Phenotypic and functional characterization of surgery induced myeloid derived suppressor cells

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ABSTRACT

Removing the bulk of tumor burden with surgery is critical for the recovery of patients with solid malignancies. Surgery, however, induces many physiological changes which impair both NK cell cytotoxicity and cytokine secretion giving circulating tumor cells an opportunity to escape and form distant metastases. The rise of MDSCs in the postoperative landscape has been identified as a major contributor of postoperative NK cell dysfunction. Lack of characterization of both SxMDSC phenotype and suppressive mechanisms are a major challenge in targeting these cells to improve long-term outcomes for patients. This work gives a previously unseen detailed phenotypic description of SxMDSCs, showing a phenotypic switch toward an "M2 like" phenotype after tumor resection. Furthermore, preliminary data from this study identifies a contact-dependent mechanism for SxMDSC suppression of NK cell cytotoxicity and indicates they are likely not a major contributor of suppression NK cell cytokine or cytotoxic granule secretion.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

ACTH	-	Adrenocorticotropic Hormone
ADCC	-	Antibody-dependent Cellular Cytotoxicity
ARG1	-	Arginase-1
BL	-	Baseline
BSA	-	Bovine Serum Albumin
BV	-	Brilliant Violet
cAMP	-	Cyclic Adenosine Monophosphate
CCR	-	C-C Motif Receptor
CD	-	Cluster of Differentiation
CHOP	-	C/EBP homologous protein
CLL	-	Chronic Lymphocytic Leukemia
CMI	-	Cell Mediated Immunity
CMP	-	Common Myeloid Progenitor
CO_2	-	Carbon Dioxide
COX2	-	Cycooxygenase-2
CP450	-	Cell Proliferation Dye 450
CRC	-	Colorectal Cancer
CRH	-	Corticotropin-Releasing Hormone
CTC	-	Circulating Tumor Cell
CXCR	-	C-X-C Motif Receptors
DAMP	-	Damage-associated molecular pattern

DC	-	Dentritic Cell
DNA	-	Deoxyribonucleic acid
DNAM	-	DNAX Accessory Molecule
EDTA	-	Ethylenediaminetetraacetic Acid
e-MDSC	-	Early/Immature MDSC
EMH	-	Extracellular Hematopoiesis
EMT	-	Epithelial-Mesenchymal Transition
ER	-	Endoplasmic Reticulum
FasL	-	Fas Ligand
FATP	-	Fatty Acid Transport Protein
FBS	-	Fetal Bovine Serum
FKLRG1	-	Killer cell lectin-like receptor G1
FSC	-	Foward Scatter
GC	-	Glucocorticoid
GM-CSF	-	Granulocyte-Monocyte Colony Stimulating Factor
GP1b-1X-V	-	Glycoprotein Ib-IX-V complex
GPIIb/IIIa	-	Glycoprotein/Integrin IIb/IIIa
Gr1	-	Granulocytic Marker 1
G-MDSC	-	Granulocytic-MDSC
HCC	-	Hepatocellular Carcinoma
HD	-	Heathy Donor
HDN	-	High Density Neutrophil
HIF	-	Hypoxia Inducible Factor
HMGB1	-	High Mobility Group Box 1
HSC	-	Hematopoietic Stem-Cell
HSP	-	Heat Shock Protein
IDO	-	Indoleamine 2,3-dioxygenase

IGF	-	Insulin-like Growth Factor
IL	-	Interleukin
IFNγ	-	Interferon-Gamma
IMC	-	Immature Myeloid Cell
iNOS	-	Inducible Nitric Oxide Synthase
K562	-	Human Leukemia target cell
KIR	-	Killer-cell immunoglobulin-like receptor
LDN	-	Low Density Neutrophil
Lin	-	Lineage Markers
LPS	-	Lipopolysaccharide
Ly6G	-	Lymphocyte Antigen 6 Complex
MAPK	-	Mitogen-Activated Protein Kinase
MDSC	-	Myeloid Derived Suppressor Cell
MFI	-	Mean Fluorescence Intensity
MHC	-	Major Histocompatibility Complex
MIPIA	-	Macrophage Inflammatory Protein
MM	-	Multiple Myeloma
M-MDSC	-	Monocytic-MDSC
MMP	-	Matrix Metallopeptidase
NCAM	-	Neural Cell Adhesion Molecule
NCR	-	Natural Cytotoxicity Receptor
NET	-	Neutrophil Extracellular Traps
NFκB	-	Nuclear Factor κ-B
NK	-	Natural Killer
NK92	-	Natural Killer-91 Cell Line
NKG2D	-	Natural Killer Group 2 D Receptor
NKp	-	Natural Killer Protein

NLR	-	Neutrophil to Lymphocyte Ratio
NLRP3	-	NOD-like Receptor Protein 3
NO	-	Nitric Oxide
NOX2	-	NADPH oxidase 2
NSAID	-	Non-steroidal Anti-inflammatory Drugs
NSCLC	-	Non-Small Cell Lung Cancer
PAMP	-	Pathogen-Associated Molecular Patterns
PBMC	-	Peripheral Blood Mononuclear Cell
PBS	-	Phosphate-Buffered Saline
PDE5	-	Phosphodiesterase type 5
PDL1	-	Programmed-death Ligand 1
PGE2	-	Prostaglandin E2
PFA	-	Paraformaldehyde Fixation
PHBSP	-	Perioperative Human Blood and Tissue Collection Program
PI3K	-	Phosphatidylinositol-3 Kinase
PMN-MDSC	-	Polymorphonuclear MDSC
POD	-	Postoperative Day
RAGE	-	Receptor for Advanced Glycation End Products
RANTES	-	Regulated upon Activation, Normally T-Expressed, and presumably Secreted (also known as CLL5)
RCC	-	Renal Cell Carcinoma
RPMI	-	Roswell Park Memorial Institute Media
ROS	-	Reactive Oxygen Species
SSC	-	Side Scatter
STAT	-	Signal Transducer and Activator of Transcription
Sx	-	Surgery
SxMDSC	-	Surgery-Induced Myeloid Derived Suppressor Cell

TAM	-	Tumor-Associated Macrophage
TAN	-	Tumor-Associated Neutrophil
TDF	-	Tumor-Derived Factors
TGFβ	-	Transforming Growth Factor-Beta
Th1	-	T helper 1
Th2	-	T helper 2
TIGIT	-	T Cell Immunoreceptor with Ig and ITIM Domains
TLR	-	Toll-like Receptor
TME	-	Tumor Microenvironment
TNFα	-	Tumor Necrosis Factor-Alpha
TRAIL	-	TNF-related apoptosis-inducing ligand
Treg	-	Regulatory T-Cell
VEGF	-	Vascular Endothelial Growth Factor

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1. INTRODUCTION

Chapter 1 explores the immune system's critical role in combatting cancer, detailed which cells defend against malignancy and which impede this defense. This chapter focuses on the role of NK cells, examining their varied functions, such as cytotoxicity and cytokine release, in managing tumor progression. Additionally, we connect NK cell activity to cancer prognosis. Chapter 2 opens by emphasizing the vital role of surgery in treating patients with solid tumors. We also explore the various physiological changes triggered by surgery and their impact on NK cell numbers, phenotype, and function. In Chapter 3, we begin by discussing how the effects of the surgical stress response on NK cells lead to a general dysfunction of these cells. Next, we explore the relationship between overall surgical stress and cancer outcomes. We then detail how postoperative NK cell dysfunction is a crucial mechanism for recurrence in surgical cancer patients and the main proposed mechanisms involved in this issue. Finally, we highlight the importance of myeloid-derived suppressor cells in contributing to postoperative dysfunction. In Chapter 4, we will thoroughly explore this diverse group of cells. We start by detailing the roles of MDSCs in pathophysiology, followed by their identification in humans. Next, we examine the conditions—related to cancer and surgical stress pathways—that trigger their expansion, development, and pathogenic activation, which can create a more conducive environment for post-operative metastases. Subsequently, we summarize the mechanisms MDSCs use to inhibit NK cells and provide an overview of current clinical strategies aimed at targeting these suppressive cells to enhance patient outcomes.

Chapter 1: The immune system is crucial to host defense against cancer development and spread

1.1 Key players in balancing antitumor immunity

The immune system is crucial in the body's natural protection against malignant cells and the fight against tumor growth and spread. Antitumor immunity encompasses both adaptive and innate immune responses which protect the host against malignancy.[1] Tumor growth triggers multiple inflammatory processes, which are recognized by key players in both innate and adaptive immunity and drive the antitumor response.[2] The presence and activity of different immune subsets can both hinder or promote tumor progression.[2]

Innate immune cells are the first response against infected or malignant cells. These cells include phagocytic cells (ex: neutrophils, mast cells, monocytes, macrophages, dendritic cells, eosinophils and basophils) as well as natural killer cells (NK cells).[2] These promote antitumor immunity using multiple mechanisms: activation of the adaptive immune response (via antigen presentation), direct killing of malignant cells or by amplifying and directing immune responses (via cytokine secretion).[2] Importantly, innate immune cells can recognize and target cancer cells quickly without need for immunological priming.[3–7]

Adaptive immune cells include B-cells, CD4+ and CD8+T-cells.[8] They are recruited to the anti-tumor response when innate antigen presenting cells (APCs) capture dying tumor cells, process and present antigens on their surface and travel to the lymph nodes to activate adaptive immunity.[8] Adaptive immune cells will promote a potent and long-lasting antitumor response: production of antitumor antibodies will block tumor growth or survival pathways. Cytotoxic T-cells can also directly target and kill tumor cells in an antigen-dependent manner.[9]

Signals from the tumor microenvironment can polarize immune cells toward a pro-tumorigenic phenotype. In this manner, tumors can alter immune responses to their own advantage: immunosuppressive cells such as regulatory T-cells (Tregs), tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) can promote cancer spread through multiple mechanisms including ECM remodeling, angiogenesis or promoting an immunosuppressive environment.[2]

1.2 NK cell subsets have different roles in the antitumor response

Natural killer (NK) cells were first identified in 1975 as cytotoxic innate immune cells capable of targeting stressed, infected, or cancerous circulating cells.[3,5,10–12] Today, we recognize NK cells as large granular lymphocytes belonging to the innate immune system. They are named after their ability to kill a target cell "naturally", meaning spontaneously without the requirement of immunological priming.[3–7] NK cells are known to be the first line of defense against virally-infected and transformed cells.[5,13–17] They are often identified through their expression of CD56, an isoform of neural cell adhesion molecule (NCAM).[6]

NK cells can recognize cell targets via changes in MHC-1 expression, ligands induced by tumor transformation, cell stress or DNA damage.[7] Many of the ligands (ex: MICA/B and ULBP1/6) recognized by NK cells are strongly upregulated by cancer cells.[4,18–20] NK cell activation itself is tightly regulated by the integration of both activating and inhibitory signals through germline-encoded receptors. When the activating signals (Natural Cytotoxicity Receptors/NCRs and NKG2D binding to upregulated ligands on target cells)[3,4,18,21–23] outweigh the inhibitory signals (KIRs, NKG2A binding to MHC molecules on non-target cells), the balance is tipped toward activation.[4,6,13,18,18,19,24,25] NK cells surveil the host for infected or malignant cells and have two major functions in response: production of cytokines to enhance the immune response and direct

cytotoxicity.[6,13,26] Traditionally, there are two recognized major NK cell subsets in literature: $CD56^{bright}$ and $CD56^{dim}$, named according to their level of expression of CD56, although recent literature is challenging this stringent definition.[3,13,18,27]

In healthy individuals, most NK cells in peripheral blood and are mostly cytotoxic in nature. [6,10,13,18,28–33] These NK cells can rapidly lyse target cells without needing antibody recognition, making them essential for the antitumor response.[6] They can accomplish this through binding of CD16 to the Fc region of immunoglobulin when immobilized on a cell surface, triggering degranulation (release of cytotoxic granules perforin and granzyme).[13] They can also use death receptor related pathways to induce target cell apoptosis (ex: Fas, TRAIL).[4,6,10,27,30,34,35]

NK cells have also been shown to produce high and constant levels of immune modulatory cytokines (IFNγ, TNFα and GM-CSF).[6,10,13,18,28–33] The production of IFNγ by NK cells is a crucial link between the human innate and adaptive immune responses.[6,29] The cytokines secreted by these cells activate APCs to upregulate MHC class I, activate macrophage killing of intracellular pathogens and have antiproliferative effects on viral and malignant cells.[6] NK cells require 2 signals to produce IFNγ: one of which being IL-12 and the other being either IL-1, IL-2, IL-15, IL-18 or engagement of an NK-activating receptor (ex: CD16 or NKG2D).[4] These cytokines can be released from other immune cells (monocytes, macrophages, dendritic cells/DCs…etc).[6,7]

1.3 NK function is correlated with cancer outcomes

It is no surprise, due to their capacity to efficiently target malignant cells, that low NK cell activity can make individuals more susceptible to infection or cancer development.[36,37] Outside the protection of the primary tumor site (where tumor cells greatly outnumber effector cells), tumor cells in circulation are much more vulnerable to attack. In circulation, their previously

developed escape mechanism (ex: downregulation of MHC-I to evade T-cell mediated immunity) leave them vulnerable to NK cells which predominate in this environment.[3,6,10,38,38–41] This makes NK cells particularly important in clearing tumor cells in peripheral blood.[42–45] Consequently, many studies both clinical and with various animal models have indicated that NK cells are critical for the antitumor response.[10,39,46–48] Murine studies have linked depletion of NK cells with increased metastases.[10,38,49–53] Human studies have linked intra-tumoral NK cell density with cancer prognosis and incidence.[38,40,54–57] Not only is the presence of NK cells crucial to clinical outcomes, but so is their function. Clinical studies have correlated the degree of functionality of NK cells with prognosis and survival.[38,58,59]

Chapter 2: Physiological changes during the surgical stress response impact NK cell function

1.4 Surgery is crucial to the recovery of patients with solid tumors

Surgical tumor removal is crucial in the recovery of patients with solid malignancies and remains the treatment of choice.[60] Eliminating the major pool of malignant cells via surgery is curative in the majority of patients.[10,38,60] Modern developments in surgical techniques such as robotic and laparoscopic surgeries have significantly lowered the risks associated with tumor resection. Ongoing research is focusing on long-term outcomes and maintaining the patient's quality of life.[61] Unfortunately, this research has struggled to improve survival rates for patients with metastasized solid tumors.[60] As it stands, surgery does not address the issue of circulating tumor cells (CTCs), leading many patients to recur.[4,10,26,42,60,62,63] Several studies in the last decade have noted that surgery can be both beneficial and have adverse effects due to the capacity of surgical stress to promote tumor growth.[64] The correlation between surgery and metastasis has been

established since the early 1900s.[5,10,65–67] Most patients undergoing major thoracic or abdominal surgery recur within 5 years and tend not to respond to frontline therapies.[26,68,69]

Animal studies using various hosts and tumor models have linked surgery and the formation of metastatic disease.[3,42,70,71] Research from our laboratory has shown that surgical stress, induced by nephrectomy, significantly increases the likelihood of pulmonary metastases in mouse models compared to mice that undergo no surgery.[42,43,62,72] In human clinical studies, it is suggested that tumor resection may promote later metastases. Even when a tumor is completely removed, cancer patients frequently face recurrence due to minimal residual disease present at the time of surgery.[73]

1.5 Surgical stress is linked to immunodeficiencies in multiple procedures

Postoperative immune dysfunction has been observed in many types of surgical procedures[74–76] such as gastric bypass surgery[77], cardiac surgery[78] or hernia repairs[28] as well as surgical resections for multiple cancers including but not limited to bone cancer[79], prostate cancer[80], ovarian cancer[81], breast cancer[82], pancreatic cancer[83,84], kidney cancer[85], esophageal cancer[84,86], lung cancer[87,88], colorectal cancer[89] and gastric cancer.[90] This immune depression is characterized by reduced *in vitro* T-cell proliferation and blastogenesis, reduced -DR expression by APCs and suppressed cytotoxicity of macrophages and NK cells.[38,63] Surgery is also associated with a decline in circulating effector cells (NKs, cytotoxic T lymphocytes and occasionally to a lesser extent B-cells) and an increase in immunomodulatory cells (ex: MDSCs).[38,54,90] Overall, postoperative immune depression peaks between 24-72h after surgery but some aspects can easily last several weeks.[26,39,43,62,91–94] In major surgery, not only is CMI shown to be suppressed for several days, but deeper and longer immune suppression is associated with more invasive procedures that can last for months.[38,68,95] In studies from our

group, postoperative NK cell dysfunction was seen regardless of age, sex or cancer stage and was correlated with surgery invasiveness.[29,54] While the concept of postoperative NK cell dysfunction is well documented, few studies have attempted to characterize the mechanism of post-op NK cell dysfunction.[62,73,92,93,96–98]

1.6 Surgical stress impacts both NK cell numbers and phenotype

Surgical stress has been shown to decrease circulating DCs, B cells[99], T cells[63], and NK cells.[28,29,100–102] Our group, along with others, have noted that the decrease in NK cell numbers is relatively modest and NK cell subsets remain consistent, meaning NK cell dysfunction cannot be solely attributed to population loss.[28,29,54,62,101] Postoperative NK cell dysfunction has also been linked to phenotypic changes in NK cells. NK cells in the postoperative landscape have greatly reduced expression of key activating receptors, including NKG2D, DNAM-1, NKG2A, FKLRG1 and TIGIT[24,62]. Not surprisingly, this is correlated with a decrease in NK cell cytotoxicity, as NK cells recognize tumor ligands using these receptors.[62] A study from our lab further demonstrated a reduction in the phosphorylation of NK signaling proteins such as STAT4, STAT5, p38 MAPK and S6, as well as a reduction in cytokine receptor expression (ex: CD212, CD132…etc).[24] Surgically stressed NK cells also express decreased expression of maturity markers CD62L and CD11b.[62]

1.7 The surgical stress response involves a cascade of events which direct the immune response after trauma

Surgical trauma induces a cascade of events and responses aimed at protecting the host and promoting wound healing. Unfortunately, many of these processes have been shown to promote an immunosuppressive environment and, subsequently, tumor growth.[10,54] Surgery, while having important curative benefits, does involve the deliberate infliction of tissue and vasculature trauma.[26] This tissue insult results in necrotic cell death, thereby triggering the release of

"alarmins" to alert nearby immune cells to the existing tissue damage, thereby triggering the surgical stress response.[26] This response is often separated into two distinct phases: the pro-inflammatory phase followed by the anti-inflammatory phase. Both of these are carefully mediated by soluble factors released by innate and adaptive immune cells.[10,26] This process is well organized and aimed at restoring homeostasis.[10,24,90] While this response overall is deemed an essential evolutionary process to survive trauma, many have argued that this response is unnecessary in the context of a sterile and controlled surgery.[4,10,90]

1.7.1 Pro-inflammatory phase

The body's immediate response to tissue injury involves the release of "alarmins" and damage-associated molecular pattern (DAMP) molecules, both of which stimulate innate immune cells through TLR, NF-κB, or other molecular pathways.[4,68,103,104] Alarmins also attract mesenchymal cells to the surgical site, which bind to monocyte receptors to induce their production of proinflammatory cytokines (ex: IL-1β, IL-6, TNFα).[4,90,105,106] Fibroblasts then produce IL-6 and IL-8 to stimulate hepatocyte production of acute phase reactants (IAP and CRP), the latter contributing to both infection control and wound healing.[90,105,106] This ultimately results in a shift in the Th1/Th2 balance: Th1 cytokines (IL-2, IL-12 and IFNγ) are reduced while Th2 cytokines (IL-6/8, IL-10 and TNFα) are increased.[28,90,90,103,103,106–109]

1.7.2 Anti-inflammatory phase

During the subsequent anti-inflammatory phase, processes aim to restrict inflammation to the site of the tissue damage, where it is most needed to promote proper wound healing.[38] IL-6 is a pleiotropic cytokine with both pro- and anti-inflammatory capabilities and it serves as the switch between both phases.[4,10] IL-6 promotes the release of various anti-inflammatory factors such as glucocorticoids (GCs), soluble TNFα receptors, PGE2-dependent IL-10, and TGFβ1.[4,10] GCs act

directly on CD4+ T cells and indirectly (via the promotion of IL-12 release) on monocytes, resulting in the decrease of Th1 cytokines and increase of Th2 cytokines.[27,103] They also enhance local clearance of foreign antigens, toxins, microorganisms and dead cells through their enhancement of macrophage phagocytic activity, protecting the host from opportunistic infections.[90,103]

1.8 The surgical stress response induces potent physiological changes which impact NK cell function

The cascade of events triggered by tissue trauma impacts many immune pathways. Shifts in cytokine concentrations, release of growth factors and stress hormones, surgery induced hypercoagulability and tissue hypoxia as well as the expansion of immune regulating suppressive cells all have documented impacts on the function of NK cells, which could explain the postoperative changes in cell numbers, phenotype and function.

1.8.1 Cytokine shift and growth factor release alters NK cell functions and pathways

Postoperative immune suppression is preceded by the release of growth factors (VEGF, PDGF, TGFβ) and a shift in cytokine levels.[3,28,38,103] Our group's studies have demonstrated that postoperative patient plasma inhibits NK IFNγ secretion in vitro, emphasizing the presence of these suppressive soluble factors.[28] During the pro-inflammatory phase of the response, monocytes downregulate cytokines that favor CMI (Th1 cytokine such as IL-2, IL-12, IFNγ).[38] Consequently, an increase in Th2 cytokines can be observed in the plasma, which are known to interfere with CMI (ex; IL-10, IL-6, IL-1 and IL-8).[38] IL-6 is known to decrease NK production of perforin and granzyme B, resulting in a reduction of cytotoxicity.[131,132] IL-10, on the other hand, has pleiotropic effects on NK cells: both inducing NK cell cytotoxicity and inhibiting NK cell cytokine secretion.[5,133,134] The surgical stress response also encompasses an increase in growth factors. TGFβ for example, is essential to wound healing following surgical trauma.[10]

TGFβ has also been shown to induce NK cell anergy, reduce NKG2D expression and IFNγ production.[11,18,46,110,111] VEGF, another growth factor increased during surgical stress, has been shown to downregulate adhesion molecules on NK cells, impairing their ability to migrate into tumors.[112] Additionally, it has been shown to downregulate NCR receptors on NK cells resulting in reduced cytotoxicity.[113]

1.8.2 Hypercoagulable state protects CTCs from NK-cell mediated immunity

Both surgery and cancer are also known to contribute to a hypercoagulable state. In this state many clotting factors such as fibrinogen, factor VIII and von Willebrand factor are upregulated.[3] Fibrinogen in particular is increased by pro-inflammatory cytokines released during the pro-inflammatory phase.[42,54] This phenomenon is linked to induction of platelet activation by tumor cells as a protection mechanism.[54,114] Platelets along with fibrin (which is converted from fibrinogen during the wound healing process), can coat and protect CTCs, allowing them to escape NK cell mediated clearance.[24,42,54,115]

1.8.3 Neuroendocrine activation releases stress hormones which alter NK cell function and phenotype

Many cytokines released during the pro-inflammatory activate the neuroendocrine system through stimulation of the hypothalamus-pituitary-adrenal axis.[27,116] This triggers the secretion of adrenocorticotropic hormone (ACTH) by the pituitary gland, resulting in significant increases of stress hormones (GCs, catecholamines and prostaglandins/PGEs).[3,28,38,103] Many stress hormones have been shown to suppress NK cells in a dose-dependent manner, resulting in increased tumor spread.[2,10,127,128]

ACTH triggers the release of GCs by the adrenal cortex, resulting in their significant increase after surgery.[27,38,90,105,110,115,116] GCs can epigenetically downregulate the expression of IL-12 receptors on NK cells, alter NK cell receptors CD16, DNAM-1, NKp46 and NKp30 and impair

cytotoxicity.[110,117,118] GCs have also been shown to reduce NK cell expression of adhesion proteins (crucial for binding to target cells), and NK cell activity.[13,95,101,102] Other stress hormones heightened postoperatively are catecholamines (norepinephrine, epinephrine/adrenaline and dopamine)[90,105,119,120] Catecholamines are known to reduce the production of Th1 cytokines (ex: IL-12, TNFα, IFNγ) while stimulating the release of Th2 cytokines (ex: IL-10).[38,103] As a result, they are capable of inhibiting both NK cell cytotoxicity and immunomodulatory functions.[13,127] Cortisol is also greatly elevated post-op.[90,105,115,120] This hormone has been shown to downregulate NCR receptors (NKp46 and NKp30), thereby inhibiting cytotoxicity.[130] Finally, Prostaglandin E2 (PGE2), is elevated post-op near surgical sites and can act directly on tumor cells to induce metastatic activity, proliferation, adhesion, migration, and resistance to apoptosis.[10,38,103] Additionally, PGE2 can suppress NK cells directly by triggering an increase of intracellular cyclic adenosine monophosphate (cAMP) levels[129].

1.8.4 Tissue hypoxia induces NK epigenetic modifications and promotes a immunosuppressive environment

Hypoxia refers to a condition in which there is a reduced supply of oxygen to the tissues. In the event of tissue injury, tissue ischemia-perfusion is known to play a role in releasing various proinflammatory factors.[103,121] Hypoxia is also capable of impairing NK cell activity through metabolic and epigenetic alterations.[7,10] Furthermore, hypoxia promotes the suppressive tumor-microenvironment through expansion of regulatory immune cells (ex: MDSCs, Tregs).[10]

1.8.5 Amino-deficiencies suppress NK cell function

Surgery is known to induce a state of amino deficiency. Plasma arginine levels are significantly depressed for days or weeks following surgery.[122] Arginine is the sole amino acid substrate to produce nitric oxide (NO), as such it is key in many wound healing processes such as

vasodilation and microcirculation.[122] NK cells rely on arginase for both their proliferation and secretion of IFNγ, therefore the lack of arginine can significantly impact their proper function.[123]

1.8.6 Immunosuppressive cells affect NK cell phenotype and function

Tissue insult is also associated with the expansion of suppressive immunomodulatory cells (MDSCs, Tregs, N2 neutrophils…etc).[124,125] Pro-inflammatory factors (ex: IL-1, IL-6, PGE…etc) can induce emergency myelopoiesis in the bone marrow, resulting in accumulation of MDSCs.[124] Neutrophils have been known to release large amounts of pro-inflammatory molecules during the surgical stress response.[115] Though they play an important role in protecting the host from opportunistic infections[54,115,126,127], the neutrophil extracellular traps (NETs, web-like structures of protein-covered nucleic acids) used to bind pathogens have been shown to sequester CTCs from circulation and, as a result, promote tumor cell invasion and metastasis.[54,68,115,126,126,127]

Chapter 3: NK cell dysfunction promotes postoperative metastases

1.9 NK dysfunction is linked to trauma and surgery

The correlation between surgical trauma and post-op NK dysfunction has only been known since the 1980s. At this time, NK cell dysfunction was noted in patients undergoing abdominal surgery and related to the degree of surgical stress.[22] Moreover, studies as early as the 1990s demonstrated how patients undergoing solid tumor resection have drastically reduced NK cell cytotoxicity and IFNγ secretion. This phenomenon was independent of age, sex, surgery length, blood loss, use of anesthetics or tumor size.[4,42] Murine studies supported this.[4,45,46,70,84,133,143,144] Few of these early studies elucidated potential mechanisms of suppression.[4,145–151] In the last several decades, this phenomenon has been observed in response to various trauma, illnesses and procedures conducive to a potent inflammatory stress response.[3,10,16,24,152] These conditions

include thermal injury[153], spinal cord injury[154], traumatic brain injury[155], staph infection[156], critically ill patients (resulting from trauma or pulmonary disease)[157] cardiopulmonary bypass surgery[145], colorectal cancer surgery[24,158], pancreatic cancer surgery[159], bone cancer surgery[160] and more.

1.10 Surgical stress is correlated with cancer outcomes

Postoperative suppression of CMI is heavily correlated with postoperative metastases.[38] This link has been established in research since the 1970s.[39,104,128–130] Coffey et al identified post-op CMI depression as an "immunological window of opportunity" perpetuating tumor growth and metastases in surgical cancer patients.[4,91] In this "window", the period of postoperative immune suppression has been seen to coincide with the period of compromised resistance to metastasis in patients.[3,38,68,115,131]

The surgical stress response is tailored to protect an individual following major tissue trauma. Unfortunately, many of the involved processes intersect with signaling pathways that contribute to cancer proliferation.[26] While tumor cells travelling away from the primary mass during early formation of metastases often do not gain their full malignant potential, the pathways triggered by surgery may very well tip the balance in their favor.[132] Various animal studies have shown that the primary tumor sometimes inhibits recruitment of blood vessels (critical for metastatic development), and this inhibition is removed when the tumor is resected.[38,131] It is also suggested that primary tumor resection eliminates a safeguard against angiogenesis and as a result can awaken dormant micro-metastases already present at distant sites.[38,131] In murine studies, surgical stress was shown to increase tumor implantation, growth rate and incidence of metastases.[133–137] In human studies, it has been shown to promote metastases and associated with worse survival.[54,105,138,139]

Numerous researchers have worked to explain how surgery may contribute to the formation of metastases. While some of the mechanisms identified earlier might have only a limited impact on tumor development when considered in isolation, their combined effect could significantly increase the likelihood of cancer recurrence in patients.[38] Our group especially has identified postoperative NK cell dysfunction as the key mechanism responsible for the pro-metastatic effects of surgery.[38,42,62,72]

1.11 Post-op NK cell dysfunction promotes metastases

Metastasis remains the principal cause of cancer death due to the resistance of metastasized tumors to most modern day treatments such as surgery, radiation or chemotherapy.[24,49,60,64,132,140,141] The process of metastasis involves primary tumor cell entry into the vasculature and their subsequent colonization at distant sites.[142] Shakar & Ben-Eliyahu further highlighted that complete remission after surgery occurs not because of full excision but when immune mechanisms can eradicate any residual tumor cells.[38] As such, the inability of immune cells to eradicate these residual tumor cells is a major contributor to recurrence.

Strong evidence in both human and murine studies identify NK dysfunction as the phenomenon responsible for the development of postoperative metastases.[5,29,54,143] Some of these, including those from our own group, utilized a murine model of surgical stress by adoptively transferring NK cells from surgically stressed or control mice into NK-deficient recipient mice to demonstrate *in vivo* the contribution of NK cells in particular to post-op tumor spread.[3,10,42,62] Models such as these were further able to confirm the metastatic effect was a result of NK dysfunction in particular, as the same phenomenon was not observed when using other immune cells. [3,10,26,62,143–145]

In terms of clinical studies, several have linked NK cells with tumor clearance in the vasculature.[42] Further studies definitively linked the impairment of NK cell cytotoxicity after surgery to cancer recurrence and metastasis.[5,29,43,91,143,144,146] Low NK activity following excision of various cancer types is associated with a higher rate of recurrence and mortality[10,26,43,72,73,146–148] while high NK cell activity has been associated with better prognosis.[53,62] More importantly, several studies have suggested that inhibiting this phenomenon could prevent patient recurrence and improve long-term outcomes.[43,62,133]

Chapter 4: Myeloid-derived suppressor cells are a major culprit in post-op NK cell dysfunction

1.12 Myeloid derived suppressor cells are immunosuppressive and expand due to inflammation

A major contributor of postoperative NK cell dysfunction is the expansion of immunomodulatory cell populations, particularly MDSCs.[26] There are two major subsets of MDSCs described in literature: “monocytic like” MDSCs (M-MDSCs) or “granulocytic like” MDSCs (G-MDSCs or PMN-MDSCs). Expansion of these cells is associated with many chronic and acute inflammatory diseases and processes such as burns[124,149–151], sepsis[152–154], autoimmune diseases[155], infection[156,157], trauma[158], surgery[73,107] and cancer[159–162], the extent of which is proportional to the degree of inflammation.[104] These cells are a heterogenous population of pathologically activated myeloid cells including immature macrophages, granulocytes, dendritic cells and myeloid progenitor cells.[14,104,124,126,163–165] It is important to note that while MDSCs resemble many classically activated immunocompetent cells, they are primarily defined by their immunosuppressive capacities.[3,10,153,163,166] Exposing mature neutrophils or monocytes to the same signals *in vitro* will not convert them to MDSCs.[163,167]

First reports of immunosuppressive regulatory cells of myeloid origin began to circulate in the 1970s.[18,163,163,168–171] In fact, one laboratory showed the emergence of a suppressive myeloid cell in response to surgical stress capable of suppressing NK cells decades before MDSCs were properly described.[4,172] Suppressive myeloid cells were then first described in cancer patients during the 1990s.[163,173–177] It was around this same time period when MDSCs were properly identified in mice as immunosuppressive $Gr1^{+}CD11b^{+}$ cells that were phenotypically similar but functionally different from mature monocytes and neutrophils.[163]

1.12 Dual nature of MDSCs: MDSCs can both protect the host and promote disease progression

The expansion of MDSCs occurs in response to inflammation as a complex balance between increased immune surveillance and dampened adaptive immunity.[124] While MDSCs are immunosuppressive in nature their presence is important in maintaining host homeostasis. Alternatively, MDSCs have been shown to worsen outcomes in some diseases due to excessive immune suppression. This highlights the pleiotropic nature of MDSCs: in some contexts, they are protective while in others they can perpetuate disease.[164]

1.12.1 MDSCs have many important physiological roles following trauma

Though MDSCs are often views as a pathological response to a tumor, they are a normal component of the inflammatory response.[124,126,154,165,178] They are among the first to respond to tissue injury and play a major role in tissue repair and wound healing.[18,179] This is accomplished by metabolizing arginine to ornithine, which stimulates the proliferation of fibroblasts and the production of collagen.[122,179] They are also essential in the prevention of uncontrolled inflammation and immune responses.[18,179] Beyond this, they are involved in protection against infection and communicate with adaptive immunity via inflammatory mediators.[10,124,163,164] Studies have outlined a protective role of MDSCs in multiple diseases and processes like

autoimmune diseases[155,164,179], type-1 diabetes[179–181] or infections[124,153,164]. For trauma patients, the potent innate immune effector cell functions of MDSCs can help protect individuals from opportunistic infections.[104,124,173,182] Even in the context of cancer, regulatory immune mechanisms are necessary to maintain a fine balance between destroying malignant cells and protecting the host from excessive collateral tissue damage and inflammation.[179] Regrettably, tumors and cancer cells are able to turn these mechanisms to their own advantage, high-jacking MDSC processes to promote their own growth and progression.[68,179]

1.12.2 MDSCs with aberrant function can promote disease progression and immunosuppression

While MDSCs have a clear evolutionary protective role, situations of high or prolonged inflammation can give rise to massive expansion of these cells with aberrant function.[68,127,179] These pathologically activated MDSCs are associated with excessive immune suppression leading to more persistent infection at the detriment of the host.[173,179,183–188] Presence of MDSCs is correlated with increased risk for infection or other complications in patients with severe injury or trauma[189], burns[190], specific autoimmune diseases[191,192], sepsis[153,154,182,193,194], major surgery[195–197], among others.[198] The prolonged activation of MDSCs can lead to tissue damage, promotion of angiogenesis and immune dysfunction, which all serve to promote tumor growth and metastasis.[179] While the prognostic value of MDSCs is clearly defined, there exist many challenges in studying them, mostly attributed to their varied phenotypes and plasticity.

1.13 Phenotypical characterization of MDSCs

The first study immunophenotyping these suppressive immature cells in humans occurred in 1995, in head and neck cancer tissues.[165] Consensus on identifying these cells began in 2007 when scientists sought to unify various descriptions under the term MDSC, focusing on their origin and suppressive capacity.[163] In the last few decades MDSCs have been described in the

bone marrow, peripheral blood, spleen, liver, lungs or tumor of mice while in humans they have been mostly described in blood or tumors with a few studies describing them in bone marrow.[163]

Lack of specific markers makes identification of these cells in humans particularly challenging: phenotypically MDSCs are almost identical to neutrophils and inflammatory monocytes, their distinguishing feature is their immunosuppressive capacity.[126,165,199,200] For this reason, phenotypic identification is not enough: functional analysis of MDSCs is necessary for them to be defined as such.[163] Despite major efforts to harmonize the field, very large inter-laboratory differences exist in reporting MDSCs and specific MDSC biomarkers are still being defined in humans.[4,124,201,202] Additionally, the lack of appropriate mechanistic studies (as many studies focus solely on flow phenotyping without evaluating immunosuppressive capacity), and lack of specific phenotypic markers and/or genomic signatures pose a major challenge.[124,203]

1.13.1 Two major MDSC subsets are recognized in humans with new markers and subsets still under investigation

In mice, MDSC subsets are mostly identified via their functionality and expression of Gr1[63]: M-MDSCs are defined as $CD11b^{+}Ly6G^{-}Ly6C^{high}$ while PMN-MDSCs are defined as $CD11b^{+}Ly6G^{+}Ly6C^{low}$.[13,124,164,165] Humans lack Gr1, therefore MDSC definitions are more arbitrary. In this instance M-MDSCs are usually defined as $CD33^{+}CD11b^{+}CD14^{+}HLADR^{-/low}CD15^{-}$ while PMN-MDSCs are defined as $CD33^{+}CD11b^{+}CD14^{-}CD15^{+}$ or $CD11b^{+}CD14^{-}CD66b^{+}$ depending on the study in question.[13,124,203] A third subset of MDSCs in humans has recently been identified with potent immunosuppressive capacities termed “early” MDSCs (e-MDSCs) and are classified as $CD33^{+}CD11b^{+}CD14^{-}CD15^{-}HLA\text{-}DR^{low}$.[13,18,163]

Beyond the major subsets, there are other features distinctive to MDSCs. Overall, MDSCs are known to have increase expression of arginase (ARG1)[203,204], inducible nitric oxide synthetase

(iNOS), PGE2, and produce large quantities of reactive oxygen species (ROS).[124,126,127,163,164,164,200,205,206] MDSCs are also known to produce many inflammatory mediators, including IL-10, TNFα or the chemokines RANTES and MIPIA (macrophage inflammatory protein).[124,152] Other MDSC surface markers under investigation include CD124, CD40, CD80 and CCR2.[14,165] Some studies have even distinguished MDSCs from neutrophils and monocytes by their elevated endoplasmic reticulum (ER) stress response.[163] Beyond this, some markers or genomic profiles are unique to the major MDSC subsets.

1.13.2 M-MDSC specific markers for identification in humans

M-MDSCs are distinguished through the downregulation of HLA class II expression (HLA-DR^{low}).[165,198] Similarly to tumor associated macrophages (TAMs), MDSCs tend to express markers for inflammatory monocytes such as F4/80 (macrophage protein), CD115 (CSF receptor) and chemokine receptor CCR2.[165] M-MDSCs are distinguishable from TAMs through their expression of various proteins including Ly6C, S100A9 and IRF8.[163,207] Other markers used to define certain M-MDSC subsets include CD83, DC-SIGN, CD124 (IL-4 receptor alpha) and CD34.[203]

1.13.3 PMN-MDSC specific markers for identification in humans

In both mice and humans, PMN-MDSCs represent the major subset of circulating and expanding MDSCs.[208,209] While Gr1 allows for relatively easy identification of this MDSC subset in mice, their identification is much more complex in humans.[124,127,203,210] Many studies rely on the fact that PMN-MDSCs are known to co-purify with PBMCs during Ficoll density centrifugation alongside degranulated neutrophils and myelocytes[127,163,165,191,200,211–213]; however, many researchers have noted that doing so may not only exclude certain PMN-MDSC subsets that

have a higher density. Furthermore, the PBMC isolation process can inadvertently affect the phenotype and functionality of MDSC cells.

Unfortunately, the existing established human PMN-MDSCs markers cannot distinguish between immature, mature or activated neutrophils.[191,214] Additionally, immunosuppressive PMN-MDSCs coexist not only with classical neutrophils but also with less activated and less suppressive PMN-MDSCs.[126,215] PMN-MDSCs, as previously defined, match the phenotypic, characteristic and functional definitions of other "granulocytic suppressive cells" in literature such as tumor associated neutrophils (TANs) or N2 neutrophils, and many studies do not even distinguish them from classically activated neutrophils.[126,200] To overcome these challenges, new identification methods and more specific markers for PMN-MDSCs are emerging.

PMN-MDSCs are often described as having high side-scatter (SSC) and low forward scatter (FSC).[127,165,191] They are also known to express high levels of CD66b, CD11b and VEGFR1 along with low levels of CD62L and CD16 in comparison to neutrophils. While some papers use CD16 to separate PMN-MDSCs from neutrophils, it is important to note that several others have highlighted how CD16 is expressed on PMN-MDSCs and essential to their phenotyping.[165,203,216,217] PMN-MDSCs also have decreased expression of markers related to neutrophil maturity such as CD10, CD13 and CD45.[191,214]

Several markers involved in lipid metabolism are recently being used to distinguish PMN-MDSCs, as it is heavily linked to their immunosuppressive function.[218] LOX1 (Lectin type oxidized receptor 1) is being considered a specific PMN-MDSC marker which could distinguish them more easily from their neutrophil counterparts.[18,126,171,201,211,219] Fatty acid transport protein 2 (FATP2) and 12-15 lipoxygenase are also being investigated to this end.[126,220] The expression of other molecules such as metalloproteinases (ex:MMP8/9), metabolic enzymes (ex: IDO1) and

peroxidases (ex: myeloperoxidase) have also been proposed in the identification of PMN-MDSCs due to their contribution to MDSC immunosuppressive pathways.[163,191,211,221] PMN-MDSCs were even reported to express CD115 and CD244, normally expressed by NK cells or immature hematopoietic stem cells.[200,206] Outside of general marker expression, PMN-MDSCs have been known to have lower phagocytic activity and production of IFNγ and TNFα compared to neutrophils.[200,206] They have also been shown to have distinct morphological features and genomic profile from neutrophils.[163,203,211,222]

1.14 Varying signals drive MDSC expansion, development and pathological activation

The molecular pathways guiding the expansion and function of MDSCs are poorly understood.[179] Generally, MDSC activity is regulated through 3 major events: myelopoiesis impairment, MDSC migration to the tumor site and finally MDSC activation.[18]

1.14.1 Signals driving MDSC expansion

The expansion of MDSCs occurs as a result of a two-signal system: the first is responsible for the expansion of immature myeloid cells and the inhibition of their terminal differentiation while the second is responsible for the pathological activation of these cells into their suppressive phenotype.[10,211,223] This first group of signals is mostly driven by tumor-derived growth factors (ex: STAT3, G-CSF, VEGF, TNFα …etc).[105,165,179,191,224–226] These can direct MDSCs to distant sites and promote the formation of pre-metastatic niches.[18,105] The second group of signals are produced by the tumor stroma or can be produced as a result of the surgical stress response such as proinflammatory cytokines (ex: IL-1, IL-6)[104], PGEs, COX2, HMGB1 and more.[10,13,68,105,124,163,166,227,228] IL-6 and VEGF signaling in particular are known to promote MDSC accumulation through STAT3 signaling.[105,164,229] S100A8/9 proteins, known to increase in response to surgical stress, can also bind to RAGE receptors on MDSCs and promote their

accumulation via NF-κB signaling.[105] This signaling induces myelopoiesis in the bone marrow. Under regular myelopoiesis, hematopoietic stem cells (HSCs) differentiate into common myeloid progenitors (CMPs) and then into immature myeloid cells (IMCs).[124,179] These IMCs are released from the bone marrow into circulation where they can mature into DCs, macrophages and granunocytes.[124,179] The exuberant signaling from tumor growth factors and inflammatory mediators results in a constant draw on the bone marrow, forcing an immense production of MDSCs.[3,104,126,230–232] This process, known as "emergency myelopoiesis", leads to the production of MDSCs with aberrant effector functions, immense immunosuppressive potential and resistance to apoptosis.[126,232] These are guided to sites of stress and inflammation by DAMPs and chemokines, where the environment results in blocking of their maturation and enhancement of their immunosuppressive capacities.[126,179] Once the inflammatory source is cleared, signals prompt MDSCs to clear from the circulation or differentiate into mature myeloid cells.[179]

1.14.2 Signals driving MDSC pathological activation

Many have attempted to explain what prevents the terminal differentiation of MDSCs.[124] It has been repeatedly shown that MDSCs retain the capacity to differentiate into mature cells but are unable to due to the environment.[124,124,152,233,234] This block leads to accumulation of MDSCs in a patient's blood, lymphoid organs or tumor site in multiple inflammatory processes.[103,126,233,235] In cancer particularly, tumor derived factors are responsible for this phenomenon[115,126,233,236], as well as other factors present in the microenvironment such as hypoxia, lactic acid build-up and adenosine accumulation.[18,208,237–240] Their activation of STAT3 pathways during inflammation halts normal cell maturation.[13] Traditionally, MDSCs are said to be pathologically activated in response to DAMPs as well as their downstream mediators (ex: PGE2, IL-10).[4,18,68,105,163,241–243] The recognition of DAMPs by certain MDSC receptors triggers a cascade of events involving

NK-κB and STAT3 which activate MDSCs and enhance their migration and immunosuppression.[68,105] Many of these factors, particularly IL-6, are known to increase expression of molecules involved in the immunosuppressive functions of these cells such as ARG1, PD-L1 and iNOS.[18,105,208,237–240] Additionally, there is emerging evidence involving epigenic modifiers in MDSC activation. These mainly affect pathways such as STAT3, PI3K, NK-κB, CHOP and even pathways of ER stress.[179] Lastly, immunosuppressive functions in MDSCs can be activated through lipid uptake.[179]

1.14.3 A MDSCs originate beyond the expansion in bone marrow

The drastic increase in MDSC numbers observed in many contexts cannot be explained by emergency myelopoiesis in the bone marrow alone.[124] It is possible MDSCs in the spleen, liver and peripheral lymph nodes originate from the organs themselves (extra-medullary hematopoiesis, frequently seen in chronic inflammatory diseases and cancer).[124] Not only this, but MDSCs have been known to arise from cell other than myeloid progenitors. M-MDSCs for example can convert into PMN-MDSCs through an epigenetic mechanism.[200,206] Classical high-density neutrophils have also been shown to undergo a phenotypic switch to PMN-MDSCs.[127,127,200] This plasticity bolsters the theory that MDSCs are not a unique cell population, but rather alternatively activated immune cells from existing lineages.[191,204,211,226,244,245]

1.15 MDSCs development and activation are impacted by surgical stress and tumor signaling

1.15.1 MDSCs expand in response to tumor signaling and are correlated with poor prognosis in cancer patients

Due to their capacity to promote tumor progression, MDSCs are described and defined as a pathological event rather than a physiological event in cancer.[165] Studies as early as the 1980s correlated MDSC numbers in cancer patients with worse prognosis, shorter survival and higher

rates of metastasis.[10,200,203,246] In the last several decades this has been observed in both hematological[14,18,247–251] and solid cancers.[173,252–261] The extent of MDSC expansion can depend on tumor type, size and growth time.[165] Additionally, the frequency of each MDSC subset is influenced by the type of tumor.[164,209] Furthermore, MDSC expansion is correlated with resistance to treatment.[18,126,262,263] M-MDSC expansion specifically has been described in the peripheral blood of patients with melanoma, hepatocellular carcinoma, renal cell and bladder carcinoma, prostate, gastrointestinal, lung, and head and neck cancer, multiple myeloma (MM), chronic lymphocytic leukemia (CLL), and glioblastoma.[165,210] It has also been noted that patients with M-MDSC tumor infiltration have significantly reduced overall survival.[126,165,264,265] In fact, a meta-analysis of studies involving over 400 patients with various solid tumors all associated MDSCs with poor progression free-survival.[163,266]

1.15.2 Tumors exploit emergency myelopoiesis to protect themselves and promote their growth

Tumors can exploit the mechanism of emergency myelopoiesis to evade immune destruction.[17,126,191,267] MDSCs both support the formation of pre-metastatic niches[200,268–271] and reinforce the survival, proliferation and extravasation of CTCs.[105,126] Zhu et al described this as both fertilizing the “soil” (niche) and the “seeds” (CTCs) at the same time.[267]A premetastatic niche is a microenvironment in a distant organ characterized by immunosuppression, inflammation, angiogenesis and vascular permeability.[105] MDSCs promote the formation of these niches by growth factors (Ex: VEGF or metalloproteinases)[18,46,105,163,272–276], cytokines and chemokines.[46,165,208] Inflammatory pathways increase chemokine secretion (including CXCLs and CCLs) which recruit MDSCs into premetastatic niches, where they can enact their immunosuppressive potential and create a fertile environment for tumor cells to thrive.[105] MDSCs can also reinforce the survival of CTCs by adhering to them and forming a CTC/PMN-

MDSC complex.[267,277] They can even directly infiltrate primary tumor tissues and induce epithelial-mesenchymal transition (EMT) of tumor cells.[71,133,200,278,279] Plus, MDSCs protect CTCs from circulating immune cells by promoting an immunosuppressive environment.[46,200,278,280–282] MDSCs were even shown to have reprogramed lipid metabolism (via FATP2), increasing their uptake and storage of lipids to be used as an energy source by metastatic tumor cells.[126,283]

1.15.3 MDSCs expand due to surgical stress signaling

Trauma and surgery have been linked to the release of MDSCs to mediate tissue repair, aid in the healing process and restore homeostasis.[179] MDSCs expand in trauma patients and are proportional to the degree of surgical manipulation.[122,173,179,189,284] MDSC expansion has been shown in response to surgical stress in both murine[43,63,285,286] and human models.[10,73,105,287,288] This expansion is driven by factors released during the surgical stress response. Stress hormones, for example, have been shown to promote MDSC expansion and promote their immunosuppressive capacities.[105,270,289–291] Likewise, growth factors (TGFβ, VEGF) and cytokines (IL-10) have a similar effect.[133] In human surgery, MDSCs are associated with septic complications and increased mortality.[189,292–295]

1.15.4 SxMDSCs perpetuate postoperative recurrence

Since both surgical stress and tumor-derived factors contribute to MDSC expansion and activation, it is no surprise massive surges in MDSC numbers have been documented in surgical tumor resection. For example, IL-10 is increased in response to surgical stress, it is also produced by MDSCs in response to the TME.[14,18,296–303] Tumor resection has been shown to induce MDSC expansion in multiple mouse models and was consistently associated with increased tumor burden and decreased survival.[54,71,133,304,305] In clinical studies, MDSC expansion following

tumor resection has been correlated with cancer stage, early recurrence and poor prognosis in patients undergoing tumor resection for esophageal cancer[306], breast cancer[307], prostate cancer[80], gastric cancer[308,309], bladder cancer[310], lung cancer[270,304], kidney cancer[69], colorectal cancer[173,311], thyroid cancer[312], pancreatic cancer and more.[68,203,284] Since MDSCs response to both surgical stress and tumor-derived factors, MDSCs expanded during solid tumor resection have been linked to enhanced immunosuppressive capacities compared to those present prior to surgery.[105,284,304,313]

1.16 MDSCs can suppress NK cells using multiple mechanisms

A few select studies have investigated the expansion of MDSCs in patients undergoing tumor resection.[26] NK dysfunction is correlated with MDSC accumulation in various tumor models[62]. MDSCs can inhibit NK cell cytotoxicity[13,18,314,315] and cytokine secretion[157,314] both *in vitro* and *in vivo* in a dose dependent manner.[4,46,157,161,314] Many studies have linked this dysfunction with MDSC potentiated Jak3/STAT5 inhibition.[11,13,18,199,316] The interactions of MDSCs and NK cells can also cause the downregulation of multiple markers/receptors crucial for their immunological function such as CD247 (key subunit for many cytotoxicity receptors) or NKG2D (important for degranulation and IFNγ secretion).[13]

MDSCs can use both contact dependent and independent mechanisms to suppress CMI: this does not mean all of them are being used at any given point in any situation, but rather likely a combination entirely dependent on the type of MSDC expanded and other pathological factors.[3,18,163,191,233] MDSCs can even utilize multiple suppression methods, sometimes even in the context of a single disease.[164] PMN-MDSCs and M-MDSCs have been found to induce T-cell dysfunction through different mechanisms.[154,208,317] While most research involving MDSCs have studied their interactions with T-cells[124], a few studies have described mechanisms by

which MDSCs suppress NK cells.[73,318] While precise mechanisms utilized by SxMDSCs to suppress NK cells remain to be characterized, it is likely that a dominant suppressive mechanism is used and the identification of this mechanism could aid in the development of treatments aimed at improving patient outcomes.[3,4,163]

1.16.1 MDSC contact dependent mechanisms

MDSCs have many receptors which allow them to bind and modulate other immune cells in a direct contact dependent manner.[314] They can interact with PD-1 on NK cells using PD-L1, resulting NK cell dysfunction.[179] MDSCs also interact with NKs using FasL[179], Gal9[319] or NCR ligands.[320] Other cell to cell interactions between NK cells and MDSCs are still being investigated and discovered. Multiple studies have also noted a contact dependent mechanism of suppression involving NK receptor NKp30 with an unknown MDSC receptor.[13,18,199] Another major suppression mechanism utilized by MDSCs in various cancer models is through the use of membrane bound TGFβ, the effects of which on NK cells were previously described.[46] Additionally, MDSCs have been shown to suppress NK cell degranulation and IFNγ secretions through direct interactions of CD155 with NK cell receptor TIGIT.[3,18,156,156]

1.16.2 MDSC contact independent mechanisms

MDSCs can produce soluble factors capable of suppressing CMI.[13,124,163] Overall, most of the contact-independent mechanisms described for these cells involve ROS[164,321], ARG1[73,123], and soluble TGFβ.[62,73,159] Production of reactive oxygen species (ROS) by MDSCs is known to induce immunosuppression.[18,234,322] These are highly reactive and unstable oxygen-containing molecules that also potentiate tissue damage and possible carcinogenesis.[126,323] The production of ROS by MDSCs is mediated through the increased activity of the membrane bound enzyme complex NOX2.[208,324] MDSCs can also metabolize L-arginine through ARG1, depleting from the

microenvironment an amino acid crucial for the function of both NK cells.[122,124,179,313] MDSCs can also uptake and deplete cysteine, another important amino acid for immune cells.[179] MDSCs can produce soluble TGFβ, a known regulator of NK cell function capable of downregulating expression of NK activating receptors (NKG2D, NKp30 and 2B4).[3,13,18,24,46,111,325–327] MDSCs can impair both Fc-receptor mediated cytotoxicity (ADCC) and cytokine production (IFNγ, TNFα and granzyme B) of NK cells through production of the molecular gaseous transmitter nitric oxide (NO) production.[3,13,18,314] NK cell ADCC inhibition in particular occurs due to nitration of receptors, thereby reducing their responsiveness to antigen-MHC complexes, reducing their binding to MHC molecules on tumor cells or blocking their migration through the nitration of chemokines.[163,208] Activated MDSCs also produce cytokines (ex: IL-10) with known immunoregulatory effects on NK cells.[62,164,179] Additionally, indoleamine 2,3-dioxygenase (IDO) is produced by MDSCs and can impair NK cells by decreasing expression of NK activating receptors (NKG2D, NCR, DNAM1) and IFNγ secretion.[18,328] IDO production is regulated through STAT3 induced NF-κB activation.[13,18,163,165,288,328,329]

1.16.3 MDSC crosstalk with other immunosuppressive cells

MDSC-derived mediators can promote the generation of other immunosuppressive cells such as M2-type macrophages, tolerogenic DCs, T helper type 17 (Th17) and regulatory T-cells (Tregs).[3,124,179,208,304,330,331] They have also been shown to promote conversion of macrophages into tumor-associated macrophages (TAMs)[115,302]

1.17 Multiple therapies targeting MDSCs are under investigation

Although surgery itself is unavoidable, some of its immunosuppressive effects can be managed or prevented.[38] The postoperative period contributes substantially to the risk of future metastasis, targeting the issue of postoperative CMI depression with standard of care surgery could

significantly improve long-term survival in surgical cancer patients.[68,284] Despite this, there are very few cancer therapies or approved drugs specifically addressing the pro-metastatic effects of surgery and their use is very stringent and limited in their success.[4,26,43,49,72,73,267,332,333]

Development of therapeutics involving MDSCS can either inhibit or induce their expansion: both may aid patients depending on the context.[179] In cancer particularly, most therapies are aimed at abrogating the suppressive effects of MDSCs.[105,154,235,334] These therapies target MDSC in various ways: including their expansion, infiltration, migration, activation, differentiation, ARG1 and iNOS induction and more, many of which showing significant therapeutic promise in both preclinical models and early phase trials.[13,105,126,267,304,335,336]

Many MDSC targeted therapies, while showing promise in many instances, are also associated with worsened outcomes, which could be explained by the lack of specificity of the some approaches.[124,337] Major challenges in defining and identifying them in humans prevent their specific targeting.[191] Depleting MDSCs could be detrimental and enable uncontrolled inflammation, or dampen important immune surveillance and microbial activity of these cells thus contributing to post-op complications.[24,124] These unfavorable outcomes highlight the need to fully characterize pathologically activated MDSCs to enable their specific targeting.

2. OBJECTIVE, RATIONALE AND HYPOTHESIS

2.1 Rationale

There is substantial evidence suggesting postoperative NK cell dysfunction is majorly responsible for recurrence and metastasis in cancer patients undergoing curative resection. Beyond this, there is a large body of literature demonstrating the major involvement of myeloid derived suppressor cells in this phenomenon, however the mechanisms used by surgery induced MDSCs in cancer patients to inhibit NK cell activity are relatively unknown. Understanding the

nature of these cells and how they operate will allow for new targeted therapies that could significantly improve patient outcomes.

2.2 Objectives

1. Phenotyping MDSCs pre- and postoperatively and correlating these results with NK cell function
2. Evaluating mechanisms by which SxMDSC inhibit NK cell cytotoxicity and activity postoperatively

2.3 Hypothesis

We propose that by identifying MDSC markers that are increased after surgery might provide insight into the mechanisms of postoperative immune suppression of NK cells by sxMDSCs. Additionally, we hypothesize that MDSCs use different mechanisms to suppress NK cells cytokine secretion and cytotoxicity.

3. MATERIALS AND METHODS

3.1 Cell lines

K562 human leukemia cells (ATCC CCL-243™) were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and maintained in maintained in HyCloneTM Roswell Park Memorial Institute (RPMI) 1640 medium (GE 29 Healthcare; Mississauga, ON) supplemented with 10% Fetal Bovine Serum (FBS; Sigma-Aldrich, St. Louis, MO), labelled as Complete RPMI (CRPMI).

NK92 MI human NK cells (human NK cell, ATCC CRL-2408™) were also obtained from ATCC and maintained in CRPMI supplemented with 10% FBS (FBS; Sigma-Aldrich, St. Louis, MO), 50 000 Units (U) of Penicillin-Streptomycin (Pen-Strep; Gibco Ref#15140-122, New

York, USA), 10mM HEPES Buffer Solution (Gibco, Ref#15630-080, New York, USA) and 55μM β-Mercaptoethanol (Gibco, Ref#21985-023, New York, USA).

Nano-Luciferase expressing K562 cells were previously transfected and generously donated by individuals from Dr. Michele Ardolino's lab[338,339] and maintained in CRPMI with 100 U/mL penicillin (Gibco, San Diego, CA, USA), 100 μg/mL streptomycin (Gibco), 10 g/mL gentamycin sulfate (Gibco), and 20 mM HEPES (Fisher, Mississauga, ON, Canada), labelled as supplement complete.

All cell lines were cultured in a 37°C incubator supplemented with 5% CO2.

3.2 Antibodies

Table 1: PROT1 optimization antibodies

CD33 PE-Cy7 (mouse)	Biolegend	366618	P67.7
CD14 APC-Cy7 (mouse)	BD Bioscience	557831	MφP9
CD15 FITC (mouse)	Biolegend	394706	MMA
CD16 AF700 (mouse)	BD Bioscience	8353738	3G8
CD11b (mouse)	R&D Systems	FAB1699N	ICRF44
CD66b (mouse)	Biolegend	392917	6/40c
HLA-DR APC (mouse)	Biolegend	307610	L243

Fixable Viability Dye 510	BD Bioscience	564406	-
CD3 FITC	Invitrogen	11-0039-42	HIT3a
CD19 FITC	Biolegend	302206	HIB19
CD56 FITC	BD Bioscience	340410	NCAM16.2
CD45 PERCP-Cy5.5	Biolegend	304028	HI30
CD193 (CCR3) PE-Dazzle594	Biolegend	310727	5E8
LOX-1 PE	Biolegend	358604	15C4

Table 2: Fresh whole blood MDSC panel antibodies

CD38 PERCP-Cy5.5	BD Bioscience	551400	HIT2
CD40 PE	BD Bioscience	555589	5C3
TREM1 BUV395	BD Bioscience	747907	174031
CD206 BV421	Biolegend	321125	15-2
CD73 PE	BD Bioscience	550257	AD2
PD-L1 PE	BD Bioscience	557924	MIH1
CD284 (TLR4) BV421	Biolegend	312811	HTA125
CD47 PERCP-Cy5.5	BD Bioscience	561261	B6H12
CD3 FITC	Invitrogen	11-0039-42	HIT3a
CD19 FITC	Biolegend	302206	HIB19
CD56 FITC	BD Bioscience	340410	NCAM16.2

CD45 PERCP-Cy5.5	Biolegend	304028	HI30
CD11b (mouse)	R&D Systems	FAB1699N	ICRF44
CD66b (mouse)	Biolegend	392917	6/40c
HLA-DR APC (mouse)	Biolegend	307610	L243
Fixable Viability Dye 510	BD Bioscience	564406	-
CD33 PE-Cy7 (mouse)	Biolegend	366618	P67.7
CD14 APC-Cy7 (mouse)	BD Bioscience	557831	MφP9

Table 3: PROT1 MDSC panel antibodies

CD33 cFluor BYG781	Cytek	R7-40010	WM53
CD14 cFluor B515	Cytek	R7-40010	MEM-18
CD15 cFluor V505	Cytek	R7-40010	W6D3
CD16 cFluor V450	Cytek	R7-40010	3G8
CD11b cFluor BYG610	Cytek	R7-40010	ICRF44
CD66b cFluor R720	Cytek	R7-40010	G10F5
HLA-DR cFluor R840	Cytek	R7-40010	L243
CD3 cFluor R685	Cytek	R7-40010	SK7

CD19 cFluor R685	Cytek	R7-40010	HIB19
CD56 cFluor R685	Cytek	R7-40010	TULY56
CD45 cFluor B548	Cytek	R7-40010	HI30
CD193 (CCR3) cFluor BYG667	Cytek	R7-40010	5E8
LOX-1 cFluor R659	Cytek	R7-40010	15C4
CD84 cFluor BYG575	Cytek	R7-40010	CD84.1.21
CD181 (CXCR1) cFluor BYG710	Cytek	R7-40010	8F 1-1-4
CD155 BUV615	BD Bioscience	752327	SK11.4
CD124 (IL-4Ra) RB780	BD Bioscience	755625	HIL4R-M57
CD62L (L-Selectin) BV786	Invitrogen	417-0629-41	DREG-56
CD192 (CCR2) BV421	Biolegend	357209	K036C2
CD182 (CXCR2) BUV737	BD Bioscience	749071	6C6
CD54 (ICAM1) BV711	BD Bioscience	564078	HA58
CD206 BV605	Biolegend	321139	15-2
CD163 APC-Cy7	Biolegend	333621	GHI/61

CD74 BV650	BD Bioscience	743734	LN2

Table 4: MDSC sort purity antibodies

CD33 PE-Cy7	Biolegend	366618	P67.7
CD14 APC-Cy7	BD Bioscience	557831	MφP9
CD11b AF700	R&D Systems	FAB1699N	ICRF44
CD19 FITC	Biolegend	302206	HIB19
CD3 FITC	Invitrogen	11-0039-42	HIT3a
CD56 FITC	BD Bioscience	340410	NCAM16.2
HLA-DR APC	Biolegend	307610	L243

Table 5: NK sort purity antibodies

CD3 FITC	Invitrogen	11-0039-42	HIT3a
CD14 APC-Cy7	BD Bioscience	557831	MφP9
CD56 BV421	BD Bioscience	562751	NCAM16.2

Table 6: NK cell extracellular staining mix antibodies

CD3 FITC	Invitrogen	11-0039-42	HIT3a
CD14 APC-Cy7	BD Bioscience	557831	MφP9
CD56 BV421	BD Bioscience	562751	NCAM16.2

Table 7: NK cell intracellular staining mix antibodies

IFNγ APC	BD Bioscience	554413	XMG1.2
Granzyme B Percp-Cy5.5	Biolegend	372212	QA16A02
Perforin PE	Biolegend	353304	B-D48

3.3 Selection criteria

Patients were consented through the *Perioperative Human Blood and Tissue Collection Program* (PHBSP), which was approved by the Ottawa Health Science Network (OHSN) Research Ethics Board (REB) under protocol number 2011884. Eligible patients were >18 years of age and had a scheduled surgical resection of the primary or metastatic tumor (cancer patients) or healthy donors who volunteered to participate. Cell mediated immune functions is also known to deteriorate with age, for this reason elderly patients (more than 80 years of age) were avoided when possible.[128] Patients with a history of autoimmune disease (Lupus, Rheumatoid Arthritis...etc) or taking immunosuppressive medications were excluded. Patients which received chemotherapy and/or radiation therapy within four weeks of their scheduled surgery were also excluded.[5] Patients with Hemoglobin (Hgb) levels below 90 were also excluded. Dyslipidemia, frequently found in obesity, has been proposed to lead to the accumulation of MDSCs and enhancement of their immunosuppressive capacities: for this reason, whenever a patient had a distinct dyslipidemia diagnosis they were excluded from the study.[179]

3.4 Sample collection

Fresh whole blood was collected from healthy donors and cancer surgery patients (Table 1) at baseline and on POD1 by venipuncture in sodium-heparin tubes (BD Vacutainer® Cat#367878/367874). Blood was drawn by registered nurses in the surgical day care unit

(SDCU) for baseline samples and by registered nurses in the surgical wards for POD1 samples. Blood was processed within 2 hours of collection. Replicate aliquots of 1 mL of patient matched blood sample were either stained as fresh whole blood or fixed with PROT-1, frozen and thawed, and lysed before staining.

3.5 Whole blood stabilization

As per the manufacturer's protocol, 1 mL of whole blood vas fixed and stabilized by the addition of 1.4 mL of PROT-1 stabilizer (SmartTube Inc., San Carlos, CA). After incubation at room temperature (RT) for 10 minutes the samples were immediately transferred to -80°C for long-term storage.

3.6 Whole blood freezing

Fresh whole blood was mixed in a 1:1 ratio with a freezing mix of RPMI 1640 medium with 10% DMSO, a freezing mix previously shown to preserve immune cell integrity and staining during cryopreservation.[340] The samples were then transferred to -80°C for long-term storage.

3.7 Flow cytometry staining

3.7.1 Fresh whole blood staining

Fresh whole blood was treated with 1X PharmLyse buffer (BD Bioscience, Cat#555899) and vortexed at full speed for 30 seconds. Samples were then incubated for 15 minutes in the dark at RT to lyse red blood cells. Samples were then centrifuged at 500xg for 5 minutes and washed once with flow buffer (FB: 0.5% BSA, 1 mM EDTA). Cells were then stained with fixable viability stain 510 (BD Bioscience, Cat#564406) and washed once with FB. Cells were then stained with antibody mix (Table 2) and incubated at 4°C for 20 minutes. Cells were then washed with flow buffer and fixed with 1% Paraformaldehyde (PFA, Fisher Scientific, Cat#AAJ19943K2) for 20 minutes at 4°C. Cells were then spun down and resuspended with FB and stored at 4°C until acquisition by flow (samples were run within 2 days).

3.7.2 Stimulation of fresh whole blood for flow cytometry staining

Certain MDSC markers of interest could not be measured in the large MDSC immunophenotyping panel due to their poor staining with PROT1 or their general low expression (Table 2). To evaluate these, fresh whole blood was plated in a tissue-culture treated V-bottom 96 well plate (Corning, Ref#3894, Kennebunk, ME, USA) and treated with 2ng/mL of LPS (LPS, eBioscience Cat #00-4976-93) for 24h. After the 24h incubation cells were washed and stained as previously described (Section 3.7.1).

3.7.3 Frozen whole blood staining

Samples frozen in RPMI/DMSO freezing media were thawed for 15 minutes on ice and washed with Phosphate Buffered Saline 1X (PBS, GE Healthcare).

3.7.4 PROT1 stabilized whole blood staining

Stabilized samples were thawed for 10-15 minutes in a -12°C water bath. Once thawed 1 mL of 1X Thaw-Lyse buffer (SmartTube Inc., San Carlos, CA) was immediately added. Content was then strained through a 70µm cell strainer (Corning®, Durham, North Carolina, USA, REF 431751) into an additional 25 mL of Thaw-Lyse buffer. Strainer was washed with 5 mL of Thaw-Lyse buffer. Samples were incubated for 10 minutes at RT. Following incubation samples were centrifuged at 600xg for 5 minutes at RT and resuspended in 25 mL Thaw-Lyse buffer. After a secondary spin at 600xg for 5 minutes and wash with 20 mL of 1X Thaw-Lyse buffer, cells were resuspended in 25µL Human Fc Block and heparin and incubated for 20 minutes at RT.

Cells were treated with Human BD Fc Block^{TM} (BD Bioscience) and 200U/mL Heparin (Sigma-Aldrich, United States) and incubated for 10 minutes at RT. Cells were then resuspended in 25uL of antibody mix (Table 1) and incubated for 20 minutes on ice. 900uL of FB.

3.8 Flow cytometry analysis

The comprehensive blood staining panel (Table 2) utilized the CytekCloud tool from Cytek Biosciences to choose markers that provide optimal resolution and to develop a panel with minimal spillover. Samples were run on the Cytek Aurora. A total of 5000 events were collected gating on $CD33^{+}CD11b^{+}CD14^{+}CD15\text{-}Lin^{-}HLA\text{-}Dr^{low}$ (see Figure 1 for gating strategy). The resulting data was analyzed with Flojo software (Version 10.1, BD Bioscience). Gating of specific cell populations or subtypes of MDSCs were done manually. The data obtained was processed using Flowjo software (Version 10.1, BD Bioscience). Gating for specific cell populations or MDSC subtypes was performed manually.

3.9 MDSC gating strategy

We developed a large gating strategy to single out M-MDSC populations in whole blood (Figure 1A). We began by removing doublets through a FSC-H vs FSC-A gate. Next, we excluded contamination from red blood cells and platelets by targeting CD45 positive white blood cells. We then filtered out eosinophils and basophils by selecting CCR3 negative cells. Following this step, NK cells, T-cells, and B-cells were excluded using a lineage cocktail (C3, CD19, CD56). Lastly, granulocytic cells were eliminated by identifying CD15 and CD66b negative cells. We characterized myeloid cell populations based on the co-expression of myeloid lineage markers CD33 and CD11b, and we confirmed CD14 expression on the myeloid cell population.

M-MDSCs are distinguished through the downregulation of HLA class II expression ($HLA\text{-}DR_{low}$).[165,198] HLA-DR gating varies significantly across studies: while some employ isotype controls, modern flow cytometry guidelines advocate using Fluorescence Minus One (FMO) controls to establish gating boundaries. It is recommended that isotype controls should be reserved for assessing non-specific binding.[341–343] Additionally, isotypes fail to consider spillover

effects from other channels, limiting their effectiveness in establishing gates within large extended panels difficult.[344] Others use an FMO[201,345] (though this is also discouraged by other groups[346]), through the use of FMOs can become inaccurate for setting positive/negative gates in the presence of background signal or spillover.[344] Certain studies have used other populations (mostly lymphocytes) to set their gate.[186,347] We chose the latter due to the massive separation/stain index seen for HLA-DR in PROT1 samples compared to fresh blood (Figure 1B). Using an FMO rendered all non-granulocytic cells "HLA-DR positive, making the use of an FMO unrealistic (Appendix A).

The gating strategy for PMN-MDSC cells differed slightly from that of M-MDSCs (Figure 1C). Gating was identical to M-MDSCs until the previously defined lineage negative gate ($CD3^-$ $CD19^-CD56^-$cells). From these lineage negative cells, $CD16^{low}$ cells were gated on to eliminate any mature neutrophils. From there myeloid cells were once again identified through co-expression of lineage markers CD33 and CD11b and PMN-MDSC cells were defined as CD15 positive.

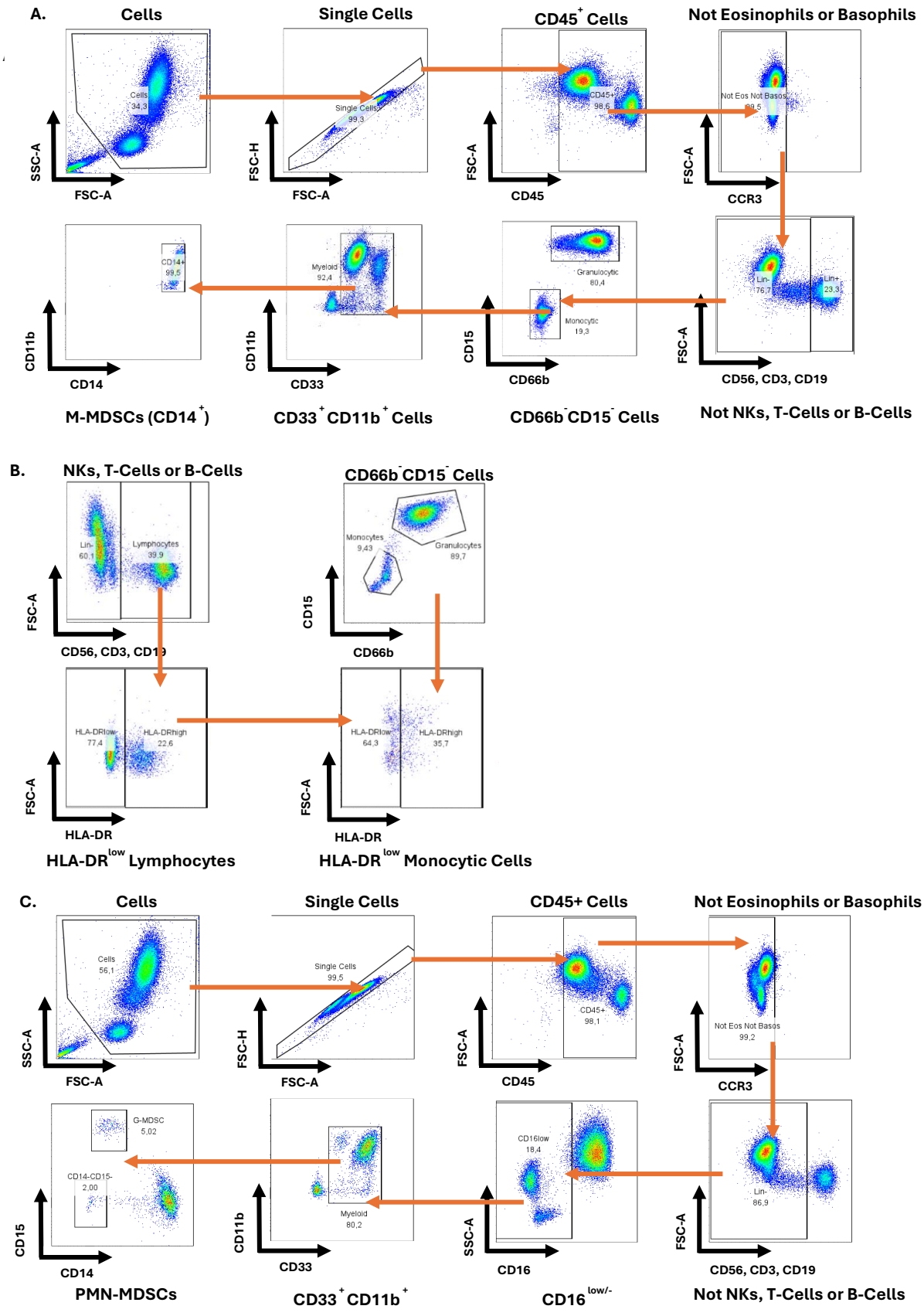
A.
Cells
Single Cells
CD45+ Cells
Not Eosinophils or Basophils
SSC-A
FSC-A
FSC-H
CD45
CCR3
CD11b
CD14
CD33
CD15
CD66b
CD56, CD3, CD19
M-MDSCs (CD14+)
CD33+CD11b+ Cells
CD66b-CD15- Cells
Not NKs, T-Cells or B-Cells
B.
NKs, T-Cells or B-Cells
CD66b-CD15- Cells
HLA-DR
HLA-DRlow Lymphocytes
HLA-DRlow Monocytic Cells
C.
Cells
Single Cells
CD45+ Cells
Not Eosinophils or Basophils
G-MDSC
PMN-MDSCs
CD33+CD11b+
CD16low/-
Not NKs, T-Cells or B-Cells

Figure 1: Gating Strategy for MDSC identification in whole blood. A) Gating strategy for identification of monocytic cells in whole blood. **B)** Gating strategy for identifying HLA-DR^{low} monocytic cells in whole blood. **C)** Gating strategy for identifying CD16^{low} PMN-MDSCs in whole blood.

3.10 Cell isolation for functional assays

3.10.1 PBMC isolation

Blood was processed within 2 hours of collection. Blood was gently overlaid on Ficoll-Paque PLUS (Cytiva, Ref#1714403, Uppsala, Sweden) as per the manufacturer's protocol. Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coat and washed with PBS (GE Healthcare) and manually counted on a hemocytometer for further experimentation.

3.10.2 MDSC isolation

Patient MDSCs were isolated from patient PBMCs by automatic magnetic bead separation with the Automacs Cell Sorter (Miltenyi, North Rhine-Westphalia, Germany) using positive selection of CD33+ immunomagnetic (IM) microbeads (Miltenyi, Ref#130-045-501, North Rhine-Westphalia, Germany) according to the manufacturer's protocol. Following separation, SxMDSCs were washed with pre-warmed CPRMI and manually counted with a hemocytometer for further experimentation. An aliquot of MDSCs were also stained with an antibody mixture (Table 4) to assess sort purity.

3.10.3 Healthy donor NK cell isolation

Healthy NK cells were isolated from non-cancer patient PBMCs by automatic magnetic bead separation with the Automacs Cell Sorter (Miltenyi, North Rhine-Westphalia, Germany) using negative selection of CD56^{+} cells using the Milteneyi NK cell isolation kit (Miltenyi, Ref# 130-092-657, North Rhine-Westphalia, Germany) according to the manufacturer's protocol. This process involves magnetically labelling T cells, B cells, stem cells, dendritic cells, monocytes, granulocytes, and erythroid cells using a cocktail of biotin-conjugated antibodies, allowing NK

cells to be isolated untouched. Following separation, NK cells were washed, counted and resuspended in FBS with 10%DMSO and stored at -80˚C until needed. An aliquot of NK cells was also stained with an antibody mixture (Table 5) to assess sort purity.

3.11 Functional assays

3.11.1 MDSC flow-Based NK cell cytotoxicity suppression assay

Freshly isolated SxMDSCs were plated in a tissue-culture V-bottom 96 well plate (Corning, Ref#3894, Kennebunk, ME, USA) at a concentration of $2x10^4$ cells per well in triplicates. IL-2 independent NK92 MI cells were washed with pre-warmed CRPMI, counted and then plated at $1x10^4$ alongside the SxMDSCs for co-culture, maintaining a 2:1 ratio of MDSCs to NK cells. Control wells in triplicates contained only NK92 cells and an equal volume of CRPMI to compare NK cell killing of tumor targets with and without SxMDSCs. The co-culture was then incubated for 24 hours.

The following day, K562 target cells were washed in PBS (GE Healthcare), resuspended to 2 $x10^6$ cells/mL and labelled with Cell Proliferation Dye eFluor™ 450 (CP450; eBioscience; San Diego, CA, USA) for 15 minutes at 37ºC. The excess dye was then removed by washing the cells several times in pre-warmed CRPMI and the cells were added to the co-culture at $5x10^4$ CP450-K562 cells per well (4:2:1 MDSC: NK: Target ratio). An aliquot of CP450-K562 cells were also heat-killed at 65ºC for 5 minutes for a dead control and plated in triplicates. A triplicate of wells containing only live CP450-K562 cells were included as well for a live control. The co-culture was then incubated for a further 4h at 37ºC. Following this incubation, cells were then washed with PBS (GE Healthcare) and then stained with eBioscience Fixable Viability Dye eFluor780 (Ref#65-0865-14, Invitrogen, USA). Following a 15-minute incubation with the dye the cells were washed with Flow Buffer comprised of PBS (GE Healthcare), 0.2% (v/v) UltraPureTM

0.5M EDTA (Thermo Fisher Scientific), and 0.5% (w/v) Bovine Serum Albumin (BSA; Bioshop Canada; Burlington, ON) and fixed with 1% Paraformaldehyde (PFA, Thermoscientific, Cat#J61899.AK). Finally, cells were resuspended in flow buffer and stored at 4ºC until flow acquisition.

Samples were analyzed using the HTS on the BD Fortessa™ (BD Biosciences) at the University of Ottawa Flow Cytometry Core Facility (Ontario, ON). NK Cell mediated cytotoxicity was measured as the percentage of dead CP450-labelled K562 target cells gated on $CP450^{+}$/$eFluor780^{+}$. %Suppression was calculated using the following equation:

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

3.11.2 MDSC nanoluciferase-based NK cell cytotoxicity suppression assay

Flow-based suppression assay was used unless specified otherwise in the results. SxMDSCs and NK92 cells were prepared and plated as previously described. After a 24h co-culture nano-luciferase transfected K562 cells were added for a 4:2:1 MDSC to NK to target ratio for a 4.5h co-culture before supernatant was transferred into a new plate and mixed with CTZ substrate. Luminescence was then measured by plate reader and NK specific lysis was assessed using the following equation:

$$\%\ specific\ lysis = \frac{(experimental\ release - spontaneous\ release)}{(maximal\ release - spontaneous\ release)} x\ 100$$

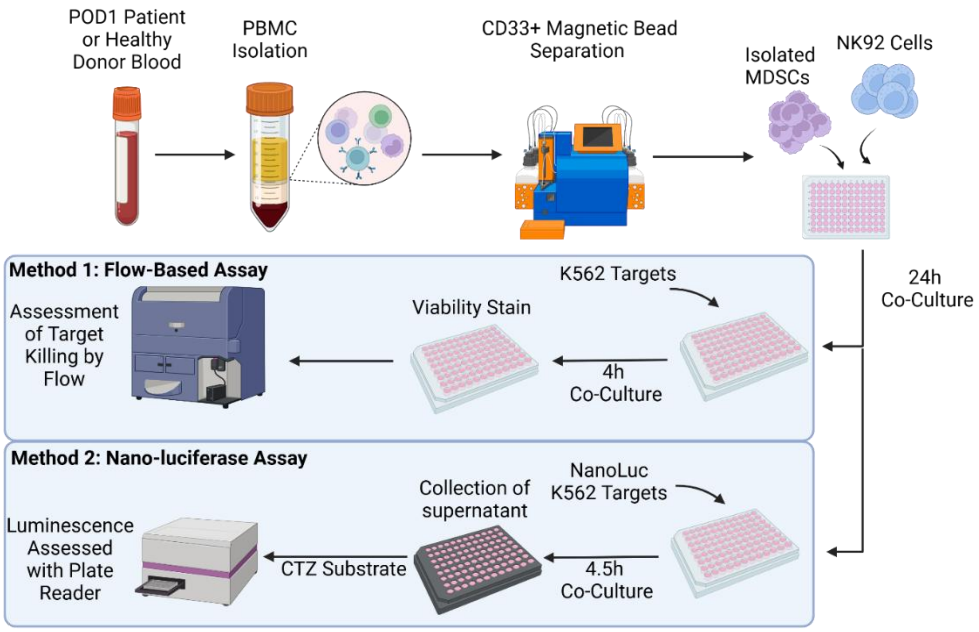

Figure 2: Suppression assay methodology.

3.11.3 MDSC NK cytokine secretion suppression assay

Healthy donor NK cells were quickly thawed in a 37˚C water bath and resuspended in CRPMI with a low dose of 1U/mL human recombinant IL-2 (rhIL-2, Cat#202-IL, R&D Systems, Minneapolis, USA). NK cells were then rested in a 37˚C incubator for 1 hour before they were washed and counted. They were then plated in a tissue-culture treated V-bottom 96 well plate (Corning, Ref#3894, Kennebunk, ME, USA) at $1x10^5$ cells per well. SxMDSC were isolated as previously described and plated alongside the healthy NK cells at $4x10^5$ cells per well (4:1 MDSC to NK ratio). SxMDSCs and NK cells were allowed to co-culture for 24h, after which a selection of wells were treated with additional low dose IL-2 (resting condition) and others with a IL2/12 stimulation encompassing 400U/mL of IL-2 with 20ng/mL of human recombinant IL-12 (rhIL-12, Cat#219-IL, R&D Systems, Minneapolis, USA). Cells were incubated for a further 20h after which brefeldin A (BD GolgiPlug™, BD biosciences, cat#555029) was added for an additional 4h of incubation.

After this, cells were washed and stained with fixable viability dye BV510 (BD biosciences, cat#564406). Following a wash and a short incubation with a non-specific Fc Receptor-mediated fluorescent antibody (BD biosciences, cat 564219), cells were stained with an extracellular staining mix identifying NK cells (Table 10). After this, cells were incubated with a fixation/permeabilization reagent mixture (BD Cytofix/Cytoperm™, BD Biosciences, cat#554714) for 20 minutes at 4˚C, and then washed with the associated washing buffer (BD Perm/Wash™, BD Biosciences, cat#554714). Once fixed, they were stained with an intracellular staining mix including IFNγ, granzyme B and perforin (Table 11). Following a wash, they were resuspended in flow buffer and stored at 4ºC until flow acquisition (gating strategy seen in Appendix C).

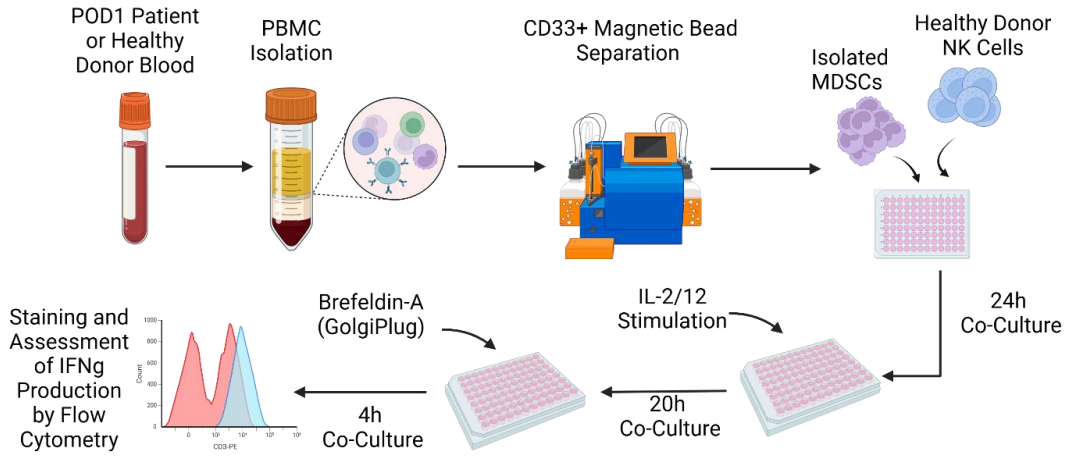

Figure 3: MDSC cytokine suppression assay methodology

3.11.4 MDSC NK cell function supernatant assays

Freshly isolated SxMDSCs were plated in triplicates in a tissue-culture treated V-bottom 96 well plate (Corning, Ref#3894, Kennebunk, ME, USA) at $2x10^4$ cells per well. These cells were then cultured for 24h or 48h, after which the cells were pelleted, and the supernatant was collected.

For evaluation of NK cell cytotoxicity, IL-2 independent NK92 MI cells were also washed with pre-warmed CRPMI, counted and plated in triplicates at $1x10^4$ cells per well. Supernatant was then added in a 1:1 SxMDSC supernatant to NK92 cell suspension ratio. A set of control wells also contained NK92 cells with 50% pre-warmed CPRMI or the supernatant of the negative fraction obtained from the MDSC isolation and cultured at the same concentration.

For evaluation of NK cell cytokine secretion, healthy donor NK cells were isolated and plated as previously described. SxMDSC supernatant was added in the same ratio for 24h before NK cells were stimulated and IFNγ secretion was assessed via intracellular staining and flow assessment.

For the concentrated supernatant assay measuring NK cell cytotoxicity, supernatant as collected and concentrated using the Amicon Ultra-4 10K Centrifugal Filter Units (REF UFC801008, Merck Millipore Ltd, Co. Cork, Ireland) and otherwise followed the same workflow.

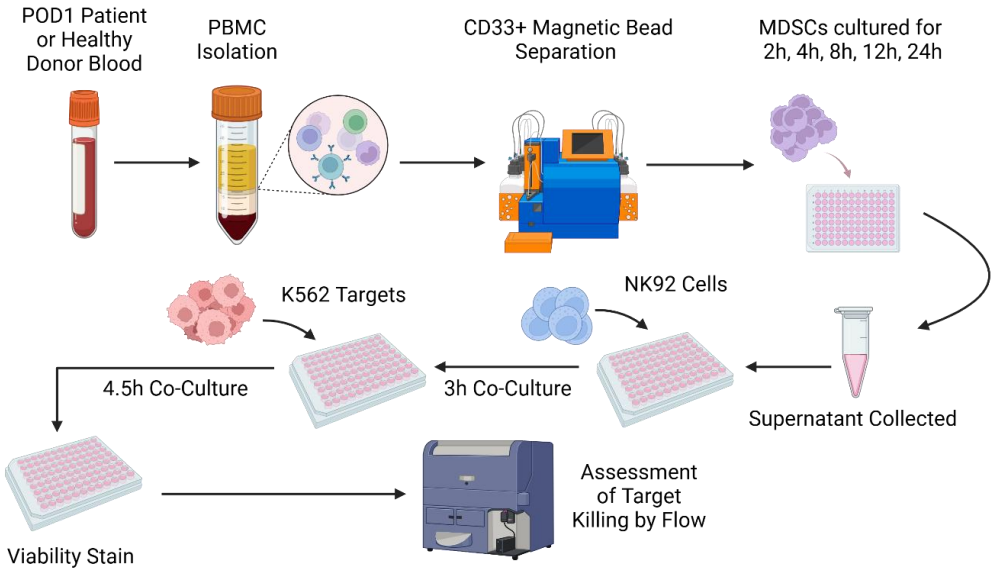

Figure 4: MDSC supernatant assay methodology

To assess how NK cell presence affects the ability of SxMDSC to generate suppressive supernatant, SxMDSCs were cultured with NK92 (for the cytotoxicity assay) or HD NK cells (for the cytokine secretion assay) for 24 hours before collecting the supernatant. The subsequent assays followed the same workflows for both assessment.

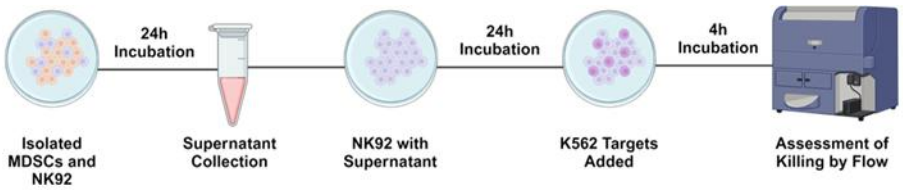

Figure 5: MDSC supernatant assay with NK co-culture

3.11.5 Plasma NK cell function suppression assays

Blood samples were obtained as described earlier and centrifuged at 1000 xg for 10 minutes. Subsequently, plasma was separated and subjected to a second centrifugation at 10,000 xg for 15 minutes to remove any residual platelets. The resulting platelet-free plasma was stored at -80˚C until the assay day (Figure 6). For assays involving concentrated plasma, samples were collected and concentrated using the Amicon Ultra-4 10K Centrifugal Filter Units (REF UFC801008, Merck Millipore Ltd, Co. Cork, Ireland) and otherwise followed the same workflow. For plasma assays investigating both NK cell cytotoxicity and cytokine secretion, NK cells were cultured in wells containing 75% CRPMI and 25% plasma. These assessments were performed in the same manner as the SxMDSC and NK cell co-culture assay.

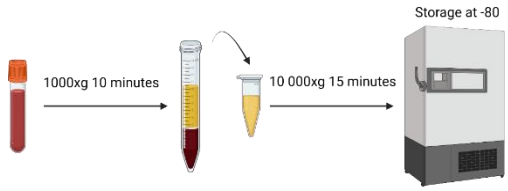

Figure 6: Plasma isolation methodology.

NK92 cells were isolated and plated as previously described in the MDSC suppression assay for all cytotoxicity plasma suppression assays. Alternatively, for plasma cytokine secretion assays, healthy donor NK cells were isolated and used as described in the MDSC cytokine suppression assay. Once NK cells were plated, patient plasma was added to reach a 1:4 plasma to CRPMI ratio and cells were cultured for 24h, after which they were assessed for either cytotoxicity (against K562 targets) or IFNγ secretion (using intracellular flow staining).

3.12 Statistical analysis

All statistical tests were performed using GraphPad Prism 9. Significance was denoted as *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001. Generally, paired and non-parametric Wilcoxon tests were performed when comparing two groups (ex: BL vs POD1 or BL vs POD3). To determine average or overall fold-changes, the fold-change between two time points was calculated for each set of patient samples and mean was calculated. Statistical comparisons were only conducted for experiments with n>6. Staining index was calculated by the following: SI = (Medianpos – Medianneg)/2x StdDevneg.

4. RESULTS

Chapter 1: Perioperative Profiling of Myeloid Derived Suppressor Cells in Surgical Cancer Patients

4.1 Demographic Data

The study cohort was 55 cancer patients and 37 non-cancer patients. The demographic data used for these patients is summarized in Table 1. Patients were diagnosed with various solid tumors, primarily colorectal and gastrointestinal (including stomach, esophageal, colorectal, pancreatic and liver, 37%), gynecological (including endometrial and ovarian, 11%), urinary (including bladder, kidney and prostate, 42%) and lung (9%). The surgical technique for tumor resection included laparotomy [open surgery, 33%), laparoscopic (24%) and robotic surgery (44%)].

Table 8: Study participant demographics

Category	Sub-Category	Cancer patients	Healthy
Total (n)	N/A	55	37
Gender (n)	Male	36	26
	Female	19	11
Age (median; range)		64; 37-*80*	56; *21-85*
Length (Duration) of surgery (median; range)		4h52min; *2h40min – 13h30min*	
Surgery type (n)	Open	18	
	Laparoscopic	13	
	Robotic Assisted	24	
Cancer Type (n)	Prostate	17	
	Colorectal/Gastrointestinal	15	
	Bladder/Kidney	6	
	Ovarian/Endometrial	6	
	Lung	5	
	Liver	3	
	Pancreas	3	
Staging (n)	I	18	

	II	15
	III	7
	IV	2
	Unknown	13

Tables 2 and 3 provide the demographics of the patients utilized for the immunophenotyping study comparing fresh whole blood to PROT1 stabilized blood (n=18) and the patients used for the MDSC immunophenotyping study (n=22), respectively.

Table 9: Study participant demographics PROT1 optimization

Category	**Sub-Category**	**Cancer patients**	**Healthy**
Total (n)	N/A	12	6
Gender (n)	Male	7	4
	Female	5	2
Age (median; range)		65; *53-79*	60; *26-74*
Duration of surgery (median; range)		4h21min; *2h40min – 7h45min*	
Surgery type (n)	Open	4	
	Laparoscopic	2	
	Robotic Assisted	6	

Cancer Type (n)	Prostate	4
	Colorectal/Gastrointestinal	3
	Bladder/Kidney	1
	Ovarian/Endometrial	2
	Pancreas	1
Staging (n)	I	4
	II	4
	III	3
	IV	0
	Unknown	1

Table 10: Study participant demographics for MDSC Immunophenotyping

Category	**Sub-Category**	**Cancer patients**	**Healthy**
Total (n)	N/A	20	2
Gender (n)	Male	12	1
	Female	8	1
Age (median; range)		63; *37-80*	56; *52-60*
Duration of surgery (median; range)		5h19min; 3*h15min – 12h*	

Surgery type (n)	Open	7
	Laparoscopic	6
	Robotic Assisted	7
Cancer Type (n)	Prostate	4
	Colorectal/Gastrointestinal	6
	Bladder/Kidney	3
	Ovarian/Endometrial	3
	Lung	1
	Liver	2
	Pancreas	1
Staging (n)	I	10
	II	4
	III	1
	IV	0
	Unknown	5

4.2 Impact of PROT1 on M-MDSC populations during cryopreservation.

MDSCs are highly sensitive to cryopreservation and therefore most studies have evaluated them in fresh blood or PBMC samples. The SmartTube proteomic stabilizer (PROT1) both fixes and preserves blood with a single buffer and has previously been used for large or complex

immunophenotyping studies.[348–350] We aimed to use PROT1 to perform a detailed immunophenotyping study investigating SxMDSCs. To determine whether the PROT1 stabilizing solution preserves the cell surface phenotype of myeloid cells, we compared the cell surface expressions following freezing with PROT1 to that of fresh blood. Whole blood was collected from 10 cancer patients at both BL and POD1, along with 6 healthy donors. Equal amounts were stained fresh, stabilized with PROT1 and stored at -80˚C, or cryopreserved using a standard cryopreservation mixture of FBS+10%DMSO (Freezing Mixture, FM)[340,351] for -80˚C storage (Figure 7A). Cryopreserved samples were later thawed and stained with the same panel.

Interestingly, both PROT1 and FM altered the distribution of major cell populations in an FSC vs SSC-A plot (Figure 7B), but the overall frequencies of major immune cell populations (granulocytes, monocytes and lymphocytes) remained consistent when using PROT1 (fold-change of 1.1 for granulocytes, 0.9 for lymphocytes and 0.95 for monocytes, Figure 7C-D). FM induced higher and more significant alterations in population frequencies (1.92-fold increase in lymphocytes, 0.7-fold decrease in granulocytes and 0.5-fold decrease in monocytes).

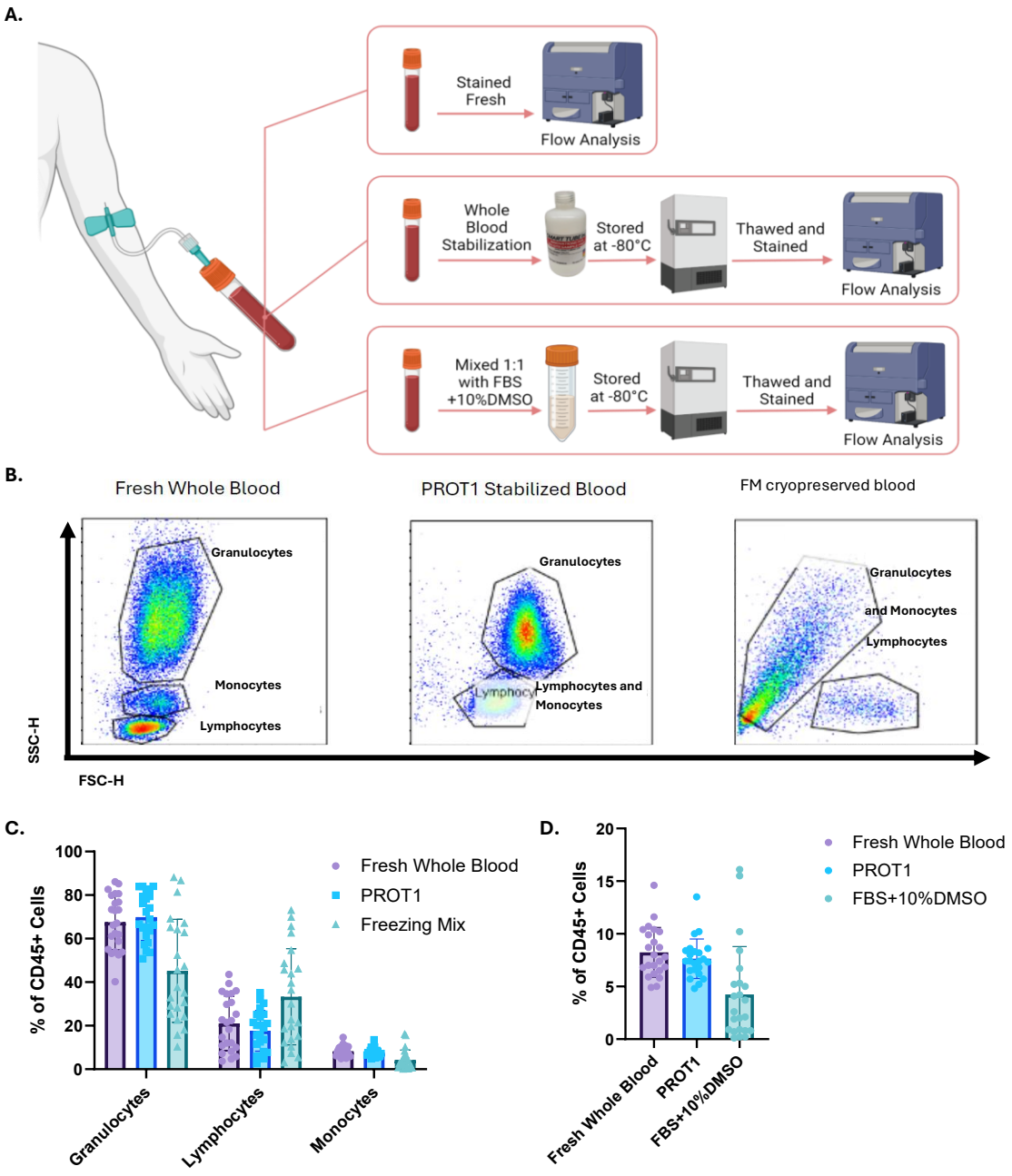

Figure 7: PROT1 preserves major immune population frequencies in whole blood after cryopreservation. A) Experimental method illustration. B) Representative flow plots showing major immune subsets distributions in fresh whole blood (left), PROT1 stabilized blood (middle) and FM cryopreserved blood (right). C) Frequencies of granulocytes, lymphocytes and monocytes in matched samples (n=23) for fresh whole blood (purple), PROT1 cryopreserved blood (blue) and FM blood (green). Paired Wilcoxon (Granulocytes Fresh vs PROT1 P=0.03 and Fresh vs FM P=0.0002; Lymphocytes Fresh vs PROT1 P=0.01 and Fresh vs FM P=0.006; Monocytes Fresh vs PROT1 P=0.14 and Fresh vs FM P=0.001). D) Monocytes frequencies in fresh whole blood (purple), PROT1 cryopreserved blood (blue) and FM blood (green).

Overall, M-MDSCs populations were well maintained in PROT1 but large cell losses were seen when freezing whole blood with FBS (Figure 8A). When looking at M-MDSC frequencies, a significant difference in this population was observed as a percentage of monocytic $CD14^+$ cells (Figure 8B) and $CD45^+$ white blood cells (Figure 8C) when comparing fresh samples with the FM. These population frequencies dropped by an average of 39% and 23%, respectively, when frozen with the FM. This difference was not observed when comparing fresh with the PROT1 samples: frequencies only dropped by 13% in all WBC and 4% in monocytes. M-MDSC populations are preserved for up to 1 year of cryopreservation using PROT1 (Appendix B, n=1). Furthermore, the M-MDSC frequencies are much more highly correlated between fresh whole blood and PROT1 compared to fresh whole blood and FM (Figure 9A-B).

Granulocytic MDSCs were subject to increased losses in population frequencies when cryopreserved, as shown in Figure 8D. A significant 64% reduction was noted in the frequency of PMN-MDSC cells among CD45+ cells when comparing fresh blood to PROT1 (Figure 8D). This drop was increased to 80% when using FM. Both PROT1 and FM were poorly correlated with fresh, but PROT1 to a lesser degree (Figure 9C).

Previous studies conducted in our lab have reported an increase in the levels of M-MDSCs following surgery.[313,352] To determine if the post-operative increase in M-MDSC frequency was still observable with PROT1 stabilized blood, the fold-change in M-MDSC frequency was evaluated by normalizing POD1 samples to BL. An increase in MDSCs at POD1 can still be seen in 80% of patients with PROT1. No significant difference was noted in the magnitude of this increase of M-MDSCs or PMN-MDSCs when normalized to BL sample (Figure 8E).

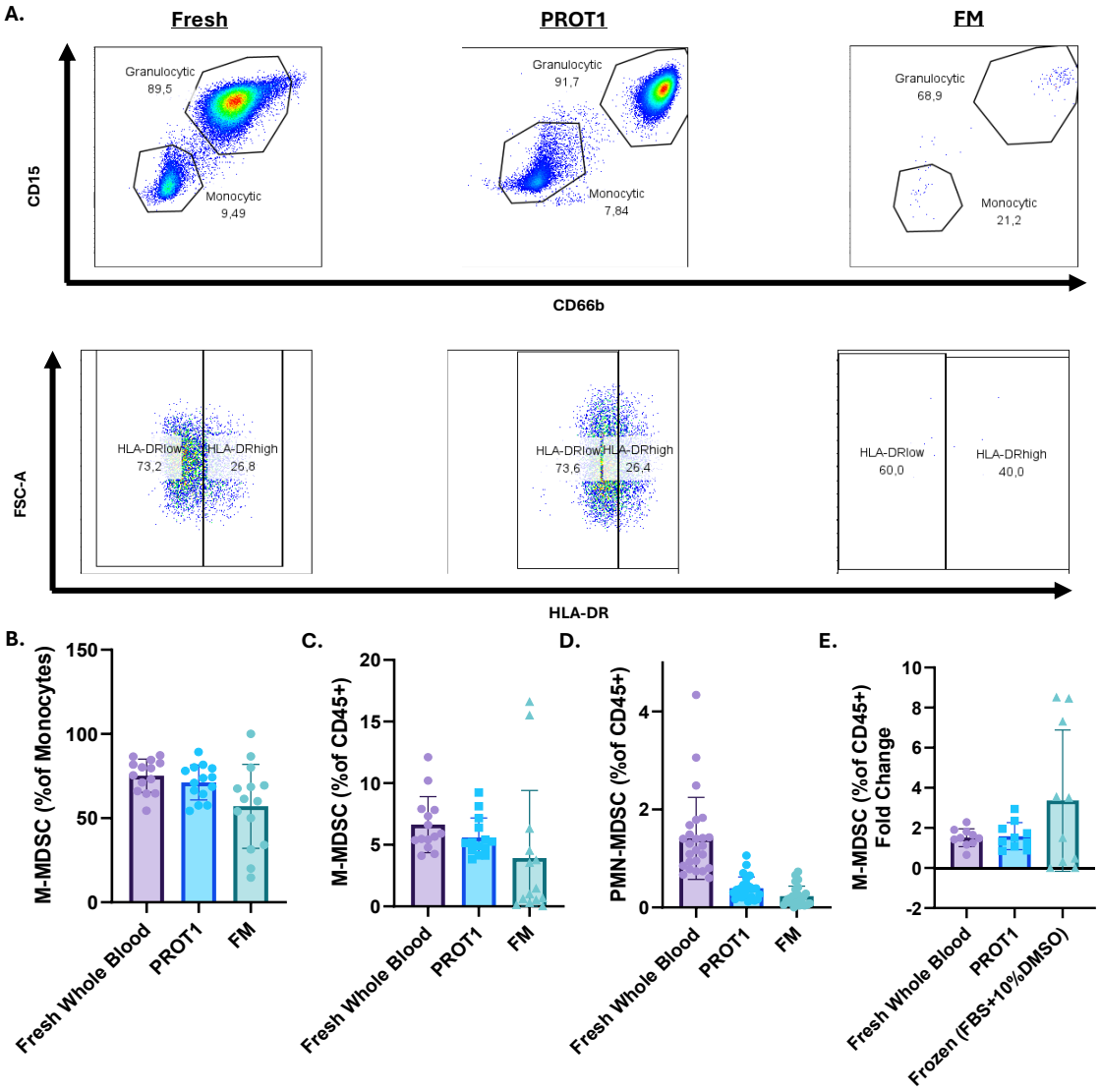

Figure 8: Changes in MDSC population frequencies following whole blood cryopreservation with PROT1. A) Representative flow plots illustrating granulocytic ($CD33^+CD11b^+CD15^+CD66b^+Lin^-$), monocytic ($CD33^+CD11b^+CD15^-CD66b^-Lin^-CD14^+$) and M-MDSC ($CD33^+CD11b^+CD14^+CD15^-Lin^-HLA\text{-}DR^{low}$) frequencies for fresh blood, PROT1 stabilized blood and FM cryopreserved blood. **B)** M-MDSC frequencies in fresh blood (purple), PROT1 stabilized blood (blue) and FM cryopreserved blood (green) as a percentage of monocytic ($CD14^+$) cells. Paired Wilcoxon test (n=23, Fresh vs PROT1 P=0.001 and Fresh vs FM P=0.0003) **C)** M-MDSC frequencies in fresh blood (purple), PROT1 stabilized blood (blue) and FM cryopreserved blood (green) as a percentage of $CD45^+$ cells. Paired Wilcoxon test (n=23, Fresh vs PROT1 P=0.002 and Fresh vs FM P=0.04). **D)** PMN-MDSC ($CD33^+CD11b^+CD15^+CD14^-Lin^-CD16^{low}$) frequencies in fresh blood (purple), PROT1 stabilized blood (blue) and FM cryopreserved blood (green) Paired Wilcoxon test (n=23, Fresh vs PROT1 P<0.0001 and Fresh vs FM P<0.0001). **E)** Histogram depicting BL to POD1 M-MDSC fold change in fresh blood (purple), PROT1 stabilized blood (blue) and FM cryopreserved blood. Paired Wilcoxon test (n=23, Fresh vs PROT 1 P=0.77 and Fresh vs FM P=0.28).

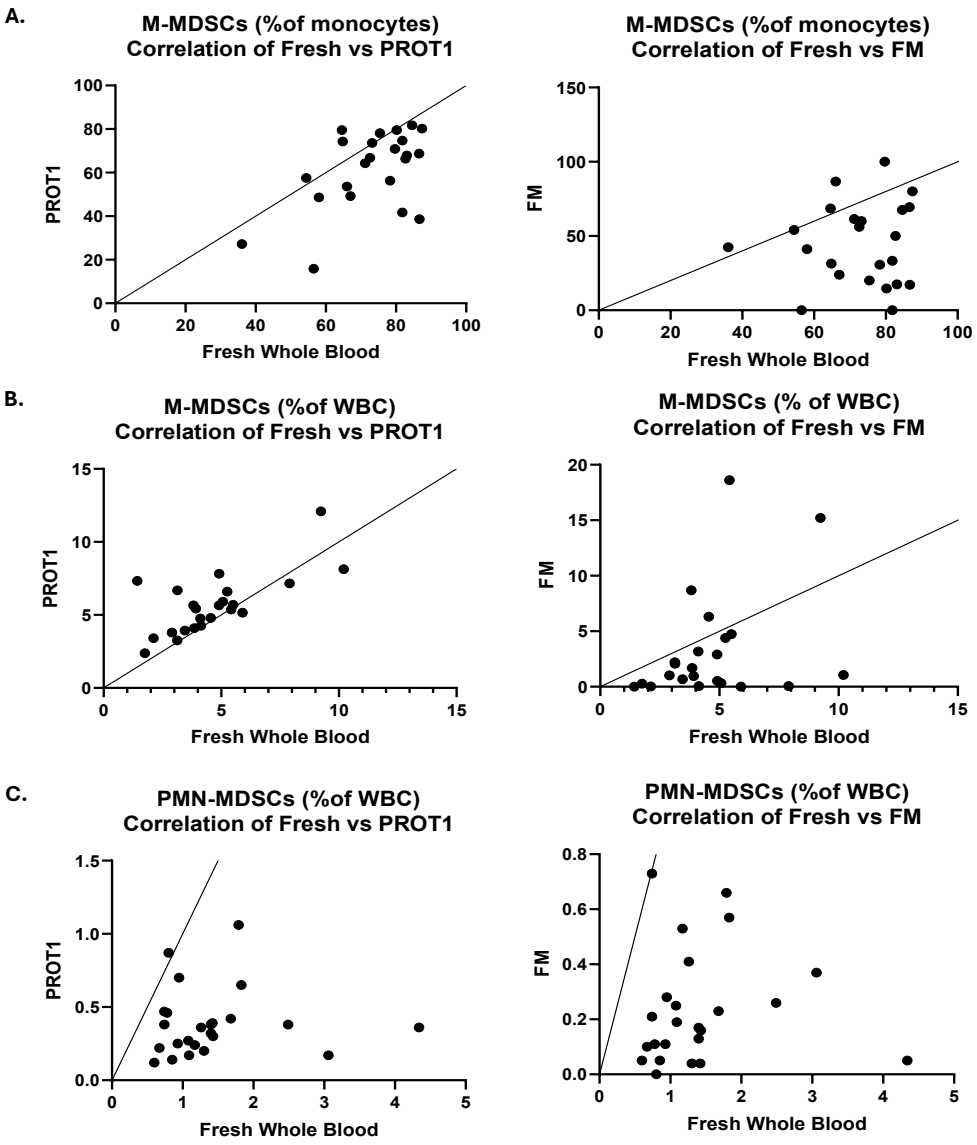

Figure 9: Correlation between MDSC frequencies with different whole blood processing methods. A) Pearson correlation between M-MDSC frequency of total monocytes ($CD14^+$) in fresh blood, PROT1 blood and FM cryopreserved blood (Fresh vs PROT1 R=0.40 and Fresh vs FM R=-0.4) **B)** Pearson correlation between M-MDSC frequency of WBC ($CD45^+$) in fresh blood, PROT1 blood and FM cryopreserved blood (Fresh vs PROT1 R=0.59 and Fresh vs FM R=0.28). **C)** Pearson correlation between PMN-MDSC frequency of WBC ($CD45^+$) in fresh blood, PROT1 blood and FM cryopreserved blood (Fresh vs PROT1 R=0.16 and Fresh vs FM R=-0.18).

4.3 Distribution of major MDSCs subsets in the whole blood of cancer patients following tumor resection

Expansion of myeloid cells in the immediate post-operative period has been previously described, including reports from our own laboratory.[54,71,133,304,305,313] Most immunophenotyping studies performed on myeloid cells are conducted using PBMCs, with a few being done with whole blood.[165] For this experiment, whole blood was collected from patients undergoing cancer surgery at baseline (BL; before surgery), 24h after surgery (post-operative day 1; POD1) and 3 days after surgery (POD3). Blood samples were stabilized in PROT1, and different myeloid populations were identified using a comprehensive, multicolor flow cytometry panel.

As previously outlined, the suppressive myeloid cells that emerge following surgical stress are defined as $CD33^{+}$ and HLA-DR^{low}, a marker of immaturity, and are termed SxMDSCs. These cells are further separated into monocytic (M-MDSC) and granulocytic/polymorphonuclear MDSCs (PMN-MDSC) SxMDSCs.

In the M-MDSC ($CD33^{+}CD11b^{+}CD14^{+}CD15^{-}Lin^{-}$HLA-$DR^{low}$) population, a 2-fold increase was observed on POD1 as a percentage of both all $CD45^{+}$ cells and a 1.9-fold increase as a percentage of $CD14^{+}$ monocytic cells (Figure 10A). When comparing BL levels to POD3, 1.3-fold and 1.6-fold changes were seen in MDSC frequencies as a percentage of all white blood cells or monocytes respectively The frequency of M-MDSCs returned to BL levels in 85% of patients by POD3 (6 out of 7 patients for which this last time point was collected).

The frequencies of PMN-MDSCs ($CD33^{+}CD11b^{+}CD14^{-}CD15^{+}CD16^{low}Lin^{-}$HLA-$DR^{low}$) measured a 1.3-fold increase on POD1 but this did not reach significance (Figure 10B).

The proportion of $CD33^{+}CD11b^{+}CD14^{-}CD15^{-}Lin^{-}HLA\text{-}DR^{low}$ cells described in the literature as "early" or e-MDSCs was significantly reduced (0.21-fold) on POD1 and on POD3 as a percentage of all CD45+ cells (Figure 10C).

Two distinct populations of M-MDSCs were observed with this panel. The first expressed high levels of CD11b (described and quantified in Figure 10A), and the second expressed lower CD11b, suggesting a more immature phenotype[201]. When gating on the $CD14^{+}HLA\text{-}DR^{low}$ portion of this population, a significant 0.8-fold decrease was noted in the frequency of these cells (expressed as a percentage of CD45+ cells) on POD1 and a non-significant 0.6-fold decrease on POD3 (Figure 10D).

CD16 is a marker commonly used to describe mature neutrophils, as such, most immature PMN-MDSCs subsets do not express it.[126,165,203,219] For this reason, PMN-MDSCs were identified as $CD16^{low}$, although this strategy may gate out any PMN-MDSC subsets that do express this marker. The neutrophil to lymphocyte ratio (NLR) was evaluated as it also holds an important prognostic role in literature, as it is correlated with poor outcomes and prognosis.[126,127,203,353,354] NLR is also negatively correlated with some MDSC subsets in cancer.[355] A very significant 3-fold increase in NLR was observed on POD1 (Figure 10E), and a subsequent 0.04-fold decrease on POD3.

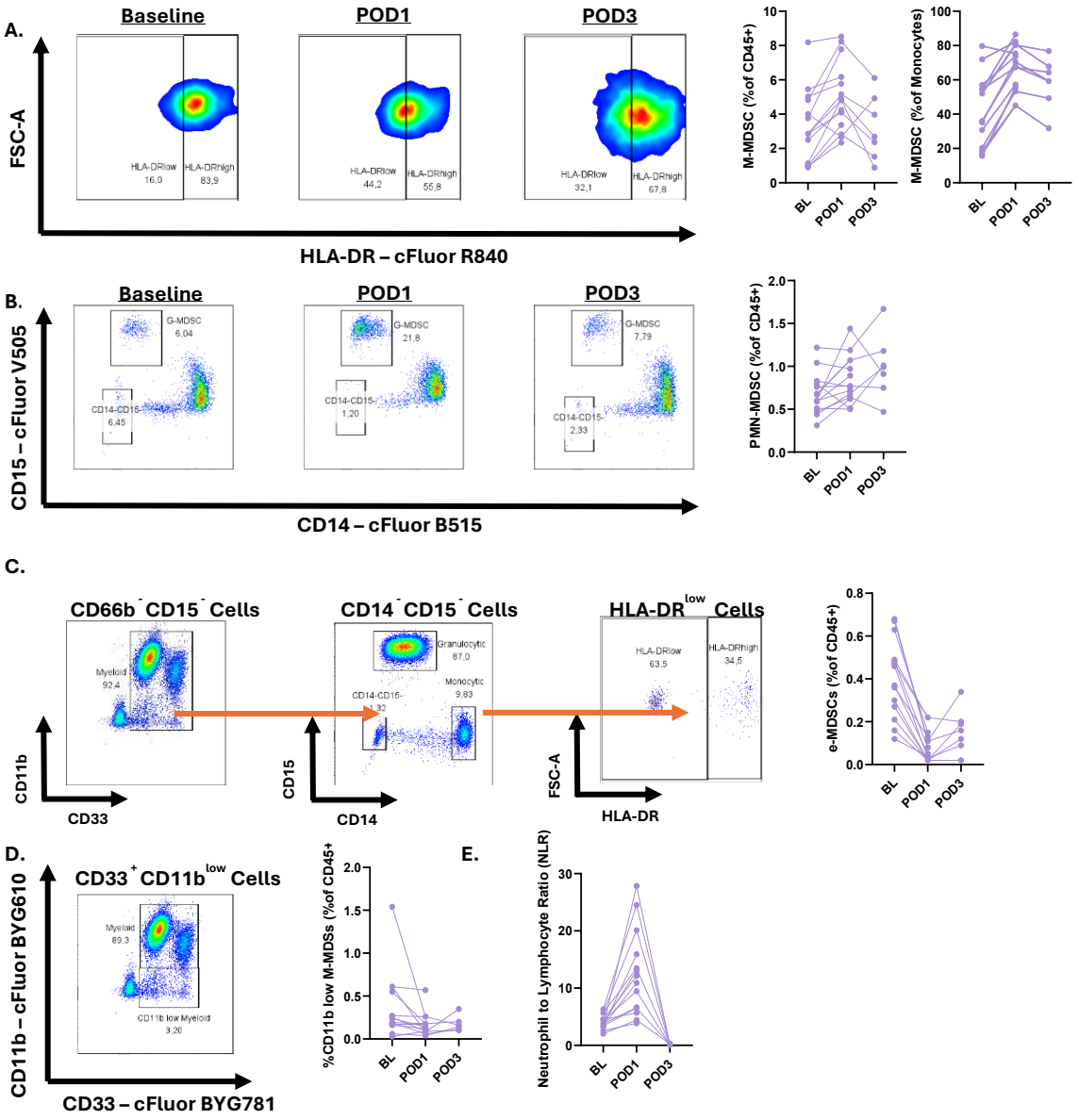

Figure 10: MDSC subset populations frequencies are altered in the post-operative landscape. Blood was collected from patients pre-operatively (BL), 24h post-op (POD1) and 3 days post-op (POD3). Blood was stabilized with PROT1 before thawing and staining with a harmonized multicolour flow cytometry panel for human MDSCs in whole blood. **A)** Frequency of M-MDSCs ($CD33^{+}CD11b^{+}CD14^{+}CD15^{-}Lin^{-}HLA\text{-}DR^{low}$ cells) for BL (n=14), POD1 (n=14) and POD3 (n=7) patient samples as a percentage of CD45+ cells and monocytic cells along with representative flow plots. Paired Wilcoxon test (%$CD45^{+}$ BL vs POD1 P=0.001 and BL vs POD3 P=0.38; %$CD14^{+}$ BL vs POD1 P=0.0002 and BL vs POD3 P=0.02). **B)** Proportion of PMN-MDSCs ($CD33^{+}CD11b^{+}CD14^{-}CD15^{+}Lin^{-}CD16^{low}$) for BL (n=14), POD1 (n=14) and POD3 (n=7) patient samples along with representative flow plots. Paired Wilcoxon test (BL vs POD1 P=0.06 and BL vs POD3 P=0.06). **C)** Proportion of e-MDSCs ($CD33^{+}CD11b^{+}CD14^{-}CD15^{-}Lin^{-}HLA\text{-}DR^{low}$ cells) for BL (n=14), POD1 (n=14) and POD3 (n=7) patient samples along with representative flow plots showing gating strategy. Paired Wilcoxon test (BL vs POD1

P=0.0001 and BL vs POD3 P=0.02). **D)** Proportion of $CD11b^{low}$ M-MDSCs ($CD33^{+}CD11b^{low}CD14^{+}CD15^{-}Lin^{-}HLA\text{-}DR^{low}$) for BL (n=14), POD1 (n=14) and POD3 (n=7) patient samples along with representative flow plots. Paired Wilcoxon test (BL vs POD1 P=0.007 and BL vs POD3 P=0.09). **E)** NLR (Neutrophil to lymphocyte ratio) for BL (n=14), POD1 (n=14) and POD3 (n=7) patient samples. Paired Wilcoxon test (BL vs POD1 P=0.0001 and BL vs POD3 P=0.02).

4.4 Investigating post-operative changes in SxMDSC phenotype using PROT1 stabilized blood

As previously demonstrated in this thesis and other research studies from our group, M-MDSCs are the most significantly increased post-operatively among all the MDSC subsets studied.[313,352] Data from our group have also shown surgery induced M-MDSCs are more suppressive than their PMN-MDSC counterparts.[352] Furthermore, prior results (Figure 8D) indicate PMN-MDSC data may not be reliable when studied in PROT1 stabilized whole blood. For these reasons, we focused our further investigations on the M-MDSC subset. Using PROT1 stabilized blood, the MFI of key MDSC markers was evaluated at BL and POD1 for either M-MDSC or PMN-MDSC subsets. These markers have been highlighted in the literature for their roles in MDSC suppressive capacity, MDSC accumulation, and changes in expression on monocytes due to surgical stress. A summary of these results can be seen in Table 4.

Among markers heavily altered in expression on MDSCs in the post-operative landscape was the scavenger receptor CD163. CD163 is a scavenger receptor associated with an inflammatory pro-tumorigenic M2 phenotype in macrophages and is known to be overexpressed on M-MDSCs.[214,356–363] It is also known to increase post-op in patients undergoing surgery or suffering from trauma.[364–366] CD163 MFI significantly increased on M-MDSCs on POD1 by an average of 5.7-fold and returned to BL levels by POD3 in all patients (Figure 11A).

Another marker whose expression (MFI) increased significantly on POD1 was the interleukin 4 receptor CD124. CD124 MFI increased significantly on M-MDSCs by an average of 1.2-fold on

POD1. An increase of 1.3-fold was noted on POD3 (Figure 11B). MDSC expressing CD124 are a subset observed in various studies and tend to have higher immunosuppressive capacities.[165,203]

Chemokines such as CCR2, CXCR1, and CXCR2 are involved in MDSC recruitment, trafficking, and promotion of pre-metastatic niches.[165,187] They are also known to be increased in the post-operative period.[105,367,368] The MFI for both chemokine receptors CCR2 and CXCR2 (Figure 11C) significantly increased on M-MDSCs on POD1, both by an average of 1.9-fold. Interestingly, CCR2 expression dropped back to BL levels on POD3 for all patients; however, CXCR2 expression was only recovered in 4 out of 7 patients and continued to increase for the others. Contrastingly, chemokine receptor CXCR1 only increased 1.5-fold on POD1 and 1.4-fold on POD3, these changes did not reach significance (Figure 11D).

CD74 is the primary macrophage inhibitory factor (MIF) receptor in M-MDSCs and is essential to their generation and penetration of the tumor-microenvironment in some cancer types.[369–371] CD74 was significantly decreased on POD1 by an average of 0.8-fold and by 0.9-fold on POD3 (Figure 11E).

Surgical trauma has been shown to induce upregulation of CD206 on the surface of TAMs and is associated with an immunosuppressive phenotype.[68] It is also expressed on MDSCs and promotes an M2 pro-tumorigenic phenotype.[372] Comparison of BL MFI on M-MDSCs to POD1 and POD3 levels revealed non-significant 1.3-fold and 1.1-fold changes (Figure 11F).

MDSCs can suppress CMI through the CD155/TIGIT axis, and its expression on MDSCs is correlated with worse clinical outcomes in multiple cancers.[373,374] A significant 1.1-fold increase in the MFI of CD155 was seen on POD1 M-MDSCs (Figure 11G). This is consistent with previous reports from our lab.[352,375] The fold-change from BL to POD3 overall was higher,

reaching 1.3. This is conducive to the fact that CD155 stayed elevated or continued to increase on POD3 for all patients.

CD54 is an intracellular adhesion molecule (intracellular adhesion molecule 1/ICAM1).[376] It is associated with calcium-based immunosuppression of MDSCs.[377] CD54 MFI on M-MDSCs remained consistent from BL to POD1 (1-fold change). CD54 did significantly increase by POD3, reaching a 1.2-fold difference (Figure 11H).

CD84 is upregulated on MDSCs and is correlated with immunosuppressive capacity.[378] 1.1-fold and 1.3-fold increases of CD84 MFI on M-MDSCs were noted on POD1 and POD3 respectively, though this did not reach significance (Figure 11I).

The adhesion molecule L-selectin (CD62L) is a known activation marker and has been investigated as a reliable PMN-MDSC identifier (due to their low expression compared to neutrophils).[379,380] Notably, CD62L has been seen to increase in expression on monocytes following colorectal cancer surgery.[381] Minimal increases of 1.1-fold were seen for CD62L on M-MDSCs from BL to POD1 and POD3, this did not reach significance (Figure 11J).

Summary of immunophenotyping results can be seen in Figure 12. Due to multiple queries being applied to this data set, a Bonferonni correction was implemented. This statistical adjustment helps ensure that the significance of the results is not overstated. After applying this correction, the increases of CD163, CCR2 and CXCR2 from BL to POD1 remained significant. This indicates that the changes in these markers are relevant meaningful and warrant further investigation.

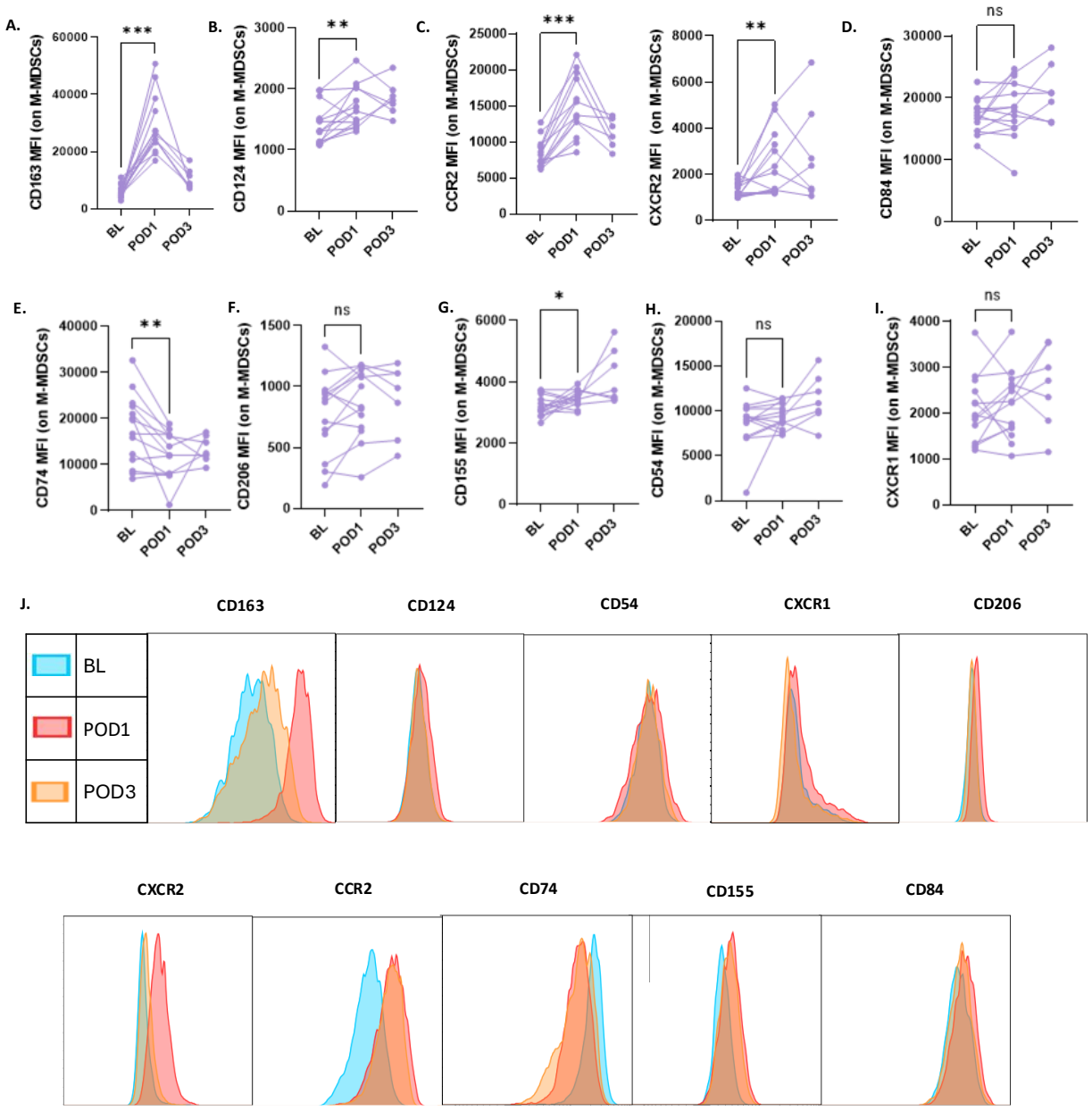

Figure 11: M-MDSC phenotype is greatly altered with surgical stress. A) CD163 expression on patient M-MDSCs at BL (n=14), POD1 (n=14) and POD3 (n=7). Paired Wilcoxon test (BL vs. POD1 P=0.0001 and BL vs. POD3 P=0.11) **B)** CD124 expression on patient M-MDSCs at BL (n=14), POD1 (n=14) and POD3 (n=7). Paired Wilcoxon test (BL vs. POD1 P=0.005 and BL vs. POD3 P=0.11). **C)** CCR2 and CXCR2 expression on patient M-MDSCs at BL (n=14), POD1 (n=14) and POD3 (n=7). Paired Wilcoxon test (CCR2 BL vs. POD1 P=0.0001 and BL vs. POD3 P=0.08; CXCR2 BL vs POD1 P=0.004 and BL vs POD3 P=0.08). **D)** CXCR1 expression on patient M-MDSCs at BL (n=14), POD1 (n=14) and POD3 (n=7). Paired Wilcoxon test (BL vs. POD1 P=0.5; BL vs POD3 P=0.17). **E)** CD74 expression on patient M-MDSCs at BL (n=14),

POD1 (n=14) and POD3 (n=7). Paired Wilcoxon test (BL vs. POD1 P=0.004; BL vs. POD3 P=0.11). **F)** CD206 expression on patient M-MDSCs at BL (n=14), POD1 (n=14) and POD3 (n=7). Paired Wilcoxon test (BL vs. POD1 P=0.12 and BL vs. POD3 P=0.58). **G)** CD155 expression on patient M-MDSCs at BL (n=14), POD1 (n=14) and POD3 (n=7). Paired Wilcoxon test (BL vs. POD1 P=0.03 and BL vs. POD3 P=0.03). **H**) CD54 expression on patient M-MDSCs at BL (n=14), POD1 (n=14) and POD3 (n=7). Paired Wilcoxon test (BL vs. POD1 P=0.22 and BL vs. POD3 P=0.02). **I)** CD84 expression on patient M-MDSCs at BL (n=14), POD1 (n=14) and POD3 (n=7). Paired Wilcoxon test (BL vs. POD1 P=0.36 and BL vs. POD3 P=0.30). **J)** CD62L expression on patient M-MDSCs at BL (n=14), POD1 (n=14) and POD3 (n=7). Paired Wilcoxon test (BL vs. POD1 P=0.15 and BL vs. POD3 P=0.38). **K)** Representative histograms illustrating MFI change from BL (blue) to POD1(red) and POD3 (orange) (n=1).

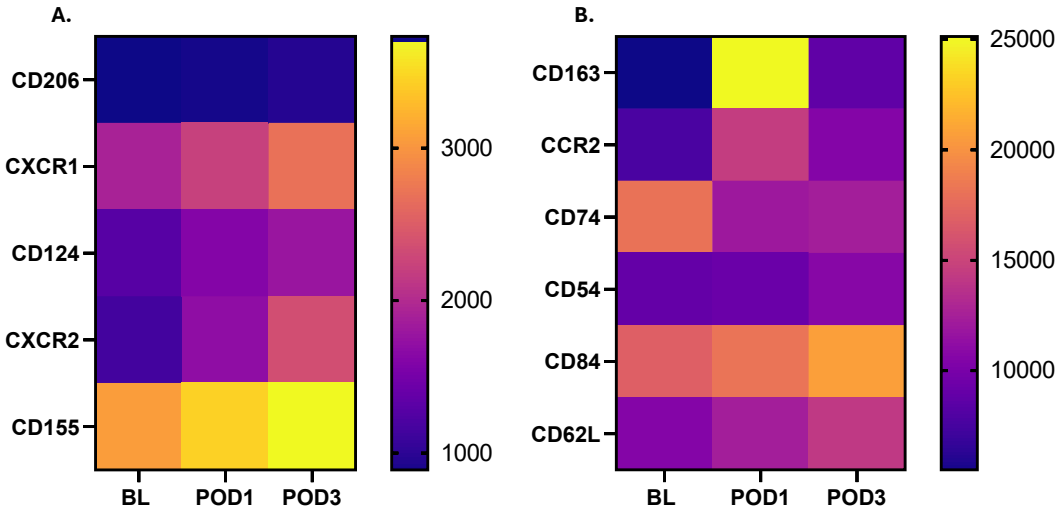

Figure 12: Heatmap illustrating the changes in MFI for various markers on M-MDSCs in PROT1 stabilized blood at BL, POD1, and POD3. A) Heatmap of lowly expressed markers (average MFI per time point below 5000) **B)** Heatmap of highly expressed markers (average MFI per time point above 5000)

4.5 Investigating SxMDSC phenotype in response to LPS stimulation using fresh whole blood

Several additional MDSC markers were considered for SxMDSC immunophenotyping, but panel optimization and preliminary experiments revealed some challenges. Fixation with PROT1 is known to affect the binding of certain markers or impact antibody staining patterns; some markers exhibited this. Additionally, certain markers associated with MDSCs are expressed at low levels, which presents challenges for their measurement without prior stimulation of the cells. These markers also could not be evaluated in the PROT1 panel since the stabilizer pre-

fixes the cells. These were then evaluated in a separate experiment on MDSC populations at BL and POD1 in fresh whole blood or fresh whole blood stimulated for 24h with LPS.

MDSCs have been identified to engage in crosstalk that promotes Tregs through the CD40/CD40L pathway.[18,208,382] Notably, on POD1, a decrease in MFI for CD40 on M-MDSCs was observed in fresh whole blood. In this analysis, 2 out of 3 patients showed CD40 levels that dropped below detectable limits (Figure 13A). However, a 1.2-fold increase in CD40 expression was noted when whole blood was stimulated for 24h with LPS.

We also aimed to measure the MFI of PD-L1, a ligand through which MDSCs can impair NK cell cytotoxicity, in the postoperative period.[13,18,288,383,384] In fresh unstimulated whole blood, PD-L1 MFI was below detectable limits, making analysis inconclusive. Upon 24h stimulation of fresh whole blood with LPS, a 0.4-fold decrease in MFI was seen when comparing BL and POD1 (Figure 13B).

TREM1 (myeloid triggering receptor) expression was also investigated due to its association with inflammation, disease progression, and MDSC function.[385] TREM-1 increase in expression on monocytes has also been discerned following surgery and correlated with post-operative complications.[386,387] No fold-change in MFI was seen on M-MDSCs in fresh whole blood. A small 1.1-fold increase was seen when whole blood was stimulated with LPS (Figure 13C). We also looked at TREM-1 expression on monocytes overall in both fresh unstimulated and LPS stimulated blood and noted no fold-change (Data not shown).

CD38 expression on MDSCs is linked to tumor growth in many models, as such it was also investigated in the context of SxMDSCs.[388] A 0.9-fold decrease in CD38 MFI on M-MDSCs was

seen on POD1 when staining fresh whole blood (Figure 13D). Contrastingly, a 2.9-fold increase was seen when performing the same experiment on whole blood stimulated with LPS.

Toll-like receptor-4 ligand (TLR4L) expression was also explored as it has been shown to promote MDSC inflammation-driven suppressive activity, and toll-like receptor pathways are heavily involved in MDSC functions.[208,209,389] A significant 1.6-fold increase in TLR4 expression was measured on M-MDSCs on POD1 in whole blood stimulated for 24h with LPS (Figure 13E).

We also studied the inhibitory receptor CD47. CD47 has been shown to suppress NK cell cytokines secretion via the JAK/STAT3 pathway.[390,391] Its expression on MDSCs is linked to inhibition of differentiation in MDSCs.[392,393] CD47 expression on M-MDSCs in LPS stimulated blood at BL and POD1 was also evaluated, a 0.9-fold decrease was noted (Figure 13F).

Changes in MFI for CD73 was investigated due to its role in the MDSC adenosine-based suppression mechanism.[13,394] Similarly to PD-L1, the expression of CD73 was below detectable levels in fresh whole blood. Unlike PD-L1, this remained the case after a 24h LPS stimulation rendering interpretation impossible (Data not shown)

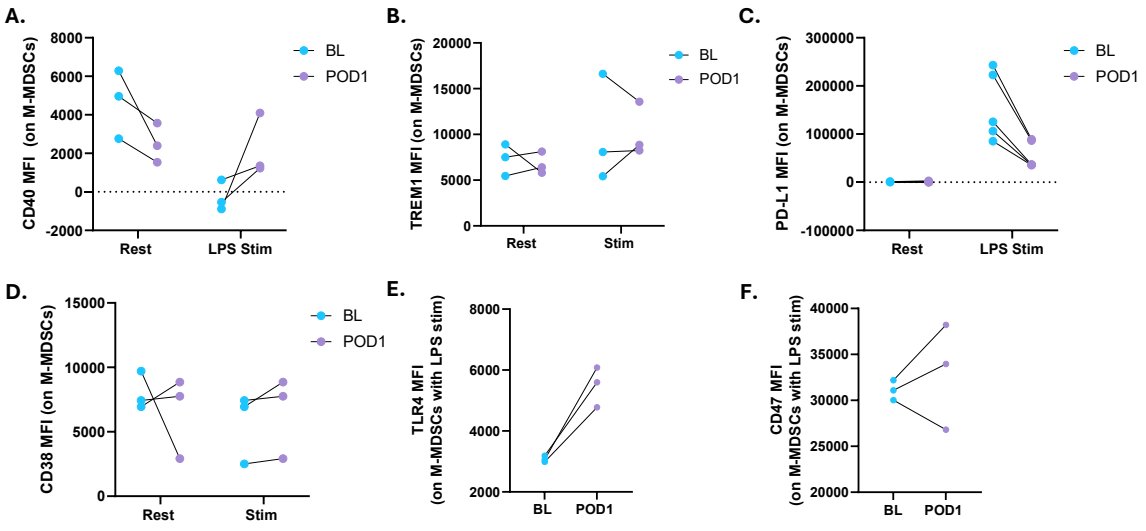

Figure 13: Postoperative alterations in MDSC phenotype and responsiveness to LPS stimulation in fresh whole blood. **A)** CD40 expression on M-MDSCs in fresh LPS stimulated and unstimulated whole blood at BL and POD1 (n=3). **B)** PD-L1 expression on M-MDSCs in fresh LPS stimulated and unstimulated whole blood at BL and POD1 (n=5). **C)** TREM1 expression on M-MDSCs in fresh LPS stimulated and unstimulated whole blood at BL and POD1 (n=3). **D)** CD38 expression on M-MDSCs in patient fresh LPS stimulated and unstimulated whole blood at BL and POD1 (n=3). **E)** TLR4 expression on M-MDSCs and in fresh stimulated blood at BL and POD1 (n=3).

Chapter 2: Analysis of Suppressive Mechanisms Utilized by Myeloid Derived Suppressor Cells in the Postoperative Period in Surgical Cancer Patients

4.6 NK cell cytotoxicity and NK cell cytokine secretion in cancer patients following tumor resection

It is well documented that surgical stress can inhibit NK cell function, affecting their cytotoxic capacity against tumor cell targets and their secretion of immunomodulatory cytokines. Prior to investigating the mechanisms and causes of this phenomenon, post-operative NK cell dysfunction was confirmed in this study cohort of Ottawa General Hospital cancer patients. To this end, blood was collected immediately prior to surgery (Baseline; BL), 24h following surgery (Postoperative Day 1; POD1) or 72h following surgery (Postoperative Day 3; POD3). A portion of these samples was incubated with a proprietary NK stimulating cocktail (NKVue) for 24h

after which the levels of IFNγ secreted by the NK cells were measured via an ELISA to assess NK cell IFNγ production. NK cells were also isolated from these samples using a negative selection of $CD56^+$ cells with magnetic bead separation. These NK cells were cultured with nano-luciferase transfected K562 target cells and target cell lysis was measured using luminescence to assess cytotoxicity. Consistent with previous reports, NK cells isolated from patients at POD1 exhibited significantly reduced cytotoxicity compared to those isolated before surgery, showing an average 50% reduction (Figure 14A). Likewise, NK cells from patients at POD1 secreted considerably less IFNγ than those at BL, with an average reduction of 116% (Figure 14B). NK cell cytotoxicity was recovered by POD3 in 4 out of 7 patients while NK cell IFNγ secretion remained low in the entire cohort and was not recovered by the POD3 timepoint.

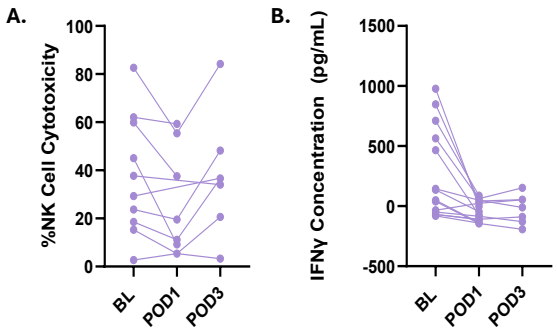

Figure 14: NK cell cytotoxicity and NK cell cytokine secretion are suppressed in patients following tumor resection. A) Patient NK cell cytotoxicity when isolated and cultured at BL (n=10), POD1 (n=8), POD3 (n=7) with K562 targets for 4.5h. Paired Wilcoxon test (BL vs PROT1 P=0.02 and BL vs POD3 P=0.08). **B)** Plasma concentration (pg/mL) of IFNγ of patient samples taken at BL (n=14), POD1 (n=14) and POD3 (n=7) when stimulated with a proprietary cytokine cocktail **(**NKVue). Paired Wilcoxon test (BL vs PROT1 P=0.0009 and BL vs POD3 P=0.08).

4.7 SxMDSC impact on NK cell cytotoxicity

Our group has previously confirmed NK cell cytotoxicity suppression by SxMDSCs through previous studies using direct co-cultures. The nature of this suppression mechanism remains to

be elucidated. We aimed to evaluate the impact of multiple co-culture factors (contact-dependency, time, dose) on the suppressive effects of SxMDSCs.

4.7.1 Impact of SxMDSC on NK cell cytotoxicity in direct co-culture

This suppressive ability of SxMDSC was confirmed using co-cultures varying in time length at a ratio of 4:2:1 (SxMDSC:NK cell:Target K562 cell). Consistent with previous reports, NK cells consistently showed reduced cytotoxicity when cultured with SxMDSCs for 24h compared to controls (Figure 15A). An average suppression of 15% was noted across BL samples, while a higher suppression averaging 19% was seen across both POD1 and POD3 samples. Contrastingly, suppression of NK cytotoxicity averaged below zero when cultured with healthy donor MDSCs. As seen in previous findings from our group, the degree of suppression observed was dose-dependent (Figure 15B). To determine whether these suppressive effects could be observed earlier or over a longer duration, additional experiments were conducted with varying incubation times. It was observed that SxMDSCs caused a 43% suppression of NK cell cytotoxicity *in vitro* as early as 3 hours into the co-culture. Furthermore, when NK cells were cultured with SxMDSCs for up to 48 hours, there was a 17% reduction in their cytotoxicity (Figure 15C).

Controls were run alongside each of the previously described co-cultures to confirm the killing of targets results from NK cytotoxicity alone. In these, SxMDSCs were cultured with K562 target cells without NK cells present to ensure SxMDSCs were not killing any of the targets. We consistently noted less than 1% cell death in these wells (Figure 15D), the values being consistent with those seen in wells containing tumor targets alone (spontaneous lysis). As further confirmation that the suppression of NK cells observed was a phenomenon unique to the MDSC cells, we included control wells that evaluated the suppressive effects of all CD33- PBMCs on

NK cell cytotoxicity. An average suppression of less than 10% was noted for these controls (Figure 15E).

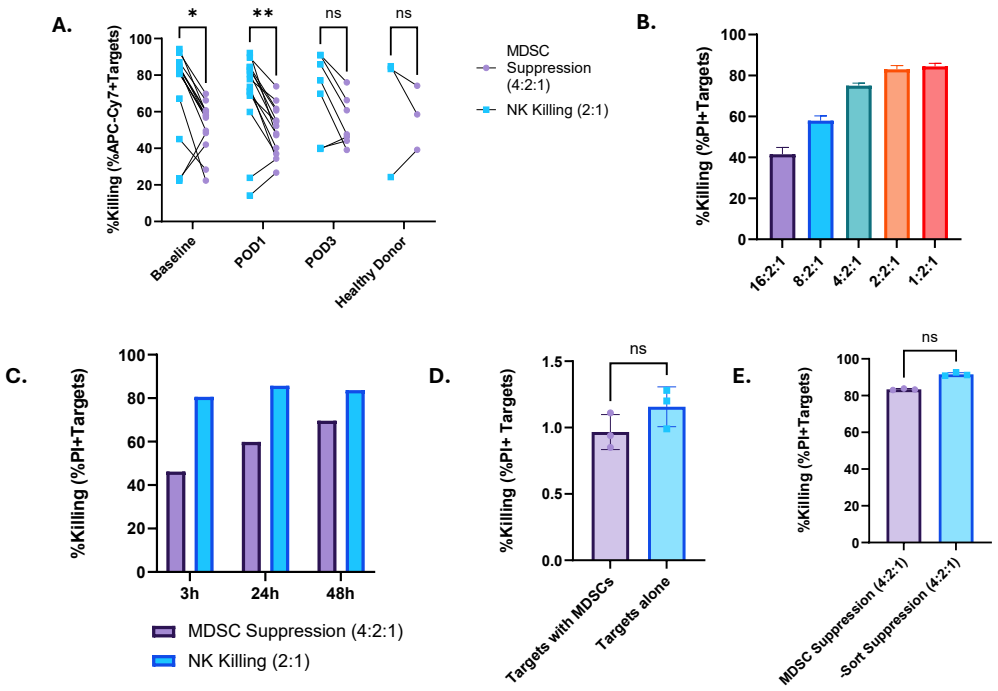

Figure 15: SxMDSCs suppress NK cell cytotoxicity in vitro. A) NK92 cell cytotoxicity when cultured with SxMDSCs isolated at BL (n=14), POD1 (n=14), POD3 (n=7) or from healthy donors (n=3) for 24h at a 4:2:1 SxMDSC to NK cell to target cell ratio. Paired Wilcoxon test (BL with vs without MDSCs P=0.008; POD1 with vs without MDSCs P=0.0009; POD1 with vs without MDSCS P=0.08) **B)** NK92 cell cytotoxicity when cultured with various quantities of SxMDSCs for 24h (n=1). **C)** NK92 cell cytotoxicity when cultured with SxMDSCs for various time points (n=1). **D)** K562 cell viability when cultured with SxMDSCs for 24h (n=3). **E)** K562 viability when cultured with CD33- POD1 PBMCs for 24h (n=3).

4.7.2 Impact of SxMDSC on NK cell cytotoxicity with indirect co-culture

An indirect co-culture with NK cells was employed to investigate whether the suppressive effects of SxMDSCs were attributable to secreted soluble factors. SxMDSCs were isolated as previously described and cultured for either 24h or 48h before supernatant was collected. NK cells were plated with the supernatant from these cell cultures for 20h before targets were added and their cytotoxic capacity was assessed by flow cytometry.

Representative flow plots illustrate the similarities in target cell death frequencies with or without MDSC supernatant (Figure 16A). Unlike the degree of suppression observed in direct co-culture, the degree of NK cell cytotoxicity suppression when cultured with SxMDSC supernatant failed to reach even 10% suppression in any of the replicates (Figure 16B).

To determine if the lack of effect was due to a reduced concentration of suppressive factors, we concentrated the SxMDSC supernatant 2X using centrifuge filters. The concentrated supernatant performed similarly to the non-concentrated: on average, the degree of suppression was below 10% (Figure 16C).

Furthermore, the lifespan of soluble secreted factors was considered a factor in the immunosuppressive capacity of SxMDSC supernatant. To ensure that the soluble factors secreted by SxMDSC did not degrade or lose their suppressive ability over time during the co-culture, we performed a time-series in which NK92 cells were cultured with SxMDSC supernatant collected at various time points. A nano-luciferase based killing assay was performed to determine NK cell lysis of targets. The results were consistent with previous supernatant co-cultures whether the SxMDSC supernatant was collected at 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, or 48 hours (Figure 16D). A 43% suppression of cytotoxicity was observed when MDSCs were directly cultured with NK cells; however, when cultured with the supernatant from these same cells at any of the previously mentioned time-points, it suppressed at most 10% of NK cytotoxicity.

Finally, we considered whether SxMDSCs needed to be in the presence of or in contact with NK cells to produce suppressive factors. To this end, we co-cultured SxMDSCs with healthy NK cells for 24h before collecting supernatant. This supernatant was plated on different healthy NK cells for a 24h incubation. The impact of SxMDSC supernatant on NK cell *in vitro* cytotoxicity appeared consistent with previous results even when co-cultured with NK cells. We noted a

similar proportion of target cell death between supernatant wells and control wells (Figure 16E) with an average drop in cytotoxicity of less than 10% overall when NK cells were cultured with the supernatant (Figure 16F).

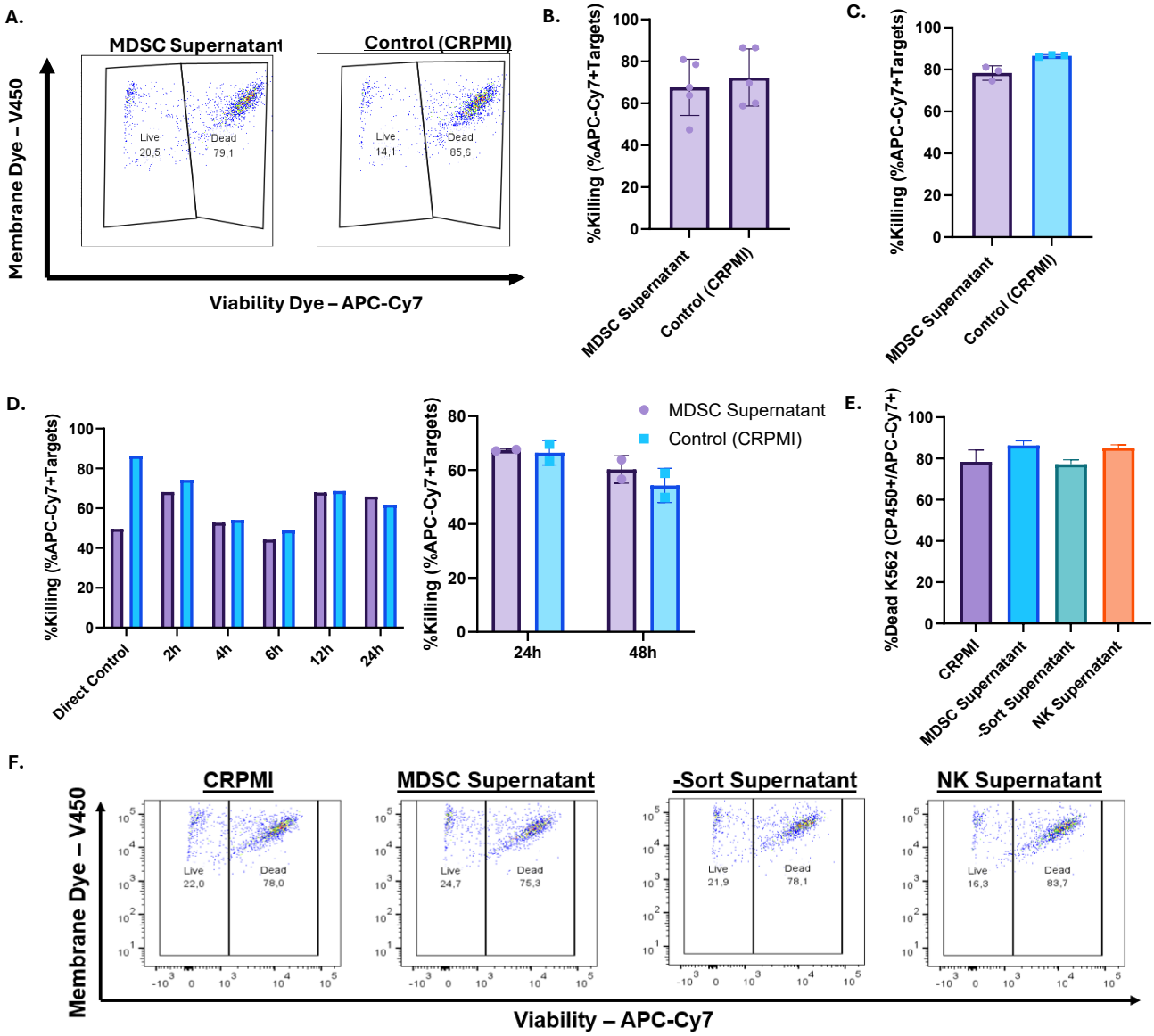

Figure 16: SxMDSC supernatant does not impair NK cytotoxicity in vitro. A) Representative flow plots illustrating the proportions of killed targets for NK92 cells cultured with SxMDSC supernatant (left) or CRPMI (right). **B)** NK92 cell cytotoxicity when cultured with supernatant collected from SxMDSCs for 24h (n=5). **C)** NK92 cell cytotoxicity when cultured with 2X concentrated supernatant collected from SxMDSCs for 24h (n=1). **D)** NK92 cell cytotoxicity when cultured for 3h with supernatant collected from SxMDSCs cultured for various time lengths (n=1). **E)** NK92 cell cytotoxicity when cultured with supernatant collected from SxMDSC/NK cell co-culture for 24h (n=1). **F)** Representative flow plots illustrating the

proportion of killed targets for NK92 cells cultured with CRPMI, SxMDSC/NK supernatant, non-MDSC POD1 PBMCs or NK supernatant.

4.7.3 Effect of post-operative patient plasma on NK cytotoxicity

Previous reports from our group have noted the suppressive effect of patient POD1 plasma on NK cell activity (IFNγ secretion).[24] We inquired whether soluble factors in patient plasma could also suppress cytotoxicity. To this end, plasma was collected from surgical cancer patients at BL (n=14), POD1 (n=14) and POD3 (n=7), as well as healthy donors (n=4) and concentrated 2X using centrifuge filters. NK92 killing of target cancer cells ranged from 48-55% whether they were cultured with patient plasma at any indicated time points, healthy donor plasma or CRPMI (Figure 17A). There was a significant 0.9-fold decrease in cytotoxicity from BL to POD1 and a non-significant 1.2-fold increase from BL to POD3. Though the increase on POD1 was significant it was low in magnitude. An average degree of suppression of less than 1% was observed when NK cells were cultured with BL plasma compared to CRPMI controls, reaching 13% and 14%, respectively, for POD1 and POD3 plasma (Figure 17B). Healthy donor plasma induced a 10% suppression in NK cell cytotoxicity when compared to CRPMI.

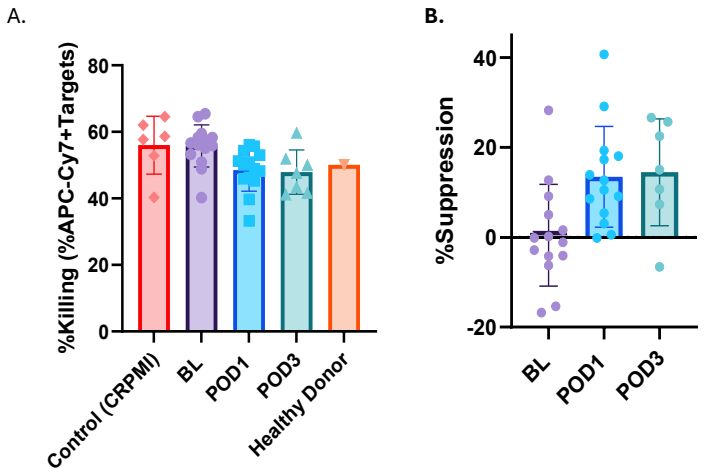

Figure 17: POD1 plasma does not suppress NK cell cytotoxicity in vitro. A) NK92 cell cytotoxicity when cultured with patient plasma collected at BL (n=14), POD1 (n=14), POD3 (n-7), healthy donor (n=4) or CRPMI for 24h. Paired Wilcoxon (BL vs POD1 P=0.0001 and BL vs

POD3 P=0.08) B) %Suppression of NK92 cell cytotoxicity in wells containing patient plasma collected at BL (n=14), POD1 (n=14), and POD3 (n-7) compared to CRPMI.

4.8 SxMDSC impact on NK cell cytokine secretion

Multiple studies from our lab (including this study) have confirmed the impact of SxMDSCs on NK cytotoxicity.[4,62,313] Additional reports from our lab also established how NK cell IFNγ secretion is profoundly suppressed post-op.[29] A link has yet to be established between the suppression of NK cell IFNγ post-op and SxMDSC.

3.2.1 Impact of SxMDSC on NK cell cytokine secretion with direct co-culture

We sought to explore the capacity of SxMDSCs to suppress NK cell activity by measuring IFNγ in NK cells in response to IL-2/12 stimulation following co-culture with SxMDSCs.

NK cells and MDSCs were co-cultured as previously described, and after a 24h co-culture, an IL-2/12 stim was added. Representative flow plots illustrate similar proportions in $IFN\gamma^+$ NK cells in response to IL-2/12 stimulation when cultured with or without SxMDSCs (Figure 18A). Data from this study showed on average a small 5% decrease in $IFN\gamma^+$ NK cells in response to stimulation when NK cells are cultured with SxMDSCs compared to control wells (Figure 18B). We also examined the effect of SxMDSCs on the production of cytotoxic granules by NK cells. The MFI levels of granzyme B were similar when comparing the SxMDSC wells to the control wells, with averages of 25334 and 22274, respectively (Figure 18C). Similarly, the MFI levels of perforin were closely aligned between the SxMDSC and control wells, at 11409 and 11253, respectively.

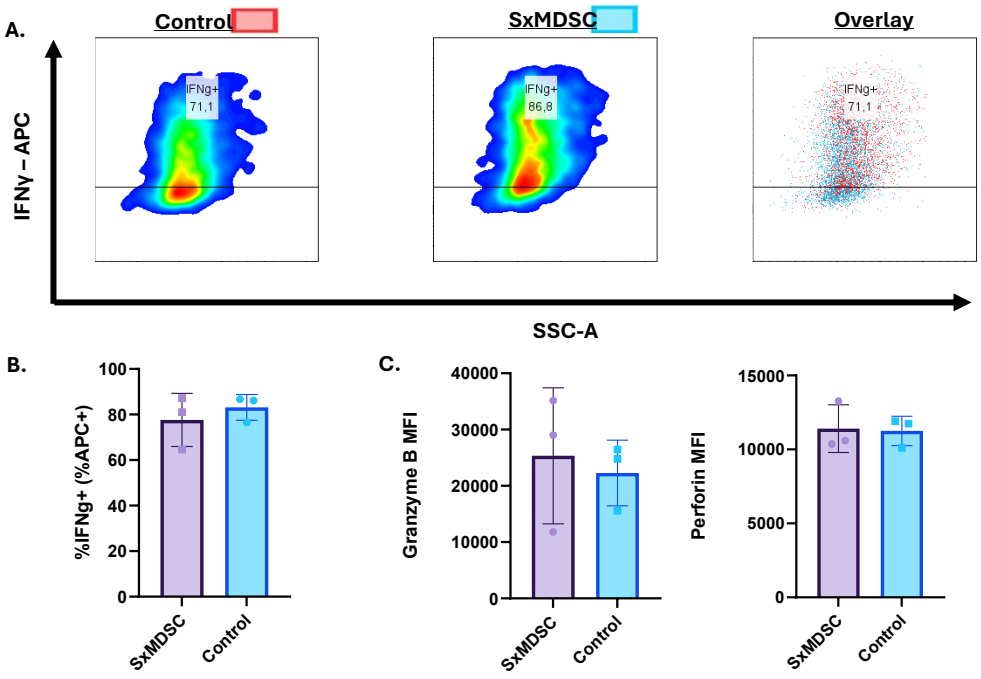

Figure 18: SxMDSCs do not suppress NK cell activity in vitro. A) Representative flow plots illustrating the proportion of IFNγ positive NK cells ($CD3^{-}CD14^{-}CD56^{+}$) cultured with SxMDSCs (right, blue) or CRPMI (left, red). **B)** IFNγ secretion by healthy donor NK cells in response to IL-2/12 stimulation when cultured with SxMDSCs (purple) or CRPMI (blue) (n=3). **C)** Granzyme B and perforin (n=3) secretion by healthy donor NK cells in response to IL-2/12 stimulation when cultured with SxMDSCs (purple) or CRPMI (blue).

3.2.2 The effect of SxMDSC on the cytokine secretion of NK cells in indirect co-culture

We also investigated the capacity of SxMDSC supernatant to impact NK cell activity. SxMDSC supernatant was collected as previously described and plated on isolated healthy donor NK cells for 24 hours. Following this, NK cells were subjected to a 24h stimulation with IL-2/12 and subsequently for flow cytometry analysis.

Results mirrored those seen with the direct SxMDSC co-culture. The overall production of IFNγ in response to IL-2/12 remained relatively unchanged, with an average 1% decrease in NK cell IFNγ secretion whether NK cells were cultured with or without SxMDSC supernatant. (Figure 19A). The MFI of cytotoxic granules was similar between conditions as well: granzyme B MFI averaged 10220 in SxMDSC wells compared to 9869 in control wells (Figure 19B). Similarly,

perforin MFI averaged 8124 in SxMDSC wells and 7733 in controls.To determine whether MDSCs needed to be in the presence of NK cells to secrete any soluble suppressive factors, SxMDSCs were co-cultured with either NK92 cells or isolated healthy donor NK cells for 24h before collecting the supernatant. There was a mere 3% drop in IFNγ production when NK cells were in the presence of SxMDSC supernatant. We did observe a 30% decrease in granzyme B MFI and a 22% increase in perforin MFI, though this is only in one replicate. (Figure 19C-D).

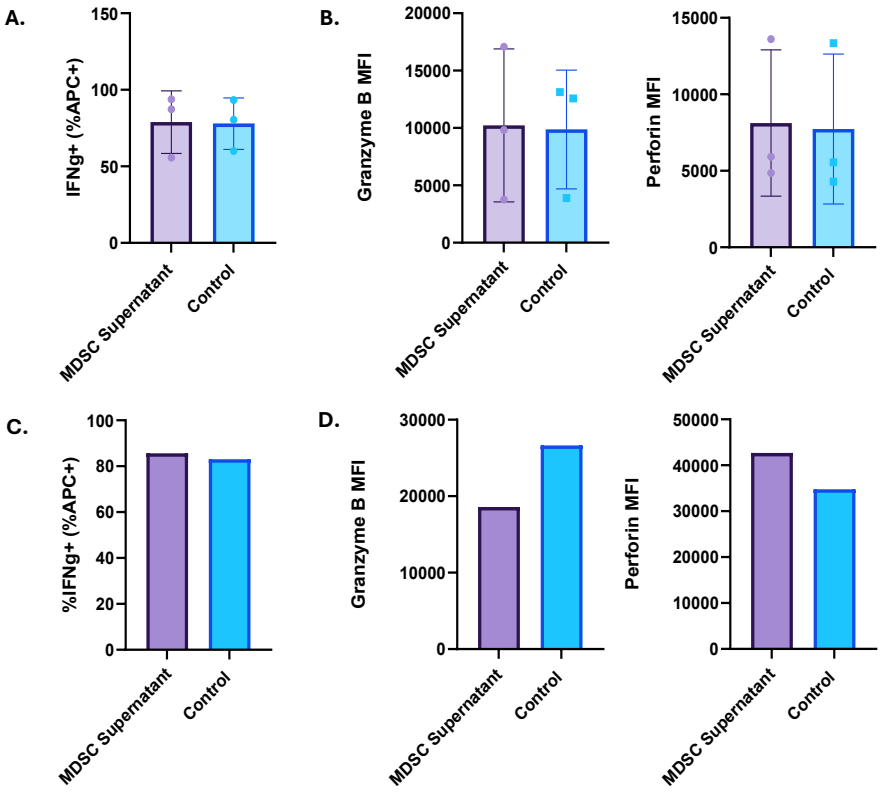

Figure 19: SxMDSC supernatant does not suppress NK cell activity in vitro. A) IFNγ secretion by healthy donor NK cells in response to IL-2/12 stimulation when cultured with SxMDSC supernatant (purple) or CRPMI (blue) (n=3) **B**) Granzyme B and perforin (n=3) secretion by healthy donor NK cells in response to IL-2/12 stimulation when cultured with SxMDSC supernatant (purple) or CRPMI (blue). **C)** IFNγ secretion by healthy donor NK cells in response to IL-2/12 stimulation when cultured with supernatant of SxMDSCs co-cultured with NK cells (purple) or CRPMI (blue) (n=1) **D)** Granzyme B and perforin (n=1) secretion by healthy donor NK cells in response to IL-2/12 stimulation when cultured with supernatant of SxMDSCs co-cultured with NK cells (purple) or CRPMI (blue).

3.2.3 Effect of post-operative patient plasma on NK cytokine secretion

It was established via studies by our group that plasma collected from surgical cancer patient at POD1 can reduce IFNγ secretion of healthy NK cells in response to IL-2/12 stimulation. To confirm this phenomenon with the 2X concentrated plasma obtained from patients in this study (the same plasma used for the cytotoxicity experiment in section 3.2.1.3), primary NK cells were isolated from healthy donors and plated with either CRPMI, healthy plasma or patient plasma collected at BL, POD1 or POD3. When culturing NK cells with BL plasma, we noted a 18% decrease in IFNγ production compared to CRPMI controls. Consistent with previous data from our lab, there was a significant 32% decrease in IFNγ secretion by NK cells when cultured with POD1 plasma (Figure 20A). IFNγ decreased on POD1 in 10 out of 13 patients. A 25% decrease was seen when comparing both POD3 plasma and healthy donor plasma to control wells. There was a significant difference when comparing BL to POD1 plasma suppression, but not when comparing BL and POD3 suppression. The MFI of granzyme B and perforin expressed by these cells remained consistent between conditions, with non-significant 1.5-fold and 1.1-fold increases (Figure 20B).

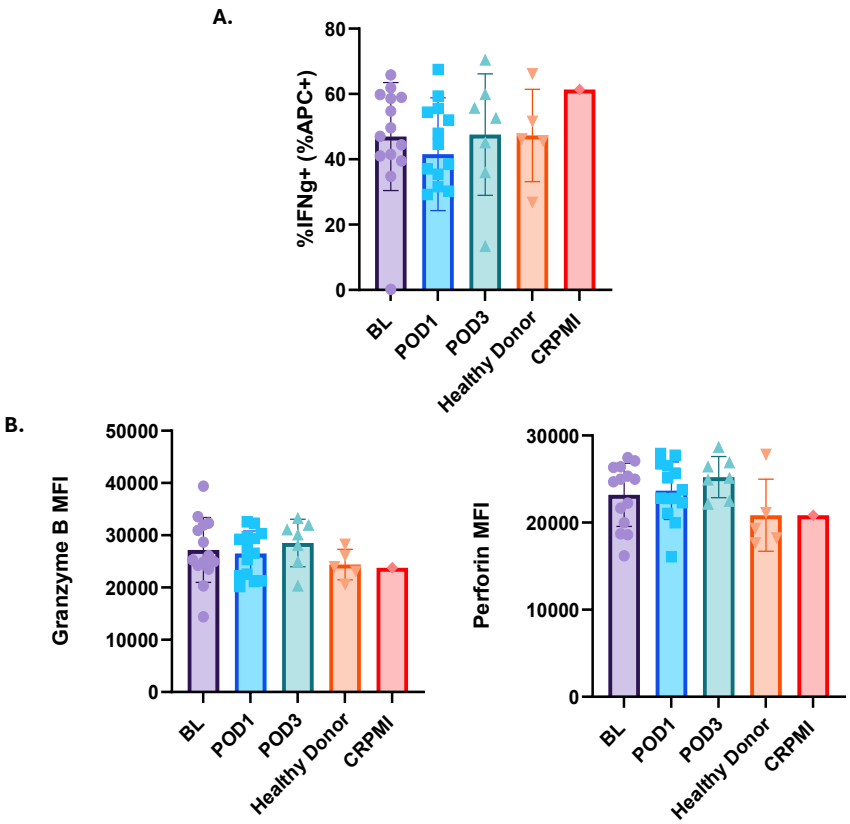

Figure 20. POD1 plasma suppresses NK cell activity in vitro. A) IFNγ secretion by healthy donor NK cells in response to IL-2/12 stimulation when cultured with patient plasma collected at BL (n=14), POD1 (n=14) and POD3 (n=7) plasma, healthy donor (n=5) plasma or CRPMI for 24h. Paired Wilcoxon (BL vs POD1 P=0.04 and BL vs POD3 P>0.99) **B)** Granzyme B and perforin secretion by healthy donor NK cells in response to IL-2/12 stimulation when cultured with patient plasma collected at BL (n=13), POD1 (n=13) and POD3 (n=7) plasma, healthy donor (n=5) plasma or CRPMI for 24h. Paired Wilcoxon (Granzyme B BL vs POD1 P >0.99 and BL vs POD3 P=0.69; Perforin BL vs POD1 P=0.50 and BL vs POD3 P=0.08).

Data from this study corroborated the suppressive effects of POD1 plasma on NK cell activity, indicating the presence of suppressive soluble factors in the plasma. Based on the results from the multiple supernatant experiments we ran, SxMDSC supernatant does not appear to suppress NK cell activity to the same degree. To confirm whether the suppressive factors present in the plasma are not attributable to SxMDSCs, we aimed to directly compare the suppressive ability of plasma to matched SxMDSC supernatant samples.

The suppressive ability of the SxMDSCs was first confirmed via a direct co-culture with NK92 cells. The SxMDSCs from these patients were all capable of suppressing NK cell cytotoxicity (Figure 21A). The plasma obtained from these patients suppressed NK cell activity to a similar degree to what was observed in previous plasma experiments (average 33% decrease, Figure 21B). When the SxMDSC supernatant from these same patients was cultured with healthy NK cells they failed to suppress their activity to the same degree as plasma (average 4.5% decrease, Figure 21C).

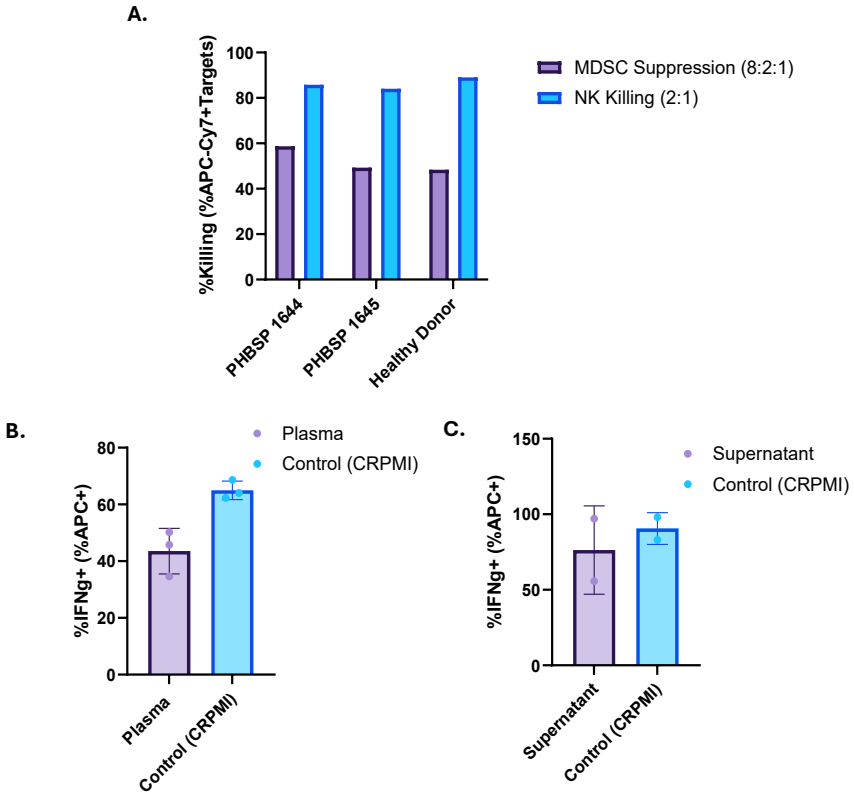

Figure 21: Direct comparison of POD1 plasma and SxMDSC suppression. A) NK92 cell cytotoxicity when cultured with SxMDSCs for 24h at a 4:2:1 SxMDSC to NK cell to target cell ratio. (n=2) **B)** IFNγ secretion by healthy donor NK cells in response to IL-2/12 stimulation when cultured with patient POD1 plasma (n=2) or CRPMI for 24h. **C)** IFNγ secretion by healthy donor NK cells in response to IL-2/12 stimulation when cultured with SxMDSC supernatant (n=2) or CRPMI for 24h.

5. DISCUSSION

5.1 PROT1 Validation for MDSC Phenotyping

Whole blood samples were used in lieu of PBMCs for immunophenotyping purposes since PBMCs are not representative of whole-blood populations *in vivo*. Furthermore, many research groups have discouraged the study of MDSCs in PBMC samples as gradient centrifugation has been shown to affect cell composition or even activate some cells.[350,395–397]

A major challenge in the clinical study of MDSCs is the cryopreservation of these cells. Cryopreserved samples are ideal for multicenter clinical studies because they guarantee the possibility of batch analyses. Stored samples decrease day to day variability that may come from staining, the instrument or sample handling.[165] Unfortunately, numerous studies have shown that MDSCs are highly sensitive to cryopreservation, resulting in significant yield loss and changes in marker expression.[340,398–402]

We opted to use stabilized whole blood (PROT1) to effectively address several challenges associated with analyzing frozen blood samples. This approach allows us to perform batch analyses while minimizing the significant yield loss that is often encountered when cryopreserving MDSCs. Freezing with PROT1 also eliminates the drawbacks associated with PBMC isolation and allows the study of granulocytic subsets excluded with density centrifugation.[348] PROT1 has been used in various immunophenotyping studies and has been shown to maintain the frequency and distribution of major leukocyte subsets.[348,403–409] Our data supports this: monocyte, lymphocyte and granulocyte ratios remained consistent when freezing whole blood after PROT1 preservation. At the same time, significant alterations in these population frequencies were seen when freezing matched blood samples in a mixture of FBS and DMSO (Figure 7). Additionally, alterations in FSC/SSC scatter were observed in PROT1

samples when compared to fresh: this is a phenomenon that has been noted throughout multiple studies using the product.[349,350] For instance, in the study by Rybakowska et al., monocytes showed decreased FSC and increased SSC, whereas granulocytes exhibited increased FSC and decreased SSC when PROT1 was compared to fresh whole blood samples.[349] It is important to note that while alterations in cell morphology affected the distribution of cell populations in an FSC/SSC plot, the frequencies of significant cell subsets remain unchanged unaltered. Through the validation of PROT1 for the study of MDSCs, it was found that freezing whole blood with this stabilizer maintained the frequency of M-MDSC subsets compared to FBS (Figure 8).Kotsakis noted in his study that M-MDSCs can be detected after cryopreservation but there is significant loss of PMN-MDSCs; however, both subsets lose their suppressive activity in functional assays after cryopreservation.[165,401] Consistent with previous reports, PMN-MDSCs had a higher scatter profile compared to M-MDSCs even with PROT1.[410]

We did note a significant reduction of PMN-MDSC frequency when using PROT1, confirming that this subset of MDSCs is more sensitive to cryopreservation than M-MDSCs.[398,399,401,402,411] Consequently, additional immunophenotyping studies focused on M-MDSCs. A 2014 study utilizing PROT1 reported a significant rise in M-MDSCs between 24 to 72 hours after surgery. Similarly, this study observed postoperative increases from baseline to POD1 in PROT1 stabilized blood, with the magnitude of increase remaining consistent between fresh and PROT1 samples (Figure 7D).

After confirming the consistency of MDSC major subset frequencies with PROT1, we sought to verify that the markers essential for M-MDSC immunophenotyping maintained optimal staining PROT1. Using a fixing/stabilizing agent like PROT1 has its limitations. Fixation with PROT1 can affect the signal or staining of specific cell markers, often resulting in a reduced stain

index.[340,348–350,395,412–414] For this reason, all MDSC markers were compared between fresh and PROT1 samples to ensure optimal staining. Some markers showed little to no signal when used with PROT1: many of these were then used on a smaller subset of patient to compare pre- and post-op expression on fresh whole blood in a separate experiment (Figure 13). Some markers such as CD14 and HLA-DR showed a reduction in stain index (Appendix A) when comparing fresh to PROT1, consistent with previous reports.[350] For these markers, a higher antibody concentration was used when staining with PROT1 to achieve comparable staining to fresh.

In summary, results from this PROT1 validation study for immunophenotyping MDSCs confirmed M-MDSC frequency is maintained after cryopreservation with PROT1, and major MDSC markers still maintain adequate binding for flow cytometry analysis of these samples. A significant drop in PMN-MDSCs was observed which may impact the reliability of PMN-MDSC studies performed using PROT1 samples. For this reason, future SxMDSC immunophenotyping experiments were only performed on the M-MDSC subset.

5.2 Flow cytometry identification of postoperative MDSC population

The plasticity of MDSCs make it difficult to characterize them under different conditions or assign specific functions to different subtypes/populations.[179] Some researchers have suggested that MDSCs should not be regarded as a unique cell population but rather just monocytes and neutrophils altered by immunosuppressive factors.[126,201] MDSCs can alter their phenotype as the disease progresses and are also capable of undergoing terminal differentiation. Not only this, but MDSCs have been known to arise from cell other than myeloid progenitors. M-MDSCs for example can convert into PMN-MDSCs through an epigenetic mechanism requiring the downregulation of retinoblastoma.[200,206] Classical high-density neutrophils have also been shown to undergo a phenotypic switch to PMN-MDSCs.[127,127,200] MDSCs fill many different roles and

they rely on signals in the microenvironment to dictate their expansion, survival, migration, phenotype and functional intensity.[179,415,416] In cancer, MDSCs phenotype, subset frequency and mechanisms of suppression have been shown to depend on the tumor type.[14,178,417] Some studies even indicated that myeloid cells have the plasticity to interconvert between various phenotypes, which may be influenced by the TME.[208,418] It is very important to note that MDSCs expressing different phenotypes have different suppression mechanisms and capacities. For example, PMN-MDSCs most commonly suppress using ROS while NO and ARG1 based suppression mechanisms are most often utilized by M-MDSCs.[154,208,317] Plus, certain MDSC subsets are more suppressive than others (ex: M-MDSCs are more suppressive than PMN-MDSCs).[208,288]

Researchers have observed that differences among various MDSC studies arise due to the type of disease investigated, its severity, and the organs involved.[153,188,191] Due to MDSC plasticity and phenotypic variability, many discrepancies exist in flow-cytometry panels, markers and gating strategies for MDSC identification, particularly in whole blood.[165]

M-MDSCs are distinguished through the downregulation of HLA class II expression (HLA-DR_{low}).[165,198] Studies differ in how they measure HLA-DR based on how they process and handle samples, flow cytometer settings, their gating strategy, and how they report it (either "% of positive monocytes" or MFI).[182,198] The gating strategy used to identify M-MDSCs (defined as $CD33^{+}CD11b^{+}CD14^{+}CD15^{-}CD66b^{-}HLA\text{-}DR^{low}$) in this study gated on $HLA\text{-}DR^{low}$ monocytic cells by using the lymphocytes as a control to set the gate, a strategy used in a few other studies.[186]Multiple factors affect the expression of HLA-DR in whole blood samples that need to be considered. Research indicates that monocytic HLA-DR expression can be influenced by the type of anticoagulant, the conditions under which samples are stored, and the handling the sample before analysis.[198] Therefore, samples should be collected with an suitable anticoagulant

and analyzed within 4 hours of blood draw, as supported by the literature recommendations.[165,198]

PMN-MDSC gating in whole blood is more challenging, with many studies opting not to differentiate them from neutrophils.[419–422]Lack of Gr1 makes PMN-MDSC identification complex in humans.[124,127,203,210] Many studies prefer using PBMCs due to the enrichment of PMN-MDSCs in the "buffy coat" during Ficoll separation, allowing them to discriminate between PMN-MDSCs and the phenotypically similar neutrophils.[163,211] Other researchers compared MDSC frequencies between whole blood and PBMCs and found them to be consistent for M-MDSCs but not for PMN-MDSCs.[165,398] Beyond this, Ficoll density gradient separation can significantly reduce the surface expression of some granulocytic markers such as CD66b and CD16 as well as receptors like TLR4 and TLR2.[165,423] While the use of PBMCs for MDSC studies has its limitations, whole blood comes with its own set of challenges. Conventional high-density neutrophils (HDN) cannot be distinguished from PMN-MDSCs when using whole blood, making their identification difficult.[165] Some used CD16 to differentiate them, a strategy we employed in this study.[346] In this thesis, PMN-MDSCs were characterized as CD33+CD11b+CD14-CD15+CD66b+CD16low. However, in this identification, PMN-MDSC subsets expressing CD16 were excluded.

5.3 Expansion of MDSCs after tumor resection

The surgical stress response involves a cascade of signals, many of which are involved in inhibiting CMI and promoting metastasis. These signals and molecules trigger emergency myelopoiesis, leading to a substantial increase in regulatory myeloid cells that both inhibit CMI and promote metastasis. Therefore, they are regarded as a potentially significant contributor to the pro-metastatic effects of surgery stress.

In this thesis, we assessed changes in MDSC subsets from a diverse cohort of surgical cancer patients with diverse diagnoses and undergoing varied surgical procedures (Table 10). We showed that the M-MDSC subset of SxMDSCs expanded significantly in the whole blood of most patients (Figure 2A). M-MDSCs as previously defined significantly expanded in all patients, while a much more moderate increase was observed in PMN-MDSCs. Within our study cohort we observed a significant 2-fold increase in M-MDSCs with PROT1 (Figure 11A). This was not seen with PMN-MDSCs (Figure 11B). This was not seen with PMN-MDSCs (Figure 11B). Likewise, other work from our group noted a 2.1-fold increase in M-MDSCs following surgical tumor resection, through this was evaluated in PBMCs[352].

Although earlier studies from our group observed a higher overall increase in PMN-MDSCs, previously outlined limitations with PROT1 may explain the differing results.[313,352,375] While the relative expansion of PMN-MDSCs was large in these studies, PMN-MDSCs accounted for less than 6% of total MDSCs and the bulk of SxMDSCs was composed of M-MDSCs. Other investigations have corroborated that M-MDSCs are the main MDSC subtype present after surgery: one of these demonstrated this in a much larger cohort of over 100 lung cancer surgery patients.[288,352]

Early M-MDSCs, a population described in a few papers defined as CD33+CD11b+Lin-CD14-CD15-HLA-DR^{low} were interestingly seen to decrease significantly post-op in all patients. Dr. Angka et al. did not observe any differences in the PBMCs. However, it's crucial to mention that the population we analyzed was significantly smaller, as we had to account for all blood cells, making observations more challenging differences.[352]

A unique population of CD11b^{low} SxMDSCs were also observed in this study. CD11b^{low} MDSCs have been shown to suppress T-cells via an arginase mechanism in human gastric cancer

patients.[424] Arginase depletion has been identified in past studies by our group as a mechanism utilized by SxMDSCs to suppress NK cell cytotoxicity, potentially implicating e-MDSCs in post-operative suppression. [313]

The overall change in MDSC subset distribution after surgery resulted in a large and significant increase in M-MDSCs, a significant decrease in CD11b^{low} M-MDSCs, a non-significant increase in PMN-MDSCs (though yield loss due to PROT1 should be considered) and a significant decrease in e-MDSCs. This data suggests M-MDSCs are the predominant subset present on POD1.

5.4 SxMDSCs undergo a phenotypic switch toward an M2 phenotype

Several key differences in the main M-MDSC phenotype present at POD1 compared to BL were discovered using our MDSC phenotyping panel. Notably, many markers used throughout the literature to distinguish M2 macrophages were more highly expressed at POD1, including CD163, CD124, CD155, CCR2, and CXCR2. It appears as though SxMDSCs are undergoing a phenotypic switch and being pushed toward an M2 phenotype because of postoperative stress signaling. Cells exhibiting an M2 macrophage phenotype have been shown to limit NK cell activation by downregulating certain receptors (ex: CD25, CD69 and CD56).[425] M-MDSCs are capable of differentiating into M2 macrophages while retaining their immunosuppressive characteristics, as seen in a study culturing MDSCs isolated from patients afflicted with lung, colon or head and neck cancer.[426] An *in vitro* study attributed this phenotypic switch to STAT4 and NF-κB pathways.[427]

M2 macrophages are also known to highly express ARG1.[428] Murine studies from our group have shown that SxMDSCs have increased ARG1 and regulate systemic arginine levels in the

postoperative period as a mechanism of NK cell dysfunction, further corroborating the "M2 like" phenotype of these cells.[313]

5.4.1 CD163

CD163 is a scavenger receptor expressed by both monocytes and macrophages and is known to play a role in immune surveillance, protection against infection, and wound healing.[358,429] CD163 is associated with a pro-tumorigenic M2 phenotype in macrophages. This protein is known to be cleaved and released by cells promoting inflammatory pathways as a result, many of which are involved with MDSCs.[214,356,357,359] Soluble CD163 has been seen to increase post-op in patients undergoing surgery, in cancer patients, or individuals suffering from various types of trauma.[359,360,362,364,365] Literature shows that over 99% of $CD14^+$ monocytes express CD163, and this expression is heightened by IL-10 and glucocorticoids, which are known to increase during the surgical stress response.[214,363,366] This corresponds with our findings, in which all $CD14^+$ monocytes expressed the protein. Though all M-MDSC expressed CD163, the MFI of CD163 was greatly increased on POD1 in response to surgical stress (Figure 11A). Interestingly, CD163 expression dropped back to POD3 levels in all patients. Monocytes expressing CD163 have even been shown to suppress activation of primary NK cell cytotoxicity when co-cultured with lymphoma targets.[430] $CD163^+$ macrophages have even been shown to promote colorectal polyps by inhibiting the local T-cell response through TGFβ production.[361] In some studies, the suppression of NK cell by M2 macrophages was linked to a contact-dependent TGFβ mechanism[425,431,432] This is notable due to previous data from our group linking TGFβ in POD1 plasma to postoperative NK cell dysfunction.[24]

5.4.2 IL-4 Receptor (CD124)

IL-4 receptor (CD124) is expressed on M-MDSCs and PMN-MDSCs in multiple cancer types[165,203] as well as sepsis patients.[154] $CD124^+$ MDSCs are a subset observed in various studies, many of which have highlighted how this marker denotes MDSC subsets with immunosuppressive potential compared to their non-suppressive counterparts.[433,434] In tumor-bearing mice, CD124 expression on inflammatory monocytes was found to be directly correlated with the suppressive abilities of these cells against T-cell proliferation.[435] Another murine studied showed CD124 supports the suppressive activity of MDSCs against T-cells via an IL4Rα/STAT6 pathway, and that blocking this abrogated the suppressive activity of these MDSCs.[436] In clinical studies involving melanoma, lung and glioma patients, CD124 was upregulated on immature monocytes when compared to healthy controls.[435,437–439] The Il-4 receptor CD124 is also a key marker for M2 macrophages, and we observed a notable increase (1.2-fold) in the MFI of M-MDSCs in our patient cohort on POD1 (Figure 11B). This elevation persisted on POD3.

5.4.3 Chemokines (CXCR1/2 and CCR2)

CCR and CXCR chemokine markers (such as CXCR1/2 and CCR2) are expressed on inflammatory monocytes, as such studies have seen their expression on M-MDSCs in particular.[165,187] These chemokines are heavily involved in the trafficking of MDSCs, particularly the migration of MDSCs to pre-metastatic niches.[440,441] In tumor-bearing mice, clusters of MDSCs were detected near common metastasis sites (alveoli and terminal bronchioles) before tumor cells even reached the area.[442–444] Additionally, the amount of MDSCs present in the pre-metastatic lung in one of these models was seen to correlate with the degree of future metastatic burden.[444] Cancer cells have been shown to secrete CCL2, which can recruit MDSCs to these metastatic sites and promote tumor spread.[18,256,445] CXCL2, on the other hand, is elevated within

hours of surgery and can chemotactically recruits MDSCs to distant sites through interactions with CXCR2.[105,367,368] In colorectal cancer patients, growth factors secreted by tumors have been shown to stimulate TAM production of CXCL1, which recruits CXCR2+ MDSCs to the liver, leading to liver metastasis.[446]Certain chemokine targeting treatments aim to block the migration of MDSCs to distant sites.[126,447] Therapies targeting CXCR2 have been shown to reduce tumor mass and enhance lymphocyte tumor infiltration[68,105,448–450] Significant increases in CXCR2 and CCR2 on M-MDSCs were seen on POD1 within our study cohort (Figure 11). CXCR1 expression was not altered on the M-MDSCs of our patient cohort (Figure 11D), though literature relates this receptor more closely with PMN-MDSCs (which we did not investigate due to PROT1 population loss).[451]

5.4.4 CD155

M2 macrophages are also known to express CD155 which can inhibit NK cells via TIGIT.[452] CD155 expression on macrophages has been shown to contribute to an M2-phenotype transition, immunosuppression and tumor progression in colorectal cancer.[453] The MFI of CD155 was increased on POD1, further confirming the polarization of M-MDSCs to an M2 phenotype. This finding is consistent with previous reports from our lab that noted a similar increase in CD155 on M-MDSCs in PBMCs.[352]

4.4.5 Macrophage migration inhibitory factor (CD74)

Interestingly, the macrophage migration inhibitory factors (MIF) CD74 was seen to decrease in expression on M-MSDCs post-op in most patients. CD74 is associated with M1-type macrophages[454,455], further confirming the polarization of the M-MDSCs in this study to a M2 phenotype.

Overall, data from this thesis supports both the expansion of M-MDSCs on POD1 in patients undergoing tumor resection, as well as their differentiation into an "M2 Macrophage like" phenotype. Results from the immunophenotyping panel in particular highlights some key markers highly expressed by this suppressive group of cells which may prove effective targets in future studies.

5.5 SxMDSC responsiveness to LPS stimulation in fresh whole blood

As previously indicated, some M-MDSCs markers were not compatible with the PROT1 stabilizer as their expression was below detectable levels (either due to poor staining with the PROT1 or low expression on the population of interest). Some of these were evaluated in a smaller subset of patients in fresh whole blood. It is important to note that due to this smaller cohort statistical significance cannot be assigned to any of the results. We also evaluated the functional capacity of these MDSCs in response to LPS stim. LPS can promote expression of proteins on MDSCs which prolong survival and potentiate their immunosuppressive functions: as such it is not only known to increase the expression of our markers of interest but also activate the cells toward an "M2 like" phenotype.[165]

5.5.1 CD40

MDSCs are known to express CD40, and targeting CD40/CD40L interactions are being considered as a target to block MDSC/Treg interactions or to force MDSC maturation.[208,456,457] The former is due to the capacity of MDSCs to cross-talk with Tregs using the CD40/CD40L axis.[18,208,382] CD40 is an established M1 macrophage marker[458], and unsurprisingly it decreased in expression on M-MDSCs in fresh unstimulated blood (Figure 13A). When M-MDSCs were stimulated with LPS for 24h, POD1 M-MDSCs appeared to be much more responsive with a

more notable expression of CD40 than their BL counterparts (though further replicates would be needed to determine statistical significance).

5.5.2 PD-L1

MDSCs can impair NK cell cytotoxicity through the PD-L1 checkpoint ligand.[13,18,288,383,384] As such, there has been an observed correlation between PD-L1+ MDSCs and disease stage/clinical outcome in various tumor types.[18,126,165,459,460] M2 macrophages are also known to express PDL-1 which can interact with NK cells via PD1.[452] For this reason treatments targeting the PD-L1 expression of MDSCs (ex: Sunitinib) is being utilized in multiple trials thus far showing much promise.[124,461] PD-L1 is known to be upregulated on MDSCs in response to LPS stim.[462] Interestingly, POD1 M-MDSCs were far less responsive to LPS stimulation in regard to PD-L1 expression: despite being stimulated to the same degree and for the same length of time as their BL counterparts, their expression of PD-L1 was far less notable (Figure 13B). Further investigation is warranted to determine the significance of these results.

5.5.3 Toll-like receptors (TLR4)

TLR4L has been shown to promote MDSC inflammation-driven suppressive activity.[208,209,389] TLR signaling genes are low in mature neutrophils but upregulated in PMN-MDSCs.[208,463] Toll-like receptor agonists (such as DAMPs released by the TME) are known to pathologically activate MDSCs, thereby increasing their generation of ROS, NO and pro-inflammatory cytokines.[464] It is, therefore, unsurprising that TLR-targeted therapeutics are a promising front for immunotherapy.[208,465] Preliminary data from this study indicates SxMDSCs may be more responsive to LPS stimulation than their pre-operative counterparts (Figure 13E).

SxMDSCs in general do appear to be more responsive to LPS stimulation than their BL counterparts (with the exception of PD-L1 increase), indicating they may show greater immunosuppressive potential. Further investigation is needed for any conclusive observations.

5.6 Postoperative NK cell dysfunction

Surgical tumor resection is essential for patient treatment and removing the bulk of the tumor burden is crucial to recovery. While the curative benefits of surgery are undeniable, the surgical stress response is also known to promote the dissemination of tumor cells in circulation and the formation of pre-metastatic niches. This phenomenon is well documented in both murine and human models, including many from our own group.[42,43,62,72,466] In the present study, both NK cell cytotoxicity (Figure 10A) and NK cell IFNγ secretion (Figure 10B) were significantly suppressed in patients at POD1, consistent with previous research from our laboratory.[4,24,29,313] Similar to other reports from our group, NK cell IFNγ secretion was suppressed to a greater magnitude than NK cell cytotoxicity on POD1.[29,352] These reports also noted the recovery of NK cell cytotoxicity within a few days of surgery and a much longer length of suppression for cytokine secretion. Consistently, over half of our patient cohort recovered NK cytotoxicity by POD3 but their secretion of IFNγ remained low for all patients even at POD3. It is important to note that when measuring NK cell activity using a proprietary cytokine cocktail targeting NK cell (NKVue), multiple samples showed IFNγ below the minimum detectable level. This is a drawback also seen within our lab's previous studies involving the use of NKVue.[24,467] While this study did not specifically address the link between postoperative NK cell dysfunction and metastases, previous experiments were conducted by our group confirming this link.[42,62,466]

Data from these two assays confirms both NK cell cytotoxicity and IFNγ secretion are suppressed in the MDSC immunophenotyping cohort. In sum, these patients had dysfunctional

NK cells as well as an expansion of M-MDSCs exhibiting a predominantly M2 phenotype following tumor resection. Precise mechanisms linking these SxMDSCs to the observed NK cel suppression remain to be elucidated.

5.7 Factors influencing NK cell cytotoxicity suppression by SxMDSCs

Two assays were used to quantify NK cell cytotoxicity suppression when cultured with SxMDSCs: a flow-based assay and a nano-luciferase based assay. Prior optimization experiments confirmed both assays showed consistent target cell lysis results by NK cells (data not shown). For both, SxMDSCs were isolated via positive magnetic bead isolation, a method found to be ideal for functional assays.[165,423] NK92 cells were used to reduce variability between assays and to circumvent the documented effects of primary NK cell cryopreservation on cytotoxicity.[28] Previous documented discrepancies for NK cell *in vivo* vs *ex vivo* cytotoxicity should be considered when interpreting results.[468]

We first confirmed the previously observed suppression of NK cell cytotoxicity by SxMDSCs. Killing of tumor targets was suppressed at all time points investigated (43% suppression of cytotoxicity after 3h, 19% after 24h, 17% after 48h). The consistent suppression from 3h to 48h illustrates how quickly SxMDSCs can inhibit cytotoxicity *in vitro* and how even 48h later either NK cells fail to recover or MDSCs can still exert their immunosuppressive effects. In our MDSC immunophenotyping cohort, there was an average 15% suppression of NK cell cytotoxicity using BL SxMDSCs and an average 19% suppression using either POD1 or POD3 SxMDSCs. BL SxMDSCs on average suppressed to a lesser degree than POD1 SxMDSCs, consistent with our lab's previous results.[313,352,469] Overall, culturing with MDSCs isolated from healthy individuals did not result in a drop in NK cell cytotoxicity.

To investigate whether the mechanism of suppression utilized by these SxMDSCs to suppress NK cell cytotoxicity were contact-dependent or independent, we cultured SxMDSCs for 24h (the culture time used for most of our previous direct co-culture assays) and 48h (time necessary for collecting exosomes, a possible mechanism)[470], collected the supernatant and plated it on NK92 cells. We observed a less than 10% suppression in killing of tumor targets overall (Figure 16). The same result was obtained was culturing with 2X concentrated SxMDSC supernatant, or when collecting supernatant from MDSCs co-cultured with NK cells (Figure 16C, 16E). Similarly, no changes were noted when MDSC supernatant was collected following a 2h, 4h, 6h or 12h SxMDSC culture (Figure 16D). Finally, we looked to see whether POD1 plasma could suppress NK cell cytotoxicity *in vitro*. The culturing of healthy NK cells with patient POD1 plasma resulted in significant but low magnitude (at most 13% suppression) changes in NK cell cytotoxicity (Figure 17), further supporting the notion that contact dependent mechanisms are mainly responsible for post-op NK cell cytotoxicity dysfunction. POD1 plasma did suppress the most out of all sample types, and this only reached a 13% reduction in cytotoxicity, much lower than the degree of suppression seen when culturing NK cells directly with SxMDSCs isolated from the same patients.

In vitro co-culture assays have confirmed the suppressive capacity of the SxMDSCs studied in the previously described immunophenotyping panel against NK cell cytotoxicity. Further preliminary data described above indicates SxMDSC suppression of NK cell cytotoxicity is predominantly contact-dependent, though further replicates would be needed to determine the significance.

5.8 Influence of SxMDSCs in Post-operative NK Cell Cytokine Secretion Dysfunction

Prior to this study, our group had documented a drop in both IFNγ secretion (using NKVue and ELISA to measure IFNγ directly in patient plasma) as well as production (stimulating isolated patient NK cells in vitro with IL2/12 and measuring IFNγ production by flow cytometry) in surgical cancer patients on POD1.[24] The mechanisms responsible for this suppression were unknown. We conducted a series of assays to verify the implication of SxMDSCs in this phenomenon.

Consistent with previous work by our group, there was a reduction in IFNγ production when healthy NK cells were cultured with POD1 plasma (Figure 20). A significant average 32% decrease was seen on POD1, which is consistent with previous publications from our laboratory.[24] It is important to note that the drop in IFNγ seen using flow cytometry was not as large in magnitude as what was seen using the NKVue cytokine cocktail and ELISA to measure IFNγ directly in the plasma from the same patients (Figure 10). This was a limitation noted in our group's prior publications.[24] Previous studies from our group have also noted the most profound suppression of IFNγ secretion on POD1, consistent with the results we obtained.[24,29] It is important to note that levels of IFNγ from *in vitro* stimulated samples have been shown not to correlate directly with *in vivo* levels of serum IFNγ.[28,29]

With the suppressive effects of POD1 plasma confirm we next investigated the contribution of SxMDSCs. To this end, IFNγ production of healthy NK cells was measured in response to IL2/12 stimulation when cultured with SxMDSCs. These preliminary experiments revealed an average 5% drop in IFNγ production when NK cells were cultured with SxMDSCs, supporting the notion that they are likely not responsible for postoperative NK cell cytokine secretion dysfunction (Figure 18). We additionally investigated the impact of SxMDSC supernatant on the

IFNγ production of healthy NK cells in response to IL2/12 stimulation. A less than 3% average drop in IFNγ production was observed across all supernatant experiments (Figure 19). Finally, when comparing plasma NK cell IFNγ suppression to SxMDSC supernatant from matched patient samples (Figure 21), suppression was much greater in response to plasma (33% decrease) compared to supernatant (4.5% decrease). This is further confirmation that the suppressive elements present in the plasma on POD1 are likely not being released by SxMDSCs.

In summary, this body of work confirms the suppressive abilities of POD1 plasma on NK cell cytokine secretion. Further preliminary data suggests SxMDSCs are not a driving factor of post-operative NK cell cytokine secretion dysfunction, though further replicates and experimentation are required to definitively confirm this.

5.9 Overall Study Limitations and Considerations

5.7.1 Assay limitations

It is well documented throughout literature that MDSC activity and phenotype is variable based on both cancer and surgery type.[164,209,304] Studies on MDSCs in general are limited due to the great phenotypic plasticity and variability of these cells. Several studies have noted the expansion of only specific MDSC subsets based on the context, disease or environment.[173,267,288] Additionally, MDSCs can alter their phenotype as the disease progresses, or can even differentiate into other cell types once they enter the TME (ex: TAMS, DCs and fibrocytes).[126,378,471–473] As such, the diversity of surgical procedures studied and patient demographics may limit the identification of results with significant statistical value; however the advantage of this strategy means consistent significant results have broad generalizability.[28]

It is important to note in terms of the MDSC co-culture data obtained in this investigation that MDSCs are highly plastic cells and this must be considered when interpreting results. MDSCs

are known to change phenotypically in culture or lose their MDSC phenotype entirely.[124,152,474] Another important limitation is due to the exclusive use of peripheral blood without including tumor tissue: some studies have shown MDSCs from lymph nodes and tumor sites are more suppressive than peripheral blood.[165,475] Furthermore, whole blood NK assays have been found to yield more profound results compared to PBMC NK assays in literature.[28] Our assays involving the use of healthy donor NK cells to investigate NK cell cytokine secretion might have shown more profound results if they had been performed in whole blood, however, such an assay has yet to be developed and optimized in this context.

An additional significant limitation to the results of this study is also attributed to incomplete data as a result of sample collection. Mainly POD3 samples (though some other time points were also affected) were unable to be collected due to inability to locate a vein, inadequate sample volume, vacutainer vacuum failure or early patient discharge. This resulted in missing time-points for many of the assays conducted during this thesis.

5.7.2 Confounding factors in the study of surgical patient samples

Several characteristics of surgery have been implicated in post-op immunosuppression, including but not limited to anesthetic/analgesic drugs, tissue damage, hypothermia, blood loss/transfusion, nociception, pain and perioperative anxiety and stress.[3,10,28,38,270,476–482] These factors were not taken into consideration for the purposes of this study; however previous research from our lab and others have dismissed many as a major contributor of post-operative NK cell dysfunction. Several other groups have demonstrated no major impact on the surgical stress response or MDSC expansion depending on the type of anesthetic used.[116,417] Our group in particular noted in a murine study that mice receiving anesthesia has similar metastatic burden to control

mice.[10,42,62] It has also been noted hypothermia did not further increase pulmonary metastases compared to mice that underwent surgical stress under normothermic conditions.[43]

Some studies have noted an important sexual dimorphism regarding immune cells (for example neutrophils) [200,483], we thus aimed to consent an even number of female and male patients to account for any differences.

Dyslipidemia, frequently found in obesity, has been proposed to lead to the accumulation of MDSCs and enhancement of their immunosuppressive capacities.[179,484] For this reason, patients with a diagnosis of dyslipidemia were excluded from this study. It is important to note that while patients were carefully vetted, there exist many conditions or treatments existing prior to surgery which may have affected the results.[28] Particularly in the case of healthy donor controls: these donors were patients with no current cancer diagnoses but were undergoing surgeries at the Ottawa General Hospital, many of which had several comorbidities.

5.10 Future Directions

As previously mentioned, MDSC phenotype and immunosuppressive capacity may vary between peripheral blood and tumor. Further investigations may warrant a comparison of the results of this study on peripheral blood with tumor-associated MDSCs. Furthermore, many of the therapies targeting the key markers evaluated in this immunophenotyping studies were discussed, but they lack research in the context of surgical stress. The use of M2 Macrophage marker inhibitors or chemokine inhibitors could show promise in reducing post-operative metastases. These remain to be investigated both *in vitro* and *in vivo* with the use of a murine surgical stress model.

5.9 Conclusion

Immunotherapy is at the forefront of ongoing research for improving cancer patient outcomes. It is clear that SxMDSCs play an important role in post-operative NK cell dysfunction. While MDSCs have clear detrimental pro-metastatic effects, they are also an essential component of the immune response and patient recovery following surgical intervention. Understanding MDSC development and plasticity is crucial in order to identify the point in patient recovery where targeting them may prove beneficial and to which extent.[124,126,485] There already exist a multiple of treatments targeting MDSC induced immunosuppression many of which are undergoing clinical trial: none of these have been evaluated in the context of surgical stress.[63] The perioperative window is key in the development of tumor progression and metastases, and yet it is greatly underutilized un terms of therapeutic interventions.[42,54] The results of this study provide an outlook on MDSC phenotype and mechanism following tumor resection and may give insight on which existing interventions may prove most beneficial for patients in this context.

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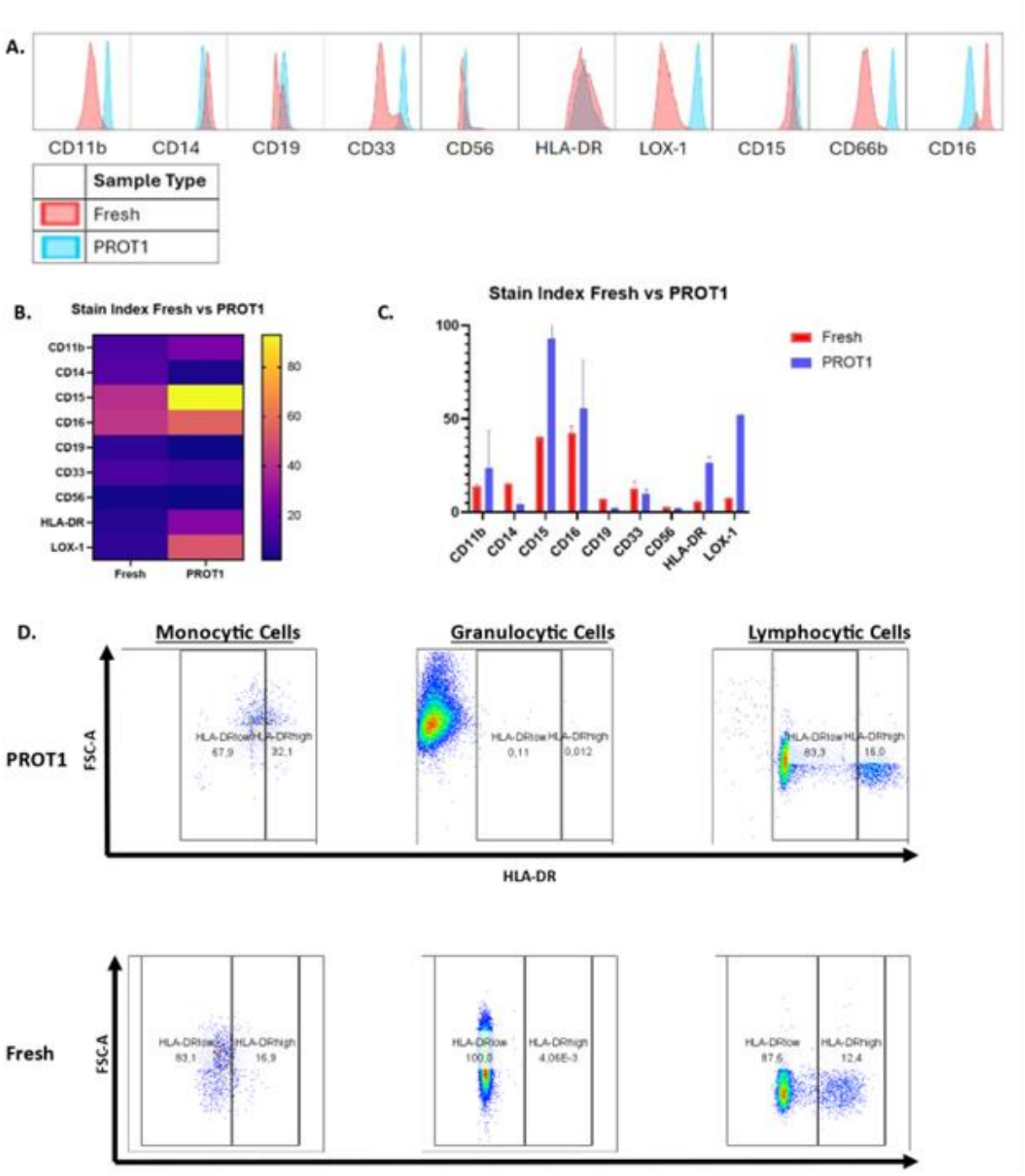
7. APPENDIX

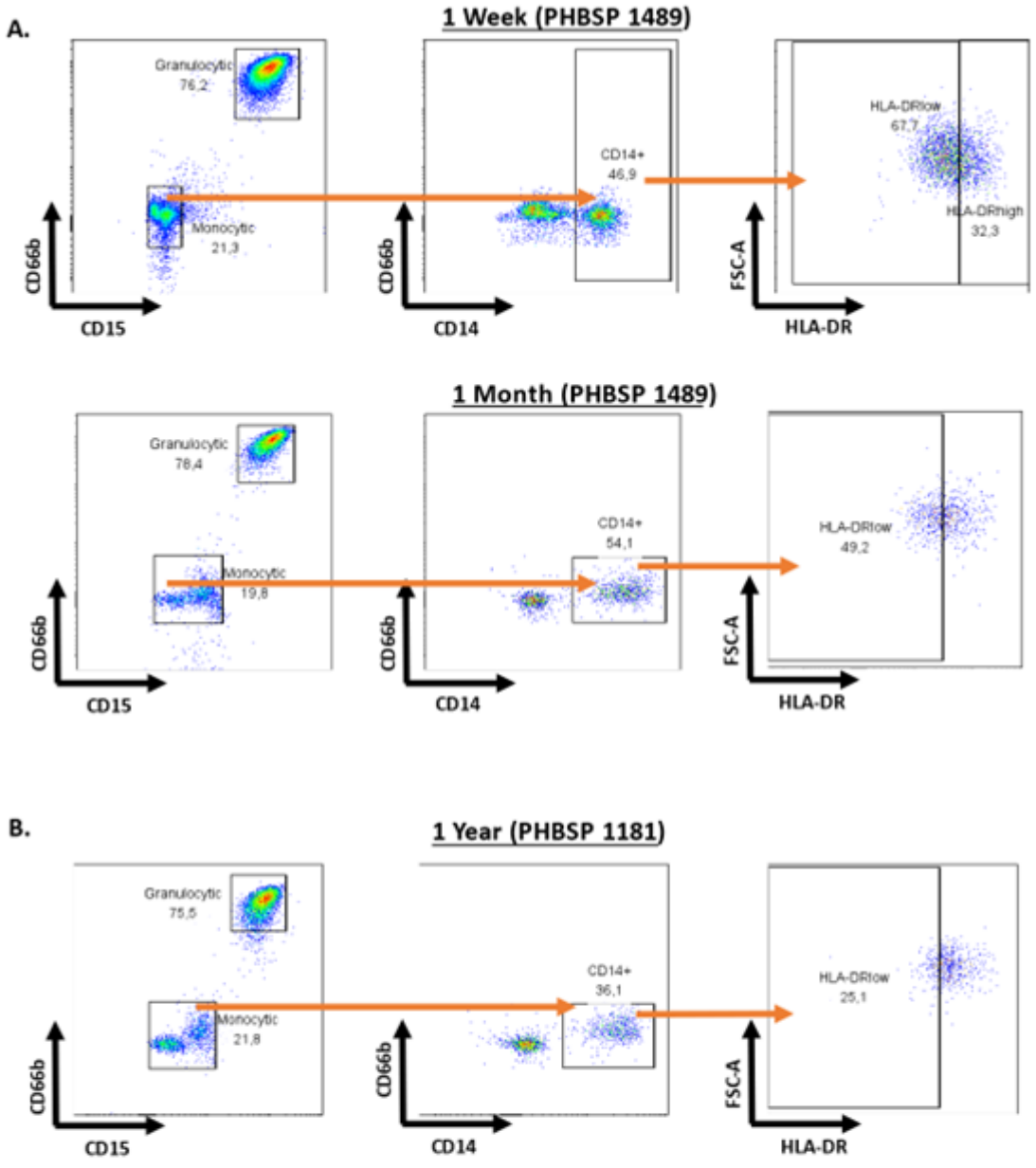
APPENDIX A: ANTIBODY STAINING DIFFERENCES BETWEEN FREH WHOLE BLOOD AND PROT1 STABILIZED WHOLE BLOOD



Appendix A: Binding of key MDSC antibodies is maintained by PROT1 while inducing changes in stain index. A) Representative histograms of the stain index of common MDSC

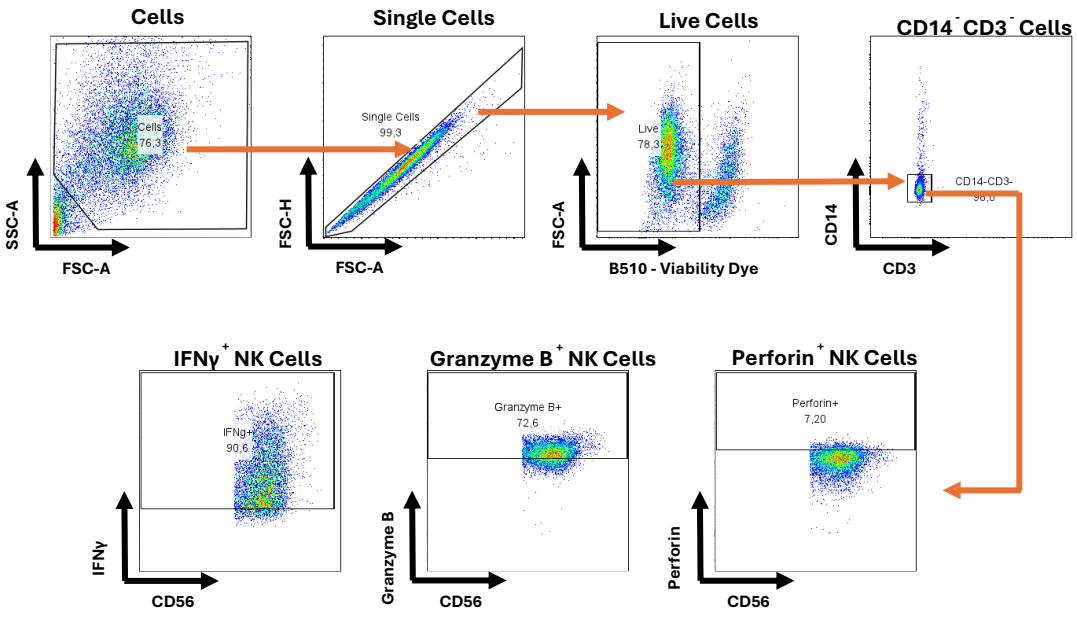
markers with fresh blood (red) or PROT1 stabilized blood (blue). **B)** Heatmap comparing stain index of common MDSC markers with fresh blood (left) or PROT1 stabilized blood (right). **C)** Stain index differences between Fresh (red) and PROT1 (blue) for common MDSC markers. **D)** Representative flow plots showing differences in HLA-DR staining for fresh and PROT1.

APPENDIX B: LONG-TERM CRYOPRESERVATION OF MDSCS WITH PROT1



Appendix B: PROT1 can preserve MDSC population for several months. A) Representative flow plots comparing M-MDSC population 1 week vs 1-month PROT1 stabilized blood. **B)** Representative flow plots for M-MDSC population 1-year PROT1 stabilized blood. **C)** Comparative histogram comparing PROT1 stabilized blood analyzed after 1 week (purple, left) and 1 month (blue, right) M-MDSC population frequencies. **D)** Comparative histogram comparing PROT1 stabilized blood analyzed after 1-week (purple, left) and 1-month (blue, right) PMN-MDSC population frequencies.

APPENDIX C: DETAILED ICS GATING STRATEGY TO DETERMINE NK CELL IFNγ, GRANZYME B AND PERFORIN PRODUCTION



Appendix C: ICS gating strategy. Representative gating for a POD1 cancer patient. After excluding doublets, debris and dead cells, CD14 and CD3 negative cells were selected. NK cells were then identified by CD56 expression and IFNγ, granzyme B and perforin were gated based on the resting condition.

APPENDIX D: OVERVIEW OF ALL FOLD-CHANGES FOR SXMDSCS IMMUNOPHENOTYPING

Sample Type	Marker	MFI Av BL	MFI Av POD1	MFI Av POD3	MFI Change (BL to POD1)	Significance
PROT1 Stabilized Blood	CD163	6135.45	28719.1	10517.4	4.68X	$P \leq 0.001$
	CD124	1415.67	1704.07	1844.38	1.2X	$P \leq 0.01$
	CCR2	8340.9	14987	11211.4	1.8X	$P \leq 0.001$
	CXCR2	1298.26	2374.07	2897.48	1.83X	$P \leq 0.01$
	CD74	17572.2	12054.1	13321.3	1.46X	$P \leq 0.01$
	CD155	3195.33	3472.05	4183.76	1.09X	$P \leq 0.05$
	CXCR1	2055.64	2188.26	2593	1.06X	P = NS
	CD206	777.406	877.8	894.2	1.13X	P = NS
	CD54	8591.69	9478.4	11331.5	1.1X	P = NS
	CD84	17004	18292.9	20979.7	1.08X	P = NS
	CD62L	12109.5	13482.8	9127.14	1.11X	P = NS
Fresh Blood	CD40	4670.67	-267.67		17.4X	$P \leq 0.05$
	CD73	124.4	-715		5.75X	P = NS
	CD206	1352.33	654.667		2X	P = NS
	PD-L1	373.8	813.08		2.17X	P = NS
	CD38	8024.67	6508.67		1.23X	P = NS
	TREM-1	7291	6779.33		1.08X	P = NS

Fresh Stim Blood	TLR4	3970.33	5485.33		1.38X	$P \leq 0.05$
	CD40	2501.33	2225.33		1.12X	P = NS
	CD73	948.2	1699		1.79X	P = NS
	CD206	919.667	1323.67		1.44X	$P \leq 0.05$
	PD-L1	156546	56892.6		2.75X	$P \leq 0.01$
	CD38	5624.67	6508.67		1.16X	P = NS
	TREM-1	10042.3	10223.7		1.02X	P = NS
	CD47	31099.7	32989		1.06X	P = NS

9. CURRICULUM VITAE

EDUCATION

2018-2022 Bachelor of Science (BSc), Biochimie, Laurentian University

AWARDS

MIC Seminar Award - BMI Seminar Days 2023

OGS Master's Award – September 2023 to April 2023

CONFERENCES AND ORAL PRESENTATIONS

Seminar: Investigating the mechanism of natural killer cell dysfunction mediated by surgery induced myeloid derived suppressor cells. BMI Seminar Day (Feb 2023), Ottawa, ON

Poster: Investigating the use of proteomic stabilizer in the phenotyping of myeloid derived suppressor cells. CanNKC Summit 4CI (Sept 2023), Hamilton, ON

Poster: Investigating the use of proteomic stabilizer in the phenotyping of myeloid derived suppressor cells. BioCanRX Summit for Cancer Immunology (Sept 2023), Ottawa, ON

Poster: Investigating the use of proteomic stabilizer in the phenotyping of myeloid derived suppressor cells. OHRI Research Day (Nov 2023), Ottawa, ON (Virtual)

SELECT WORK EXPERIENCE

2024 – Booking Clerk, Northeastern Cancer Centre, Sudbury, ON

2023 – Teaching Assistant, University of Ottawa, Ottawa, ON

2021 – Research Assistant, Health Sciences North Research Institute (HSNRI), Sudbury, ON

2019-2021 – Laboratory Teaching Assistant, Laurentian University, Sudbury, ON

COURSES

MIC5100 – Pathogen Interactions and Host (Grade: A+)

MED8166 – Professionalism and Professional Skills (Grade: S)

MIC8120 – Advanced Topics in Immunometabolism (Grade: A+)

PROFESSIONAL DEVELOPMENT AND TRAINING

2022 Good Clinical Practice (GCP), OHRI, Ottawa, ON

2022 Flow Cytometry Training, Flow Core Facility, University of Ottawa, Ottawa, ON

2022 Laboratory and Safety Training, OHRI, Ottawa, ON

2022 Research Ethics based on the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2: CORE 2022), OHRI, Ottawa, ON

2023 CANTRAIN Common Core Foundation Course, OHRI, Ottawa, ON

LANGUAGES

French and English – fluently spoken and written