro-inflammatory cytokines (e.g., IL-1β, IL-6, and TNF-α) through NFκB activity. IL-6 has dual pro- and anti-inflammatory roles [22], but following the acute pro-inflammatory phase the prevailing anti-inflammatory properties of IL-6 set the stage for the next phase of surgical inflammation.

It has been shown that the magnitude of the pro-inflammatory phase dictates the magnitude of anti-inflammatory phase [19]. The anti-inflammatory phase is characterized by: *i)* the negative feedback of IL-1 and TNF-α by IL-6; *ii)* the accumulation of C-reactive protein (CRP); *iii)* the downregulation of HLA-DR expression on monocytes; and *iv)* the induction of IL-10 through prostaglandin E2 (PGE2) [17,19,23,24]. Narita et al. compared the level of IL-6 in prostate cancer surgery patients undergoing a laparoscopic vs open surgery and reported that serum IL-6 was significantly lower in patients undergoing the less invasive, laparoscopic surgery [25]. Therefore, IL-6 levels can be used as a surrogate marker for the magnitude of surgical stress. While on the topic of "minimally invasive surgeries", laparoscopic surgeries were shown to have similar 3 year and 10 year outcomes in terms of disease-free and overall survival when compared to open surgeries [26]. This highlights that the size of skin incision does not determine the magnitude of stress, and that the surgical stress response can occur during any operation. The Ebb and Flow relationship between the pro- and anti-inflammatory phases exists to maintain a balanced inflammatory response to surgery in order to protect against the damaging effects of excessive inflammation [19].

Postoperative Arginine Depletion

The *Flow* phase after surgery is a hypermetabolic state that can persist for weeks after severe injury. Increased oxygen consumption, body temperature, tachycardia, insulin

resistance, hyperglycemia, protein catabolism, and lipolysis after surgery result in a high nutritional demand for postoperative cellular processes [27,28]. Thus, basal metabolic rates are increased to restore homeostasis in the patient but a prolonged period of nutrient deficiency can increase postoperative complications and should be counteracted perioperatively [27] (Section 1.4.2). One amino acid that is particularly affected and reduced during the hypermetabolic phase of surgical stress is arginine. Arginine deprivation after surgery has many implications on immunity that will be described in subsequent sections [29].

Postoperative Immunosuppression

The degree of induced stress and the invasive nature of the procedure are correlated with the magnitude of postoperative immune suppression [19,30]. After surgery, a state of emergency myelopoiesis and granulopoiesis ensues to respond to the systemic redistribution of lymphocytes out of the bone marrow and towards the site of injury [21,31]. This results in a large increase in monocytes and neutrophils which have been shown to be subject to phenotype switching due to the anti-inflammatory, T helper 2 (Th2) cytokine milieu [30]. Therefore, the dominant immunosuppressive cell type post-surgery are immature, heterogeneous, innate myeloid cells [32]. Using the expression of HLA-DR as a marker of immune competence, antigen presentation, and maturation state, monocytes have been shown to have significantly decreased expression of HLA-DR post-surgery [19,32–34]. These postoperative HLA-DR^{lo} immature myeloid cells have been described to resemble myeloid derived suppressor cells (MDSCs), and have a key role in mediating immunosuppression that will be discussed in greater detail in Section 1.3. But first, I will describe the immense suppressive effect that surgery has on Natural Killer (NK) cells, which our lab [35,36] and others [37,38] have shown drives postoperative metastasis.

1.2 NATURAL KILLER CELLS

In 1975, Kiessling et al. [39] made an observation that red blood cell (RBC)-lysed splenocytes were able to naturally kill leukemic cells without prior *in vivo* sensitization. This was the first observation that would ultimately define a cell type that is now known as "Natural Killer" cells. NK cells are the cytolytic cells of the innate immune system that surveil the body for stressed, virally-infected or malignant cells [40]. Human NK cells account for 1-17% of all circulating lymphocytes in peripheral blood [41], and have an average half-life of 12 days [42]. They are broadly identified as $CD3^{-}CD56^{+}$ but can be further sub-grouped based on their expression level of CD56. $CD56^{dim}$ NK cells make up the vast majority of circulating NK cells (~90%) [43] and harbor granules containing perforin and granzyme. Additionally, $CD56^{dim}$ NK cells are able to engage in antibody dependent cellular cytotoxicity (ADCC) through their expression of the CD16, the FcγRIII [41,43]. These characteristics define $CD56^{dim}$ NK cells as the cytotoxic subset. The subset of NK cells that make up the remaining ~10% are the $CD56^{bright}CD16^{-}$ cytokine secreting NK cells [43]. These cells can secrete large amounts of cytokines including interferon-gamma (IFN-γ), TNF-α, IL-10, IL-13, and Granulocyte-macrophage colony-stimulating factor (GM-CSF) [43]. Notably, while these subgroups of NK cells exist, their functions are not mutually exclusive as $CD56^{dim}$ NK cells are able to produce cytokines (to a lesser extent) and $CD56^{bright}$ NK cells can be stimulated to become cytotoxic [43]. Evolutionarily, NK cells are thought to be a transitional immune cell type that bridges the innate and adaptive immune response in higher-level organisms [44]. These cells differ from adaptive immune effector cells due to their defining characteristic of naturally being ready to perform effector functions without antigen-specific clonogenic expansion. Through the integration of inhibitory and activating signals or the engagement of FAS-ligand or TRAIL, NK cells can lyse a cell within minutes of target cell recognition and control the establishment of

metastases [5]. Furthermore, NK cells can efficiently produce cytokines due to the reserve of IFN-γ transcripts that are primed for translation and secretion [44].

NK cell Target Recognition

Through the integration of inhibitory and activating stimuli, NK cells determine which cells to eliminate and which to spare. The main ligands that inhibit NK cells are the major histocompatibility (MHC) class I and MHC class I-like molecules, which are expressed at high levels on normal cells to prevent autoreactivity [45]. Inhibitory receptors on human NK cells include the killer Ig-like receptors (KIRs) and the CD94-NKG2A heterodimer which recognizes HLA-class I or HLA-E molecules, respectively [46]. Murine NK cells express Ly49 lectin-like inhibitory receptors that bind to MHC Class I [45]. Importantly, the inhibitory receptor repertoire that each individual NK cell expresses is an indication of their potential responsiveness. NK cells with the greatest cytotoxic potential express the greatest level of inhibitory receptors, while hyporesponsive NK cells have no expression of inhibitory receptors [47]. This concept forms the basis of the *rheostat* model whereby NK cells are tuned to a specific threshold of responsiveness during development that is dependent on the level of inhibitory receptor expression [47]. This model likens NK cell decision making more to an analog response as opposed to a binary, kill-or-not-kill, response during NK cell recognition [44]. This is likely another evolutionary safeguard that prevents NK cells with no inhibitory receptors from causing unregulated self damage [46,47]. On the flip side, potential target cells that do not express any inhibitory self-ligands are not automatically targeted for destruction by NK cells as the ultimate decision also requires input from activating receptor engagement at the NK-target cell synapse [44].

Activating receptors on NK cells bind to stress-induced ligands on target cells which then signals through phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) associated with the activating receptors through adaptor proteins such as DAP10

and DAP12 [48]. Once phosphorylated, ITAMs recruit and activate tyrosine kinases (Syk and Zap70) to perform their downstream signaling events resulting in the reorganization of the actin cytoskeleton for the release of cytolytic granules or the transcription/translation of cytokines [48]. The expression of conserved activating receptors on NK cells, as opposed to highly antigen-specific T cell receptors that are not present on all T cells (but are enriched for after clonal expansion) [49], enable NK cells to mount a strong response without prior sensitization to the foreign antigen. NK cell activating receptors include: *i)* NKG2D which binds the MHC-class I polypeptide-related sequence (MIC)-A, MIC-B, and UL16-binding proteins (ULB) 1-6 [44,48]; *ii)* the natural cytotoxicity receptors (NCRs) NKp30, NKp44, and NKp46 which binds to various heparan sulfate structural motifs [50]; and *iii)* 2B4 (CD244) which binds CD48 [44,48,51]. Costimulatory receptors, such as DNAM1, are also expressed by NK cells to allow further stimulation but do not lead to NK cell activation alone [51]. Therefore, coupling the fact that tumours often down regulate their MHC-I expression to evade T cell recognition and that activating ligand expression is often upregulated on tumour cells, NK cells are especially effective anti-tumour surveillance [48].

Although classically, NK cells are prided for not going through the process of antigen-specific clonal expansion, accumulating evidence in viral infection models has challenged this notion and describe a process of NK cell "training" or "memory" [52–54]. However, clonal expansion of NK cells in the context of cancer has yet to be fully investigated [55] but would serve to improve and inform the use of adoptive NK cell transfer cancer therapies.

1.2.1 Natural Killer Cells, Cancer Prognosis and Metastases

NK cells are critical for anti-tumour immunity. Imai et al. conducted a prospective, 11-year follow-up study and showed that the risk of cancer incidence was associated with the responsiveness of their peripheral blood lymphocytes (PBL) against NK cell specific K562

target erythroleukemia cells [56]. Medium to high cytotoxicity of PBLs against K562 was associated with significantly reduced risk of cancer. In a subsequent study, they showed that high cytotoxicity of NK cells can be traced back to a set of single nucleotide polymorphisms (SNPs) found in the natural killer complex on chromosome 12p, with the majority of SNPs located in the NKG2D gene region [57]. The high NK activity haplotype (HNK1/HNK1) strongly correlated with cytotoxicity and reduced the risk of colorectal cancer (CRC) [57,58]. Furthermore, multiple reports have shown that either infiltration of NK cells [59,60] or increased expression of NKG2D ligands (MIC or ULPBs) [61] can be used as a prognostic marker in various carcinomas. Conversely, reduced NK cell function can also serve as an accurate prognostic marker that is correlated with advanced cancer stage, recurrence and mortality [62].

As outlined in Section 1.1, metastases can occur at early and late stages of primary tumour growth, but in both cases, CTCs must be able to establish themselves in a new tissue environment. Therefore, both circulating and tissue resident NK cells are critical components for preventing disseminated CTCs from forming metastatic niches. The presence of highly active NK cells in circulation has been inversely correlated to metastatic disease [5]. This posits that when NK cells are suppressed, there is an increased risk for metastases.

1.2.2 Natural Killer Cells and Dysfunction after Surgery

The physiological toll and consequences of surgery has direct effects on NK cell cytotoxicity (NKC), NK cell activity (NKA; the ability to secrete IFN-γ in response to stimulation), and receptor expression. Our group [35,63,64] and others [38,65] have reported that surgery results in pronounced suppression of NKC which lasts on average for 1 week after surgery, but normalizes after 1 month. It has also been reported that on POD1 NK cells suffer a marked reduction in their cytokine secretion in response to stimulation, or

NKA [66,67]. Reinhardt et al. showed that following surgery, $CD56^{bright}$ NK cells have exceptionally reduced IFN-γ production when stimulated with staphylococcus aureus or recombinant IL-12. Although the $CD56^{bright}$ NK cells have reduced IL-12R after surgery, this did not result in changes in pSTAT4, which receives signals from the IL-12 pathway to regulate the transcription of IFN-γ [68]. Therefore, surgery creates an opening for CTCs to establish metastatic niches in the postoperative period of NK cell dysfunction.

The events that lead to NK cell suppression after surgery are many. As described in Section 1.1.1, surgery results in an anti-inflammatory cytokine milieu which systemically affects the immune system. Additionally, MDSCs expand and suppress NK cells in *ex vivo* experiments. Lastly, surgery leads to a sharp reduction in arginine, which is necessary for NK cell function.

NK cell suppression due to Postoperative Inflammation

The postoperative cytokine milieu is dominated by elevated IL-6 in the peripheral blood, but even more so at the site of surgical trauma [69]. The results from a Phase I clinical study in 20 patients with advanced cancer (colon and pancreatic) receiving recombinant IL-6 (rIL-6) showed the suppressive effects of rIL-6 on NKC [70]. Patients given rIL-6 suffered a 50% drop in NKC on day 7 after treatment which gradually returned by day 20. Importantly, a second administration of rIL-6 at day 22 also decreased NKC on day 30 confirming that rIL-6 administration negatively affects NKC [70]. Recent studies have demonstrated that IL-6 inhibits the cytotoxic activity of NK cells by decreasing perforin and granzyme production via the modulation of SHP-2 expression [71–73]. IL-6 can also lead to the production of PGE2 which has been reported to have direct effects on NK cells [74]. Two day cultures of isolated human NK cells with rIL-15 ± PGE2 resulted in a dose dependent decrease in NKC and NKA.

Postoperative TGF-β levels were shown to be associated with increased metastasis to lymph nodes after prostate removal [75]. TGF-β is known to suppress NK cell function and responsiveness to activating cytokines [76–78]. Of the different signaling cascades affected by TGF-β, inhibition of the mammalian target of rapamycin (mTOR) pathway has been directly implicated in suppressing the anti-tumour efficacy of NK cells [79]. In particular, increased engagement of TGF-β with its receptor on NK cells reduces their proliferation, metabolism and cytotoxicity by countering IL-2 or IL-15 mediated mTOR activation. In cancer patients, TGF-β has been shown to drive the downregulation of NKG2D resulting in unregulated tumour proliferation [80,81]. Since IL-6 drives TGF-β production from various cell types and TGF-β itself may enhance IL-6 release, a resulting feedback loop may perpetuate post-surgical immune suppression [82–84]. Furthermore, platelets [85] and shear stress [86] have been reported to increase TGF-β. Thus, the prolonged elevated presence of IL-6, PGE2, and TGF-β in the postoperative period would result in reduced NK cell responsiveness.

NK cell suppression due to Postoperative Arginine Depletion

Arginine is necessary for proper immune cell function [29] but is rapidly reduced after surgical trauma [87]. Arginine was shown to be necessary for NKC decades earlier by Xiao et al. [88]. Primary NK cells had a 70% reduction in their cytotoxic potential when cultured in “deficient media” (devoid of amino acids, serum, pyruvate, vitamins) compared to “complete media” (RPMI 1640, 10% FCS, penicillin, and streptomycin). Importantly, the addition of L-arginine (1mM) back into the deficient media restored NKC but adding the other components (serum, glutamine and vitamins) resulted in minimal recovery. Even reconstituting the deficient media with everything except for arginine did not increase the recovery of NKC above that attained by the addition of arginine alone [88]. These

experiments provided the initial evidence that arginine is required for NK cell function and has since been confirmed by subsequent groups [89–91].

Insufficient arginine impairs NK cell proliferation, NKC, and IFN-γ production [92]. Furthermore, expression of NK cell receptor NKp46 and to a lesser extent NKp30, is decreased in arginine depleted conditions [90]. Reduced arginine can trigger the amino acid deprivation response that can be sensed by two main pathways, the GCN2 (general control non-depressible 2) and the mTOR pathway [93,94].

The GCN2 pathway becomes activated when there is an abundance of uncharged tRNA amino acid residues which lead to the phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α) in order to halt the process of translation. Both the GCN2 and mTOR pathways have been shown to be highly attuned to fluctuations in arginine availability, in multiple cell lines [94]. Oberlies et al. showed that culturing IL-2 stimmed human NK cells in arginine depleted media results in reduced proliferation and impaired IFN-γ translation, however, this was independent of GCN2 activation [89]. Importantly, they showed that culturing NK cells in tryptophan depleted media did not result in NK cell dysfunction, suggesting that the absence of arginine in particular mediates these effects. Similarly, Goh et al. showed that arginine deprivation also results in impaired IFN-γ and does not alter viability [91]. Their study assessed how arginine deprivation affects the mTOR pathway and showed that the phosphorylation and activity of mTOR and its downstream target, 4EBP1 were reduced in NK cells cultured in arginine depleted conditions [91]. These provide support for the hypothesis that arginine depletion after surgery contributes to postoperative NK cell suppression by the disruption of mTOR signalling.

1.3 MYELOID DERIVED SUPPRESSOR CELLS

Myeloid derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells that are characterized by their ability to suppress the immune system. While these suppressive cells were initially described in relation to the tumour microenvironment in cancer, their existence is seen throughout acute inflammatory states such as bacterial infection [95], trauma [96] and surgery patients [97]. Large inter-laboratory differences in reporting MDSCs have complicated the scientific literature [98], however, Bronte et al. [99] has made a harmonized guideline that includes descriptions of phenotypic, functional, and biochemical/molecular attributes that must be met to classify MDSCs. In humans, phenotypic characterizations of MDSCs fall under three myeloid specific ($CD11b^+$ or $CD33^+$), lineage negative ($CD3^-CD56^-CD19^-$) subsets: (1) $CD14^+CD15^-$ monocytic MDSCs (M-MDSCs); (2) $CD14^-CD15^+$ polymorphonuclear MDSCs (PMN-MDSCs) or; (3) $CD14^-CD15^-HLA\text{-}DR^-$ early/immature-MDSCs (eMDSCs) [99]. In mice, the total population of MDSCs can be identified as $CD11b^+Gr1^+$ cells with the subtypes being (1) $CD11b^+Ly6G^-Ly6C^{hi}$ M-MDSCs and (2) $CD11b^+Ly6G^+Ly6C^+$ PMN-MDSCs (synonymously referred to as granulocytic MDSCs, G-MDSCs) [99]. Regardless of the species, a functional test assessing suppressive activity must be shown to classify bona fide MDSCs. Most functional reports on MDSCs use an *in vitro* co-culture of MDSCs and T cells stimmed with anti-CD3/anti-CD28 and assess T cell proliferation, however, it is also known and accepted that MDSCs can suppress NKC and IFN-γ production [91,100,101].

It is hypothesized that MDSCs arise from common myeloid progenitor cells that have been polarized by cytokines from the tumour microenvironment. The known signaling pathways that regulate MDSC suppressive machinery are the Ras/Raf/MAPK, Phosphoinositide 3-kinases (PI3K)/Akt and Jak/Stat signaling pathways [102]. In particular, the PI3K/Akt pathway has been shown to control the suppressive phenotype of “M2” macrophages [103],

in addition to controlling myeloid cell accumulation and trafficking [102]. The Class IB PI3K isoform, p110γ, is the major catalytic PI3K subunit expressed specifically in myeloid cells [104]. Studies using *p110γ*$^{-/-}$ mice or PI3K-γ specific inhibitors have shown that disabling p110γ signalling leads to reduced tumour progression via a mechanism that prevents the recruitment and activation of suppressive CD11b^{+}Gr1^{+} myeloid cells [103,104].

MDSCs have numerous mechanisms of suppression at their disposal, which are differentially expressed in specific disease contexts or by specific MDSC subsets. The main suppressive mechanisms are the production of reactive oxygen species (ROS) and the activity of arginase-1 (Arg1) or inducer of nitric oxide synthase (iNOS) [105]. MDSCs have upregulated NADPH oxidase activity which allows MDSCs to produce large amounts of ROS which can disrupt T cell antigen responses [106,107]. Arg1 and iNOS are two enzymes that metabolize arginine, leading to the production of urea/ornithine or nitric oxide (NO)/citrulline, respectively [108].

In general, PMN/G-MDSCs are known to produce higher levels of ROS and have lower levels of iNOS activity compared to their M-MDSCs counterparts which have lower ROS production, but higher iNOS activity [108,109]. Both subsets express Arg1 and arginine depletion by MDSCs is a major reason for T-cell immunosuppression. MDSC iNOS activity results in NO production that can lead to CD3ζ chain nitrosylation and JAK3/STAT5 signalling inhibition [108]. Arg1 can result in rapid arginine reductions which can impact cellular immunity in multiple ways (described below). In addition, MDSCs can sequester cysteine (another amino acid important for immune cells) as well as produce immunoregulatory cytokines such as IL-6, IL-10, and TGF-β [109]. The number of suppressive mechanisms that MDSCs possess are a glimpse of their heterogeneity and potential as immune regulators.

1.3.1 Myeloid Derived Suppressor Cells and Suppression of Natural Killer Cells

Various groups have reported on different mechanisms of NK cell suppression likely owing to the context-dependent, heterogeneous nature of MDSCs. Studies have shown that MDSCs can suppress NK cells in both a contact dependent or independent manner. Sarhan and colleagues demonstrated that the suppressive interaction of MDSC and NK cells involved contact dependent signalling through the immune checkpoint, TIGIT [110]. Culturing NK cells with MDSCs isolated from patients with myelodysplastic syndrome resulted in a reduction in NK cell degranulation (%CD107a) and IFN-γ production. However, using α-TIGIT blocking antibodies or separating cells via a transwell abrogated NK cell suppression [110]. Other groups have also demonstrated the ability to prevent MDSC-NK cell suppression by blocking their interaction via α-TGF-β [111] and α-NKp30 [101] antibodies, or adenosine A2A receptor blockade [112].

While the aforementioned studies reported on contact dependent suppression, numerous groups have described the ability of MDSCs to inhibit NK cells through secreted factors or by nutrient depleting mechanisms. In chronic Hepatitis C viral infection, NK cells were suppressed in a contact- and ROS-independent mechanism [91]. A recent study by Stiff et al. showed that MDSCs can impair Fc-receptor mediated function of NK cells (i.e. ADCC and the production of cytokines) through NO production [113]. They were able to rescue NK cell ADCC function by treating 4T1 tumour bearing mice with the iNOS inhibitor, NIL. Furthermore, culturing patient MDSCs and NK cells in the presence of NIL restored NK cell ADCC lysis and IFN-γ production. Interestingly, neutralizing antibodies against TGF-β and IL-10 were not effective, but they showed that nor-NOHA, the Arg1 inhibitor, could improve NK cell ADCC in 4T1 bearing mice [113].

An early study by Uchida and colleagues showed that PBMCs isolated from 16/21 breast cancer patients undergoing radical mastectomies had a reduction in NK cell-specific killing

that was significantly less on POD7 compared to preoperative levels. The number of large granular lymphocytes did not change after surgery but culturing post-operation PBMCs for 24 h in monocyte depleted conditions recovered NK cell-specific cytotoxicity while undepleted cultures still suffered a >50% drop in cytotoxicity [114]. Using normal NK cells cultured with monocytes from surgery patients, but not conditioned media of monocytes, suppressed NK cell killing and they concluded that the emergence of a suppressive myeloid cell in response to surgical stress caused NK cell dysfunction [114]. Decades later, it is possible that the suppressive cells described by Uchida et al. were in fact surgery-induced MDSCs (Sx-MDSCs).

1.3.2 Myeloid Derived Suppressor Cells, Surgery and Arginine depletion

Many factors contribute to the generation of Sx-MDSCs. Prostaglandins, cyclooxygenase-2 (COX2), IL-6, IL-4, VEGF, GM-CSF and the process of emergency myelopoiesis could contribute to the expansion of not-fully mature myeloid cells into the bloodstream to restore homeostasis in lieu of surgical stress [32,115]. These cells are released from the bone marrow as immature myeloid cells and systemically contribute to the anti-inflammatory state following surgery [116]. In an experimental model of colorectal cancer, Xu et al. showed that surgery results in an increase in CT26 tumour growth with a concomitant increase in MDSCs and decrease in CXCL4 [117]. Interestingly, inoculating CXCL4 over-expressing CT26 tumours resulted in a decrease in MDSC infiltration *in vivo* and CXCL4 reduced MDSC migration in an *in vitro* transwell assay. Previously, our group has reported an increase in monocyte chemoattractants (MCP-1 and eotaxin-1) and a decrease in lymphocyte chemokines (6C-kine, IP10, and SDF-1) following surgery in mice [35]. These studies highlight the systemic rearrangement of immune cells and accumulation of MDSCs in response to surgical stress. In addition, since MDSCs are associated with a tumour microenvironment, cancer surgery patients have a pre-existing pool of suppressive MDSCs

which Yuan et al. [34] reported to be a potential prognostic marker for disease free survival (DFS) and local recurrence free survival (LRFS) after curative surgery in 64 rectal cancer patients. MDSCs in this study were defined as HLA-DR^{-}Lin^{-}CD33^{+}CD11b^{+} cells with the ability to suppress T cell proliferation [34]. The MDSC population in these surgery patients increased from 3.89% before surgery to 7.1% on POD7 before contracting to 4.39% on POD14 and finally to 2.21% on POD21. Importantly, they showed that in patients with higher numbers of MDSCs before surgery as well as on POD21, DFS and LRFS were significantly decreased ($p < 0.05$).

T-helper (TH) 1 or TH2 cytokines have been shown to induce iNOS or Arg-1, respectively [118,119]. Therefore, since the postoperative period is characterized by the increased presence of TH2 cytokines (IL-4, IL-6, IL-10, TGF-β) which play a role in wound healing [120], the TH2 cytokine response would also induce the expression of Arg1 in Sx-MDSCs [29,118,119,121]. A study of 17 car accident trauma patients showed a dramatic spike in plasma IL-10 within the first 24hrs of injury and this correlated significantly with the patients' Arg1 activity [122]. Furthermore, a correlation between Arg1 in colorectal cancer tissues has been associated with advanced stages of disease and prognosis [123]. Therefore, an increase in Sx-MDSC postoperatively with elevated Arg1 has been hypothesized to be a major contributor of arginine depletion in the postoperative period [97].

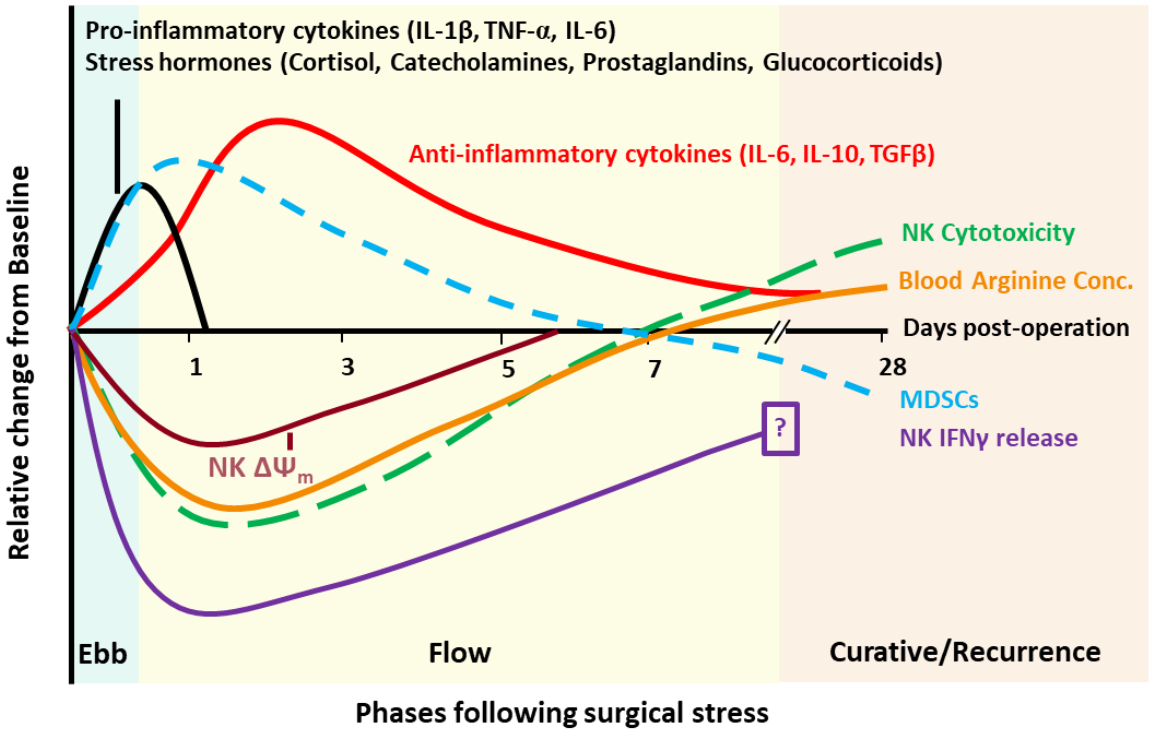

Figure 1.1. Aligning NK cell dysfunction with the Ebb and Flow of surgical stress.

Figure 1.1. Aligning NK cell dysfunction with the Ebb and Flow of surgical stress.

Within hours following surgical incision, an acute and robust increase in pro-inflammatory cytokines accumulate which characterizes the "Ebb" phase of the surgical stress response. This first phase subsides within ~12hrs as compensatory mechanisms take over to regulate excessive inflammation after surgery. This phase is the "Flow" phase which is characterized by the release of anti-inflammatory cytokines, hypermetabolism, increased cardiac output, and surgery-induced MDSCs. During the flow phase NKC, cytokine production and mitochondrial membrane potential ($\Delta\Psi m$; not described in this thesis) are at its lowest on POD1, but cytotoxicity and $\Delta\Psi m$ gradually normalize by POD7. IFN-γ production, however, may have a longer course of recovery. Whether cancer surgery results in a curative or recurrence phase is unknown but I hypothesize that it will depend on the magnitude and duration of the surgical stress response which forms a window of opportunity for perioperative intervention. This figure was published in my review article [1] and reformatted for use in this thesis.

1.4 PERIOPERATIVE CANCER IMMUNOTHERAPY

The perioperative window of opportunity describes the period of time where cancer patients are at their most vulnerable [10], and it is hypothesized that preventing immune suppression preoperatively or improving immune function postoperatively would reduce the rate of metastatic relapse [124]. Currently, there are no clinically approved therapeutics given to cancer surgery patients to combat postoperative immune suppression but many groups have tried (Table 1). The final sections of Chapter 1 will describe the perioperative therapies that have been investigated which target either Sx-MDSCs or the reduction in arginine after surgery.

Table 1.1. Clinical trials assessing the use of Perioperative immunotherapies to combat immunosuppression after surgery

Perioperative Strategy	Intervention	Trial ID	Phase
NK Cell Stimulation and PDE5 Inhibition	Influenza and Cialis	NCT02998736	Phase I
Adoptive Cell Transfer	Autologous NK cells	NCT02725996	Phase II
Cytokine Therapy	IFN-α	NCT00276523	Phase II
	IL-2	NCT00053807	Phase III
Immunonutrition	Dietary Arginine	NCT02987296	Phase II
Beta-adrenergic blockers and COX2 Inhibitor	Propranolol and etodolac	NCT00502684, NCT00888797	Phase II, Phase III
Hyper-coagulation	Tinzaparin	NCT01455831	Phase III

1.4.1 Perioperative therapies that target Sx-MDSCs

The presence of MDSCs have been correlated with worse cancer progression and metastases [125], likely due to their ability to alter the inflammatory milieu within the tumour microenvironment and cause immune suppression. Phosphodiesterase-5 (PDE5) inhibitors such as sildenafil and tadalafil have been shown by our group [64] and others [126] to disable the suppressive machinery of MDSCs in preclinical murine studies. We showed that treating mice perioperatively with PDE5 inhibitors prevented NK cell suppression and attenuated lung metastases after surgery [64]. Furthermore, combining PDE5 inhibitors with a preoperative dose of influenza to stimulate NK cells resulted in an even greater control of metastasis [64]. PDE5 inhibitors prevent the immunosuppressive effects of MDSC via decreasing Arg1 and iNOS expression [64,126]. Given these promising preclinical results, our group has initiated a Phase Ib clinical trial to investigate the ability of a preoperative influenza shot coupled with perioperative tadalafil treatment to improve NK cell function (NKC and NKA) and inhibit Sx-MDSCs in CRC patients (NCT02998736). When translating preclinical findings to clinical trials, assessing the safety and tolerability of the investigational therapy is of utmost importance. One complicating feature of cancer surgery is that each cancer patient may be on different types of medication, therefore, certain drug classes are suspended during the perioperative period [127]. Non-steroidal anti-inflammatory drugs (NSAIDs), for example, are generally stopped seven days before surgery as they can bind irreversibly to COX enzymes which would prevent the synthesis of clotting factors, important for regulating vasodilation and coagulation [128,129]. Interestingly, the specific COX2 inhibitor, etodolac, can be used perioperatively because it will not lead to vasodilation. Recent results from a Phase II randomized controlled clinical trial assessing etodolac in combination with the β-blocker, propranolol, was well tolerated and showed promising results in early stage breast cancer patients (NCT00502684) [130].

The authors report that this combination of perioperative drugs decreased molecular biomarkers and transcriptional pathways (GATA-1, GATA-2 and EGR3) of EMT, attenuated the perioperative increases in serum IL-6 and CRP levels (markers of surgical stress), reduced the number of tumour infiltrating monocytes and also increased NK cell activation markers (CD11a) following surgery [130]. Further clinical testing of these potential MDSC antagonists will shed greater insight into the mechanism and importance of Sx-MDSC in the perioperative period and more research into this is warranted.

1.4.2 Perioperative immunonutrition

Immunonutritional formulas have been investigated perioperatively as a way to overcome the catabolic effects of surgery with the most common components of these formulations being arginine, omega-3 fatty acids, nucleotides and glutamine [131]. The use of perioperative immunonutritional formulas have been reported to have a substantial impact on surgical outcomes - reducing infectious complications by 40-48% and hospital length of stay by up to 1.2-2.4 days in multiple meta-analyses [131–133]. While these findings are surely encouraging, a mechanism explaining how perioperative immunonutrition leads to these postoperative benefits has not been clearly elucidated. The prevailing hypothesis is that perioperative immunonutrition is working as intended - by improving immune responses after surgery - but there is little to no clinical evidence for this. Therefore, knowing which cells perioperative immunonutrition impacts, and which components of perioperative immunonutrition are necessary for these effects are critical questions to answer before this therapy can move forward as part of the standard protocol for cancer surgeries.

1.5 OBJECTIVE, RATIONALE, HYPOTHESIS, AND CHAPTER OVERVIEW

1.5.1 Overarching Objective

The overarching objective of my doctoral thesis was to identify perioperative therapies that could either prevent NK cell dysfunction or improve NK cell function after surgery to ultimately attenuate postoperative metastatic disease.

1.5.2 Rationale

The prometastatic consequence of surgery is in large part due to the profound suppression of NK cells. The postoperative period is characterized by increased anti-inflammatory cytokines and decreased arginine bioavailability, both of which may be caused by Sx-MDSCs and both of which would result in NK cell dysfunction. Furthermore, Sx-MDSCs have not been thoroughly characterized in the literature, and a deeper understanding of Sx-MDSCs will help inform future investigations in the field. Lastly, the mechanism of NK cell suppression by Sx-MDSCs has not yet been confirmed or exploited during the perioperative period.

1.5.3 Hypothesis

I hypothesize that preventing the immunosuppressive effects of surgery on NK cells through the inhibition of Sx-MDSC or through perioperative arginine immunonutrition, will result in reduced metastatic disease after surgery.

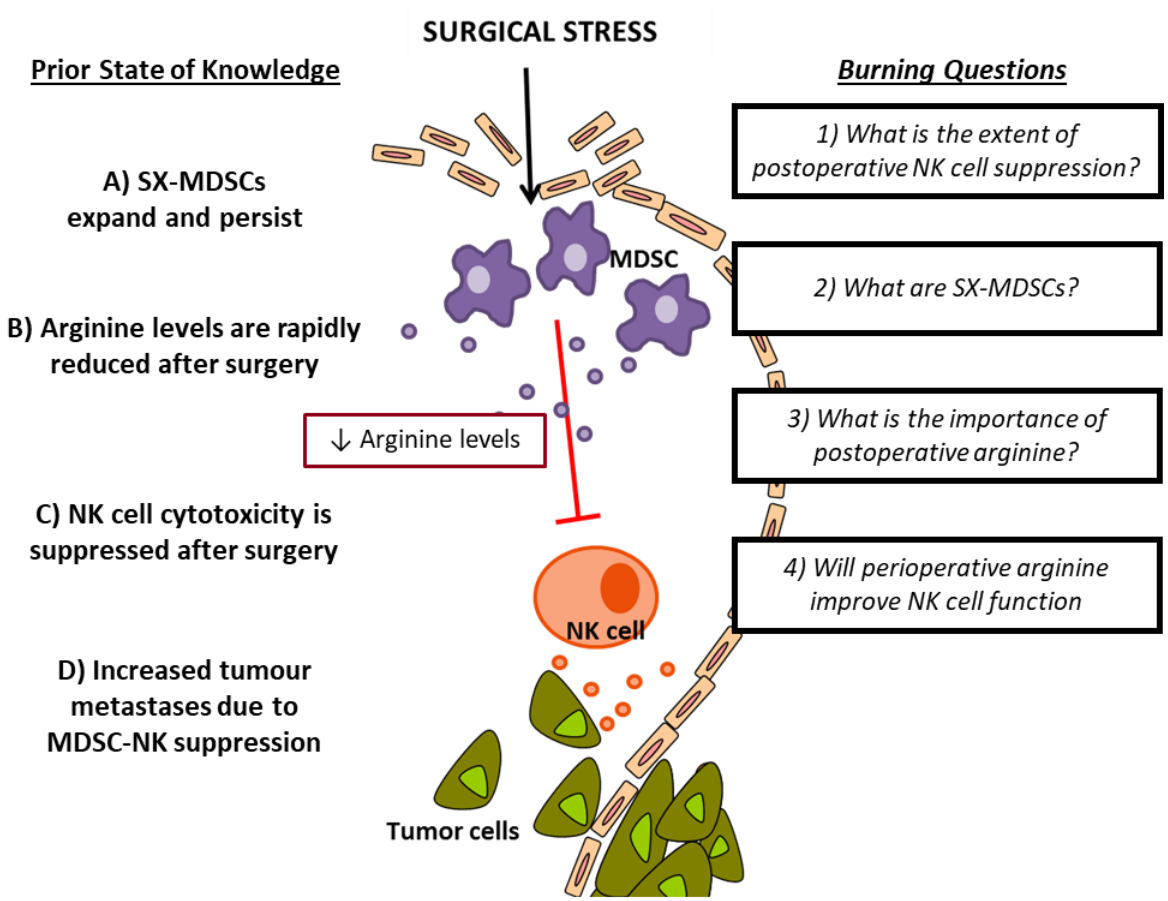

Figure 1.2. A model of surgical stress.

Figure 1.2. A model of surgical stress.

The state of knowledge prior to the beginning of this thesis project. **A)** Surgical stress leads to the expansion and persistence of Sx-MDSCs that have been poorly characterized in human cancer surgery patients. **B)** The cause and impact of reduced arginine bioavailability on NK cell function and metastases after surgery is unknown. **C)** NKC is suppressed after surgery, however the extent of NK cell IFN-γ secretion after surgery has not been described. **D)** Sx-MDSC:NK cell mediated suppression contributes to postoperative metastases and perioperative therapies that target Sx-MDSCs should be investigated. **1-4)** The burning questions that Chapters 2-5 will address, respectively.

1.5.4 Chapter Overview

In Chapter 2, I set out to further characterize human NK cell dysfunction with a focus on understanding the effect of surgery on NK cell IFN-γ secretion a.k.a. NK cell activity (NKA).

In Chapter 3, I characterize Sx-MDSCs from cancer surgery patients to understand more about their suppressive characteristics and to screen a library of small molecule inhibitors to identify cellular pathways that regulate the suppressive phenotype of Sx-MDSCs.

In Chapter 4, using preclinical models of surgical stress, I assess the ability of perioperative arginine immunonutrition to attenuate surgery-induced metastases by improving postoperative NK cell function.

In Chapter 5, I present the results from our Phase II clinical trial in which I assessed whether a perioperative arginine enriched supplement would impact NK cell function after surgery in CRC patients at The Ottawa Hospital.

In Chapter 6, a discussion on the thesis as a whole is presented which will focus on overarching themes and topics not covered in the individual chapter discussions.

Chapter 2

Surgery Results in Profound NK cell Dysfunction

2.0 PREFACE

This chapter was published in the Annals of Surgical Oncology on September 5, 2018.

Angka L, Martel, AB, Kilgour MK, Jeong A, Sadiq M, Souza CT, Baker L, Kennedy MA, Kekre N, and Auer RC. Natural Killer Cell IFNγ Secretion is Profoundly Suppressed Following Colorectal Cancer Surgery. Annals of Surgical Oncology. 25, 3747–3754 (2018). https://doi.org/10.1245/s10434-018-6691-3

This manuscript has the following accolades:

- Outstanding Research Accomplishments and Discoveries. 2019 Annual Report for the University of Ottawa.
- Best Abstract. Canadian Society of Surgical Oncology 24th Annual Meeting. 2018.
- Best Basic/Translational Paper. 70th Society of Surgical Oncology Meeting. 2017.

Author contributions for this work are as follows:

Angka L: Performed the cytotoxicity assays, processed the majority of the patient samples, and designed the flow cytometry panels. Analysed all of the data and created the figures. Wrote the Introduction, Methods, and Results. Worked with Auer RC on the discussion.

Martel AB: Gathered the clinical and demographic data. Presented this work at the SSO meetings. Assisted in editing and proofing.

Kilgour MK: Performed the majority of the NK characterization experiments by flow cytometry.

Jeong A: Performed a minority of the NK characterization experiments by flow cytometry and coordinated patient enrolment as a clinical research assistant.

Sadiq M: Clinical research assistant who coordinated patient enrolment. Wrote the clinical study protocol with Baker L and Auer RC.

Souza CT: Assisted in the NK-Vue^{TM} protocol development, sample processing and organization, in addition to reagent ordering.

Baker L: Assisted in writing the clinical study protocol with Sadiq M and Auer RC.

Kennedy MA: Performed the majority of the NK-Vue^{TM} ELISA experiments. Assisted in the interpretation of results, in addition to editing and proofing.

Kekre N: Assisted in the interpretation of results, in addition to editing and proofing.

Auer RC: Conceived of the study, wrote the clinical study protocol, provided guidance and oversight throughout the study.

The manuscript has been reformatted for the purpose of thesis.

The title page and permissions of use for this work can be found in Appendix B.

2.1 TITLE PAGE

Natural Killer Cell IFN-γ Secretion is Profoundly Suppressed Following Colorectal Cancer Surgery

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2.2 ABSTRACT

Background: Surgical stress results in a significant reduction in natural killer (NK) cell cytotoxicity (NKC), which has been linked to postoperative cancer metastases. However, few studies have measured the impact of surgical stress upon NK cell IFN-γ secretion (NKA), a cytokine with essential roles in controlling infection and metastases. The objective of this study was to investigate the impact of surgical stress on NKA in colorectal cancer (CRC) surgery patients.

Methods: Peripheral blood was collected from CRC surgery patients (n = 42) preoperatively and on postoperative day (POD) 1, 3, 5, 28, and 56. Healthy donor blood (n = 27) was collected for controls. We assessed NKA by production of IFN-γ following whole blood cytokine stimulation, NKC by ^{51}Cr-release assay, and immune cell profiling by flow cytometry.

Results: The mean reduction in NKA on POD1 compared with baseline was 83.1% (standard deviation 25.2%; confidence interval 75–91), and therefore the study met the primary endpoint of demonstrating a > 75% decrease in a cohort of CRC surgery patients ($p < 0.0001$). The profound and universal suppression of NKA persisted with 65.5% (19/29) and 33.3% (4/12) of patients with levels measuring < 75% of baseline on POD28 and POD56 respectively. The NKC was significantly reduced on POD1, but the degree was less pronounced (24.6%, $p = 0.0024$). Immune cell profiling did not reveal differences in the absolute number of NK cells ($CD3^{-}CD56^{+}$) or the ratio of $CD56^{dim}$-to-$CD56^{bright}$ subsets.

Conclusions: NKA is significantly suppressed for up to two months following surgery in CRC patients, a degree of surgery-induced immunosuppression far worse than previously reported.

2.3 INTRODUCTION

Natural killer (NK) cells are cytotoxic lymphocytes of the innate immune system. Immunosurveillance of malignant and infected cells by NK cells results in direct cytotoxicity and the production of immune stimulating cytokines. NK cells play a central role in the control of cancer metastases [44]. Clinical studies have linked intratumoral NK cell infiltration [59,60], as well as peripheral blood NK cell effector functions with cancer prognosis and incidence [56,134–136]. Transient impairment in NK cell cytotoxicity (NKC) following surgery has been linked to cancer recurrence and metastases in both animal models [137–139] and human studies [15,140,141]. Several clinical studies have been designed to attenuate the detrimental effects of surgical stress on NK cells with the goal of improving prognosis [15,130,140–143].

Beyond NKC, cytokine secretion represents an additional NK cell effector function critically involved in the antitumour immune response. The two main subsets of human NK cells can be distinguished based on expression of CD56 and CD16. While cytotoxic activity in the resting state is primarily assigned to $CD56^{dim}/CD16^{+}$ NK cells, the stimulated production of cytokines, in particular interferon-gamma (IFN-γ) [144], is attributed to both the $CD56^{dim}/CD16^{+}$ and $CD56^{bright}/CD16^{-}$ populations. Moreover, NK-secreted IFN-γ is responsible for shaping the adaptive immune response [145], and correlated with tumour stage and cancer prognosis [135,146–148]. This suggests that measurement of IFN-γ may provide a more comprehensive overview of NK cell activity (NKA) [149].

The present study is the first to investigate the effects of major surgery on NK cell IFN-γ secretion (or "NKA") in cancer patients. To measure NKA, we used an assay, which has been shown specifically to stimulate NK cell-IFN-γ production from whole blood [147]. We report that NKA is universally and profoundly suppressed in the postoperative period, with a greater magnitude and duration compared with NKC. This alternative assay of NK cell

dysfunction has important implications for understanding how the immunosuppressive effects of surgery impact the development of subsequent metastases, which will aid in the design of perioperative immunotherapies.

2.4 METHODS

Patient Characteristics and Clinical Protocol

This single center, prospective, translational study, approved by the Ottawa Health Science Research Ethics Board (20160012-01H), was conducted between August 2016 and June 2017. Eligible patients were > 40 years of age and had a histologically confirmed diagnosis of primary colorectal cancer and a planned surgical resection of the primary tumour (CRC cohort) or healthy donors who volunteered to participate (HD cohort). Exclusion criteria included a history of active viral or bacterial infection or known HIV or Hepatitis B or C, autoimmune diseases, use of immunosuppressive medications, or prior anticancer treatments. All subjects provided written, informed consent.

The primary objective was to compare the reduction in NKA on postoperative day (POD) 1 compared with preoperative (baseline) in the CRC cohort. The secondary objective was to compare NKA at baseline in the CRC cohort to the HD cohort. Exploratory studies included measurement of NKA secretion on POD3, POD5 (optional and only if patient still hospitalized), POD28, and POD56, comparing NKA and NKC across different CRC AJCC cancer stages.

Blood Processing

Patient and HD blood was drawn into BD Vacutainer Sodium-Heparin coated tubes (~ 20 mL/blood draw). One milliliter of whole blood was aliquoted into vacutainer tubes containing Promoca™ and from the remaining whole blood sample peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density centrifugation before cryopreservation.

Immune Monitoring

NKA Assay

NK-secreted IFN-γ levels were measured by ELISA following stimulation of 1 ml of whole blood, as described by the manufacturer (ATGen, NK-Vue™) and in prior publications, with the exception that blood was not drawn directly into tubes containing the proprietary stimulating cytokine cocktail (Promoca™) [150]. This may result in slight differences in absolute NKA compared with prior studies (ATGen, NK-Vue™). All plasma samples were stored at − 80 °C. The upper and lower limits of detection of the assay were 4000 pg/mL and 15.6 pg/mL respectively, and values exceeding these were assigned the cutoff values.

NKC Assay

Cytotoxicity was measured using the ^{51}Cr-release assay as previously described [139]. Briefly, K562 target cells were labelled with 51Cr before co-incubation with PBMCs at increasing concentrations (PBMC:Targets = 10:1, 50:1, and 100:1) in triplicate from cryopreserved samples. Following incubation, supernatants were collected and 51Cr was determined by a gamma counter.

NK Cell Immunophenotyping

Cryopreserved PBMCs (106) were thawed and stained with BV510 Fixable viability dye, CD3, CD56, CD16, and CD14 in Brilliant Stain Buffer (BD). Samples were run on the BD FACSCelesta and analysed with FlowJo v10.

Statistical Analysis

The primary endpoint was to determine if NKA is significantly decreased on POD1 compared with baseline in the CRC surgery cohort. The secondary endpoint was to determine if there is a significant reduction in NKA in the baseline CRC patients compared with HD. Based on our previous studies of postoperative suppression of NKC, we

anticipated > 75% reduction in NKA from baseline [35]. Given the expected mean for CRC patients (mean = 263.6, standard deviation [SD] = 349), we have an 80% power to detect this difference using a two-sided paired Wilcoxon rank-sum test ($\alpha = 0.05$) with a sample size of 40 CRC patients (measured at baseline and POD1) [147]. For the secondary endpoint, we have > 90% power to detect a difference between the CRC patient cohort (baseline) and the HD cohort using a two-sided Mann–Whitney test ($\alpha = 0.05$), with a sample size of 20 HD. Demographic data was summarized using the median values and 95% confidence interval (CI). Unpaired, nonparametric Mann–Whitney U tests were performed when comparing two groups and paired Wilcoxon rank-sum was used to compare two paired samples (i.e., different timepoints or different assays at a single time point). Multiple comparisons were tested with nonparametric Kruskal–Wallis tests. P values < 0.05 were considered significant.

2.5 RESULTS

Demographic Data

The demographic data for 27 HD and 42 CRC surgery patients is summarized in Table 1. There was no significant difference in the median age between the HD and CRC groups. A similar number of patients undergoing open (n = 20) or laparoscopic (n = 22) surgery were included, with an even distribution between Stage I, II, and III patients (33.3, 26.2, and 28.6% respectively). Five (11.9%) patients were Stage IV undergoing combined resection of primary and metastatic tumours.

Scheduled preoperative and postoperative blood draws were collected when possible. The optional POD5 blood draw was collected for only 12 due to earlier discharge. The POD56 collection was added as an amendment following initial results, demonstrating a persistent decrease in NKA at POD28, resulting in a sample size of 12.

NKA is Decreased in CRC Patients

In agreement with previously reported findings, CRC patients had a significantly lower NKA (median: 299.5 pg/mL; CI: 159–391) compared with HD (median: 966 pg/mL; CI: 398–2027; $p = 0.0006$; Fig. 1) [147]. Notably, NKA was decreased with advancing age and was significantly reduced in patients > 70 years old (median: 111 pg/mL, CI: 39–629) compared with patients < 60 years old (median: 391 pg/mL, CI: 337–2575; $p = 0.04$; Supplemental Figure 1).

Table 2.1. Demographic data

Category	Subcategory	Healthy donors	Patients	Postop complications
Total (*n*)		27	42	9/42
Blood draws	Baseline (*n*)	27	42	–
	POD1 (*n*)	–	41	–
	POD3 (*n*)	–	37	–
	POD5 (*n*)	–	12	–
	POD28 (*n*)	–	29	–
	POD56 (*n*)	–	12	–
Gender	Male (*n*)	16	22	7/22
	Female (*n*)	11	20	2/20
Patient age (median years; *95% CI*)		63; *58–67*	65; *60–72*	–
	<60 (*n*)	10	13	3/13
	60–69 (*n*)	13	11	2/11
	>70 (*n*)	4	18	4/18
Stage	I (*n*)	–	14	3/14
	II (*n*)	–	11	1/11
	III (*n*)	–	12	5/12
	IV (*n*)	–	5	0/5
Type of surgery	Open (*n*)	–	20	6/20
	Laparoscopic (*n*)	–	22	3/22

Italic values indicate the 95% Confidence Intervals (CI)

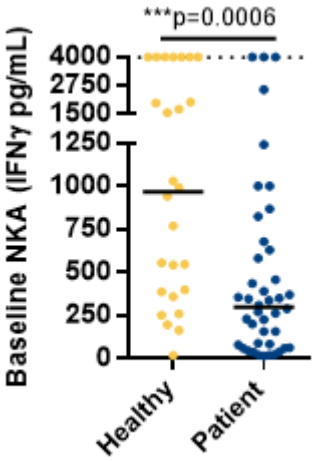


Figure 2.1. NK cell IFN-γ secretion (NKA) is reduced in CRC patients.

Figure 2.1. NK cell IFN-γ secretion (NKA) is reduced in CRC patients.

NKA from healthy donors (n = 27) and CRC patients (n = 42) before surgery (baseline) was assessed following a 24 h stimulation with Promoca™ cytokine cocktail. Upper limit of detection for the NK Vue™ assay is 4,000 pg/mL. Median indicated by solid line. Mann–Whitney U test.

NKA is Impaired After Surgery

We measured NKA on POD1, 3, 5, 28, and 56 in our CRC patient population (Fig. 2). Astoundingly, 90.2% (37/41) had IFN-γ levels below detectable levels (15.6 pg/mL) on POD1 with the highest value being only 53 pg/mL. The mean reduction in NKA on POD1 compared with baseline was 83.1% (SD 25.2%, CI: 75–91), and therefore the study met the primary endpoint of demonstrating a > 75% decrease in a cohort of CRC surgery patients ($p < 0.0001$). The profound and universal suppression of NKA persisted with 65.5% (19/29) and 33.3% (4/12) maintaining levels reduced > 25% from baseline on POD28 and POD56 respectively.

While no significant difference in NKA based on cancer stage was observed at baseline ($p = 0.6$; Supplemental Figure 2a), the impact of the pathological stage was noticeable in postoperative recovery of NKA (Supplemental Figure 2b). On POD28, patients with no systemic or regional metastases (Stage I and II) had a significantly lower median suppression from baseline (36.3%; CI: − 5.4–70) compared with Stage III and IV patients (85.14%; CI: − 13.15–93.22; $p = 0.035$). Examining the impact of open versus laparoscopic surgery on NKA did not reveal any effect of invasiveness of the surgical procedure on the degree of NKA suppression or recovery (Supplemental Figure 3). Lastly, there was a lower baseline and POD28 NKA (although not statistically significant) observed in patients who had a postoperative complication ($n = 9$ complications vs. $n = 32$ no complications; Supplemental Figure 4).

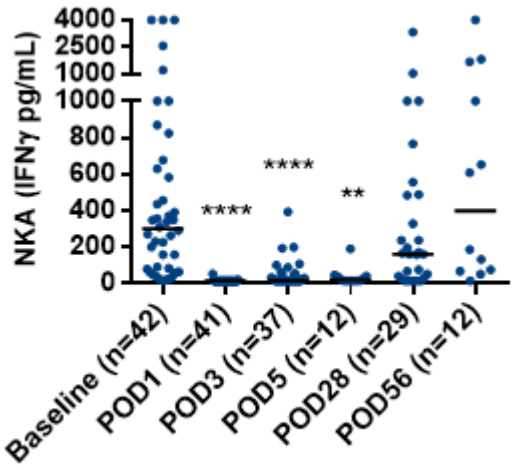


Figure 2.2. NK cell IFN-γ secretion (NKA) is reduced following surgery.

Figure 2.2. NK cell IFN-γ secretion (NKA) is reduced following surgery.

NKA measured in CRC surgery patients on the indicated post-operative days (POD). Solid line indicating the median; Kruskal–Wallis tests (**$p = 0.0035$ and ****$p < 0.0001$).

NKC is Suppressed to a Lesser Degree Postoperatively

Next, we compared NKA with NKC, using the ^{51}Cr-release assay, in a subset of HD (n = 12) and CRC patients (n = 13) at baseline and POD1 (Fig. 3a). The median NKC at an E:T ratio of 100:1 was 30% (CI: 24.2–32.3) for HD, 41% (CI: 16.28–43.34) for CRC patients at baseline, and 26% (CI: 12.2–42.2) on POD1. Similar to our results with NKA, NKC was significantly reduced from baseline following surgical stress ($p = 0.0024$), but the magnitude of suppression was not as profound (24.6% suppression; range: 50.2–104.3%).

Impaired NKA is Not Due to Changes in NK Cell Number or Distribution

We examined the impact of surgery on the absolute number of circulating NK cells by flow cytometry in 23 CRC patients as well as in 15 HD (Fig. 3b; Supplemental Figure 5a). There was a modest but nonsignificant reduction in the number of NK cells/mL ($CD3^{-}CD56^{+}$) compared with the HD group (median 0.268×10^6 cells/mL to 0.175×10^6 cells/mL; $p = 0.2$). Furthermore, there was no significant reduction in the number of $CD56^{bright}$ or $CD56^{dim}$ NK cell subsets after surgery (Table 2). Previous studies of cancer patients observed a significant correlation between the ratio of $CD56^{dim}$-to-$CD56^{bright}$ NK cells and NK function [147,151]. In the present study, the relative ratio of these populations remained constant following surgery (Supplemental Figure 5b).

Lastly, we detected a significant increase in the number of $CD14^{+}$ monocytes on POD1 (Table 2; Fig. 3c; $p = 0.0006$) and these were predominantly classical and intermediate monocytes (Supplemental Figure 5c), consistent with an immediate release of immature monocytes from the bone marrow during acute injury [152,153].

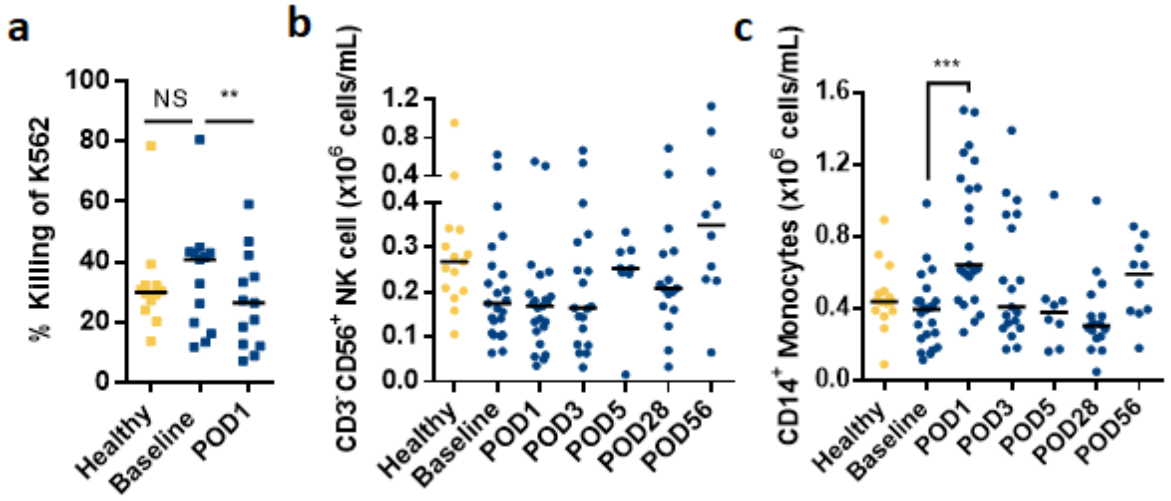


Figure 2.3. NK cell cytotoxicity but not cell number is reduced following surgery.

Figure 2.3. NK cell cytotoxicity but not cell number is reduced following surgery.

a NK cell cytotoxicity in healthy donors (n = 12) and CRC patients before and after surgery (n = 13). Paired Wilcoxon test (Baseline vs. POD1). Unpaired Mann–Whitney test (Healthy vs. Baseline). **b** Circulating NK cells ($CD3^{-}CD56^{+}$) and **(c)** monocytes ($CD14^{+}$) in healthy donors (n = 15) and CRC surgery patients (n = 16) at multiple time points following surgery; Kruskal–Wallis tests.

Table 2.2. Median NK cell IFN-γ secretion, NK cell cytotoxicity, and immune cell profiling data.

Variable	HD	Baseline	POD1	POD3	POD5	POD28	POD56
NK cell IFNγ secretion (*n*)	**27**	**42**	**41**	**37**	**12**	**29**	**12**
IFNγ pg/mL	966	299.5	15.6	15.6	19.5	161	398
95% CI	*398–2027*	*159–391*	*15.6–15.6*	*15.6–26*	*13–42*	*42–329*	*68–1703*
NK cell cytotoxicity (*n*)	**12**	**13**	**13**				
% Killing of K562 Target Cells	29.95	40.76	26.44				
95% CI	*24.2–32.3*	*16.3–43.3*	*12.2–42.2*				
Immune cell profiling (*n*)	**15**	**23**	**23**	**19**	**8**	**16**	**10**
$CD3^{-}CD56^{+}$ NK cell ($\times 10^6$ cells/mL)	0.268	0.175	0.169	0.165	0.2535	0.2085	0.3505
95% CI	*0.2–0.34*	*0.14–0.24*	*0.12–0.19*	*0.08–0.31*	*0.02–0.34*	*0.16–0.29*	*0.23–0.86*
$CD56^{dim}$ NK cell ($\times 10^6$ cells/mL)	0.249	0.144	0.131	0.145	0.213	0.1645	0.304
95% CI	*0.18–0.28*	*0.10–0.21*	*0.1–0.17*	*0.06–0.21*	*0.01–0.25*	*0.11–0.27*	*0.1–0.43*
$CD56^{bright}$ NK cell ($\times 10^6$ cells/mL)	0.01	0.011	0.007	0.009	0.017	0.0135	0.012
95% CI	*0.007–0.015*	*0.006–0.013*	*0.004–0.009*	*0.005–0.015*	*0.001–0.033*	*0.004–0.019*	*0.007–0.023*
$CD14^{+}$ Monocyte ($\times 10^6$ cells/mL)	0.438	0.396	0.643	0.41	0.3795	0.3045	0.591
95% CI	*0.39–0.5*	*0.26–0.44*	*0.58–1.07*	*0.29–0.92*	*0.16–1.03*	*0.24–0.48*	*0.37–0.81*

Median values, *italicized 95% Confidence Intervals (CI) of the median*

Sample size (*n*) is bolded

2.6 DISCUSSION

In the present study, we demonstrated that NKA is severely suppressed following cancer surgery, with more than 90% of patients below the limit of detection on POD1 (Fig. 2). The effect was present in every patient, regardless of gender, age, cancer stage or type of surgery. The duration of suppression also was unexpectedly present until POD28 in 65.5% of patients. The prolonged suppression suggests a degree of immunoparalysis that has implications for the development of postoperative infections and cancer recurrence.

The impaired immune response to bacterial pathogens following surgery is well documented, and although the role of NK cells is less clear, studies have shown that IFN-γ secretion by PBMCs in response to *Staphylococcus aureus* is reduced following severe injury [154,155] due to impairment of $CD56^{bright}$ NK cells [67]. Coordinated immune responses against bacterial infection requires the secretion of proinflammatory cytokines (e.g., IL-12, TNF-α) by innate immune cells to stimulate NK cell IFN-γ secretion [156], which promotes an effective T cell response to several pathogens, providing strong evidence for the importance of NK-cell dysfunction in postoperative susceptibility to infections [157–159].

Impairment of NKA also may have implications for the development of postoperative metastases [130], although we are not aware of any studies exploring the effect of NKA on cancer outcomes. We and others have previously shown that surgical stress results in a significant impairment in NKC, leading to the formation of cancer metastases in animal models [35,63,64,139,160]. A clinical study demonstrated that postoperative autologous tumour cell killing is correlated with lung cancer survival [141]. T-cell dysfunction has been well described following surgical stress [161,162], and we have previously published that surgery can render a protective cancer vaccine completely ineffective in a murine model secondary to T-cell suppression [36]. Interestingly, Wirsdorfer et al. demonstrated that

surgery-induced impairment of NK-cell IFN-γ secretion was responsible for T-cell suppression, using OVA-specific T cells [163]. This provides a provocative hypothesis for the effects of surgery on T-cell mediated antitumor immunity and has implications for perioperative vaccination and immunotherapy strategies.

A second objective of the study was to compare NKA in a cohort of CRC patients to HD of a similar age (Fig. 1). We confirmed that cancer patients have a significant defect in the IFN-γ secretory capacity as previously reported [135,147]. However, likely due to our small sample size, we did not observe a significant association between cancer stage and NKA at baseline. We did observe a significant reduction in NKA in patients > 70 years of age, which is consistent with the known effects of aging upon NK-cell function [164,165]. Because of its reproducibility and simplicity, this assay is currently under development as a screening tool for CRC detection [150], with preliminary results suggesting similar negative predictive value but higher sensitivity compared with fecal immunochemical tests [166]. NKA was measured from *in vitro* stimulated samples, which cannot be directly extrapolated to in vivo levels of serum IFN-γ as recently demonstrated by other groups [130,167].

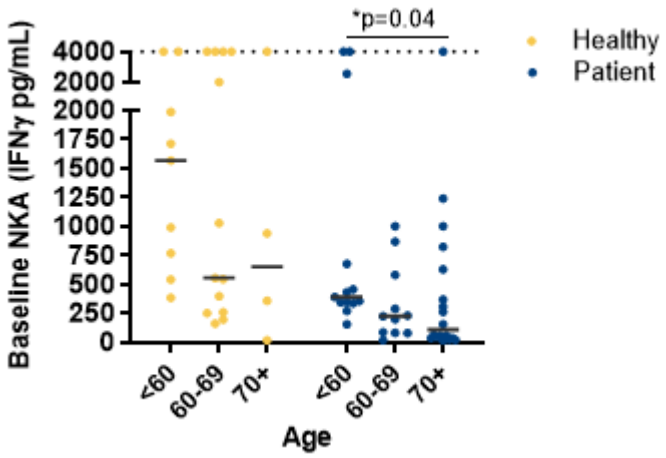
When comparing NKA to NKC, we observed three main differences. First, no significant decrease in baseline NKC of CRC patients compared with HD was detected. This is in contrast to other studies [168–170] and may be explained by the preponderance (25/42 patients, 60%) of early-stage patients in our study, which is consistent with findings that NKC is correlated with disease burden in cancer patients [135,146–148]. Second, the degree of NKC impairment on POD1 (Fig. 3a) was much less pronounced compared with NKA and was not universally present. This may be a result of using cryopreserved PBMCs to measure NKC, because cryopreservation may have unintended effects on NK-cell function [167]. Finally, suppression of NKA was still present in the majority of patients at POD28, a time when we have previously reported that NKC is normalized to preoperative levels [65,139].

One possible explanation is that with NKC, NK cells are stimulated with K562 cells, which is known to measure only the activity of $CD56^{dim}$ [171], whereas with NKA, stimulation is with cytokines, which measures function of both the $CD56^{bright}$ and $CD56^{dim}$ NK subsets [172].

To explore this further, we assessed the numbers and subsets of circulating NK cells by flow cytometry. We report that there were no significant changes in the absolute number of NK cells (Fig. 3b) or ratio of $CD56^{dim}$-to-$CD56^{bright}$ NK cells, which confirms that, as expected, NK cells are present but dysfunctional following surgery. Koo et al. reported that NKA was inversely correlated with the $CD56^{dim}$-to-$CD56^{bright}$ ratio in prostate cancer patients [135]. Unfortunately, we were unable to explore such a correlation in the postoperative period, because the NKA was below assay detection in the majority of patients following surgery. Interestingly, there was a significant increase in the number of $CD14^{+}$ monocytes on POD1 compared with baseline (Fig. 3c). Our group and others have shown that there is a large increase in myeloid-derived suppressor cells (MDSCs) following surgery in humans and in mice [36,115,173]. We suspect that the large increase in $CD14^{+}$ monocytic cells is likely the expansion of surgery-induced MDSCs; however, we did not assess suppressive function in this study.

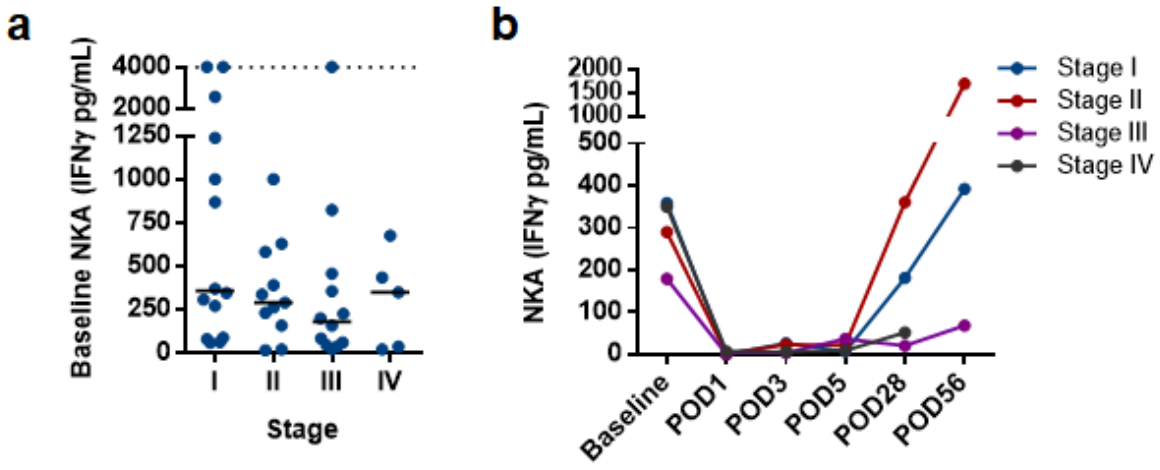
NK cells are a main source of IFN-γ during the initial stages of an innate immune response and are central to the development of an adaptive immune response [174]. Thus, postoperative suppression of NKA is potentially a major contributor to postoperative cancer recurrence, the early formation of micrometastases, and, more broadly, postoperative infectious complications. We hypothesize that perioperative therapies positioned to prevent or minimize postoperative NK cell dysfunction may improve postoperative cancer outcomes. Currently, we have two ongoing clinical trials of perioperative immunotherapy, which include NKA as a correlative endpoint [143,175]

2.7 SUPPLEMENTARY FIGURES



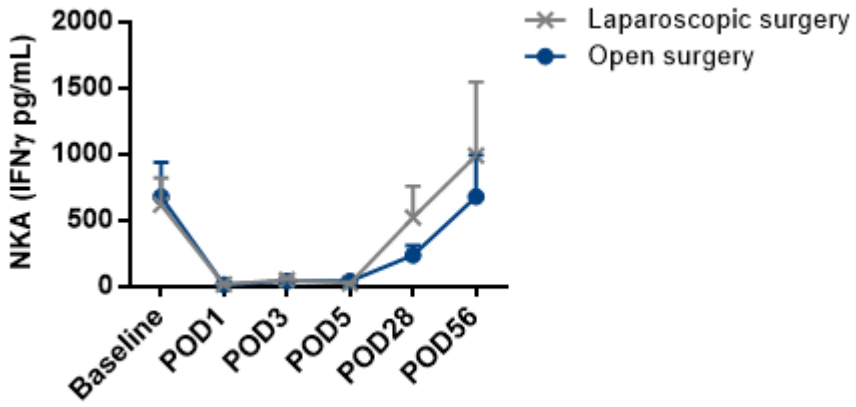
Supplemental Figure 2.1. The effect of age on NKA

NKA in healthy donors and CRC patients at baseline; Kruskal-Wallis tests. Median indicated by solid line.



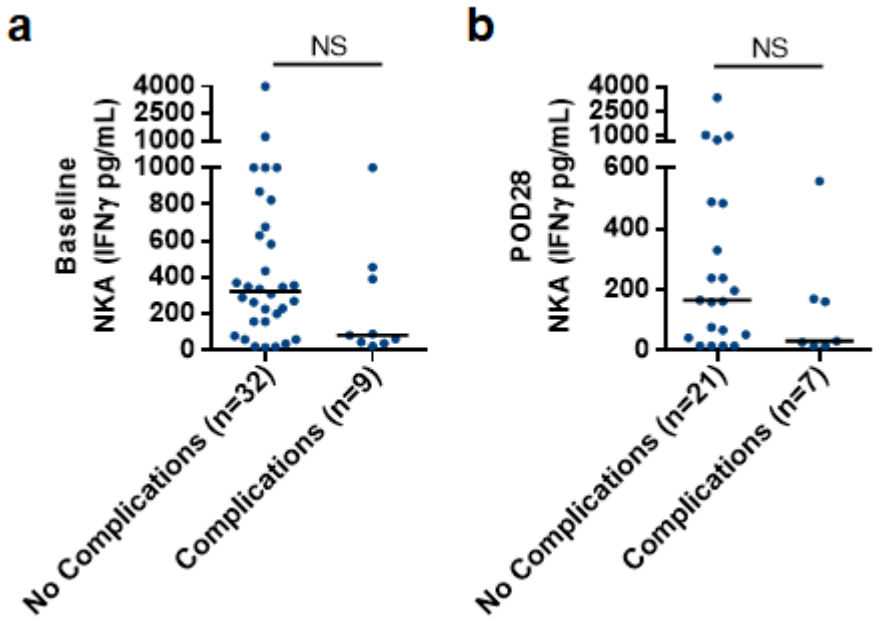
Supplemental Figure 2.2. The effect of cancer stage on NKA

(a) NKA at baseline; Kruskal-Wallis tests. Median indicated by solid line**. (b)** Median NKA of patients categorized by Stage at Baseline, POD1, 3, 5, 28, and 56.



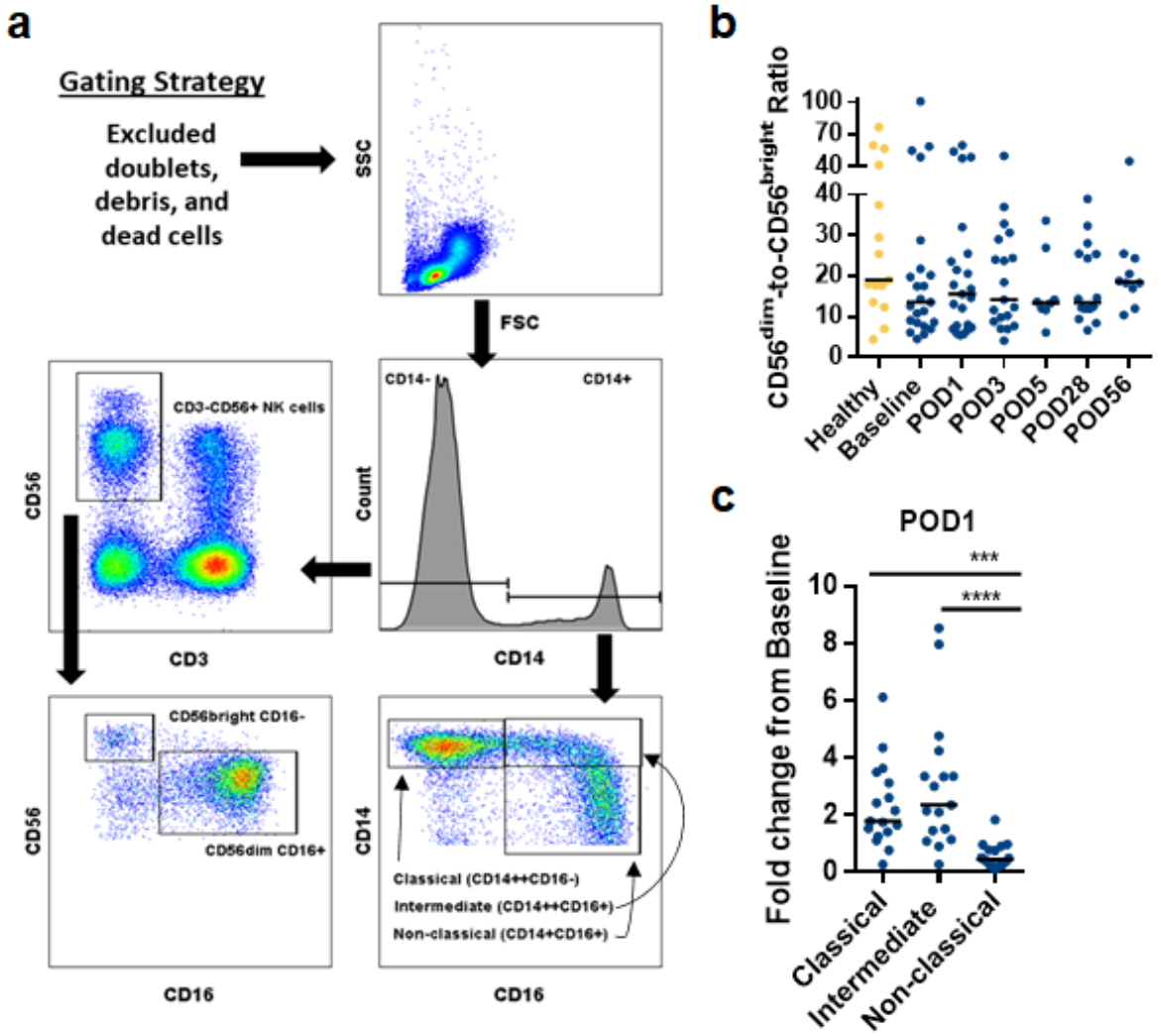
Supplemental Figure 2.3. Comparison of NKA between patients undergoing open vs laparoscopic surgery.

NKA of patients did not differ significantly between groups at Baseline (p=0.857), POD1 (p=0.54), POD3 (p=0.495), POD5 (p>0.99), POD28 (p=0.674), and POD56 (p=0.71) using multiple Mann-Whitney U tests. On POD28, 7/14 patients in the laparoscopic group (n=22) and 12/15 patients in the open group (n=20) remained suppressed. On POD56, 2/7 patients in the laparoscopic group and 2/5 patients in the open group remained suppressed. Mean and SEM (error bars) indicated.



Supplemental Figure 2.4. Baseline and POD28 NKA of patients grouped by development of postoperative complications.

Patients who develop a postoperative complication (n=9) had a slightly lower NKA as compared to those patients who did not develop a postoperative complication at baseline (323pg/mL vs 84pg/mL; p=0.2) and when measured at POD28 (165pg/mL vs 31pg/mL; p=0.2), but the difference did not reach statistical significance. Mann Whitney U test. Median indicated by solid line.



Supplemental Figure 2.5. Immune cell profiling.

(a) A representative gating strategy for identifying NK cells ($CD3^{-}CD56^{+}$), $CD56^{bright}$ ($CD56^{bright}$ $CD16^{-}$), $CD56^{dim}$ ($CD56^{dim}$ $CD16^{+}$), and monocyte ($CD14^{+}$) subpopulations (classical, intermediate, and non-classical). **(b)** Ratio of $CD56^{dim}$-to-$CD56^{bright}$ NK cells in healthy donors and in CRC patients at indicated timepoint. **(c)** The fold change of classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and non-classical ($CD14^{+}CD16^{+}$) monocytes on POD1 compared to baseline. Kruskal-Wallis tests (***p=0.0002, ****p<0.0001).

Chapter 3

Surgery-Induced Myeloid Derived Suppressor Cells in Cancer Patients

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3.0 PREFACE

This chapter has not yet been submitted due to delays in experiments because of COVID-19.

Angka L, Cook D, Tennakoon G, Martel AB, Market M, Ng J, Souza CT, Cummins E, Kekre N, Vanderhyden B, Kennedy MA, and Auer RC. Characterizing Surgery-Induced Myeloid Derived Suppressor Cells in Cancer Patients. Unpublished 2020.

Author contributions for this work are as follows:

Angka L: Performed all experiments with the exception of scRNA-Seq and some PI3K-γ inhibitor co-cultures. Wrote the manuscript.

Cook D: Performed the scRNA-seq analysis with input given by LA MK and RCA. Wrote the majority of the methods for scRNA-seq, edited the results and discussion points for scientific accuracy, and provided expertise in all things scRNA-seq/gene expression related.

Tennakoon G: Performed the phospho-flow cytometry experiments and some PI3K-γ inhibitor co-cultures.

Martel AB: Assisted in MDSC characterization experiments and obtaining clinical demographic data.

Market M: Assisted in MDSC characterization experiments and phosphoflow.

Ng J: Clinical research coordinator for patient enrolment and clinical demographic data.

Souza CT: Assisted in protocol development, reagent acquisition and blood sample processing/storage.

Cummins E: Collaborated on project and provided materials for the compound screen

Kekre N: Provided insight and expertise in analysing blood smears and MDSC characterization throughout the project.

Vanderhyden B: Provided insight and expertise in RNA-seq analysis.

Kennedy MA: Assisted with all of the protocol development, project management, and interpretation of data analysis. Edited and proofed the manuscript.

Auer RC: Oversaw the entire project. Edited and proofed the manuscript.

3.1 TITLE PAGE

Characterizing Surgery-Induced Myeloid Derived Suppressor Cells in Cancer Patients.

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Disclosures

adMare Bioinnovations provided the materials for the small molecule compound screen.

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3.2 ABSTRACT

Myeloid derived suppressor cells (MDSCs) have a dominating presence in the postoperative period and mediate the suppression of the immune system after surgery. However, their functional characteristics and effect on cellular immunity after surgery have not been comprehensively investigated. Here, we characterized the expansion of surgery-induced (Sx) MDSCs via multi-colour flow cytometry, single cell RNA-sequencing, and functional *ex vivo* Natural Killer (NK) cell suppression assays. Furthermore, we screened 147 small molecules in our Sx-MDSC:NK cell suppression assay to identify pathways that could be targeted to antagonize Sx-MDSCs. From this screen, PI3K inhibitors were identified to reduce MDSC mediated NK cell suppression. Lastly, inhibiting the specific PI3K-γ isoform proved more potent than pan-PI3K inhibition, revealing a potential pathway amenable to therapeutic targeting.

3.3 INTRODUCTION

The pronounced postoperative immune suppression that each cancer surgery patient is confronted with can determine their quality of life during recovery, susceptibility to infections, and likelihood of metastatic relapse [12]. In sterile surgical environments, the disruption of endothelial cell barriers results in the release of damage associated molecular patterns (DAMPs) or "alarmins", which serve to alert and mobilize immune cells to the wound site to remove damaged cells and protect from foreign pathogens [19,30]. This inflammation is perpetuated further by the recruitment of monocytes from bone marrow reserves which release cytokines such as IL-1, IL-6 and TNF-α. To compensate for the large egress of cells towards the site of trauma, emergency myelopoiesis ensues which results in the persistent release of immature myeloid cells [31].

While monocytes and neutrophils are the main players in establishing the initial proinflammatory response, they are also critical for its resolution [30]. In particular, monocytes have been shown to undergo phenotypic reprogramming into anti-inflammatory and immunosuppressive-like cells with altered cytokine profiles (IL-10, TGF-β secretion) and reduced antigen presentation (downregulation of HLA-DR). Large perturbations in myeloid populations following surgery have been observed including the expansion and persistence of a diaspora of immature myeloid cells that phenotypically resemble myeloid derived suppressor cells (MDSCs) and craft a state of immunosuppression [32,34,115,173]. It is thought that the magnitude of this immunosuppressive response in the immediate postoperative period can predict the development of cancer metastases, which has yet to be targeted perioperatively [176,177].

In order to alleviate postoperative immune suppression by MDSCs, we must first characterize the cells involved. In cancer surgery patients, a preoperative population of

MDSCs exists with direct ties to the primary tumour [109,178,179]. Generally, MDSCs are described as being either polymorphonuclear (PMN-), monocytic (M-), or early stage (E-) MDSCs [98]. The phenotypic markers used to define these subtypes are not mutually exclusive which has complicated the reporting of these cell types by various groups [180]. Nevertheless, the defining feature of an MDSC is their ability to suppress immune effector cells, whether they be Natural Killer (NK) cells or T cells [179,180].

In this study multicolour flow cytometry and single cell RNA sequencing (scRNA-seq) were used to phenotypically describe the MDSCs that accumulate following surgery. Given the importance of NK cell antitumor activity in the postoperative period, we focused on defining MDSCs based on their ability to suppress NK cell cytotoxicity. Furthermore, we screened a library of 147 small molecules to identify potential therapies to antagonize postoperative MDSCs. This led us to understand the signalling pathways governing their suppressive machinery. In the end, we have shown that "surgery-induced" (Sx-) MDSCs expand and are amenable to therapeutic targeting.

3.4 MATERIALS AND METHODS

Study Design

The objective of this study was to characterize human Sx-MDSCs for the purpose of identifying a pathway amenable to therapeutic targeting. We first used multicolour flow cytometry to phenotypically characterize the myeloid cells from PBMCs or ACK lysed whole blood from patients (n=32) enrolled under the Perioperative Human Blood and Tissue Specimen Collection Program (PHBSP; OHSN-REB# 2011884-01H). To be enrolled, participants had to be over the age of 18, undergoing a surgical procedure for their cancer treatment, and be able to sign an informed consent form. We only enrolled patients who were not previously treated with chemotherapy, radiation, or immunotherapy, and those that

did not receive blood transfusions during surgery. The patient samples collected under this study protocol were used in all experiments other than the scRNA-seq (Table 1).

To control for interassay variability, antibodies were titrated and stained following a standard flow cytometry staining protocol using previously determined gates and voltages. Voltages for excitation/emission spectra were routinely assessed to ensure that MFI values could be compared between baseline and POD1, and across different patient samples on different days of experimentation. Lastly, for flow cytometric immunophenotyping, a core panel of antibodies was used to phenotype MDSCs with the capacity to expand the panel to include optional exploratory markers. Patient samples were also used in multiple experiments to ensure the ethical and efficient use of patient materials.

We also performed scRNA-seq from six cryopreserved peripheral blood mononuclear cells (PBMCs) from abdominal cancer surgery patients (Supplemental Table 1) who were enrolled in the PERIOP-02 clinical trial (NCT02987296; OHSN-REB# 20160732-01H). These patients were instructed to take a supplement enriched in arginine (n=3) or an isocaloric/isonitrogenous control supplement (n=3) TID for 5 days as part of the trial. To account for the potential impact of the differences in treatments, we performed a separate analysis comparing the transcriptional changes between groups and did not find any effect attributable to the supplement given (fig. S1). Therefore, we opted to combine all six patients in our final analysis. The patient samples selected for the scRNA-seq had similar demographic, procedural and surgical outcome data (Table S1).

Table 3.1. – Patient Demographics for Flow Cytometry

Category	Subcategory	Cancer patients (n=32)	Healthy (n=6)
Sex	Male (n)	23	4
	Female (n)	9	2
Age - median; range		65; 36-81	30; 28-42
	< 50 years (n)	2	6
	50-69 years (n)	19	0
	> 70 years (n)	11	0
Cancer Type - n	GI	9	N/A
	GU	8	
	Prostate*	8	
	Lung	6	
	Sarcoma	1	
Cancer Stage - n (%)	Stage 0	4	N/A
	Stage 1	6	
	Stage 2	8	
	Stage 3	12	
	Stage 4	2	
Length of Operation – median; range		3.25; 2-10	N/A

GI – Gastrointestinal (upper and lower GI, & Pancreatic cancer)
GU – Genitourinary (kidney, bladder, ureteral, Prostate uterine/endometrial)

Human Blood Sample Collection and Processing

Patient (Baseline and POD1) and healthy volunteer peripheral blood samples (20-40mL) were drawn at The Ottawa Hospital after receiving approved informed consent under the following clinical protocols: *i)* OHSN-REB# 20160732-01H and *ii)* OHSN-REB# 2011884-01H. Blood was drawn into BD Vacutainer Sodium-Heparin coated tubes and processed within 2 hours by Ficoll density centrifugation (GE Healthcare #17-1440-03).

Antibodies for Phenotyping by Flow Cytometry

The panel of MDSC markers were chosen based on published guidelines to harmonize human MDSC reporting led by the Association for Cancer Immunotherapy, Cancer Immunoguiding Program [98] - with slight modifications. Freshly isolated PBMCs were first labelled with a fixable viability stain (BV510; BD #564406) in PBS at room temperature for 10 minutes. Next, an extracellular MDSC antibody mastermix was added to the PBMCs and simultaneously stained with the viability dye for an additional 20 minutes at 4°C. The antibodies in the MDSC mastermix were used at individually titrated dilutions and included: CD33 clone P67.6 (Pe-Cy7, Biolegend #366617), CD14 clone MθP9 (APC-Cy7, BD #561709), CD11b clone ICRF44 (AF700, Novus #FAB1699N), HLA-DR clone L243 (APC, Biolegend #307609), CD15 clone MMA (efluor450, eBioscience #48-0158-41), CD124 clone 25463 (PE, R&D systems #FAB230P-025), and lineage markers CD3 clone UCHT1 (FITC, Biolegend #300440), CD56 clone NCAM16.2 (FITC, BD Biosciences #340410) and CD19 clone HIB19 (FITC, Biolegend #302205) [98]. Exploratory extracellular markers included LOX-1 clone 15C4 (PE, Biolegend #358603), VISTA clone 730804 (AF700, R&D systems #FAB71261N), and PD-L1 clone MIH1 (PE, eBioscience #12-5983-42). Following viability and extracellular staining, PBMCs were washed and resuspended in 1% PFA and acquired by flow cytometry within 48 hours. For intracellular staining, the BD Cytofix/Cytoperm™ kit (BD #554714) was used after viability and

extracellular staining and cells were incubated in Perm/Wash buffer with Arginase-1 (APC, R&D systems #IC5868A) for 30 minutes at 4°C. Samples were acquired on the BD Fortessa LSRII and analyzed with FlowJo v10.

Cell lines and Isolation of Sx-MDSC subsets and High density neutrophils

K562 and NK92-MI cell lines were purchased from ATCC and maintained in cRPMI. Bulk Sx-MDSCs (M-MDSCs and PMN-MDSCs) were sorted from freshly isolated PBMCs on POD1 by magnetic bead separation with CD33 microbeads (Miltenyi #130-045-501). To sort for PMN-MDSCs and M-MDSCs, PBMCs were first magnetically sorted with CD15 microbeads (Miltenyi #130-046-601) to obtain a $CD33^{lo}CD15^{+}$ PMN-MDSC fraction with a purity of 80-85%. The CD15 negative fraction was then incubated with CD33 microbeads (Miltenyi #130-045-501) resulting in a positive fraction that was $CD33^{+}CD15^{-}$ and mainly $CD14^{+}$ M-MDSCs (>95% purity) (fig. S2). High density neutrophils (HDNs) were isolated via double density centrifugation by overlaying 6mL whole blood on top of 3mL Histopaque 1077, which was first overlaid on top of 3mL Histopaque 1119. After centrifugation at 700g for 30min with brakes off, the PBMCs can be collected from the upper interface, followed by HDN collection of the bottom interface.

Giemsa- Wright Staining and Microscopy

Freshly isolated PBMCs were washed twice in MilliQ H_2O and resuspended in ~20µL of MilliQ H_2O. 5-10µL of PBMCs were pipetted onto a glass slide and gently smeared with the long edge of a 20uL pipette tip. Slides were fixed with 100% MeOH and air dried for 2 minutes. At this point, 0.5 to 1mL of undiluted stock Giemsa-Wright stain (Sigma #WG80-2.5L) was pipetted onto the slides and left for 2 minutes to stain. Afterwards, excess Giemsa-Wright stain was poured off and the slides were submerged into a diluted Giemsa-Wright stain (1:20, stain:MilliQ H_2O) for 5 minutes. Slides were then gently submerged into

MilliQ H_2O to rinse and left to air dry. Slides were viewed and imaged the same day (Nikon Te2000e, The Ottawa Hospital Research Institute).

MDSC:NK92 Suppression Assay

MDSC mediated NK cell suppression was measured by isolating MDSCs from surgery patients on POD1 and incubating them in cRPMI at increasing ratios with the IL-2 independent NK cell line, NK92-MI for 20 hours at 37°C. Following incubation, K562 target cells labelled with Cell Proliferation Dye efluor 450 (eBioscience #65-0842-90) were added to the MDSC:NK co-cultures for 4 hours. NK cell cytotoxicity (NKC) was then measured by adding Ethidium homodimer (EtHD, Invitrogen™ E1169) to each well just prior to acquiring the samples by flow cytometry. NKC was reported as %dead K562 (EtHD+ CP450+). To calculate %MDSC suppression, we used the following equation:

$$\left(1 - \frac{(\%\text{dead K562}_{\text{MDSC: NK}})}{(\%\text{dead K562}_{\text{NK alone}})}\right) \times 100\% = \%\text{ MDSC suppression}$$

Single cell RNA sequencing library preparation and sequencing

Cryopreserved and matched Baseline/POD1 PBMC patient samples (n=6) were used for single-cell RNA-sequencing (scRNA-seq) on the 10x Genomics Chromium Single Cell 3' platform (StemCore Laboratories, the Ottawa Hospital Research Institute). Baseline and POD1 samples were multiplexed into separate pools using MULTI-seq before being processed on two lanes of the Chromium platform superloaded to target a 20,000 cell yield per lane. Briefly, each sample was labelled for 10 minutes with 200nM of a unique DNA barcode hybridized to complementary lipid-modified DNA enabling anchoring to the cells' plasma membrane. Each sample was washed twice with PBS+1% BSA prior to pooling. Gene expression libraries were prepared following the standard 10x Genomics protocol and separate MULTI-seq barcode libraries were isolated by size selection and PCR amplified as described by McGinnis et al. (https://github.com/chris-mcginnis-ucsf/MULTI-seq).

Libraries were sequenced using NextSeq500 (Illumina) high-output 75-cycle runs. Gene expression libraries were sequenced to a depth of approximately 20,000 reads/cell and MULTI-seq barcode libraries were sequenced to a depth of approximately 1000 reads per cell, which is sufficient for sample annotation.

scRNA-seq Data processing and pipeline

Following sequencing, fastq files were generated using the Cell Ranger mkfastq tool (10x Genomics) and resulting fastq files. Gene expression libraries were processed using the Cell Ranger count function, aligning to the human transcriptome (GRCh38) with default parameters other than explicitly setting --expect-cells 20000. MULTI-seq barcode fastq files were processed using the deMULTIplex tool developed for MULTI-seq (https://github.com/chris-mcginnis-ucsf/MULTI-seq), providing a sample annotation for each cell barcode.

Gene expression libraries were imported into R and processed with Seurat [181]. Cells containing high proportions of mitochondrial reads were first removed before splitting the data into separate 12 Seurat objects representing each unique sample. Each sample was normalized using SCTransform prior to integration using Seurat's SCTransform integration pipeline [181,182]. The integrated data was then processed with principal component analysis (PCA) and clustered using the Louvain algorithm (resolution=0.15) on a neighbor graph derived from the first 30 principal components. Deriving cluster identities based on the integrated data ensured that clusters were not associated with variability of each patient or experimental groupings (ie. baseline/POD1). Each cluster was annotated based on expression of canonical markers of PBMC populations.

Following clustering, unintegrated data was re-processed using SCTransform, PCA, and UMAP embedding, providing a low-dimensional representation of the data capturing biological variability of interest while retaining cluster annotations that represent the

underlying cell type. We then used the R tool muscat to perform differential state analysis between POD1 and baseline samples within each cluster [183].

Signalling pathway activity inference

The R package PROGENy was used to infer the activity of 14 signalling pathways in each cell of the data [184]. PROGENy derives activity scores using a regression model trained on data from transcriptional profiles from hundreds of signalling perturbation experiments. After inferring activity scores, we tested for pathways with differential activity between cells from POD1 and baseline samples using a simple linear model.

Gene set enrichment analysis

The R package fgsea was used to perform gene set enrichment analysis on genes ranked by their average log fold change from the differential state analysis for individual cell types [185]. Lists of all GO terms, KEGG pathways, Reactome pathways, and Hallmark gene sets were acquired from the Molecular Signatures Database (MSigDB)[186,187]. All gene sets presented in the manuscript are significantly enriched (adjusted p-value < 0.05) and normalized enrichment scores (NES) are presented.

Identification of coordinated gene expression programs

To identify coordinated gene expression programs that represent continuous phenotypic gradients, we applied consensus non-negative matrix factorization (NMF) to normalized gene expression counts for the top 2000 variable genes (based on variance computed by SCTransform), as implemented in the NMF tool described by Kotliar et al.[188]. To identify the appropriate number of programs to identify (factors; k), we used NMF to perform 100 iterations of NMF for each k from 2-10. k=7 provided stable factorizations with low error and was used for downstream analysis (fig. S3). Consensus NMF then identifies a consensus factorization based on the 100 iterations for a given k. The “cell usage” and coefficient

matrices resulting from the factorization were then imported into R. As a gene's coefficient is ultimately dependent on its average expression level, we Z-score-transformed each gene's coefficients across the NMF programs and ranked genes by their Z-score value for each NMF program. To gain insight into which biological phenotypes may be associated with each NMF program, we calculated correlated cells' NMF program usages with gene set scores from MSigDB and publications of interest.

Gene set scoring and autocorrelation

Gene set scoring was performed using Vision [189]. Similar to complementary tools, Vision computes a score for query gene sets based on the average expression of each gene relative to background control gene sets. Vision also calculates an autocorrelation score (1-Geary's C) for each gene set, providing insight into whether gene set activity represents coordinated variation in latent space (kNN graph from 50-dimensional PCA space) or if the scores are randomly distributed.

Small Molecule Screen for MDSC Antagonists

Our MDSC antagonists screens were separated into 2 screens (Screen #1 and Screen #2) which were each performed twice. Screen #1 included 147 unique small molecule compounds plated in singlets at 1μM (Supplemental Table 2). Screen #1 was created in collaboration with adMare Bioinnovations (British Columbia, Canada). The library was plated in 96 v-bottom plates and shipped on dry ice via priority courier to the OHRI where the suppression assays were done immediately upon receiving the compounds. Each plate contained their own DMSO and media only wells in which control samples were plated. Sx-MDSCs were isolated from cancer surgery patients on POD1 and plated at a 4:1 Sx-MDSC:NK92 cell ratio +/- drug compounds for 24hrs prior to adding CP450-labelled K562 cells. The top hits from Screen#1 informed the creation of Screen #2 which included a

refined list of only 40 compounds, plated in duplicates, at 1μM and 10μM (Supplemental Table 3). Drugs were plated in a randomized order and kept blinded from experimenters until after completing the analysis.

Statistical Analysis

Statistical analysis was performed in GraphPad Prism 8. The unpaired, nonparametric Mann-Whitney U tests were used when comparing two groups and paired Wilcoxon rank-sum tests were used to compare two matched samples (i.e., baseline vs POD1). Multiple comparisons were tested with nonparametric Kruskal-Wallis tests. P values were considered significant when $p < 0.05$. Bonferroni corrections were applied when multiple queries were applied to a data set.

3.5 RESULTS

Surgery-induced Myeloid Derived Suppressor Cells in Cancer patients.

The expansion and persistence of MDSC-like cells in the postoperative period has been observed and reported previously [32,64]. To corroborate these findings we profiled a cohort of cancer surgery patients (n=32; Table 1) from various cancer histologies, procedures, sex and ages for changes immediately following surgery (POD1) using a harmonized multicolour flow cytometry panel for human MDSCs [98] (Fig. 1A and B; fig. S4, S5). We specifically focused our analysis on the expansion and characterization of myeloid cells ($CD33^{+}Lin^{-}$) which we observed to be elevated prior to surgery in cancer patients compared to healthy volunteers. On POD1, there was a significant 1.9-fold increase in the mean proportion of $CD33^{+}Lin^{-}$ myeloid cells (Fig. 1C, $p<0.0001$).

In humans, MDSCs have been broadly categorized into monocytic (M-MDSC; $CD14^{+}CD15^{lo}HLA\text{-}DR^{lo}$), polymorphonuclear (PMN-MDSC; $CD14^{-}CD15^{hi}HLA\text{-}DR^{-}$), or early stage MDSCs (E-MDSC; $CD14^{-}CD15^{-}$) [99]. In our cancer surgery cohort, the M-

MDSC and PMN-MDSC subtypes expanded 2.1-fold and 2.9-fold, respectively, and there was no observed change in the frequency of E-MDSCs after surgery (Fig. 1C). Importantly, the majority of Sx-MDSCs on POD1 were M-MDSCs which accounted for 39.6% (95%CI: 34.8-44.5) of PBMCs, while PMN-MDSCs and E-MDSCs accounted for 6.0% (95%CI: 4.2-8.0) and 4.4% (95%CI: 3.3-5.5), respectively. Therefore, although the relative expansion of PMN-MDSCs was larger, M-MDSCs had the greatest pre- and postoperative presence of PBMCs. The MFI of HLA-DR on M-MDSCs decreased significantly and likewise the proportion of HLA-DR^{lo} M-MDSCs increased significantly after surgery (Fig. 1D, fig. S4).

In addition to the harmonized panel of MDSC markers, we also assessed the effect of surgery on previously reported markers of MDSCs such as arginase-1 (Arg1), CD124 (IL-4Rα) [99], and Lox-1 [190], as well as exploratory immune checkpoint markers VISTA [191] and PD-L1 [192] and observed M-MDSCs and PMN-MDSCs express different levels of these markers, but their expression was not increased after surgery (Fig. 1E). Next we wanted to confirm the suppressive functions of the different Sx-MDSC subtypes.

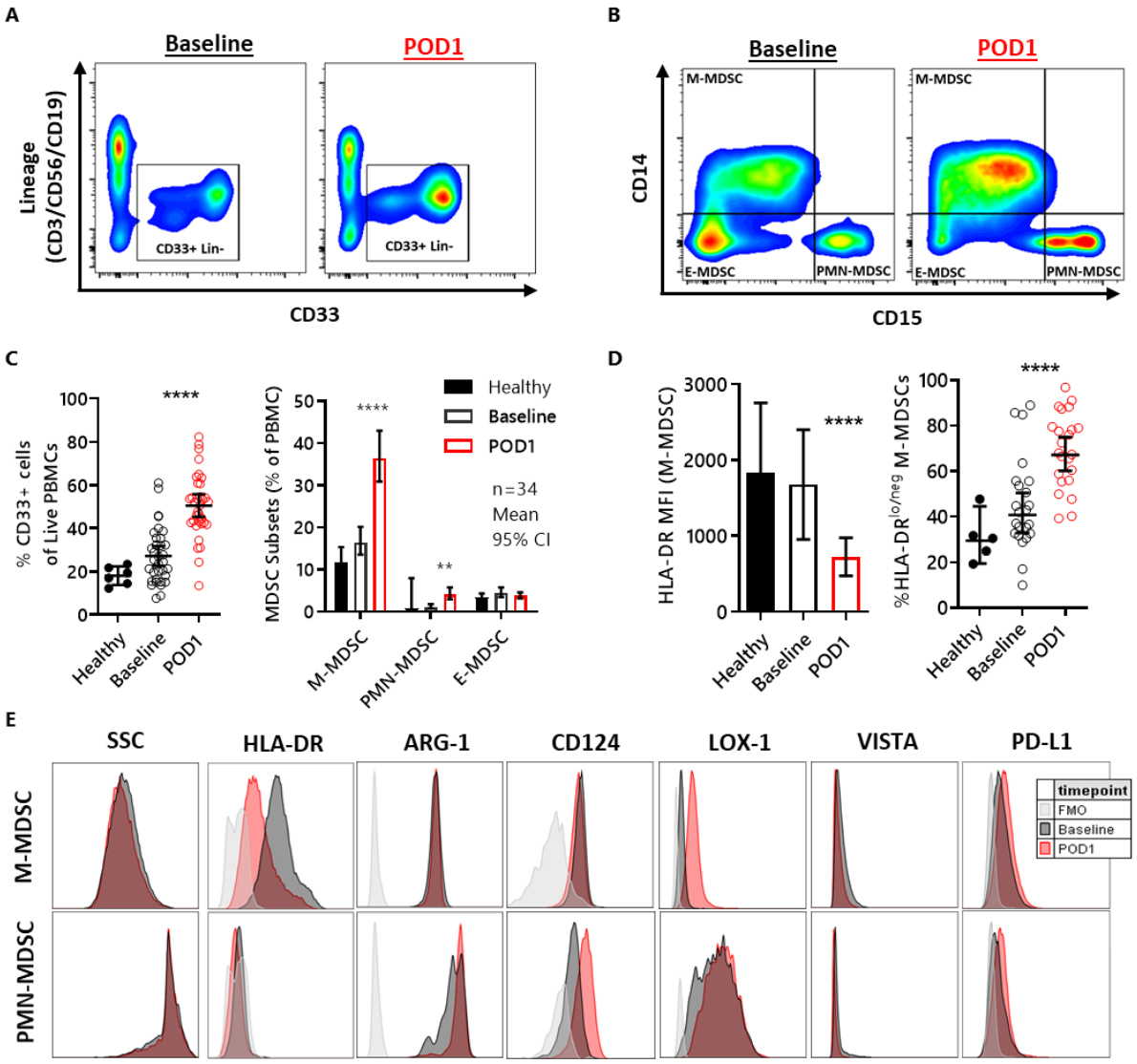

Figure 3.1. Large expansion of phenotypically defined Sx-MDSCs consisted mainly of M-MDSC and PMN-MDSCs immediately after surgery (POD1).

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A) Representative flow plots showing CD33 vs Lineage (CD3/CD56/CD19) for Baseline (left) and POD1 (right) PBMCs. **B)** Representative flow plots gated down on CD33+Lin- cells showing CD15 vs CD14 expression. **C)** Healthy controls (n=6), Baseline and POD1 patients (n=34) proportion of CD33+Lin- cells (left) and MDSC subsets (right). **D)** Proportion of M-MDSCs that are HLA-DRlo (left) and the MFI of HLA-DR gated on M-MDSCs (right) in healthy controls, Baseline and POD1 cancer patients. **E)** Representative histograms of common M-MDSC and PMN-MDSC markers, before and after surgery.

Sx-MDSCs isolated from cancer patients on POD1 (fig. S2) suppressed the cytotoxic potential of NK92-MI cells against K562 target cells (Fig. 2A and B). This was not due to $CD33^+$ cells being targeted by NK92s because the viability of $CD33^+$ cells was not affected following 6 or 24hr co-cultures with NK92 alone (Fig. 2B). Sx-MDSCs on POD1 had a greater NK cell suppressive capacity compared to MDSCs isolated at Baseline (Fig. 2C). To further clarify the contribution of the different subsets in mediating suppression, we isolated PMN-MDSCs and M-MDSCs on POD1 (fig. S2B and C). Additionally, HDNs were isolated from the same patients on POD1. We confirmed that the sorts yielded the intended cell types via Giemsa-Wright staining for cellular morphology and flow cytometry (Fig. 2D). Expectedly, M-MDSCs were mononuclear while PMN-MDSCs and HDN differed based on their density and state of nuclei hypersegmentation [193]. These sorted cell populations were then used in the MDSC:NK92 suppression assay (Fig. 2E). Importantly, while both MDSC subsets were able to suppress the cytotoxicity of NK cells, co-culturing POD1 HDNs with NK92-MI did not result in any suppression. Therefore, on POD1, Sx-MDSCs are a mixture of M-MDSC (majority) and PMN-MDSC (minority) subsets with different phenotypic features, but both have the ability to suppress NK cell functions.

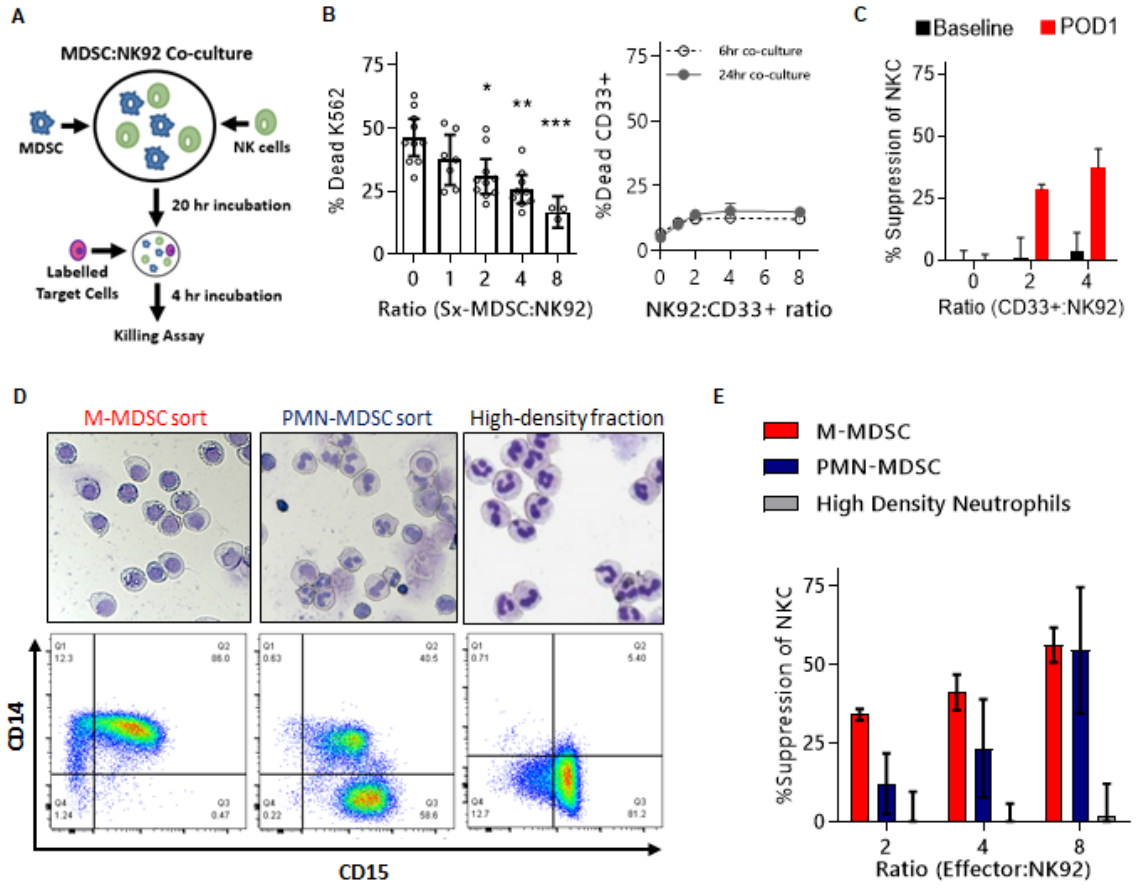


Figure 3.2. Sx-MDSCs suppress NK92 cytotoxicity.

Figure 3.2. Sx-MDSCs suppress NK92 cytotoxicity.

A) Schematic of MDSC-NK92 co-culture suppression assay with CP450-labelled K562 target cells. **B)** NK92s were co-cultured at increasing ratios with CD33+ cells for 6 or 24hrs to assess CD33 death by NK92s (left). Sx-MDSCs from n=10 POD1 patient samples were cocultured with NK92 cells and NK cytotoxicity was measured as % dead K562 (right). **C)** % suppression was calculated as the reduction of NK cytotoxicity, normalized to NK cells alone (ratio = 0). CD33+ cells are more suppressive on POD1 than Baseline. **D)** Representative images of sorted cells stained with Giemsa Wright and their matched flow plots (gated on CD33+Lin- cells). **E)** Sx-MDSCs (M-MDSCs and PMN-MDSCs) suppress NK cytotoxicity (NKC) but high-density neutrophils do not (n=3 POD1 patients).

Single-cell RNA sequencing captures significant transcriptional perturbations in monocytes after surgery resembling immunosuppressive MDSCs.

To understand the transcriptional changes occurring in the myeloid population after surgery, we performed scRNA-seq on cryopreserved PBMCs from matched baseline and POD1 patient samples (n=6) (Fig. 3A; fig. S1; Table S1). After removing cells with low gene detection (<200 detected genes) and high mitochondrial gene content (>25%), we integrated and clustered the remaining 30,773 cells by underlying cell type (Fig. 3B), resulting in the identification of 10 cell types (Fig. 3C and D). Interestingly, we observed reproducible shifts in the transcriptional profiles of all immune cell types after surgery. Although there were no differences on POD1 in the proportion of NK cells (p=0.8), there was a significant decrease in the %CD3+ cells (0.6 fold-change, p=0.03) and increase in the %CD14+ monocytes (2.0 fold-change, p=0.03) of PBMCs measured by flow cytometry (fig. S1B-D). As the monocytic Sx-MDSCs make up the majority of the PBMCs postoperatively (fig. S1D, Fig. 3D and E), we performed differential state analysis [183] followed by gene set enrichment analysis (GSEA) using a collection of query gene sets from the Molecular Signatures Database (GO terms, KEGG pathways, Reactome pathways, and Hallmark gene sets) [186,187] on the monocytic populations. This revealed an upregulation of expected/known responses to surgery such as IL-1 signaling, coagulation, and wound healing (Fig. 3F). Interestingly, there was an increase in the "TNF-α signaling via NF-κB" Hallmark gene set which has been described to be upregulated in immunosuppressive monocytes and MDSCs [194]. Furthermore, the downregulation of the Reactome pathway "MHC class II antigen presentation" has been described previously [32], and supports our flow cytometry observations (Fig. 1D).

Although the monocyte clusters separated into two distinct populations (Baseline and POD1, Fig. 3E), we observed that each population retained patterns of phenotypic gradients

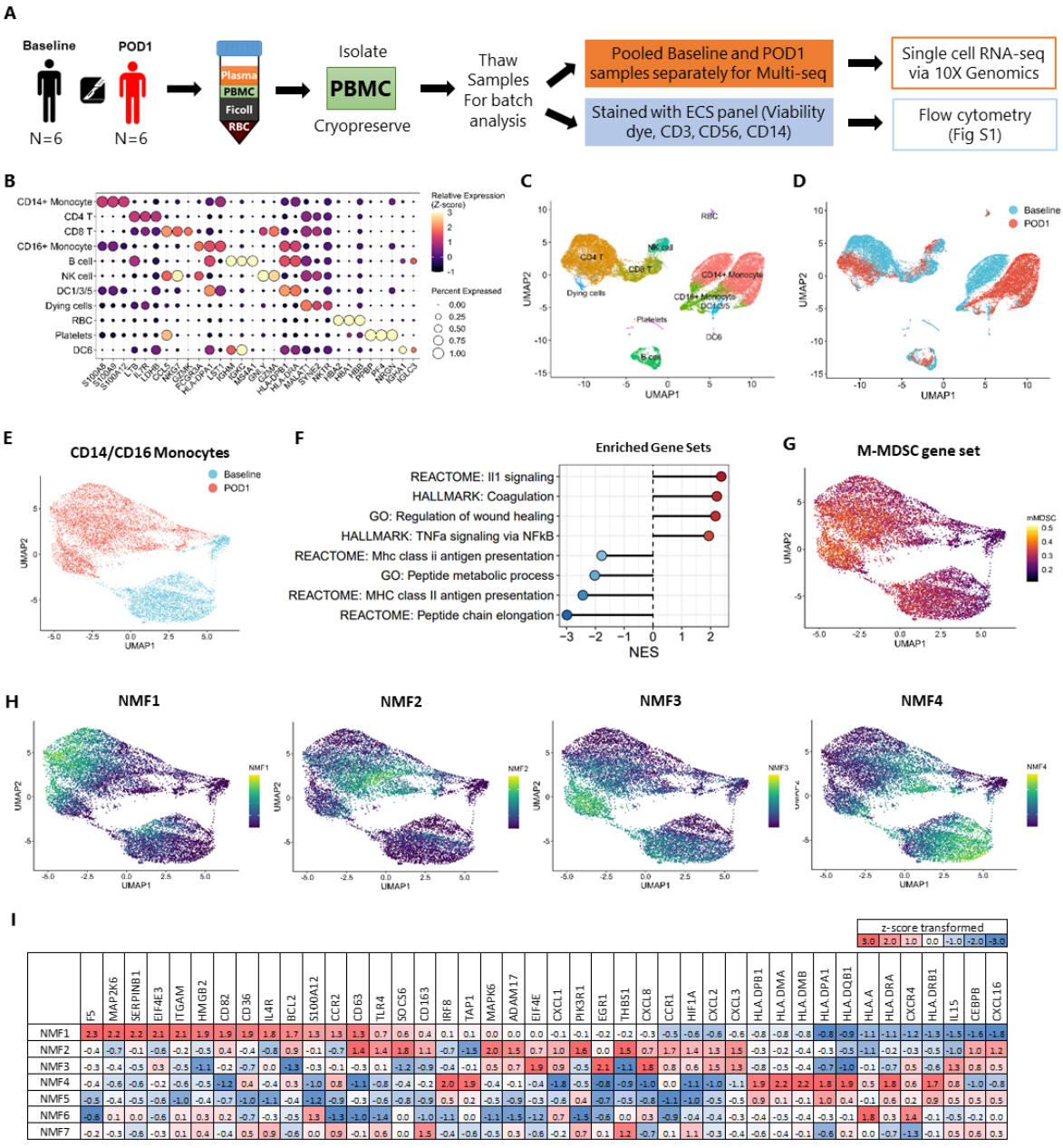
in their gene expression profiles, suggesting that the monocytes are on a spectrum of differentiation before and after surgery as opposed to being clearly defined postoperative monocytic cell subtypes. This is best exemplified by the observation that similar gradients of gene expression programs can be found in both Baseline and POD1 states. To understand the differences in genes driving a global shift vs. a gradient effect, we used non-negative matrix factorization (NMF) to learn distinct modules of coordinated gene expression [188]. This resulted in the identification of seven gene expression programs with variable activity in monocytes on Baseline and POD1 (NMF1-7; fig. S2). Some NMF programs (NMF1, NMF2 and NMF7) were mainly expressed in monocytes on POD1 while other NMF programs (NMF3, NMF4 and NMF6) had a similar expression at Baseline and POD1. Lastly, NMF5 was densely expressed in the CD16+ monocyte populations (fig. S2).

We observed that M-MDSC gene signatures recently identified by scRNAseq [195] were most expressed in the cells using NMF1 and 2 gene expression programs (Fig. 3G and H). Genes contributing to NMF1 and 2 included MDSC-related genes such as *S100a12, Hmgb2, Adam17, Hif1a, Ccr2, Il4r and Serpinb1* [195–197]. Notably, NMF4 activity was inversely correlated with this signature and driven by expression of genes involved in antigen presentation, including the HLA family genes (e.g. *Hla.dra, Hla.drb1, Hla.dma, Hla.dpa1*) and *Tap1*, corroborating our GSEA results. Lastly, cells highly expressing NMF3 and 7 gene expression programs may be driven by individual patients, ARG12 and ARG19, respectively (fig. S1E and S3A). These NMF programs were also more expressed by POD1 cells and had high expression of genes related to MDSCs (*CEBPB, EIF4E, Trem1*, *Il1b*, *Tnf*, and *Ptgs2*) *[198,199]*. Therefore, our NMF analysis showed that monocytes have varying levels of activity for each gene expression program, but that there was higher activity of NMF1, 2, 3, and 7 on POD1 with similarities to MDSC-related genes (Fig. 3I). These results implicate 4

different activity states of Sx-MDSC which each contribute uniquely to the immunosuppressed period after surgery.

PI3K regulates Sx-MDSCs suppressive machinery and is amenable to targeting.

As an alternative approach to identifying pathways regulating the suppressive activity of MDSCs, we performed a series of compound screens of small molecules that affect major biological signalling pathways including PI3K/AKT, TGF-β, VEGF, NFkB, COX, NOS, MMPs, and pathways governing apoptosis and autophagy (adMare Bioinnovations; Fig. 4A). Each compound was incubated in MDSC:NK92 co-cultures for 24hr at a final concentration of 1μM and MDSC-mediated suppression of NK cell cytotoxicity was evaluated. In the first set of screens (Table S2), 147 compounds were assessed at 1uM and the compounds which prevent NK cell suppression by >50% were identified and informed which compounds to use in the second set of screens (Table S3). The top hits can be found in Supplemental Table 4. Interestingly, in the second set of screens (Screen #2), compounds that targeted the PI3K pathway, such as LY294002 and AS-252424, were among the compounds that inhibited MDSC:NK92 suppression the most, suggesting a potential role for PI3K signaling in Sx-MDSCs. Numerous reports have shown an important role for PI3K signalling in MDSCs [103,200,201]. Therefore, we next sought to validate the role of PI3K in mediating the suppressive activity of Sx-MDSC (Fig. 4B and C).



	F5	MAP2K6	SERPINB1	EIF4E3	ITGAM	HMGB2	CD82	CD36	IL4R	BCL2	S100A12	CCR2	CD63	TLR4	SOCS6	CD163	IRF8	TAP1	MAPK6	ADAM17	EIF4E	CXCL1	PIK3R1	EGR1	THBS1	CXCL8	CCR1	HIF1A	CXCL2	CXCL3	HLA.DPB1	HLA.DMA	HLA.DMB	HLA.DPA1	HLA.DQB1	HLA.A	HLA.DRA	CXCR4	HLA.DRB1	IL15	CEBPB	CXCL16
NMF1	2.3	2.2	2.2	2.1	2.1	1.9	1.9	1.9	1.8	1.7	1.3	1.3	1.3	0.7	0.6	0.4	0.1	0.1	0.0	0.0	0.0	-0.1	-0.1	-0.2	-0.2	-0.3	-0.5	-0.6	-0.6	-0.6	-0.8	-0.8	-0.8	-0.8	-0.9	-1.1	-1.1	-1.2	-1.3	-1.5	-1.6	-1.8
NMF2	-0.4	-0.7	-0.1	-0.6	-0.2	-0.5	0.4	-0.4	-0.8	0.9	-0.1	-0.7	1.4	1.4	1.8	1.1	-0.7	-1.5	2.0	1.5	0.7	1.0	1.6	0.0	1.5	0.7	1.7	1.4	1.3	1.5	-0.3	-0.2	-0.4	-0.3	-0.5	-1.1	-0.2	-0.3	-0.5	-1.0	1.0	1.2
NMF3	-0.3	-0.4	-0.5	0.3	-0.5	-1.1	-0.2	-0.7	0.0	-1.3	-0.3	-0.1	-0.3	0.1	-1.2	-0.9	-0.4	-0.1	0.5	0.7	1.9	0.9	-0.5	2.1	-1.1	1.8	0.8	0.6	1.5	1.3	-0.8	-0.5	-0.4	-0.7	-1.0	-0.1	-0.7	0.5	-0.6	1.3	0.8	0.5
NMF4	-0.4	-0.6	-0.6	-0.2	-0.6	-0.5	-1.2	0.4	-0.4	0.3	-1.0	0.8	-1.1	-0.8	-0.4	-0.2	2.0	1.9	-0.4	-0.1	-0.4	-1.8	-0.5	-0.8	-0.9	-1.0	0.0	-1.1	-1.0	-1.1	1.9	2.2	2.2	1.8	1.9	0.5	1.8	0.6	1.7	0.8	-1.0	-0.8
NMF5	-0.5	-0.4	-0.5	-0.6	-1.0	-0.4	-0.7	-1.0	-1.1	-0.4	-1.2	-0.9	-0.3	-0.6	-0.8	-0.9	0.5	0.2	-0.5	-0.9	-0.3	-0.6	0.4	-0.7	-0.5	-0.8	-1.1	-1.0	-0.5	-0.5	0.9	0.1	-0.1	1.0	0.4	-0.1	0.6	0.2	0.9	0.5	0.5	0.5
NMF6	-0.6	0.1	0.0	-0.6	0.1	0.3	0.2	-0.7	-0.3	-0.5	1.3	-1.3	-1.0	-1.4	0.0	-1.0	-1.1	0.0	-1.1	-1.5	-1.2	0.7	-1.5	-0.6	0.0	0.3	-0.9	-0.4	-0.4	-0.3	-0.4	-0.5	-0.4	-0.3	-0.1	1.8	0.3	1.4	-0.1	-0.5	-0.2	0.0
NMF7	-0.2	-0.3	-0.6	-0.3	0.1	0.4	-0.4	0.5	0.9	-0.6	0.0	0.9	-0.1	0.6	0.0	1.5	-0.4	-0.6	-0.5	0.3	-0.6	-0.2	0.7	0.1	1.2	-0.7	0.1	1.1	-0.3	-0.4	-0.6	-0.4	-0.1	-0.6	0.2	0.0	-0.7	-1.3	-0.1	0.5	0.6	0.3

Figure 3.3. Single cell RNA seq of cryopreserved PBMCs before and after surgery reveals drastically altered monocyte/myeloid cell expression profiles on POD1.

Figure 3.3. Single cell RNA seq of cryopreserved PBMCs before and after surgery reveals drastically altered monocyte/myeloid cell expression profiles on POD1.

A) Schematic showing PBMCs (cryopreserved) from 6 matched Baseline and POD1 patients were processed for multiplexed scRNA-seq with the 10x Genomics Chromium platform. **B)** Dot plot displaying the relative expression of the top 3 marker genes (x-axis) of each cluster (y-axis). **C)** UMAP plot of scRNA-seq data. Each point corresponds to a single cell and is coloured by cluster. **D)** Identical UMAP embedding as in (C), with Baseline and POD1 cells labelled. **E)** UMAP plot of the CD14+ and CD16+ monocyte population at Baseline and POD1. **F)** Gene set enrichment analysis (GSEA) showing the normalised enrichment scores (NES) of the top upregulated and downregulated pathways. All gene sets are significantly enriched (FDR < 0.05). **G)** UMAP plot showing activity of an M-MDSC gene set from Alshetaiwai *et al.*; in monocyte populations in Baseline and POD1 samples. **H)** Non-negative matrix factorization (NMF) plots of gene expression programs for NMF 1, 2, 3 and 4 (see fig. S2 for all NMF programs). **I)** A heatmap of selected genes driving the various NMF gene expression programs (z-score transformed; ranked according to NMF1).

Although LY294002 is able to bind to multiple catalytic subunits of class I PI3K heterodimers, the p110-γ subunit is preferentially expressed in myeloid cells [104] and is known to regulate immunosuppressive profiles of tumour associated macrophages [103]. To specifically validate the role of PI3K-γ, we used inhibitors with increased specificity for this isoform, namely, IPI-549, TG100-115 and 3,3′-(2,4-Diaminopteridine-6,7-diyl)diphenol ("Compound 17") [202] in our co-culture assays (Fig. 4D and E). Similar to our findings with LY294002, each of these inhibitors improved NKC and inhibited Akt phosphorylation in Sx-MDSC (fig. S6A). PI3K-γ specific inhibitors were more effective than LY294002 at reducing phospho-Akt in *ex vivo* whole blood experiments with and without IL-4 stimulation (fig. S6B). Notably, TG100-115 was also able to increase the MFI of HLA-DR when gated on Sx-MDSCs in a dose dependent manner, as has been reported by Kaneda et al. (fig. S6B, n=1) [103].

Signalling through the PI3K pathway is increased following surgery

Given the results from the small molecule screen, we used PROGENy to infer relative differences in the signalling pathway activity of monocytic cells in our scRNA-seq. On POD1, there was significant upregulation in several signalling pathways in the majority of patients: VEGF, PI3K, hypoxia, MAPK, androgen, NF-κB, and TNF-α. Conversely, there was a decrease in JAK-STAT and WNT signalling, while TGF-β, p53 and Trail signaling did not differ much after surgery (Fig. 4F). Importantly, PI3K signalling pathway activity was upregulated in all patients (Fig. 4G). Consistent with our functional data, PI3K-regulated transcripts were significantly altered in monocytes after surgery. To determine which NMF program(s) in our monocyte population were associated with elevated PI3K activity, we queried our scRNA-seq against publicly available gene sets of PI3K-regulated genes [203] (Fig. 4H). The NMF programs that had the highest correlation with PI3K-regulated genes were NMF1 (r = 0.22) and 2 (r=0.32), suggesting that these putative Sx-

MDSC gene programs are also associated with elevated PI3K activity. Therefore, the PI3K signaling pathway is transcriptionally elevated in Sx-MDSCs, which may explain why targeting this pathway with PI3K inhibitors attenuates their suppressive machinery.

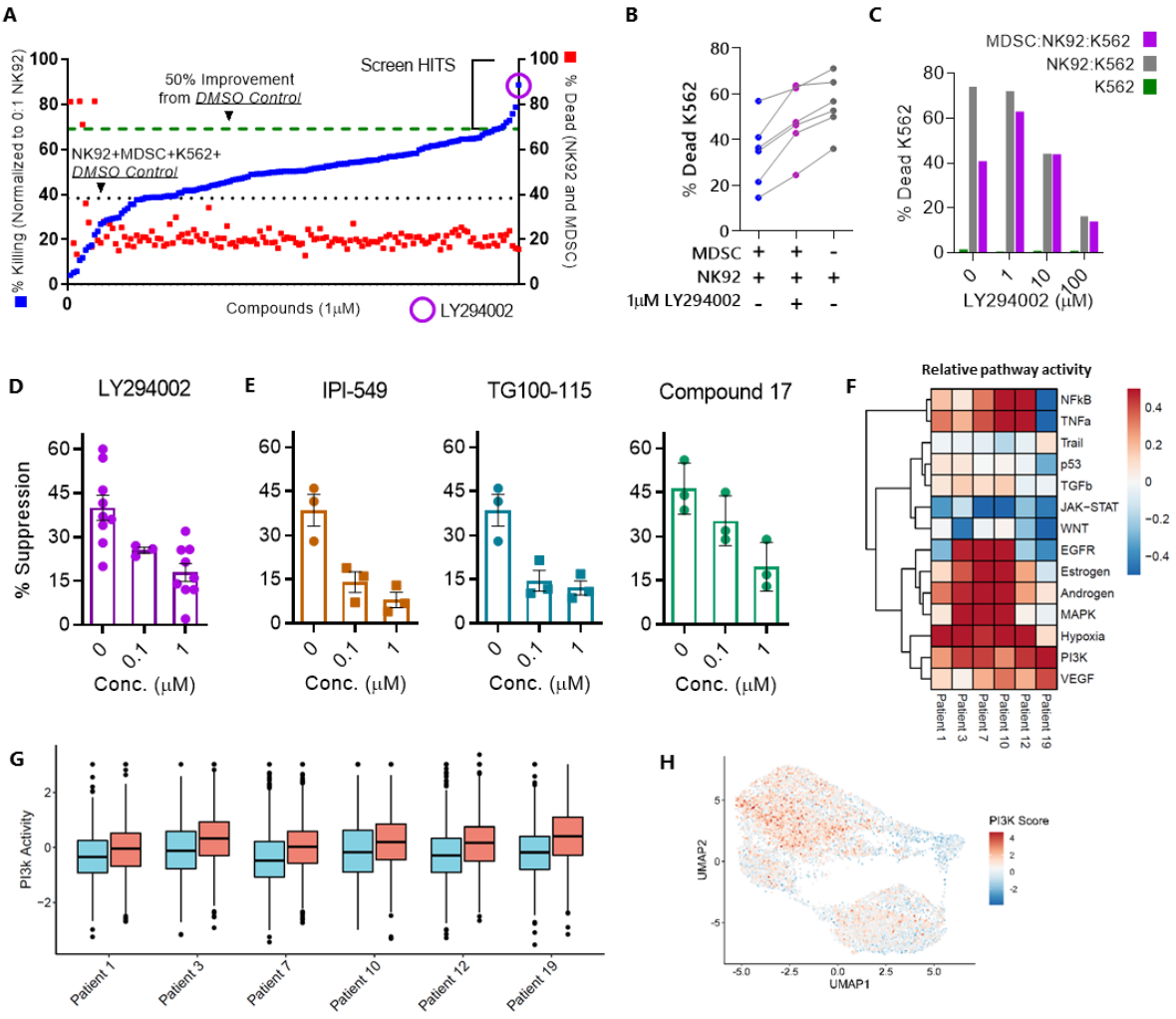


Figure 3.4. PI3K inhibitors reverse the suppressive effects of Sx-MDSCs on NK cells.

Figure 3.4. PI3K inhibitors reverse the suppressive effects of Sx-MDSCs on NK cells.

A) Representative results from Screen #1A of 147 compounds plated at 1uM in the MDSC-NK cell coculture assay. Compounds that improved NK cell cytotoxicity (blue, left y-axis) by >50% from DMSO control (black dotted line), without impacting NK cell viability (red, right y-axis), were considered hits. LY294002 (purple circle) improved cytotoxicity in all screens and was the top hit in 3/4 screens. **B)** LY294002 improves NK cell cytotoxicity (n=6). **C)** Dose response of LY294002 and the effect on NK92 cytotoxicity and K562 viability. **D)** pan-PI3K and **(E)** PI3K-γ specific inhibitors (IPI-549, TG100-115, and Compound 17) on MDSC-NK cell suppression. **F)** Relative activity of pathway signalling inferred from scRNA-seq data using PROGENy shows the main pathways that are upregulated after surgery in monocytes. **G)** PI3K pathway signalling from individual patients inferred from scRNA-seq data using PROGENy. **H)** UMAP of gene set scores for PI3K-dependent genes in monocytes' response to GM-CSF [203]. Points are coloured by Z-score transformed gene set scores.

3.6 DISCUSSION

The earliest report of a suppressive monocytic cell population with the capacity to suppress NK cells after surgery was published in 1982 by Uchida and colleagues [114]. Decades later, as technology has advanced our understanding of these suppressive cells, it is almost certain that Uchida et al. was investigating Sx-MDSCs. Only recently has the umbrella term "MDSC" become an accepted nomenclature for the heterogeneous population of immunoregulatory myeloid cells [204]. Numerous context dependent identifiers have been used to further categorize MDSCs in an attempt to bring greater clarity and accuracy to the field. This is because factors such as tissue localization, cancer subtype, disease setting, and inflammatory conditions can all contribute to determining the prevailing MDSC subtype [205,206].

The term "Surgery-induced MDSCs" has been explicitly used in previous publications by our group [64] and others [173], to define the population of immunosuppressive cells that arise from sterile (non-microbial), surgical inflammatory conditions (i.e., inflammatory responses triggered through the release of host-derived DAMPs) [207]. Other groups have used the term "trauma-induced MDSCs" [208] which may be synonymously interchanged with Sx-MDSCs if the context is sterile inflammation. Interestingly, Go et al. measured Arg1 activity in trauma-induced MDSCs after PGE2 stimulation with or without an additional TLR4 lipopolysaccharide (LPS) stimulation (a pathogen-associated molecular pattern; PAMP) and showed that LPS synergistically increased the activity of Arg1 [208]. This suggests that DAMPs and PAMPs activate different suppressive pathways in MDSCs. Furthermore, Th1 cytokines push MDSCs towards increased expression of inducer of nitric oxide synthase (iNOS) while Th2 cytokines lead to increased expression of Arg1 [118,119], lending further support that the inflammatory context MDSCs arise from determines their resulting suppressive machinery.

In our study, we assessed the changes in MDSC populations from a diverse cohort of cancer surgery patients differing in their primary cancer, and thus surgical procedures (Table 1). This allowed us to assess the universal effect of surgery on postoperative MDSCs to determine the dominant MDSC subtype. When breaking down the expansion of M-MDSCs on POD1 by cancer type, even though there were varying starting M-MDSC levels at baseline, the median fold-change was higher in M-MDSCs than PMN-MDSCs across all cancer types, with the exception of lung cancer surgery patients (fig. S5). Interestingly, Wang et al. [173] observed that the M-MDSCs were the main MDSC subtype after surgery in a much larger cohort of 111 lung cancer surgery patients, which supports the evidence that M-MDSCs are the main Sx-MDSC subtype.

In agreement with previously reported expression patterns of PMN-MDSCs and M-MDSCs [109], PMN-MDSCs had a higher scatter profile compared to M-MDSCs, in addition to greater expression of Arg1, CD124, and Lox1. Lox1 was recently described as a distinguishing biomarker for PMN-MDSCs [190]. M-MDSCs also expressed Arg1 and CD124 but they did not increase after surgery in the M-MDSC subset. Regardless of the magnitude of expansion or subtype specific MDSC markers, both M-MDSCs and PMN-MDSCs were able to suppress NK cells when separately enriched for on POD1 (Fig. 3E).

Single-cell mass cytometry profiling in patients undergoing hip arthroplasty surgery, showed the greatest immune cell perturbations occurred in $CD14^{+}$ monocytes, which resembled the phenotype of immunosuppressive MDSCs [32]. Similarly, our study showed the most striking differences in the monocytic compartments after surgery. We observed that gradients of gene expression patterns were present at baseline and POD1, suggesting that Sx-MDSCs are on a continuum of differentiation as opposed to distinct terminal stages of differentiation or maturity (Fig. 3). Querying our data set against a publicly available gene set of M-MDSCs [195], we saw that the gene expression profile overlapped with

NMF1 and NMF2. The genes that were highly expressed in these programs were increased DAMPs (e.g. *Hmgb2, S100a8/9/12*) and chemokines (e.g. *Ccr2*) in addition to decreased HLA gene expression (Fig. 3). Interestingly, NMF4 had the inverse gene expression program of NMF 1 and 2, with very high expression of HLA-DR. Since NMF4 activity was mainly expressed in monocytes at baseline (fig. S3B), this supports the hypothesis that after surgery, emergency myelopoiesis results in an expansion of immature myeloid cells that gain a suppressive phenotype as a result of the postoperative inflammatory context.

An important limitation of our scRNA-seq experiments to note is that we are unable to make firm inferences about PMN-MDSCs due to the negative effect of cryopreservation on PBMC viability and recovery of PMN-MDSCs/LDNs [98,209]. Furthermore, specific genes that define MDSC suppressive mechanisms, Arg1 in particular, are untraceable upon freeze-thawing [109].

To identify which pathways were regulating Sx-MDSC suppression, we used a small molecule compound screen with bulk Sx-MDSCs. The screen included compounds that targeted TGF-β, VEGF, Raf/MAPK, and NFκB/TNF-α pathways which the scRNA-seq showed were upregulated after surgery in monocytes. Furthermore, the compounds GW5074 and Bay11-0785 which inhibit the Raf/MAPK and NKκB/TNF-α, respectively, were among the top compounds that inhibited Sx-MDSCs (Table S4); however, these were not pursued further because of their direct inhibitory effects on NK cell viability and cytotoxicity (fig. S7). The PI3K pathway has been reported as a regulator for macrophage polarization and immunoregulatory programs [103,200], and the p110-γ catalytic subunit of PI3K in particular is preferentially expressed by myeloid cells [104]. Therefore, we used the PI3K-γ specific inhibitors and found that they were more potent in inhibiting Sx-MDSCs. In our data set, the PI3K pathway was upregulated in monocytes on POD1 (Fig. 4F and G). To confirm that basal levels of PI3K activity was increased after surgery, we performed

phospho-flow for pAKT (Ser473 and Thr308) and pS6 (Ser235/236), which are downstream targets of PI3K activity [103]. Although we did not see an increase in pAKT (n=3; fig. S6C), pS6 levels were slightly increased on POD1 (1.6-fold, p=0.2, n=11; fig. S6D and E). Therefore, further studies should aim to repeat this in a larger subset of surgery patients, and in the presence of stimulating cytokines to determine if Sx-MDSCs are more permissive to PI3K activity after surgery.

In summary, the perioperative period is a dynamic landscape occupied by immunosuppressive monocytic Sx-MDSCs which have heterogeneous transcriptional profiles. Scoring gene sets from MDSCs generated in different inflammatory settings will help identify the context specific attributes of MDSCs and bring greater clarity and accuracy to the field. Currently, no perioperative therapy exists to target Sx-MDSCs, but we have shown preliminary evidence that Sx-MDSCs may rely on PI3K signalling for their suppressive activity and NK cell suppression, warranting further investigation.

3.7 SUPPLEMENTARY TABLES AND FIGURES

Supplemental Table 3.1. Patient data for samples used in scRNA-Seq

Patient ID	Sex	Age	Stage	CCI	Trial Arm ***	Type of surgery	OR Pathology details	NKC (POD1/BL)	SX-MDSCs (POD1/BL)
ARG01	M	56	II	2	A	Lap	Adenocarcinoma	0.93	2.46
ARG03	M	66	I	4	A	Lap	Adenocarcinoma	0.36	1.95
ARG19	M	66	II	4	A	Lap	Adenocarcinoma	N/A	2.23
ARG07	M	65	II	4	B	Open	Adenocarcinoma	0.67	1.36
ARG10	M	57	I	3	B	Lap	Invasive carcinoma in ascending polyp	0.18	2.46
ARG12	M	79	II	5	B	Lap	Adenocarcinoma	0.40	1.29

*Footnote text
**Data taken from the PERIOP-02 Clinical Trial Registered (NCT02987296)
***Participants enrolled were randomized to receive either an arginine enriched supplement ("A") or isocaloric/isonitrogenous control supplement ("B") for 5 days prior to surgery.

Supplemental Table 3.2. Screen #1 compound list

Compounds #1-75	Chemical name	Targeted Pathway
1	CGP 57380	MAPK inhibitor
2	Tyrphostin A9	PDGF
3	I-OMe-Tyrphostin AG 538	IGF1R Inhibitor
4	AC-55649	RARβ2 receptor agonist
5	U-73122	Phospholipase C inhibitor
6	Imiquimod	TLR7 agonist
7	SQ 22536	Adenylyl Cyclase inhibitor
8	Tyrphostin AG 808	tyrosine kinase inhibitor
9	Pifithrin-mu	Bcl-xL and Bcl-2
10	Tyrphostin AG 490	Jak-2 protein tyrosine kinase inhibitor
11	DMSO	Control
12	SU 4312	Neuronal NOS inhibitor
13	SCH-202676 hydrobromide	GPCR ligand inhibitor
14	Sulindac	NSAID
15	Retinoic acid	enhances cell maturation
16	Rottlerin	Protein kinase Cδ (PKCδ) inhibitor.
17	Stattic	STAT3 inhibitor
18	A3 hydrochloride	Non-selective casein kinase inhibitor.
19	SMER28	enhancer of rapamycin
20	Bay 11-7082	inhibitor of IκBα phosphorylation
21	Quercetin dihydrate	antioxidant flavonoid
22	ARP 101	MMP-2 inhibitor
23	SB 216763	GSK-3 inhibitor
24	Piceatannol	promotes glucose uptake
25	Olomoucine	CDK inhibitor
26	Nimesulide	NSAID
27	NG-Nitro-L-arginine methyl ester hydrochloride	iNOS inhibitor
28	GW9662	PPARγ antagonist
29	Myricetin	Flavonoid antioxidant
30	Meclofenamic acid sodium	COX inhibitor
31	beta-Lapachone	Modulates NAD metabolism
32	SB-525334	TGFβ receptor I inhibitor
33	LY-294,002 hydrochloride	pan-PI3K inhibitor
34	SD-169	MAPK inhibitor
35	1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine dihydrochloride	PKC inhibitor
36	Olvanil	synthetic analogue of capsaicin
37	LFM-A13	BTK inhibitor
38	3-Isobutyl-1-methylxanthine	cAMP and cGMP inhibitor
39	ML-7	Myosin light chain kinase inhibitor
40	Imazodan	phoshodiesterase III inhibitor
41	DMSO	Control
42	JFD00244	SIRT2 inhibitor
43	Ibudilast	PDE4 inhibitor
44	MNS	tyrosine kinase inhibitor
45	HA-1004 hydrochloride	inhibitor of protein kinase G (PKG) and PKA
46	HA-100	PKA, PKC, and PKG inhibitor
47	Emodin	tyrosine kinase inhibitor
48	JX401	p38 MAP Kinase Inhibitor VI
49	GW7647	PPARα agonist
50	Genipin	Prevents NO production
51	GW5074	cRaf1 kinase inhibitor
52	GW2974	EGFR / ErbB-2 inhibitor
53	AS 604850	ATP-competitive PI3Kγ inhibitor
54	GW1929	PPAR-γ activator
55	S-Ethylisothiourea hydrobromide	iNOS, eNOS, and nNOS inhibitors
56	Forskolin	Protein kinase A agonist
57	SP600125	JNK2 inhibitor
58	7-Cyclopentyl-5-(4-phenoxy)phenyl-7H-pyrrolo[2,3-d]pyrimidin-4-ylamine	Lck inhibitor
59	Diclofenac sodium	NSAID
60	Cytidine 5'-diphosphocholine sodium salt hydrate	synthesis of phosphatidylcholine
61	3,7-Dimethyl-1-propargylxanthine	A2 adenosine receptor antagonist
62	Imperatorin	acetylcholinesterase inhibitor
63	Enoximone	PDE3 Inhibitor
64	PD 169316	p38 MAP kinase inhibitor
65	Clodronic acid	Calcium modulator
66	SANT-1	Inhibits Sonic hedgehog signalling
67	1,7-Dimethylxanthine	Adenosine receptor ligand
68	Cambinol	inhibits NAD-dependant deacetylases
69	Capsazepine	TRPV1 receptor antagonist
70	Diacylglycerol Kinase Inhibitor II	Diacylglycerol Kinase Inhibitor II
71	Y-27632 dihydrochloride	Rho kinase inhibitor
72	Daphnetin	EGFR , PKA, and PKC inhibtor
73	CGS-15943	adenosine receptors A1 and A2A antagonist
74	2-Chloroadenosine	adenosine receptor agonist.
75	Cilostamide	PDE3 Inhibitor

Supplemental Table 3.2. Screen #1 compound list(continued)

Compounds #76-150	Chemical name	Targeted Pathway
76	9-cyclopentyladenine	adenylyl cyclase inhibitor
77	Chelerythrine chloride	inhibitor of protein kinase C
78	BRL 50481	PDE7 Inhibitor
79	ML-9	Myosin light chain kinase inhibitor
80	BWB70C	5-lipoxygenase inhibitor
81	L-Cycloserine	serine palmitoyltransferase inhibitor
82	BTO-1	polo-like kinase
83	CP55940	non-selective cannabinoid receptor agonist
84	Bay 11-7085	inhibitor of IκBα phosphorylation
85	SB 202190	5-lipoxygenase inhibitor
86	AS-252424	Pi3K-gamma inhibitor
87	Bromoenol lactone	inhibitor of calcium-independent phospholipase A2
88	TBBz	Casein Kinase-2 (CK2) inhibitor
89	Diacylglycerol kinase inhibitor I	Diacylglycerol kinase inhibitor I
90	DMSO	Control
91	2-(2-Aminoethyl)isothiourea dihydrobromide	iNOS inhibitor
92	N-arachidonylglycine	GPR18 agonist
93	Tryptamine hydrochloride	antagonizes 5-HT serotonin receptors
94	Aminoguanidine hemisulfate	iNOS inhibitor
95	Acetylsalicylic acid	COX1/2 inhibitor
96	5-azacytidine	cytidine analog
97	YM 976	PDE4 inhibitor
98	DL-alpha-Methyl-p-tyrosine	tyrosine hydroxylase enzyme inhibitor
99	6-Methoxy-1,2,3,4-tetrahydro-9H-pyrido[3,4b] indole	indole
100	Acetamide	anti-microbial, antifungal
101	Amantadine hydrochloride	nicotinic antagonist
102	GABA	neurotransmitter
103	Gabaculine hydrochloride	analog of GABA
104	O-(Carboxymethyl)hydroxylamine hemihydrochloride	forms oximes
105	(±)-2-Amino-7-phosphonoheptanoic acid	NMDA glutamate receptor antagonist
106	N-Acetylprocainamide hydrochloride	Class III antiarrhythmic
107	Actinonin	MMP inhibitor
108	S(-)-p-Bromotetramisole oxalate	inhibitor of alkaline phosphatase
109	TMB-8 hydrochloride	antagonist of nicotinic acetylcholine receptors
110	L-azetidine-2-carboxylic acid	inhibitor of collagen synthesis
111	S-(p-Azidophenacyl)glutathione	inhibit glyoxalase and glutathione S-transferase
112	Acetyl-beta-methylcholine chloride	Muscarinic acetylcholine receptor antagonist
113	AA-861	5-Lipoxygenase inhibitor
114	Azathioprine	prodrug of 6-mercaptopurine
115	L-732,138	Neurokinin-1 receptor antagonist
116	Amifostine	free radical scavenger and detoxifier
117	Atropine methyl bromide	muscarinic receptor (mAChR) antagonist
118	Azelaic acid	tyrosinase inhibitor
119	Atropine methyl nitrate	Muscarinic acetylcholine receptor antagonist
120	(±)-Norepinephrine (+)bitartrate	alpha- and beta-adrenergic receptors
121	Aurintricarboxylic acid	inhibitor of ribonuclease and topoisomerase II
122	3-Aminopropionitrile fumarate	lysyl oxidase (LOX) inhibitor
123	1-Aminobenzotriazole	inhibitor of cytochrome P450 and chloroperoxidase
124	Sandoz 58-035	Acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor
125	Acetylthiocholine chloride	nicotinic acetylcholine receptor agonist
126	A-315456	α1D-adrenoceptor antagonist
127	Agmatine sulfate	Nitric oxide modulator, NADPH oxidase activator
128	Arcaine sulfate	NMDA glutamate receptor antagonist
129	4-Amino-1,8-naphthalimide	PARP inhibitor
130	(±)-2-Amino-4-phosphonobutyric acid	agonist for the group III metabotropic glutamate receptors
131	Apigenin	Flavonoid, inhibits MAPK, ERK, JNK and p38
132	3-Amino-1-propanesulfonic acid sodium	GABAA receptor agonist
133	(±)-2-Amino-3-phosphonopropionic acid	Metabotropic glutamate receptor antagonist
134	4-Androsten-4-ol-3,17-dione	unknown
135	GR 46611	5-HT1D agonist
136	4-Aminobenzamidine dihydrochloride	fibrinogen receptor antagonists
137	(±)-Nipecotic acid	inhibitor of uptake of γ-aminobutyric acid (GABA)
138	Atropine sulfate	antagonist of muscarinic receptors
139	3-aminobenzamide	PARP inhibitor
140	N-Acetyl-5-hydroxytryptamine	precursor of melatonin
141	5-(N-Ethyl-N-isopropyl)amiloride	Sodium-hydrogen exchanger inhibitor
142	10058-F4	c-Myc inhibitor
143	Amiprilose hydrochloride	Inhibits PLA2 substrate availabiliity
144	5-(N-Methyl-N-isobutyl)amiloride	nhibitor of Na+/H+ antiporter
145	Arecoline hydrobromide	agonist of the muscarinic acetylcholine receptors
146	N-Phenylanthranilic acid	NSAID
147	S-(4-Nitrobenzyl)-6-thioguanosine	Potent adenosine uptake inhibitor
148	TLR7 Agonist	TLR7 Agonist
149	ABT-737	inhibits Bcl-2 and Bcl-xL
150	AT406	IAP Inhibitor

Supplemental Table 3.3. Screen #2 compound list.

Compounds #1-40	Chemical name	Targeted Pathway	Compound # from Screen 1
1	Salubrinal	inhibitor of eIF2α phosphatase	N/A
2	Luteolin	antioxidant flavonoid	N/A
3	Ebselen	glutathione peroxidase mimetic	N/A
4	4BPA	ER stress inhibitor	N/A
5	LY294002	pan-PI3K inhibitor	33
6	LCK Inhibitor	Lck inhibitor	N/A
7	ABT-737	inhibits Bcl-2 and Bcl-xL	149
8	AT406	IAP Inhibitor	150
9	Tyrphostin A9	PDGF	2
10	U-73122	PLC inhibitor	5
11	DMSO	Control	11
12	ARP 101	MMP-2 inhibitor	22
13	Quercetin dihydrate	antioxidant flavonoid	21
14	Imazodan	phoshodiesterase III inhibitor	40
15	MNS	tyrosine kinase inhibitor	44
16	HA-1004 hydrochloride	inhibitor of protein kinase G (PKG) and PKA	45
17	GW5074	cRaf1 kinase inhibitor	51
18	GW2974	EGFR / ErbB-2 inhibitor	52
19	AS 604850	ATP-competitive PI3Kγ inhibitor	53
20	GW1929	PPAR-γ activator	54
21	Clodronic acid	Calcium modulator, used to deplete BM macrophages	65
22	SANT-1	Inhibits Sonic hedgehog signalling	66
23	1,7-Dimethylxanthine	Adenosine receptor ligand	67
24	Cambinol	inhibits NAD-dependant deacetylases	68
25	L-Cycloserine	serine palmitoyltransferase inhibitor	81
26	BTO-1	polo-like kinase	82
27	CP55940	non-selective cannabinoid receptor agonist	83
28	Bay 11-7085	inhibitor of IκBα phosphorylation	84
29	AS-252424	PI3K-gamma inhibitor	86
30	Bromoenol lactone	inhibitor of calcium-independent phospholipase A2	87
31	TBBz	Casein Kinase-2 (CK2) inhibitor	88
32	Diacylglycerol kinase inhibitor I	Diacylglycerol kinase inhibitor I	89
33	Aurintricarboxylic acid	inhibitor of ribonuclease and topoisomerase II	121
34	3-Aminopropionitrile fumarate	lysyl oxidase (LOX) inhibitor	122
35	1-Aminobenzotriazole	inhibitor of cytochrome P450 and chloroperoxidase	123
36	Sandoz 58-035	Acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor	124
37	Acetylthiocholine chloride	nicotinic acetylcholine receptor agonist	125
38	A-315456	α1D-adrenoceptor antagonist	126
39	Agmatine sulfate	Nitric oxide modulator, NADPH oxidase activator	127
40	Arcaine sulfate	NMDA glutamate receptor antagonist	128

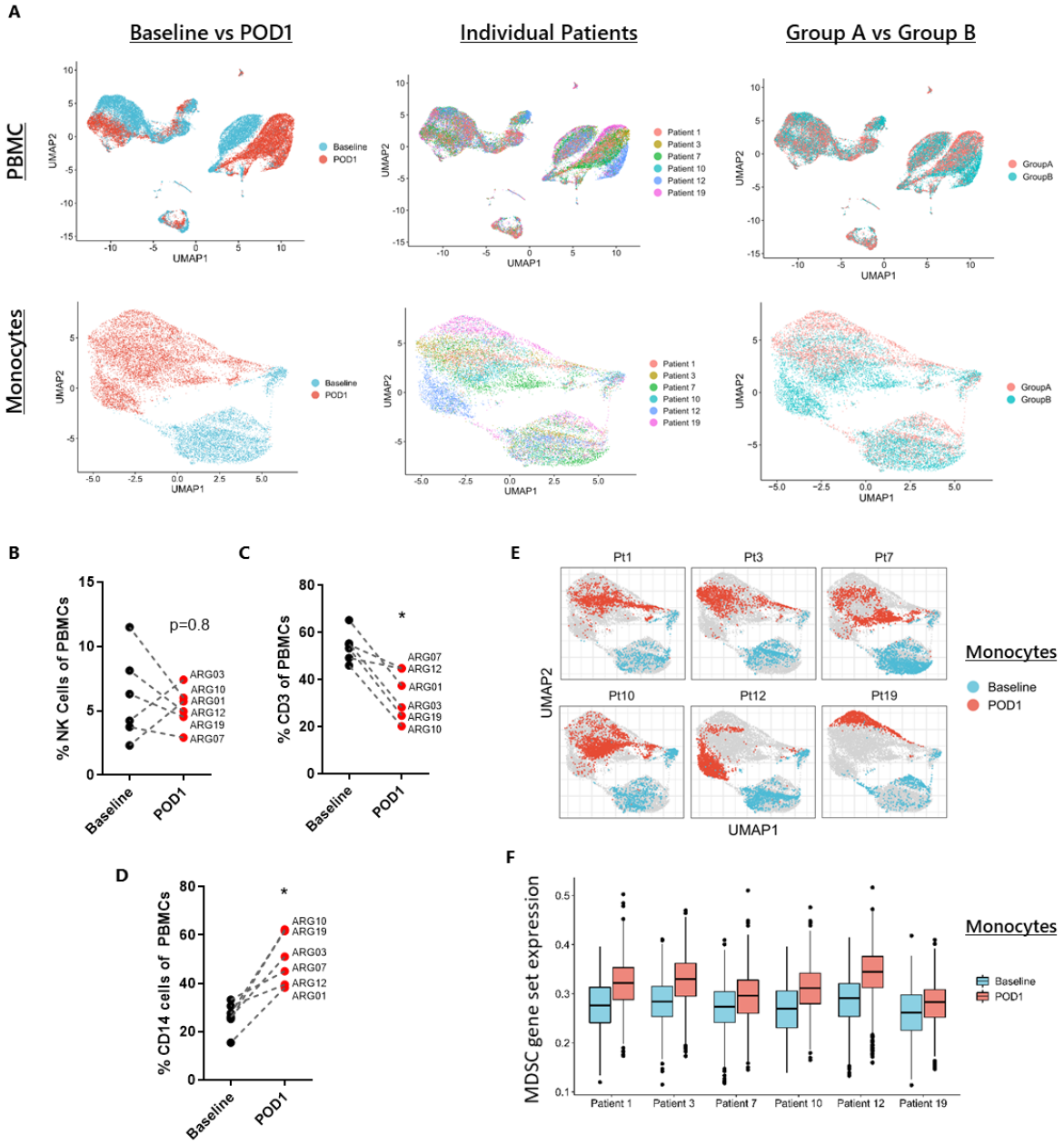
Supplemental Table 3.4. List of top hits from Screens #1A, 1B, 2A, and 2B.

Screen #1 (Supplemental Table 2)

Screen	Compound # Screen 1	Compound	Target	Avg % Dead K562	Dead normalized	% suppression	>50% improved?
S1A	Controls	NK92:K562 (max killing)		44.3	100%	0%	
S1A	Controls	NK92:MDSC:K562 (MDSC suppression)		17.0	38%	62%	Cutoff = 31%
S1A	33	LY-294,002 hydrochloride	pan-PI3K inhibitor	39.3	89%	11%	yes
S1A	58	7-Cyclopentyl-5-(4-phenoxy)phenyl-7H-pyrrolo[2,3-d]pyrimidin-4-ylamine	Lck inhibitor	34.9	79%	21%	yes
S1A	52	GW2974	EGFR / ErbB-2 inhibitor	33.6	76%	24%	yes
S1A	143	Amiprilose hydrochloride	Inhibits PLA2 substrate availability	32.3	73%	27%	yes
S1A	51	GW5074	cRaf1 kinase inhibitor	31.9	72%	28%	yes
S1A	132	3-Amino-1-propanesulfonic acid sodium	GABAA receptor agonist	31.2	70%	30%	yes
S1A	69	Capsazepine	TRPV1 receptor antagonist	30.8	69%	31%	yes
S1B	Controls	NK92:K562 (max killing)		53.7	100%	0%	
S1B	Controls	NK92:MDSC:K562 (MDSC suppression)		28.1	52%	48%	Cutoff = 24%
S1B	20	Bay 11-7082	inhibitor of IκBα phosphorylation	46.6	87%	13%	yes
S1B	139	3-aminobenzamide	PARP inhibitor	45.9	85%	15%	yes
S1B	71	Y-27632 dihydrochloride	Rho kinase inhibitor	44.3	83%	17%	yes
S1B	74	2-Chloroadenosine	adenosine receptor agonist	41.7	78%	22%	yes
S1B	133	(±)-2-Amino-3-phosphonopropionic acid	metabotropic glutamate receptor agonist	41.3	77%	23%	yes
S1B	141	5-(N-Ethyl-N-isopropyl)amiloride	Sodium-hydrogen exchanger inhibitor	40.9	76%	24%	no
S1B	33	LY-294,002 hydrochloride	pan-PI3K inhibitor	39.6	74%	26%	no

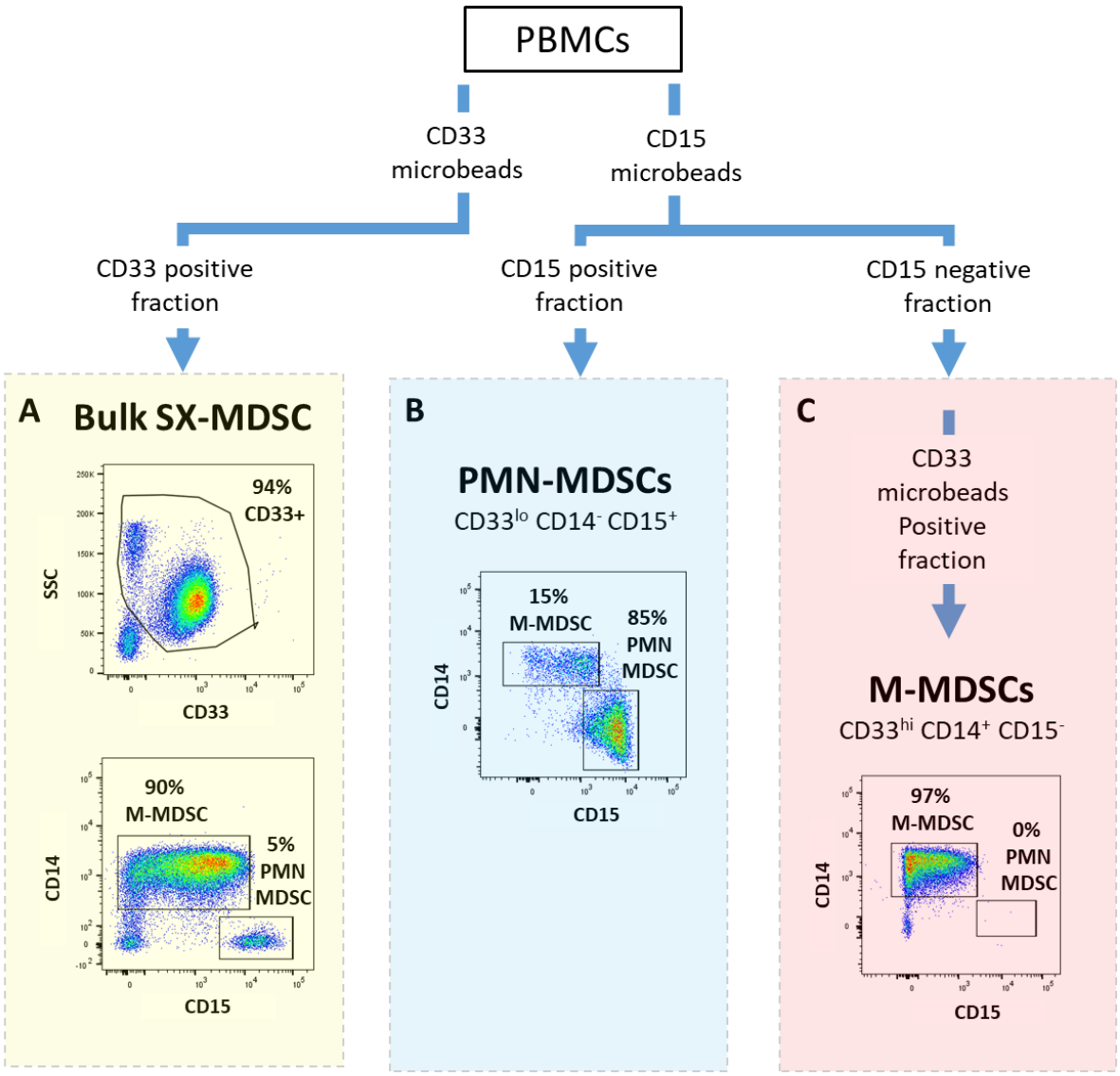
Screen #2 (Supplemental Table 3)

Screen	Compound # Screen 2	Compound	Target	Avg % Dead K562	Dead normalized	% suppression	>50% improvement?
S2A	Controls	NK92:K562 (max killing)		56.7	100%	0%	
S2A	Controls	NK92:MDSC:K562 (MDSC suppression)		29.8	53%	47%	Cutoff = 23.5%
S2A	17	GW5074	cRaf1 kinase inhibitor	44.9	79%	21%	yes
S2A	5	LY294002	pan-PI3K inhibitor	44.7	79%	21%	yes
S2A	10	U-73122	PLC inhibitor	44.1	78%	22%	yes
S2A	*28*	*Bay 11-7085*	*inhibitor of IκBα phosphorylation*	*42.5*	*75%*	*25%*	no
S2A	*4*	*4BPA*	*ER stress inhibitor*	*42.0*	*74%*	*26%*	no
S2A	*21*	*Clodronic acid*	*Calcium modulator, depletes BM macrophages*	*41.3*	*73%*	*27%*	no
S2B	Controls	NK92:K562 (max killing)		35	100%	0%	
S2B	Controls	NK92:MDSC:K562 (MDSC suppression)		11.5	33%	67%	Cutoff = 33.5%
S2B	5	LY294002	pan-PI3K inhibitor	26.9	77%	23%	yes
S2B	29	AS-252424	PI3K-gamma inhibitor	25.1	72%	28%	yes
S2B	22	SANT-1	Inhibits Sonic hedgehog signalling	23.3	67%	33%	yes
S2B	*21*	*Clodronic acid*	*Calcium modulator, depletes BM macrophages*	*23.2*	*66%*	*34%*	no
S2B	*28*	*Bay 11-7085*	*inhibitor of IκBα phosphorylation*	*23.2*	*66%*	*34%*	no
S2B	*10*	*U-73122*	*PLC inhibitor*	*23.0*	*66%*	*34%*	no



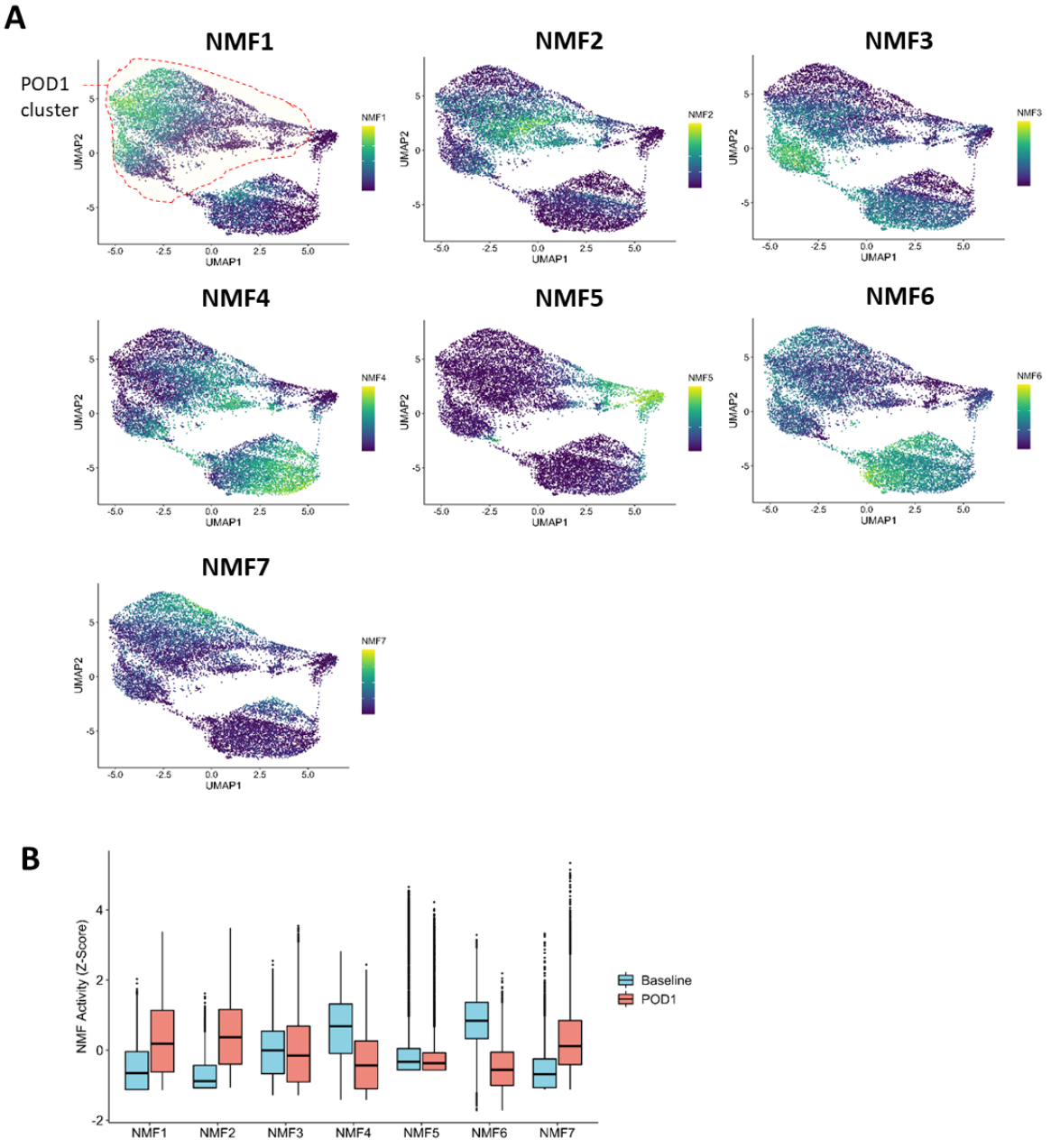
Supplemental Figure 3.1. scRNA-seq extended data 1.

A) UMAP plots of all PBMCs (upper) or monocyte (bottom) populations grouped by: Baseline vs POD1 (left); individual patient (middle); or Group A vs Group B (right). **B-D)** PBMC samples used for scRNA-seq were simultaneously phenotyped by flow cytometry for changes in the proportion of **(B)** CD3-CD56+ NK cells, **(C)** CD3 T cells, and **(D)** CD14 monocytes before and after surgery. **E)** Overlaying the contribution of each individual patient set as grouped by Baseline vs POD1 in the PBMC UMAP plots. **F)** Comparing the expression of MDSC gene set scores for individual patient monocyte populations on Baseline and POD1 (greater = more MDSC-like).



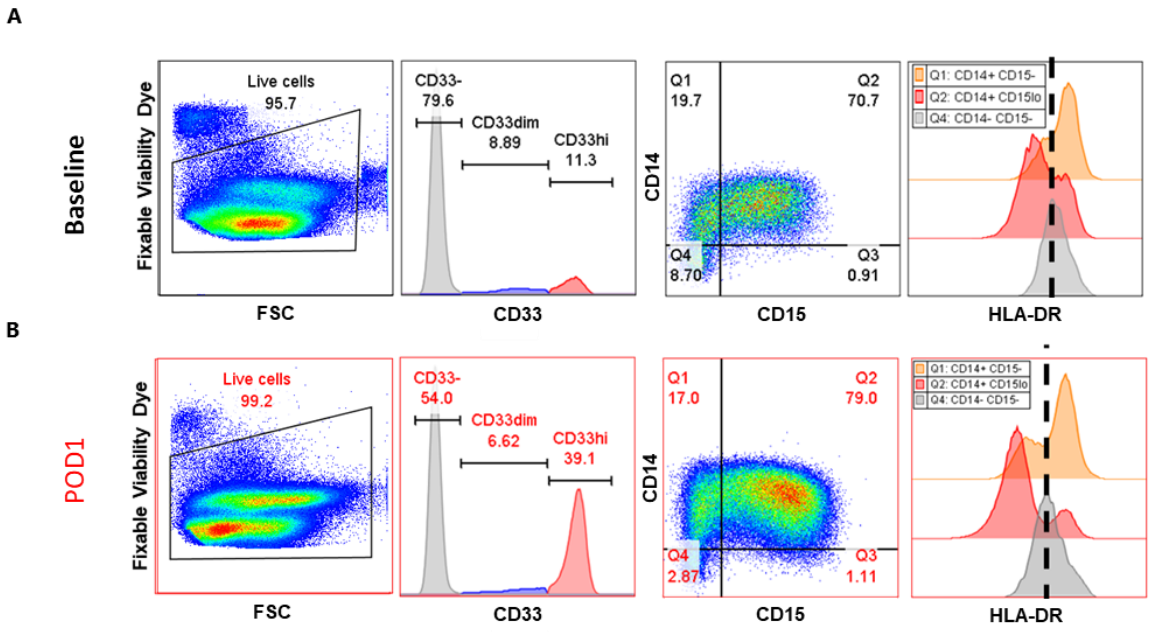
Supplemental Figure 3.2. Workflow for isolating Sx-MDSC subtypes.

A) Bulk Sx-MDSCs had a purity of ~95% and contains both M-MDSCs and PMN-MDSCs. **B)** PMN-MDSCs were sorted with a purity of 80-85%. **C)** The M-MDSC sort had a purity of >95%.



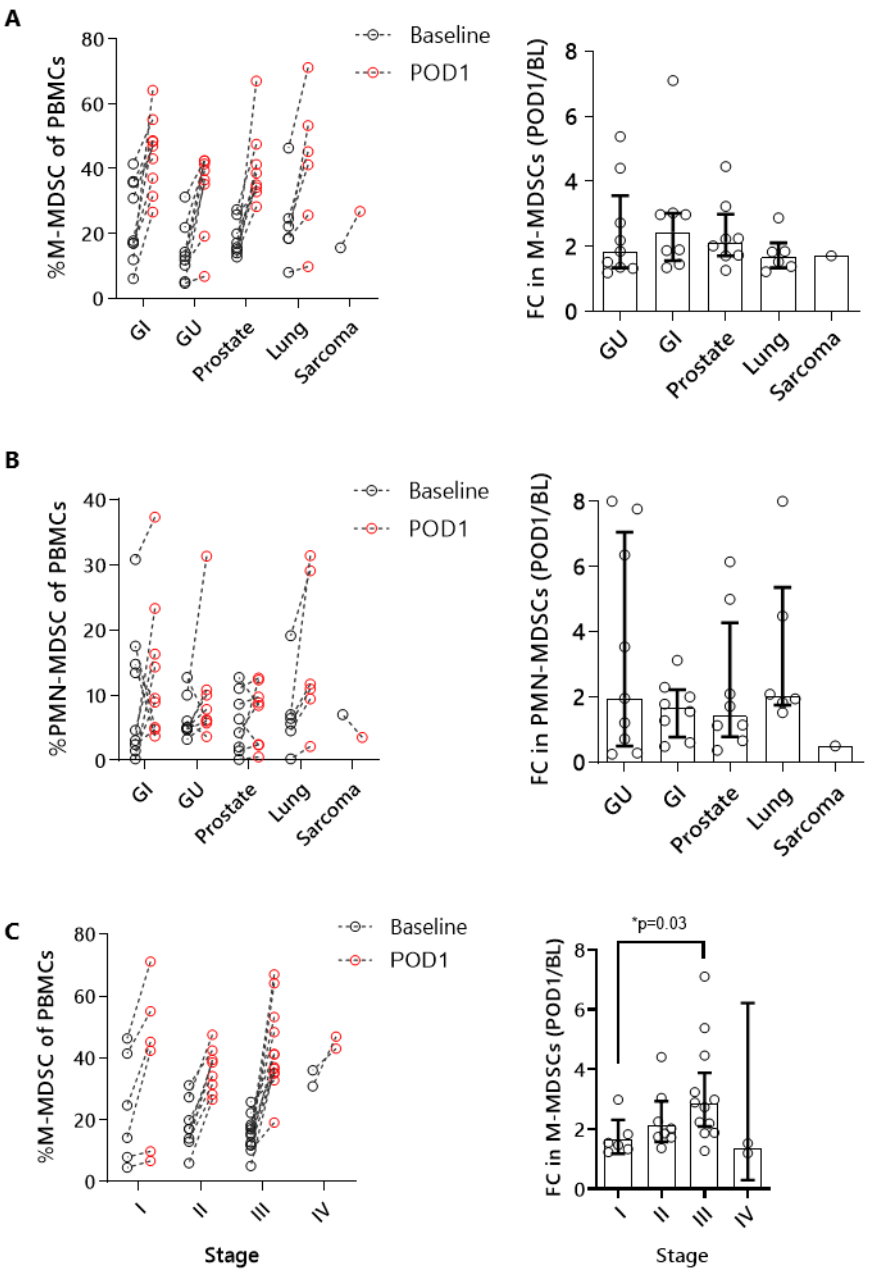
Supplemental Figure 3.3. scRNA-seq extended data 2 – NMF plots.

A) NMF plots of all 7 gene expression profiles and which monocytic cells express them. The highlighted, red circled population in NMF1 demarcates where the POD1 monocytes clustered to help with the interpretation. **B)** Histograms displaying the number cells that express a certain NMF program either on Baseline or POD1.



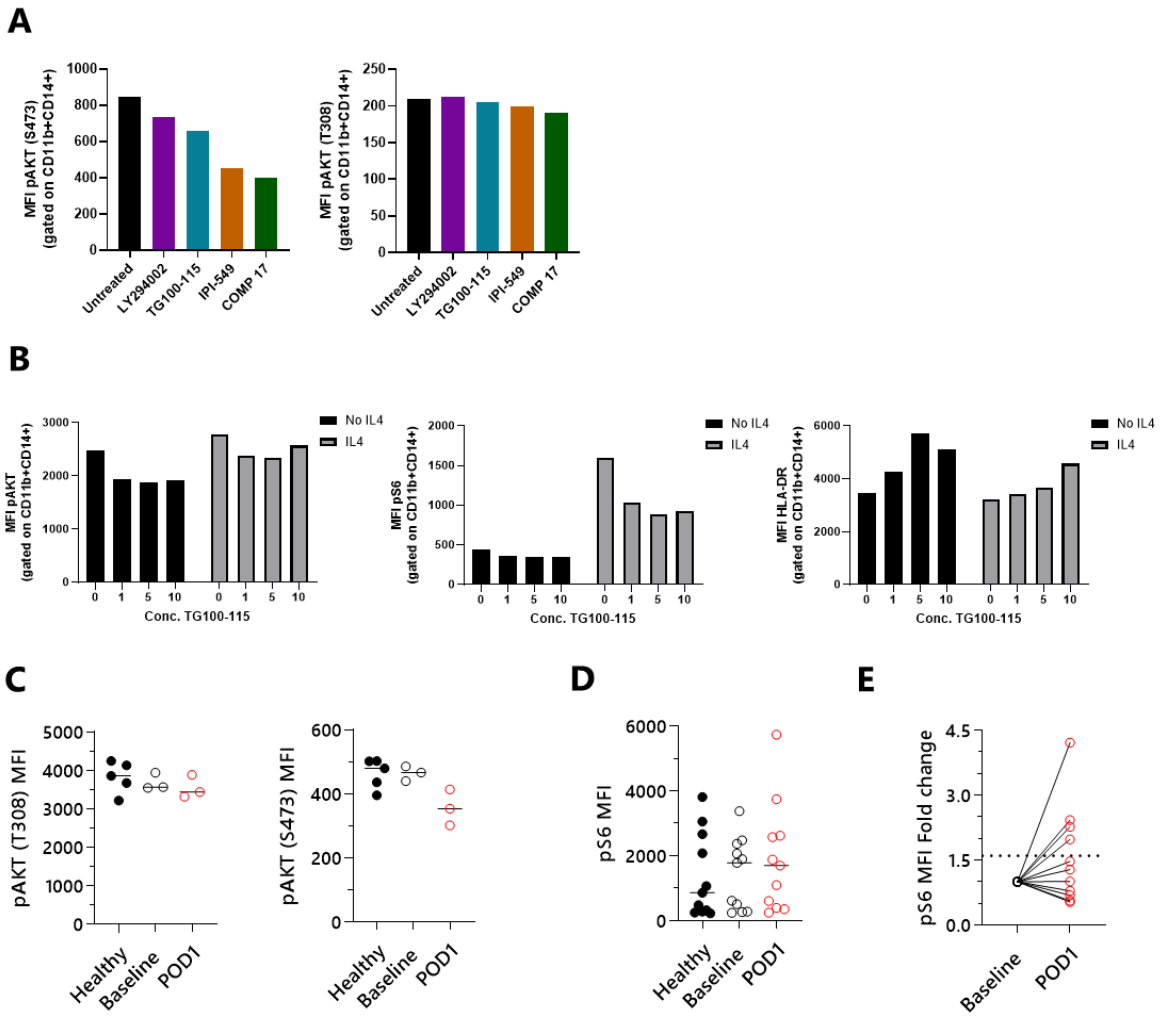
Supplemental Figure 3.4. Gating strategy for MDSC immunophenotyping.

Patient PBMCs (baseline or POD1) or murine splenocytes (no surgery or surgery) were stained for phenotypic characterization by flow cytometry immediately after isolation. **(A-B)** Doublets, debris, and dead cells were excluded and live cells were analysed. $CD33^{hi}$ cells were gated on and then analysed for CD14 vs CD15 and HLA-DR expression. The $HLA\text{-}DR^{lo}$ cutoff is shown as a black dotted line. **(C-D)** Doublets, debris, and dead cells were excluded and live cells were analysed for NK cells ($CD3^{-}DX5^{+}$), T cells ($CD3^{+}$) and myeloid cells ($CD3^{-}DX5^{-}$). Myeloid cells were further classified by their expression of CD11b and Ly6G/Ly6C.



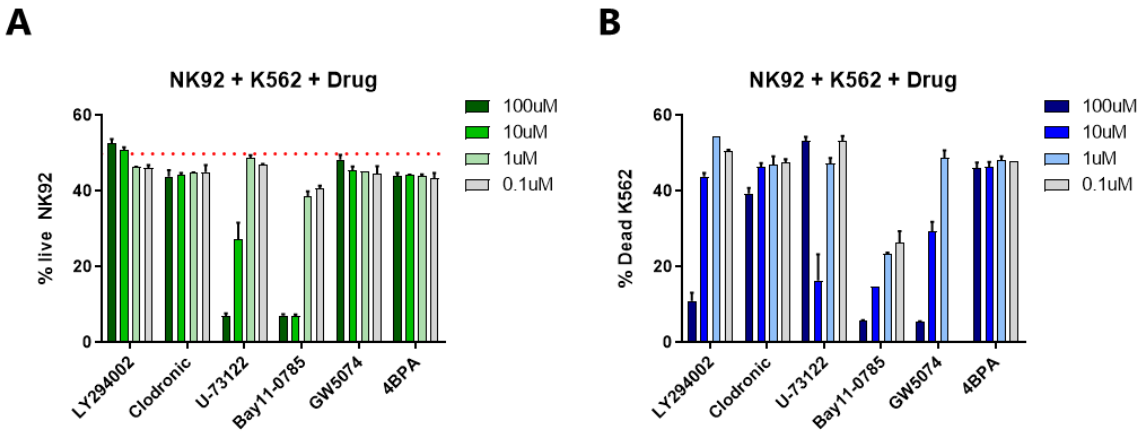
Supplemental Figure 3.5. Subgroup analysis on expansion of M-MDSCs.

A) The effect of various cancer surgery on the %M-MDSCs (left, $CD33^{+}Lin^{-}CD14^{+}CD15^{lo}HLA\text{-}DR^{lo}$) and the fold change on POD1 (right). **B)** %PMN-MDSCs (left, $CD33^{+}Lin^{-}CD14^{-}CD15^{hi}$) and the fold change on POD1 (right). **C)** Stratifying by %M-MDSCs (left) and the FC in M-MDSC according to cancer stage.



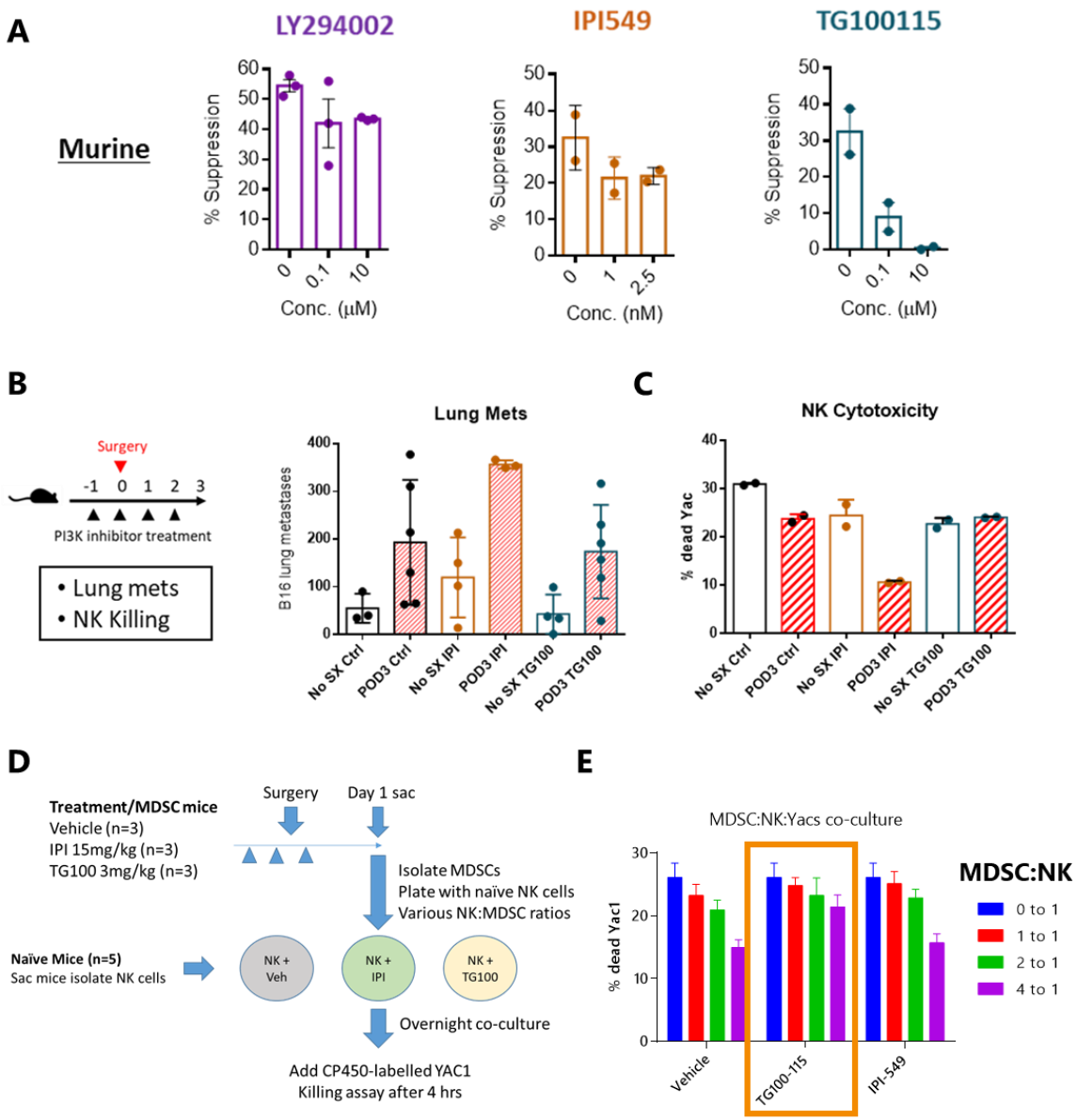
Supplemental Figure 3.6. Phospho-flow cytometry for pAKT and pS6 gated on Sx-MDSCs +/- PI3K inhibitors.

A) Effect of PI3K inhibitors (each at 10uM for 40minutes) on AKT phosphorylation (S473 and T308) in unstimulated whole blood gated on Sx-MDSCs (n=1). **B)** Dose response of TG100-115 on HLA-DR (left), pAKT (mid) and pS6 (right) gated on Sx-MDSCs (n=1). **C)** AKT phosphorylation at T308 (right) and S473 (left) were measured from healthy donors (n=5), and matched cancer surgery patients at Baseline and POD1 (n=3). **D)** pS6 MFI and **E)** Fold change in pS6.



Supplemental Figure 3.7. Validation of top candidate compounds following compound screen.

NK92s were incubated for 24 hrs with increasing concentrations of inhibitors. **A)** Effect of inhibitors on NK cell viability and **B)** NK cell cytotoxicity.



Supplemental Figure 3.8. Systemic delivery of PI3K-γ inhibitors does not prevent metastases in our B16F10LacZ murine model of surgical stress.

A) Ex vivo MDSC:NK suppression assay with murine POD1 MDSCs and naïve NK cells +/- PI3K inhibitors. **B)** Outline and experimental endpoints (right). Lung metastases after surgery +/- inhibitors (right). **C)** NK cell function on POD3 +/- inhibitors. **D)** Schematic of murine MDSC suppression assay. MDSCs were isolated on POD1 from mice following in vivo treatment with PI3K inhibitors and seeded with naïve murine NK cells. **E)** Results of suppression assay.

*This figure will be discussed in Chapter 6, General Discussion

Chapter 4

Perioperative Arginine Accelerates NK cell recovery after Surgery

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4.0 PREFACE

This chapter was submitted to Science Translational Medicine on February 9, 2021.

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Author contributions for this work are as follows:

Angka L: Performed the majority of the experiments. Analysed and created all of the figures. Wrote the manuscript in its entirety with editing help from Kennedy MA and Auer RC.

Souza CT: Performed all tumour inoculations and animal surgeries in addition to assisting with animal endpoints. Laboratory manager.

Baxter KE: Helped with animal endpoints and many flow cytometry experiments.

Khan S: Helped with animal endpoints and many flow cytometry experiments.

Market M: Helped with flow experiments and NK cell surface marker phenotyping after surgery.

Martel AB: Helped with experimental endpoints.

Tai L-H: Performed the NK cytotoxicity assay and lung metastases after MDSC-depletion experiments.

Kennedy MA: Provided oversight and guidance throughout the project. Helped with animal endpoint experiments. Assisted in the writing, editing and proofing of the manuscript.

Auer RC: Oversaw the entire project. Edited and proofed the manuscript.

The manuscript has been reformatted for the purpose of this thesis.

4.1 TITLE PAGE

Perioperative Arginine Prevents Metastases by Accelerating Natural Killer Cell Recovery After Surgery

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Disclosures

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Funding

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4.2 ABSTRACT

Profound Natural Killer (NK) cell suppression after cancer surgery is a main driver of metastases and recurrence, for which there is no clinically approved intervention available. Surgical stress is known to cause systemic postoperative changes that negatively modulate NK cell function including the expansion of surgery-induced Myeloid Derived Suppressor Cells (Sx-MDSCs) and a marked reduction in arginine bioavailability. In this study, we determine that Sx-MDSCs regulate systemic arginine levels in the postoperative period and that restoring arginine imbalance after surgery by dietary intake alone was sufficient to significantly reduce surgery-induced metastases in our preclinical murine models. Importantly, the effects of perioperative arginine were dependent upon NK cells. Although perioperative arginine did not prevent immediate NK cell immunoparalysis after surgery, it did accelerate their return to preoperative cytotoxicity, IFN-γ secretion, and activating receptor expression. Finally, in a cohort of colorectal cancer patients, postoperative arginine levels were shown to correlate with their Sx-MDSC levels. Therefore, this study lends further support for the use of perioperative arginine supplementation by improving NK cell recovery after surgery.

4.3 INTRODUCTION

Natural killer (NK) cells are the cytolytic cells of the innate immune system which are central for controlling metastatic disease [40]. Our group and others have shown that surgery results in a profound impairment in NK cell cytotoxicity and IFN-γ secretion in both cancer patients [38,65,67,210] and murine studies [35,63,139]. This period of diminished NK cell function after surgery is directly linked to increased postoperative lung metastases [35] and is the result of physiological changes that occur in response to surgery [1]. Notably, there is an expansion of Myeloid Derived Suppressor Cells (MDSCs) [211] in response to the proinflammatory state that ensues immediately after surgical insult [19,64]. These immature myeloid cells are known to express the arginine consuming enzymes, arginase-1 (Arg1) or inducer of nitric oxide synthase (iNOS) [108], depending on the inflammatory environment. Since the postoperative period is characterized by elevated IL-10, a Th2 cytokine known to induce Arg1 [122], we hypothesize that the large accumulation of Arg1-expressing MDSCs leads to the systemic depletion of arginine after surgery.

Arginine is a conditionally essential amino acid that is necessary for proper immune cell function [29] but is rapidly reduced after surgical trauma [1,87]. Insufficient arginine is known to impair T cells [212–214] and NK cells [89–91]. In the absence of arginine, T cells are less responsive due to reduced T cell receptor expression [29] and NK cells have decreased proliferation, cytotoxicity, and IFNγ production [92]. To combat the catabolic effects of surgery, perioperative arginine supplements have been investigated and shown to reduce the length of patient recovery time and the number of postoperative infections and complications [131–133,215]. Whether or not arginine supplementation has anti-metastatic properties as a result of beneficial immunomodulatory effects on NK cells has yet to be studied.

Here, we investigated the cause of postoperative arginine depletion and assessed the therapeutic potential of an arginine enriched diet (AED) to modulate perioperative arginine levels and prevent surgery induced metastases. We report that surgery-induced MDSCs (Sx-MDSCs) regulate postoperative arginine bioavailability and that a perioperative AED attenuates metastases by accelerating NK cell recovery after surgery, a phenomenon and mechanism that has not been previously reported.

4.4 MATERIALS AND METHODS

Study Design

The objective of this research was to determine a therapeutic effect for a perioperative diet enriched in arginine in reducing postoperative metastases. For all animal experiments, initial experiments were done using at minimum, n=3 mice per group. Experiments were performed multiple times on different days in order to replicate initial findings and increase the sample size. Two murine models were used which allowed us to study perioperative arginine supplementation from two unique perspectives. In the intravenously (i.v.) injected B16F10 model in C57BL/6 mice and the orthotopic 4T1 model in Balb/c mice. In both models, all samples were normalized to the average number of metastasis quantified in the no surgery control group. This allowed us to compare the increase in metastases, as a fold-change, between different experimental runs. For the 4T1 model, we prospectively determined that lung metastases from mice with primary tumours ≥ 0.3g would be analysed in a separate analysis from primary tumours <0.3g. For cytotoxicity and suppression assays, samples were plated in triplicates and replicated in subsequent experiments under the same conditions. To ensure we collected as much data as possible from our animal experiments, we would often perform many experimental endpoints simultaneously such quantifying as

lung metastases, characterizing immune cells by flow cytometry, and collecting blood samples for amino acid measurements.

We also used data that was collected from the Phase II clinical trial (NCT02987296) to assess if arginine levels correlated with the number of Sx-MDSCs or NK cell cytotoxicity.

Mice and cell lines

Six week-old female C57BL/6 and Balb/c mice were purchased from Charles Rivers Laboratory and housed in pathogen-free conditions. All studies were done in compliance with the guidelines of the Animal Care Veterinary Service facility (University of Ottawa). The B16F10LacZ melanoma cell line was obtained from Dr. K Graham (London, Ontario) and maintained in cDMEM. 4T1, YAC-1, K562, NK92-MI cell lines were purchased from ATCC and maintained in cRPMI.

Murine models of surgical stress and treatment regimens

The experimental metastasis model was performed in C57BL/6 mice by inoculating $3x10^5$ B16F10LacZ melanoma cells via tail vein injection 30 minutes prior to inflicting surgical stress by abdominal laparotomy and left nephrectomy. On postoperative day (POD) 3, lungs from euthanized mice were stained with X-gal to visualize the tumor metastases for quantification [35]. The AED (4% arginine + Teklad Global 16% protein rodent diet, ENVIGO) in the B16F10 model were given for 14 days prior to surgery and on all postoperative days. Balb/c mice were used in the orthotopic 4T1 breast cancer model which leads to spontaneous lung metastases. $1x10^5$ 4T1 cells were inoculated orthotopically and resected 14 days later, with or without major surgical stress (abdominal laparotomy and left nephrectomy). Mice were left to recover for another 14 days and then euthanized in order to quantify the macroscopically visible 4T1 lung metastases [216]. AEDs were given 5 days before and 5 days after surgery (perioperatively) in the 4T1 model. The Arg1 inhibitor, CB-

1158 (Calithera Biosciences) [217], was given twice daily by oral gavage starting 3 days before surgery.

Flow cytometry staining and analysis

Mouse spleen and saphenous blood samples were collected and RBCs were lysed with Ammonia Chloride Potassium (ACK) lysis buffer. $1x10^6$ cells were stained with Fixable Viability Stain 510 (BD Bioscience #564406) and Mouse BD Fc Block™ (BD Bioscience #553142) prior to extracellular staining. Anti-mouse antibodies used in this study included: CD3e (Clone 500A2, AF700 BD), CD11b (Clone M1/70, FITC, PeCy7 Biolegend), Ly6G (Clone 1A8; BD; PE-CF594, PeCy7), Ly6C (Clone AL-21 PerCP-Cy5.5 BD), Gr-1 (Clone RB6-8C5; Biolegend; FITC, Pe-Cy7), Arginase-1 (R&D systems APC, Catalogue #IC5868A), DX5/CD49b (PE BD), NK1.1 (PE-CF594 BD), DNAM-1/CD226 (BV421 Biolegend), and NKG2D/CD314 (FITC Biolegend). Samples were acquired with the BD Fortessa LSRII and analyzed with FlowJo v10.

Isolation of Surgery-induced MDSCs

Sx-MDSCs (CD11b+Ly6G+ cells) were isolated from mouse splenocytes on POD1 by magnetic separation using the Myeloid-Derived Suppressor Cell Isolation kit (Miltenyi #130-094-538). Human Sx-MDSCs were isolated from human peripheral blood mononuclear cell (PBMC) samples on POD1 by magnetic separation using anti-CD33 microbeads (Miltenyi #130-045-501) which results in a purified population of Sx-MDSCs (Lin-CD33+CD14+CD15+). The Miltenyi autoMACS was used to obtain both sorted cell populations.

NK Cell isolation

Murine NK cells were isolated with the EasySep™ Mouse NK cell Isolation Kit (StemCell #19855) via magnetic bead separation from splenocytes. Human NK cells were isolated with the StraightFrom® Whole Blood CD56 MicroBeads magnetic separation kit (Miltenyi

#130-090-875).

Natural Killer cell Cytotoxicity and MDSC:NK cell Suppression Assays

NK cell cytotoxic potential was determined by isolating NK cells from surgery or no surgery, treated or untreated, mice and incubating them for 4 hours with CP450-labelled (eBioscience) NK specific target tumour cells (either Yac1 or K562 for murine or human studies, respectively) in a 96-V bottom plate in triplicates. Following incubation, Ethidium homodimer (Invitrogen™ E1169) was added to each sample well before acquiring by flow cytometry. NK cell cytotoxicity was measured by quantifying the proportion of dead EtHD+ CP450+ target cells. To measure the suppressive effects of MDSCs on NK cell cytotoxicity, MDSCs were isolated before surgery or on POD1 and seeded together with naive NK cells at increasing MDSC:NK cell ratios for 24 hrs at 37°C. The following day, CP450-labelled target cells were added to the MDSC:NK co-cultures for 4 hours and NK cell cytotoxicity was quantified as described above.

Arg1 activity assay

Arg1 activity in MDSC lysates was determined by measuring the conversion of arginine to urea as previously described [218]. Briefly, lysates, collected by lysing $1x10^5$ magnetically sorted MDSCs with 0.1% Triton X-100 + 1X EDTA-free Protease inhibitor, were heat activated by incubating at 50°C for 15 min. L-arginine was then added to the lysates for 2 hours at 37°C and Arg1 activity quantified by the amount of urea produced (detected by adding α-isonitrosopropiophenone and measuring absorbance at 490nm).

Peripheral blood Amino Acid measurements

Murine and human blood samples (75µl) were spotted onto Whatman 903 Protein Saver Cards (Sigma-Aldrich) and immediately stored at -20ºC until analysis by LC-MS/MS at Newborn Screening Ontario, Children's Hospital of Eastern Ontario as previously described

[219]. The amino acids measured included arginine, ornithine, citrulline, argininosuccinate, leucine, valine, phenylalanine, alanine, glycine, methionine, and tyrosine.

In vivo MDSC, NK cell, or T cell depletion experiments

MDSCs were depleted prior to surgery by 3 preoperative intraperitoneal (i.p.) injections of 100µg/mouse InVivoMab anti-mouse Ly6G/Ly6C (Gr-1; RB6-8C5) (BioxCell; BE0075-5MG-A) on days -3, -1, and the day of surgery. MDSC depletion was confirmed by flow cytometry staining with the Ly6G clone 1A8 (PeCy7 Biolegend). NK cells were depleted by injecting 200ug of anti-NK1.1 (PK136) i.p. on POD -4, -1, and +1, as previously described [35]. Double depletion of CD4 and CD8 T cells were performed on POD -2, -1, and +1 via 100ug of anti-CD4 (GK1.5) and 50ug of anti-CD8β2 (53-5.8) injected i.p.

Natural Killer cell activity assay (NK Vue)

The murine NK-Vue^{TM} kit (ATGen Canada/NKMax) was used to measure the amount of IFN-γ secreted (ELISA) by NK cells after a whole blood stim with using a proprietary NK cell-specific cytokine cocktail (Promoca).

Sx-MDSC Conditioned media/arginine depleted media

RPMI-1640 without arginine, leucine, lysine, and phenol red (R1780, Sigma-Aldrich) was reconstituted with L-leucine (0.05g/L, Sigma), L-lysine (0.04g/L, Sigma), and increasing amounts of L-arginine (Sigma). Sx-MDSCs were isolated from cancer surgery patients (n=3) on POD1 via CD33+ microbead magnetic sorting (Miltenyi) and plated in reconstituted 50uM Arginine media for 24hrs. Following the incubation, Sx-MDSCs were removed by pelleting and collecting the supernatants two times. NK92-MI cells were counted and plated in PBS and immediately spun down to remove the PBS. The NK92-MI cells were resuspended in the Sx-MDSC conditioned media/arginine depleted media and

incubated for 24hrs. CP450-labelled K562 target cells were then added to the plates and NK cell cytotoxicity was measured by flow cytometry after 4 hrs of incubation.

Human Patient Data Set

All patients that participated in this study have given informed consent and the research ethics board has approved our study under Ottawa Health Science Network Research Ethics Board (20160732-01H). For the human colorectal cancer surgery patient studies we used the patient data set from the PERIOP-02 clinical trial (NCT02987296).

Statistical Analysis

Statistical tests were performed using GraphPad Prism 8 and described within the figure legends. Generally, unpaired, non-parametric Mann-Whitney U tests were performed when comparing between two groups (e.g. No surgery vs surgery). When assessing changes over time from the same animal (e.g. amino acid concentrations time course), a paired, non-parametric, Friedman test was used. For the Sx-MDSC depletion studies we used a matched two-way ANOVA, with the Dunnett's multiple comparisons test. Wilcoxon rank-sum tests were used to compare matched patient samples at different time points. Significance was assigned when *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001.

4.5 RESULTS

Surgery-induced MDSCs expand and persist postoperatively with increased Arg1 expression and activity

To assess the prometastatic effects of surgery we utilized two models of surgical stress: *i)* the experimental B16F10 melanoma metastasis model in C57BL6 mice (Fig. 1A) and *ii)* the spontaneous orthotopic 4T1 breast cancer model in Balb/c mice (Fig. 1B). As previously reported, lung metastases significantly increased (B1610, n=18/group, P <0.0001; 4T1, n=12/group, P = 0.0008) in response to surgical stress [35,63,64]. Surgical stress resulted in

a significant expansion of monocytic (SSC^{low}, $CD3^{-}CD11b^{+}Ly6C^{hi}Ly6G^{neg}$) and polymorphonuclear (PMN; SSC^{hi}, $CD3^{-}CD11b^{+}Ly6G^{hi}Ly6C^{+}$) MDSCs, referred to now collectively as "Sx-MDSCs" (Fig. 1C and D; fig. S1). On postoperative day (POD) 1, PMN Sx-MDSCs had already significantly increased in both spleen and blood samples ($P < 0.0001$). In contrast, although circulating monocytic Sx-MDSCs were increased on POD1 ($P = 0.0006$) and POD3 ($P = 0.0002$), significant increases in the spleen were not observed until POD3.

Since the majority of Sx-MDSCs on POD1 were the PMN subtype, we measured their ability to suppress NK cells. Consistent with previous reports [64], on POD1, magnetically sorted PMN Sx-MDSCs significantly suppressed NK cell cytotoxicity (P = 0.03) compared to MDSCs isolated from non-surgically stressed mice (Fig 1E and F). Arg1 has been postulated to contribute to the suppressive function of PMN Sx-MDSCs [29,99,220], therefore, we measured the expression and enzymatic activity Arg1. We observed a >2-fold increase in Arg1 MFI in PMN Sx-MDSCs after surgery (Fig. 1G; n=8, $P = 0.0002$). Likewise, the lysates of Sx-MDSCs converted exogenous arginine into urea [218] at a >2-fold rate compared to naive mice (Fig. 1H; n=4, $P = 0.03$). Therefore, the large increase in Sx-MDSCs after surgery coupled with increased Arg1 activity may heavily contribute to the substantial drop in arginine levels documented after surgery [87,122,221].

Sx-MDSCs regulate systemic arginine bioavailability

Arginine, ornithine, citrulline, and argininosuccinate (Fig. 2A), as well as other amino acids (fig. S2) were measured at 0, 4, and 18hrs postoperatively in the B16F10 surgery model. Consistent with other reports [221], we observed a significant decrease in blood arginine at 4hrs post-surgery that had normalized by 18hrs post-surgery (Fig. 2B; n=17, $P = 0.0002$). Importantly, increased ornithine levels were evident at 18hrs post-surgery suggesting the

conversion of arginine into ornithine by Arg1. To determine whether Sx-MDSCs regulate systemic arginine levels, we depleted MDSCs before surgery and measured blood arginine levels at 4 and 18hrs after surgery (Fig. 2C). MDSC depletion attenuated the decrease in arginine concentrations after surgery without affecting preoperative arginine levels (Fig 2D), supporting the hypothesis that Sx-MDSCs are responsible for the postoperative reduction in systemic arginine availability. The NK cells isolated from MDSC depleted mice had greater cytotoxic potential compared to NK cells isolated from vehicle control mice on POD1 (Fig. 2E), however, cytotoxicity was not fully restored suggesting that other mechanisms of NK cell dysfunction likely exist. Lastly, the prometastatic effects of surgery were completely abrogated on POD3 in MDSC depleted mice (Fig. 2F). Together these findings suggest that Sx-MDSCs mediates arginine depletion and contributes to NK cell dysfunction postoperatively, and since arginine is necessary for NK cell function [89], we hypothesized that restoring the arginine imbalance after surgery will improve NK cell function and reduce metastases.

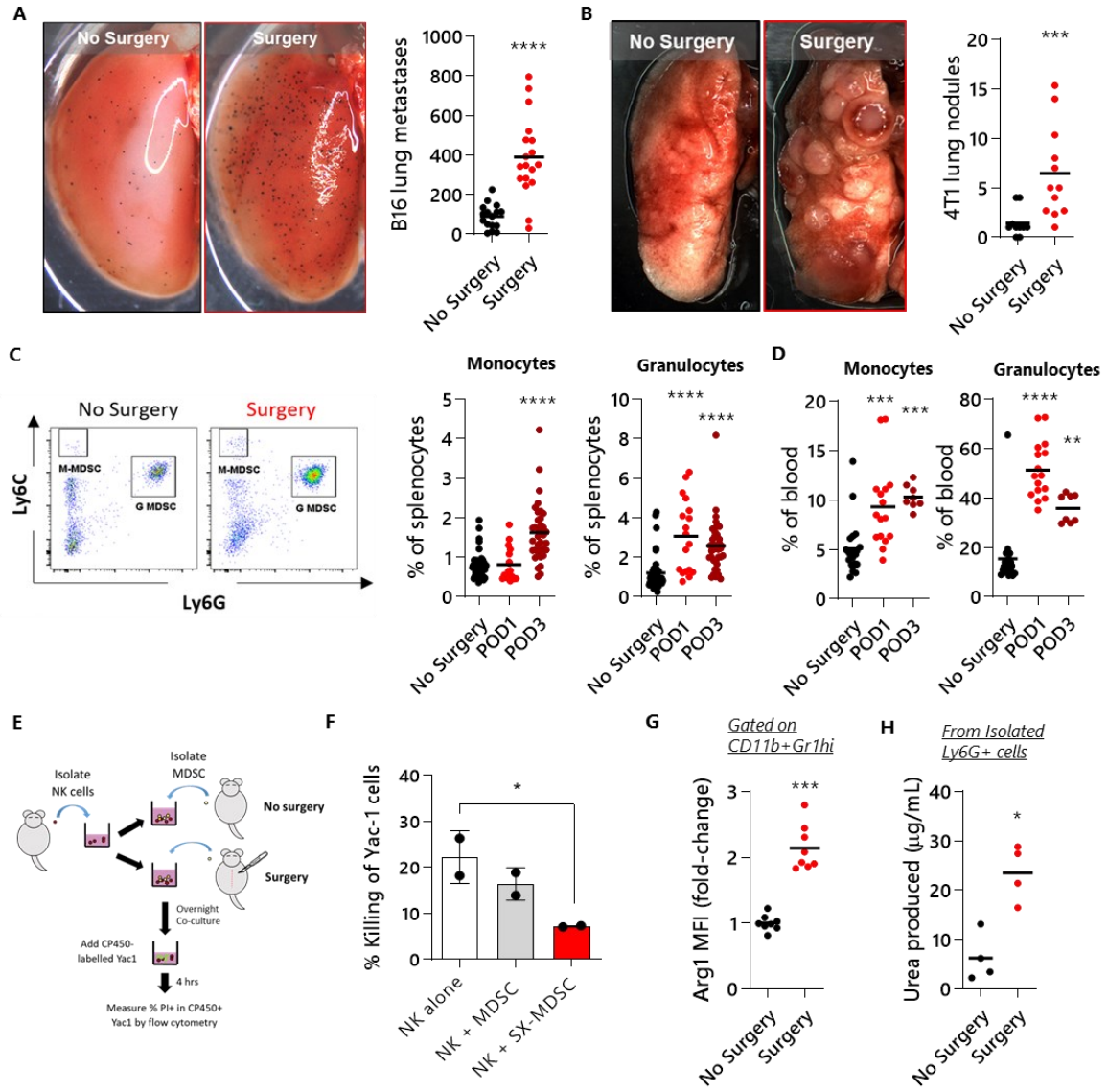


Figure 4.1. Surgery increases post-operative metastases and MDSCs.

Figure 4.1. Surgery increases post-operative metastases and MDSCs.

A) Representative lung images of mice injected with B16F10lacZ cell IV prior to receiving abdominal surgery (left). The lungs were harvested from the mice 3 days after surgery and lung metastases were enumerated (right; n=18/group from 3 experiments; Mann Whitney U tests). **B)** Orthotopic 4T1 tumors were inoculated in Balb/c mice and resected after 14 days +/- nephrectomy. Lung metastases were enumerated 14 days after resection (right; n=12/group from 2 experiments; Mann Whitney U tests). Monocytic and granulocytic cells were quantified by flow cytometry from **(C)** no surgery (n=43), POD1 (n=17), and POD3 (n=36) splenocytes (pooled from 11 experiments) or **(D)** no surgery (n=22), POD1 (n=16), and POD3 (n=8) blood (pooled from 4 experiments, Kruskal-Wallis test) of C57Bl/6 mice. **E)** Schematic of fluorescence based MDSC:NK cell co-culture and suppression assay. **F)** NK cell killing of Yac1 (n=2 mice/group, average of triplicates, Kruskal-Wallis test). **G)** Fold increase of Arginase-1 MFI gated on granulocytic cells ($CD11b^{+}Gr1^{hi}$) (n=8, Mann-Whitney U test). **H)** Arginase-1 activity from isolated $Ly6G^{+}$ cells as measured by urea production from no surgery or surgery mouse cohorts (n=4, Mann-Whitney U test). *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

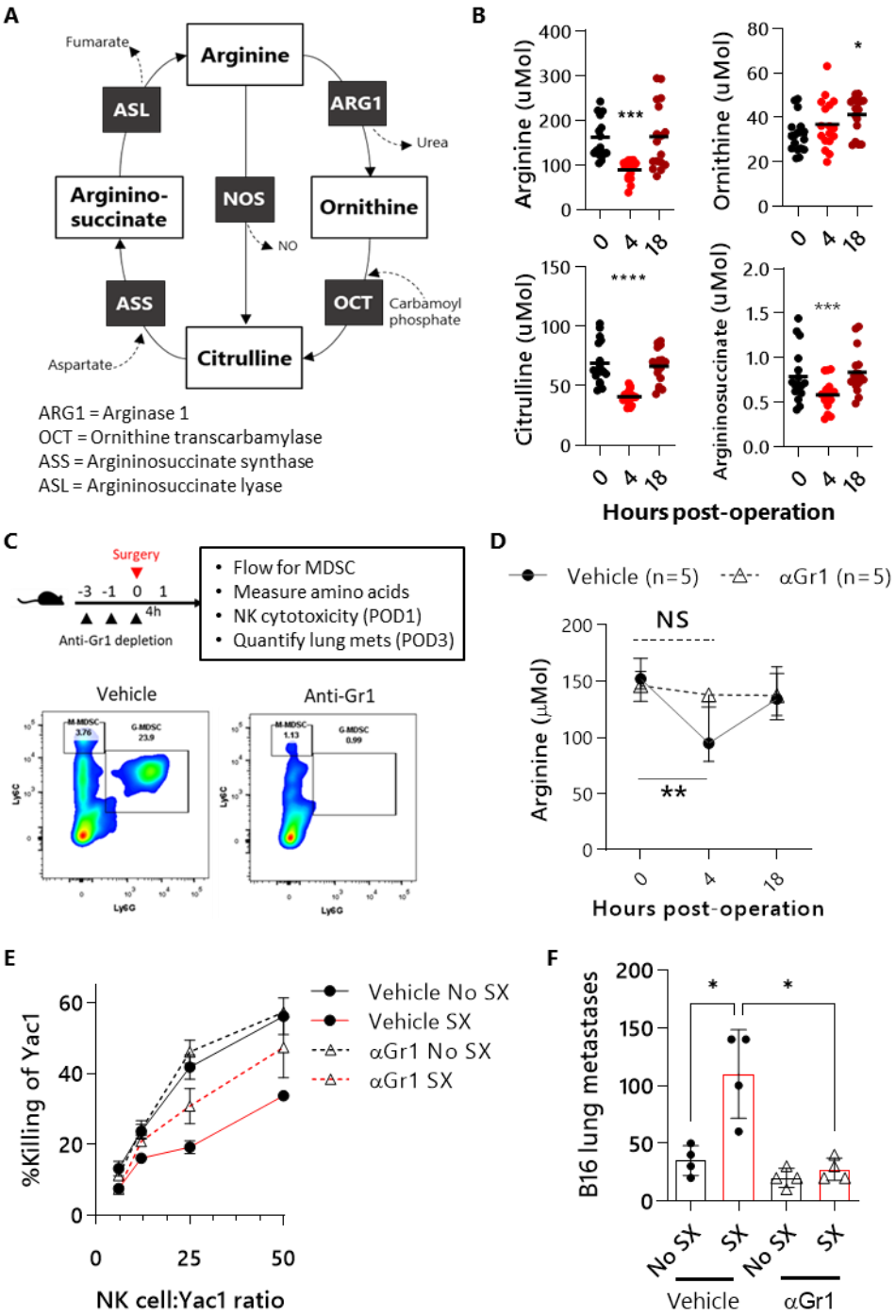


Figure 4.2. Surgery results in a rapid decrease in systemic arginine levels mediated by Sx-MDSCs.

Figure 4.2. Surgery results in a rapid decrease in systemic arginine levels mediated by Sx-MDSCs.

A) The Arginine cycle. **B)** Amino acid levels quantified by LCMS from murine blood samples at 0, 4, and 18 hours post-surgery (Friedman test). **C)** Anti-Gr1 (clone RB6-8C5) depletion time line and experimental endpoints (upper panels). Representative flow plots from spleens of vehicle or anti-Gr1 treated mice gated on live CD11b+ CD3- cells (lower panels). **D)** Blood arginine levels from vehicle or anti-Gr1 depleted mice at 0, 4, and 18 hours post-operation (Two way ANOVA, Dunnett's post test). **E)** NK cells were isolated from MDSC depleted and control animals after surgery and cytotoxicity was measured against Yac1 target cells. **F)** Depleting MDSCs prior to surgery attenuates post-operative metastases (Mann-Whitney U test). *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

Perioperative arginine supplementation controls metastatic disease

Although arginine can be produced *de novo*, in times of physiological stress arginine must be obtained externally through dietary sources [222]. Dietary arginine has been shown to have beneficial effects when given to patients perioperatively [132,223]. Therefore, to assess the effects of a perioperative arginine diet on postoperative metastases, we fed mice either an AED or control diet ad libitum (Fig. 3A and D; Supplemental Table 1). After 14 days of feeding in the B16F10 model, blood arginine levels from AED fed mice were significantly higher than control diet fed mice (1.5-fold at 0hrs; Fig. 3B). Unexpectedly, at 4hrs post-surgery the AED fed mice had similar levels of arginine as the control fed mice, despite being significantly higher preoperatively. However, the AED fed mice had a quicker return to baseline levels and were significantly higher (>1.5-fold) than control diet fed mice by 72hrs post-surgery. While there were no significant differences in the number of Sx-MDSC in AED compared to control diet fed mice (fig. S3A), AED fed mice had significantly reduced metastatic lung tumour burden following surgery (Fig. 3C). Additionally, we assessed whether an ornithine enriched diet could protect against surgery-induced metastases (Fig. 3D). The ornithine enriched diet was able to increase perioperative ornithine levels without affecting arginine, but there was no protection against surgery induced metastases (Fig. 3E and F). This suggests that arginine, rather than the downstream metabolite, ornithine, is required postoperatively.

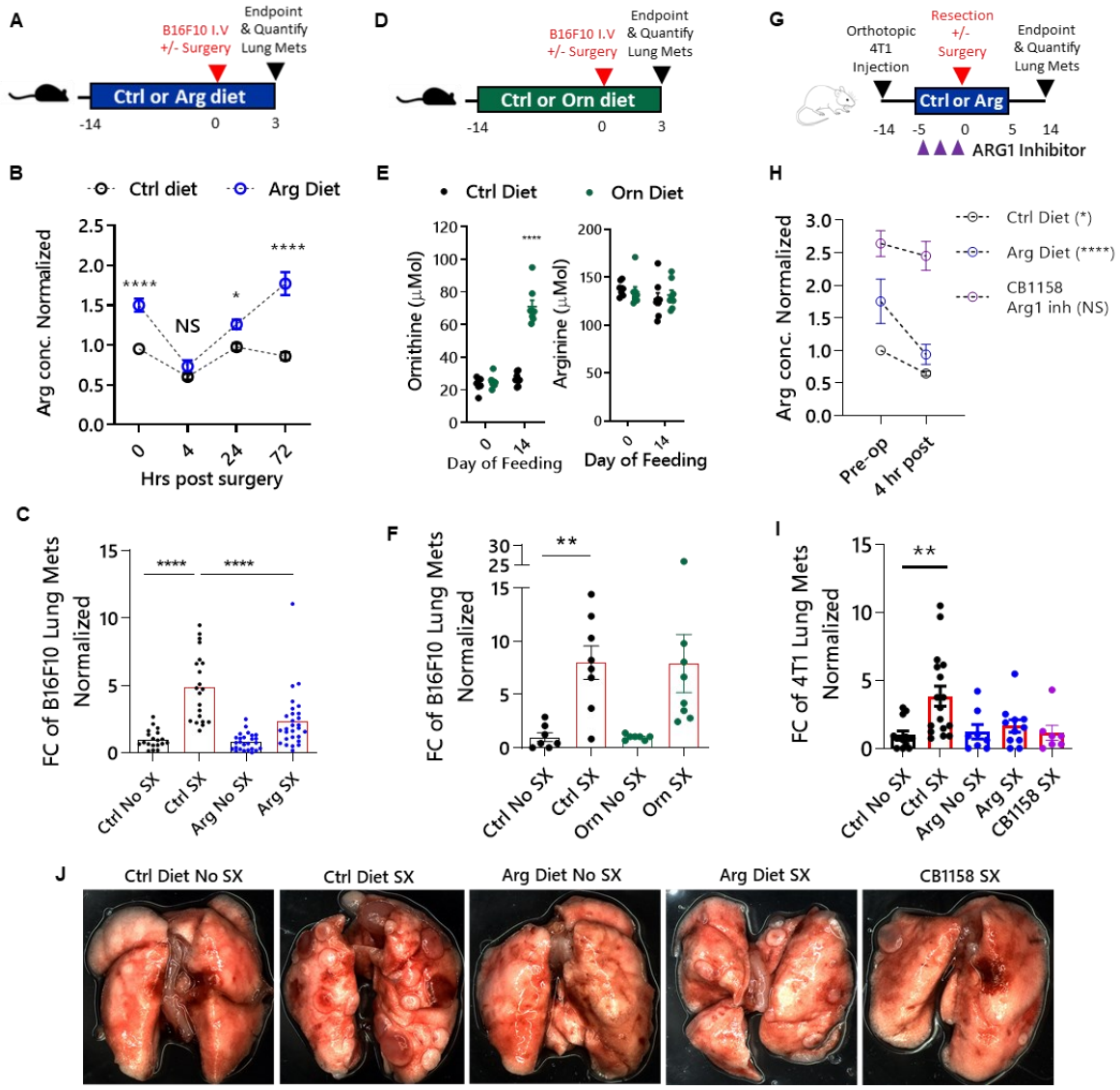


Figure 4.3. Dietary arginine supplementation increases arginine levels and reduces post-operative metastases in two murine models of surgical stress.

Figure 4.3. Dietary arginine supplementation increases arginine levels and reduces post-operative metastases in two murine models of surgical stress.

A) Schematic of the arginine diet regimen in B16F10 model of surgical stress. **B)** Arginine concentrations after 14 days of feeding, normalized to their baseline levels (n=12-28 mice/group, Mixed-effects model with Bonferroni's multiple comparisons test). **C)** Lung metastases were quantified on POD3 and normalized to the control no surgery group (n=19 to 28 mice from 6 experiments, Mann-Whitney test). **D)** Schematic of ornithine diet treatment in B16F10 model of surgical stress. **E)** Ornithine and arginine concentrations following ornithine enriched diet. **F)** Lung metastases were quantified on POD3 and normalized to the control no surgery group (n=8/group, Mann-Whitney U test). **G)** Schematic of 4T1 model of surgical stress. The ARG1 inhibitor, CB1158, was given BID for 3 days pre-operatively with control diet (n=6 mice/group; Mixed-effects model with Bonferroni's multiple comparisons test). **H)** Arginine concentrations after pre-op treatment in Balb/c mice normalized to baseline (Compared pre-op vs. **I)** Lung metastases were quantified on POD14 and normalized to the control no surgery group (n=8 to 17 mice from 3 experiments, Kruskal-Wallis). **J)** Representative images of lungs from the orthotopic 4T1 surgery model. *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001.

To corroborate this observation in a secondary model of surgical stress, we used a therapeutically relevant feeding schedule in the 4T1 model in Balb/c mice (Fig. 3G). In this model, the AED was given perioperatively (5 days before and 5 days after surgery). This feeding regimen was sufficient to increase preoperative arginine levels of AED fed mice 1.5-fold higher than control diet fed mice (Fig. 3H) and had no effect on the primary 4T1 tumour growth (fig. S3B and C), but was able to prevent the increase in spontaneous metastases after surgery (Fig. 3I). Furthermore, we assessed the ability of an Arg1 inhibitor, CB1158, to attenuate postoperative metastases (Fig. 3G). Notably, oral gavage of CB1158 b.i.d. was able to attain much higher levels of pre- and postoperative arginine levels compared to AED feeding, and there was no decrease in arginine at 4hrs post-surgery (Fig 3H), further supporting that Sx-MDSC Arg1 regulates postoperative arginine levels. Lastly, CB1158 attenuated postoperative metastases to a similar extent as the AED (Fig. 3I). Since these results show that therapies which improve arginine levels perioperatively can protect against postoperative metastases, we next sought to determine whether this was due to immunomodulatory effects on NK cell function.

Arginine accelerates NK cell recovery

To identify the cellular target mediating the anti-metastatic effects of dietary arginine supplementation, we depleted CD4 (clone: GK1.5) and CD8 (clone: 53-5.8) T-cells (fig. S4) or NK cells (anti-NK1.1) in the B16F10 model of surgical stress. While T cell depletion did not alter the number of surgery-induced metastases, the anti-metastatic effect of the AED was completely abrogated in the absence of NK cells, suggesting an NK-dependent mechanism (Fig. 4A). Since NK cells are critical for controlling metastases and are suppressed after surgery, we investigated whether the AED exerts its anti-metastatic effects by preserving NK cell function after surgery. To that end, we first quantified the amount of lung metastases at 4, 24, and 72hrs after surgery in AED fed mice (Fig 4B). Contrary to our

initial hypothesis, there was no significant difference in the number of early lung metastases (4 or 24hrs post-surgery) between the AED and control diet groups, however, lung metastases were reduced at 72hrs post-surgery in the AED group (Fig 4B and 3C). Therefore, while the AED does not prevent NK cell dysfunction or influence lung tumour seeding immediately after surgery, the beneficial effects are observed during the extended postoperative recovery period.

To confirm this finding, we evaluated the impact of the AED on NK cell cytotoxicity (NKC) and NK cell activity (NKA; the ability to secrete IFN-γ after stimulation) at 24 and 72hrs postoperatively. Consistent with our observation that Sx-MDSCs accumulate (fig. S3) and arginine levels are reduced to a similar extent in both control and AED fed mice at 4hr post-operation (Fig. 3B and H), NKC and NKA were also suppressed to the same extent in both groups on POD1 (Fig. 4C). However, both NKC and NKA were significantly improved in AED fed mice compared to control diet fed mice by POD3 suggesting a beneficial effect of perioperative arginine supplementation on NK cell function (Fig. 4C). In agreement with these functional assays we also observed higher expression of the activating receptors DNAX accessory molecule 1 (DNAM1) and NKG2D on POD3, but not on POD1 (Fig. 4D), further supporting that perioperative arginine supplementation does not prevent NK cell dysfunction, but accelerates their recovery from surgical stress.

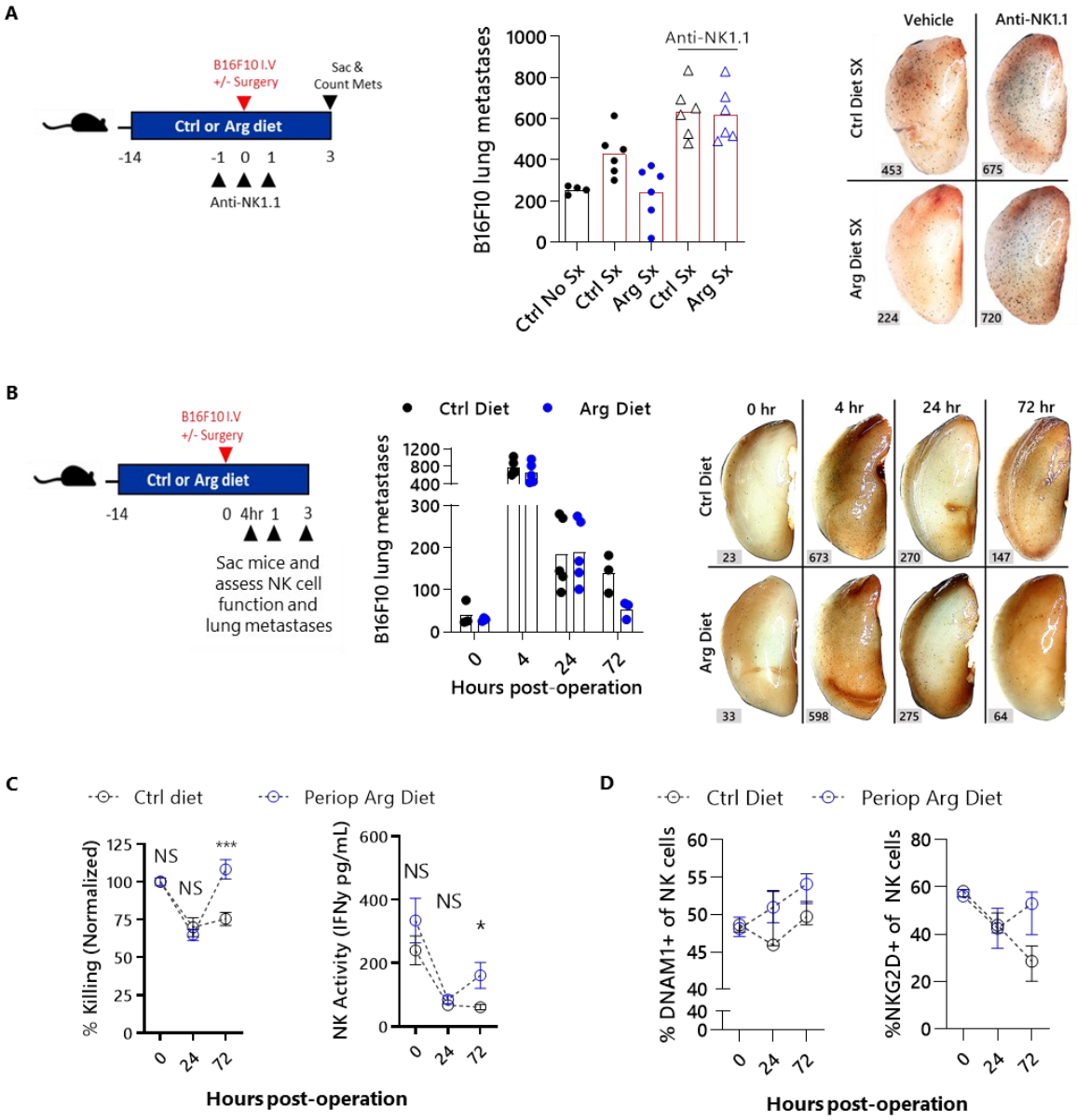


Figure 4.4. Arginine accelerates NK cell recovery from surgery-induced dysfunction.

Figure 4.4. Arginine accelerates NK cell recovery from surgery-induced dysfunction.

A) Schematic of NK cell depletion (anti-NK1.1) in C57Bl/6 mice fed a perioperative arginine diet (left panel). Lung metastases were quantified on POD3 (right panel). **B-D)** Experimental outline assessing NK cell function at 4, 24, and 72hrs post surgery (left panel). C57Bl/6 mice were fed an arginine enriched diet or control diet and metastatic lung tumour burden was enumerated at 4, 24, and 72 hrs post-operation (right panel). **C)** Isolated NK cell cytotoxicity against Yac1 targets (left; n=11-15 mice/group). Murine NK cell IFNγ from whole blood following overnight stimulation with NK Vue^{TM} (right panel; n=7-18 mice/group). **D)** Proportion of splenic NK cells that are DNAM1+ (left) and NKG2D+ (right). *$p \leq 0.05$; ***$p \leq 0.001$.

Arginine supplementation can restore the function of human NK cells cultured in Sx-MDSC conditioned media

Since arginine is required for NKC [90], we first sought to establish the minimal concentration of arginine required for NKC activity of the NK cell line, NK92-MI. By supplementing arginine-free media with increasing concentrations of arginine prior to measuring NKC against CP450-labelled K562 target cells [224], we observed that NKC is impacted when cultured in media containing <50μM arginine (Fig. 5A). Primary healthy donor NK cells (n=3) were similarly affected (Fig. 5B). Next, we investigated whether Sx-MDSCs isolated from cancer surgery patients could mediate NK cell suppression through the depletion of arginine from the culture media. To accomplish this, cell-free conditioned media was collected following the incubation of Sx-MDSCs in media containing the minimal concentration of arginine required for maximal NKC (50μM) for 24hrs (Fig. 5C). Culturing NK92 cells in media conditioned by human Sx-MDSCs (CD33+) on POD1 is sufficient to significantly reduce NKC. This inhibitory effect was not present when the media was conditioned with PBMCs depleted of Sx-MDSCs (CD33-) from the same cancer patient on POD1 (Fig. 5D). Together these findings confirm that the suppressive activity of Sx-MDSCs is not strictly dependent upon direct cell contact, which is consistent with previous findings [113]. To determine whether the inhibitory effects of Sx-MDSC under these conditions is due to a depletion of arginine from the culture media we supplemented the conditioned media with exogenous 200μM of arginine. Importantly, arginine supplementation is sufficient to significantly increase NKC under these conditions (Fig. 5E; n=3 experiments, $P = 0.008$).

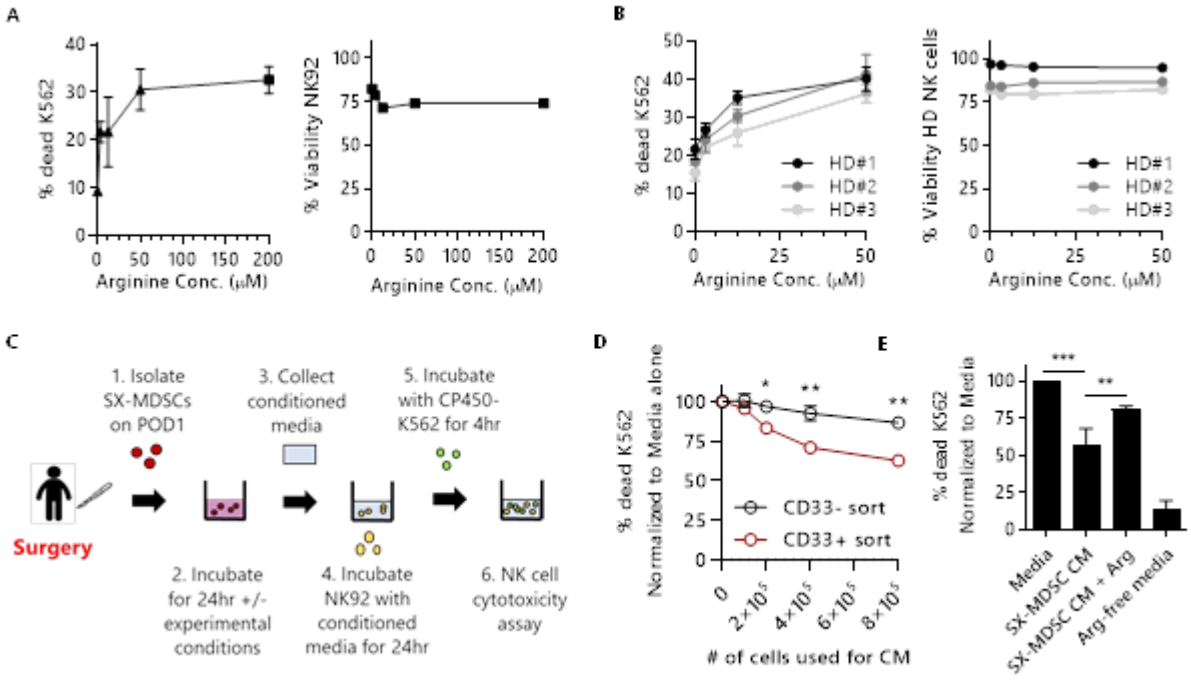


Figure 4.5. NK cells require arginine for cytotoxicity.

Figure 4.5. NK cells require arginine for cytotoxicity.

NK cells were incubated for 24hrs in arginine-free media reconstituted with increasing amounts of arginine. **A)** NK92-MI cytotoxicity (left) and viability (right). Representative results from n=2 separate experiments done in triplicates. **B)** Healthy donor primary NK cells (n=3) cytotoxicity (left) and viability (right). C) Schematic of Sx-MDSC conditioned media (CM) transfer onto NK92 cells and subsequent NK cytotoxicity assay. D) Increasing number of Sx-MDSCs (CD33+ sort) or CD33- cells from a POD1 cancer patient were used to condition media. NK92s were subsequently incubated with CM and tested for their cytotoxic potential (Two-way ANOVA with Bonferroni's multiple comparisons test, CD33- vs CD33+ at each cell concentration). **E)** NK cell cytotoxicity following conditioned media transfers. *p≤0.05; **p≤0.01; ***p≤0.001.

Arginine levels correlate to NK cell function in colorectal cancer surgery patients

The combined preclinical murine data and *ex vivo* co-culture experiments suggest that surgical stress results in the postoperative expansion of Arg1-expressing Sx-MDSCs which suppress NK cell activity in part through the depletion of systemic arginine concentrations. To explore whether Sx-MDSCs similarly impact arginine levels in cancer surgery patients we analysed the amino acid levels in a cohort of colorectal cancer patients (n=22, NCT02987296) preoperatively on the morning of surgery (POD0), and on POD1, POD3 and POD35 as well as in healthy donors (n=9). Consistent with our preclinical data, we observed a significant decrease in arginine on POD1 (Fig. 6A). Furthermore, there was a significant inverse correlation between the absolute number of Sx-MDSCs and the concentration of arginine on POD1 (Fig. 6B; $P = 0.03$, $R^2 = 0.2$). Additionally, we observed a significant positive correlation between arginine concentration and NKC on POD0 (Fig. 6C; $P = 0.006$, $R^2 = 0.4$). Therefore, in colorectal cancer surgery patients, arginine concentrations may hint at the magnitude of Sx-MDSCs present on POD1 which can indicate the severity of postoperative immunosuppression.

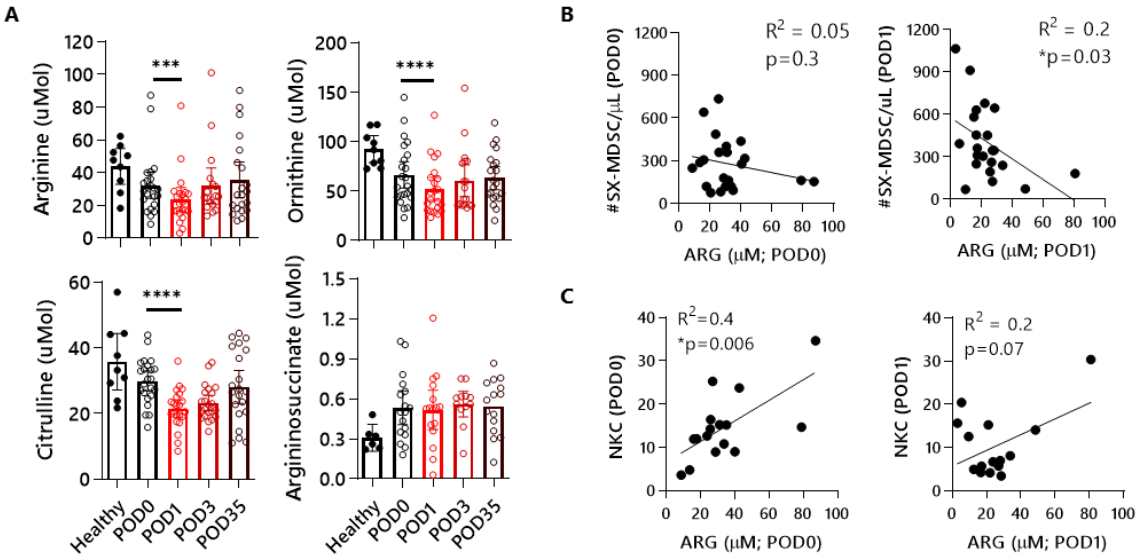


Figure 4.6. Arginine levels correlate with Sx-MDSC number and NK cytotoxicity in colorectal cancer patients.

Figure 4.6. Arginine levels correlate with Sx-MDSC number and NK cytotoxicity in colorectal cancer patients.

A) Blood amino acid concentrations from healthy donors (n=9) and CRC patients (n=22). **B)** Correlations between the absolute number of circulating Sx-MDSCs vs arginine concentrations on POD0 (left) and POD1 (right). **C)** Correlations between raw NKC and arginine concentrations on POD0 (left) and POD1 (right). ***$p \leq 0.001$; ****$p \leq 0.0001$.

4.6 DISCUSSION

It is well established that surgery results in a systemic decrease in the concentration of arginine, however, the impact of reduced arginine after surgery on immunity and in particular, metastases, have not been investigated previously. Here, we have shown that following surgical insult, Sx-MDSCs expand and reduce blood concentrations of arginine via increased Sx-MDSC Arg1 activity. While MDSCs are also known to express high levels of iNOS under proinflammatory conditions [118,225], iNOS is less effective than Arg1 in reducing arginine bioavailability [226,227]. Furthermore, we have previously shown that Sx-MDSC iNOS activity does not increase after surgery [64], and in this study we observed an increase in blood ornithine and decrease in citrulline levels after surgery which are the downstream metabolites of the Arg1 and iNOS pathways, respectively. It is worth noting that in our murine models, postoperative arginine levels were normalized by POD1, but Sx-MDSCs remained significantly elevated until POD3. This discrepancy may be explained by the multiple ways that arginine can be restored, such as through *de novo* synthesis from citrulline, amino acid recycling from the breakdown of proteins, or directly from dietary sources [225]. Since western diets can contribute up to 30% of the arginine in circulation [228], dietary arginine supplementation is an effective way of replenishing arginine bioavailability.

Although the use of perioperative arginine has been recommended by product manufactures for many years, it has not found widespread acceptance in surgical practice and the mechanisms underlying its efficacy are poorly understood [229]. Interestingly, despite the 1.5-fold increase in arginine after preoperative AED feeding, arginine levels were reduced to similar levels as the control diet group immediately (4hrs) after surgery. Since the number of Sx-MDSCs did not differ between groups, this suggests that either Sx-MDSCs have an excessive capacity to deplete arginine levels, or the excess supplemental arginine is being

metabolised by other immune cells. It has been shown that following CD3/CD28 stimulation, T cells have enhanced arginine uptake compared to unstimulated T cells due to increased transcription and translation of the cationic amino acid transport-1 (CAT-1; gene *SLC7A1*) [230]. While the same conditions have not been replicated in NK cells, NK cells are known to metabolically adapt from glycolysis during quiescence to aerobic glycolysis and oxidative phosphorylation under stimulating conditions [231–233]. Thus, it is possible that NK cells upregulate amino acid transporters in response to surgical stress, and the supplemental arginine accelerates recovery and function by fueling their metabolic needs when they encounter micrometastases.

Interestingly, unstimulated NK92 cells have been shown to upregulate CAT-1 and CAT-2B transcription in the absence of arginine [90]. Therefore, in times of immune stimulation or arginine depletion, immune effector cells respond by increasing their capacity for arginine uptake. This may explain why, in the absence of surgery, we observed no benefit of arginine supplementation on NK cell function. Since supraphysiological levels of arginine do not improve immune cell function beyond normal levels [29], immune cells may only need to meet a threshold of arginine and any excess arginine would not necessarily lead to better immune cell function. This threshold for human NK cytotoxicity was between 12.5 - 50μM of arginine in our *ex vivo* culture conditions. Given that the blood concentrations of arginine did not reach a level below this minimum threshold in control or AED fed mice, the reduction in arginine bioavailability alone after surgery cannot explain surgery-induced NK cell dysfunction.

Reduced arginine after traumatic stress/injury may be evolutionarily conserved to aid in wound healing via Arg1 to produce polyamines and proline which enhance collagen synthesis and wound closure [234]. Furthermore, Arg1 may be used by myeloid cells to limit the amount of available substrate for NO production, thus regulating the pro-

inflammatory phase after surgery. This resembles what is observed in preterm newborns, where reduced arginine bioavailability prevents excessive pro-inflammatory responses to foreign pathogens newborns are exposed to at birth and commensal colonization [235]. Thus, in the context of cancer surgery where postoperative immune surveillance for micrometastases is suppressed but needed, it would be more advantageous to quickly re-establish anti-tumour immunity.

It has been shown that postoperative and perioperative arginine supplementation schedules are associated with better postoperative outcomes compared to preoperative supplementation alone [132,133,229]. This does not discredit the use of preoperative arginine supplementation, since cancer patients present with lower arginine levels than healthy individuals [213]. Furthermore, preoperative arginine levels were significantly correlated to preoperative NK cell cytotoxicity in our patient cohort. Since NK cell activity is associated with cancer prognosis [56], and Arg1 expression increases with advanced stages of colorectal cancer [123], arginine status may also indicate the severity of cancer progression.

The therapeutic necessity of preoperative arginine supplementation should still be confirmed because of the potential for fueling cancer outgrowth in tumours that are auxotrophic for arginine [236]. Although we did not see an effect on primary tumour growth in our experimental murine models, arginine is known to have a role in tumour growth and proliferation [237]. This is why another strategy for cancer therapy is through arginine deprivation via pegylated-Arg1 [237,238]. Amino acid deprivation therapies (mainly targeting glutamine, asparagine, or arginine) have been devised to starve growing tumours of essential amino acids [239]. For example, acute lymphoblastic leukemias (ALL) are auxotrophic for L-asparagine (they cannot synthesize adequate amounts) and therefore depend on exogenous sources for cell growth [240], and asparaginase therapy to limit the

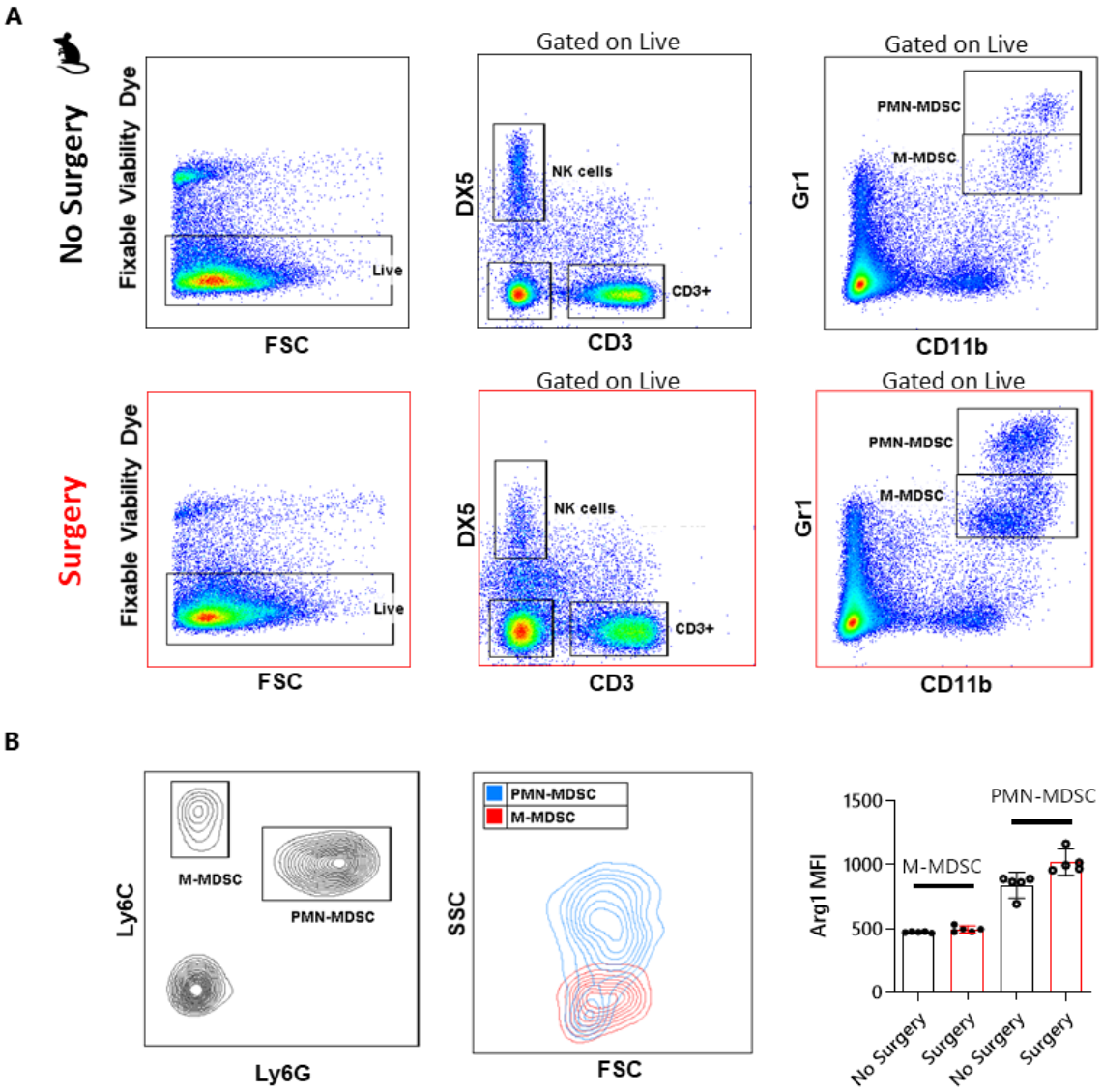
amount of available asparagine for cancer growth is an approved treatment option for ALL patients [241].

The efficacy of perioperative dietary arginine was dependent on the presence of NK cells as there was no effect on metastases when the AED was given in NK cell depleted mice. Other studies have demonstrated a role for arginine in the expression of cell surface receptors such as CD25 (IL-2 receptor) in neonatal lymphocytes [242], and NKp46 and NKp30 on the human NK cell line NK92 [90]. Many of these receptors are already reduced in cancer patients in the absence of surgical stress [243] and their reduction after surgery may be the combined result of reduced arginine levels and the increase in anti-inflammatory cytokines such as TGF-β, which has been shown to inversely correlate with the expression of NKG2D, DNAM1, NKp30, and NKp46 on NK cells [35,244–246]. In our study, AED resulted in a quicker recovery of NK cell cytotoxicity, IFN-γ secretion, and the expression of the activating receptors DNAM1 and NKG2D.

If the efficacy of perioperative arginine supplementation is only seen postoperatively, dietary intake may be limited immediately following surgery, especially for procedures involving the gastrointestinal tract, where patients are often not able to tolerate a diet in the early postoperative period. In these situations parenteral arginine administration (such as intravenous) or small molecule inhibitors of Arg1 activity may be more effective in preserving arginine levels after surgery. The Arg1 inhibitor, CB1158, has been shown to have antitumor and antimetastatic effects in tumour mouse models as a monotherapy or in combination with checkpoint inhibitors [217]. This remains a promising avenue for perioperative investigation, however, it should be noted that Arg1 plays an important role in wound healing via the production of ornithine which is a substrate for polyamines and tissue repair [247].

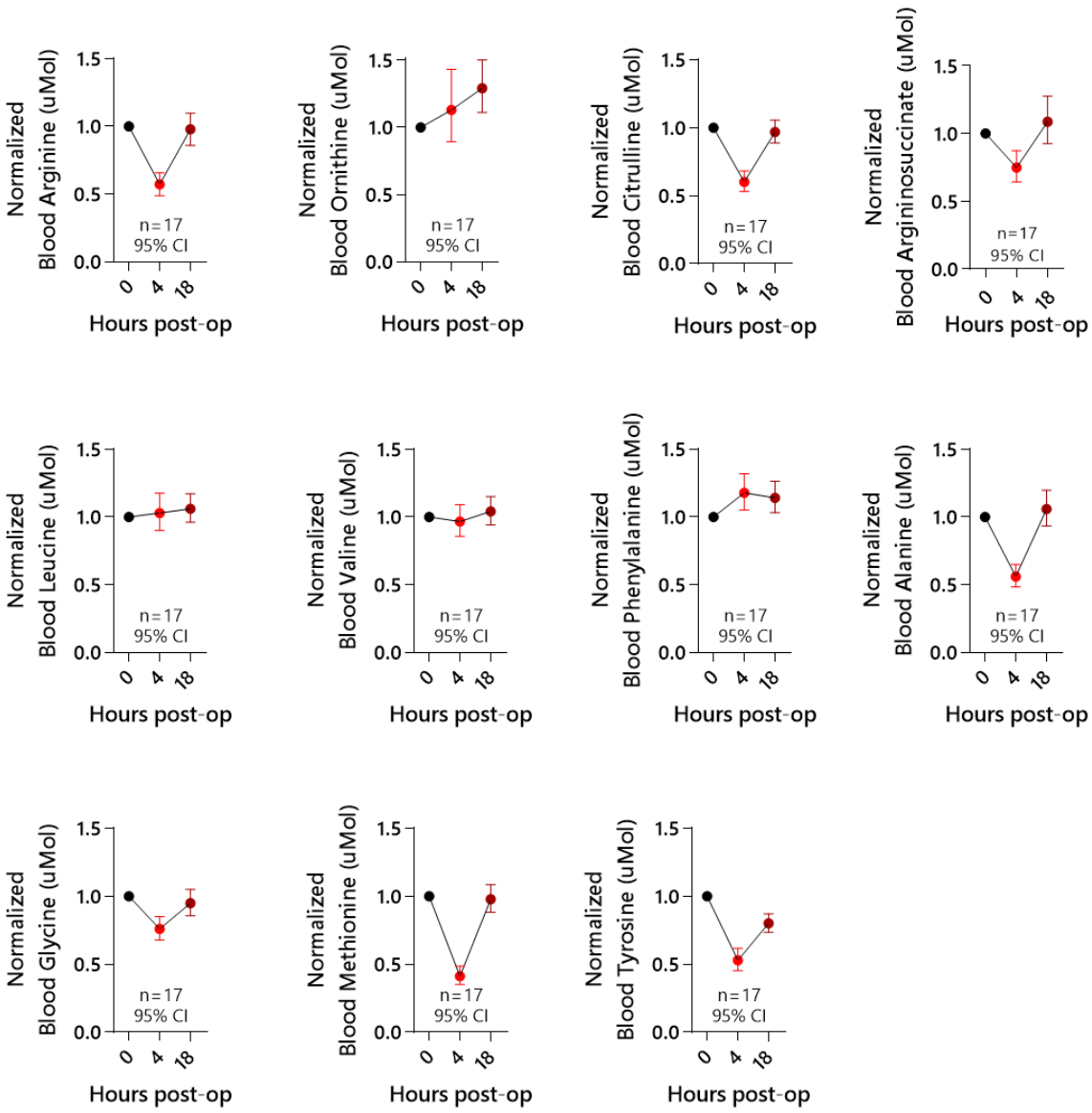
The immunosuppressive effects of surgery disarms the immune system's ability to destroy invading tumour cells at metastatic sites, particularly through NK cell immunoparalysis. In our models of surgical stress, we have elucidated a role for Sx-MDSCs in facilitating the systemic reduction in arginine bioavailability, necessary for NK cell function. However, this is only one piece of a multifactorial reaction to surgery as simultaneous immunosuppressive mechanisms in the postoperative period are likely at play. Although depleting Sx-MDSCs in our murine models was an effective strategy in reversing these effects, this is not a viable option for surgery patients as a marker for Sx-MDSCs has not yet been identified. Total ablation of CD33+ myeloid cells in cancer patients would in theory prevent Sx-MDSC accumulation, however, this would also remove myeloid progenitor cells which give rise to neutrophils that are vital to fighting postoperative infections [248]. Therefore, as a non-invasive approach to restoring arginine balance postoperatively, we investigated the potential of perioperative arginine supplementation, which has shown promising results on short-term surgical outcomes [132]. Much is still unknown about the complex relationship between nutrition and immunity, however, through this study we have outlined how perioperative arginine can positively impact innate immunity through improved NK cell recovery after surgery.

4.7 SUPPLEMENTARY FIGURES

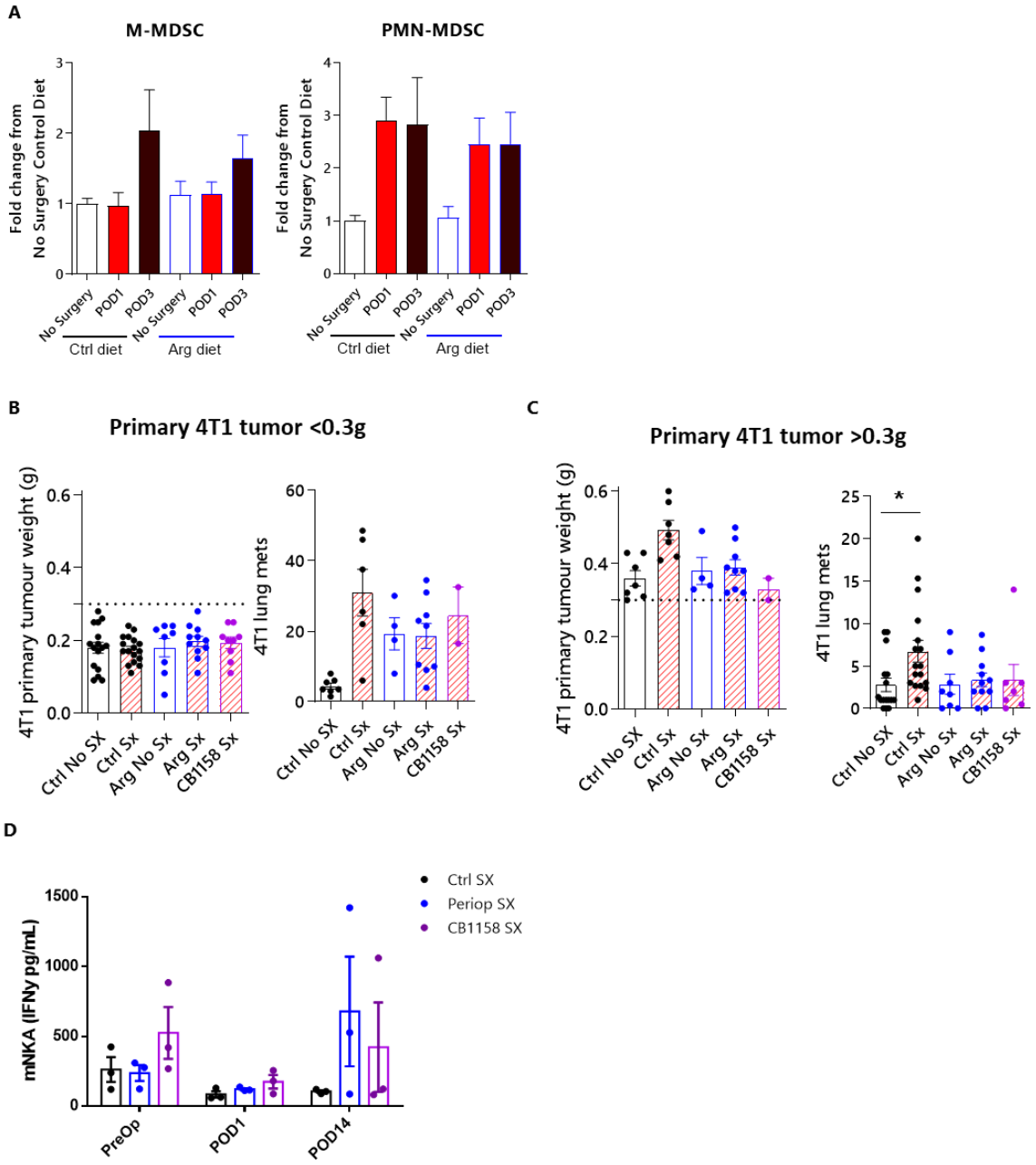


Supplemental Figure 4.1. Murine MDSC Flow Cytometry Gating Scheme.

A) Representative flow plots from no surgery (top) and surgery (bottom) groups from mouse splenocytes. **B)** Gating on MDSC subsets (left; M-MDSC: Ly6Chi Ly6G-; PMN-MDSC: Ly6Clo Ly6Ghi) and their FSC vs SSC profile (right).

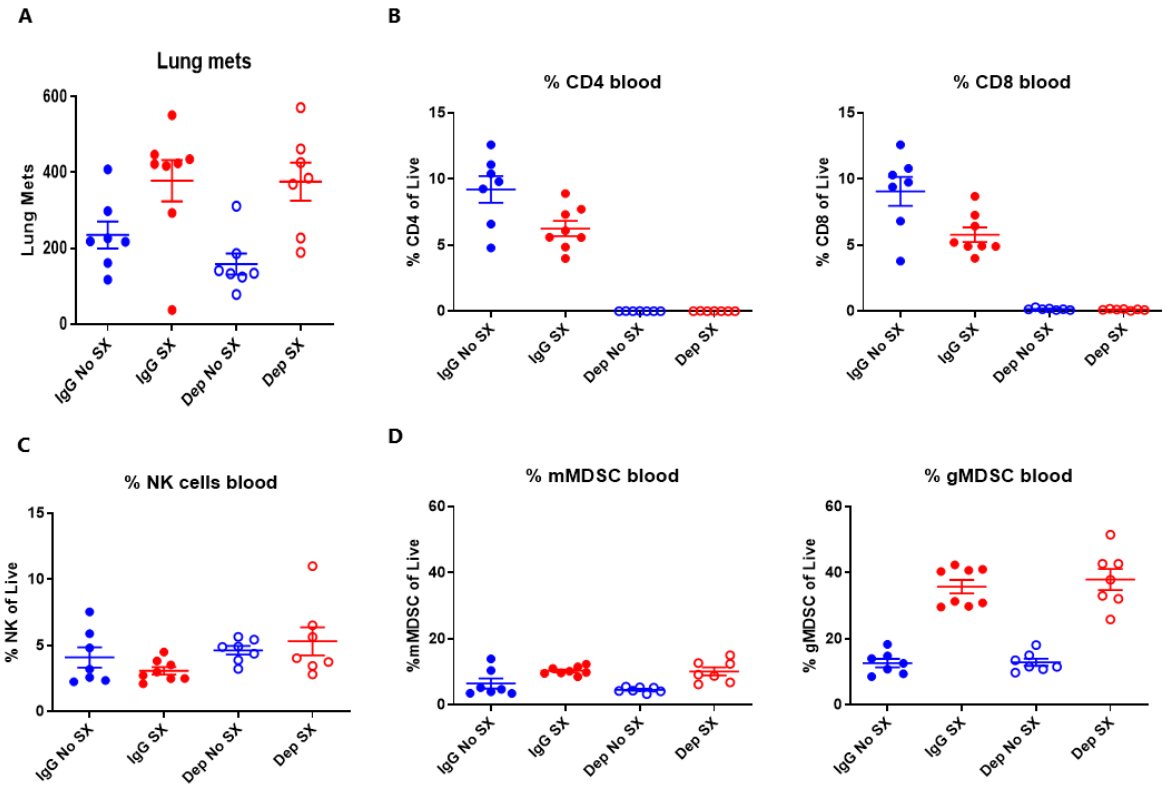


Supplemental Figure 4.2. Changes in amino acid concentrations after surgery in mice. Values are normalized to their preoperative levels. Mean and 95% CI.



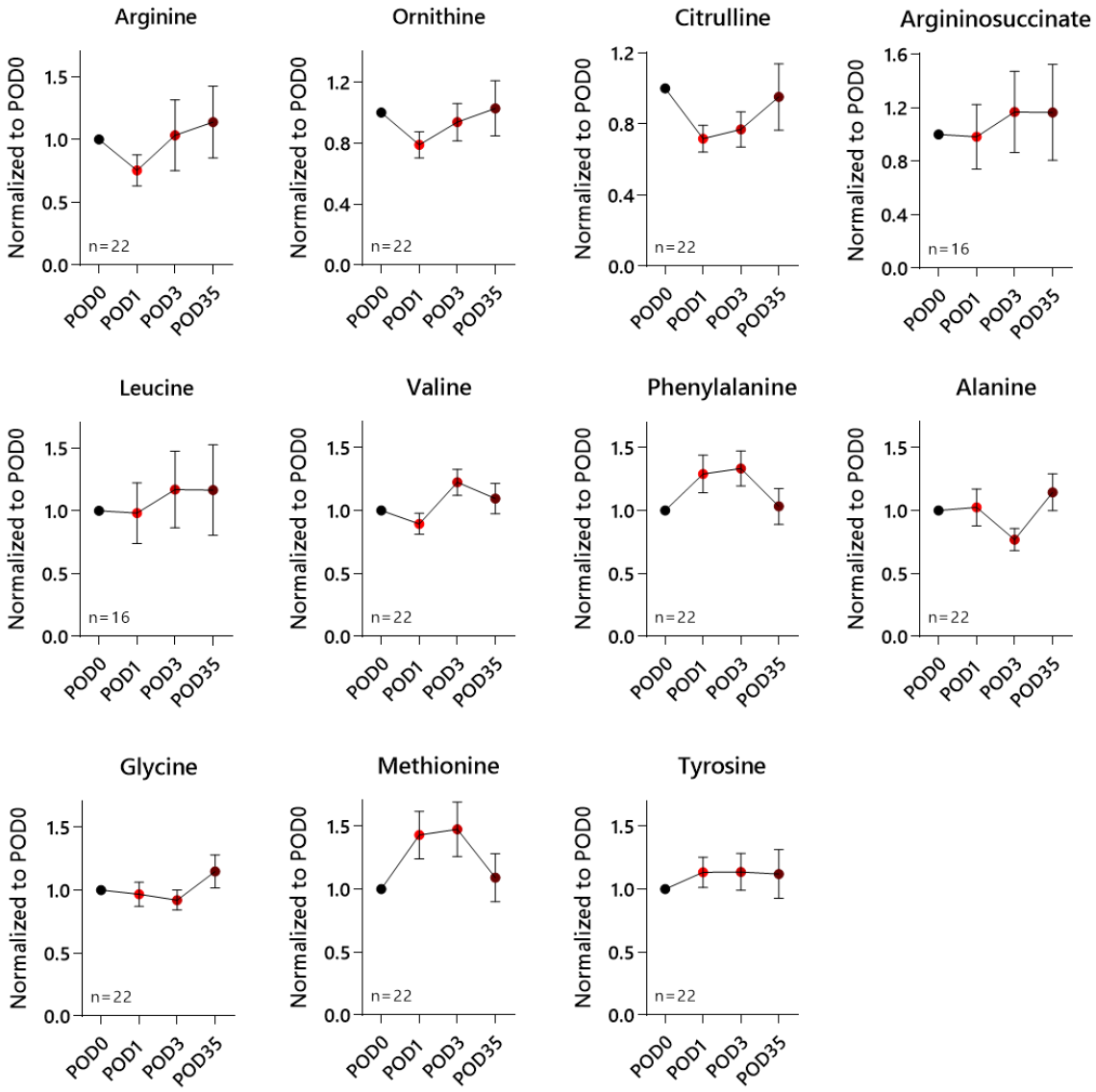
Supplemental Figure 4.3. Changes in MDSCs and lung mets following surgery.

A) M-MDSCs (left) and PMN-MDSCs (right) after surgery in the B16F10 model following perioperative immunonutrition. Primary tumor weight (left) and resultant number of 4T1 lung metastases (right) for **(B)** tumors <0.3g or **(C)** tumors >0.3g. **D)** The effect of perioperative Arginine and Arg1 inhibition on mNKA values in the 4T1 model of surgical stress.



Supplemental Figure 4.4. Effect of surgery in the B16 model in mice depleted of both CD4/CD8 T cell populations.

A) Quantifying lung metastases in IgG treated vs CD4/CD8 double depleted mice after surgery. **(B)** CD4 (left) and CD8 (right) cells were quantified by flow cytometry from blood samples. **C)** NK cells and **(D)** M-MDSCs/G-MDSC populations are not changed by CD4/CD8 depletion after surgery.



Supplemental Figure 4.5. Changes in blood amino acid concentrations after surgery in colorectal cancer surgery patients.

Values are normalized to their preoperative levels. Data from the PERIOP-02 clinical trial (NCT02987296). Mean and 95% CI (n=16-22).

Chapter 5

Effect of perioperative arginine on NK cell function in cancer surgery patients

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5.0 PREFACE

This chapter is submitted and currently under review in the Journal of Clinical Investigation Insights (JCI Insights) (Submission #144254-INS-CMED-1 on September 25, 2020).

Angka L, Martel AB, Ng J, Pecarskie A, Sadiq M, Jeong A, Scaffidi M, Souza CT, Kennedy MA, Tadros S, and Auer RC. A translational randomized trial of perioperative arginine on Natural Killer cells in colorectal cancer patients. Submitted to JCI Insights. September 25, 2020.

Author contributions for this work are as follows:

Angka L: Planned (with oversight from Kennedy MA and Auer RC), performed and analysed all of the experiments. Wrote the manuscript in its entirety.

Martel AB: Obtained and analysed the clinical demographic data. Assisted in editing.

Ng J: Clinical Sample Coordinator and helped with sample processing

Pecarskie A: Clinical Patient Coordinator for the study.

Sadiq M: Clinical sample coordinator and helped with sample processing.

Jeong A: Clinical sample coordinator and helped with sample processing.

Scaffidi M: Clinical sample coordinator and helped with sample processing.

Souza CT: Laboratory manager, protocol development and sample storage assistance.

Kennedy MA: Assisted with experiments and provided guidance on data interpretation as well as manuscript editing assistance.

Tadros S: Operating surgeon

Auer RC: Conceived of the study, operating surgeon, and gave oversight for the entire study.

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5.1 TITLE PAGE

A translational randomized trial of perioperative arginine on Natural Killer cells in colorectal cancer patients.

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Disclosures

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5.2 ABSTRACT

Background: Surgery results in severe impairment of Natural Killer (NK) cell cytotoxicity (NKC) and activity (NKA, cytokine secretion) and a dramatic drop in arginine levels. Postoperative immunosuppression is associated with increased complications and recurrence. Perioperative arginine is reported to reduce postoperative complications. Since arginine modulates NK cell function, we set out to determine if perioperative consumption of arginine-enriched supplements (AES) can improve NK cell function in colorectal cancer (CRC) surgery patients.

Methods: Twenty-four CRC patients were randomized to receive the AES or isocaloric/isonitrogenous control supplement three times a day for five days pre- and post-surgery. The primary objective was to determine if AES improves NKC by 50% compared to the control group post-surgery.

Results: NKC was significantly reduced postoperatively on surgery day (SD) 1 in the control group by 50% (IQR: 36-55%; *p=0.02) but not in the AES group (25% reduction; IQR: -28-75%; p=0.3). Furthermore, there was no benefit of AES on NKA or NK cell number. Compliance was much greater preoperatively (>91%) than postoperatively (<46%). However, despite excellent preoperative compliance, arginine was rapidly cleared from the blood within 4hrs of consumption and therefore, did not prevent the postoperative drop in arginine.

Conclusions: Oral consumption of arginine immunonutrition resulted in a modest improvement in NKC after surgery but was unable to prevent postoperative arginine depletion or the suppression of NKA.

Trial Registration Number: ClinicalTrials.gov NCT02987296

5.3 INTRODUCTION

Surgical resection is the primary curative treatment modality for most solid malignancies, including colorectal cancer (CRC). Postoperative infectious complications are a major source of morbidity, resulting in an increased length of stay (LOS), a delay in chemotherapy and is ultimately associated with a worse cancer prognosis [249]. The underlying cause for postoperative infections is the profound immune suppression in response to surgery-induced changes, such as nutritional deficiencies and inflammatory signals [1,19]. This critical period of immunosuppression also enables micrometastases to establish metastatic niches, which ultimately lead to cancer recurrence, a phenomenon that has been definitely established in animal models [1]. In these models, surgery-induced Natural Killer (NK) cell dysfunction plays a key role in determining the outcomes of surgical stress on lung metastases [35,63,64,250,251]. NK cells are the effector cells of the innate immune system and their function can critically determine prognosis for CRC patients [150,252,253]. Furthermore, NK cells are crucial frontline defenders against infections, underscoring their importance for recovering surgery patients [19,254]. Therefore, perioperative therapies to reverse or prevent NK cell dysfunction have the potential to attenuate the negative sequelae of surgery-induced immunosuppression [1].

In response to surgical stress, the body enters a state of emergency myelopoiesis which results in a large expansion of immature myeloid cells shown to have immunosuppressive characteristics [32,64]. These surgery-induced Myeloid Derived Suppressor Cells (Sx-MDSCs) express high levels of intracellular arginase-1 (Arg1) [64,255], an enzyme that metabolizes arginine into urea and ornithine [222]. As a result, surgery leads to arginine deprivation which is detrimental to the immune system [29,87,256]. For T cells, insufficient arginine limits proliferation and their response to antigens by reducing cell surface T-cell receptor/CD3 zeta-chain expression [29,257]. More recently it was shown that T cells adapt

metabolically to changes in L-arginine concentrations, and elevated L-arginine enhanced T cell survivability and maintained memory T cell populations [258]. For NK cells, arginine deprivation impairs NK cell proliferation [92], NK cytotoxicity (NKC) [88,90], interferon-gamma (IFN-γ) production (referred to now as NK activity or NKA) [89–91], and the expression of NK cell activating receptors such as NKp46 and NKp30 [90]. Given the important role of arginine for cellular immunity, it is hypothesized that the postoperative drop in arginine is responsible for postoperative immune suppression and impaired recovery, including infections complications.

Since arginine is a conditionally essential amino acid which means in times of physiological stress arginine must be obtained externally through dietary sources [222], many clinical trials have investigated perioperative "immunonutrition" that generally include supraphysiological levels of arginine within the formulations [133]. Several meta-analyses have confirmed an association between perioperative immunonutrition and improved surgical outcomes, including a 40-48% decrease in postoperative complications and a ~2-day reduction in hospital LOS [131–133]. In our own preclinical animal studies, perioperative arginine-enriched food increased blood arginine levels, improved NK cell recovery and reduced metastatic burden after surgery [259]. Therefore, given the strong preclinical and clinical rationale, we initiated a Phase II randomized controlled clinical trial (NCT02987296) to determine the direct impact of a perioperative arginine-enriched supplement (AES) on NK cell function in CRC patients (Figure 1).

5.4 METHODS

Trial Design

This single-center, placebo-controlled, double-blind, randomized interventional trial was approved by the Ottawa Health Science Network Research Ethics Board (20160732-01H) and conducted between March 2017 to July 2019 at The Ottawa Hospital. The trial was prospectively registered at clinicaltrials.gov (NCT02987296). Twenty-four CRC patients were enrolled and their baseline blood collection was obtained upon written consent. Participants were then randomized to receive the isocaloric/isonitrogenous control supplement or the AES at a one-to-one ratio and instructed to take three doses/day (TID) for five days before and after their scheduled surgery day (Figure 1). Surgeries were completed by two surgical oncologists (RA and ST).

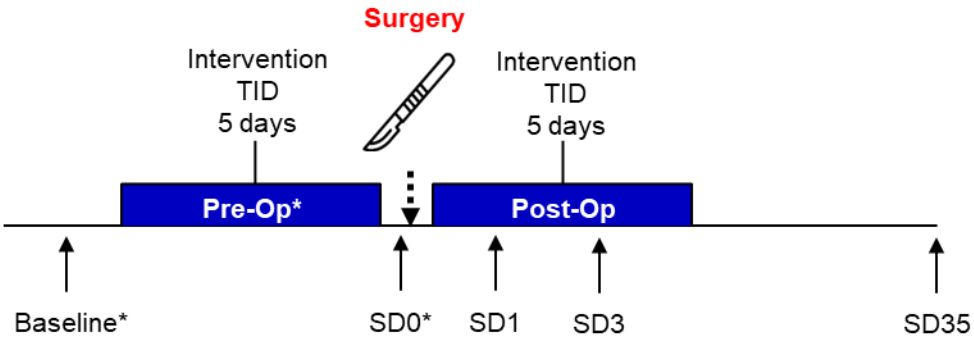


Figure 5.1. Trial Design.

Figure 5.1. Trial Design.

Participants were randomized to either Control or AES arm and given 15 doses before and 15 doses after their operations. Blood was collected at multiple time points: Baseline (BL), Surgery day (SD) 0, SD1, SD3, and SD35. *Pre-operation.

Participants

Patients over the age of 18 with primary CRC undergoing surgical resection (including malignant polyps) with adequate kidney function (creatinine clearance >30mL/min), hemoglobin levels (>90mg/dL) and capable of giving written consent were eligible. Patients were ineligible if they met any of the following exclusion criteria: recent neoadjuvant chemotherapy or radiation therapy within 8 weeks of surgery; significant immunodeficiency due to illness or medication; hypotension (resting blood pressure < 90/50mmHg); history of autoimmune disease; active infection; pregnant or nursing; severe asthma; inherited guanidinoacetate methyltransferase deficiency (inability to convert arginine to creatine); history of liver cirrhosis; heart failure or significant cardiac disease (myocardial infarct or life-threatening arrhythmia within the last 6 months); or medical or psychological illness that would preclude expected compliance with the protocol. One patient in the control supplement cohort withdrew their consent before consuming any preoperative doses and was removed from the analysis. Another patient in the control arm declined to have their blood drawn at all postoperative time points (Figure 1).

Outcomes

The primary outcome was to compare the reduction in NKC immediately following surgery (SD1 to SD0) between the control supplement and AES cohorts. Secondary outcomes compared arginine levels at all time points and the level of pre- and postoperative compliance between the groups. Additionally, we assessed the level of NKA and the changes in NK and MDSC frequencies at all time points.

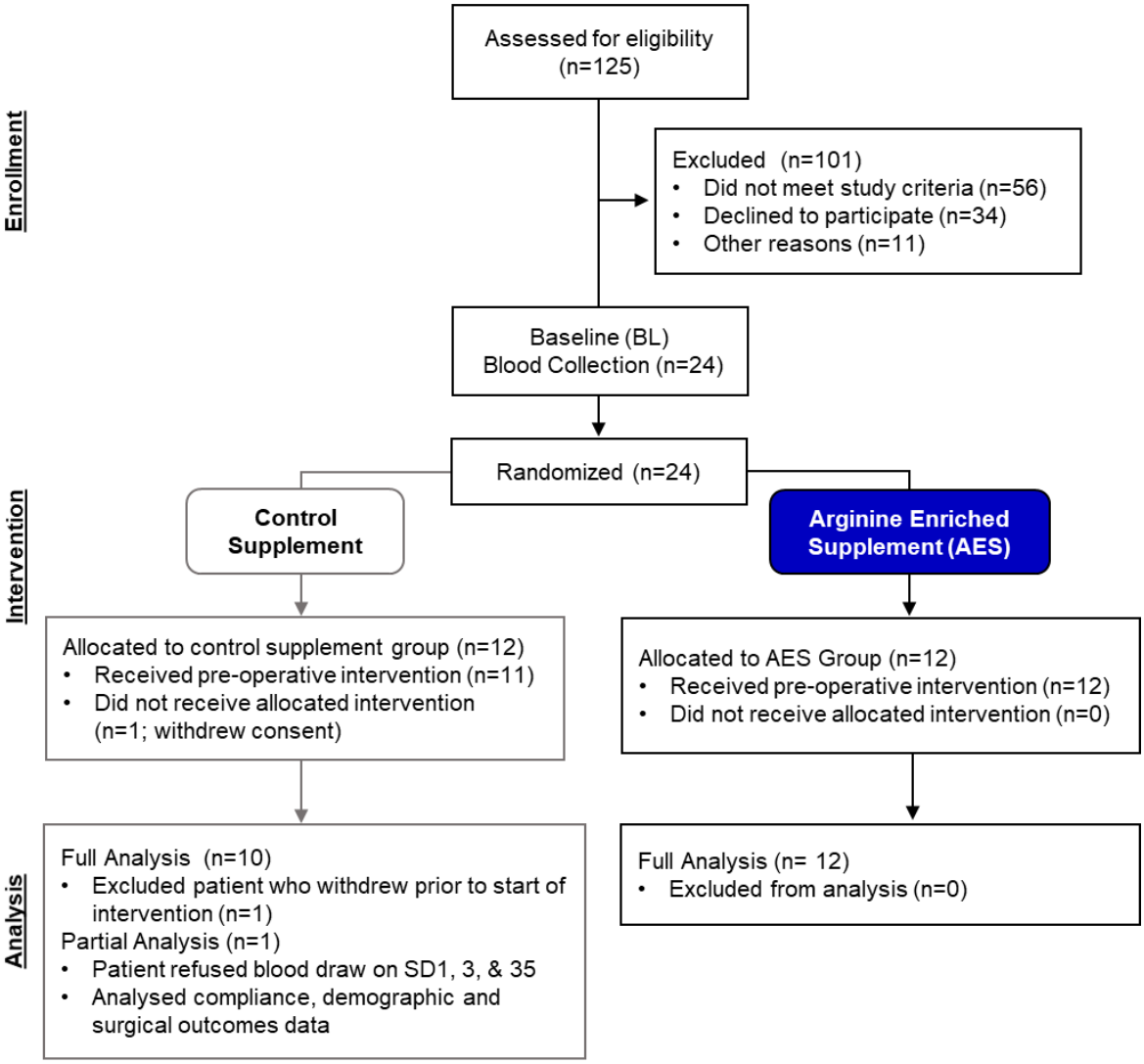


Figure 5.2. CONSORT Flow Chart.

Intervention, Randomization and Blinding

The AES and the placebo isocaloric/isonitrogenous supplement were provided by *Enhanced Medical Nutrition* (EMN) and their composition can be found in Supplemental Figure 1. EMN randomized the order (www.random.org) that either AES (n=12) or control (n=12) supplements would be given as participants enrolled in the study. Both investigators and participants were blinded to the allocation. Participants received the study intervention in unmarked packages and were instructed to record and return any unused doses. The master list was only unblinded after the completion of all correlative assays and data collection.

Patient Blood Processing and Storage.

Patient and healthy volunteer blood was drawn into BD Vacutainer Sodium-Heparin coated tubes (~40mL/blood draw). Of the whole blood, 1mL was aliquoted into vacutainer tubes containing Promoca™ [210], and the remaining was used to isolate PBMCs by Ficoll density centrifugation prior to cryopreservation.

Immune assays

Natural Killer Cytotoxicity (NKC) assay:

NKC was measured from cryopreserved PBMCs at all time points via a fluorescence-based cytotoxicity assay against K562 targets labelled with CP450 (eBioscience) [224]. Cryopreserved PBMCs were thawed then incubated with 0.1mg/mL DNAse-1 (STEMCELL technologies) for 15min at room temperature and then rested overnight at 37°C in 0.025mg/mL DNAse-1, 10% FBS RPMI1640 media [260]. The following day PBMCs were washed, counted and plated in triplicates with CP450-K562 target cells for 4hrs at 37ºC at increasing effector to target ratios (10:1, 40:1, and 80:1). Killing assay results are represented either as “NK cell cytotoxicity” quantified by %Dead K562, or “normalized cytotoxicity” calculated by normalizing to matched patient SD0 values.

Natural Killer Activity (NKA) assay:

NKA, or the ability of NK cells to secrete IFN-γ in response to stimuli, was measured by ELISA (ATGen Canada/NKMax) after overnight incubation in vacutainer tubes containing a proprietary cytokine cocktail, NK-Vue^{TM} Tubes (ATGen Canada/NKMax), as previously described [210]. Samples were given a value equal to the upper or lower limit of detection of 4,000pg/mL and 11pg/mL, respectively, if values fell outside of the assay parameters.

Flow cytometry profiling of immune cell populations:

Freshly isolated PBMCs were used to characterize the expansion of Sx-MDSCs by flow cytometry using CD33, CD11b, CD14, CD15, HLA-DR, and Lineage markers (CD3, CD56, CD19) [99]. Cryopreserved PBMCs were used to characterize NK cells and monocytes using CD3, CD56, CD16 (BD Horizon), and CD14 (eBioscience) [210]. Samples were run on the BD Fortessa LSRII (immunophenotyping) or FACsCelesta (NKC) and analyzed with FlowJo v10.

Blood Amino Acid Testing:

Fresh blood samples were collected on Whatman 903 Protein Saver Cards (Sigma-Aldrich) at all time points and immediately stored at -20ºC until batch analysis. A panel of amino acids (arginine, ornithine, citrulline, argininosuccinate, and leucine) were then measured via LC-MS/MS at Newborn Screening Ontario, Children's Hospital of Eastern Ontario [219].

Statistics and Sample Size

All figures and reported values are the median and interquartile ranges (IQR), unless otherwise specified. Unpaired, non-parametric Mann-Whitney U tests were performed when comparing between AES and control supplement groups at any given time point, and paired Wilcoxon rank-sum tests were used to compare matched patient samples at different time points. We hypothesized that a clinically relevant difference between the AES and control

group would be a 50% improvement in the degree of NK cell suppression on SD1. Based on previous *ex-vivo* studies, the estimated reduction in NKC on SD1 is 0.54 (s.d. 0.22) in the control cohort. A 50% improvement would be a reduction of 0.27 in the AES group. Given this baseline data, the sample size of 12 patients per group provides 80% power to detect a 50% improvement in the experimental cohort at a significance level (alpha) of 0.05. Significance was assigned when *$p<0.05$, **$p<0.005$ and ***$p<0.0005$.

5.5 RESULTS

Demographic, Clinical, and Operative Outcomes

The patient demographics, surgical data, and postoperative outcomes are summarized in Table 1. Control supplement and AES cohorts were similar across multiple demographic categories (age, cancer stage and type of surgery). A greater number of male participants were enrolled than females and there were no Stage IV CRC patients enrolled. From the control supplement arm, one patient withdrew from the study, and another patient had no residual cancer on surgical pathology (malignant polyp). The consort diagram is shown in Figure 2. There was no substantial difference in postoperative complications (infectious or non-infectious) or LOS, however, the study was not powered to detect significant differences in these outcomes.

Table 5.1. Clinical Demographics and Operation Outcomes

Category	Subcategory	Control Supplement (n=11)	Arginine Enriched Supplement, AES (n=12)
Sex	Male (n)	8	11
	Female (n)	3	1
Age - median; range		65; 48-82	67.5; 23-81
	< 50 years (n)	1	2
	50-69 years (n)	7	5
	> 70 years (n)	3	5
Type of Cancer - n (%)	Rectal	0 (0 %)	3 (25%)
	Left colon/Sigmoid	2 (18%)	3 (25%)
	Right colon	9 (82%)	5 (42%)
	Colon cancer NOS	0 (0 %)	1 (8 %)
Stage of Cancer - n (%)	Stage 0	1 (9 %)	0 (0 %)
	Stage 1	3 (27%)	5 (42%)
	Stage 2	4 (36%)	4 (33%)
	Stage 3	3 (27%)	3 (25%)
	Stage 4	0 (0 %)	0 (0 %)
Operation - n (%)	LAR / AR	1 (9 %)	3 (25%)
	Left hemicolectomy	1 (9 %)	2 (17%)
	Right hemicolectomy	9 (82%)	6 (50%)
	Proctocolectomy	0 (0 %)	1 (8 %)
Type of Surgery - n (%)	Open	4 (36%)	5 (42%)
	Laparoscopic	7 (64%)	7 (58%)
* Postoperative Complications - n (%)		6 (55%)	4 (33%)
	† Infectious (n)	2	1
	‡ Non-infectious (n)	3	2
	Postoperative Ileus (n)	3	3
Median Length of Stay - days; range		4; 3-12	6; 3-22
Re-Hospitalization - n (%)		2 (18 %)	1 (8.3 %)

NOS – Not otherwise specified; LAR / AR – lower anterior resection / anterior resection
* Some patients had more than one type of complication
† Infectious complications: pneumonia, urinary tract and wound infection
‡ Non-infectious complications: pulmonary embolism, non-ST elevated myocardial infarction, anastomotic leak and pancreatic leak

AES on Natural Killer Cell Function

To determine if perioperative arginine immunonutrition has a beneficial effect on NK cell function we measured NKC and NKA at all time points (Figure 3). There were no significant differences between the control supplement and AES groups at any time points. As expected, immediately after surgery (from Surgery Day (SD) 0 to SD1) NKC and NKA were profoundly suppressed [63,64,67,210,250]. There was a median reduction in NKC of 50% (IQR: 36-55%) on SD1 in the control supplement group. Although NKC did not decrease significantly on SD1 in the AES group (median reduction of 25%, IQR: -28-75%), NKC was further reduced in the AES group (median reduction of 47% IQR: 28-69%) on SD3. Lastly, the paralyzing effects of surgery on NKA were profound and similar to what we have seen previously [210], and the AES did not provide any improvement in NKA.

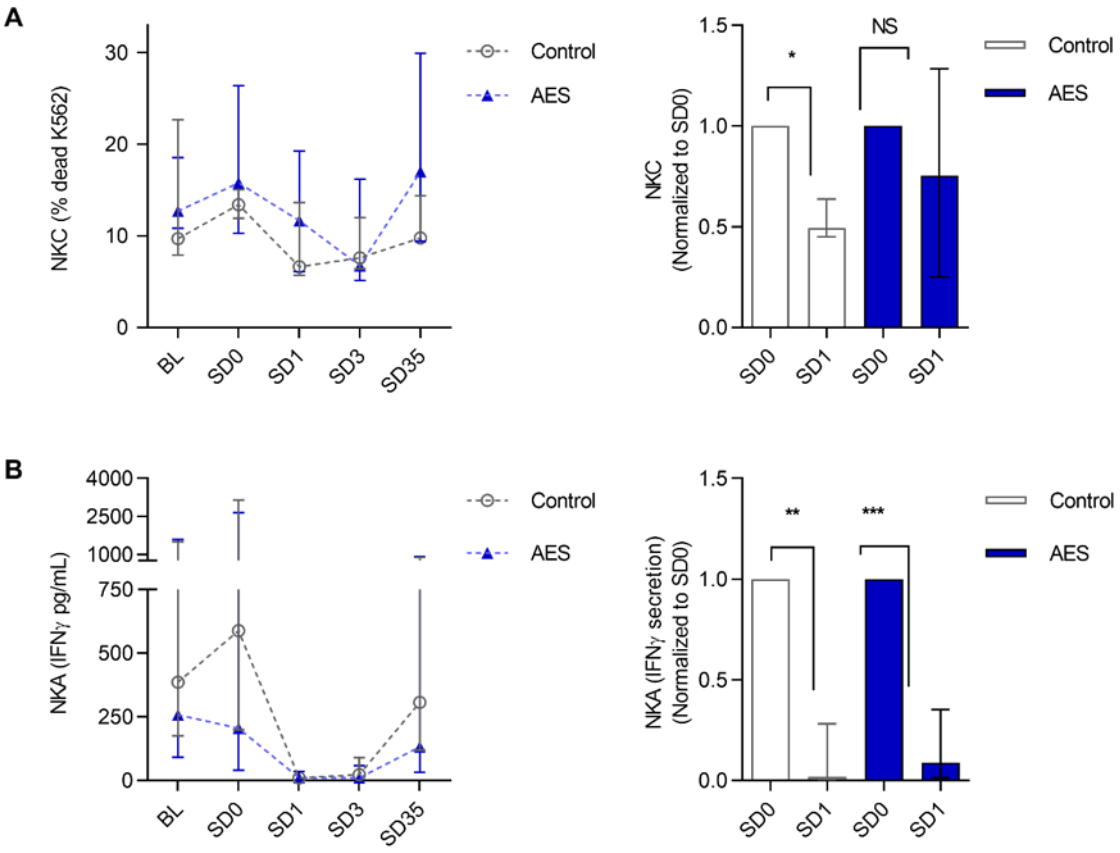


Figure 5.3. Effects of perioperative arginine immunonutrition on NK cell function.

Figure 5.3. Effects of perioperative arginine immunonutrition on NK cell function.

A) NK cell cytotoxicity (NKC) against NK-specific target cells (K562) does not differ between groups at all time points (left panel). Immediately after surgery (from SD0 to SD1), NKC significantly decreases in the control arm (n=9), but not the AES arm (n=7; right panel). **B)** NK cell activity (NKA; IFN-γ secretion) after overnight blood stimulation does not differ between groups at all time points (left panel). NKA is significantly suppressed postoperatively in both treatment groups (Control n=10, AES n=12; right panel).

Pre vs Postoperative Compliance and Blood Arginine Kinetics

Compliance was measured by tabulating self-reported patient logs of all preoperative and postoperative doses as well as the number of returned supplement doses (Figure 4). We defined "compliant" as taking ≥80% (12/15) of the required doses. Participants were not excluded from the study due to inadequate compliance. Preoperative compliance did not differ between groups with 91% (10/11 control supplement) and 92% (11/12 AES), and postoperative compliance was significantly reduced in both treatment arms. After surgery, only 46% (5/11 control supplement) and 33% (4/12 AES) of patients remained compliant. The main reason for non-compliance after surgery was postoperative nausea and ileus (intestinal paralysis).

Amino acid levels were measured at each time point (Figure 4 and Supplemental Figure 2). In both groups arginine levels decreased significantly from SD0 to SD1 (**$p<0.005$) as has been previously reported [87], and did not differ between groups at each time point. To determine the kinetics of arginine levels after 1 dose of control supplement or AES, amino acid levels were measured before and at 0.5, 1, 2, 4, and 12 hours after dosing (n=3 per group). While the AES was able to specifically increase arginine levels greater than the control supplement, the spike in arginine was transient - only persisting above normal resting levels for 2-4 hours. Ornithine levels were also significantly increased only in the AES group, but leucine levels spiked in both supplement groups immediately after dosing (Supplemental Figure 3).

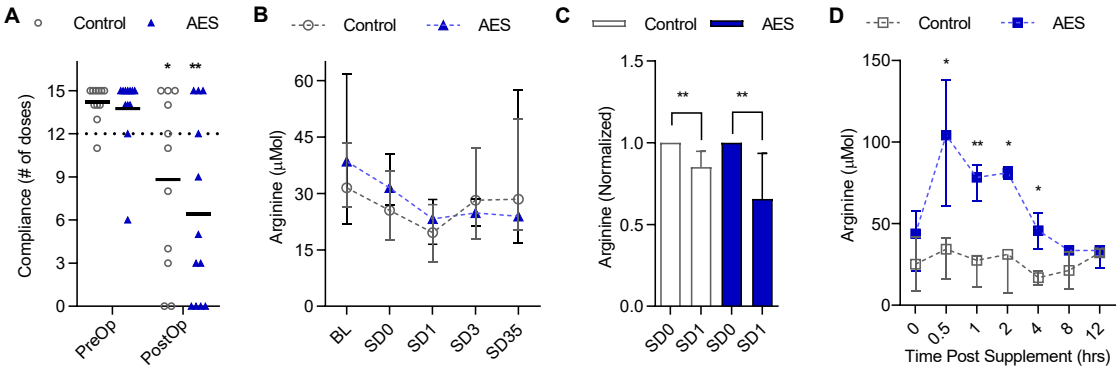


Figure 5.4. Compliance and arginine levels before and after perioperative immunonutrition.

Figure 5.4. Compliance and arginine levels before and after perioperative immunonutrition.

A) A total of 15 pre- and 15 postoperative doses were given to each participant. They were "compliant" if they consumed ≥80% (12/15) of the perioperative oral intervention (black dotted line). Solid bars represent the group mean. **B)** Blood arginine levels at each time point. **C)** Arginine levels normalized to SD0 in the control supplement and AES group. Paired, non-parametric t-tests. **D)** Kinetics of arginine levels following 1 dose of control supplement or AES (n=3/group).

Effects of AES on circulating NK cell and Sx-MDSC Frequencies

NK cells from cryopreserved peripheral blood mononuclear cells (PBMCs) and Sx-MDSCs from freshly isolated PBMCs were assessed for changes in absolute immune cell numbers and cell surface markers [210]. The number of circulating NK cells ($CD3^{-}CD14^{-}$ $CD56^{+}CD16^{+}$) remained unchanged and within reported ranges [261] irrespective of AES (Supplemental Figure 4). Furthermore, we did not observe any significant differences between the groups in the number of $CD56^{bright}$ or $CD56^{dim}$ NK cell subsets (Figure 5). The number of monocytes ($CD3^{-}CD14^{+}$) in the cryopreserved samples increased in both groups on SD1, as previously described [210]. Sx-MDSC can suppress NK cells both *in vitro* [64] and *in vivo [262]*. In order to quantify the number of circulating Sx-MDSCs (Lin^{-} $CD33^{+}$ $CD11b^{+}$ $CD14^{+}$ $CD15^{lo/-}$ $HLA\text{-}DR^{low}$), PBMCs were phenotyped fresh (before cryopreservation) [99]. These were found to be significantly increased on SD1 ($p=0.004$), as we have reported previously [64]. However, there were no significant differences in Sx-MDSC expansion between the perioperative supplement arms. Some studies have shown that immunonutrition increases the HLA-DR expression on monocytes, suggesting they are less immature [263]. An increase in HLA-DR expression after preoperative immunonutrition was not observed, but there was a substantial decrease in expression following surgery in both groups. Lastly, there was a significant increase in the number of circulating low density neutrophils (LDNs) postoperatively which was unaffected by AES. While both mature “high-density” neutrophils and LDNs are known to expand following surgery, only the LDNs have been shown to suppress NK cell function [193].

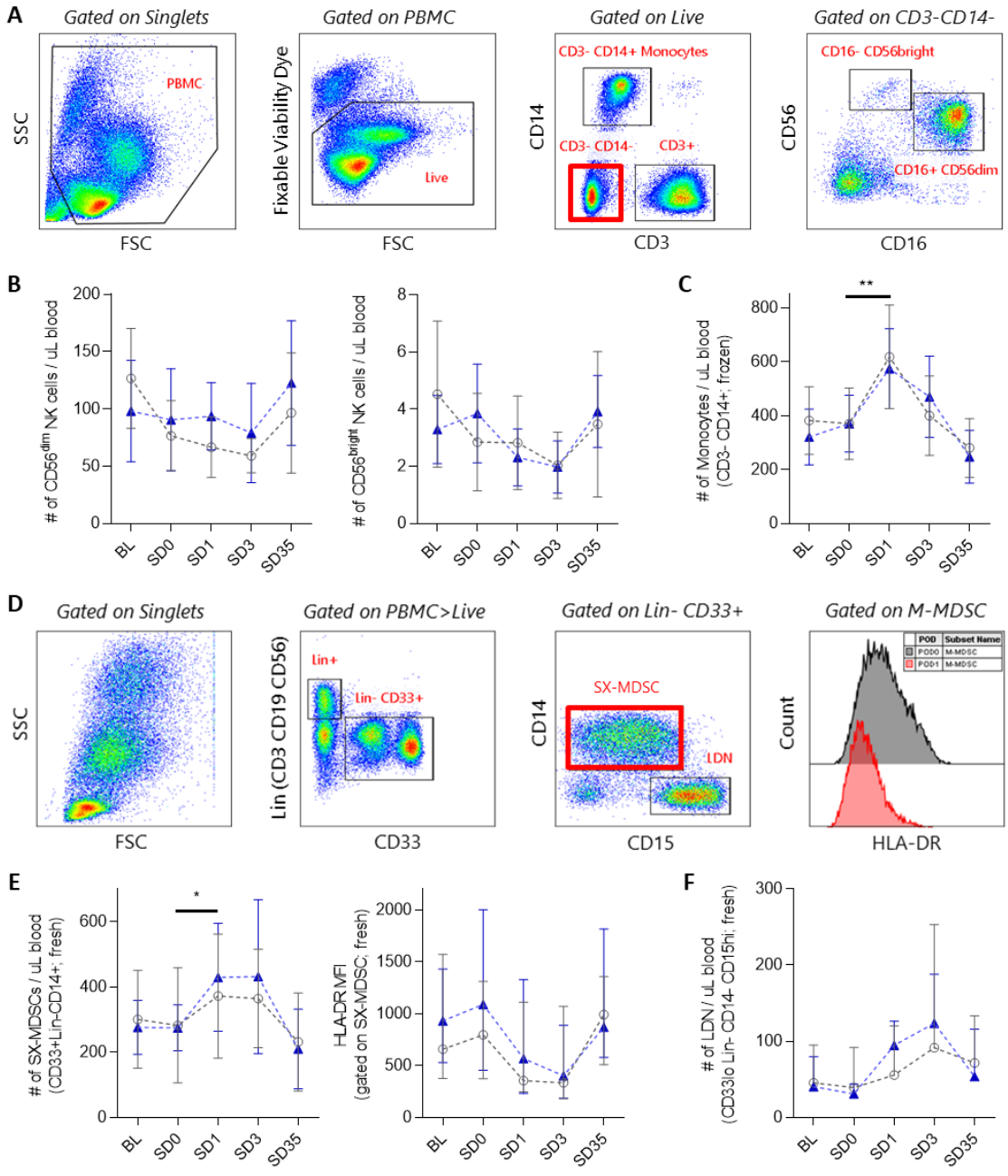


Figure 5.5. Effects of perioperative arginine immunonutrition on circulating NK cell and Sx-MDSC populations.

Figure 5.5. Effects of perioperative arginine immunonutrition on circulating NK cell and Sx-MDSC populations.

A) Gating strategy for identifying NK cells by flow cytometry from cryopreserved samples (control n=10; AES n=12). **B)** Absolute number of circulating $CD56^{dim}$ (left panel) and $CD56^{bright}$ (right) NK cells/μL of blood. **C)** The absolute number of circulating $CD3^{-}CD14^{+}$ monocytes/μL of blood from cryopreserved PBMCs. **D)** Gating strategy for identifying M-MDSCs and low density neutrophils (LDNs) by flow cytometry from freshly isolated PBMCs. **E)** Absolute number of Sx-MDSCs ($CD33^{+}Lin^{-}CD14^{+}CD15^{lo/-}$, left panel) and MFI of HLA-DR expression on Sx-MDSCs (right panel). **F)** Absolute number LDNs ($CD33^{lo}Lin^{-}CD14^{-}CD15^{hi}$).

5.6 DISCUSSION

To our knowledge, this is the first study to assess changes in postoperative NK cell function (i.e. NKC, NKA and cell frequencies) after perioperative arginine-based immunonutrition in surgery patients. Reports on the impact of perioperative immunonutrition have been primarily limited to assessments of the overall changes in systemic inflammatory and immunological markers. While these immunological parameters are important to capture, it is equally important to measure whether these postoperative changes impact immune effector cell functions. The AES group had a 50% improvement in NKC on SD1 compared to control, however, this benefit was not seen at later postoperative time points. Furthermore, perioperative AES did not influence NKA suppression induced by surgery (Figure 3) and the frequency of NK cell subtypes or Sx-MDSC populations did not differ between the groups at any time point. The sum of these results revealed that perioperative arginine immunonutrition results in, at best, a modest improvement in NK cell function immediately after surgery. However, it is unclear from this study how much of this can be attributed to poor postoperative compliance, primarily due to gastrointestinal intolerance (Figure 4).

Multiple meta-analyses have demonstrated improved postoperative outcomes associated with perioperative arginine immunonutrition [131–133]. Notably, regarding the timing of immunonutritional support, these meta-analyses conclude that full perioperative immunonutrition (pre and post) provides the best and most consistent efficacy compared to immunonutrition given only pre- or postoperatively. Indeed, a lack of efficacy has been reported in numerous studies that administered only preoperative immunonutrition in CRC patients [264,265]. Thornblade *et al.* analyzed the postoperative outcomes of CRC surgery patients receiving 5 days of preoperative immunonutrition (n=632) vs no immunonutrition (n=2743) and found no differences in infectious complications or median LOS [265].

Therefore, although the participants in our study had optimal levels of preoperative compliance, accumulating evidence supports postoperative immunonutrition as a requisite for improved surgical outcomes, which makes sense given the rapid clearance of arginine from the blood following oral administration that we observed (Figure 4). It is however interesting that both study arms had an improvement in NKC on SD0 (preoperatively), and it is possible that this reflects benefits associated with an overall improvement in protein intake leading up to surgery [266].

Given that AES is promoted to be an immunonutrition supplement, we conducted a comprehensive evaluation of all clinical trials reported across three systematic reviews [131–133] in order to determine whether these trials reported on compliance, arginine concentrations, systemic inflammatory/immune markers, and functional immune assays. Unexpectedly, we found a very low reporting frequency of these important endpoints (Table 2).

Only 6/22 (27%) studies administering postoperative enteral immunonutrition reported on compliance (Table 2). In these studies, postoperative compliance was adequate due to the use of enteral feeding tubes, a practice that has been abandoned following the widespread adoption of Enhanced Recovery After Surgery (ERAS) [229]. Only 18% (9/50 studies) measured the effect of immunonutritional supplementation on arginine concentrations, with 56% (5/9 studies) reporting a significant increase after arginine immunonutrition. Notably, while only 4/18 studies assessing preoperative immunonutrition alone reported on arginine concentrations, none of these reports measured arginine concentrations after surgery [267–270]. This is a critical time point as the therapeutic benefit of arginine may lie only in its ability to exert immunomodulatory effects during the postoperative period.

Table 5.2. Review of immune correlate reporting found in clinical reports using perioperative immunonutrition.

Category	Preop	Postop	Periop	All studies
Studies (n)	18	22	10	50
CRC only (n, %)	*10 (56%)*	*3 (14%)*	*2 (20%)*	*15 (30%)*
Non CRC Cancer (n, %)	*8 (44%)*	*19 (86%)*	*6 (60%)*	*33 (66%)*
Non Cancer (n, %)	*0 (0%)*	*0 (0%)*	*2 (20%)*	*2 (4%)*
Reported compliance* (n, %)	8/18 (44%)	6/22 (27%)	4/10 (40%)	18/50 (36%)
Reported on Arginine levels (n, %)	4/18 (22%)**	2/22 (9%)	3/10 (30%)	9/50 (18%)
Reports where arginine increased (n, %)	*3/4 (75%)*	*2/2 (100%)*	*1/3 (33%)*	*5/9 (56%)*
Reported on infectious comps (n, %)	16/18 (89%)	17/22 (77%)	7/10 (70%)	40/50 (80%)
Reported a significant decrease in infectious comps (n, %)	*7/16 (44%)*	*10/17 (59%)*	*7/7 (100%)*	*24/40 (60%)*
Reported on systemic characterization of immune outcome measures***	12/18 (67%)	13/22 (59%)	8/10 (80%)	33/50 (66%)
Reported on functional immune outcome measures	5/18 (28%)	6/22 (27%)	1/10 (10%)	12/50 (24%)
Mitogenesis/lymphocyte proliferation	*3/5 (60%)*	*1/6 (17%)*	*0/1 (0%)*	*4/12 (33%)*
Neutrophil/Monocyte Phagocytosis assay	*2/5 (40%)*	*3/6 (50%)*	*0/1 (0%)*	*5/12 (42%)*
Antigen response	*0/5 (0%)*	*2/6 (33%)*	*1/1 (100%)*	*3/12 (25%)*
Cytotoxic potential	*0/5 (0%)*	*0/6 (0%)*	*0/1 (0%)*	*0/12 (0%)*

Studies found in Drover et al, 2011; Osland et al, 2014; and Adiamah et al, 2019

*these studies used postoperative feeding tubes to administer immunonutrition

**Only preoperative arginine levels reported

***systemic changes in inflammatory markers, immune cell numbers, and/or cell surface phenotypic changes

Our primary rationale for the present study was to explore the ability of AES to restore or prevent postoperative NK cell dysfunction because this dysfunction results in increased cancer metastases in experimental models. It should be noted that T cells and other non-immunological factors may have been positively affected by the AES immunonutrition, but that was beyond the scope of our study. We focused on NK cells because in clinical studies, NK cell function can be used as a prognostic marker for cancer outcomes in CRC patients [150,252]. A retrospective study in 447 CRC patients showed that NKC was an accurate predictor for overall survival, especially when combined with other prognostic factors such as blood CEA [253]. NK cells are also one of the main producers of IFN-γ, a cytokine which choreographs the anti-tumour adaptive immune response, but has been shown to be severely suppressed after surgery [67,210,271]. Numerous groups have shown that arginine deprivation results in impaired IFN-γ secretion which was not due to reduced NK cell viability [89,91]. Evaluating the amino acid deprivation response via the phosphorylation and activity of mTOR and its downstream target, 4EBP1, Goh et al. showed that these targets were reduced in NK cells cultured in arginine depleted conditions [91]. The mTOR pathway is able to sense nutrient availability and halt translational processes in their absence which may be a potential mechanism for impaired NKA after surgery. Our study showed that oral arginine supplementation alone is not sufficient to prevent the paralyzing effects of surgery on NKA. We also measured the expression levels of HLA-DR on Sx-MDSCs following immunonutrition as this has been used as an indicator of the antigen presenting capacity and the maturation status of myeloid cells, which has been shown to decrease following surgery [263]. In our study, there was no specific benefit of AES on HLA-DR expression from baseline (BL) to SD0, and was reduced immediately after surgery in both groups (Figure 5).

Despite strong preclinical rationale for perioperative AES in reducing postoperative NK cell dysfunction [259], this study was unable to confirm this effect. Our review of relevant clinical trials (Table 2) suggests that additional studies are needed to determine the mechanism of action of AES before it can be clearly labelled "immunonutrition". Moreover, alternative strategies for arginine delivery to improve compliance and pharmacokinetics (such as intravenous delivery) should be undertaken given the issues with postoperative compliance. In the absence of these studies, it is unclear how AES is working to provide benefit, particularly given that it adds significant costs compared to the standard of care [272].

The results of this single-centre, double-blind, randomized control clinical trial highlights the need for translational studies to complement clinical trials in reporting efficacy outcomes. Rigorous testing combined with thoughtful clinical trial design that focus on the direct effect of AES on functional immune parameters is pertinent to substantiating improved postoperative immune function due to AES. In the present study, we tested the hypothesis that arginine supplementation improves NK cell function and increases arginine levels. The results of this Phase II trial revealed that *i)* postoperative NK cell dysfunction, as measured by NKC was modestly improved with no effect on NKA and circulating NK cell numbers; *ii)* compliance with postoperative oral supplements is quite poor overall in CRC patients; and *iii)* elevated blood arginine level was not maintained at a steady state. In conclusion, oral consumption of AES in CRC patients is not able to reverse all of the immunosuppressive effects surgery has on NK cells and postoperative intolerance of oral supplements may be a factor.

5.7 SUPPLEMENTARY FIGURES

EMN - Formulated Liquid Diet (Citrus/Vanilla)
Placebo
Nutrition Facts
Valeur nutritive
Per 1 pouch(81g)/Pour 1 poche (81g)

Amount Teneur	% Daily Value % valeur quotidienne
Calories / Calories 300	
Fat / Lipides 0 g	**0%**
Saturated / satures 0.2 g + Trans / trans 0 g	**1%**
Cholesterol / Cholersterol 4 mg	
Sodium / Sodium 160 mg	**7%**
Carbohydrate / Glucides 55g	**18%**
Fibre / Fibres 0.0 g	**0%**
Sugars / Sucres 21g	
Proteins / Proteines 20 g	
Vitamin A / Vitamine A	**68%**
Vitamin C / Vitamine C	**12%**
Calcium / Calcium	**16%**
Iron / Fer	**21%**

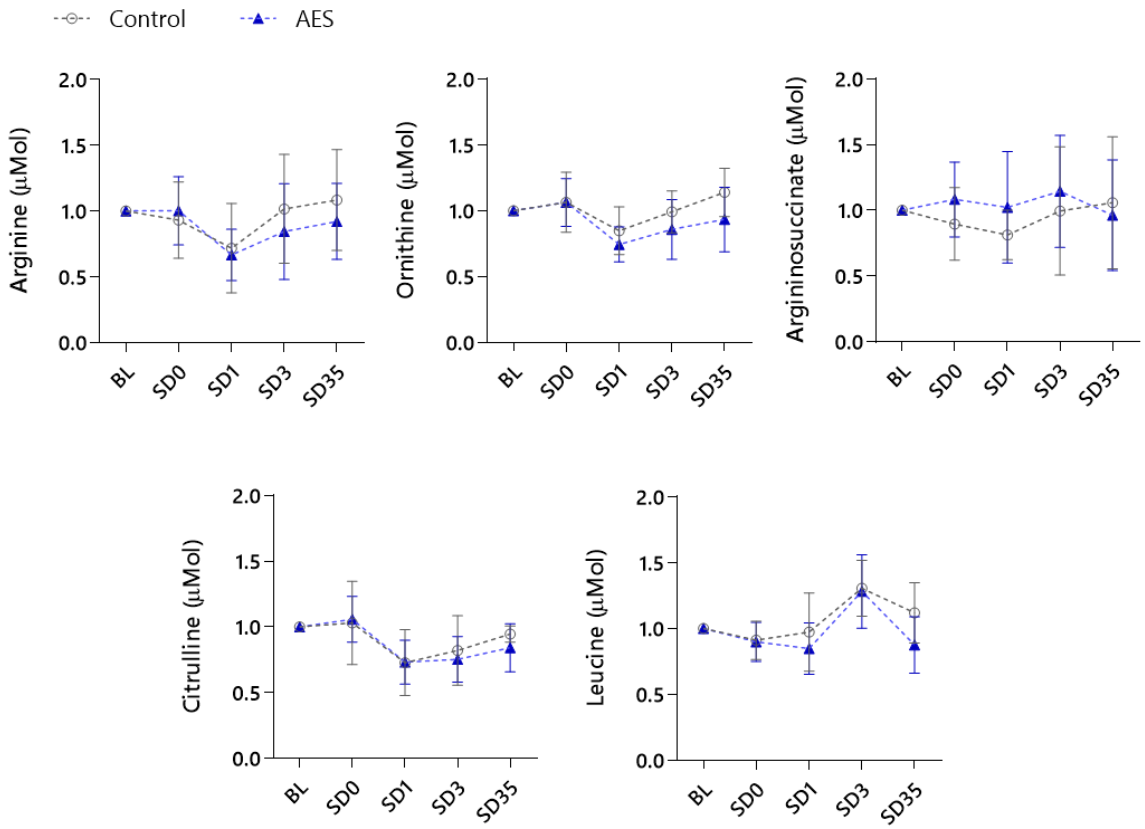
Ingredients / Ingrédients
Maltodextrin, Whey Protein Isolate, Sucrose, Magnesium Gluconate, Natural Flavours, Citric Acid, Calcium Phosphate Tribasic, Ferrous Gluconate, Zinc Sulfate, Ascorbic Acid, Retinyl Palmitate, Copper Gluconate, D-Alpha Tocopheryl Acetate, Nicacinamide, D-Calcium Pantherate, Pyridoxine HCL, Chloecalciferol, Riboflavin, Thiamine Mononitrate, Cyanocobalamin With Mannitol, Folic Acid, Potasium Iodide

EMN - Formulated Liquid Diet (Citrus/Vanilla)
Active
Nutrition Facts
Valeur nutritive
Per 1 pouch(85g)/Pour 1 poche (85g)

Amount Teneur	% Daily Value % valeur quotidienne
Calories / Calories 300	
Fat / Lipides 0 g	**0%**
Saturated / satures 0.2 g + Trans / trans 0 g	**1%**
Cholesterol / Cholersterol 4 mg	
Sodium / Sodium 160 mg	**7%**
Carbohydrate / Glucides 55g	**18%**
Fibre / Fibres 0.0 g	**0%**
Sugars / Sucres 21g	
Proteins / Proteines 20 g	
Vitamin A / Vitamine A	**68%**
Vitamin C / Vitamine C	**12%**
Calcium / Calcium	**16%**
Iron / Fer	**21%**

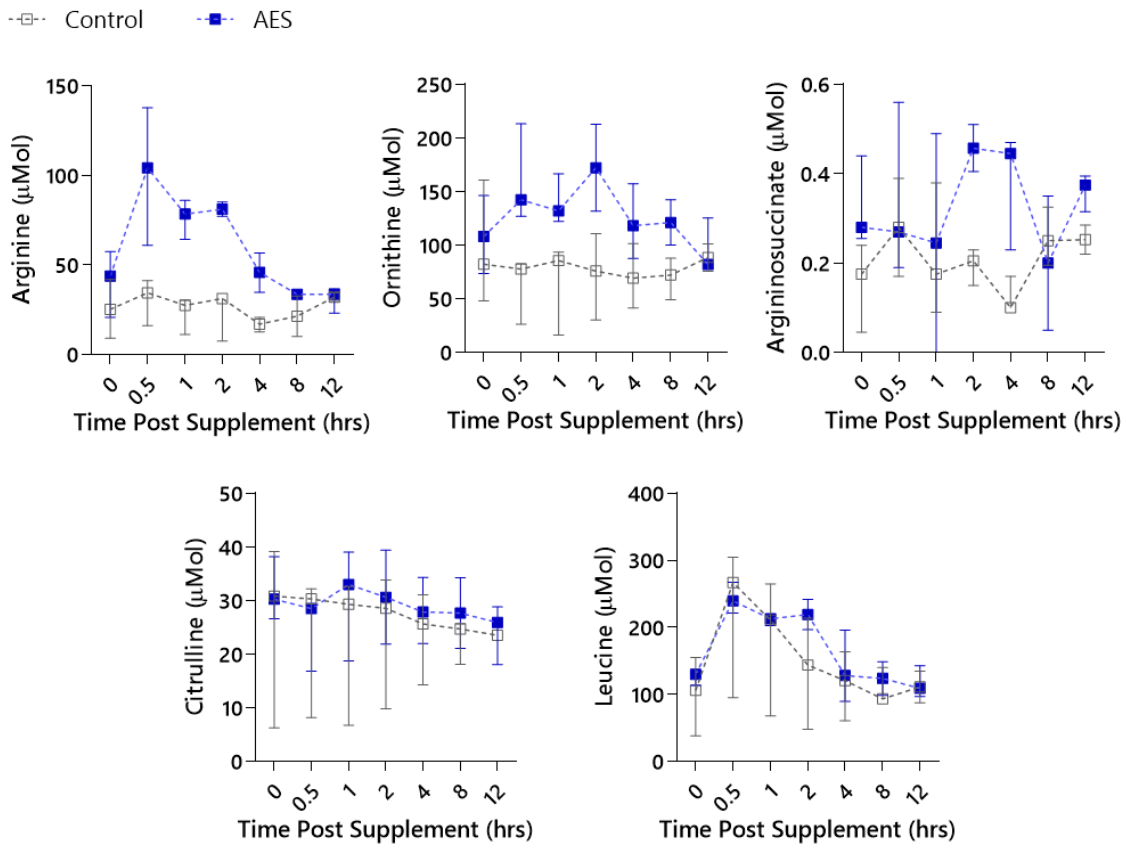
Ingredients / Ingrédients
Maltodextrin, Whey Protein Isolate, Sucrose, L-Arginine, Magnesium Gluconate, Natural Flavours, Citric Acid, Calcium Phosphate Tribasic, Ferrous Gluconate, Zinc Sulfate, Ascorbic Acid, Retinyl Palmitate, Copper Gluconate, D-Alpha Tocopheryl Acetate, Nicacinamide, D-Calcium Pantherate, Pyridoxine HCL, Chloecalciferol, Riboflavin, Thiamine Mononitrate, Cyanocobalamin With Mannitol, Folic Acid, Potasium Iodide

Supplemental Figure 5.1. Composition of Control and Arginine Enriched Supplement.



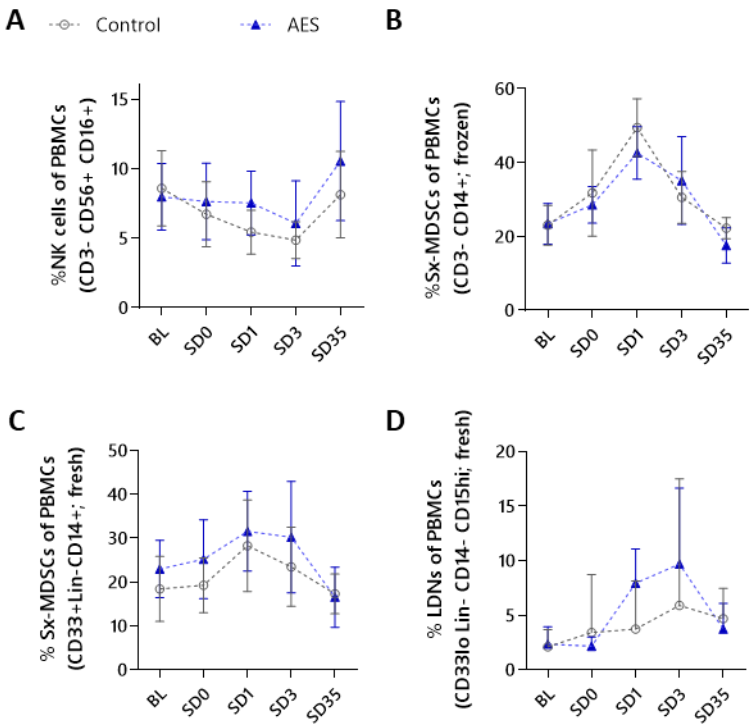
Supplemental Figure 5.2. Patient blood amino acid levels normalized to Baseline (BL).

In all amino acids measured, there was no significant increase on SD0 (after preoperative intervention) when normalized to their baseline levels. From SD0 to SD1, there were observable drops in arginine, ornithine, and citrulline, but not in argininosuccinate or leucine. By SD35, amino acid levels returned to their baseline level. Control n=11, AES n=12.



Supplemental Figure 5.3. Blood amino acid kinetics following one dose of either control supplement or AES from healthy volunteers (no surgery; n=3).

AES consumption led to a transient increase in certain amino acids that are involved in the arginine/Arginase-1 metabolism pathway (arginine, ornithine, and argininosuccinate). In both perioperative intervention arms citrulline levels remained unchanged while leucine levels had a transient increase.



Supplemental Figure 5.4. Immune cell populations as a percentage of live PBMCs.

Proportion of **A)** NK cells ($CD3^-$ $CD56^+$ $CD16^+$) and **B)** Sx-MDSCs ($CD3^-$ $CD14^+$) of PBMCs from cryopreserved samples (Control n=10; AES n=12). Proportion of **C)** Sx-MDSCs ($CD33^+$ Lin^- $CD14^+$) and **D)** low density neutrophils (LDNs; $CD33^{lo}$ Lin^- $CD14^-$ $CD15^{hi}$) from freshly isolated PBMCs (Control n=10; AES n=12).

Chapter 6

General Discussion

The different bodies of work presented within this thesis have been organized to first provide a clearer picture of the perioperative immune landscape and surgery induced NK cell dysfunction (Chapters 2 and 3) before investigating the use of rationally conceived perioperative immunotherapies (Chapter 3, 4 and 5). As each chapter has their own specific discussion, this general discussion will focus on bridging these findings, expanding on discussion points, and presenting the limitations and future areas of investigation.

6.1 NK cell dysfunction is worse than previously described.

While NK cell suppression after surgery has been well established, most studies have focused on the detrimental effect of surgery on NKC [38]. Groups have shown that whole blood stimulation after surgery results in impaired IFN-γ production [66,67,273,274], but these studies did not assess IFN-γ secretion past POD7. Furthermore, these studies used stimulatory cytokines and biologics such as Concanavalin A or LPS, which would surely stimulate cells other than NK cells present within the blood. Thus, the advantage of our study [210] was the use of the NK cell-specific cytokine cocktail (PROMOCA) paired with a focused inquiry on NK cell derived IFN-γ over an extended postoperative follow-up time (up to POD56).

Measuring NKA through the NK Vue^{TM} assay was, at the time, a novel application of a new technology/assay that had been shown to accurately predict colorectal cancer risk when compared to the patients' colonoscopy results [150]. Now, NK Vue^{TM} has been utilized in subsequent clinical studies, adding to the prognostic value of this assay. Hansen et al.

showed that palliative cancer patients who had NKA values of <200 pg/mL had a median progression free survival (PFS) of only 2.6 months (n=35) compared to patients with >200 pg/mL NKA who had a significantly longer median PFS of 10 months (n=28) [275,276]. Another study applied the NK Vue assay in the context of robot-assisted laparoscopic radical prostatectomy and showed that positive surgical margins (where the cancer is incompletely removed) resulted in significantly lower 4-6 week postoperative NKA values compared surgeries with negative margins (557 vs. 1921 pg/mL, respectively) [277]. An interesting follow up to our studies would be to assess if there was any correlation between NKA (on POD28 or POD56) and recurrence free / overall survival in our cancer surgery cohort especially given the observation that 50% (6/12) of the CRC patients on POD56 had an NKA of <200 pg/mL (Fig. 2.2 and Table 2.2).

It is amazing how long the suppressive effects of surgery on NKA persists in both human surgery patients (Fig 2.2 and Fig 5.3) and in our murine studies (Fig 4.4 and fig S4.3). IFN-γ results in multiple downstream antitumour effects such as increased antigen presentation and processing by APCs, activation of cytotoxic functions in NK and CD8 T cells, and the further propagation of Th1 responses and inhibition of Th2 signals [278]. This underscores how the effects of surgery can result in a window of opportunity for cancer cells to establish themselves into metastatic niches in the absence of fully functional NK cells. The anti-inflammatory cytokines (IL-4, IL-10, and TGF-β) that characterize the postoperative period are known to suppress IFN-γ secretion in NK cells [19,24,79,279,280]. IL-4 and IL-10 plasma concentrations are elevated after surgery and CRC patients who develop postoperative infectious complications were shown to have significantly higher IL-4 and IL-10 [281]. In our murine models of surgical stress, TGF-β was significantly increased after surgery, but IL-4 and IL-10 were not [35]. Dr. Market (recent MD/PhD graduate from the Auer lab) has shown in her thesis that recombinant TGF-β recapitulates the phenotype of

NK cell dysfunction after surgery (reduced IFN-γ, activating receptor expression, and downstream signalling). Furthermore, TGF-β was also found to be elevated in POD1 plasma, but TGF-β mAbs prevented NK cells suppression in *ex vivo* co-cultures. TGF-β has been associated with a higher rate of mortality and shorter disease free survival after surgery in CRC patients [282]. Lastly, it has been shown that TGF-β mRNA is still elevated by POD14 in mice [283] which may perpetuate the immunoparalysis of NKA. Therefore, to comprehensively know the extent of NK cell dysfunction in patients, one should assess NKA at later time points after surgery, to ensure that the recovery of NKA has occurred.

6.2 Characterizing Sx-MDSCs

There is a long-standing debate on whether MDSCs are distinct immunoregulatory cell-types or if they are pleiotropic neutrophils and monocytes at different stages of differentiation that have both anti- and pro-tumoral mechanisms [193,195,284]. Some groups define M-MDSCs as the precursors to tumor associated macrophages (TAMs) [206] with the accepted understanding that M-MDSC still have the potential for further (albeit reduced) differentiation into terminal monocytic cell types. Similarly, PMN-MDSCs have been compared to tumor associated neutrophils (TANs) [285]. Therefore, distinguishing between M-MDSCs, TAMs, and M2 macrophages or PMN-MDSCs, TANs, and N2 neutrophils would provide much needed clarity when reporting on these cells.

The minimum characteristics needed to identify an MDSC is a combination of phenotypic myeloid markers (described in Section 1.3) and a functional assay demonstrating their suppressive activity [99]. The monocytic Sx-MDSCs in humans represented a continuum of differentiation, exemplified in the scRNA-seq data and further supported by the range of HLA-DR expression. Gaudilliere et al. also showed that monocytes after surgery could be clustered according to their level of HLA-DR expression [32], and we see similar trends in our non-negative matrix factorization (NMF) programs where the gene expression programs

that best matched a putative MDSC gene set had lower HLA-DR expression (Fig 3.1). Often in the literature, flow cytometry analysis of M-MDSCs set very stringent gates on HLA-DR expression levels, which may limit the identification of M-MDSCs to only include cells that are HLA-DR^{neg} (for example, see fig 1. [286]). The gating strategy that I used in my characterization of Sx-MDSCs assessed HLA-DR expression from the whole monocytic population (CD14^{+}CD33^{+}Lin^{-}) to show that there is a decrease in the total POD1 monocyte population, similar to the guidelines found in Mandruzzato et al. (MDSC4 subtype) [98]. This gating strategy provides more information on the magnitude of surgical stress on all monocytic cells depending on the decrease of HLA-DR after surgery. A limitation of our suppression assays with MDSCs is that the sorting method used does not discriminate between HLA-DR status, but I hypothesize that the suppression would be even greater in a sample containing only HLA-DR$^{lo/neg}$ Sx-MDSCs.

Density centrifugation is a method that must be used to successfully identify PMN-MDSCs in the population of low density neutrophils (LDNs) that fractionate within the PBMC buffy coat layer [99]. Recently, Condamine et al. showed that LDN PMN-MDSCs vs high density neutrophils (HDNs) had increased expression of the gene OLR1 and its protein product LOX1 on PMN-MDSCs [190]. Furthermore, they showed that PMN-MDSCs and not HDNs were able to suppress T cells. Encouragingly, I was able to confirm their findings by showing that PMN-MDSCs, and not HDNs, were able to suppress NK cells (Fig 3.3E). LOX-1 expression was also confined to the PMN-MDSC subset, but did not increase after surgery (Fig 3.2). The identification of unique markers to identify the various MDSC subsets after surgery would be a game changer that would allow for specific targeting of Sx-MDSCs. Dr. Martel (MSc/surgical resident from the Auer lab) has been investigating a putative marker that is upregulated specifically on M-MDSCs after surgery.

6.2.1 Unexplored aspects of Sx-MDSCs

Unexpectedly, many markers of exosomes (CD63, CD82, and THBS1) were identified to be highly upregulated in our MDSC gene expression programs (Chapter 3). MDSCs have been shown to produce and carry exosomes that contain immunosuppressive and chemotactic factors [109,287]. Few have reported on postoperative exosomes, but they have been observed to be increased in the serum of coronary bypass surgery patients [288] and in the context of surgical sepsis [289]. It would be interesting to investigate whether Sx-MDSCs utilize exosomes to exert their immunosuppressive mechanisms. Additionally, Wang et al. showed that postoperative M-MDSCs had a greater ability to induce the expansion of $CD4^+CD25^+Foxp3^+$ Tregs in *in vitro* settings [173]. Notably, there were also large transcriptional changes in the CD4 populations on POD1 (Fig. 3.1C). It would be interesting to determine what those changes are, and whether that transcriptional shift represents a phenotype switching or emergence of CD4 T cells to Tregs or an alternative T-helper cell cytokine profile (Th1, Th2, Th17 etc.).

6.3 Targeting MDSCs Perioperatively

From the MDSC depletion studies (Fig. 4.2) it is clear that these cells play a critical role in mediating postoperative NK cell suppression, arginine depletion, and lung metastases. However, taking this approach in cancer surgery patients is not a feasible option due to the negative consequences of ablating ones myeloid progenitors. Therefore, in the absence of a defining Sx-MDSC marker, targeted perioperative therapies are limited. Furthermore, systemic delivery of MDSC antagonists can have off target effects on other cells of the immune system which I have seen in our *ex vivo* validation studies of other candidate compounds. Even pan-PI3K inhibition resulted in reduced NK cell cytotoxicity at concentrations >1μM (Fig. 3.4C). For this reason we assessed the ability of PI3K-γ specific inhibitors to target Sx-MDSCs specifically since the catalytic p110-γ subunit is

preferentially expressed in myeloid cells [104,290]. Unfortunately, systemic delivery of PI3K-γ specific inhibitors (TG100-115 and IPI-549) did not attenuate metastases (Fig S3.8B), instead, IPI-549 treatment actually worsened metastatic disease before and after surgery due to off target effects on NK cells (Fig S3.8C). Furthermore, only TG100-115 was able to attenuate Sx-MDSC mediated NK cell suppression when these PI3K-γ specific inhibitors were delivered systemically (Fig S3.8D and E). Therefore, given the off target potential of this class of compounds, designing a targeted therapy against Sx-MDSCs would greatly increase the therapeutic potential of these compounds. To that end, we have collaborated with adMare to design an antibody drug conjugate (ADC) using Compound 17 (PI3K-γ specific inhibitor) [202] bound to a putative cell surface marker that has been identified to be increased on monocytic Sx-MDSCs by Dr. Martel.

6.4 Perioperative Arginine

As an alternative to direct targeting Sx-MDSCs, I investigated the ability of arginine supplementation to mitigate effects of reduced postoperative arginine [87,122], which also was shown to be regulated by Sx-MDSCs (Fig 4.2). Normal physiological concentrations of plasma arginine range from ~84 to 111μM [213]. In my experiments, arginine was measured from dried blood spots and not plasma, which resulted in lower arginine levels in our studies (Chapter 4/5). Regardless, blood arginine levels were still significantly reduced in cancer patients vs healthy donors (Fig 4.6) and on POD1 vs matched BL samples (Fig 5.4). Healthy volunteers (n=9) had a median arginine level of 47.7μM (95% CI: 27.5-56.4μM) and the CRC patient cohort (n=22) had a lower preoperative arginine level of 30μM (95% CI: 23.7-35μM). On POD1 their arginine levels dropped to a median of 21.5μM (95% CI: 16.5-27μM).

In order to underscore the importance of arginine for NKC, I performed *in vitro* experiments where NK cells were cultured in decreasing amounts of arginine. At supraphysiological

conditions of arginine (>50μM), NKC was unaffected, but in arginine depleted conditions (<50μM) NKC was suppressed (Fig. 4.5). Although the *in vitro* experiments support the necessity of arginine for NK cell function, they do not fully recapitulate the events following surgical stress. The main disparities that need to be considered are the length of time that NK cells spend in arginine depleted conditions, the simultaneous contribution of anti-inflammatory cytokines and the potential additional suppressive mechanisms harbored by Sx-MDSCs that were not present in the *in vitro* experiments. In the Sx-MDSC conditioned media experiments, adding exogenous arginine back into the media did not fully prevent the reduction in NK cell cytotoxicity which supports that other Sx-MDSC derived factors (e.g. TGF-β) contribute to reduced NK cell function after surgery.

6.4.1 Considerations for perioperative immunonutritional therapies

Perioperative restriction of certain amino acids is a strategy based on the rationale that tumours also rely on certain amino acids to drive tumorigenesis [237]. Therefore, amino acid deprivation therapies (mainly targeting glutamine, asparagine, and arginine) have been devised to starve growing tumours of essential amino acids [239]. For example, acute lymphoblastic leukemias (ALL) are auxotrophic for L-asparagine (they cannot synthesize adequate amounts) and therefore depend on exogenous sources for cell growth [240]. Asparaginase therapy to limit the amount of available asparagine for cancer growth is an approved treatment option for ALL patients [241]. Glutamine is also an important fuel source for tumour cells and interestingly glutamine deprivation does not impair NK cell IFN-γ production from murine NK cells [291]. Lastly, arginine is also known to have a role in tumour growth and proliferation [237], which is why arginine deprivation via pegylated Arg1 is under investigation [237,238]. Therefore, in addition to fortifying perioperative formulations with immune-boosting components, understanding the postoperative

importance of certain amino acids and limiting the presence of certain amino acids in perioperative formulas would further improve their therapeutic benefit.

Although briefly discussed in Chapter 5.6, I wanted to highlight here that supraphysiological levels of arginine did not result in enhanced or improved NK cell potential above homeostatic levels. Furthermore, since arginine levels were similar immediately after surgery regardless of the nutritional diet (Fig 4.3 and Fig 5.4), it is uncertain what postoperative benefit resulted from immunonutrition before surgery. Apart from ensuring patients are not malnutritious going into surgery, preoperative arginine supplementation in mice and in humans did not have any perceived benefit on NK cell function immediately after surgery (Fig 4.4 and Fig 5.3). Therefore, studies on the timing of perioperative immunonutrition should be undertaken to determine specifically if preoperative treatment confers any immunological benefit as increased arginine levels may enhance the growth of the primary tumour or micrometastases [239].

6.4.2 Determining a mechanism for postoperative NK cell recovery with arginine

As for determining a mechanism to explain the accelerated recovery of NK cells with supplemental arginine observed in the murine studies (Chapter 4), a starting point would be to assess the effect of supplemental arginine on the mTOR pathway. As described in section 1.2.2, previous studies have shown that the GCN2 pathway is not affected by reduced postoperative arginine [89]. Instead, the phosphorylation of 4EBP1 [91], a downstream phosphorylation target of mTOR, is reduced after surgery. Downstream phosphorylation of S6-kinase (pS6K) is also an indicator of mTORC1 activation [292] which we have seen is significantly reduced in NK cells from patients after surgery (Market et al. manuscript in preparation). Therefore, it would be exciting to see if arginine supplementation enhances the phosphorylation of these two targets (4EBP1 and S6K) in recovering postoperative NK cells compared to control fed animals.

6.5 Concluding thoughts

The perioperative period is unique because it superimposes an acute inflammatory response on top of a chronic inflammatory setting established previously by the primary cancer [293]. Since the need to operate often outweighs the risks associated with surgery, minimizing the adverse sequelae of cancer surgery is the ultimate goal of this research. Decades of research has shown that NK cells are particularly affected by surgery [140]. Here, I have shown that the suppression extends for much longer than previously thought [210]. A key mediator of NK suppression are Sx-MDSCs which boast an array of suppressive machinery that may need to be targeted simultaneously to fully prevent their immunosuppressive activity (Chapter 3). Although our MDSC-antagonist compound screen identified a biological pathway amenable to therapeutic targeting (PI3K), further studies will need to be done to determine the best method of *in vivo* delivery. Likewise, a barrier to perioperative arginine immunonutrition in CRC surgery patients was postoperative compliance, which indicates that this therapy may benefit from alternative parenteral routes of delivery (Chapter 4 and 5). Furthermore, while we only saw a modest beneficial effect on NK cells in our clinical trial, we did not assess the potential benefits on adaptive immune responses, which could provide valuable insight into the mechanism of perioperative arginine.

The paradoxical nature of cancer surgery is fascinating, frustrating, and deserving of further investigation to ensure the best course of perioperative care for cancer surgery patients. Historically, perioperative therapies have been slow to adopt due to concerns about safety and efficacy in the recovering surgery patient. However, decades of research have clarified our understanding of the perioperative period. Therefore, research should now be focused on exploiting the perioperative period to generate novel, targeted approaches that complement and improve surgical outcomes. This will ensure that “curative” cancer surgeries remain *curative*.

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APPENDIX A

Open Access Review

Dysfunctional Natural Killer Cells in the Aftermath of Cancer Surgery

by Leonard Angka [1,2], Sarwat T. Khan [1,2], Marisa K. Kilgour [3], Rebecca Xu [1], Michael A. Kennedy [1] and Rebecca C. Auer [1,4,*]

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Abstract

The physiological changes that occur immediately following cancer surgeries initiate a chain of events that ultimately result in a short pro-, followed by a prolonged anti-, inflammatory period. Natural Killer (NK) cells are severely affected during this period in the recovering cancer patient. NK cells play a crucial role in anti-tumour immunity because of their innate ability to differentiate between malignant versus normal cells. Therefore, an opportunity arises in the aftermath of cancer surgery for residual cancer cells, including distant metastases, to gain a foothold in the absence of NK cell surveillance. Here, we describe the post-operative environment and how the release of sympathetic stress-related factors (e.g., cortisol, prostaglandins, catecholamines), anti-inflammatory cytokines (e.g., IL-6, TGF-β), and myeloid derived suppressor cells, mediate NK cell dysfunction. A snapshot of current and recently completed clinical trials specifically addressing NK cell dysfunction post-surgery is also discussed. In collecting and summarizing results from these different aspects of the surgical stress response, a comprehensive view of the NK cell suppressive effects of surgery is presented. Peri-operative therapies to mitigate NK cell suppression in the post-operative period could improve curative outcomes following cancer surgery. View Full-Text

Keywords: Natural Killer (NK) cells; surgery; immunosuppression; peri-operative therapies; immunity; immunotherapy

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APPENDIX B

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SPRINGER NATURE

Natural Killer Cell IFNγ Secretion is Profoundly Suppressed Following Colorectal Cancer Surgery

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PhD Publications

First Authored:

1. **Leonard Angka**, Christiano T. De Souza, Katherine E. Baxter, Sarwat Khan, Marisa Market. Perioperative Arginine Prevents Metastases by Accelerating Recovery of Natural Killer Cell Function After Surgery. Submitted to Sci Trans Med.
2. **Leonard Angka;** Andre B. Martel; Juliana Ng; Amanda Pecarskie; Manahil Sadiq et al. A translational randomized controlled trial of perioperative arginine immunonutrition on Natural Killer cell function in colorectal cancer surgery patients. Submitted to JCI Insights, #144254-INS-CMED-1
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2. Lee EA, **Angka L,** Rota SG, Hanlon T, Mitchell A, Hurren R, Wang XM, Gronda M, Boyaci E, Bojko B, Minden M, Sriskanthadevan S, Datti A, Wrana JL, Edginton A, Pawliszyn J, Joseph JW, Quadrilatero J, Schimmer A, Spagnuolo PA. Targeting mitochondria with avocatin B induces selective leukemia cell death. Cancer Research. June 2015. Original Research.
3. **Angka L** and Spagnuolo PA. From food to clinical medicine – nutraceuticals as clinical therapeutics for hematological malignancies. Current opinion in food science. March 2015. Review Article.
4. **Angka L,** Spagnuolo, P.A. Interactions between nutraceutical supplements and standard acute myeloid leukemia chemotherapeutics. Journal of Pharmacy and Pharmaceutical Sciences. August 2015. Original Research.
5. Rogers MA, Spagnuolo PA, Wang T-M, and **Angka L.** A Potential Bioactive Hard-Stock Fat Replacer Comprised of a Molecular Gel. Food Science and Nutrition. September 2016. Original Research.

Honours and Awards

PhD awards:

1. BioCanRX Summit 4CI Conference for Best Speed Poster (2019)
2. Faculty of Medicine Graduate Mentorship Award (2019)
3. 3rd Place award for BMI Seminar day (2017)
4. Ontario Graduate Scholarship (2016-2019)
5. University of Ottawa Excellence Scholarship and Tuition Scholarships (2015-2020)
6. Queen Elizabeth II Graduate Scholarship Science and Technology (2015-2016)

MSc awards:

7. M.Sc. Dean of Science Award for the Department of Pharmacy (2015)
8. Donald J. & Kathleen D. McDougall Graduate Scholarship (2014)
9. Association of Faculties of Pharmacy of Canada RX&D Poster Award (2014)
10. Special Merit Award for Impact on Pharmacy Research Mission (2013)

Conference Presentations

1. Oral talk. SSO 2020. Boston, MA. Aug 2020. Virtual presentation due to COVID-19.
2. Poster. 5th Annual Canadian Cancer Research Conference. Ottawa, ON. Nov 2019.
3. Poster. 4th Annual Summit for Cancer Immunotherapy. Victoria, BC. Oct 2019.
    a. Member of the HQP working group responsible for planning the conference
4. Oral talk. 25th Canadian Society of Surgical Oncology. Toronto, ON. May 2019.
5. Poster. 31st Canadian Student Health Research Forum. Winnipeg, MB. June 2018.
6. Oral Presentation. TOH Surgery Research Day. Ottawa, ON. April 2018.
7. Oral Presentation. OHRI Research Day. Ottawa, ON. November 2017.
8. Poster. 2nd Annual Summit 4CI. Gatineau, QC. June 2017.
9. Oral Presentation. TOH Surgery Research Day. Ottawa, ON. April 2017.
10. Poster. Keystone Cancer Immunology and Immunotherapy. Whistler, BC. March 2017.
11. Poster. OHRI Research Day. Ottawa, ON. November 2016.

Clinical Trial Involvement

1. **NCT04442048 "COV-IMMUNO" -** Immunization With IMM-101 vs Observation for Prevention of Respiratory and Severe COVID-19 Related Infections in Cancer Patients
2. **NCT02987296 "PERIOP-02"** – Perioperative Immunonutrition in Colorectal Cancer Patients Undergoing Abdominal Surgery**.** Phase II
3. **NCT03422120 -** Human blood specimen collection to evaluate immune cell function.
4. **NCT00668707 "AMPLCaRe" –** Adjuvant Melatonin for Prevention of Lung Cancer Recurrence/Mortality. Phase III
5. **NCT02998736 "PERIOP-04" –** Trial of Perioperative Tadalafil and Influenza Vaccination in Cancer Patients Undergoing Major Surgical Resection of a Primary Abdominal Malignancy. Phase I
6. **PRIMe/PERIOP-05** - A Randomized Phase II Trial of the Impact of Perioperative Immuno-modulation on Immune Function following Resection for Pancreatic or Periampullary Adenocarcinoma.

Committees, Involvement and Internships

1. OHRI Ximbassador Intern, Ximbio (Jan 2020 - September 2020)
2. BioCanRx HQP Working Group (2019)
3. Let's Talk Science volunteer (for Let's Talk Cancer events) (2017, 2018)
4. Open Doors Ottawa (2018)

Courses

1. MIC 8125 – Special Topics in Microbiology and Immunology – Grade: A-
2. MIC 8122 – Advanced Topics in Immunology – Grade: A
3. ESG5310 – Community Outreach and Media Relations in the Sciences – Grade: S

Certificates and Training

1. Biotech Primer Courses on Drug Discovery (2 courses), Preclinical (1 course) and Clinical Development (3 courses) (2020)
2. BioCanRX Navigating the Regulatory Steps in Biotherapeutic Translation 3 Day Workshop (2020)
3. Bench, Bix & Beyond: The Basics of Research Commercialization. University of Ottawa Innovation Support Services, 7 Workshops (2019)
4. Transportation of Dangerous Goods (Classes 6.2 & 9). Ottawa Hospital Research Institute (2019-21)
5. Radiation Safety Training. The Ottawa Hospital Research Institute. (July 2018)
6. Canada GCP – Stage 1 Basic. Ottawa Hospital Research Institute / CITI Program. (November 2017-20)
7. Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans Course on Research Ethics (TCPS 2: CORE). (Sept 2015)
8. National Institutional Animal User Training (NIAUT) Program. Canadian Council on Animal Care, University of Ottawa. (Sept 2015)