TGFβ CAUSES POSTOPERATIVE NATURAL KILLER CELL PARALYSIS THROUGH MTOR INHIBITION

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Abstract

Background: Life-prolonging tumour removal surgery is associated with increased metastasis and disease recurrence. Natural Killer (NK) cells are critical for the anti-tumour immune response. Postoperatively, NK cell cytotoxicity and interferon-gamma (IFNγ) production are profoundly suppressed and this dysfunction has been linked to increased metastases/poor patient outcomes. NK cell activity depends on the integration of signals through receptors and can be modulated by soluble factors, including transforming growth factor-beta (TGFβ). The postoperative period is characterized by the expansion of myeloid-derived suppressor cells (sxMDSCs), which inhibit NK cell effector functions. I hypothesize that ***impaired NK cell IFNγ production is due to altered signaling pathways caused by sxMDSC-derived TGFβ***.

Methods: Postoperative changes in NK cell receptor expression, receptor-dependent phosphorylation of downstream targets, and rIL-2/12-stimulated IFNγ production were assessed using newly developed whole blood assays utilizing peripheral blood samples from cancer surgery patients. Isolated healthy NK cells were incubated in the presence of healthy/baseline/postoperative day (POD) 1 plasma or isolated sxMDSCs and NK cell phenotype and function were assessed. NK cells were also cultured with plasma in the presence/absence of a TGFβ blocking monoclonal antibody (mAb) or a TGFβ RI small molecule inhibitor (smi). Single-cell RNA-sequencing was performed on six colorectal cancer surgery patients at baseline and on POD1. S6 phosphorylation was used as a proxy for mammalian target of rapamycin complex (mTORC) 1 activity to investigate the mechanism of TGFβ-mediated NK cell dysfunction.

Results: Intracellular NK cell IFNγ, activating receptors CD132 (IL-2R), CD212 (IL-12R), NKG2D, and DNAM-1, and the phosphorylation of downstream targets STAT5, STAT4, p38 MAPK, and S6 were significantly reduced on POD1. TGFβ was increased in patient plasma on POD1. The dysfunctional phenotype could be phenocopied in healthy NK cells through the addition of rTGFβ1 or by incubation with POD1 plasma. This dysfunctional phenotype could be prevented with the addition of an anti-TGFβ mAb or a TGFβ RI smi in culture. RNA-sequencing revealed a reduction in transcripts associated with mTOR effector functions, suggesting an impairment in mTOR. S6 phosphorylation was maintained with the addition of TGFβ-specific therapies. The hyporesponsive NK cell phenotype was reproduced upon culture of healthy NK cells with sxMDSCs and sxMDSCs were shown to produce soluble TGFβ in culture.

Conclusion: Surgically stressed NK cells display a dysfunctional phenotype, which could be prevented *in vitro* through the addition of TGFβ-specific blocking therapies. sxMDSCs produced TGFβ and co-incubation induced dysfunction in healthy NK cells. The recovery of impaired S6 phosphorylation with TGFβ-specific therapies suggests that TGFβ is inducing NK cell dysfunction via inhibition of mTORC1 activity. The perioperative period of immunosuppression presents a window of opportunity for novel therapeutics to prevent metastases and cancer recurrence among cancer surgery patients.

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Table of Contents

Abstract ii
Acknowledgements iii
List of Figures viii
List of Abbreviations xi
Chapter 1 Introduction 1
1.1 Natural Killer Cells 1
1.1.1 Activating/inhibitory signal integration controls NK cell activity 2
1.1.2 NK cell activity is also modulated by cytokines 4
1.1.2.1 A note on mTOR 11
1.1.3 NK cell role in tumour immunity 12
1.2 Natural Killer-T cells 14
1.3 A brief history of cancer surgery 16
1.4 The Surgery and Metastasis Paradox 17
1.4.1 Manipulation of the tumour 18
1.4.2 Tissue hypoxia and angiogenesis 18
1.4.3 Neuroendocrine activation 20
1.4.4 The pro-inflammatory phase 21
1.4.5 The hypercoagulable state 23
1.4.6 The anti-inflammatory phase 24
1.4.6.1 Anti-inflammatory cytokines 25
1.4.6.2 Immunosuppressive populations 28
1.5 Anesthesia, analgesia, and blood transfusions 33
1.5.1 The effect of blood transfusions 34
1.5.2 The effect of anesthesia 34
1.5.3 The effect of analgesia 35
1.6 NK cell suppression and postoperative metastasis 37
Chapter 2 Hypothesis 42
Chapter 3 Materials and Methods 43
3.1 Patient characteristics and clinical protocol 43
3.2 Blood processing 43
3.3 Antibodies and FACS analysis 44
3.4 *In vitro* cryopreserved PBMC phenotyping and functionality assays 44

3.4.1 Thawing cryopreserved PBMCs 44
3.4.2 Extracellular receptor phenotyping assay 44
3.4.3 IFNγ functionality assay 45
3.5 Whole blood phenotyping and functionality assays 46
3.5.1 Extracellular receptor phenotyping assay 46
3.5.2 Phospho-signaling protein functionality assay 47
3.5.3 IFNγ functionality assay 47
3.6 Cell lines 48
3.7 Cell isolation 48
3.7.1 $CD56^+$ cell isolation 48
3.7.2 $CD33^+$ cell isolation 49
3.8 *In vitro* NK-92:K562 co-culture cytotoxicity assay 49
3.9 Platelet-free plasma collection 50
3.10 Extracellular cytokine quantification 50
3.10.1 Human IFNγ ELISA 50
3.10.2 Human TGFβ ELISA 50
3.11 *In vitro* rTGFβ1 time-course assay 51
3.12 *In vitro* plasma culture assay 51
3.13 *In vitro* isolated sxMDSC: NK cell co-culture assay 53
3.14 Single-cell RNA-sequencing 53
3.15 Statistical analyses 54
Chapter 4 Results 56
4.1 Patient demographics 56
4.2 IFNγ production is suppressed in cryopreserved NK cells on POD1 56
4.3 Whole blood staining protocol development 58
4.4 IFNγ production is suppressed in whole blood NK cells on POD1 61
4.5 IFNγ production following surgical stress is more impaired in $CD56^{Bright}$ NK cells 64
4.6 IL-2 and IL-12 receptor subunit expression is reduced in whole blood NK cells on POD1 69
4.7 Postoperative NK cells have significantly reduced activating receptor expression 73
4.8 Downstream signaling molecule phosphorylation is significantly impaired on POD1 following rIL-2/12 stimulation 77
4.9 $CD56^+CD3^+$ cells are also dysfunctional on POD1 81
4.10 TGFβ is increased in the plasma of cancer patients following surgery 81

4.11 Short exposure to TGFβ1 can induce long lasting NK cell impairment 83
4.12 TGFβ1, but not IL-10, can induce NKG2D downregulation in baseline NK cells 85
4.13 Healthy NK cells cultured with rTGFβ1 show reduced receptor expression and IFNγ production 90
4.14 Healthy NK cells cultured with POD1 plasma show reduced receptor expression and IFNγ production 94
4.15 TGFβ-specific therapeutics can prevent dysfunction in healthy NK cells incubated with rTGFβ1 or POD1 plasma 94
4.16 NKG2D expression and IFNγ production show a strong correlation *in vitro* 98
4.17 TGFβ mediates NK cell dysfunction through mTOR 98
4.18 Co-culture with sxMDSCs can induce healthy NK cell dysfunction 108
Chapter 5 Discussion 116
5.1 Soluble TGFβ acts by inhibiting mTORC1 to reduce receptor expression and suppress IFNγ production on POD1 116
5.2 Limitations 118
5.2.1 Whole blood assays 118
5.2.2 Working with human specimens 118
5.2.2.1 Patient heterogeneity 118
5.2.2.2 Sample size 119
5.2.3 COVID-19 Pandemic 120
5.3 Areas of further investigation 120
5.3.1 The timeline of NK cell dysfunction in the postoperative period 120
5.3.2 Elucidating the postoperative NKT cell phenotype 122
5.3.3 Investigating the TGFβ-mTOR relationship 123
5.3.4 Assessing the impact TGFβ blockade *in vivo* 126
5.4 Clinical applications 127
5.4.1 Tracking NKG2D expression as a diagnostic tool 127
5.4.2 Targeting surgery-induced MDSCs and TGFβ 128
5.4.3 The making of an antibody-drug conjugate 130
Significance 132
Contribution of Collaborators 133
Chapter 6 References 142
Appendix A 168
Appendix B 169

Appendix C 170
Appendix D 171
Appendix E 172
Appendix F 173
Appendix G 174
Curriculum Vitae 174

List of Figures

Figure 1. Signaling pathways downstream of IL-2/12/15/18 and activating receptors NKG2D and DNAM-1. 7

Figure 2. A summary of NK cell activation and IFNγ-mediated anti-tumour effector functions. 15

Figure 3. Potential mechanisms of postoperative cancer recurrence. 19

Figure 4. Graphical depiction of hypothesis. 41

Figure 5. Dose response to stimulation in healthy donor cryopreserved NK cells. 57

Figure 6. IFNγ production is suppressed in cryopreserved NK cells on POD1. 59

Figure 7. Proportion of granulocytes is not related to NK cell IFNγ production. 62

Figure 8. NK cell extracellular receptor expression in cryopreserved vs. whole blood-stained NK cells.. 63

Figure 9. Whole blood IFNγ staining protocol and gating strategy. 65

Figure 10. IFNγ production is suppressed in whole blood NK cells on POD1. 66

Figure 11. $CD56^{Bright}$ cells produce more IFNγ upon stimulation than their $CD56^{Dim}$ counterparts. 67

Figure 12. $CD56^{bright}$ NK cells have impaired IFNγ production on POD1. 68

Figure 13. IL-2 and IL-12 receptor subunit expression is reduced in whole blood NK cells on POD1. 70

Figure 14. $CD56^{Bright}$ cells have increased cytokine receptor expression as compared to $CD56^{Dim}$ cells. .. 71

Figure 15. $CD56^{bright}$ and $CD56^{dim}$ NK cells have reduced cytokine receptor expression. 72

Figure 16. Postoperative NK cells have significantly reduced activating receptor expression. 74

Figure 17. Differential expression of receptors at baseline in $CD56^{Bright/Dim}$ populations. 75

Figure 18. Both $CD56^{Bright}$ and $CD56^{Dim}$ NK cells have reduced activating receptor expression on POD1. 76

Figure 19. Downstream signaling protein phosphorylation is significantly impaired on POD1 following rIL-2/12 stimulation. 78

Figure 20. $CD56^{Bright}$ cells have increased phosphorylation of signaling proteins at baseline. 79

Figure 21. Downstream signaling protein phosphorylation is significantly impaired in both $CD56^{Bright/Dim}$ populations on POD1 in response to rIL-2/12. 80

Figure 22. $CD56^{+}CD3^{+}$ cells display a suppressed phenotype on POD1. 82

Figure 23. TGFβ is increased in the plasma of cancer patients following surgery. 84

Figure 24. Healthy $CD56^{+}$ cells incubated with rTGFβ1 show long-term changes in NKG2D expression. 86

Figure 25. TGFβ1, but not IL-10, can induce NKG2D downregulation in baseline NK cells. 88

Figure 26. rTGFβ1 inhibits NK-92 cytotoxicity against L562 leukemia cells *in vitro*. 89

Figure 27. Combined plasma culture assay protocol schematic. 91
Figure 28. Culture of healthy NK cells with heterologous combined healthy plasma does not alter NK cell phenotype or function.. 92
Figure 29. Healthy NK cells cultured with rTGFβ1 have reduced receptor expression and IFNγ production.. 93
Figure 30. Healthy NK cells cultured with POD1 plasma show reduced receptor expression and IFNγ production.. 95
Figure 31. A TGFβ mAb and TGFβ RI small molecule inhibitor prevents a dysfunctional phenotype in healthy NK cells incubated with rTGFβ1 or POD1 plasma.. 97
Figure 32. NKG2D expression and IFNγ production show a moderate correlation *in vitro*. 99
Figure 33. Single-cell RNA-sequencing reveals transcriptomic differences between baseline and POD1 populations. 100
Figure 34. Postoperative NK cells display transcriptomic shift.. 101
Figure 35. POD1 NK cells are associated with reduced mTOR activity. 102
Figure 36. pS6 is reduced in the absence of stimulation on POD1. 104
Figure 37. Baseline phosphorylation of Akt and S6 is reduced in whole blood on POD1. 105
Figure 38. pS6 is reduced in healthy NK cells in the presence of rTGFβ1 or POD1 plasma. 106
Figure 39. TGFβ-specific therapeutics can maintain S6 phosphorylation in the presence of rTGFβ1 or POD1 plasma.. 107
Figure 40. sxMDSC: NK cell co-culture schematic and gating strategy. 109
Figure 41. Co-culture with sxMDSCs can induce NK cell dysfunction. 110
Figure 42. The effect of sxMDSCs on healthy NK cells is titratable.. 112
Figure 43. sxMDSCs express LAP-TGFβ1 on their cell surface. 113
Figure 44. sxMDSCs produce TGFβ in culture. 114
Figure 45. Phosphorylation of S6 is reduced in the presence of sxMDSCs.. 115
Figure 46. Hypothesized TGFβ signaling pathways in postoperative NK cells. 125

List of Tables

Table 1. FACS monoclonal antibodies. 134
Table 2. Reagents. 135
Table 3. Patient information for cryopreserved samples used to characterized cytokine and activating/ inhibitory receptors and intracellular/ extracellular IFNγ. 136
Table 4. Patient information for whole blood samples used to characterized cytokine and activating/ inhibitory receptors, phospho-signaling proteins, and intracellular/ extracellular IFNγ. 137
Table 5. Patient information for TGFβ ELISA. 138
Table 6. Demographics for healthy $CD56^+$ donors and healthy/ cancer patient plasma used in plasma culture experiments. 139
Table 7. Patient information for cryopreserved samples used in single-cell RNA-sequencing. 140
Table 8. Demographics for healthy $CD56^+$ donors and POD1 $CD33^+$ patients used in sxMDSC: NK cell co-culture assays. 141

List of Abbreviations

ACTH = Adrenocorticotropic hormone

ADCC = Antibody-dependent cell-mediated cytotoxicity

APC = Antigen presenting cell

AP-1 = Activator protein-1

ARE = Adenylate/uridylate-rich elements

ARG1= Arginase

atRA = All trans retinoid acid

Bcl-2 = B cell lymphoma-2

BSA = Bovine serum albumin

B-TCGF = B cell-derived T cell growth factor

CAF = Cancer-associated fibroblast

CARS = Compensatory anti-inflammatory response syndrome

CD = cluster of differentiation

CLP = Common lymphoid progenitor

COX-2 = Cyclooxygenase-2

CPI = Checkpoint inhibitor

CRC = Colorectal cancer

CRP = C-reactive protein

CSIF = Cytokine synthesis inhibitor factor

CTC = Circulating tumour cells

CTL = Cytotoxic T lymphocyte

CTLA-4 = Cytotoxic T-lymphocyte associated protein 4

CXCL4 = Chemokine (C-X-C motif) ligand 4

DAG = Diacylglycerol

DAP10 = DNAX activating protein 10

DAMPs = Danger associated molecular patterns

DC = Dendritic cell

DED = Death effector domain

DFS = Disease-free survival

DMSO = Dimethyl sulfoxide

DNAM-1 = DNAX-accessory molecule -1

ECS = Extracellular staining

EDTA = Ethylenediaminetetraacetic acid

Elk-1 = ETS-like 1 protein

EMT = Epithelial-to-mesenchymal transition

ERK = Extracellular receptor kinase

ERM = Ezrin-radixin-moesin proteins

FACS = Fluorescence-activated cell sorting

FB = Flow buffer

FBS = Fetal bovine serum

FKBP12 = FK506-binding protein-12

FoxP3 = Forkhead box P3

G-CSF = Granulocyte-colony stimulating factor

GM-CSF = Granulocyte monocyte-colony stimulating factor

GMP = Granulocyte-monocyte progenitor

Grb2 = Growth factor receptor-bound protein 2

GSEA = Gene set enrichment analysis

GvHD = Graft versus host disease

HIF = Hypoxia-inducible factor

HLA = Human leukocyte antigen

HPA = Hypothalamic-pituitary-adrenal

HSC = Hematopoietic stem cell

IC = Intracellular

ICS = Intracellular staining

IFNγ = Interferon-gamma

Ig = Immunoglobulin

IL = Interleukin

IL-2R = Interleukin-2 receptor

IL-6R = Interleukin-6 receptor

IL-10R = Interleukin-10 receptor

IL-12R = Interleukin-12 receptor

IL-15R = Interleukin-15 receptor

IL-18R = Interleukin-18 receptor

iNKT = Invariant NKT

iNOS = Inducible nitric oxide synthase

IRAK4 = IL-1 associated receptor kinase 4

IRF1 = Interferon response factor 1

ITT = Immunoglobulin tail tyrosine

i.v. = Intravenous

JAK = Janus kinase

KIR = Killer Immunoglobulin-like Receptor

LAMP = Lysosomal-associated membrane protein

LAP = Latency-associated peptide

LDL-R = Low-density lipoprotein receptor

LFA-1 = Leukocyte function associated antigen -1

LMWH = Low molecular weight heparin

LTBP = Latent TGFβ binding protein

Mab = Monoclonal antibody

MAPK = Mitogen-activated protein kinase

M-CSF = Myeloid-colony stimulating factor

MDSC = Myeloid-derived suppressor cell

MEK = Mitogen-activated protein kinase kinase

MHC = Major histocompatibility complex

MICA/B = MHC class I chain-relate protein A/B

MIP-1β = Macrophage inflammatory protein-1 beta

miRNA = Micro RNA

MK2 = MAPK kinase-activated protein kinase 2

MKK = MAP kinase kinase

M-MDSC = Myeloid-MDSC

MOA = Mechanism of action

MOR = Mu-opioid receptor

MT-MMP = Membrane-type matrix metalloprotease

mTOR = Mammalian target of rapamycin

mTORC1 = Mammalian target of rapamycin complex 1

MyD88 = Myeloid differentiation primary response 88

NCR = Natural Cytotoxicity Receptor

NES = Normalized enrichment scores

NFκB = Nuclear factor kappa-light-chain-enhancer of activated B cells

NK = Natural Killer

NKG2A/C/D = Natural Killer Group 2 member A/C/D

NKT = Natural Killer-T cell

NKP = Natural Killer progenitor

NSAID = Non-steroidal anti-inflammatory drugs

NSCLC = Non-small cell lung cancer

OS = Overall survival

PAMP = Pathogen associated molecular pattern

PBMC = Peripheral blood mononuclear cell

PBS = Phosphate buffered saline

PD-1 = Programmed cell death protein 1

PDE5 = Phosphodiesterase type 5

PFA = Paraformaldehyde

PGE = Prostaglandin

PI3K = Phosphoinositide-3 kinase

PKC = Protein kinase C

PLC-γ = Phospholipase C-gamma

PMN-MDSC = Polymorphonuclear-MDSC

POD = Postoperative day

PRR = Pattern recognition receptor

PTP = Protein tyrosine phosphatase

PVR = Polio virus receptor

Raptor = Regulatory associated protein of mTOR

RBC = Red blood cell

RCT = Randomized control trial

RFS = Recurrence-free survival

RICTOR = Raptor-independent companion of mTOR

ROS = Reactive oxygen species

RPMI = Roswell Park Memorial Institutes

scRNA-seq = Single-cell RNA-sequencing

SH2 = Src homology 2

Shp-1 = Src homology region 2 domain-containing phosphatase-1

SLP-76 = SH2 domain-containing leukocyte protein of 76 kD

STAT = Signal Transducer and Activator of Transcription

sxMDSC = Surgery-induced MDSC

TAM = Tumour-associated macrophage

T-bet = T-box protein expressed in T cells

TCE = Tumour cell emboli

TCGF = T cell growth factor

TCR = T cell receptor

TGFβ = Transforming growth factor-β

TGFβR = Transforming growth factor-beta receptor

T_h cell = T helper cell

TIGIT = T cell immunoreceptor with Ig and ITIM domains

TIM-3 = T cell immunoglobulin and mucin domain-containing 3

TLR = Toll-like receptor

TME = Tumour microenvironment

TNF = Tumour necrosis factor

TNFα = Tumour necrosis factor-alpha

TRAF6 = Tumour necrosis factor-associated factor 6

TRAIL = TNF-related apoptosis-inducing ligand

TRAMP = TNF receptor-related apoptosis-mediating protein

T_{reg} = Regulatory T cell

TRIM = Transfusion-related immunomodulation

TYK2 = Tyrosine protein kinase 2

ULBP1-6 = UL16 binding protein 1-6

UTR = Untranslated region

VEGF = Vascular endothelial growth factor

Chapter 1 Introduction

1.1 Natural Killer Cells

Natural Killer (NK) cells, first identified by Kiessling et al. in 1975, are cytotoxic lymphocytes that play a critical role in the innate immune response by destroying circulating stressed, infected, or transformed (cancerous) cells[1–3]. NK cells derive from hematopoietic stem cells (HSCs) in the bone marrow, although recent studies suggest that NK cells can also develop in the liver and spleen[4]. HSCs then become committed to lymphoid cell lineage differentiation (common lymphoid progenitor/ CLP), followed by a bipotential NK/T progenitor phase before becoming classified as NK progenitors (NKP)[5]. These immature NK cells go through five developmental stages characterized by proliferation and the acquisition of specific cell surface markers as well as the ability to exert appropriate effector functions[5]. The cytokines interleukin (IL)-2, IL-7, and IL-15 are critical for NK cell development, homeostasis, and functional maturation[4,6]. Mature NK cells comprise 10-15% of total peripheral blood leukocytes,[7] and after B and T cells are the third largest population of lymphocytes[4]. They are described morphologically as large, granular lymphocytes and phenotypically as cluster of differentiation $(CD)3^{-}$ $CD14^{-}$ $CD19^{-}$ $CD56^{+}$ $CD16^{+/-}$[4]. In addition to being found in the periphery, NK cells are also found in the lymph nodes, spleen, thymus, peritoneal cavity, lungs, liver, and uterus during gestation[4].

Two functional subsets of NK cells can be distinguished based on the cell surface density of CD56 and the low-affinity Fc-receptor CD16[8,9]. $CD56^{dim}CD16^{+}$ cells make up 90% of peripheral blood and spleen NK cells and are preferentially cytotoxic, whereas most lymph node NK cells are $CD56^{bright}CD16^{dim/-}$ and readily produce cytokines, although each subtype can exert both effector functions[8,9]. While NK cells do not undergo clonal selection, they instead express a limited number of germline-encoded receptors[10]. Their activity is thus regulated by the integration of activating and inhibitory ligands through these receptors[10,11]. In addition, NK cells express different combinations

of these receptors, resulting in a heterogeneous population capable of responding to a variety of stimuli[4]. NK cells mobilized for the immune response carry out three specific functions: (1) they induce apoptosis through the expression of death receptor ligands, (2) they are directly cytotoxic through Ca^{2+}-dependent exocytosis of cytolytic granules, and (3) they secrete cytokines[9,12–15]. The expression of soluble or transmembrane death receptor ligands, such as TNF family molecules FasL and TRAIL, initiate programmed cell death by binding to their respective receptors, Fas receptor (CD95) and TRAILR-1/R-2[12]. NK cells release the membrane-disrupting protein perforin and serine proteases called granzymes, which jointly result in the perforation and subsequent apoptosis of target cells[4,14]. In addition, NK cells secrete numerous cytokines, including interferon-gamma (IFNγ), tumour necrosis factor-alpha (TNFα), and granulocyte monocyte-colony stimulating factor (GM-CSF), which serve to modulate the immune response[14,16]. Finally, NK cells also participate in antibody-dependent cell-mediated cytotoxicity (ADCC) through CD16[17].

1.1.1 Activating/inhibitory signal integration controls NK cell activity

NK cell activating receptors recognize pathogen-derived antigens as well as stress-induced ligands in what is termed the "induced-self recognition model"[14]. Upon cellular stress, the expression of these ligands are upregulated, resulting in the binding and activation of NK cells[14,18].

Natural Cytotoxicity Receptors (NCRs) include NKp30, NKp44, NKp46, and NKp80, and are important in the recognition of virally infected and transformed cells[19]. Only recently have some NCR ligands been identified and these include: immune cell surface molecules, viral antigens, tumour-specific ligands, stress-induced proteins, and soluble growth factors[19–25]. Other significant activating receptors include C-type lectin-like receptors NKG2D and CD94-NKG2C[4].

NKG2D is composed of two homodimer subunits, which bind stress-induced ligands MHC class I chain-related protein (MIC)A/B and UL16 binding protein (ULBP)1-6[9,26–28]. NKG2D itself is not a kinase and therefore receptor signaling requires association with the tyrosine kinase adaptor protein DNAX activating protein 10 (DAP10) to propagate activation signals. Ligand binding

results in the recruitment of growth factor receptor-bound protein 2 (Grb2) and the phosphorylation of Vav1 leading to Src homology 2 (SH2) domain-containing leukocyte protein of 76 kD (SLP-76) activation, phospholipase C-gamma (PLC-γ) activation, Ca^{2+} influx, degranulation, and secretion[29]. NKG2D ligation also results in the activation of the phosphatidyl-inositol-3-kinase (PI3K)/ Akt pathway to promote survival[29]. NKG2D is expressed early in NK cell development and remains expressed by most mature peripheral NK cells[29]. In murine models, NKG2D has been implicated in NK cell education, effector cell functions, and peripheral tolerance. In addition, NKG2D-deficient animals have impaired anti-tumour functions[335]. The impact of NKG2D on human NK cell function is more difficult to determine, partly due to a lack of NKG2D deficiency syndromes[29]. NKG2D is a C-type lectin-like receptor that binds the MHC-I-like ligands MICA/B and ULBP1-6 in humans[9,26–28]. Evidence supports a role for the involvement of NKG2D in human NK cell development, potentially dependent upon an interaction with the IL-15R and the activation of the downstream PI3K pathway[336,337]. In addition to mediating NK cell activity through stress-induced ligand binding, NKG2D can function in synergy with NCRs as well as the activating receptor 2B4 and has also been shown to promote CD16-mediated ADCC[338–340]. Finally, NKG2D may be involved in the development of peripheral tolerance, as prolonged exposure to NKG2D ligands results in the downregulation of NKG2D and reduced responsiveness through NKG2D and other receptors including CD16 and the NCR NKp46[341,342]. This is thought to prevent hyper-responsiveness against peripheral ligands either due to incomplete education in the bone marrow or physiologically during pregnancy[343].

In addition to these activating receptors there are co-activating receptors, which serve to fine-tune NK cell activity[31–33]. DNAX-accessory molecule (DNAM-1, CD226) is a highly expressed transmembrane protein with an internal immunoglobulin (Ig) tail tyrosine (ITT) and functions as an adhesion molecule to trigger NK cell cytotoxicity upon binding its ligands, CD155 (necl-5, PVR) and CD112 (Nectin-2). As NK cells mature, DNAM-1 is down-regulated resulting

in DNAM-1$^+$ and DNAM-1$^-$ populations, although DNAM-1$^+$ cells exert increased cytokine secretion[33]. DNAM-1 works in synergy with other activating receptors, including Ig-like receptor 2B4 (CD244) to stimulate NK cell activity. Upon binding of its ligands, DNAM-1 is phosphorylated by protein kinase C and Fyn src kinase to induce leukocyte function associated antigen (LFA-1) cross-linking, resulting in the formation of an immune synapse. In addition, DNAM-1 works in conjunction with other activating receptors to induce the phosphorylation of SLP-76, Vav1 and PLC-γ, ultimately resulting in NK cell degranulation and secretion[34,35].

These activating signals are antagonized by inhibitory receptors, some of which function through the "missing-self hypothesis"; NK cells are inhibited by the recognition of constitutively expressed self-molecules. Specifically, inhibitory Killer Immunoglobulin-like Receptors (KIRs) and C-type lectin-like receptor CD94-NKG2A recognize HLA Class I and HLA-E, respectively[9,26]. However, alterations in the balance of these receptors can easily result in hyporesponsive or dysfunctional NK cells. For example, the co-inhibitory receptor T cell immunoreceptor with Ig and ITIM domains (TIGIT) competes with DNAM-1 and binds more strongly to their common ligands CD155 and CD112[27]. Moreover, dysfunctional NK cells have also been observed to upregulate immune checkpoint molecules such as T cell immunoglobulin mucin-3 (TIM-3) and programmed cell death protein 1 (PD-1, CD279)[36,37]. TIM-3 has both activating and inhibitory effects on NK cells, which may be explained by its promiscuous ligand specificity[37].

1.1.2 NK cell activity is also modulated by cytokines

Similar to the integration of activating and inhibitory cell surface receptors, NK cells must also integrate signals from activating and inhibitory cytokines. NK cell effector functions, specifically cytokine secretion, is strongly mediated by IL-2, IL-12, IL-15, and IL-18, which are secreted exclusively by leukocytes and act primarily on T and NK cells[38,39]. IL-2, IL-15, and IL-12 have structurally similar receptors of the type 1 cytokine receptor family, while the IL-18 receptor is part of the immunoglobulin superfamily[38,40].

IL-2 and its receptor (IL-2R) were the first cytokine and associated receptor to be cloned in the 1980s[41]. IL-2R was originally named T cell growth factor (TCGF) due to its ability to enhance T cell proliferation. The high-affinity IL-2R is composed of three subunits: α (CD25), β (CD122), and γ (CD132). Although CD25 alone can bind IL-2 with low affinity, it lacks the cytoplasmic domain responsible for signal transduction[41]. CD132 is known as the "common cytokine receptor γ chain", as it is shared by the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21[6]. In fact, IL-2R and IL-15R share both the β and γ subunits with the α subunits accounting for their specificity. The signal transduction of both receptors is mediated by the cytoplasmic domains of the β and γ chains[41]. Both receptors share Janus kinase (JAK)/ Signal Transducer and Activator of Transcription (STAT) activity, which results in the recruitment of Grb2 and signal transduction through mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways[39,41–44]. These signaling cascades lead to the activation of various transcription factors, including STAT5, c-myc, AP-1, Bcl-2, NFκB, and Elk-1, which are responsible for regulating gene expression, most notably IFNγ production[39,43,45]. In addition, the PI3K/Akt pathway potentiates mammalian target of rapamycin complex 1/2 (mTORC1/mTORC2) formation, affecting transcriptional machinery to control cell growth and proliferation[46,47].

The IL-18R is composed of two subunits: α and β (AcPL). The α subunit can bind IL-18 independently but forms a functionally high affinity receptor complex with the β subunit[40]. IL-18R recruits the canonical adaptor protein Myeloid Differentiation primary response gene 88 (MyD88), which initiates signaling through IL-1 associated receptor kinase 4 (IRAK4) and tumour necrosis factor-associated factor 6 (TRAF6) to activate the transcription factor NFκB as well as p38 MAPK[40,48]. IL-12 and IL-18 act synergistically to enhance cytokine production, albeit through differing signaling cascades[40,49,50].

The IL-12 receptor is composed of a β1 (CD212) and β2 subunit, each independently exhibiting a low affinity for IL-12. CD212 is constitutively expressed and physically associates with p40, a

subunit shared by IL-12 and IL-23[51]. CD212 associates with non-receptor tyrosine protein kinase 2 (TYK2) and IL-12R β2 associates with JAK2, the phosphorylation of which results in the activation and translocation of STAT4 and the activation of transcription factors ERM, T-bet, and c-jun[38,52,53]. IL-12 also activates p38 MAPK via MAPK Kinase 3 and 6 (MEK3/6)[54]. p38 MAPK activity downstream of IL-18 and IL-12 signaling is critical for IFNγ production through posttranslational mRNA stabilization[50,54,55]. This stabilization is mediated by MAP kinase-activated protein kinase 2 (MK2) activity upon adenylate/uridylate-rich elements (AREs), of which human IFNγ contains five copies in its 3' untranslated region (UTR)[50] (**Figure 1**).

IL-10 is a critical immunoregulatory cytokine produced by T cells, B cells, NK cells, macrophages, and dendritic cells (DCs)[56]. It has been described both as "cytokine synthesis inhibitory factor" (CSIF) due to its role as an inhibitor of T helper cell (T_h) 1 activation and cytokine secretion[56], and as "B cell-derived T cell growth factor" (B-TCGF) due to its ability to stimulate $CD8^+$ T cells[57]. IL-10 has since been reported to also inhibit macrophage cytokine and chemokine secretion, cytotoxic T lymphocyte (CTL) CD28 expression and cytokine production, and T_h1 proliferation as well as to antagonize DC activity[58,59]. IL-10 is thus essential to the prevention of autoimmunity. While still widely regarded as a strictly anti-inflammatory cytokine, IL-10 has recently been shown to have pleiotropic effects on NK cells, including increasing *in vitro* NK cell cytotoxicity[59–65]. However, IL-10 seems to inhibit the production of cytokines such as IFNγ and TNFα[58,66]. The IL-10 receptor is composed of two subunits: IL-10R1 and IL-10R2. In NK cells, IL-10 has been shown to activate JAK1 and non-receptor tyrosine kinase 2 (TYK2), resulting in the phosphorylation and activation of STAT1, 3, and 5[56]. The exact pathways by which IL-10 exerts activating versus inhibitory effects on NK cells require further investigation and may be influenced by the activity of other immune cells, as well as the cytokine milieu.

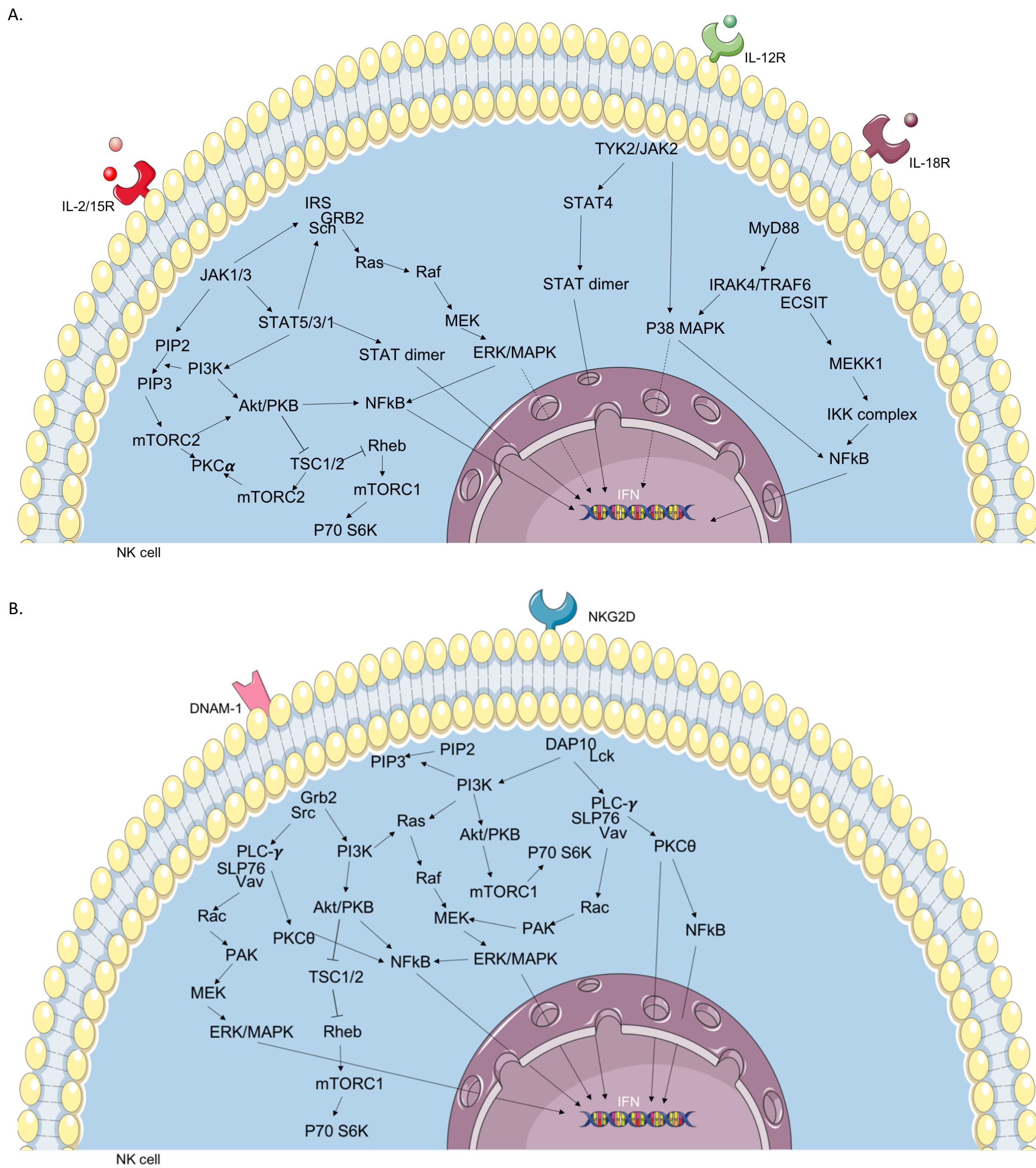

Figure 1. Signaling pathways downstream of IL-2/12/15/18 and activating receptors NKG2D and DNAM-1.

(A) In NK cells, interleukin (IL)-2 and IL-15 signal through convergent pathways; IL-12 and IL-18 signal through different pathways but synergize to induce IFNγ production. (B) NKG2D and DNAM-1 signal through convergent pathways to regulate IFNγ production.

IL-6 was initially identified as a B cell stimulatory factor and is produced by monocytes, macrophages, T cells, and non-immune cells including endothelial cells and fibroblasts[67]. It plays a critical role in inflammation by promoting plasma cell differentiation and antibody production[68], inhibiting regulatory T cell (T_{reg}) formation, inducing the differentiation of T_h17 cells[69], and promoting CTL differentiation[67]. The IL-6 receptor consists of two subunits: IL-6Rα (gp80, CD126) and IL-6Rβ (gp130, CD130). Signaling through this receptor can occur via classical signaling or trans-signaling[69]. In classical signaling, IL-6 binds to IL-6Rα, which triggers its association with gp130. This promotes gp130 dimerization inducing the formation of a heterohexameric complex consisting of IL-6, IL-6 Rα, and gp130. Classical signaling therefore relies on the expression of IL-6Rα. IL-6Rα expression is limited to hepatocytes, macrophages, neutrophils, and resting lymphocytes[69,70]. Trans-signaling allows IL-6 to exert effects on a wider range of cell types in the absence of IL-6Rα expression. In trans-signaling, IL-6 binds to soluble IL-6Rα (sIL-6Rα) and is presented to cell surface gp130. sIL-6Rα can either be produced by proteolytic cleavage from the surface or secretion from neutrophils and monocytes[68]. Regardless of initial pathway activation, IL-6 signal transduction occurs via the activation of many intracellular pathways including: Jak1/TYK2 and downstream STAT1, 3, and 5, the PI3K/Akt pathway, the MAPK pathway, and the MEK- extracellular receptor kinase (ERK)5 pathway[67,69,71].

It is now clear that IL-6 is highly pleiotropic and has both pro- and anti-inflammatory effects. Interestingly, recent studies suggest that pro-inflammatory IL-6 responses are mediated by trans-signaling while regenerative and anti-inflammatory responses are mediated via classical signaling[72]. Specifically, research focusing on NK cells has proven IL-6 to be a potent inhibitor of cytotoxicity. Cifaldi et al. reported reduced expression of perforin and granzyme B in human peripheral NK cells exposed to IL-6[73]. In patients with heart failure, NK cell cytotoxicity was reduced and this corelated with increased levels of IL-6 produced by unstimulated peripheral blood mononuclear cells (PBMCs)[74]. Kang and colleagues examined NK cell function in the context of

endometriosis and found that IL-6 in peritoneal fluid was able to suppress NK cell differentiation and cytotoxicity via the adapter protein tyrosine phosphatase SHP-2[67,75]. More research is needed to clearly elucidate the role of IL-6 as an anti-inflammatory cytokine, and specifically in the regulation of NK cell activity.

Transforming growth factor-β (TGFβ) is a highly pleiotropic cytokine of the bone morphogenic protein (BMP)-activin family and a well-described suppressor of immune activation, including NK cell activity. There are three isoforms of TGFβ: TGFβ1, TGFβ2, and TGFβ3, with TGFβ1 being the primary isoform expressed by immune cells[56,76]. TGFβ is produced as an inactive latent complex with an N-terminal latency-associated peptide (LAP). LAP is cleaved from the mature TGFβ precursor by furin proteases in the trans-golgi networks but remains non-covalently bonded via disulfide links, forming TGFβ-LAP homodimers known as the “small latent TGFβ complex”[77]. The “large latent TGFβ complex” is formed by the addition of latent TGFβ binding proteins (LTBPs)[71,77]. Thus, TGFβ bioavailability is controlled by the regulation of these complexes via proteases and integrins[71]. In addition, it is thought that LTBPs play a role in facilitating protease and integrin activity[77]. TGFβ can also be membrane-bound and is expressed on the surface of T_{regs}, endothelial cells, platelets, and monocytes/ macrophages[30].

The TGFβ receptor binds active TGFβ and is composed of two heterodimer TGFβRI (ALK5)/TGFβRII complexes[76]. TGFβRII is responsible for ligand binding, while the RI subunit possesses cytoplasmic serine/threonine kinase activity responsible for signal propagation[78,79]. In addition to the type I and II receptor subunits, there are two co-receptors, which mediate TGFβ binding, accessibility, and signaling: the membrane glycoprotein endoglin and the proteoglycan betaglycan, formerly known as TGFβRIII[79–81]. Endoglin is expressed only in hematopoietic and endothelial cells, only binds TGFβ1 and 3, and interacts with both TGFβRI and RII[81]. Betaglycan is a ubiquitous co-receptor that can be either membrane-bound or soluble and can bind to all three TGFβ isoforms. It is not required for TGFβ-responsiveness; however, it forms a high affinity

ternary complex with TGFβRI/II and functions to overcome limited RI/II binding availability, acting as a TGFβ repository to control cellular access to TGFβ. Furthermore, the expression of membrane-bound betaglycan correlates with increased TGFβ-TGFβRII binding[79]. However, membrane-bound betaglycan can undergo ectoderm cleavage or "shedding" and exist in a soluble form (sTGFβRIII), although the mechanism of cleavage is poorly understood. Recent research suggests a role for pervanadate and membrane-type matrix metalloproteases (MT-MMPs) in the shedding process[82]. Interestingly, sTGFβRIII serves to sequester TGFβ and prevent its binding and effector functions, having the opposite effect of its membrane-bound counterpart[82,83].

Once bound to its receptor, TGFβ can signal through canonical and non-canonical pathways. The canonical pathway consists of transcription factor Smad2/3 recruitment and phosphorylation, which translocates to the nucleus in complex with Smad4 and directly affects transcription[71,76]. The non-canonical pathways are numerous and include: PI3K/Akt, p38 MAPK, and MAPK, among others[56,76]. The non-canonical pathways may depend on extracellular factors and vary from cell to cell.

TGFβ1 is critical for maintaining homeostasis and preventing autoimmunity. This is evident in TGFβ or TGFβR deficient mice that rapidly succumb to systemic T cell-mediated autoimmunity[56,76]. In fact, TGFβ1 promotes the development of T_{regs} and suppressive myeloid-derived suppressor cells (MDSCs), prevents the activation and differentiation of $CD4^+$ and $CD8^+$ T cells, inhibits the maturation and antigen presenting capacity of DCs via downregulation of MHC II, and dampens NK cell proliferation, cytotoxicity, and IFNγ secretion[30,76,84]. In culture, TGFβ1 can override IL-2 activation of NK cells and induce the downregulation of activating receptors NKp30, NKp46, DNAM-1, and NKG2D[85]. TGFβ1 directly inhibits DNAM-1 at the mRNA and protein levels and causes NKG2D downregulation by increasing miRNA-1245 levels and inhibiting transcription of the critical adaptor protein DAP10[30,86–88]. In addition to the downregulation of NK cell receptors, TGFβ1 is reported to suppress IFNγ production through Smad-dependent repression

of “master regulator” transcription factor T-bet[89]. TGFβ signaling also opposed the IL-15-induced phosphorylation of both S6 and 4EBP1 (mTORC1 substrates) and Akt (an mTORC2 substrate). Moreover, the effect of TGFβ1 was comparable to the mTORC1 inhibitor rapamycin[76]. TGFβ1 was also able to reduce the production of perforin, granzyme B, IFNγ, and macrophage inflammatory protein (MIP)-1β in response to stimulation by K562 human leukemia cells in the presence of IL-2. *In vivo*, NK cell development was arrested by TGFβ1 signaling or mTOR depletion while mTOR activity and NK cell cytotoxicity were enhanced by TGFβRII depletion[78]. Taken together, this indicates a critical role for mTOR in mediating the effects of TGFβ1 in NK cells.

1.1.2.1 A note on mTOR

The serine/threonine kinase mTOR was first discovered in 1992 by Sabatini et al.[90–92] as a target of FK506-binding protein-12 (FKBP12) and rapamycin, a compound with remarkable anti-fungal, immunosuppressive, and anti-cancer effects purified from soil bacteria in 1975[93]. Schreiber and colleagues demonstrated its autophosphorylation while Abraham et al. identified 4E-BP1 as its first substrate[94,95]. mTOR was then identified to form two distinct complexes; with regulatory associated protein of mTOR (Raptor) to form mTORC1, and Raptor-independent companion of mTOR (RICTOR) to form mTORC2[96]. mTORC1 has been shown to play a central role in controlling NK cell metabolism, education, and effector functions[38,97]. Basal metabolic activity (specifically oxidative phosphorylation), is necessary for NK cell metabolic fitness, degranulation, and IFNγ secretion in response to activating receptor ligation and stimulatory cytokines in both mice and humans[98]. mTORC2 regulates actin cytoskeleton remodeling for establishment of immunological synapses[99]. There are no specific inhibitors of mTORC2, which limits the investigations into the roles played by mTORC2[98], although they likely contribute to similar metabolic and effector functions. Thus, mTOR functions as a “molecular rheostat” to control NK cell metabolism, education, and activity[97].

1.1.3 NK cell role in tumour immunity

The functional features of NK cells perfectly position them as natural cancer killers with the ability to respond to tumour cells in the absence of immunological priming. For example, numerous induced self-ligands are upregulated during oncogenic transformation, including NKG2D ligands MICA/B and ULBP1-6[100] and DNAM-1 ligands CD155 and CD112[101]. Death receptors, including TRAIL-R1/2, are also widely expressed on tumour cells[102,103]. Furthermore, tumour cells downregulate HLA Class I in an attempt to escape T cell mediated responses[104], simultaneously releasing the inhibitory "break" on NK cells. By tipping the balance towards activation, NK cells are then able to directly kill cancer cells (via death receptors and cytotoxic granules) and to rally other immune cells to do the same (via immunomodulatory cytokines).

NK cells are able to induce cancer cell apoptosis via two pathways: death receptor engagement and cytotoxic granule secretion. Currently, six different death receptors have been described: CD95, TNF receptor-1, TNF receptor-related apoptosis-mediating protein (TRAMP), death receptor-6 (DR6), and two TRAIL-binding receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5)[105]. The ligation of these receptors induces the recruitment of intracellular adapter molecules, which initiate caspase-dependent programmed cell death via death effector domains (DED) or caspase recruitment domains[106]. Cytotoxic granule secretion by NK cells requires formation of an immunological synapse and reorganization of the actin cytoskeleton to facilitate polarization of secretory lysosomes for degranulation and fusion with the target cell plasma membrane[107]. Perforin polymerizes to form pores in the target cell membrane that facilitate the entry of granzymes, which activate caspase-dependent and -independent apoptotic pathways[106,107]. During degranulation, lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) and -2 (LAMP-2 or CD107b) transiently appear on the NK cell surface and can therefore be used to indirectly measure NK cell cytotoxicity[106].

In addition to directly killing tumour cells, NK cells secrete a plethora of cytokines. Specifically, IFNγ is of particular interest due to its critical role in the anti-tumour response. IFNγ

is a type II interferon produced predominantly by NK and T lymphocytes[108]. NK cells contain constitutive IFNγ cytokine transcripts to facilitate early and rapid cytokine production[109]. IFNγ signals through its heterodimeric receptor to activate the transcription factor interferon response factor 1 (IRF1), which regulates the expression of secondary response genes, accounting for its pleiotropic effects[16]. Tumour cell induction of IFNγ production can be indirect through interactions with activated immune cells, including dendritic cells, macrophages and B cells, as well as soluble factors such as IL-2, IL-12, IL-15 and IL-18, derived from a plethora of immune cells and found systemically in circulation[38,39,108,110–113]. Direct mechanisms of activation include cell-cell contact (NK cells and NK cell recognition of tumour specific ligands and decreased constitutive self-signals) as well as through ADCC[13,17,114]. As an integral part of the anti-tumour response, IFNγ has significant immunologic and non-immunologic actions. Its non-immunologic actions include anti-proliferative, pro-apoptotic, and anti-angiogenic effects that inhibit tumour cell growth[108]. Furthermore, IFNγ increases tumour cell immunogenicity through the upregulation of MHC Class I genes[16]. The truly immunologic effects of IFNγ are immunomodulatory and involve endocrine and autocrine regulation of immune cell differentiation, activation, and homeostasis[16,108,113]. The ability to secrete IFNγ is therefore critical, as it is produced in response to tumour and immune stimuli and facilitates communication between the innate and adaptive branches of the immune system (**Figure 2**).

Unsurprisingly, circulating NK cell function is correlated with clinical cancer outcomes in both solid and hematological malignancies[115]. Reduced activating receptor expression and decreased cytolytic activity is associated with increased tumour incidence and decreased overall survival (OS)/recurrence-free survival (RFS) in both murine models and human patients. Furthermore, in murine metastasis models, NK cell depletion and genetic deficiency of IFNγ or perforin results in increased metastasis[115]. In addition, TME-associated NK cells display compromised IL-15 signaling, decreased expression of key transcription factors including Eomes

and T-bet, lower effector molecule expression, defective maturation, and dysregulated metabolism in addition to reduced activating receptor and increased inhibitory receptor expression[115]. The mechanisms of suppression by cancer cells include: upregulation of immune checkpoint expression, including PD-1, prolonged exposure to MHC class 1-deficient tumour cells and/or NK cell activating receptor antagonists[27,30], the secretion of tumour cell-derived exosomes[107], as well as the secretion of inhibitory tumour-derived cytokines, such as IL-10 and TGFβ[115–117]. In addition, tumour cells employ a variety of mechanisms to down-modulate surface activating receptors such as NKG2D, including: the proteolytic-cleavage or exosomal release of soluble ligands to modulate expression through multivalent cross-linking (ligation) and receptor internalization, downregulation of ligands via miRNAs, epigenetic changes and transcriptional repression, as well as signaling downstream of soluble inhibitory factors, such as TGFβ[30]. Thus, despite their incredible ability to recognize and target tumour cells, NK cell function can be profoundly suppressed by tumour cells.

1.2 Natural Killer-T cells

Natural Killer-T (NKT) cells are a subset of CD1d-restricted T lymphocytes that express a rearranged T cell receptor (TCR)[118]. NKT cells make up 0.01-0.1% of peripheral blood lymphocytes[119–121]. Their NK cell-like features include early activation and the rapid production of cytokines, such as IFNγ, in response to stimulation, including stimulation with IL-2 and IL-12 cytokines[122–125]. Type I NKTs are also called invariant NKT (iNKT) cells due to their expression of an invariant TCR α-chain and restricted β-chain repertoire. Type II NKT (NKT_II) cells express a broad range of TCR chain combinations and therefore encompass a more heterogenous population of cells[118,120]. In patients with hematological and solid malignancies iNKT cell frequency and function are reduced which has been shown to correlate with poor OS. Furthermore, elevated iNKT frequency in the TME is a positive prognostic indicator[126]. For these reasons, iNKT cells are being studied as possible immunotherapeutic targets to increase iNKT cell activation and anti-tumour

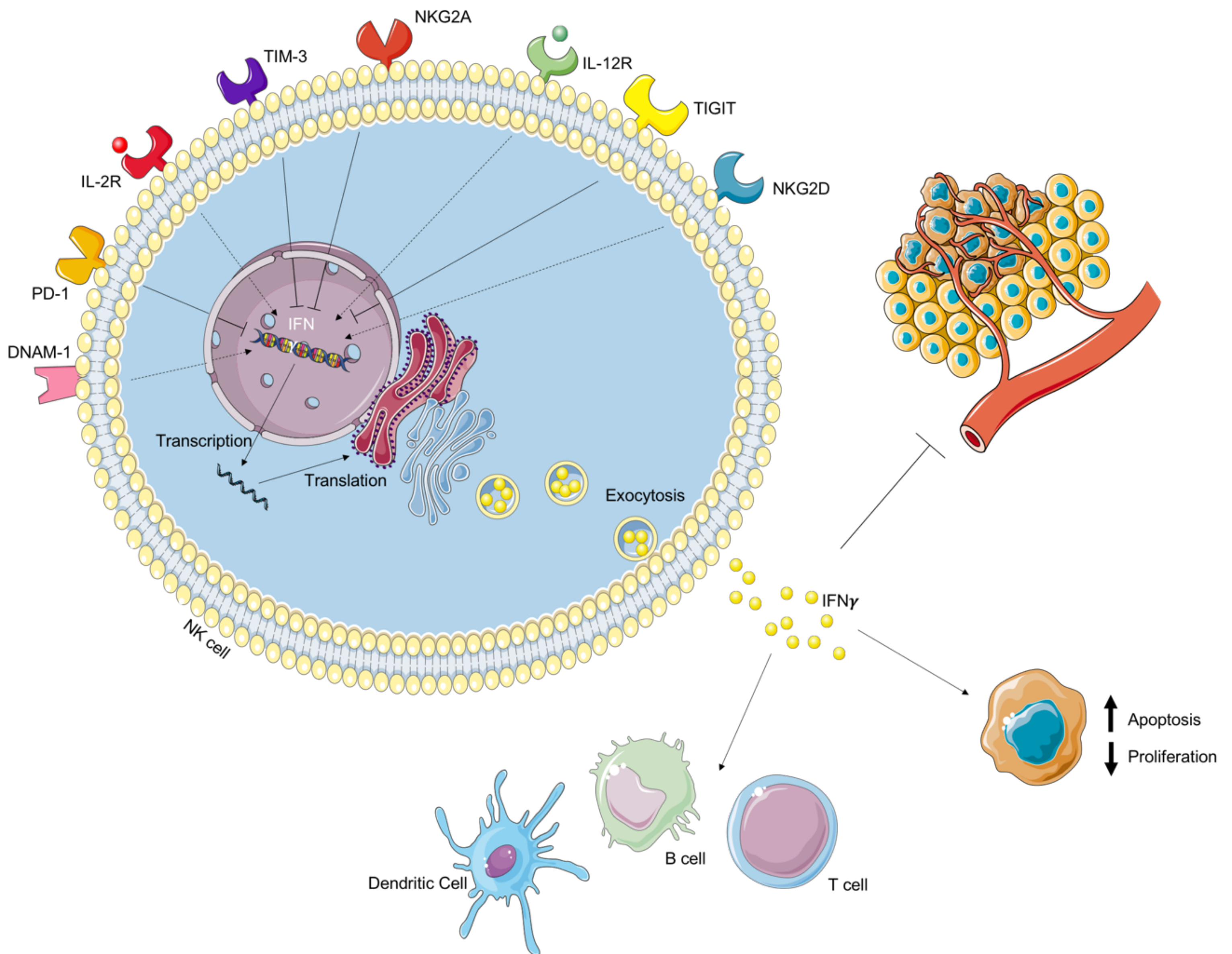

Figure 2. A summary of NK cell activation and IFNγ-mediated anti-tumour effector functions.
NK cell IFNγ production in controlled by the integration of activating/inhibitory signals through germline-encoded receptors. Soluble IFNγ exerts direct anti-tumour effects by inhibiting angiogenesis and proliferation and inducing tumour cell apoptosis and indirect anti-tumour effects through modulation of innate and adaptive immune cells.

function. Interventions currently being investigated include: iNKT cell agonists (α-GalCer), iNKT cell transfer, or antigen presenting cells (APCs) pulsed with α-GalCel[126]. Thus, iNKT cells may also important in the postoperative period, although there is currently no known literature on this subject.

1.3 A brief history of cancer surgery

The treatment of cancer has long been a medical challenge, as has been recorded in the earliest chronologies of human existence. Although the word "cancer" was not used, the oldest description of this disease can be found in the Edwin Smith Papyrus, an ancient Egyptian text that dates back to 3,000-2,500 B.C[127–129]. The text describes breast tumours that were surgically removed using primitive tools, such as the "fire drill"[128,129]. These treatments proved futile and this record concluded that "there is no cure" [128,130]. Surgery continued to be provided as a largely unsuccessful treatment for a variety of cancers, until the development of anesthesia in 1846 radically changed the field of surgery and allowed for longer, more complex surgeries[131]. In 1890, William Halsted developed the Halstedian model for cancer progression and proposed that cancer (specifically breast cancer), spreads through the lymphatic system. This resulted in a strong emphasis on aggressive locoregional treatment in an attempt to avoid cancer recurrence[132,133]. There was a rapid development of cancer surgery techniques ranging from abdominoperineal resection, to pneumonectomy, to radical hysterectomy, and radical suprapubic prostatectomy in the early 20th century[132,134]. Halstedian surgical techniques were later shown to provide little benefit over less invasive procedures and have since been replaced by less radical, modern procedures that have significantly improved patient quality of life[132,135,136]. Surgery provides undeniable benefits to cancer patients as the removal of the primary tumour relieves mass effect, prevents the release of tumour-associated factors, and allows for the pathological assessment of the cancer to inform treatment, among others[137]. In addition to surgery, the evolution of radiotherapy and chemotherapy continued into the late 20th century and remain critical pillars in the treatment of solid cancer

today[132]. However, despite the advancements in cancer treatment, remission rates have plateaued, due mostly to our inability to effectively eradicate micrometastases[132,134]. In effect, what the Roman physician Celsus wrote in 100 BC still holds true: "After excision, even when a scar has formed, none the less the disease has returned."[138]

1.4 The Surgery and Metastasis Paradox

Paradoxically, in contrast with the life-prolonging advantages of surgery, surgical resection has long been linked to increased metastases and cancer recurrence[139]. This link was first made more than a century ago when Marie and Clunet found that implanted tumours that rarely developed spontaneous metastases had increased rates of metastasis if the primary tumour was incompletely excised[140]. This was followed by Tyzzer, who showed that high-metastasizing tumour implants developed larger metastases as a result of tumour excision[141]. While Tyzzer discussed the potential for micrometastatic spread as a direct result of tumour manipulation, both phenomena were ultimately attributed to the "athrepsia hypothesis", whereby tumour proliferation was dependent upon competition for host-derived nutrients[142]. By the mid-20th century more modern hypotheses on cancer growth and metastasis developed, leading to the idea of the "dormant" cancer cell: cancer cells that remain quiescent while still retaining their ability to proliferate[143]. Various investigators wrote about animal models in which dormant tumour cells could be triggered to grow by some unknown mechanism in response to surgery[139,144]. The question still remains: ***What is this mechanism?*** **(Figure 3)**

The answer to this question is complex, in that there are many mechanisms which may contribute to increased postoperative cancer recurrence. These include manipulation of the tumour, tissue hypoxia and angiogenesis, neuroendocrine activation, the pro-inflammatory phase, a hypercoagulable state, and the anti-inflammatory phase, each of which will be discussed below. Ultimately, however, the majority of these mechanisms impact immune cell function, culminating in suppressed cellular immunity. Surgical stress has been shown to cause a decrease in circulating

DCs, and significant dysfunction in T cells and NK cells[145–152]. Cellular immunity is critical for the anti-tumour response and thus, its dysfunction may be essential for the development of postoperative metastases.

1.4.1 Manipulation of the tumour

Partial or gross total resection of solid tumours results in an increase in circulating tumour cells (CTCs) and is associated with local and systemic tumour cell seeding and metastases[152,153]. The risk of tumour cell dissemination increases with tumour grade and stage, tumour manipulation or rupture during surgery, and involvement of tumour margins[152]. Strategies to combat this include the strict implementation of oncologic surgical techniques as well as adjuvant chemotherapy and radiation therapy to target micrometastases[153,154].

1.4.2 Tissue hypoxia and angiogenesis

Intra- and postoperative hypoxia and hypoxemia are common and persistent consequences of surgery[155–158]. Tumour cells have evolved to use hypoxic stress to their advantage. The result is advanced but dysfunctional vascularization characterized by leaky vessels that facilitate tumour cell escape, acquisition of an epithelial-to-mesenchymal transition (EMT) phenotype, and abnormal cell signaling, including the activation of Hypoxia-Inducible Factor (HIF) pathways[159,160]. HIFs are "master regulator" transcription factors that influence gene expression, most notably the production of vascular-endothelial growth factor (VEGF)[160]. VEGF signals through VEGFRs, tyrosine kinase receptors, resulting in the activation of the MAPK/ERK and PI3K/Akt pathways[160]. Through the activation of these pathways, VEGF plays a critical role in promoting tumour cell growth and survival through angiogenesis, reducing tolerance to immune surveillance, and immunosuppression[159,160]. HIF pathways and VEGF can also contribute to immune suppression and tolerance to immune surveillance by promoting MDSC accumulation, inhibiting DC maturation, recruiting T_{regs}, and impairing NK cell functions[160–162].

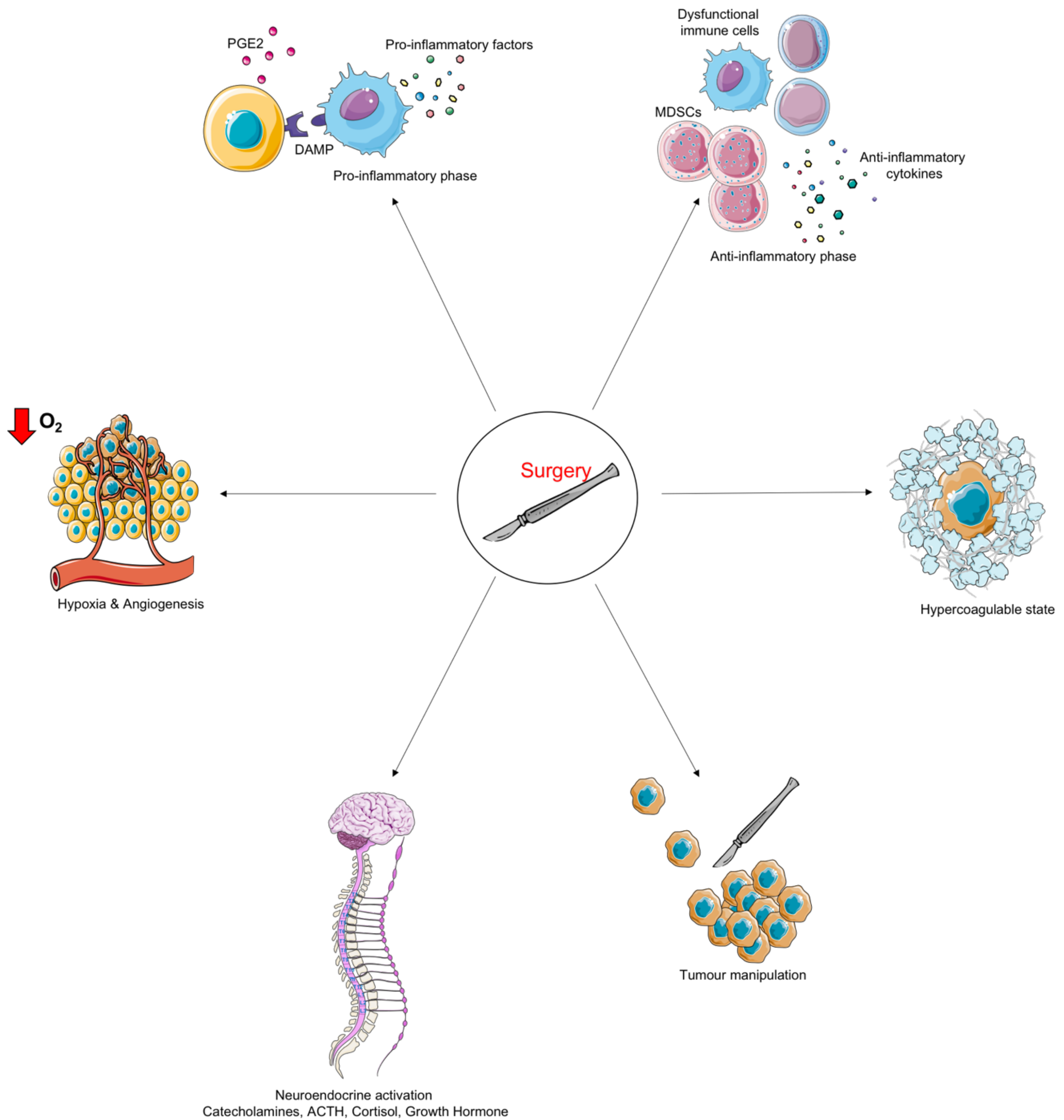

Figure 3. Potential mechanisms of postoperative cancer recurrence.

Numerous changes occurring in the postoperative period have been hypothesized to be responsible for postoperative metastasis and cancer recurrence. These include physical manipulation of the tumour, tissue hypoxia and angiogenesis, neuroendocrine activation, a pro-inflammatory phase, a hypercoagulable state, and an anti-inflammatory phase characterized by the release of anti-inflammatory cytokines and the expansion of immunosuppression populations in addition to cellular immune suppression.

While it remains to be investigated, hypoxia as a result of surgery may therefore play a role in postoperative metastases and cancer recurrence[163,164]. Potential therapies to target surgery-induced hypoxia include the anti-VEGF monoclonal antibody (mAb) bevacizumab, the tyrosine kinase small molecule inhibitors sorafenib and sunitinib, and the antineoplastic compound everolimus, which inhibits mTORC1 formation downstream of PI3K/Akt activation[160]. These therapeutics have been tested with some success in both mice and humans, although early clinical benefit has been followed by tumour progression and the induction of compensatory hypoxia, in addition to wound healing complications after surgery[160,165].

1.4.3 Neuroendocrine activation

Initially described in response to accidental injury or trauma, the "stress response" is a series of complex physiological events that include neuroendocrine and hypothalamic-pituitary-adrenal (HPA) axis activation leading to significant metabolic and immunological changes[166,167]. The stress response is activated by efferent neuronal impulses from the site of injury, which travel to the medulla to stimulate the HPA axis[166]. This results in the activation of the sympathetic autonomic nervous system, the release of catecholamines from the adrenal medulla and norepinephrine from presynaptic nerve terminals[167]. Activation of adrenergic receptors located throughout the body lead to tachycardia, hypertension, and changes in organ function[167]. In addition, corticotrophin (ACTH) released by the pituitary stimulates the adrenal cortex to release the stress hormone cortisol. Growth hormone is also released due to HPA activation during the stress response and levels are directly related to the severity of the injury[166].

Surgery is one of the most potent activators of ACTH/cortisol and inhibits the normal negative feedback mechanism, resulting in increasing plasma levels of both hormones[166]. ACTH, cortisol, and growth hormone have complex metabolic effects that are evolutionarily essential for survival post-trauma, although it has been argued that this response to sterile surgery is unnecessary[166].

Interestingly, cancer cells also respond to adrenergic stimulation resulting in increased tumour growth and invasive potential, increased matrix metalloproteinase production, and the production of VEGF[167]. Furthermore, multiple studies have highlighted cortisol and growth hormone, due to chronic stress or tumour cell-derived, as key regulators of tumour cell growth, proliferation, and metastasis either directly or through immune suppression[166,168–174]. In fact, cortisol has been shown to inhibit macrophage and neutrophil accumulation and to inhibit the synthesis of inflammatory mediators, including prostaglandin-E2 (PGE2)[166]. In addition, HPA axis activation has been shown to inhibit NK cell activity[175–179]. Current research has focused on targeting adrenergic receptors using β-blockers with promising results showing reduced invasive potential and tumour growth *in vitro* and reduced cancer recurrence or severity in cancer patients[166,167,180]. In addition, the cortisol response can be modified by anesthetic (opioids, including morphine) through inhibition at the hypothalamic level[181]. Thus, neuroendocrine activation and the stress response may play a role in postoperative cancer recurrence by directly stimulating tumourigenesis in addition to suppressing the cellular immune response.

1.4.4 The pro-inflammatory phase

Despite advancements in surgical technique, there is always injury to healthy tissue resulting in an inflammatory response[163]. More specifically, surgical stress induces an acute pro-inflammatory response, mediated by various cytokines and the pro-inflammatory mediator PGE2. This response is initiated by the release of damage-associated molecule patterns (DAMPs) as a result of cellular injury, which are recognized by pattern recognition receptors (PRRs) on monocytes. Activated macrophages and monocytes then release IL-1 and TNFα and this initiates the cascade of pro-inflammatory cytokines which serve to recruit immune cells to the site of injury[163,182,183]. While necessary for wound healing, these soluble factors also stimulate cancer cell proliferation and migration and when combined with disrupted endothelium constitute an ideal

location for CTC seeding after surgery[184]. This phenomenon has been termed "inflammatory oncotaxis"[184].

One soluble factor of particular importance is monocyte-derived IL-6[166,183]. IL-6 increases within 30-60 minutes of tissue damage, peaks at 24 hours, and remains high for 72 hours post-trauma. In addition, the magnitude of IL-6 production reflects the degree of tissue damage and therefore, less is produced in response to laparoscopic versus open surgery[166,182,185]. IL-6 is responsible for inducing the "acute phase response" characterized by the production of liver proteins such as C-reactive protein (CRP), α_2 microglobulin, and other proteinases. IL-6 also plays a critical role in tumourigenesis, in addition to mediating tumour therapeutic resistance[186]. IL-6 can be secreted into the TME by tumour cells and tumour-associated fibroblasts and increased levels of IL-6 are correlated with poorer prognosis in many cancer types[186–190]. Specifically, IL-6 has been shown to act on cancer cells through JAK1/2/STAT3, making the blockade of this pathway a promising therapeutic approach for many cancers associated with the overexpression of IL-6. IL-6 is a critical factor responsible for mediating the anti-inflammatory switch postoperatively and contributes to immune suppression, specifically acting to potently suppress NK cell cytotoxicity[73,182]. Currently, there are clinical trials investigating the safety and efficacy of IL-6 direct inhibitors (e.g. siltuximab), IL-6Rα direct inhibitors (e.g. tocilizumab), IL-6R gp130 direct inhibitors (e.g. raloxifene, bazdoxfene), and JAK inhibitors (e.g. ruxolitinib). Trials to date show mixed results with the majority of therapeutics being well-tolerated but ineffective[186,191]. This is perhaps due to the dual pro- and anti-inflammatory effects of IL-6, partially mediated by differences in classical signaling versus trans presentation via soluble IL-6Rα[175,182,186].

Prostaglandins also mediate the pro-inflammatory response to surgery. PGE2 is synthesized from plasma membrane arachidonic acid by the enzyme cyclooxygenase-2 (COX-2) and is released in response to tissue damage[180]. It is essential for wound healing, however, it can also act directly on malignant cells to induce metastatic activity, proliferation, adhesion, migration,

extracellular matrix invasion, resistance to apoptosis, and the secretion of proangiogenic factors[180]. Interestingly, PGE2 can also be released by tumour cells and is associated with increased tumour size and stage, recurrence, and decreased OS[180]. In fact, COX-2 inhibition has been shown to increase tumour cell apoptosis, decrease proangiogenic agents, and reduce tumour microvascular density[192–194]. For these reasons, non-steroidal anti-inflammatory drugs (NSAIDs), most notably COX-2 inhibitors, have been studied as long-term chemopreventers of malignancy, but are now an enticing therapeutic option for the prevention of surgical stress-induced metastases[167,180,195]. PGE2 also suppresses anti-tumour immunity through promoting macrophage differentiation to the pro-tumoural M2 phenotype, increasing T_h2 dominance, and directly inhibiting NK cell effector functions through four endogenous PGE2 receptors, EP1-4[175,180]. Interestingly, the small molecule inhibitor RQ-15986 has been shown to block EP4-mediated NK cell suppression and inhibit breast cancer metastasis *in vivo*[196], and therefore may prove to be a viable therapeutic to combat surgery-induced NK cell suppression and metastasis. Thus, although short-lived, the postoperative pro-inflammatory phase may contribute to metastatic formation via the release of soluble factors like IL-6 and prostaglandins, which have been shown to directly induce tumourigenesis and contribute to cellular immune suppression.

1.4.5 The hypercoagulable state

Surgery and cancer independently have been associated with a hypercoagulable state. This is characterized by increased tissue factor, fibrin, and thrombin, platelet activation, and the formation of clots around tumour cell emboli (TCE)[197,198]. Platelet-fibrin coating of TCE may facilitate micrometastases through the dysregulation of matrix metalloproteases, which promote tumour cell migration and metastasis, the secretion of proangiogenic and mitogenic factors including plasmin and VEGF[199–205], and mediating tumour cell adherence to endothelial cells[197]. In murine studies, platelet depletion and P-selectin deficiency reduced experimental metastases and fibrinogen-deficient mice demonstrated significantly reduced implanted and spontaneous

metastatic foci[200,201,206–209]. Clinically, perioperative anticoagulation has been associated with increased cancer-specific survival in cancer surgery patients[210,211]. Seth et al. investigated the role of surgery-induced hypercoagulation on metastases in a murine model and found that treatment with low-molecular weight heparin (LMWH) was able to reduce metastases through the inhibition of peritumoural clot formation[197]. Furthermore, this effect was NK cell-mediated in that LMWH was unable to attenuate metastasis in NK cell-depleted surgically stressed mice[197]. The ability of LMWH to reduce metastases and increase survival has been documented in both murine models and patients with solid malignancies[210–214]. Thus, evidence suggests that the formation of a hypercoagulable state postoperatively can potentiate metastases and prevent NK cell recognition of tumour cells. For these reasons, our group is conducting a Phase 3 clinical trial in which perioperative LMWH is given to colorectal cancer surgery patients in an effort to reduce postoperative metastasis (PERIOP-01; NCT01455831)[215,216].

1.4.6 The anti-inflammatory phase

The prolonged anti-inflammatory phase that is now known to follow the acute pro-inflammatory phase postoperatively was first described by Bone et al. in 1997 as “CARS” or “compensatory anti-inflammatory response syndrome” in the context of sepsis[217]. They described a compensatory reaction that could be as great or greater than the initial pro-inflammatory response, the purpose of which was to restore homeostasis[217]. It is now understood that there are concurrent pro- and anti-inflammatory responses throughout the postoperative period, with the degree of inflammatory cytokine production reflecting the extent of surgical trauma[183]. The anti-inflammatory phase is characterized by the release of anti-inflammatory cytokines as well as the expansion of immunosuppressive populations.

1.4.6.1 Anti-inflammatory cytokines

As described above, NK cell activity is determined by signal integration, including via cytokines. Cytokines such as IL-6, IL-10, and TGFβ have pleiotropic effects on a plethora of cell types and can act to inhibit NK cell activity.

As described in 1.3.4, IL-6 is a critical cytokine that is increased in response to surgery and mediates postoperative inflammation. In addition to this role, IL-6 also serves as the switch between pro- and anti-inflammatory phases by inducing anti-inflammatory factors including glucocorticoids, soluble TNFα receptors, TGFβ, and PGE2, which acts as an immunosuppressant to negatively regulate monocyte and T cell function, induce IL-10 production, and induce arginase-1 (ARG1) expression in MDSCs[183,218]. Furthermore, IL-6 has pro-tumourigenic effects that make it a potential therapeutic target for reducing postoperative cancer recurrence.

IL-10 has also been shown to increase significantly following surgical stress[219–221], which may have detrimental effects for patients in terms of cancer recurrence. The impact of IL-10 on NK cell function is complex and somewhat unclear and unsurprisingly, *in vitro* and *in vivo* studies have also reported conflicting evidence as to the role of IL-10 in cancer progression. In animal models, IL-10-expressing tumours are rejected in immunocompetent mice and this is dependent upon $CD8^+$ T cells[57]. In addition, IL-10 has been shown to inhibit cancer growth in murine models by hampering tumour angiogenesis and invasiveness and increasing the recruitment of macrophages and neutrophils. Cell lines derived from mouse mammary adenocarcinoma, ovarian carcinoma, and melanoma transfected with IL-10 show increased immunogenicity and reduced tumourigenicity in conjunction with strong immune memory[59,60]. In humans, IL-10 deficiency results in the development of lymphomas[57]. Conversely, circulating IL-10 has been associated with worse prognosis in patients with solid and hematological malignancies[60,222]. In addition, *in vitro* IL-10 can act as a tumour growth factor to enhance human melanoma cell proliferation[59,60]. Thus, the exact role of IL-10 in mediating tumouigenesis is controversial and remains incompletely characterized.

While anti-IL-10 therapies have shown some success in the treatment of inflammatory diseases such as Crohn's, psoriasis, and ulcerative colitis, its pleiotropic effects in the context of cancer make it a difficult therapeutic candidate[57].

TGFβ is essential to wound healing. While overwhelmingly anti-inflammatory, early in wound healing TGFβ does act to recruit immune cells to the trauma site, although this induces a negative feedback loop via the release of superoxide from macrophages. During granulation tissue formation, TGFβ induces the expression of fibronectin, collagen I and III, and VEGF in addition to improving the angiogenic properties of endothelial progenitor cells, promoting keratinocyte migration, and stimulating contraction of fibroblasts[223]. TGFβ is therefore important in postoperative wound healing in response to surgical trauma, however there is a paucity of research investigating postoperative TGFβ. Our group profiled 26 cytokines and chemokines using a multianalyte protein array in B16LacZ tumour-bearing surgically stressed and untreated mice[224]. At 18 hours post-operation, surgically stressed mice showed a significant increase in plasma TGFβ, IL-5, and IL-6[224]. In the context of cancer, TGFβ plays a dual role. TGFβ acts a tumour suppressor mainly through the inhibition of cell cycle progression through G1-arrest, the "cytostatic/cell proliferation" pathway. In addition, TGFβ can both induce and suppress apoptosis, maintain genomic stability thereby preventing cellular immortality, modulate growth factors produced in the stroma, and suppress immune and inflammatory processes[81]. However, tumour cells can evolve to evade these mechanisms through inactivating mutations in core TGFβ signaling proteins, including receptors and Smads, in addition to downregulating TGFβ receptor expression on tumour cell membranes[81,225]. In fact, mutations in these pathways have been found in a plethora of solid and hematological malignancies[81]. Through this loss of the cytostatic pathway, TGFβ takes on a paradoxical pro-tumourigenic role. TGFβ induces EMT, contributes to a favourable microenvironment for tumour growth, and facilitates immune system evasion[81,225]. In addition to the inhibition of NK cells through the downregulation of activating receptor

expression, TGFβ also suppresses the transcription of cytolytic factors and IFNγ in NK cells and CTLs and inhibits APC effector functions[81,117]. Unsurprisingly then, TGFβ can be secreted by tumour cells and is pathologically upregulated in humans as a result of tumour cell proliferation (ex: hepatocellular carcinoma, glioma, gastric cancer, prostate cancer, lung cancer, and CRC) and is a negative predictor of DFS and OS[81,225–228]. Moreover, there is a direct relationship between TGFβ1 levels and metastatic burden in a variety of cancers[81,229]. Interestingly, Viel et al. showed that suppression of TGFβ signaling in NK cells reduced metastases in two murine models of cancer[76]. Furthermore, in cancer patients NK cells exhibit suppressed phenotypes similar to that of postoperative NK cells, including reduced IFNγ production and cytotoxicity[230–232].

While some studies have quantified TGFβ postoperatively, none have assessed TGFβ in the plasma immediately pre- and post-operatively. Shariat et al. investigated levels of IL-6, IL-6sR, and TGFβ preoperatively and at 6-8 weeks postoperatively in prostate cancer patients and found increased levels of TGFβ postoperatively[226]. Scherer et al. reported high levels of TGFβ in wound fluid from low-risk breast cancer patients collected for 24 hours after breast-conserving surgery[233]. Therefore, although there exists a knowledge-gap in the functions of TGFβ in the postoperative period, it is known to have anti-inflammatory and pro-tumourigenic properties and may therefore be a provocative mechanism to target to prevent postoperative immune suppression and prevent cancer recurrence. Potential therapies that could be used against TGFβ postoperatively can be divided into three categories: (1) preventing TGFβ synthesis, (2) preventing ligand-receptor interactions using ligand traps (soluble receptors and mAbs) and anti-receptor mAbs, and (3) preventing TGFβ signal transduction[30,81]. However, due to its pro-tumourigenic potential and its effects on the wound-healing process, understanding TGFβ's role in postoperative immune suppression and metastases is crucial for the development of effective therapeutics.

1.4.6.2 Immunosuppressive populations

The major postoperative suppressive cell populations consist of T_{regs} and MDSCs[175,182].

1.4.6.2.1 Regulatory T Cells

The existence of suppressor T cells was first proposed in the early 1970s, however a lack of molecular markers prevented research into these cells until the mid 1990s when Sakaguchi et al. identified CD25 as a phenotypic marker in mice[234]. $CD4^+$ T cells have since been divided into two lineages: conventional T helper cells (T_h) and regulatory T cells (T_{regs})[235]. T_{regs} are now defined broadly as $CD4^+CD25^+$ and transcription factor forkhead box P3 $(FoxP3)^+$ [235,236]. FoxP3 was discovered as a lineage-defining transcription factor in mice, as 90% of T_{regs} express FoxP3 while conventional T cells do not. However, activated human conventional T cells transiently express FoxP3, resulting in some controversy as to whether this should be used as a human T_{reg} marker[236]. T_{regs} are a heterogeneous population which can be divided into: $CD45RA^+FoxP3^{low}$ resting T_{regs}, $CD45RA^-FoxP3^{high}$ activated T_{regs}, and $CD45RA^-FoxP3^{low}$ cytokine-secreting T_{regs}[237]. Natural T_{regs} (nT_{regs}) develop in the thymus driven by the need to control autoantigen reaction and autoimmunity, while inducible T_{regs} (iT_{regs}) derive from T_h cells in the presence of IL-2, TGFβ, and IL-10[238]. Finally, T_{reg} function and proliferation are promoted by catecholamines and prostaglandins[239–243].

T_{regs} play a critical role in the prevention of autoimmunity, allergy, infection-induced organ pathology, transplant rejection, and graft versus host disease (GvHD) through the suppression of $CD4^+$ and $CD8^+$ T cells, B cells, NK and NKT cells, monocytes, and DCs[235,236]. T_{regs} were initially studied for their ability to suppress conventional T cell TCR-induced proliferation and IL-2 transcription through contact-dependent and -independent mechanisms[236]. In addition to antigen-specific suppression, T_{regs} have also been shown to suppress immune cell function in the absence of antigen recognition through the secretion of soluble factors, termed "bystander suppression"[244]. Conventional T cells are also suppressed by T_{regs} indirectly through modulation of the activation status of APCs[236]. T_{regs} constitutively express cytotoxic T-lymphocyte associated protein 4 (CTLA-

4), an immune checkpoint receptor capable of inhibiting APC effector functions by binding its ligands CD80 and CD86[236]. Numerous T_{reg}-mediated NK cell suppressive mechanisms have been identified, most notably: cytokine and soluble factor release[236,245], membrane-bound TGFβ[236,246], and competitive IL-2 consumption[245]. T_{regs} have been reported to secrete the suppressive cytokines IL-10, TGFβ, and IL-35 in addition to serine proteases granzyme A and B, and perforin, resulting in target cell apoptosis[236]. Membrane-bound TGFβ mediates contact-dependent mechanisms of suppression, with studies showing that blocking this interaction can prevent immune cell inhibition[236]. Finally, T_{reg}-mediated IL-2 consumption and competition has been suggested to induce immune cell apoptosis, although this remains controversial[236].

T_{regs} are essential for maintaining self-tolerance to prevent autoimmunity, however they also exert detrimental effects on anti-tmour immunity[236,247]. Specifically, T_{regs} are reported to infiltrate the TME in many murine and human tumours, and high infiltration is associated with poor prognosis[248–252]. For this reason, T_{reg}-specific immunotherapies have emerged as a promising therapeutic option for cancer patients[175]. In terms of surgical stress, T_{regs} were reported to decrease immediately following surgery, due to their association with the TME[175]. However, Saito et al. collected blood from cancer patients preoperatively and postoperatively until postoperative day (POD) 6 and found that regulatory T cell subsets increased to higher levels than those observed preoperatively and that this increase was proportional to surgical stress and invasiveness of the surgery[253], revealing T_{regs} to be a novel marker of surgical stress. Thus, the expansion of T_{regs} in the postoperative period may play a critical role in the maintenance of an anti-inflammatory state resulting in cellular immune suppression and cancer recurrence. Potential T_{reg}-specific therapeutics that could be utilized in the perioperative period include: checkpoint blockade, inhibitory cytokine antagonists, preoperative administration of IL-2, and COX-2/β-blocker combination therapy aimed at blocking T_{reg} proliferation and function[175].

1.4.6.2.2 Myeloid-Derived Suppressor Cells

Evolutionarily, myeloid cells are important host protectors, acting to prevent infection and aid in tissue remodeling[254]. The myeloid cell population is diverse, and under physiological conditions myelopoiesis, driven by GM-CSF, results in granulocyte-monocyte progenitors (GMPs)[255]. GMPs can then differentiate into either granulocytes (polymorphonuclear neutrophils, eosinophils, basophils, or mast cells) or mononuclear myeloid cells (monocytes, macrophages, and DCs) via G-CSF or M-CSF stimulation, respectively[254,256]. Myelopoiesis is an essential mechanism to protect the host from pathogenic stimuli, as it results in the expansion of activated neutrophils and monocytes[254]. Classical activation of these cells occurs via toll-like receptor (TLR) ligands, PAMPs and DAMPs, resulting in an acute but robust response to infection. This response consists of increased phagocytosis, respiratory burst, production of pro-inflammatory cytokines, and upregulated MHC II and costimulatory molecules[254,256]. However, chronic inflammation, infection, and cancer result in persistent myelopoiesis that generates myeloid cells with aberrant genomic profiles and suppressive activity[254,255]. This mechanism could have evolved to counteract and protect against tissue damage caused by unresolved inflammation[254]. These suppressive cells are termed “myeloid-derived suppressor cells”.

The first reports of suppressive myeloid populations were made in the 1970s, although it was not until the late 1990s that the cell markers Gr1 and CD11b were used to define these populations in murine splenocytes[254]. In fact, the term “MDSC” was not introduced until 2007, with the goal of unifying these suppressive populations under one umbrella[254,256]. MDSCs are therefore a heterogenous population of immature myeloid lineage immunoregulatory cells. MDSCs are hypothesized to develop via a two-signal model. The first signal serves to inhibit terminal differentiation of myeloid progenitors and the second signal induces their pathological activation[254]. The first signal is produced in response to chronic inflammation and includes stimulation with GM-CSF, G-CSF, M-CSF, VEGF, and polyunsaturated fatty acids. The second signal is mediated by

pro-inflammatory cytokines and DAMPs and includes stimulation with IFNγ, IL-1β, IL-4, IL-6, IL-13, TNF, and HMGB1[256]. MDSCs are made up of granulocytic or polymorphonuclear- MDSCs (PMN-MDSCs) and monocytic-MDSCs (M-MDSCs), with M-MDSCs being more suppressive on a per cell basis. A third population composed of more immature progenitors with colony-forming activity has been identified in humans and termed "early-stage" MDSCs[254,256]. PMN-MDSCs are phenotypically and morphologically similar to neutrophils, while M-MDSCs are similar to monocytes. Perhaps due to their relatively recent coming of age in the immunology world, there is still some controversy around how to define these cells, what their specific functions are, and the mechanisms by which they accomplish them. Phenotypically, these cells are Lin^{-}, meaning they are negative for lineage markers CD3, CD56, and CD19. In mice, PMN-MDSCs are defined as $CD11b^{+}Ly6G^{+}Ly6C^{lo}$ and M-MDSCs are defined as $CD11b^{+}Ly6G^{-}Ly6C^{hi}$[254]. In humans, they express myeloid specific markers, such as CD33 and CD11b, and have near-absent expression of HLA-DR[254,256,257]. Human PMN-MDSCs are identified as $CD11b^{+}CD14^{-}CD15^{+}CD33^{+}$ and M-MDSCs are identified as $CD11b^{+}CD14^{+}CD15^{-}CD33^{+}HLA\text{-}DR^{-/lo}$[256]. Recently, LDL receptor 1 (LOX-1) has emerged as a marker which allows for better distinction between human PMN-MDSCs and neutrophils[258]. In addition, MDSCs can be distinguished from monocytes and neutrophils via the upregulation and activity of intracellular proteins including STAT3 and C/EBP-β[256].

Regardless of this nomenclature, MDSCs are ultimately defined by their ability to suppress immune cell function and were initially described as potent T cell suppressors. Due to this suppressive ability, MDSCs have been implicated in chronic inflammation, infectious disease, autoimmune disease, GvHD, obesity, pregnancy, trauma, and cancer[254,256]. M-MDSC inhibition of immune cell function is both antigen-dependent and -independent, while PMN-MDSCs can only suppress in an antigen-specific manner[254]. Morphologically, MDSCs display weak phagocytic activity, increased reactive oxygen species (ROS) formation, high expression of ARG1, inducible

nitric oxide synthase (iNOS), COX-2, and anti-inflammatory cytokines TGFβ and IL-10[254,256,259]. It is important to note that these mechanisms are not engaged simultaneously, and instead the dominant mechanism is dependent upon the expanded MDSC population and the pathologic condition.

Cancer results in chronic inflammation and many of the "first signals" in the two-signal model of MDSC development are produced by tumour cells[255]. As such, MDSCs play a critical role in mediating tumourigenesis and immune evasion. MDSCs can directly promote tumour progression by affecting TME remodeling and angiogenesis via soluble factors like VEGF and can inhibit tumour cell senescence by antagonizing IL-1α[254,259]. Moreover, MDSCs induce immune cell tolerance via suppression of immune cell function through the various mechanisms described above. Cancer establishes itself by evading immune surveillance and subverting the patient's immune system to its advantage through the expansion of TME-related immune cells. TME MDSCs have been described as "the cornerstone of the immunosuppressive shield that protects the tumour"[255], as they promote T_{reg} development, can mature into tumour-associated macrophages (TAMs), and promote fibroblast differentiation into cancer-associated fibroblasts (CAFs)[255]. Clinically, peripheral MDSCs are an independent indicator of poor prognosis and poor outcome in solid and hematological malignancies and can help predict response to cancer therapies[256,260]. The role of MDSCs in immunotherapy resistance was not recognized until 2016, when MDSCs were shown to predict resistance to checkpoint inhibitors (CPIs)[255]. Therefore, the presence of MDSCs is detrimental for cancer patients and provides a complex target for cancer immunotherapies.

MDSCs have recently been shown to expand rapidly in response to surgical stress in both murine models[261–263] and in humans[148,264,265]. In a 4T1 breast cancer model, Ma et al. showed a postoperative increase in MDSCs that preferentially infiltrated the TME and promoted metastasis. MDSCs promoted EMT of tumour cells through TGFβ, VEGF, and IL-10. In addition, anti-Gr1 antibody treatment reduced postoperative pulmonary metastases[262]. Similarly, Xu et al. showed that

surgery results in an increase in MDSCs and concomitant increase in colorectal cancer CT26 tumour cell growth via chemokine (C-X-C motif) ligand 4 (CXCL4) downregulation. Inoculation using a CXCL4 over-expressing CT26 tumour abrogated MDSC infiltration and reduced MDSC migration *in vitro*[263]. In addition, Wang et al. demonstrated a significant increase in M-MDSCs in lung cancer patients after thoracotomy as compared to preoperative levels. Furthermore, M-MDSC expansion positively corelated with T_{reg} expansion[264]. While some mechanisms of MDSC-mediated immune suppression and tumour progression have been elucidated, the mechanism(s) by which surgery-induced MDSCs (sxMDSCs) mediate postoperative cancer recurrence are currently incomplete. sxM-MDSCs were reported to interact in a contact-dependent manner with $CD4^+$ T cells to induce the expansion of $FoxP3^+$ T_{regs}. Furthermore, in a murine model, perioperative all-trans retinoid acid (atRA), known to induce the differentiation of immature cells, reduced postoperative sxM-MDSCs and T_{regs} and resulted in fewer lung nodules[264]. Taken together, this strongly implicates sxMDSCs and T_{regs} in the development of postoperative metastasis. In addition, our group studied the effects of the phosphodiesterase type 5 (PDE5) inhibitor sildenafil in a murine model of surgical stress and found that sildenafil was able to abrogate the suppressive effects of sxMDSCs on NK cells and reduce lung metastases postoperatively[261]. We and others have shown that PDE5 inhibitors can decrease MDSC ARG1 and iNOS expression, and we hypothesize that this is the mechanism responsible for the observed effects *in vivo*[266]. We are currently testing the effects of the PDE5 inhibitor tadalafil in conjunction with an influenza vaccine on MDSC function in a Phase 1b clinical trial (PERIOP-04)[267].

1.5 Anesthesia, analgesia, and blood transfusions

Surgical stress results in profound and complex postoperative changes. These changes directly and indirectly, through cellular immune suppression, affect cancer cell growth, proliferation, and migration leading to increased metastasis and cancer recurrence. However, other perioperative interventions may also have an impact on postoperative metastasis. Blood

transfusions, anesthesia, and analgesia are known to modulate the inflammatory response and have recently been shown to impact cancer progression as well[163].

1.5.1 The effect of blood transfusions

The transfusion of red blood cells (RBCs) and other blood products may be required during surgery. Blood transfusions have consistently been associated with immunosuppression and inflammation *in vitro.* In the context of "pure" RBC transfusions, transfusion-related immunomodulation (TRIM) is thought to be mediated by monocytes/ macrophages. Acutely, macrophage death after erythrophagocytosis results in fewer phagocytic cells. Sub-acutely the polarization of macrophages may potentiate immune suppression. Chronically there is the potential for the development of suppressive "memory" macrophages[268]. Clinically, blood transfusions have been associated with increased cancer recurrence (decreased RFS), postoperative infection, and increased morbidity in a variety of cancer types, including gastric, esophageal, prostate, and bladder[269–273]. A 2006 Cochrane meta-analysis of CRC patients reported an overall odds ratio for recurrence of 1.42 in patients that have received a blood transfusion (95% CI: 1.2-1.67)[274]. Interestingly, there may be differences between autologous and allogeneic blood transfusions with one study showing that the transfusion of autologous salvaged blood resulted in increased NK cell precursors and IFNγ production as compared to allogeneic transfusion[275]. However, not all surgery patients receive a transfusion. Despite this, there is a consistent suppression of the cellular immune response and increased metastasis and cancer recurrence in non-transfused cancer surgery patients. This strongly suggests that blood transfusions may contribute to postoperative immunosuppression and metastasis, however, is not the primary mechanism.

1.5.2 The effect of anesthesia

Propofol is the most commonly used intravenous (i.v.) anesthetic for induction and maintenance of anesthesia. Preclinical studies investigating its impact on immune cells have been mixed, although the majority of preclinical studies support an anti-cancer role for propofol through

inhibition of oncogenes and the downregulation of HIF-1α and subsequent inhibition of angiogenesis[276]. Retrospective clinical trials have overwhelmingly shown reduced cancer risk and increased OS with perioperative propofol, compared to volatile agents. Numerous randomized control trials (RCTs) are currently underway[163].

Volatile anesthetic agents are administered by inhalation and are the most commonly used method for maintenance of anesthesia. Results from preclinical studies using serum from patients receiving volatile agents have also been mixed with some showing reduced immune activity and others showing no difference between volatile agents and other anesthetics. Volatile agents have been shown to stimulate proliferation and migration of cancer cells *in vitro*[163], however this may be cancer type-specific, in addition to inducing HIF-1α expression[277], therefore promoting cancer recurrence. Clinical studies are similarly unclear with some showing no difference compared to i.v. anesthetics and others showing increasing cancer recurrence and reduced OS.

Local/ regional anesthesia acts directly to inhibit the induction of the neuroendocrine stress response. Attenuating the pain response indirectly prevents immune suppression in addition to lessening the need for volatile anesthetics and opioids, which have potentially detrimental effects. However, local/regional anesthesia has little effect on cytokine production and the inflammatory response to surgery because it does not influence tissue trauma[166]. Amide local anesthetic may exert direct inhibitory effects on tumour cells by inhibiting cancer cell viability, proliferation, and migration, although this mechanism is unknown. Despite retrospective clinical studies published over a decade ago showing a protective effect against cancer, more recent clinical studies have yielded mixed results with randomized control trials (RCTs) still ongoing[163].

1.5.3 The effect of analgesia

Opioids are potent analgesics that can also be used as anesthetic at higher doses or adjunct to other anesthetics perioperatively. Opioids act indirectly on the nervous system resulting in the release of biological amines that inhibit NK cell cytotoxicity and the stimulation of the HPA axis

and subsequent glucocorticoid release, indirectly inhibiting immune cell function[163,166]. They also act directly on immune cells, including NK cells, through mu-opioid receptors (MOR) and non-opioid receptors. However, there may be a discrepancy between the effects of different opioids, with fentanyl and morphine showing the most potent immunosuppressive effects[163,166]. The majority of preclinical studies suggest that opioids have immunosuppressive and pro-cancer effects. In addition, opioid antagonists, such as naloxone, have anti-cancer effects[163]. Cancer cells can overexpress MOR, therefore enabling the stimulation of tumourigenic processes. Furthermore, clinically MOR expression is associated with worse outcomes for patients. Retrospective reviews have associated opioid administration with increased cancer recurrence; however, it has been postulated that the pro-tumourigenic properties associated with opioid use may actually be the result of uncontrolled pain causing nervous system and HPA activation[163].

There is growing evidence to support the anti-cancer effects of NSAIDs, which act by inhibiting the activity of COX-1/COX-2 to reduce prostaglandin production and subsequently inhibit the inflammatory response. The mechanism behind their anti-cancer effects is thought to involve reduced PGE2 synthesis, as well as other unclear mechanisms including opioid-sparing effects and altered signaling cascades. Preclinical studies have reported reduced cancer cell viability, proliferation, and migration through COX-dependent and -independent mechanisms while animal models suggest potential mechanisms may include reduced VEGF and downregulation of oncogenes. Long-term NSAID use in patients has been associated with reduced risk of colorectal cancer (CRC) and increased RFS after surgery in a variety of cancer types. Studies assessing the effects of perioperative NSAIDs have shown either improved outcomes or no difference, however combination therapy may be more fruitful. Combined NSAID (Flubiprofen Axetil)-dexamethasone resulted in enhanced survival in a retrospective study of NSCLC patients[61] and is currently being investigated in a Phase 4 clinical trial (NCT03172988). In addition, a Phase 2 randomized trial of perioperative propranolol and etolodac in breast cancer patients reported

normalization of IL-6 and CRP, decreased EMT, and decreased tumour-infiltrating monocytes[278]. A multicenter Phase 3 clinical trial to assess immune suppression and cancer recurrence in CRC surgery patients treated with propranolol and etolodac is currently underway (NCT00888767)[279]. Thus, while anesthetics/analgesics may contribute to cancer recurrence and metastasis postoperatively, their use and effects are so varied that they are unlikely to be the unifying mechanism across all cancer surgery patients.

1.6 NK cell suppression and postoperative metastasis

Despite being a necessary intervention in the treatment of solid cancers, surgical stress is associated with impaired metastatic clearance and increased cancer recurrence[197,224,280–287]. Although numerous mechanisms have been proposed, cellular immune suppression is a common consequence of the pathways that are activated in response to surgery and immune suppression has been linked to metastasis and cancer recurrence. Specifically, postoperative NK cell suppression, including dysfunctional cytotoxicity and cytokine secretion, is correlated with increased metastases in animal models[224,285,286], and in human studies reduced NK cell activity is associated with increased rates of cancer recurrence and death[288–290].

In our lab, we developed a reproducible mouse model of surgical stress. Mice (B6 or Balb/c) underwent tumour cell injection (B16F10lacZ or CT26LacZ), followed by a surgical procedure (laparotomy with partial hepatectomy or left nephrectomy) and lungs were harvested and visualized for metastases at 3 days post-procedure [197,224]. Regardless of mouse strain, tumour cell type, or surgical procedure, surgery was reliably able to increase lung metastases postoperatively compared to untreated mice. Furthermore, animals receiving anesthesia (0.05 mg/kg buprenorphine) alone had similar metastatic burden to untreated controls suggesting that the pro-metastatic effect seen postoperatively is anesthesia/analgesia-independent[197,224]. Using this model, we have showed that the NK cell population is responsible for inhibiting metastasis formation through an adoptive transfer system. $DX5^+$ splenic NK cells were isolated from

surgically stressed and untreated mice and adoptively transferred into pharmacologically NK cell-depleted mice followed by tumour cell inoculation[224]. Mice that had received surgically stressed NK cells had significantly increased lung tumour burden compared to mice that had received NK cells from untreated controls[224]. These experiments established NK cells as the critical effector cells responsible for mediating postoperative metastasis.

In humans, Iannone et al. studied NK cells in pancreatic cancer surgery patients and found a significant decrease in cytotoxicity on POD7, which was restored by POD30[147]. Velásquez et al. also reported that NK cell *in vitro* killing of K562 leukemia cells was suppressed up to five days after surgery in patients with primary bone cancer[149]. We have recently shown that postoperative NK cells from CRC surgery patients have suppressed *in vitro* cytotoxic killing of tumour cells (K562 leukemic cells), which was most profoundly suppressed on POD1 and returned to preoperative levels ("baseline") by POD28. Postoperative samples also had reduced IFNγ production in response to cytokine stimulation using NKVue™ [291]. In this study, 1 mL of whole blood was stimulated using a proprietary cytokine cocktail (Promoca™) for 24 hours, plasma was collected, and extracellular IFNγ was measured by ELISA. The most profound suppression was observed on POD1 where we saw 90.2 % (37/41) of patients had IFNγ levels below the minimum detectable level (15.6 pg/mL). As compared to baseline, the mean reduction in IFNγ production was 83.1% (s.d. 25.2%; CI: 75-91). Astoundingly, this suppression persisted until POD28 in 65.5% (19/29) of patients and POD56 in 33.3% (4/12) of patients[291]. This is to say that one third of patients still had reduced NK cell activity at nearly two months post-surgery. Additionally, Reinhardt et al. investigated IFNγ production in response to *Staphylococcus aureus* or IL-12 stimulation in the $CD56^{Bright}$ population of surgery patients and found a significant decrease in IL-12R (CD212) expression and an impairment in IFNγ production on POD1 up to 7 days postoperatively[150].

The inability of surgically stressed NK cells to secrete IFNγ is devastating for the anti-tumour immune response due to its critical effects of autocrine, endocrine, and paracrine immune

modulation and direct anti-tumour functions. Defective IFNγ production in the postoperative period may be a main contributor to metastatic formation and understanding the changes in NK cell biology responsible for impaired IFNγ will allow for immunotherapeutic development and application in cancer surgery patients. While other studies have described NK cell function in the postoperative period[2,150,292], few have investigated the mechanisms of this dysfunction. In addition to postoperative immune suppression, surgical stress also induces the expansion of immunosuppressive populations, including sxMDSCs[224,262,263,265,293], and the release of inhibitory factors, including TGFβ[224]. MDSCs are known to suppress NK cell function through a variety of mechanisms, including the release of the soluble inhibitory factor TGFβ. TGFβ can suppress NK cell IFNγ production through mTOR-mediated reduction in receptor expression[76].

Hence, the relationship between surgical stress and NK cell dysfunction, with an emphasis on IFNγ production, will be the focus of this dissertation. This relationship was evaluated by assessing receptor expression and signaling protein activity upstream of IFNγ production, in addition to quantifying intra- and extracellular IFNγ. This work was done almost entirely using whole blood from healthy donors and surgery patients with a variety of cancer types. Significant reductions in critical cytokine and activating receptors in addition to reductions in the phosphorylation of associated downstream signaling proteins were observed on POD1. Upon phenotypic and functional characterization of postoperative NK cells, potential mechanisms of suppression were explored. The postoperative period is also characterized by the release of anti-inflammatory factors, including the release of TGFβ1, which we have previously shown to increase postoperatively in mice. I evaluated soluble TGFβ in patient plasma to reveal an increase on POD1. Changes in receptor expression, signaling, and IFNγ production could be phenocopied in healthy NK cells through in the presence of rTGFβ1 or by incubation with POD1 plasma. This dysfunction was nearly completely prevented with the addition of an anti-TGFβ mAb or a TGFβ RI small molecule inhibitor (smi) in culture. TGFβ has been shown to impair NK cell effector functions and

receptor expression via inhibition of mTOR activity. Upon RNA-sequencing of colorectal cancer surgery patient PBMCs at baseline and on POD1 I identified transcriptomic shifts in the NK cell population with a significant reduction in transcripts associated with downstream mTOR effector functions, namely oxidative phosphorylation and protein translation. In light of this, I assessed S6, a component of the 40S ribosomal subunit and a proxy for mTORC1 activity. I found that phosphorylation was significantly reduced in healthy isolated NK cells cultured with sxMDSCs or POD1 plasma. Additionally, phosphorylation could be maintained with the addition of an anti-TGFβ mAb or a TGFβ RI smi. Finally, I hypothesized that sxMDSCs may be responsible for TGFβ production, given that MDSCs can inhibit NK cell activity via TGFβ. Culture of healthy NK cells with sxMDSCs induced a downregulation of receptor expression and signaling protein phosphorylation and suppressed IFNγ production. The results of my graduate work suggest that sxMDSC-derived TGFβ suppresses NK cell IFNγ production and alters receptor expression in an mTOR-dependent manner (**Figure 4**). These new insights into NK cell biology in the postoperative period could provide the foundation for a novel perioperative therapeutic aimed at disrupting postoperative immune suppression. The perioperative period of immunosuppression presents a window of opportunity for such therapeutics to prevent metastases and cancer recurrence for the 65, 000 Canadians who undergo tumour resection each year[294].

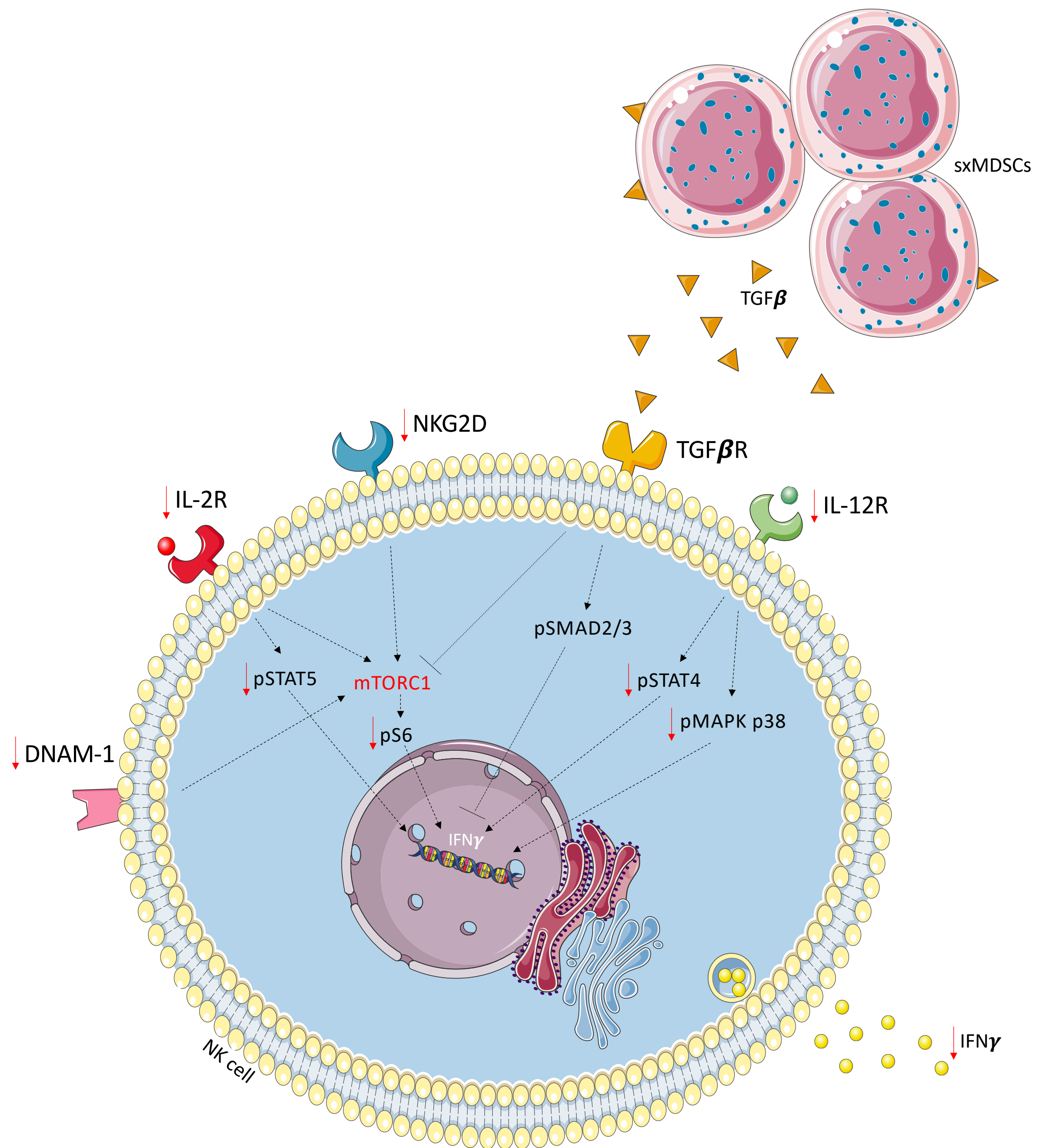

Figure 4. Graphical depiction of hypothesis.
Surgery-induced MDSCs express and release TGFβ, which binds to its receptor and signals through Smad-dependent and -independent pathways to downregulate receptor expression and impair IFNγ production in postoperative NK cells.

Chapter 2 Hypothesis

I hypothesize that ***TGFβ, released by surgery-induced myeloid-derived suppressor cells in response to surgical stress, induces postoperative NK cell dysfunction via inhibition of mTOR.***

Chapter 3 Materials and Methods

3.1 Patient characteristics and clinical protocol

This protocol was approved by the Ottawa Health Science Research Ethics Board (Perioperative Human Blood and Tissue Specimen Collection Program (PHBSP) Protocol no. 2011884-01H). All subjects gave written informed consent in accordance with the Declaration of Helsinki. Eligible patients were >18 years of age and had a planned surgical resection of the primary or metastatic tumour (cancer patients) or were healthy donors who volunteered to participate. Exclusion criteria included a history of active viral or bacterial infection or known HIV or Hepatitis B or C, autoimmune diseases, or use of immunosuppressive medications.

The primary objective was to immunophenotype NK cells from healthy donors and cancer patients prior to surgery at baseline and after surgery on POD1.

3.2 Blood processing

Healthy donor and cancer patient blood was drawn into BD Vacutainer Sodium-Heparin coated tubes (~10-30 mL/blood draw). 20-25 mL of blood diluted with phosphate-buffered saline (PBS; Hyclone) was carefully layered over 20 mL of Ficoll-Plaque (GE Healthcare) in a 50 mL conical tube and centrifuged at 400 *xg* for 30 minutes (ACC 2/DCC 2). The mononuclear cell layer was then carefully transferred to a new 50 mL conical tube and filled with PBS prior to centrifuging again at 500 *xg* for 5 minutes. Supernatant was aspirated and the pellet was resuspended in 20 mL of PBS; 1 mL was used to quantify PBMCs and assess viability and quantity using a Vi-Cell® XR Cell Counter (Beckman Coulter). PBMCs were then resuspended in a freezing buffer (10% dimethyl sulfoxide (DMSO; Fisher Scientific) and 90% fetal bovine serum (FBS; GE Healthcare)) and stored at -80°C[295]. The freezing buffer composition acted as a cryoprotectant, which allowed a slower rate of cooling leading to improved cell viability prior to phenotype assessment. The low

temperature allowed cells to enter a quiescent state in which cellular functions were suspended[296,297].

3.3 Antibodies and FACS analysis

For cryopreserved PBMC, whole blood, and isolated $CD56^+$ or $CD33^+$ cell staining, the monoclonal antibodies used are summarized in **Table 1**. Reagents are summarized in **Table 2.**

Fluorescence-activated cell sorting (FACS) acquisitions were conducted on a BD LSRFortessa using BD FACSDiva™ version 8.0.3 software (BD Biosciences, San Diego, CA, USA) and data were analyzed with FlowJo, LLC version 10.6.1 software (FlowJo LLC, Ashland, Oregon, USA).

3.4 *In vitro* cryopreserved PBMC phenotyping and functionality assays

3.4.1 Thawing cryopreserved PBMCs

Cryopreserved PBMCs were thawed at room temperature (RT) and added to 10 mL Roswell Park Memorial Institutes (RPMI; Sigma-Aldrich) prior to being centrifuged at 500 *xg* for 5 minutes. Cells were resuspended in 20.5 mL of PBS and 0.5 mL was used to quantify cell number and viability using the Vi-Cell® XR Cell Counter.

3.4.2 Extracellular receptor phenotyping assay

To stain for extracellular receptor expression (NKG2D, DNAM-1, NKG2A, PD-1, TIGIT, TIM-3, CD25, CD212), PBMCs were resuspended in 2% complete (C)RPMI and plated at $1e^6$ cells/well in 200 µL total into a 96 well v-bottom plate and rested overnight at 37°C. Cells were then resuspended in 100 µL of diluted Fc block (human Trustain (Biolegend); 5 µL in 100 µL/well) and incubated at RT for 5 minutes. Cells were washed with 150 µL of Flow Buffer (FB; 500 mL PBS, 2.5 g Bovine serum albumin (BSA; Sigma-Aldrich), 1 mL 0.5M Ethylenediaminetetraacetic acid (EDTA; Invitrogen)) and centrifuged at 500 *xg* for 5 minutes. Cells were resuspended in 80 µL of PBS and Live/Dead fixable viability dye (0.5 µL/well) and incubated at RT for 10 minutes

in the dark. Cells were resuspended in 40 μL of extracellular staining (ECS) mix and incubated at 4°C for 20 minutes. One hundred μL of FB was added, cells were centrifuged at 500 *xg* for 5 minutes, and resuspended in 200 μL of 1% paraformaldehyde (PFA; Thermo Scientific). Samples were stored at 4°C for up to 72 hours prior to acquisition and analysis by flow cytometry.

3.4.3 IFNγ functionality assay

To stain for intracellular IFNγ, PBMCs were resuspended in 10% CRPMI and plated at $1e^6$ cells/well in 200 μL total into a 96 well v-bottom plate. Cells were either incubated at 37°C for 5 hours in the presence of a control (0.2% DMSO) or PMA-ionomycin (Sigma-Aldrich) (250 ng/ 3.75 μg/mL), or for 24 hours in the presence of a control (0.2% DMSO) or three concentrations of recombinant (r)IL-2 (Tecin™ Teceleukin)/ rIL-12 (R&D Systems) ([1] 1000 U/50 ng/mL, [2] 200 U/50 ng/mL, [3] 20 U/ 50ng/mL). PMA-ionomycin non-specifically stimulates cytokine release in lymphocytes (including IFNγ). Ionomycin is a calcium ionophore produced by *S. conglobatus* that induces the hydrolysis of phosphoinositides and activates PKC via calcium ligation. PMA (phorbol 12-myristate 13-acetate) activates protein kinase C (PKC) as a result of its structural similarity to diacylglycerol (DAG), the product of phosphoinositide hydrolysis by PLC. PKC is a signal transducer that influences transcription factor activity[298]. For each incubation period, Golgiplug™ (Brefeldin A (BD Biosciences); 0.2 μL/well) was added for the final four hours and incubated at 37°C. Golgiplug™ is a protein transport inhibitor containing Brefeldin A, which allows for the intracellular quantification of secreted products by inhibiting protein transport from the endoplasmic reticulum to the golgi complex by preventing the association of coatomer proteinCOP1 with the golgi membrane[299]. The plate was centrifuged at 500 *xg* for 5 minutes, 150 μL of supernatant was collected and stored at -80°C for subsequent protein quantification by ELISA, and cells were washed with 200 μL of FB. Cells were resuspended in 100 μL of diluted Fc block (human Trustain; 5 μL in 100 μL/well) and incubated at RT for 5 minutes. Cells were washed

with 150 μL of FB and centrifuged at 500 *xg* for 5 minutes. Cells were resuspended in 80 μL of PBS and Live/Dead fixable viability dye (0.5 μL/well) and incubated at RT for 10 minutes in the dark. Cells were resuspended in 40 μL of ECS mix and incubated at 4°C for 20 minutes. One hundred μL of FB was added, cells were centrifuged at 500 *xg* for 5 minutes, resuspended in 200 μL intracellular (IC) fixation buffer (Invitrogen), and incubated at RT for 30 minutes. The plate was centrifuged at 500 *xg* for 5 minutes, cells were resuspended in 150 μL chilled Perm III Buffer (BD Biosciences) and incubated at 4°C for 30 minutes. The plate was centrifuged at 500 *xg* for 5 minutes and washed with 100 μL of FB. Cells were resuspended in 100 μL of intracellular staining (ICS) mix and incubated at 4°C for 25 minutes in the dark. One-hundred and fifty μL of FB was added, cells were centrifuged at 500 *xg* for 5 minutes, and resuspended in 1% PFA. Samples were stored at 4°C for up to 72 hours prior to acquisition and analysis by flow cytometry.

3.5 Whole blood phenotyping and functionality assays

3.5.1 Extracellular receptor phenotyping assay

Blood was collected from healthy donors and cancer surgery patients at baseline and on POD1. 200 μL of whole blood was aliquoted per flow cytometry panel into a 15 mL conical tube. 40 μL of extracellular staining (ECS) mix was added and mixed by pipetting. Blood was incubated for 15 minutes at RT prior to adding 4 mL of BD FACS lyse/fix buffer (BD Biosciences; 1:5 dilution with diH_2O). Tubes were shaken vigorously to ensure red blood cell (RBC) lysis. Tubes were incubated for 10 minutes in a 37°C water bath and centrifuged at 500 *xg* for 8 minutes. Supernatant was carefully aspirated, and the cell pellet was resuspended in 1 mL FB. Cells were immediately centrifuged at 500 *xg* for 5 minutes and supernatant was aspirated prior to resuspending in 200 μL of 1% PFA. Samples were stored at 4°C for up to 72 hours prior to acquisition and analysis by flow cytometry.

3.5.2 Phospho-signaling protein functionality assay

Blood was collected from healthy donors and cancer surgery patients at baseline and on POD1. One mL of whole blood was aliquoted per flow cytometry panel into new Sodium-Heparin tubes. Control (PBS) or rIL-2/12 stimulation (400 U/ 20 ng/mL) and 40 µL of ECS mix was added and mixed by pipetting. Blood was incubated for 20 minutes in a 37°C water bath and then transferred to a 50 mL conical tube prior to adding 20 mL of BD FACS lyse/fix buffer (1:5 dilution with diH_2O). Tubes were shaken vigorously to ensure RBC lysis. Tubes were incubated for 10 minutes in a 37°C water bath and centrifuged at 500 *xg* for 8 minutes. Supernatant was carefully aspirated, and the cell pellet was resuspended in 1 mL FB. Cells were centrifuged at 500 *xg* for 5 minutes and supernatant was aspirated prior to resuspending in 500 µL chilled BD Perm III buffer. Cells were incubated on ice in the dark for 30 minutes and centrifuged at 300 *xg* for 10 minutes. Supernatant was aspirated and the pellet was resuspended in 400 µL FB; 200 µL per well was then transferred into a 96 well v-bottom plate and centrifuged at 500 *xg* for 5 minutes. The plate was decanted, and cells were resuspended in 200 µL of appropriate ICS mix and incubated at RT in the dark for 1 hour. The plate was spun at 500 *xg* for 5 minutes and cells were resuspended in 1 % PFA. Samples were stored at 4°C for up to 72 hours prior to acquisition and analysis by flow cytometry.

3.5.3 IFNγ functionality assay

Blood was collected from healthy donors and cancer surgery patients at baseline and on POD1. One mL of whole blood was aliquoted per flow cytometry panel into new Sodium-Heparin tubes. Whole blood was incubated with PBS (control) or PMA-ionomycin (50 ng/ 750 ng/mL) for 5 hours or rIL-2/12 (400 U/20 ng/mL) for 24 hours at 37°C. Ten µg/mL Golgiplug™ (Brefeldin A) per tube was added, tubes were inverted 10 times to mix, and incubated at 37°C for the remaining two hours of each incubation. 600 µL of whole blood was then collected in an Eppendorf tube, centrifuged at 500 *xg* for 5 minutes, and plasma was collected and stored at -80°C for an IFNγ

ELISA. The remaining 400 μL of whole blood was transferred to a new 15 mL conical tube and incubated with Fc block (Human Trustain) for 5 minutes at RT. The ECS mix was then added and mixed by pipetting. Blood was incubated for 15 minutes at RT prior to adding 20 mL of BD FACS lyse/fix buffer (1:5 dilution with diH_2O). Tubes were shaken vigorously to ensure RBC lysis. Tubes were incubated for 10 minutes in a 37°C water bath and centrifuged at 500 *xg* for 8 minutes. Supernatant was carefully aspirated, and the cell pellet was resuspended in 1 mL FB. Cells were centrifuged at 500 *xg* for 5 minutes and supernatant was aspirated prior to resuspending in 500 μL chilled BD Perm III buffer. Cells were incubated on ice in the dark for 30 minutes and centrifuged at 300 *xg* for 10 minutes. Supernatant was aspirated and the pellet was resuspended in 400 μL FB; 200 μL per well was then transferred into a 96 well v-bottom plate and centrifuged at 500 *xg* for 5 minutes. The plate was decanted, and cells were resuspended in 200 μL of appropriate ICS mix and incubated at 4°C for 30 minutes in the dark for 1 hour. The plate was spun at 500 *xg* for 5 minutes and cells were resuspended in 1% PFA. Samples were stored at 4°C for up to 72 hours prior to acquisition and analysis by flow cytometry.

3.6 Cell lines

Human NK-92MI (IL-2 independent) cell lines (American Type Tissue Collection) were propagated in RPMI media (Hyclone) supplemented with 10% FBS (Cansera, Etobicoke, Ontario, Canada). Human K562 leukemic cell lines (American Type Tissue Collection) were propagated in RPMI media supplemented with 10% FBS.

3.7 Cell isolation

3.7.1 $CD56^+$ cell isolation

Blood was collected from healthy donors and cancer surgery patients at baseline and on POD1. 50 μL of Straight From™ Whole Blood CD56 magnetic microbeads (Miltenyi) were added per 1 mL of whole blood and incubated at 4°C for 15 minutes. Cells were then sorted on an

autoMACs® Pro Separator using the PosselWB protocol (Miltenyi). Sorted cells were centrifuged at 500 *xg* for 5 minutes, the supernatant was discarded, and the pellet was resuspended in 1 mL FB. Cells were counted using a hemocytometer before being resuspended at the desired concentration for use in other assays.

3.7.2 CD33$^+$ cell isolation

Blood was collected from healthy donors and cancer surgery patients at baseline and on POD1. The blood processing protocol was followed to isolate PBMCs. Up to 1e^7 PBMCs were resuspended in 80 µL of sorting buffer (500 mL PBS + 2 mL EDTA (2mM) + 2.5 g BSA) and 20 µL of CD33 magnetic microbeads (Miltenyi) and incubated at 4°C for 15 minutes. Two mL of sorting buffer was added to the suspension and cells were centrifuged at 300 *xg* for 10 minutes. The supernatant was discarded, and cells were resuspended in 500 µL of sorting buffer. Cells were then sorted on an autoMACs® Pro Separator using the Possel/S protocol (Miltenyi). Sorted cells were centrifuged at 500 *xg* for 5 minutes, the supernatant was discarded, and the pellet was resuspended in 1 mL FB. Cells were counted using a hemocytometer before being resuspended at the desired concentration for use in other assays.

3.8 *In vitro* NK-92:K562 co-culture cytotoxicity assay

NK-92 cells were plated in a 96-well v-bottom plate and incubated in the absence (control) or presence of rTGFβ1 at 1 ng/mL, 10 ng/mL, and 100 ng/mL for 24 hours at 37°C. At 24 hours, cells were washed with 200 µL PBS prior to the addition of CP450-labeled K562 cells. 2e^6 K562 cells/mL were centrifuged at 500 *xg* for 3 minutes and cells were combined with Cytochrome P450 (CP450) dye (Thermo Fisher Scientific) diluted in PBS. Cells were incubated in a 37°C water bath for 15 minutes prior to being diluted with 10 mL of media to stop the dye reaction. K562 cells were then counted using the Vi-Cell® XR Cell Counter and centrifuged at 500 *xg* for 3 minutes. K562 cells were added to NK-92 cells in culture and incubated for 4 hours prior to flow cytometric

analysis. Prior to analysis, 1 mM/well Ethidium Bromide homodimer (EthD; Thermo Fisher Scientific) was added per well. EthD is readily taken up by dead cells and stains DNA and was therefore used as a dead cell marker.

3.9 Platelet-free plasma collection

Blood was collected in Sodium-Heparin tubes from healthy donors and cancer surgery patients at baseline and on POD1. Whole blood was centrifuged at 1,000 *xg* for 10 minutes, plasma was collected and centrifuged again at 10,000 *xg* for 15 minutes to collect platelet-free plasma. Platelet-free plasma was stored at -80°C to be used either for quantification of protein by ELISA or for plasma culture assays.

3.10 Extracellular cytokine quantification

3.10.1 Human IFNγ ELISA

The R&D Quantikine® ELISA Human IFNγ Immunoassay (R&D Systems) was used to quantify extracellular IFNγ from cell culture supernatant and patient plasma. Samples were thawed at RT and either run undiluted or at a dilution of 5x or 10x with appropriate buffer. This assay was run following the R&D Quantikine® ELISA protocol. The minimum detectable dose (MDD) for the assay is < 8.0 pg/mL.

3.10.2 Human TGFβ ELISA

The R&D Quantikine® ELISA Human TGFβ Immunoassay (R&D Systems) was used to quantify extracellular IFNγ from cell culture supernatant and patient platelet-free plasma. Samples were thawed at RT and latent TGFβ was acid activated using 1 N HCl neutralized by 1.2 N NaOH/0.5 M HEPES and either run undiluted or at a dilution of 5x or 10x with appropriate buffer. This assay was run following the R&D Quantikine® ELISA protocol. The MDD for the assay ranged from 1.7-15.4 pg/mL.

3.11 *In vitro* rTGFβ1 time-course assay

CD56$^+$ cells were isolated using the cell isolation protocols. CD56$^+$ cells were plated at 1.5e^5 cells/well and incubated overnight at 37°C. Cells were incubated in 200 μL of 2% CRPMI supplemented with either 100 U/well rIL-2 or with rIL-2 and 10 ng/mL rTGFβ1 (Abcam Biochemicals). At 20 minutes, 2 hours, or 24 hours the plate was centrifuged at 500 *xg* for 5 minutes. Cells were then resuspended in appropriate ECS mix and incubated at 4°C for 20 minutes. One-hundred and fifty μL of FB was added, the plate was centrifuged at 500 *xg* for 5 minutes and cells were resuspended in 200 μL 1% PFA and stored at 4°C for up to 72 hours prior to acquisition and analysis by flow cytometry.

3.12 *In vitro* plasma culture assay

Platelet-free plasma was thawed and healthy, baseline, or POD1 samples were combined to make master healthy, baseline, or POD1 plasma mixes. CD56$^+$ cells were isolated using the cell isolation protocols. CD56$^+$ cells were plated at 1.5e^5 cells/well and incubated overnight at 37°C in 200 μL of 2% CRPMI supplemented with low-dose rIL-2 (100 U/well). CD56$^+$ cells either received media only or media supplemented with 25% healthy combined plasma, baseline combined plasma, or POD1 combined plasma. As a positive control, some cells incubated with healthy combined plasma also received 10 ng/mL rTGFβ1. When testing the ability of a monoclonal blocking antibody against TGFβ to recover NK cell receptor expression and function, combined plasma was incubated for 30 minutes at 37°C with 100 μg/mL of anti-TGFβ mouse monoclonal antibody (Bio X Cell; clone 1D11) prior to being added to CD56$^+$ cells in culture. When testing the ability of the small molecule TGFβ RI smi SB525334 (Selleck Chemicals) to recover NK cell receptor expression and function, CD56$^+$ cells were incubated with the smi (2 μM/well) for 1 hour at 37°C prior to adding combined plasma.

At 24 hours of incubation, cells were stained for surface receptors NKG2D, DNAM-1, CD212, and CD132 or stained intracellularly for pS6. After centrifuging the 96 well plate at 500 *xg* for 5 minutes, culture supernatant was collected for use in later experiments and stored at -80°C. After decanting, wells were resuspended in 40 μL ECS mix and incubated at 4°C for 20 minutes in the dark. 200 μL of FB was added prior to centrifuging at 500 *xg* for 5 minutes. Cells stained for extracellular receptor targets were then resuspended in 200 μL 1% PFA and stored at 4°C for up to 72 hours prior to acquisition and analysis by flow cytometry.

Cells used for intracellular staining were instead resuspended in 200 μL pre-warmed BD Cytofix buffer (BD Biosciences) and incubated for 10 minutes at 37°C. The plate was centrifuged at 500 *xg* for 5 minutes and wells were resuspended in 100 μL chilled BD Perm III buffer and incubated for 30 minutes at 4°C. One-hundred and fifty μL of FB was added prior to centrifuging at 500 *xg* for 5 minutes. Cells were washed once with 200 μL of FB, resuspended in 100 μL ICS mix, and incubated at 4°C for 1 hour. The plate was centrifuged at 500 *xg* for 5 minutes and cells were resuspended in 200 μL 1% PFA and stored at 4°C for up to 72 hours prior to acquisition and analysis by flow cytometry.

Cells used to assess intracellular IFNγ production were stimulated with rIL-2/12 stimulation media (2% CRPMI + 400U/well rIL-2 + 20ng/well rIL-12) at 6 hours in culture and incubated at 37°C until 24 hours. BD Golgiplug^{TM} (Brefeldin A) was added (2 μL/well) for the remaining two hours of incubation. The plate was centrifuged at 500 *xg* for 5 minutes, decanted, and washed with 200 μL of FB prior to resuspending in 40 μL of ECS mix. Cells were incubated at 4°C for 20 minutes, 100 μL of FB was added to each well and the plate was centrifuged again at 500 *xg* for 5 minutes. Cells were resuspended in 200 μL IC fixation buffer and incubated at RT for 30 minutes. Cells were centrifuged at 500 *xg* for 5 minutes, resuspended in chilled BD Perm III Buffer, and incubated at 4°C for 30 minutes in the dark. Cells were washed once with 100 μL of

FB, resuspended in 100 μL of ICS mix, and incubated at 4°C for 30 minutes in the dark. The plate was centrifuged at 500 *xg* for 5 minutes and cells were resuspended in 200 μL 1% PFA and stored at 4°C for up to 72 hours prior to acquisition and analysis by flow cytometry.

3.13 *In vitro* isolated sxMDSC: NK cell co-culture assay

CD56$^+$ and CD33$^+$ cells were isolated using the cell isolation protocols. CD56$^+$ (1.5e^5) and CD33$^+$ (3.0e^5) cells were either plated alone or at a ratio of 2:1 (CD33$^+$:CD56$^+$). Cells were incubated overnight at 37°C in 200 μL of 2% CRPMI supplemented with low-dose rIL-2 (100 U/well). Receptor expression, S6 phosphorylation, and intracellular IFNγ were assessed after 24 hours of incubation as described in 3.12.

3.14 Single-cell RNA-sequencing

Blood was collected from colorectal cancer surgery patients recruited to the PERIOP-02 (NCT02987296) clinical trial. PBMCs were isolated by Ficoll-Plaque density centrifugation and cryopreserved in liquid nitrogen in freezing buffer. Six patient samples (3 control and 3 arginine-treated) were thawed and stained for immunophenotyping and to assess viability (with Leonard Angka, Auer lab). Each sample was labelled with a unique lipid-modified DNA barcode to enable multiplexed single-cell RNA-sequencing (scRNA-seq) (with David Cook, Vanderhyden lab). Baseline and POD1 samples were separately pooled and processed for scRNA-seq using the 10x Genomics 3' RNA-seq platform (v3; StemCore, Ottawa). Barcode and cDNA libraries were prepared separately, as outlined by McGinnis et al.[300], and sequenced on an Illumina NextSeq500 to a depth of > 20,000 reads per cell. Gene expression libraries were aligned to the GRCh38 build of the human genome using cellranger v3.1.0. Individual cells from the pooled data were assigned to specific samples by aligning sequencing reads to the list of possible barcode sequences using the deMULTIplex R package.

Gene expression data was processed using the standard pipeline provided by the R package Seurat[301]. To allow clustering to group cells on the basis of cell type rather than experimental condition, we performed data integration using the R package harmony[302]. Differential expression between POD1 and baseline were then calculated using the R package muscat[303], which generates "pseudobulk" expression profiles of each sample in each cluster, performs differential expression between conditions for each cluster (cell type), and then controls for multiple hypothesis testing using the Benjamini-Hochberg false detection rate method.

Functional annotation of the differential expression results was performed using the gene set enrichment analysis (GSEA) implementation in the R package fgsea[304]. Differential expression results were ranked by log fold change and assessed for enrichment of various gene sets at the extremes of the ranked list (e.g. in the upregulated or downregulated genes). A collection of gene sets was used to query the results, including GO terms, KEGG pathways, Reactome pathways, and the MSigDB Hallmark gene sets. These gene sets were all accessed from the Molecular Signatures Database[305,306]. All gene sets discussed in this thesis were significantly enriched (adjusted p-value < 0.05) and normalized enrichment scores (NES) are presented. Positive NES values correspond to gene sets enriched in genes upregulated on POD1, while negative NES values represent gene sets with reduced expression on POD1.

3.15 Statistical analyses

Descriptive statistics were used to summarize data collected on extracellular receptors, phospho-signaling proteins, and IFNγ production (median with interquartile range (IQR)). Wilcoxon matched-pairs signed rank test was used to determine if there were significant changes in target protein expression/secretion between baseline and POD1 samples. Mann-Whitney U test was used to determine if there were significant differences between unpaired samples. All statistical analyses were determined using GraphPad Prism version 8.0 software (La Jolla, California). The

level for statistical significance was set a priori at ≤ 0.05 (*$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$, ****$p \leq 0.00005$).

Chapter 4 Results

4.1 Patient demographics

Cryopreserved NK cell phenotype and function were assessed in 12 healthy donors and 32 cancer surgery patients. Sixty-seven percent of healthy donors were female and 100% were < 60 years old. Twenty-five percent of cancer surgery patients were female and 47% were between 60-69 years old. Of 32 patients, 13 patients had prostate cancer and 10 patients had lung cancer. Other cancer types included: renal, colorectal, endometrial, pancreatic, or esophageal. Twelve patients had Stage III cancer and 10 patients had Stage I cancer, confirmed by pathology (**Table 3**).

NK cell phenotype and function were assessed in whole blood in 42 healthy donors and 39 cancer surgery patients. Sixty-two percent of healthy donors were female and 62% were < 60 years old. Forty-six percent of cancer surgery patients were female and 49% were between 60-69 years old. Eleven of 39 patients had prostate cancer, 7/39 had lung cancer, and 6/39 had colorectal cancer. Other cancer types included: renal, ovarian, sarcoma, pancreatic, parathyroid, neuroendocrine, endometrial, duodenal, or uterine. Of 39 cancer patients, 15 patients had Stage III cancer and 14 patients had Stage I cancer, confirmed by pathology (**Table 4**).

Patient populations were representative of different cancer types and disease burden, thus supporting the generalizability of these findings.

4.2 IFNγ production is suppressed in cryopreserved NK cells on POD1

Our lab has previously reported that cytokine-induced NK cell IFNγ secretion is significantly impaired following surgery in colorectal cancer patients[307]. I initially sought to better delineate the defects within surgically stressed NK cells that contribute to the observed impairment; are these observations a result of impaired synthesis, secretion, or both? IFNγ was assessed in healthy donors (n=5) by flow cytometry using a range of stimulant concentrations which provided a dose-response in healthy volunteers (**Figure 5**). Healthy, baseline, and POD1 cryopreserved PBMCs were thawed and stimulated with media (control/no stim), PMA-ionomycin, and three

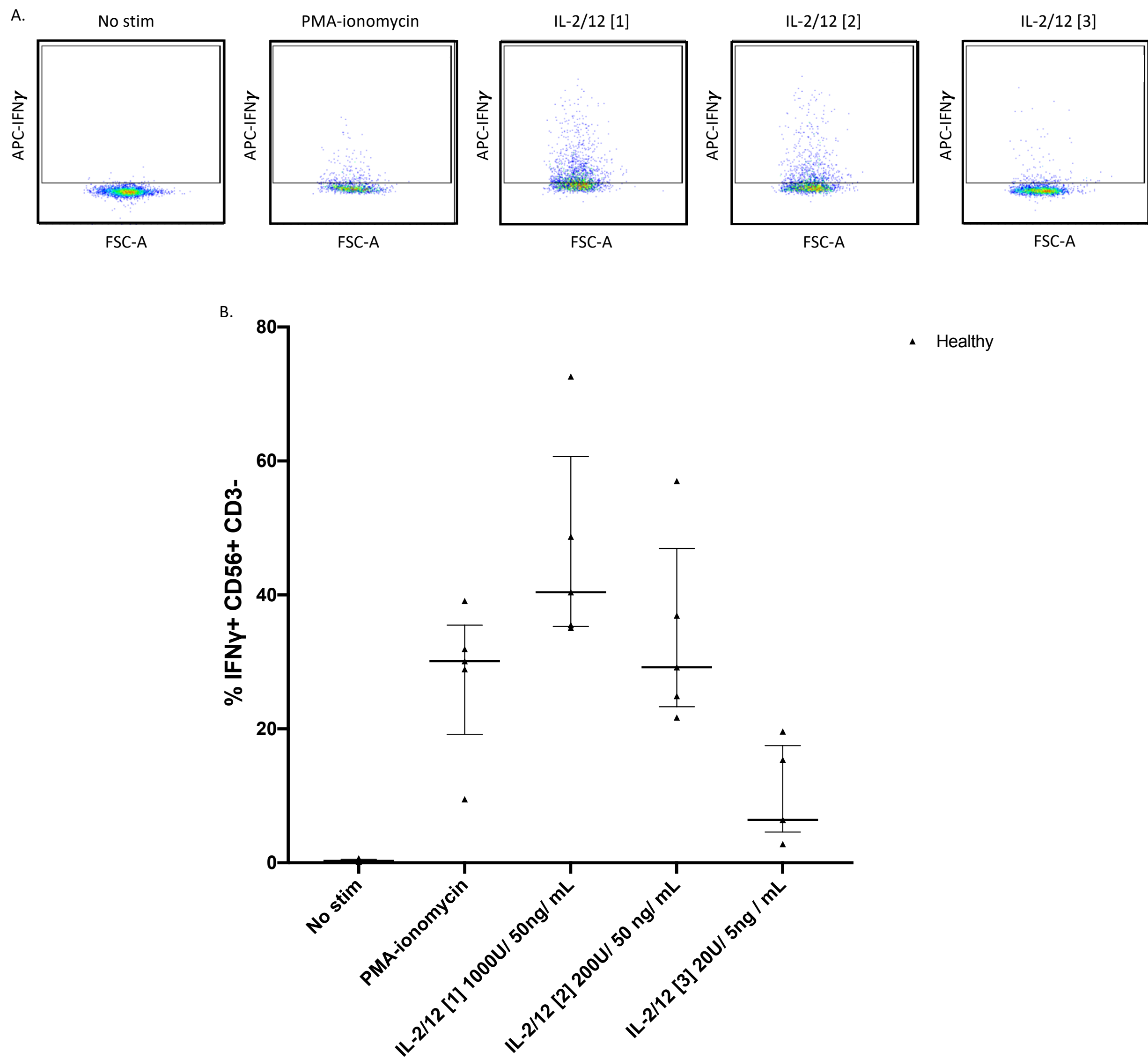

Figure 5. Dose response to stimulation in healthy donor cryopreserved NK cells.
(A) Representative flow cytometry plots of intracellular IFNγ, gated on $CD56^+CD3^-$ cells. (B) Intracellular IFNγ was assessed in healthy donors (n=5) by flow cytometry in response to PMA-ionomycin and a range of rIL-2/12 stimulant concentrations, which provided a dose-response in healthy volunteers. Shown are the median values ± IQR.

concentrations of rIL-2/12 ([1] high, [2] moderate and [3] low). Assessment of IFNγ production in $CD56^{+}CD3^{-}$ NK cells using the indicated gating strategy (**Figure 6A**) revealed, as expected, that no IFNγ was measured in the absence of stimulation and that healthy donors produced significantly more IFNγ in response to activating stimuli as compared to baseline cancer patient samples ($p<0.05$, rIL-2/12 [1], rIL-2/12 [3]) (**Figure 6B**). Furthermore, a significant reduction in both intracellular (**Figure 6B**) and extracellular (**Figure 6C**) IFNγ was observed on POD1 in response to PMA-ionomycin and all three concentrations of rIL-2/12, with the greatest reduction observed in response to rIL-2/12 [2], ($p<0.05$). Extracellular IFNγ was quantified in cancer surgery patients (n=13) and measured by ELISA. Of note, it is difficult to correlate intracellular and extracellular IFNγ as the ELISA was performed on cell culture supernatants from stimulated PBMCs and not isolated NK cells. Therefore, the IFNγ measured in the ELISA includes IFNγ produced by non-NK cell populations (namely T and NKT cells). Taken together, these results with rIL-2/12 confirm and extend our previous observations in CRC patients stimulated with a proprietary cytokine cocktail formulation. Furthermore, NK cells from cancer patients at baseline had reduced IFNγ production as compared to healthy donors, indicative of a suppressed phenotype in keeping with what has been previously reported in the literature[117,224,291,308]. Importantly, these results suggest that NK cell IFNγ production, and not secretion, is impaired postoperatively indicating that defects in cytokine responsiveness are impacted by surgical stress.

4.3 Whole blood staining protocol development

Next, I sought to directly identify the impairments in IL-2 and IL-12 signaling events upstream of IFNγ production by quantifying receptor and signaling proteins via flow cytometry. However, assessment of phosphorylation status in cryopreserved PBMCs[309] was challenging and optimizations of rest periods, FBS concentrations, and cytokine concentrations, among other obstacles limited the reproducibility of cryopreserved PBMC assays. After a careful review of the literature, I developed and optimized protocols for the assessment of extracellular receptor express-

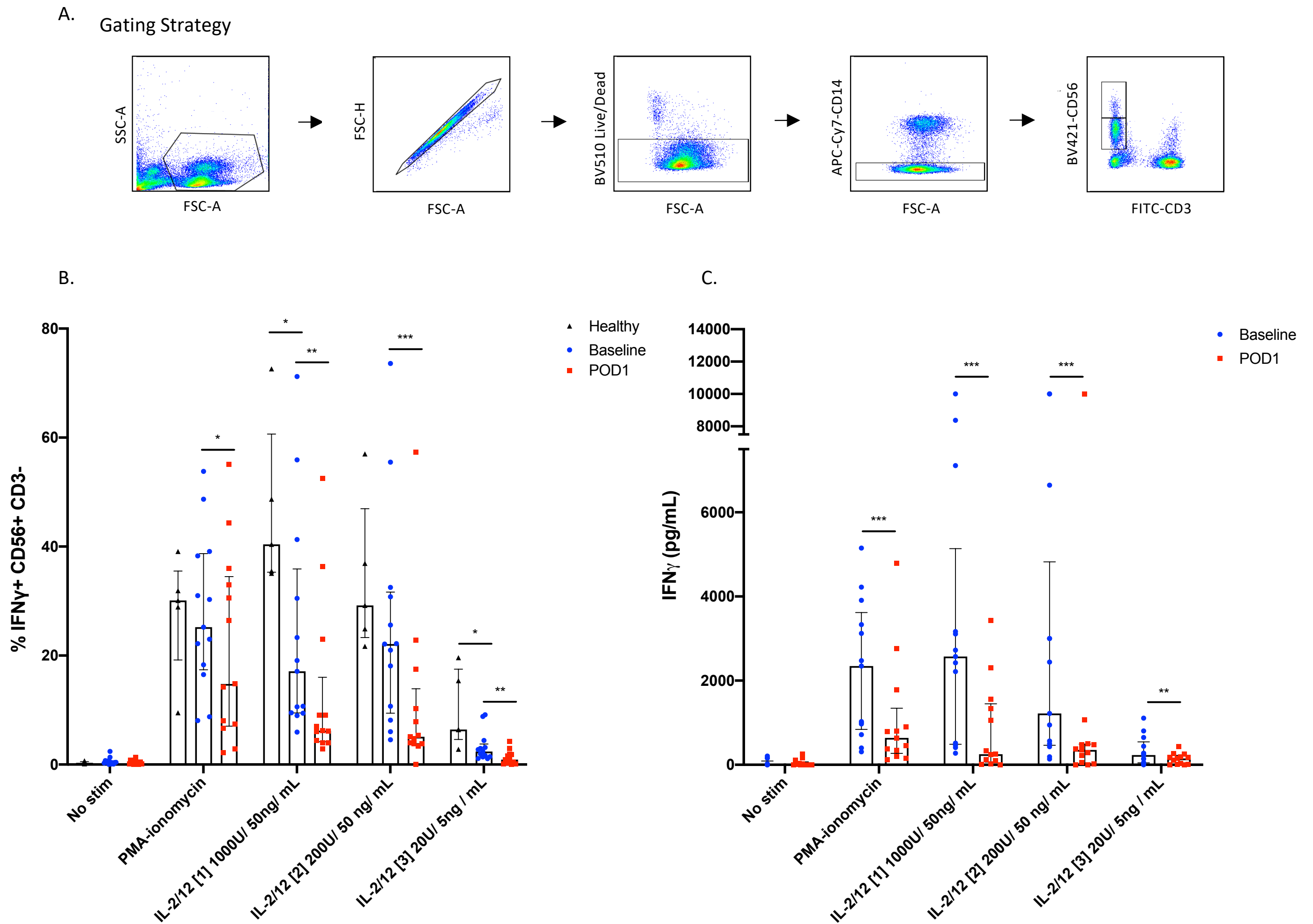

Figure 6. IFNγ production is suppressed in cryopreserved NK cells on POD1.
(A) Cryopreserved PBMCs from healthy donors (n=5) and cancer surgery patients (n=13) were thawed, stimulated with PMA-ionomycin for 5 hours or rIL-2/12 for 24 hours, and stained using a standard protocol. Debris, doublets, and dead cells were excluded before gating on $CD14^-$ $CD56^+CD3^-$ cells to assess intracellular IFNγ production. (B) There was a significant impairment in IFNγ production in baseline NK cells, as compared to healthy NK cells upon stimulation with rIL-2/12 [1] and [3] (Mann-Whitney test). A significant reduction in intracellular IFNγ was observed in POD1 NK cells in all stimulation conditions. (C) Supernatant was collected from cells in culture at 5 or 24 hours and extracellular IFNγ was quantified by ELISA. Similarly, a significant reduction in extracellular IFNγ was observed in all stimulation conditions. The Wilcoxon matched-pairs signed rank test was used to assess statistical significance between baseline and POD1 samples. $p \leq 0.05$ (*$p\leq0.05$, **$p\leq0.005$, ***$p\leq0.0005$, ****$p\leq0.00005$). Shown are the median values ± IQR.

ion, signaling molecule phosphorylation, and intracellular and extracellular IFNγ in whole blood resulting in a publication outlining this methodology[310]. In short, blood was collected from healthy donors and cancer surgery patients at baseline and on POD1. An ECS mix was added directly to whole blood for extracellular receptor staining. Whole blood was stimulated in the presence of an ECS mix for 20 minutes followed by ICS to quantify signaling protein phosphorylation. Whole blood was also stimulated for 5 hours with PMA-ionomycin or 24 hours with rIL-2/12 or in NKVue™ tubes, followed by plasma collection to quantify extracellular IFNγ or ECS/ICS to quantify intracellular IFNγ by flow cytometry.

In comparison to analyses using cryopreserved PBMCs, these assays require minimal volumes of blood (200 μL-1 mL per sample). This allows for the simultaneous assessment of many targets and the use of whole blood from one patient for multiple assays. Although I have used these assays to assess NK cells, the use of whole blood allows for the assessment of any immune cell, including high-density neutrophils which would otherwise be excluded in PBMC isolation protocols. Finally, I hypothesize that assessing immune cell phenotypes using whole blood may be more biologically relevant as these protocols minimize the time between blood draw and cell staining and reduce the manipulation of cells that may otherwise impact target expression/activity.

Granulocyte populations are present in relatively high frequencies in whole blood, however, are absent in isolated PBMCs. Granulocytes are large lymphocytes that contain cytoplasmic granules and include neutrophils, basophils, and eosinophils, with neutrophils being the most abundant leukocyte in human blood[311]. Neutrophils have been shown to have pleotropic effects on NK cells ranging from inducing NK cell licensing, to inhibiting proliferation and IFNγ production, to enhancing cytotoxic activity[312,313]. In order to discern whether these cells were having an impact on NK cell cytokine production, the percentage of $CD14^{-}FSC\text{-}A^{hi}SSC\text{-}A^{hi}$ cells (**Figure 7A**) was quantified and plotted against intracellular IFNγ in healthy donor and baseline/POD1 cancer surgery patient populations. No correlation was observed that would suggest

that granulocytes impact NK cell IFNγ production (**Figure 7B**). However, the activation of a large and variable number of granulocytes present in whole blood samples may impact results depending on the immunological phenotypes and cell populations of interest.

Comparisons of immunologic assays using cryopreserved PBMCs and whole blood samples have previously been reported with conflicting results[314–316]. I compared extracellular activating/inhibitory receptor (NKG2D, DNAM-1, NKG2A, PD-1, TIGIT, and TIM-3) and cytokine receptor (CD25 and CD212) expression in cryopreserved NK cells (n=10, n=11, respectively) and whole blood NK cells (n=20, n=9, respectively) and consistent with previous publications, found significant differences in both the percentage of positive cells as well as the per cell expression of receptors (median fluorescence intensity; MFI) gated on $CD56^{+}CD3^{-}$ cells[317,318]. Significant differences were observed in NKG2D (% and MFI), DNAM-1 (%), NKG2A (% and MFI), PD-1 (% and MFI), TIGIT (% and MFI), and TIM-3 (% and MFI) (**Figure 8A/B**) and CD25 (% and MFI) and CD212 (MFI) (**Figure 8C/D**) ($p<0.05$). This discrepancy may lead to misinterpreted conclusions about altered immune cell phenotype and function. For this reason, in addition to the aforementioned advantages of using whole blood, I decided to implement the use of these whole blood protocols for the remainder of my thesis.

4.4 IFNγ production is suppressed in whole blood NK cells on POD1

Whole blood was collected from healthy donors or cancer surgery patients at baseline and on POD1 and was incubated without stimulation (control/no stim), for 5 hours with PMA-ionomycin, or for 24 hours with rIL-2/12 (400U/ 20 ng/ mL) or in NK Vue™ tubes. Golgiplug™ was added for the last two hours of incubation. Two hundred uL of whole blood was then transferred to a conical tube for staining (**Figure 9A**). Prior to staining, 600 uL of blood was transferred to an Eppendorf tube and centrifuged to isolate plasma for quantification of extracellular IFNγ by ELISA (**Figure 9B**). Intracellular IFNγ was assessed in healthy donors (n=11) and cancer

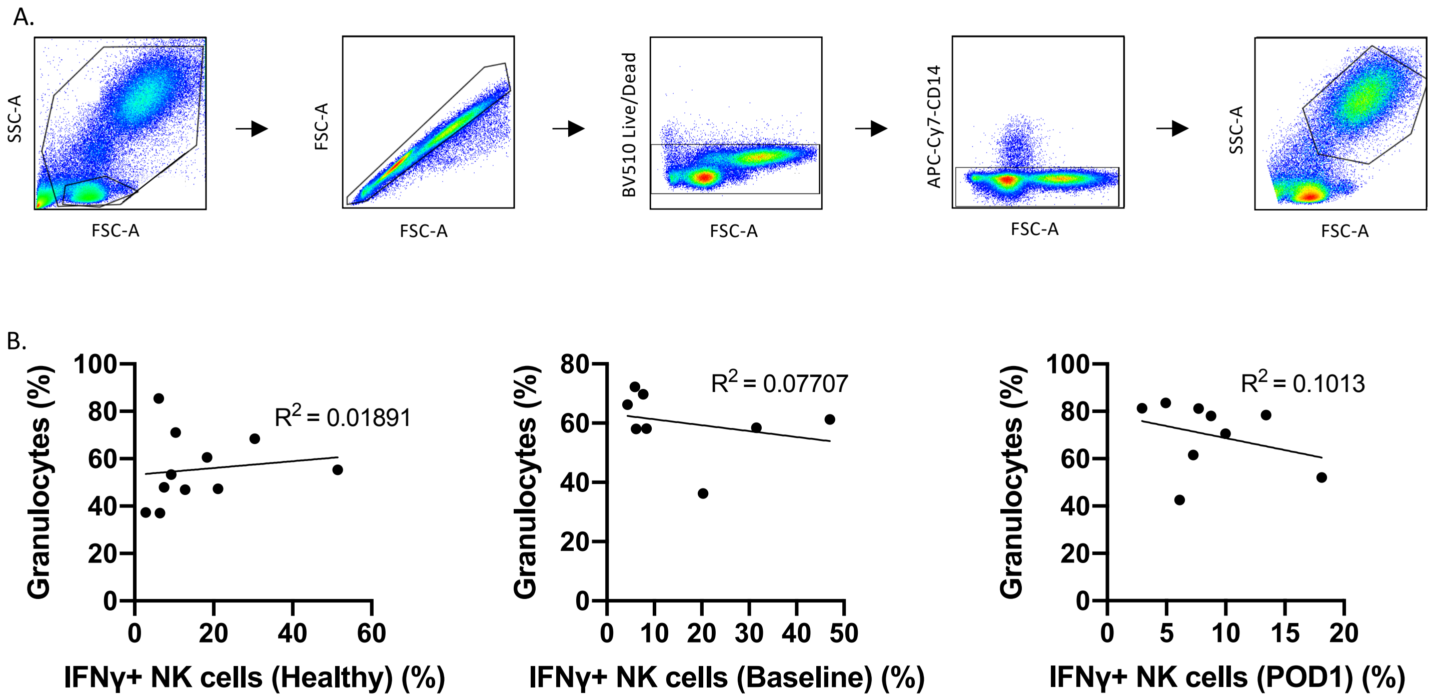

Figure 7. Proportion of granulocytes is not related to NK cell IFNγ production.
(A) Cells were gated on to exclude debris, doublets, dead cells, and $CD14^+$ cells prior to gating on $SSC\text{-}A^{hi}FSC\text{-}A^{hi}$ cells. (B) The percentage of $SSC\text{-}A^{hi}FSC\text{-}A^{hi}$ cells was plotted against intracellular IFNγ produced in the same patient samples (healthy donors (n=11) and baseline/POD1 patient samples (n=9)). No correlation was found in any population upon performing a linear regression.

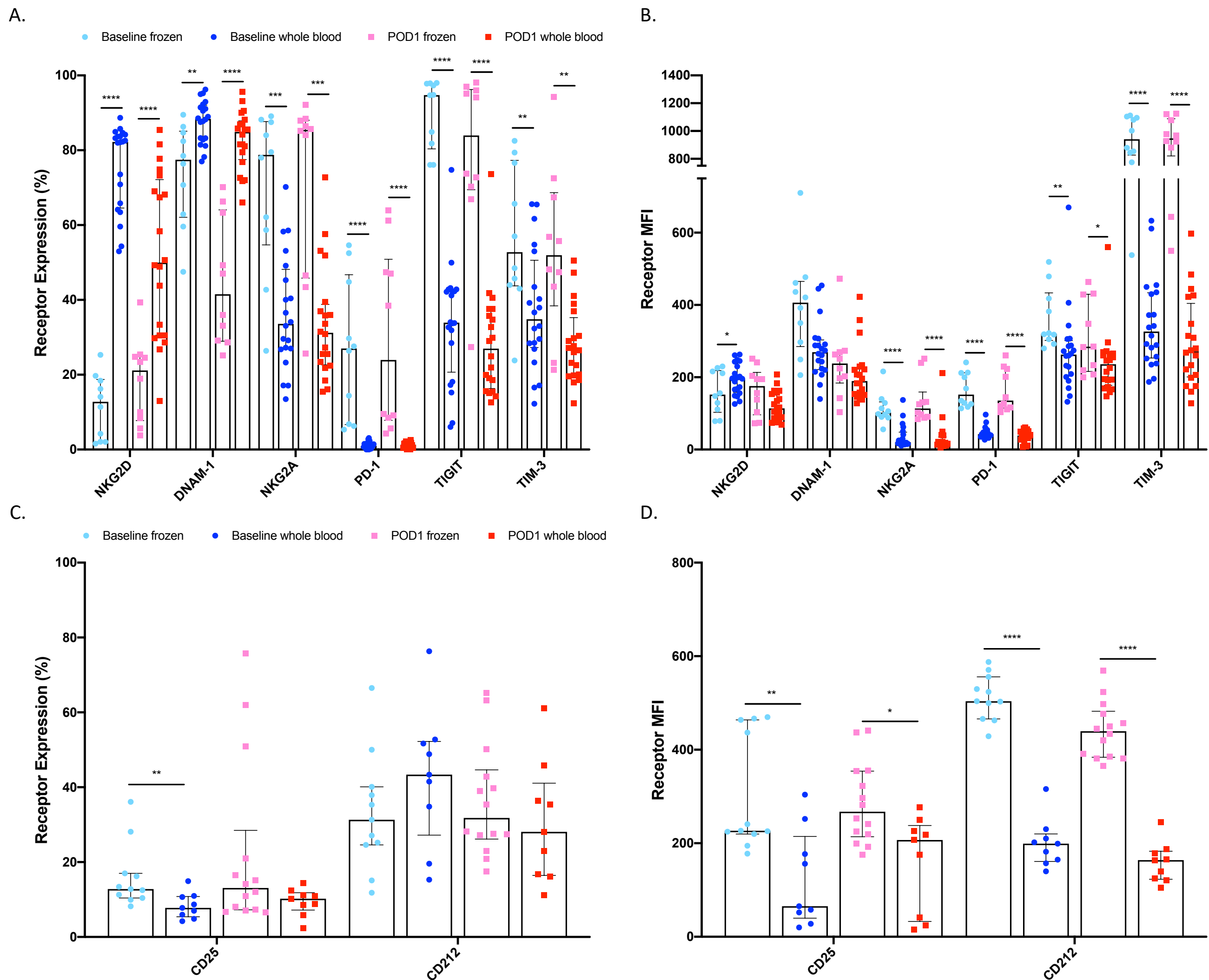

Figure 8. NK cell extracellular receptor expression in cryopreserved vs. whole blood-stained NK cells.

Activating/inhibitory receptor expression (NKG2D, DNAM-1, NKG2A, PD-1, TIGIT, and TIM-3) and cytokine receptor subunit expression (CD25 and CD212) was quantified in NK cells from both cryopreserved PBMCs (n=10, n=11, respectively) and whole blood (n=20, n=9, respectively) from cancer surgery patients at baseline and on POD1. (A/C) Significant differences were observed in the percentage of $CD56^{+}CD3^{-}$ cells expressing these receptors between cryopreserved and whole blood samples. (B/D) Significant differences were also observed in receptor MFI between cryopreserved and whole blood samples. The Mann-Whitney test was used to assess statistical significance between cryopreserved and whole blood samples. $p \leq 0.05$ (*$p\leq0.05$, **$p\leq0.005$, ***$p\leq0.0005$, ****$p\leq0.00005$). Shown are the median values ± IQR.

surgery patients (n=9) and measured by flow cytometry. The previous gating strategy was used and IFNγ gating was set based on unstimulated controls (**Appendix A**) to determine whether IFNγ production in $CD56^{+}CD3^{-}$ cells in whole blood was similar to the results from cryopreserved PBMCs (**Figure 9C**). As previously done, extracellular IFNγ was also quantified in healthy donors (n=13) and cancer surgery patients (n=10) and measured by ELISA. Similar to results using cryopreserved PBMCs, there was a suppression of intracellular IFNγ production assessed by flow cytometry in POD1 samples ($p<0.05$, rIL-2/12 stimulation) (**Figure 10A**). In addition, there was a significant reduction in extracellular IFNγ in rIL-2/12 and NKVue™ stimulated samples ($p<0.05$) (**Figure 10B**). However, lower levels of IFNγ were measured in the whole blood assay when compared to the previous cryopreserved assay which may reflect the different stimulation conditions, a shorter Golgiplug™ incubation period (2 hours versus 4 hours) or inherent inter-patient heterogeneity. These whole blood results in combination with the cryopreserved data allowed us to be confident of the suppressed postoperative NK cell phenotype.

4.5 IFNγ production following surgical stress is more impaired in $CD56^{Bright}$ NK cells

Next, I sought to determine whether surgical stress differentially impacts IFNγ production from distinct NK cell subsets. $CD56^{Bright}CD16^{dim/-}$ cells make up 10% of peripheral blood and spleen NK cells, whereas most lymph node NK cells are $CD56^{Bright}CD16^{dim/-}$. Although they can exert cytotoxic effector functions, $CD56^{Bright}$ NK cells readily produce cytokines[8,9] and are therefore a population of interest in terms of assessing IFNγ production. Although not statistically significant, $CD56^{Bright}$ cells trended towards producing more IFNγ than their $CD56^{Dim}$ counterparts (**Figure 11**). However, the $CD56^{Bright}$ population did exhibit a greater reduction in intracellular IFNγ on POD1 in response to rIL-2/12 ($p<0.05$) (**Figure 12**).

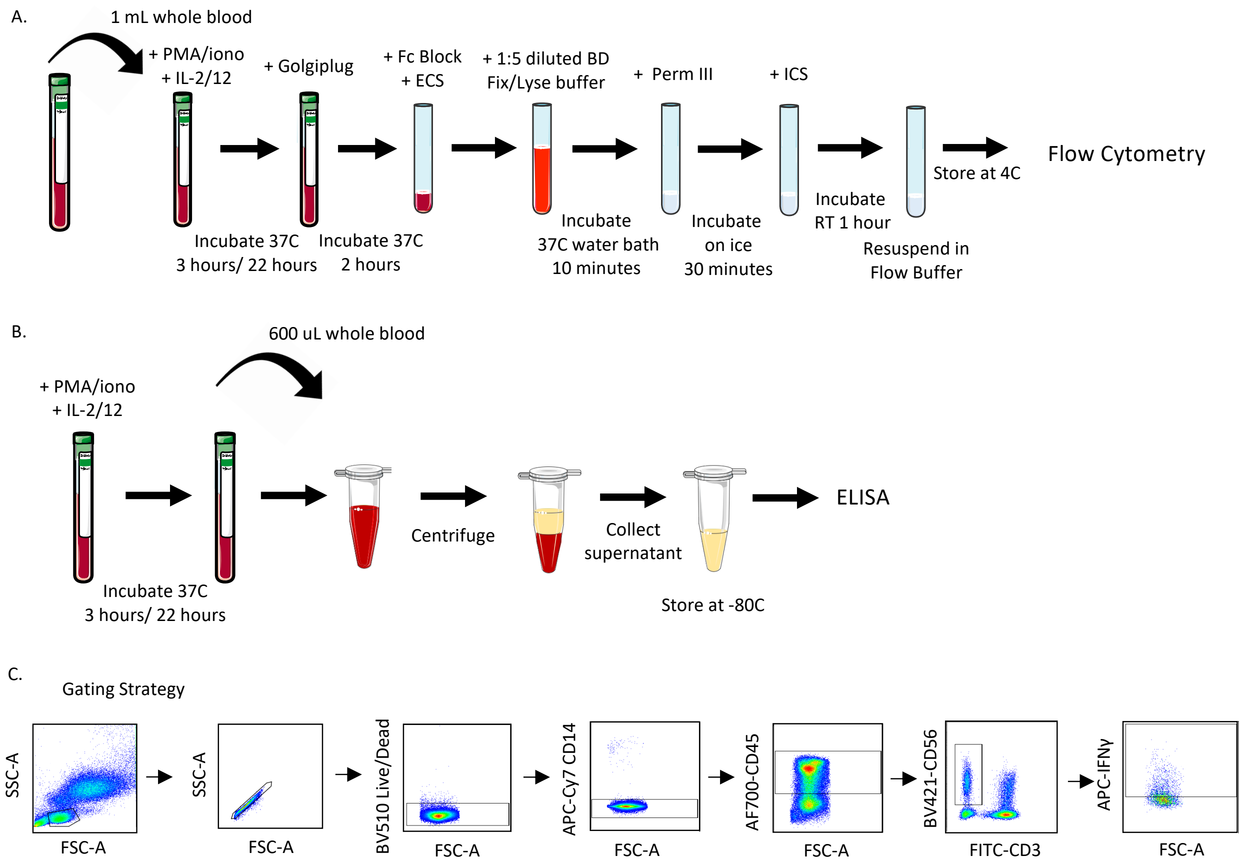

Figure 9. Whole blood IFNγ staining protocol and gating strategy.
(A) Whole blood was collected and stimulated with PMA-ionomycin for 5 hours or rIL-2/12/in NKVue^{TM} tubes for 24 hours prior to intracellular staining for IFNγ. (B) After stimulation, prior to staining, whole blood was collected and centrifuged to collect platelet-free plasma for quantification of extracellular protein by ELISA. (C) Cells were gated on to exclude debris, doublets, dead cells, and CD14^{+} cells prior to gating on CD45^{+}CD56^{+}CD3^{-} cells to assess intracellular IFNγ.

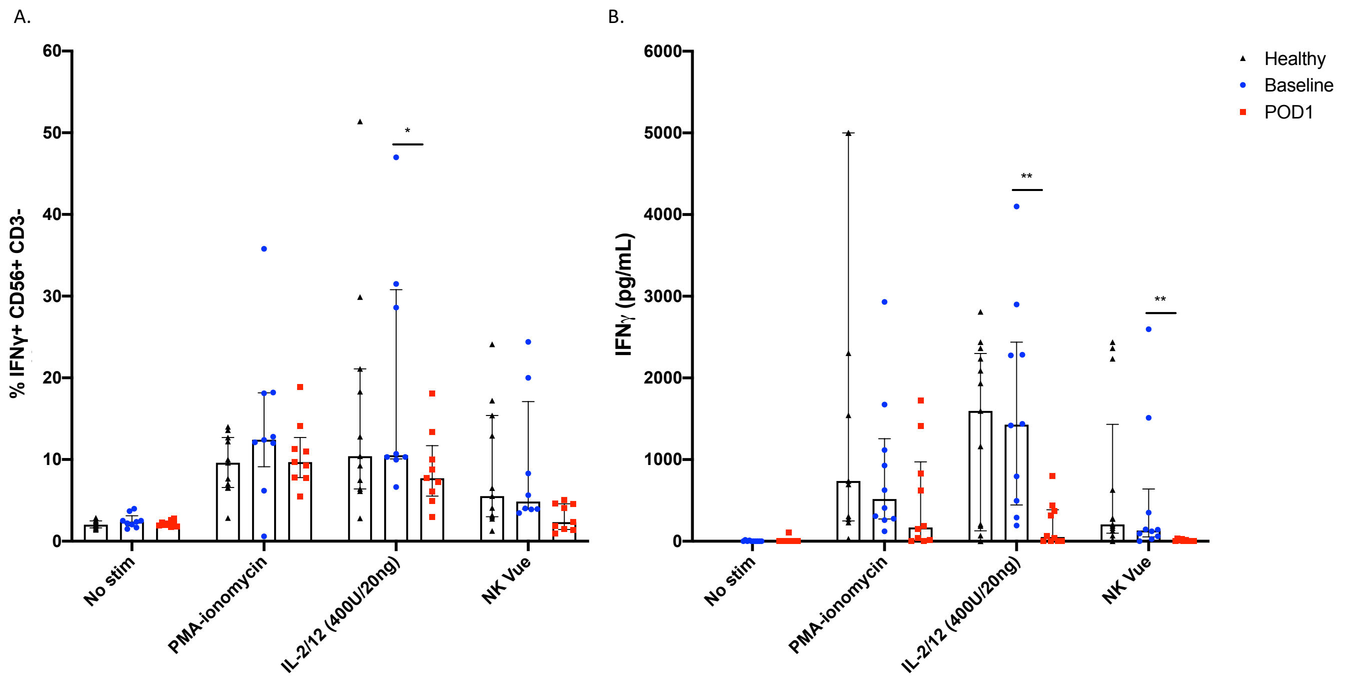

Figure 10. IFNγ production is suppressed in whole blood NK cells on POD1.
(A) There was a significant impairment in intracellular IFNγ in POD1 NK cells (n=9) upon 24-hour stimulation with rIL-2/12. (B) A significant reduction in extracellular IFNγ on POD1 (n=10) was observed upon stimulation with rIL-2/12 or with NKVue™. The Wilcoxon matched-pairs signed rank test was used to assess statistical significance between baseline and POD1 samples. $p \leq 0.05$ (*p≤0.05, **p≤0.005, ***p≤0.0005, ****p≤0.00005). Shown are the median values ± IQR.

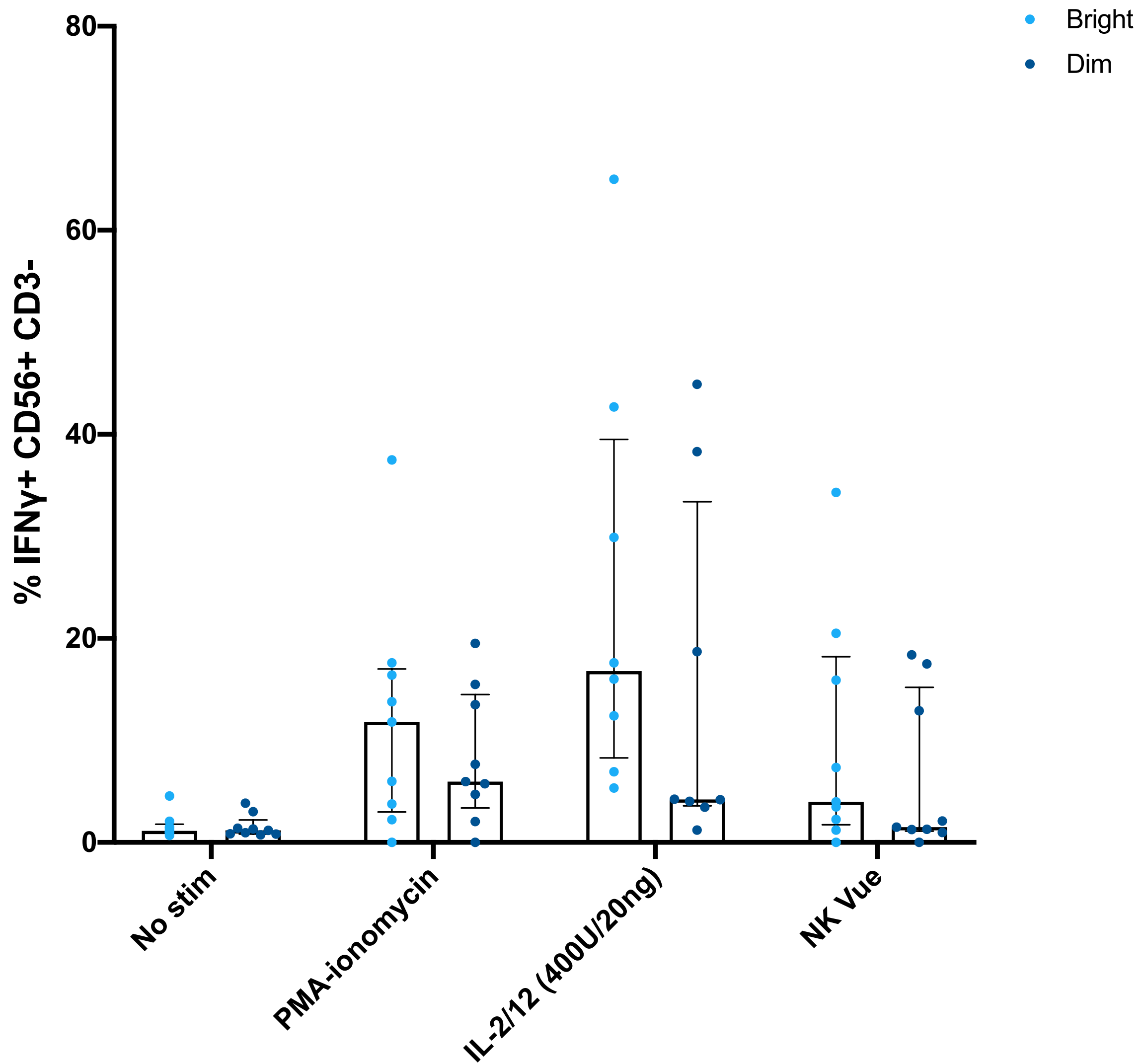

Figure 11. CD56^{Bright} cells produce more IFNγ upon stimulation than their CD56^{Dim} counterparts.

Baseline whole blood stimulated with PMA-ionomycin for 5 hours or rIL-2/12/in NKVue^{TM} tubes for 24 hours was stained for intracellular IFNγ (n=9). CD56^{Bright} cells secreted more IFNγ in response to all stimuli as compared to CD56^{Dim} cells from the same baseline patient samples (p=ns). The Mann-Whitney test was used to assess statistical significance. Shown are the median values ± IQR.

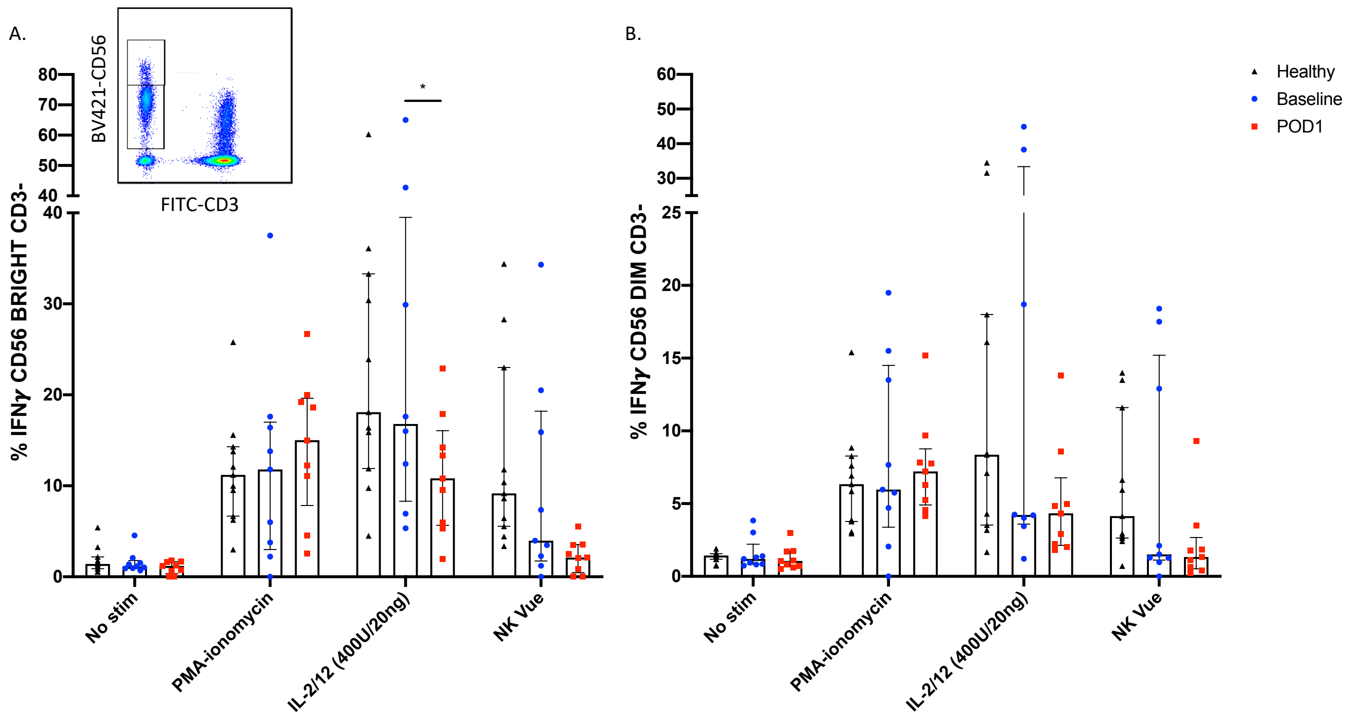

Figure 12. CD56^{bright} NK cells have impaired IFNγ production on POD1.
Baseline whole blood stimulated with PMA-ionomycin for 5 hours or rIL-2/12/ in NKVue^{TM} tubes for 24 hours was stained for intracellular IFNγ (n=9). CD56$^{Bright/Dim}$ CD3^{-} cells were gated on. (A/B) CD56^{Bright} cells exhibited a statistically significant reduction in IFNγ production, while CD56^{Dim} cells did not. The Wilcoxon matched-pairs signed rank test was used to assess statistical significance between baseline and POD1 samples. $p \leq 0.05$ (*p≤0.05, **p≤0.005, ***p≤0.0005, ****p≤0.00005). Shown are the median values ± IQR.

4.6 IL-2 and IL-12 receptor subunit expression is reduced in whole blood NK cells on POD1

As a first step in determining the defect in NK cell responses to cytokine stimulation I sought to investigate the effects of surgical stress on cytokine receptor expression. Whole blood was collected from healthy donors (n=13) or cancer surgery patients (n=9) at baseline and on POD1 and stained for IL-2 receptor subunits CD25 (α), CD122 (β), and CD132 (γ), and IL-12 subunit CD212 (β1) (**Figure 13A**). Using the indicated gating strategy (**Figure 13B**), set based on isotype controls (**Appendix B**), I observed a significant reduction in the expression of CD212 (MFI ($p<0.05$)) and CD132 (MFI ($p<0.05$)) on POD1 in the $CD56^{+}CD3^{-}$ cell population (**Figure 13C/D**). Receptor expression was also assessed in $CD56^{Bright/Dim}CD3^{-}$ populations based on CD56 staining. Baseline $CD56^{Bright}$ cells expressed significantly more CD25 (%), CD132 (%, MFI), and CD212 (MFI) as compared to their $CD56^{Dim}$ counterparts ($p<0.05$) (**Figure 14**). Interestingly, a reduction in CD132 and CD212 expression was observed in both the bright and dim populations on POD1 with significant reductions occurring in the $CD56^{Dim}$ population ($p<0.05$). No differences were observed in CD25 or CD122 expression between baseline and POD1 samples (**Figure 15**). Together these studies indicate that there is a reduction in the expression of a subset of the cytokine receptors for IL-2 and IL-12 which is correlated/associated with reduced IFNγ production on POD1. CD132 is essential for high affinity IL-2 receptor formation and is a shared subunit among other cytokine receptors, including IL-15[6]. CD212 is the constitutively expressed subunit of the IL-12 receptor and is the subunit which physically associates with IL-12[51]. These subunits are therefore essential for NK cell response to extracellular cytokines and a reduction in their expression could have significant consequences for the activation of downstream signaling cascades. Finally, although $CD56^{Bright}$ cells appear to be more impaired in their ability to secrete IFNγ and show significant reductions in cytokine receptor subunits, cytokine receptor expression is also significantly reduced in $CD56^{Dim}$ cells.

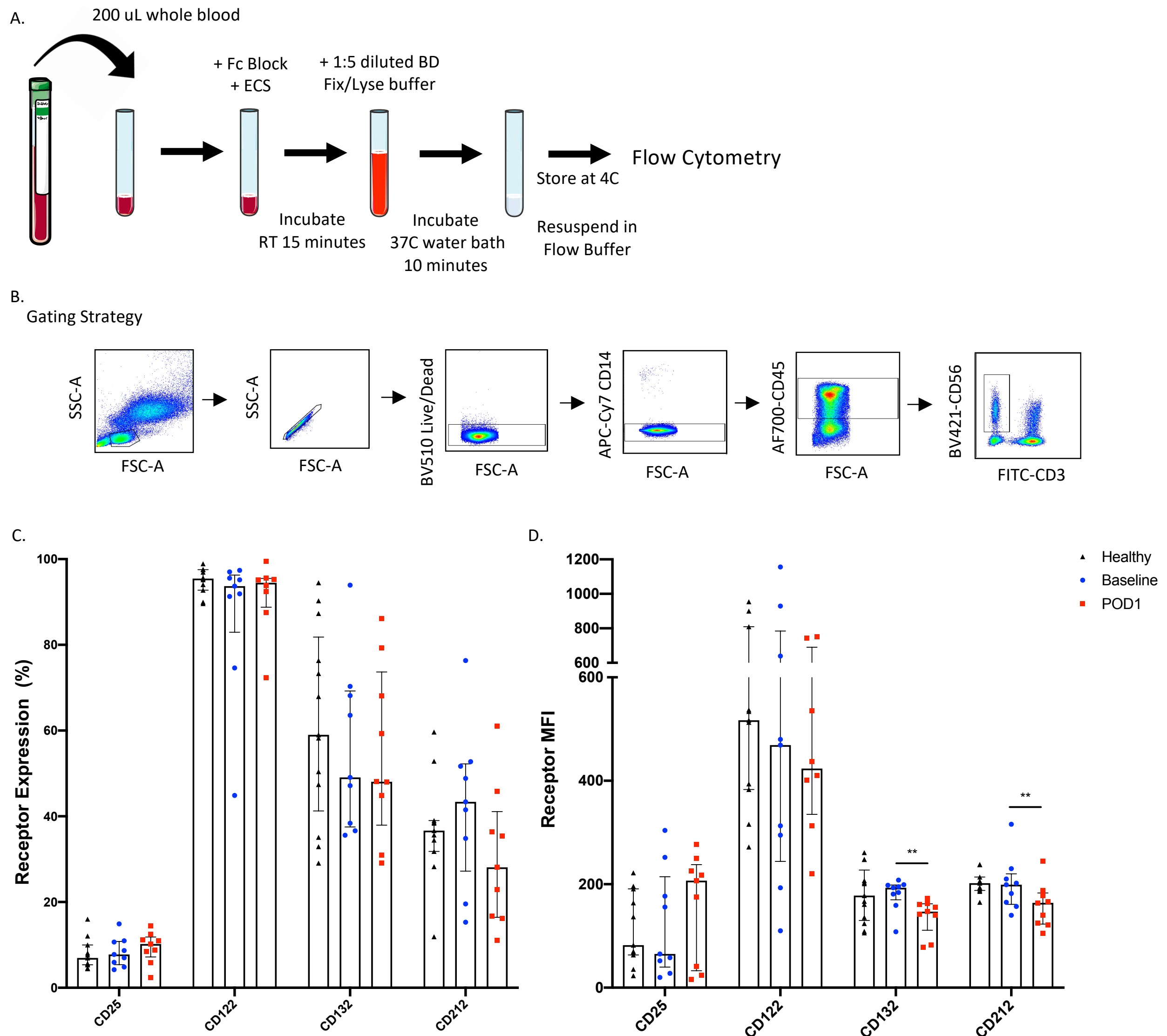

Figure 13. IL-2 and IL-12 receptor subunit expression is reduced in whole blood NK cells on POD1.

(A) Whole blood was collected from healthy donors (n=13) and cancer surgery patients (n=9) and stained for extracellular receptor expression. (B) Cells were gated on to exclude debris, doublets, dead cells, and $CD14^+$ cells prior to gating on $CD45^+CD56^+CD3^-$ cells to assess receptor expression. (C) There was a trend towards reduced percentage of $CD56^+CD3^-$ cells expressing CD212. (D) A statistically significant reduction in the MFI of CD212 and CD132 was observed in NK cells from POD1 samples. The Wilcoxon matched-pairs signed rank test was used to assess statistical significance between baseline and POD1 samples. $p \leq 0.05$ (*p≤0.05, **p≤0.005, ***p≤0.0005, ****p≤0.00005). Shown are the median values ± IQR.

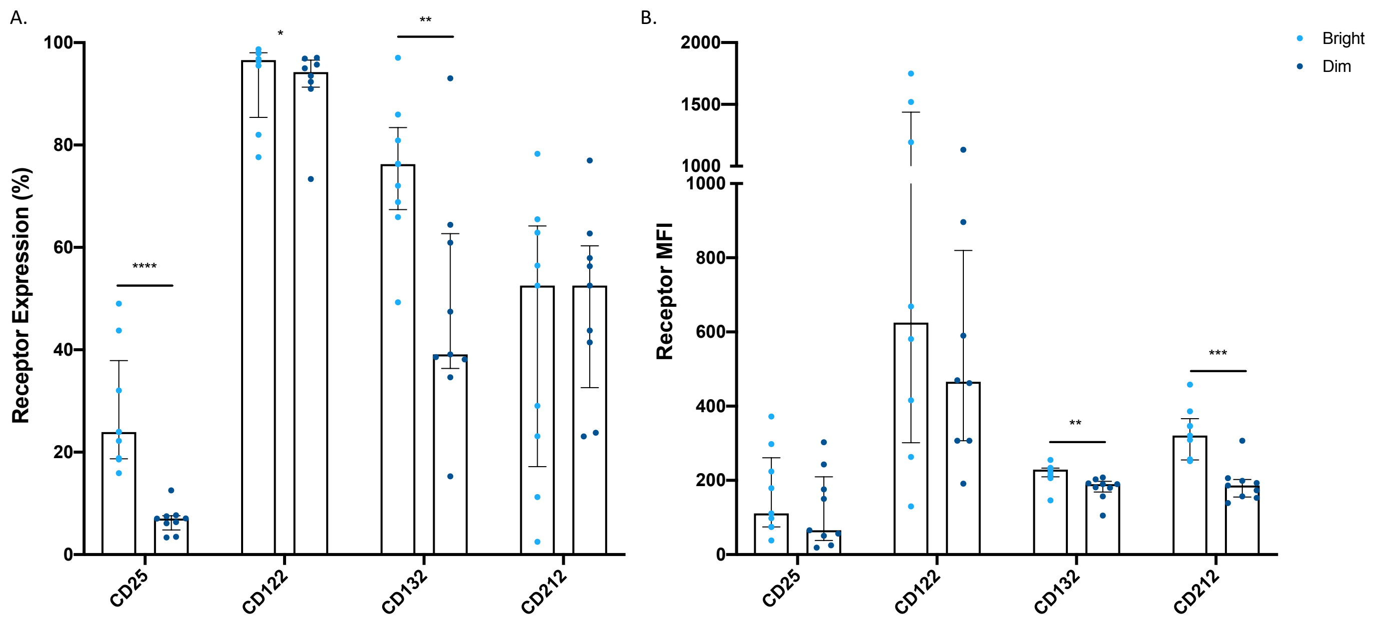

Figure 14. CD56^{Bright} cells have increased cytokine receptor expression as compared to CD56^{Dim} cells.

Baseline whole blood was stained for extracellular cytokine receptors and gated on CD56$^{Bright/Dim}$CD3^{-} populations (n=9). CD56^{Bright} cells expressed significantly more CD25 (%), CD132 (% and MFI), and CD212 (MFI) as compared to CD56^{Dim} cells. The Mann-Whitney test was used to assess statistical significance. $p \leq 0.05$ (*p≤0.05, **p≤0.005, ***p≤0.0005, ****p≤0.00005). Shown are the median values ± IQR.

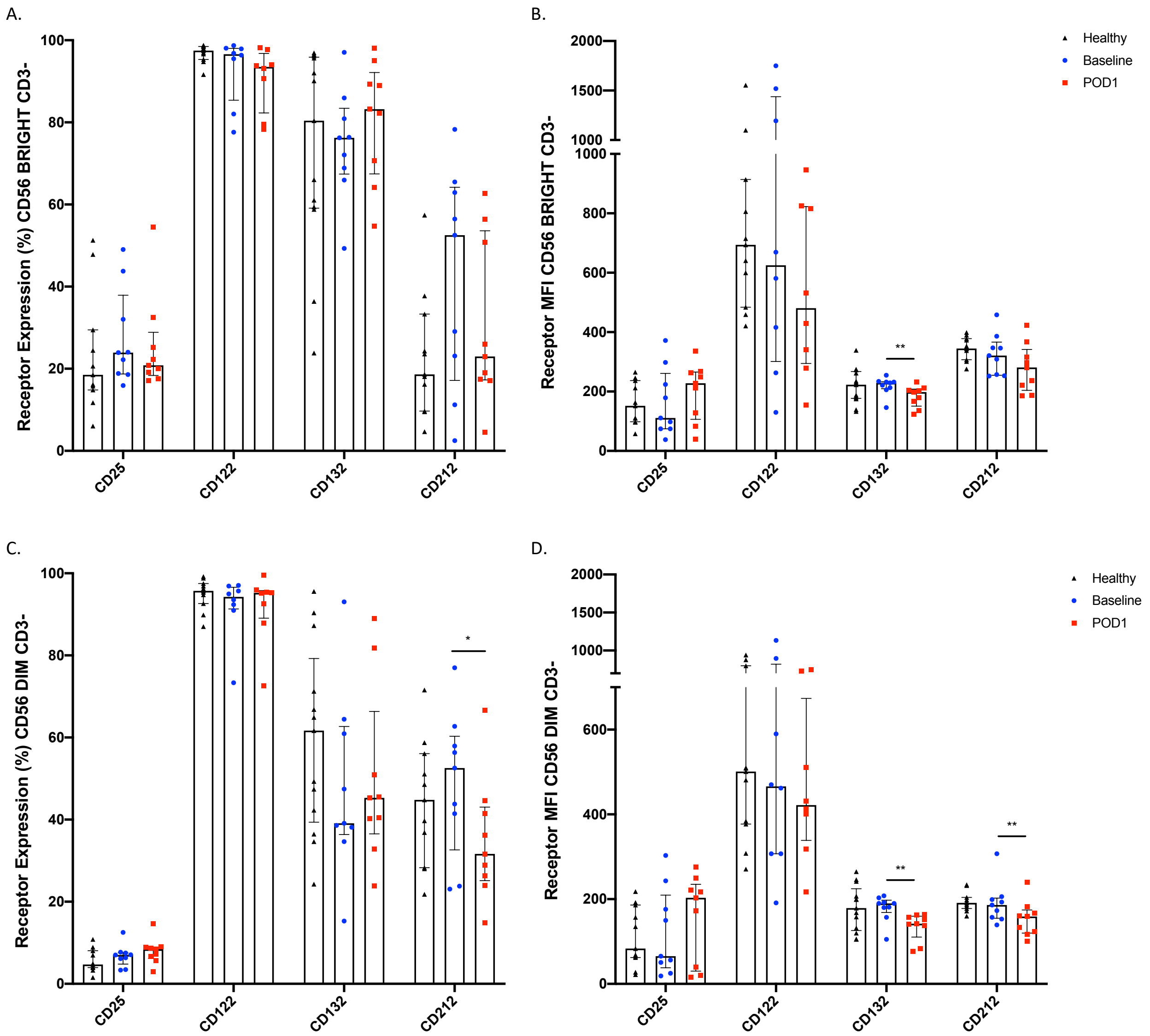

Figure 15. CD56^{bright} and CD56^{dim} NK cells have reduced cytokine receptor expression. Whole blood from healthy donors (n=13) and cancer surgery patients (n=9) was stained for extracellular cytokine receptors and gated on CD56$^{Bright/Dim}$CD3^{-} populations. (A/B) CD56^{Bright} cells had significantly reduced CD132 (MFI). (C/D) CD56^{Dim} cells had significantly reduced CD132 (MFI) and CD212 (percentage and MFI). The Wilcoxon matched-pairs signed rank test was used to assess statistical significance between baseline and POD1 samples. $p \leq 0.05$ (*p≤0.05, **p≤0.005, ***p≤0.0005, ****p≤0.00005). Shown are the median values ± IQR.

4.7 Postoperative NK cells have significantly reduced activating receptor expression

While cytokine receptors are critical for maintaining NK cell viability and function, the response of NK cells to the environment and recognition of target cells is dependent upon integration of signals through additional activating and inhibitory receptors. As above, whole blood was collected from healthy donors (n=16) and cancer surgery patients (n=20) at baseline and on POD1 and stained for two activating receptors (NKG2D and DNAM-1) and four inhibitory receptors (NKG2A, PD-1, TIGIT, and TIM-3) and assessed by flow cytometry. The indicated gating strategy allowed for the assessment of receptor expression in $CD56^{+}CD3^{-}$ NK cells and gating was set based on paired isotype controls (**Appendix C**). I hypothesized that dysfunctional POD1 NK cells would exhibit reduced activating receptor expression and increased inhibitory receptor expression, however I observed a significant reduction in both activating and inhibitory receptors with the most profound and consistent reductions being observed in NKG2D and DNAM-1 expression ($p<0.05$) (**Figure 16**). Postoperative reductions in inhibitory receptor expression (NKG2A, TIGIT, and TIM-3) was variable, although statistically significant. Receptor expression was also assessed in $CD56^{Bright/Dim}CD3^{-}$ populations based on CD56 staining. When comparing receptor expression in $CD56^{Bright}$ and $CD56^{Dim}$ populations in baseline samples, the $CD56^{Dim}$ population expressed NKG2D, TIGIT, and TIM-3 at a higher frequency ($p<0.05$), while they expressed DNAM-1 and NKG2A at a lower frequency ($p<0.05$) (**Figure 17**). This may reflect the predominantly cytotoxic effector functions of this population. The NK cell population is heterogenous due to the heterologous expression of germline-encoded receptors. Thus, the variation in activating/inhibitory receptor expression may reflect this heterogeneity.

Similar to the phenotypes observed with cytokine receptor expression in $CD56^{Bright/Dim}$ populations, $CD56^{Dim}CD3^{-}$ cells showed more significant reductions in receptor expression, as compared to $CD56^{Bright}$ cells, although both populations had significantly reduced NKG2D and DNAM-1 expression on POD1 (**Figure 18**).

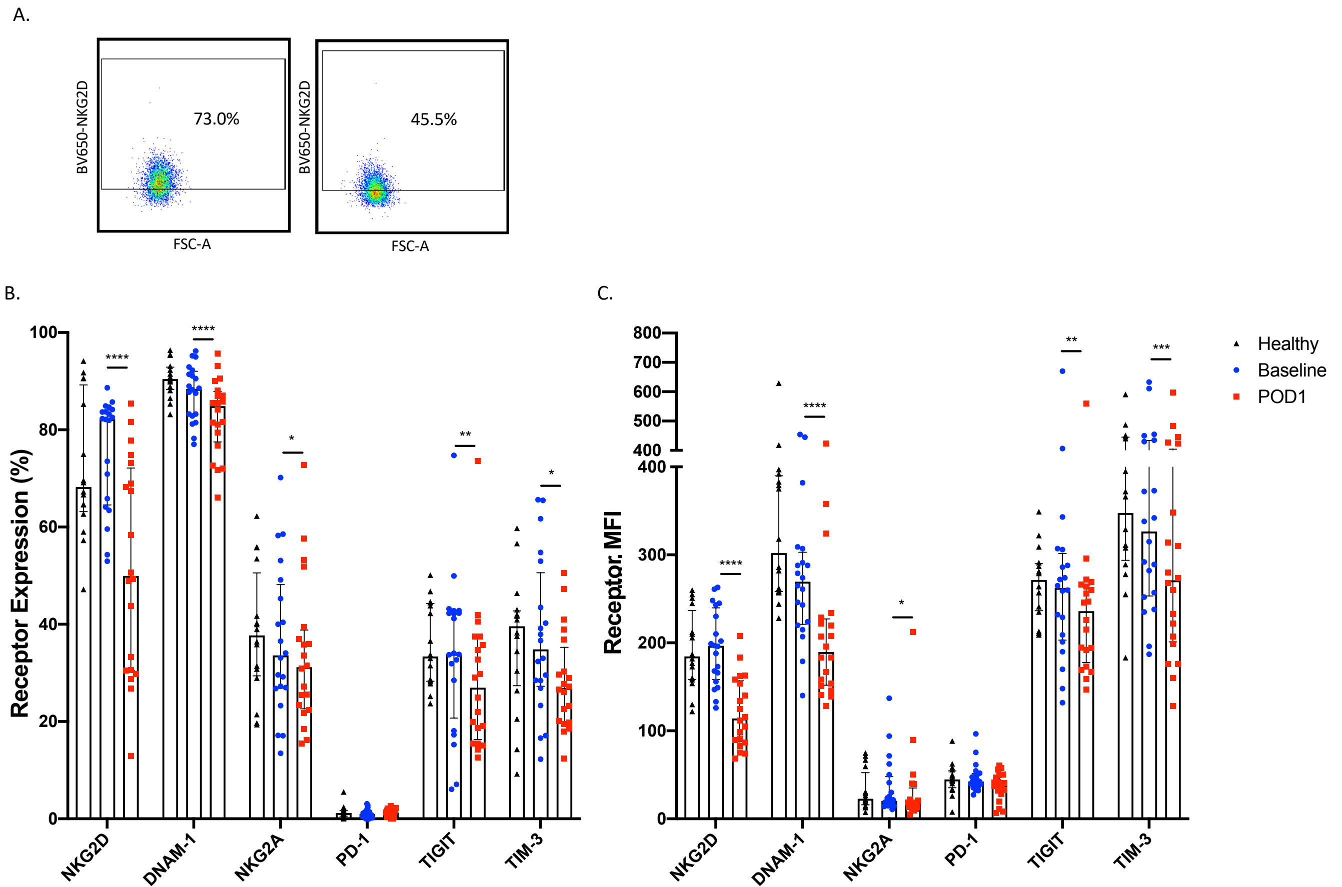

Figure 16. Postoperative NK cells have significantly reduced activating receptor expression. Whole blood was collected from healthy donors (n=16) and cancer surgery patients (n=20) and stained for extracellular receptor expression. (A/B) Postoperative NK cells express significantly less NKG2D and DNAM-1 (% and MFI) and have reduced inhibitory receptor expression (NKG2A, TIGIT, TIM-3 (% and MFI)). Wilcoxon matched-pairs signed rank test was used to assess statistical significance between baseline and POD1 samples. $p \leq 0.05$ (*$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$, ****$p \leq 0.00005$). Shown are the median values ± IQR.

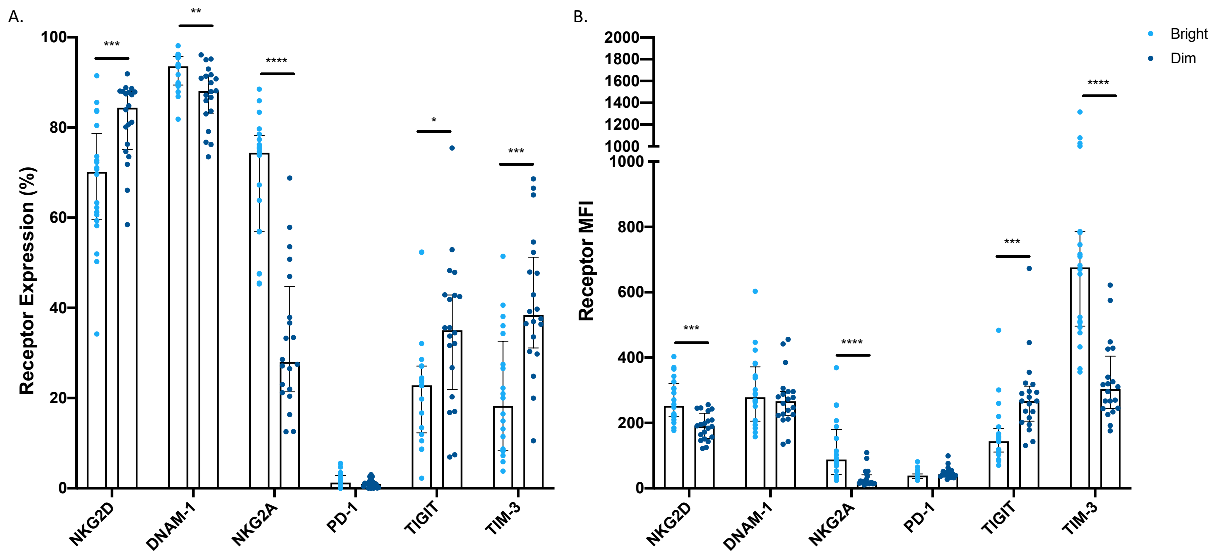

Figure 17. Differential expression of receptors at baseline in CD56$^{Bright/Dim}$ populations. Baseline whole blood was stained for extracellular cytokine receptors and gated on CD56$^{Bright/Dim}$CD3^{-} populations (n=20). CD56^{Bright} cells expressed significantly more NKG2D (MFI), DNAM-1 (%), NKG2A (% and MFI), and TIM-3 (MFI) as compared to CD56^{Dim} cells. The Mann-Whitney test was used to assess statistical significance. $p \leq 0.05$ (*p≤0.05, **p≤0.005, ***p≤0.0005, ****p≤0.00005). Shown are the median values ± IQR.

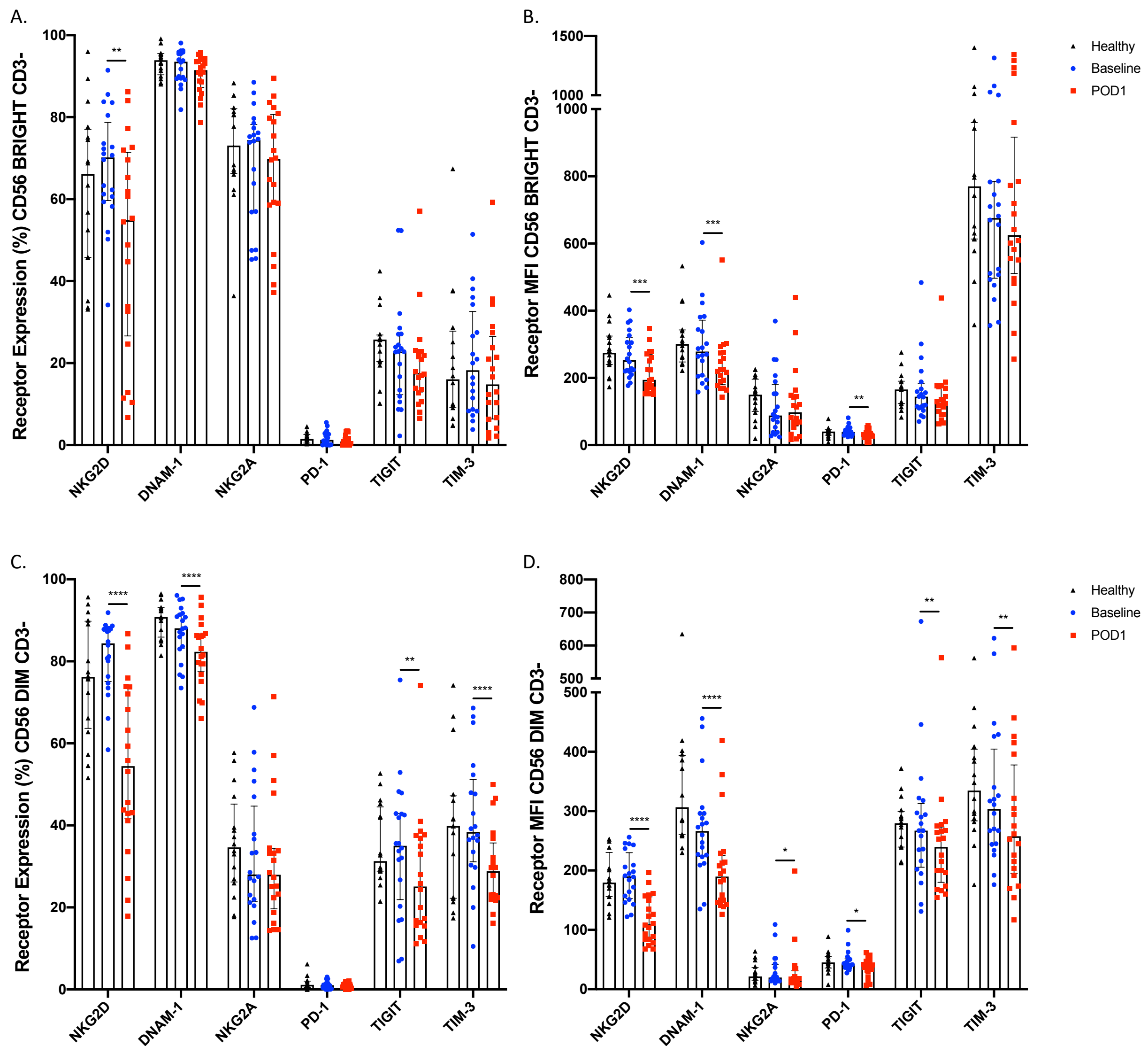

Figure 18. Both CD56^{Bright} and CD56^{Dim} NK cells have reduced activating receptor expression on POD1.

Whole blood was collected from healthy donors (n=16) and cancer surgery patients (n=20) and stained for extracellular activating/inhibitory receptors and gated on CD56$^{Bright/Dim}$CD3^{-} populations. (A/B) CD56^{Bright} cells had significantly reduced NKG2D (% and MFI), DNAM-1 (MFI), and PD-1 (MFI) expression. (C/D) CD56^{Dim} cells had significantly reduced NKG2D (% and MFI), DNAM-1 (% and MFI), TIGIT (% and MFI) and TIM-3 (% and MFI) expression. The Wilcoxon matched-pairs signed rank test was used to assess statistical significance between baseline and POD1 samples. $p \leq 0.05$ (*p≤0.05, **p≤0.005, ***p≤0.0005, ****p≤0.00005). Shown are the median values ± IQR.

These activating receptors are critical for NK cell recognition of cancer cells and anti-tumour cytotoxic activity. Given the importance of activating receptor expression in the recognition of tumour cells, this is consistent with our previous findings that NK cell cytotoxicity is impaired postoperatively[291]. Furthermore, due to the profound and universal reduction in NKG2D and DNAM-1 expression and the knowledge that postoperative immune suppression is universal, these activating receptors were investigated in following experiments.

4.8 Downstream signaling molecule phosphorylation is significantly impaired on POD1 following rIL-2/12 stimulation

Next I sought to determine whether reductions in IL-2 and IL-12 receptor expression led to impaired responses to these cytokines in whole blood collected from healthy donors (n=11) and cancer surgery patients (n=9) at baseline and on POD1. Samples were simultaneously stimulated with rIL-2/12 and stained with an ECS mix for 20 minutes prior to cell fixation, permeabilization, and intracellular staining (**Figure 19A**). Intracellular phosphorylation (MFI gated on $CD56^{+}CD3^{-}$ cells) of signaling proteins STAT4, STAT5, S6, and p38 MAPK was assessed by flow cytometry (**Figure 19B**). IFNγ production in response to rIL-2/12 is dependent upon the phosphorylation of STAT5, STAT4, p38 MAPK, and S6[45]. In addition, S6 is activated downstream of NKG2D and DNAM-1[34,319]. Without exception, all of these signaling proteins had significantly reduced phosphorylation in response to rIL-2/12 stimulation on POD1 in all patients ($p<0.05$) (**Figure 19C**). Interestingly, $CD56^{Bright}$ cells had higher levels of phosphorylated signaling proteins than $CD56^{Dim}$ cells in response to rIL-2/12 stimulation, with differences in pSTAT4 and pS6 achieving significance ($p<0.05$) (**Figure 20**). This may reflect their ability to respond to cytokine stimulation, resulting predominantly in the secretion of cytokines like IFNγ. A significant impairment in phosphorylation was observed in both $CD56^{Bright}$ and $CD56^{Dim}$ populations, although the impairment in STAT4/5 activity was more significant in the $CD56^{Bright}$ population (**Figure 21**). Together, these data suggest that surgical stress is associated with a reduction in the expression of

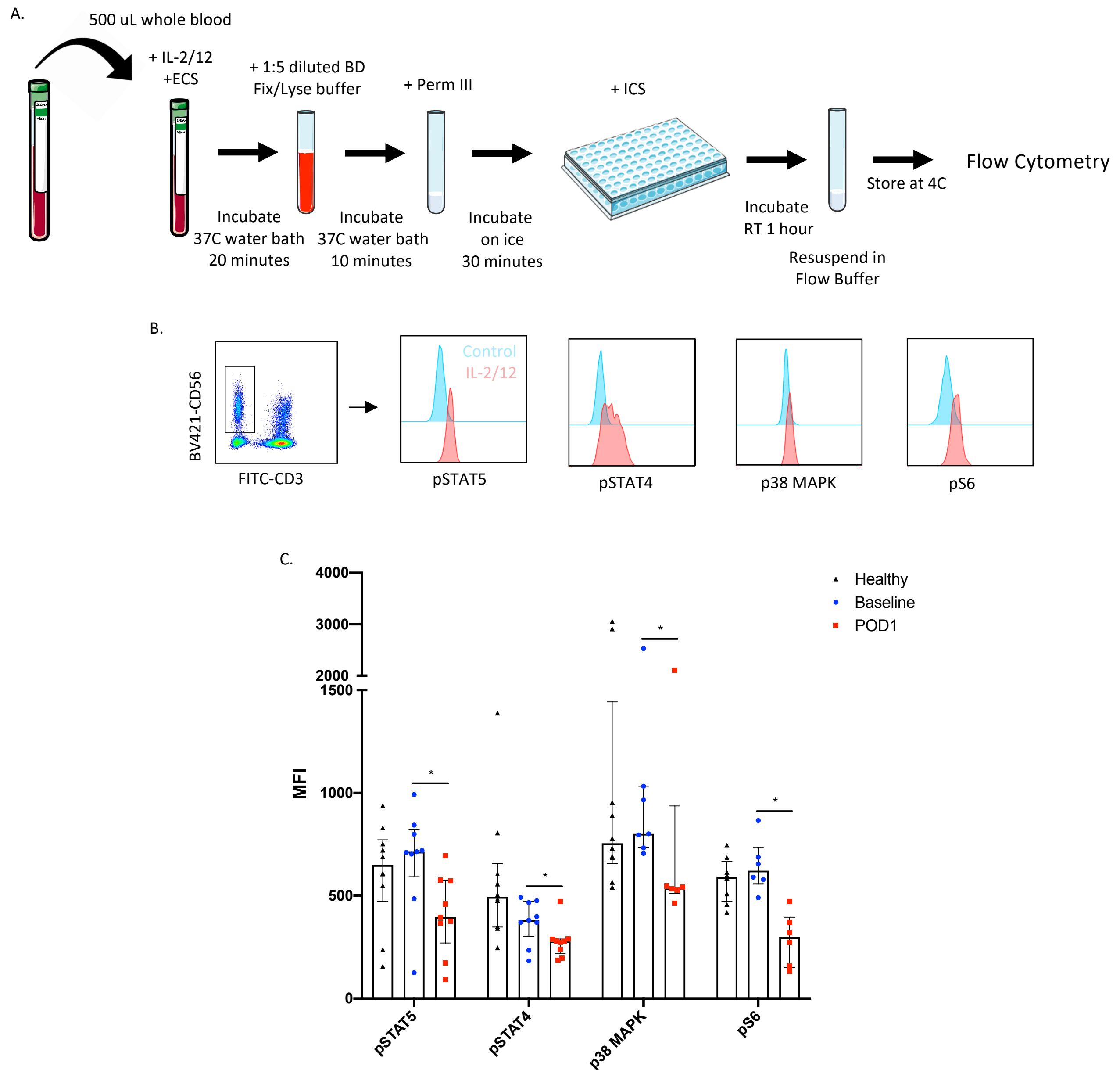

Figure 19. Downstream signaling protein phosphorylation is significantly impaired on POD1 following rIL-2/12 stimulation.

(A) Whole blood was collected from healthy donors (n=11) and cancer surgery patients (n=9) and stimulated for 20 minutes with rIL-2/12 in the presence of an ECS mix, followed by intracellular staining for signaling proteins (pSTAT5, pSTAT4, pP38 MAPK, and pS6). (B) $CD56^{+}CD3^{-}$ cells were gated on after excluding debris, doublets, dead cells, $CD14^{+}$ cells, and $CD45^{-}$ cells, to assess NK cell signaling protein MFI. (C) Postoperative NK cell target signaling protein phosphorylation was significantly impaired. The Wilcoxon matched-pairs signed rank test was used to assess statistical significance between baseline and POD1 samples. $p \leq 0.05$ (*p≤0.05, **p≤0.005, ***p≤0.0005, ****p≤0.00005). Shown are the median values ± IQR.

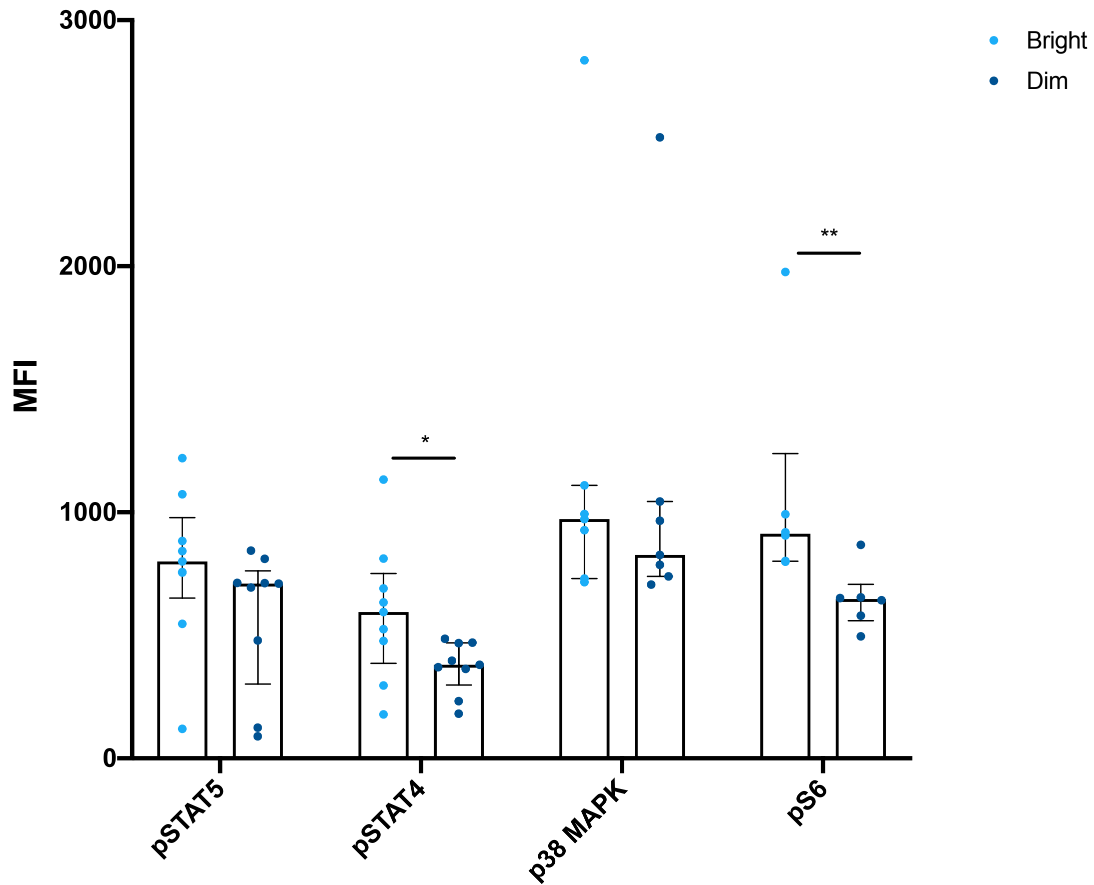

Figure 20. CD56^{Bright} cells have increased phosphorylation of signaling proteins at baseline. Baseline whole blood was stimulated with rIL-2/12 and stained to quantify intracellular signaling protein phosphorylation in CD56$^{Bright/Dim}$CD3^{-} populations (n=9). CD56^{Bright} cells expressed significantly more pSTAT4 and pS6 as compared to CD56^{Dim} cells. The Mann-Whitney test was used to assess statistical significance. $p \leq 0.05$ (*p≤0.05, **p≤0.005, ***p≤0.0005, ****p≤0.00005). Shown are the median values ± IQR.

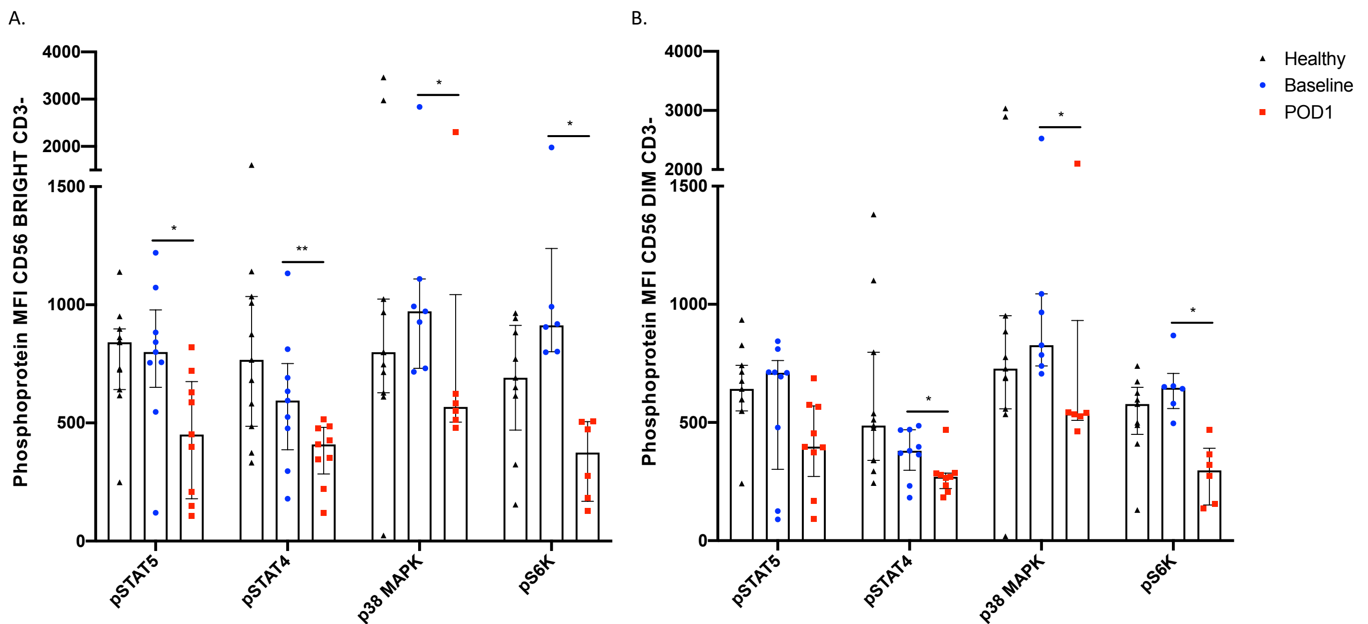

Figure 21. Downstream signaling protein phosphorylation is significantly impaired in both CD56$^{Bright/Dim}$ populations on POD1 in response to rIL-2/12.
Whole blood was stimulated and stained to quantify intracellular signaling protein phosphorylation in CD56$^{Bright/Dim}$CD3^{-} populations (n=9). (A) CD56^{Bright} cells had significantly reduced pSTAT5, pSTAT4, pP38 MAPK, and pS6 MFI on POD1. (B) CD56^{Dim} cells had significantly reduced pSTAT4, pP38 MAPK, and pS6 MFI on POD1. The Wilcoxon matched-pairs signed rank test was used to assess statistical significance between baseline and POD1 samples. $p \leq 0.05$ (*p≤0.05, **p≤0.005, ***p≤0.0005, ****p≤0.00005). Shown are the median values ± IQR.

cytokine receptors and impairment in the activation of the downstream signaling cascades ultimately contributing to impaired IFNγ production in response to cytokine stimulation in postoperative NK cells. I postulate that reduced receptor expression is partly responsible for an impairment in signaling protein activity as well as impaired IFNγ production by NK cells on postoperative day 1.

4.9 $CD56^+CD3^+$ cells are also dysfunctional on POD1

Utilization of whole blood protocols allows for the analysis of any immune cell type present in whole blood. $CD56^+CD3^+$ cells from whole blood samples were analyzed to assess whether NKT cells exhibited a similar dysfunctional phenotype. Intracellular IFNγ was assessed in 11 healthy donors and 9 cancer surgery patients. IFNγ (%) was reduced in NKT cells on POD1 in response to rIL-2/12, although this did not achieve statistical significance. Cytokine receptors were assessed in 13 healthy donors and 9 cancer surgery patients and activating/inhibitory receptor expression was assessed in 16 healthy donors and 20 cancer surgery patients. Interestingly, $CD56^+CD3^+$ cells displayed a significant reduction in CD122 (%), CD212 (% and MFI), NKG2D (% and MFI), DNAM-1 (MFI), and PD-1 (%), and an increase in CD25 (%) and the percentage of $NKG2A^+$ and $TIGIT^+$ cells on POD1 ($p<0.05$). In addition, signaling protein activity was assessed in 10 healthy donors and 9 cancer patients. Downstream signaling proteins STAT5, p38 MAPK, and S6 showed significantly reduced phosphorylation on POD1 in response to rIL-2/12 ($p<0.05$) (**Figure 22**). Thus, while not the focus of this study, whole blood protocols allowed us to identify a similar dysfunctional phenotype in postoperative NKT cells.

4.10 TGFβ is increased in the plasma of cancer patients following surgery

TGFβ is known to cause NK cell dysfunction (impaired cytotoxicity and IFNγ production) via the downregulation of activating receptors, including NKG2D and DNAM-1[76,85,89]. Previous work in our lab showed an increase in postoperative TGFβ 18-hours post-surgery in B16LacZ tu-

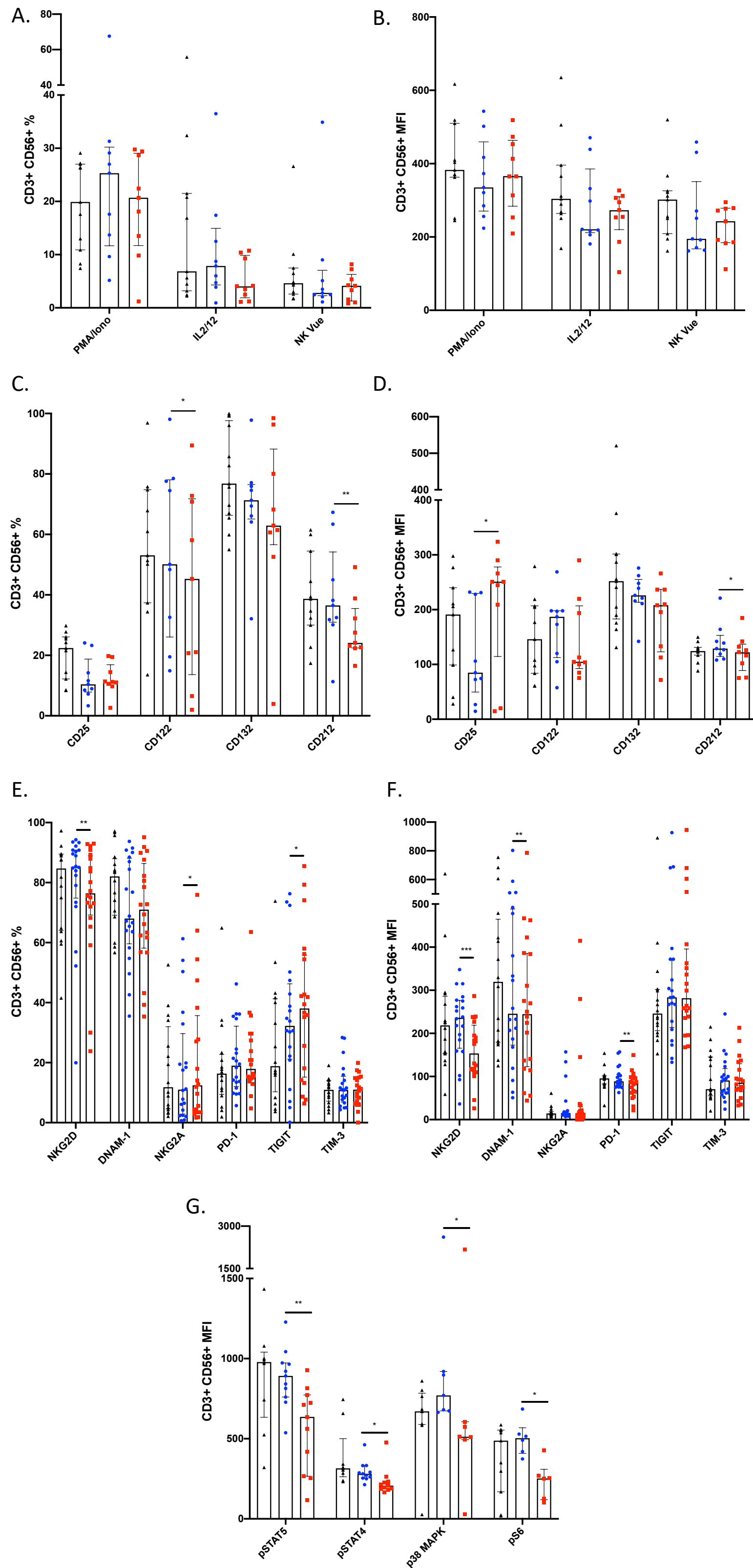

Figure 22. CD56$^+$CD3$^+$ cells display a suppressed phenotype on POD1.

CD56$^+$CD3$^+$ cells were assessed in whole blood for IFNγ production, receptor expression, and signaling protein activity. (A/B) IFNγ was assessed in healthy donors (n=11) and cancer surgery patients (n=9). Cells displayed impaired IFNγ production (%) in response to rIL-2/12 stimulation (p=ns). (C/D) Cytokine receptors were assessed in healthy donors (n=13) and cancer surgery patients (n=9). A significant reduction in CD122 (%) and CD212 (% and MFI) and increase in CD25 (MFI) was quantified on POD1. (E/F) Activating/inhibitory receptor expression was assessed in healthy donors (n=16) and cancer surgery patients (=20). A significant reduction in NKG2D (% and MFI), DNAM-1 (MFI), and PD-1 (MFI) and an increase in NKG2A and TIGIT (%) was observed on POD1. (G) Signaling protein phosphorylation was quantified in healthy donors (n=10) and cancer surgery patients (n=9). A significant reduction in phosphorylation was observed for all targets. Wilcoxon matched-pairs signed rank test was used to assess statistical significance between baseline and POD1 samples. $p \leq 0.05$ (*p≤0.05, **p≤0.005, ***p≤0.0005, ****p≤0.00005). Shown are the median values ± IQR.

mour-bearing mice as compared to non-surgical controls[224]. For these reasons, TGFβ was a top candidate for the mechanism behind NK cell suppression observed on POD1.

To investigate whether TGFβ is increased in cancer surgery patients postoperatively, platelet-free plasma was collected from healthy donors (n=3) and cancer surgery patients at baseline and on POD1 (n=11) (**Table 5**). Platelet-free plasma was acid activated and diluted with appropriate buffer prior to quantification by R&D Quantikine® Human TGFβ ELISA. An increase was observed in plasma-derived TGFβ on POD1 (median 6337.7 to 7947.7 pg/mL), although this did not achieve statistical significance (**Figure 23**). It is important to note that the issue of quantifying TGFβ is a complex one. Firstly, TGFβ is secreted in a latent form and the processing required for its activation is multi-step[71,77]. In addition, latent TGFβ has a reported half-life of >100 minutes, while active TGFβ has a reported half-life of 2-3 minutes[320]. All known ELISA kits measure active TGFβ, achieved via prior acid-activation. Thus, in using platelet-free plasma to quantify TGFβ by ELISA, both latent and active TGFβ are quantified, which may not be representative of the biological activity of TGFβ in the postoperative period. In addition, due to its short half-life, TGFβ levels could peak earlier than the 24-hour POD1 blood draw, as the kinetics of postoperative TGFβ production are currently unknown. Finally, TGFβ is known to be secreted by tumour cells[226,321,322]. Thus, with tumour-removal surgeries it is possible that TGFβ levels are being influenced by the tumour size and stage and the removal of the tumour during surgery. The following assays attempt to clarify the role of TGFβ in the context of postoperative immune suppression.

4.11 Short exposure to TGFβ1 can induce long lasting NK cell impairment

In an attempt to assess the kinetics of TGFβ-induced NK cell dysfunction, healthy isolated $CD56^+$ cells were incubated in low-dose rIL-2 2% CRPMI in the absence (control/no stim) or pres-

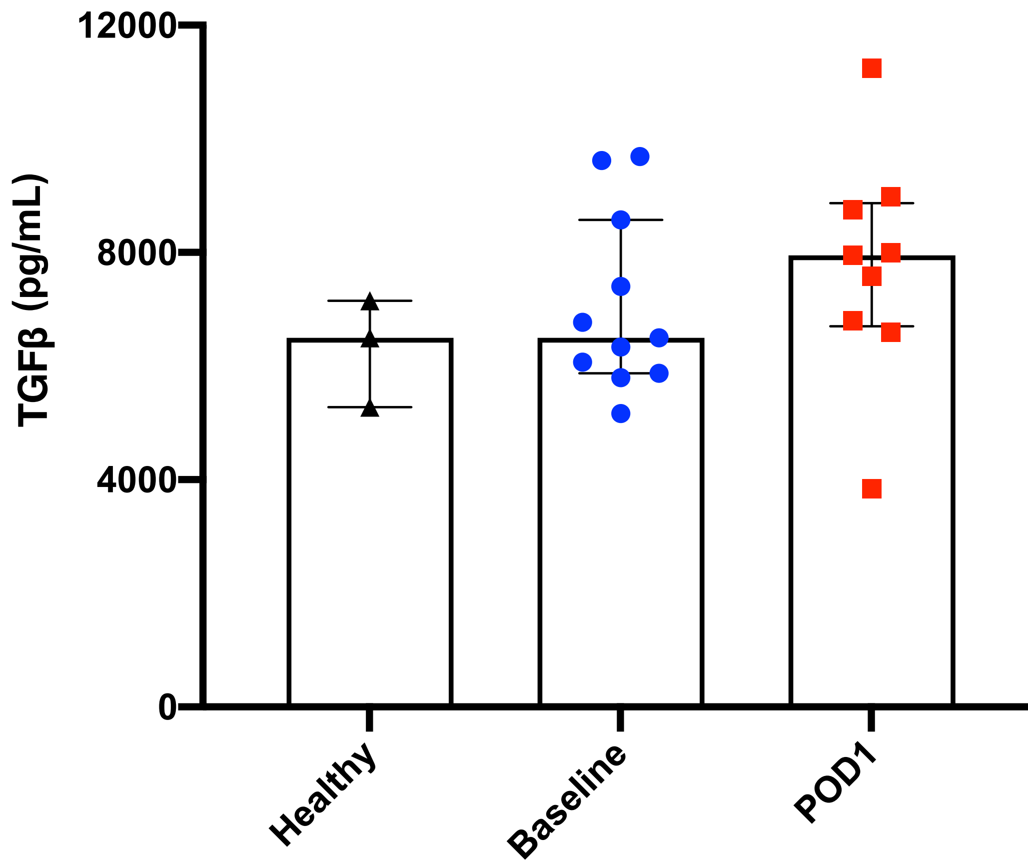

Figure 23. TGFβ is increased in the plasma of cancer patients following surgery.
Platelet-free plasma was collected from healthy donors (n=3) and cancer surgery patients at baseline and on POD1 (n=11). An increase in plasma derived TGFβ from median 6337.7 pg/mL to 7947.7 pg/mL was quantified on POD1 (p=ns). The Wilcoxon matched-pairs signed rank test was used to assess statistical significance between baseline and POD1 samples. Shown are the median values ± IQR.

ence of rTGFβ1 for 20 minutes, 2 hours, or 24 hours. After the 20-minute and 2-hour incubations, cells were washed and resuspended in low-dose rIL-2 media for the remainder of the 24-hour incubation period. The objective of this assay was to assess if a transient (20 minutes or 2 hours) exposure of NK cells to rTGFβ1 could have consequences for NK cell receptor expression and activity at 24 hours (a pseudo-POD1 time point). As compared to NK cells that were not exposed to rTGFβ1, NK cells exposed to rTGFβ1 showed a reduction in NKG2D expression after 20 minutes (30.4% reduction in NKG2D expression and 34.2% reduction in NKG2D intensity/MFI) and 2 hours (16.3% reduction in NKG2D expression and 21.3% reduction in NKG2D intensity/MFI) (n=1) (**Figure 24**). While this reduction was not as profound as that seen with 24 hour rTGFβ1 incubation (58.7% reduction in NKG2D expression and 52.8% reduction in NKG2D intensity/MFI), these results indicate that a short-lived increase in TGFβ as a result of surgical stress could have a lasting impact on NK cell function well after TGFβ levels have returned to baseline.

4.12 TGFβ1, but not IL-10, can induce NKG2D downregulation in baseline NK cells

The postoperative anti-inflammatory phase is characterized by the release of anti-inflammatory soluble factors that are known to induce NK cell dysfunction. It is well reported in the literature that TGFβ can induce NK cell dysfunction via reduced receptor expression (NKG2D, among others) resulting in impaired effector functions, including IFNγ production[78,89], and my results and others suggest that TGFβ may be released in response to surgical stress. IL-10 is another cytokine that is released in response to surgical stress[219–221] and can inhibit NK cell effector functions[58,66]. I wanted to compare the effects of rTGFβ1 and rIL-10 (R&D Systems) on NK cell receptor expression and function. To achieve this, whole blood was collected from cancer surgery patients at baseline and on POD1 and was stained immediately using the whole blood receptor staining protocol. In addition, baseline blood was incubated with low-dose rIL-2 (100U/mL, con-

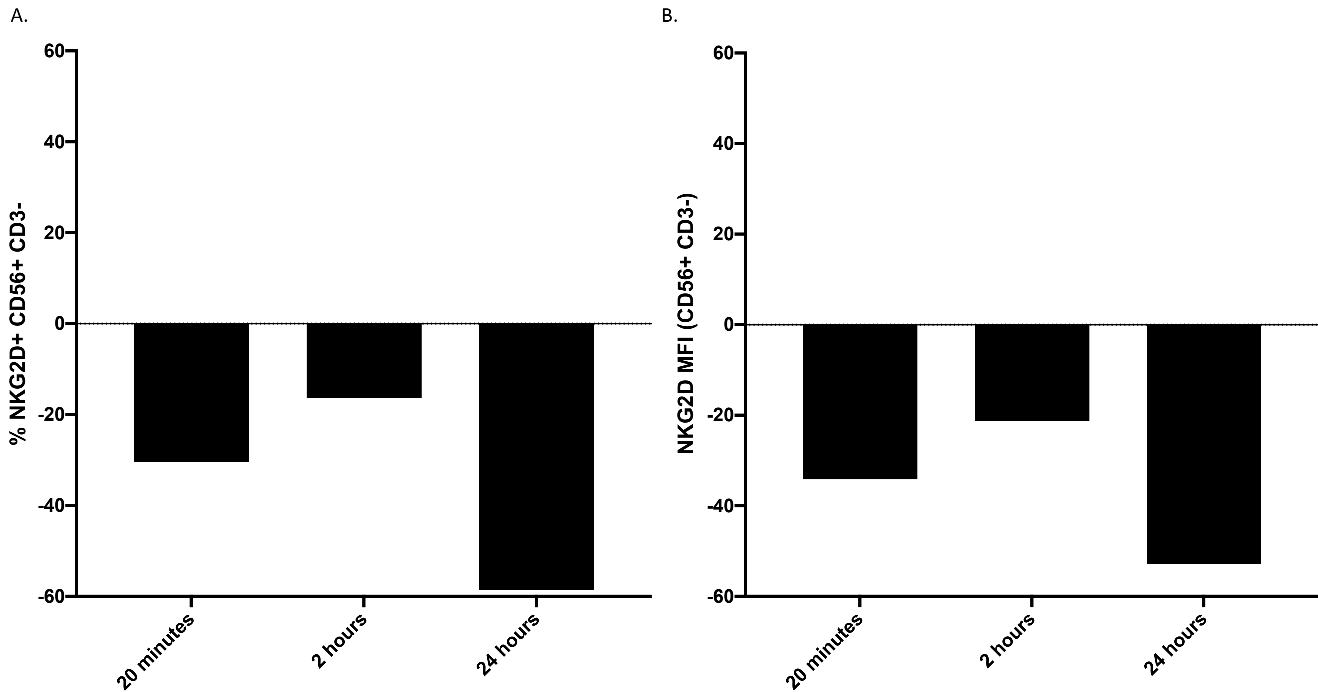

Figure 24. Healthy $CD56^+$ cells incubated with rTGFβ1 show long-term changes in NKG2D expression.

$CD56^+$ cells were isolated from a healthy donor (n=1; female, < 60 years old) and stimulated in culture with rTGFβ1 (10 ng/mL) for either 20 minutes, 2 hours, or 24 hours and then stained at 24 hours for extracellular NKG2D expression. (A/B) A reduction in NKG2D expression (% and MFI) was observed at all three time points. Shown is percentage normalized to baseline.

trol) with low-dose rIL-2 + rTGFβ1 (10 ng/mL), or with low-dose rIL-2 + rIL-10 (10 ng/mL). After 24 hours of incubation at 37°C, whole blood cells were stained and NKG2D expression (% and MFI) was assessed by flow cytometry following whole blood receptor staining. As compared to whole blood incubated in the presence of low-dose rIL-2 or low-dose rIL-2 + rIL-10, NKG2D expression was reduced only upon incubation with low-dose rIL-2 + rTGFβ1 (n=3) (**Figure 25**). In addition, NKG2D expression in baseline NK cells incubated with low-dose rIL-2 + rTGFβ1 mirrored NKG2D expression in paired POD1 NK cells.

NKG2D expression is critical for tumour cell recognition and the induction of NK cell cytotoxicity. The literature suggests that TGFβ can inhibit NK cell cytotoxicity and although this is not the focus of my thesis, I conducted an experiment to investigate the effect of rTGFβ1 on NK-92 cytotoxicity using a cytotoxicity assay. NK-92 cells are immortalized NK cells originally derived from a human non-Hodgkin's lymphoma that can be propagated in culture[323]. Following 24-hour incubation in media or with 1 ng/mL, 10 ng/mL, or 100 ng/ mL of rTGFβ1, NK-92 cells were co-cultured alone or with K562 leukemia cells at 1:1, 2:1, and 4:1 ratios and cytotoxicity was determined by percentage of PI positive K562 cells (K562 death) by flow cytometry (n=3). All concentrations of rTGFβ1 were able to reduce the percentage of PI^+ K562 cells, thus representing a reduction in the ability of NK-92 cells to kill in the presence of TGFβ1 (**Figure 26**). Furthermore, 10 ng/mL and 100 ng/mL achieved similar levels of NK-92 suppression indicating that maximal suppression of NK-92s can be achieved with 10 ng/mL of rTGFβ1. Taken together, these experiments support what is presented in the literature; TGFβ1 can both reduce receptor expression in baseline NK cells and inhibit NK-92 cell killing of target cells *in vivo*.

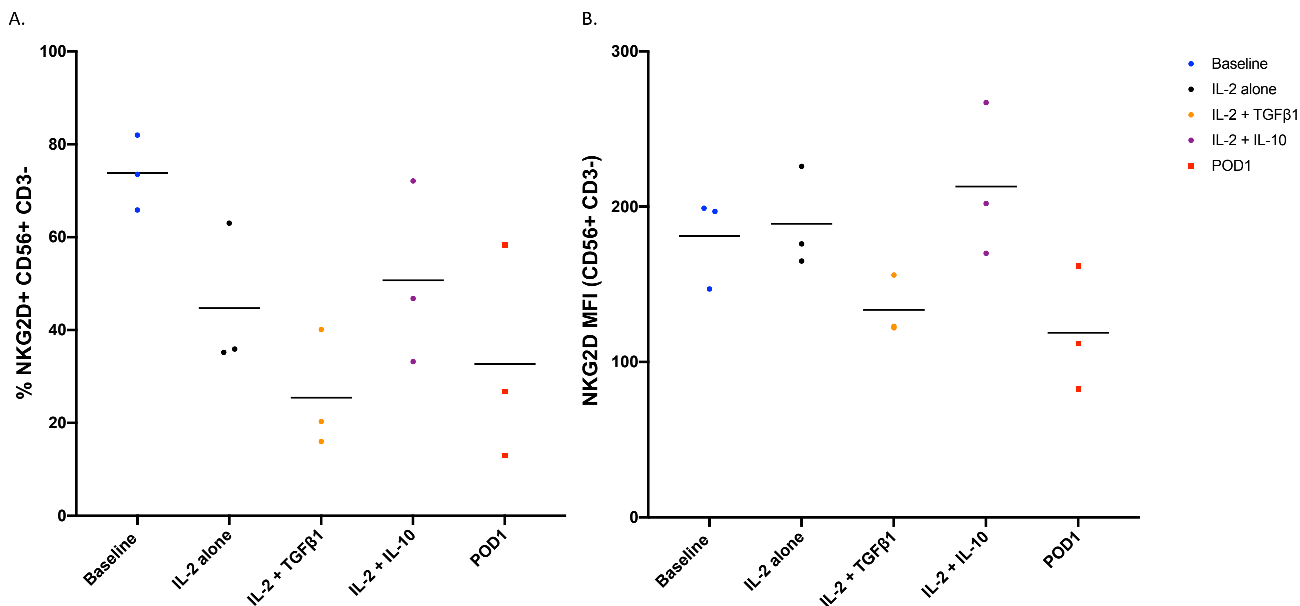

Figure 25. TGFβ1, but not IL-10, can induce NKG2D downregulation in baseline NK cells. Whole blood was collected from cancer surgery patients (n=3) at baseline and on POD1. One of three patients was female, the average age was 66 years, and cancer types included renal, prostate, and lung cancer. Baseline whole blood was incubated for 24 hours in the presence of rIL-2 +/- rTGFβ1 or rIL-10 and stained at 24 hours for NKG2D expression. (A/B) NKG2D was reduced (% and MFI) in the presence of rTGFβ1, but not rIL-10, as compared to whole blood stimulated with rIL-2 alone at 24 hours.

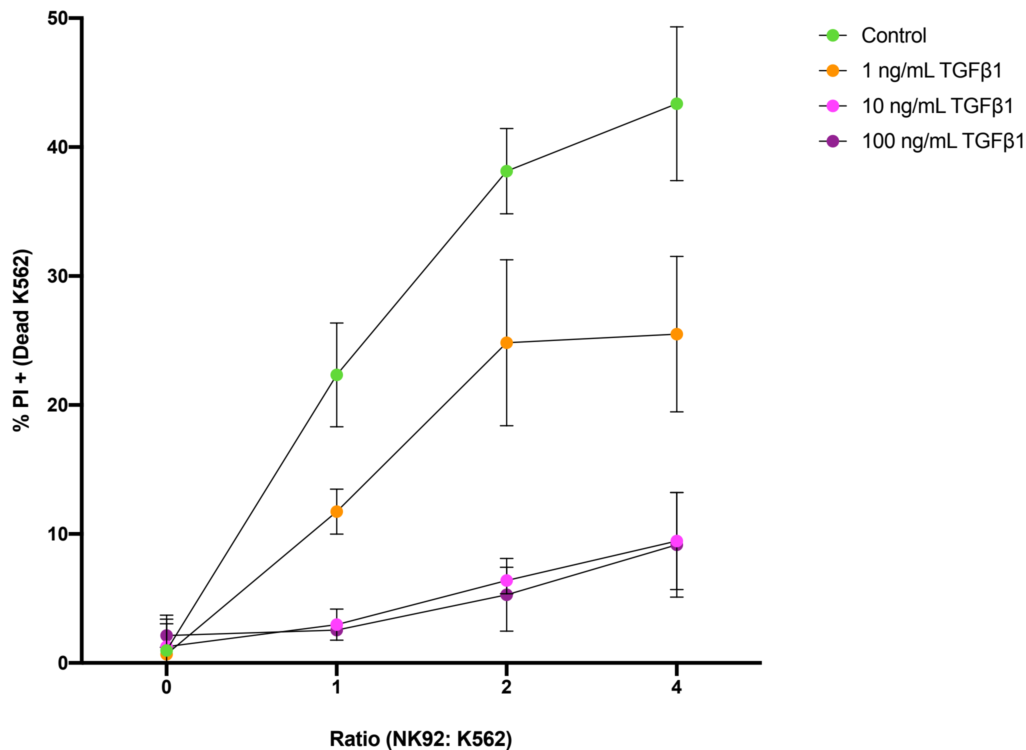

Figure 26. rTGFβ1 inhibits NK-92 cytotoxicity against L562 leukemia cells *in vitro*.

Following 24-hour incubation in 10% CRPMI or with 1 ng/mL, 10 ng/mL, or 100 ng/mL of rTGFβ1, NK-92 cells were cultured alone or with K562 leukemia cells at 1:1, 2:1, and 4:1 ratios and cytotoxicity was determined by percentage of PI positive K562 cells (K562 death) by flow cytometry (n=3). All concentrations of rTGFβ1 were able to reduce the percentage of PI^+ K562 cells, suggesting an impairment in NK-92 cytotoxicity in the presence of TGFβ.

4.13 Healthy NK cells cultured with rTGFβ1 show reduced receptor expression and IFNγ production

Due to the ability of TGFβ to induce NK cell dysfunction and in light of the increase in TGFβ in patient plasma on POD1, I hypothesized that soluble TGFβ in patient plasma could be partly responsible for NK cell dysfunction in the postoperative period. To investigate this, I first tested the ability of rTGFβ1 to reduce receptor expression and IFNγ production *in vitro.* A total of 10 healthy donors' $CD56^{+}$ cells, 19 healthy plasma samples, and 25 paired baseline and POD1 patient plasma samples were used for the assays in sections 3.15 through 3.17 (**Table 6**). Healthy isolated $CD56^{+}$ cells were incubated for 24 hours in the presence of low-dose rIL-2 and combined heterologous healthy plasma with or without rTGFβ1 (1 ng/mL) and either stained for receptor expression (NKG2D, DNAM-1, CD132, and CD212) or stimulated with rIL-2/12 (400 U/ 20 ng/well) prior to assessment of intracellular IFNγ production. The optimal concentration of rTGFβ1 was determined by a previous titration assay (**Appendix D**). Target protein expression was quantified in $CD56^{+}CD3^{-}$ cells via flow cytometry (**Figure 27**). Healthy $CD56^{+}$ cells incubated with combined healthy donor plasma from different donors served as the negative control. When comparing healthy NK cells cultured only in low-dose rIL-2 media and media with combined healthy plasma, no difference was observed in receptor expression or intracellular IFNγ (% and MFI) (**Figure 28**). Furthermore, incubation in the presence rTGFβ1 resulted in a significant reduction in NKG2D, CD132, and CD212 expression and IFNγ production and a downward trend in DNAM-1 expression, as compared to cells cultured with combined healthy plasma (**Figure 29**). These results show that healthy $CD56^{+}$ cells are not activated or suppressed when cultured with heterologous healthy plasma, suggesting that healthy NK cells could be cultured successfully in the presence of patient plasma in future assays. Furthermore, healthy NK cells exposed to rTGFβ1 *in vitro* phenocopied NK cells from POD1 whole blood, suggesting that TGFβ may contribute to the dysfunctional phenotype observe in NK cells in patients on POD1.

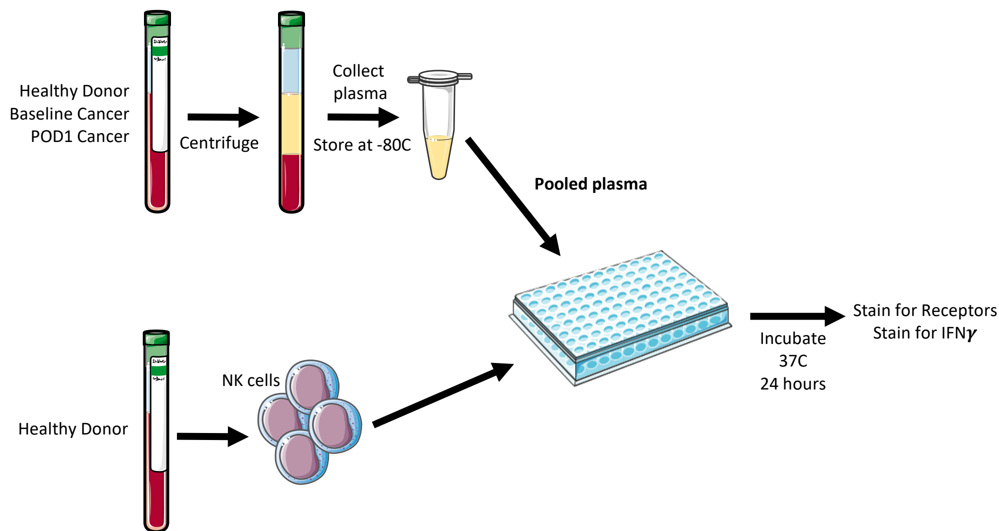

Figure 27. Combined plasma culture assay protocol schematic.
Whole blood was drawn from healthy donors or cancer surgery patients at baseline or on POD1. Platelet-free plasma was collected from whole blood and stored at -80°C. $CD56^+$ cells were isolated from healthy donors and incubated for 24 hours in the presence of combined healthy, baseline, or POD1 plasma. Cells were then either stained immediately for receptor expression or at 24 hours for IFNγ after stimulation with rIL-2/12.

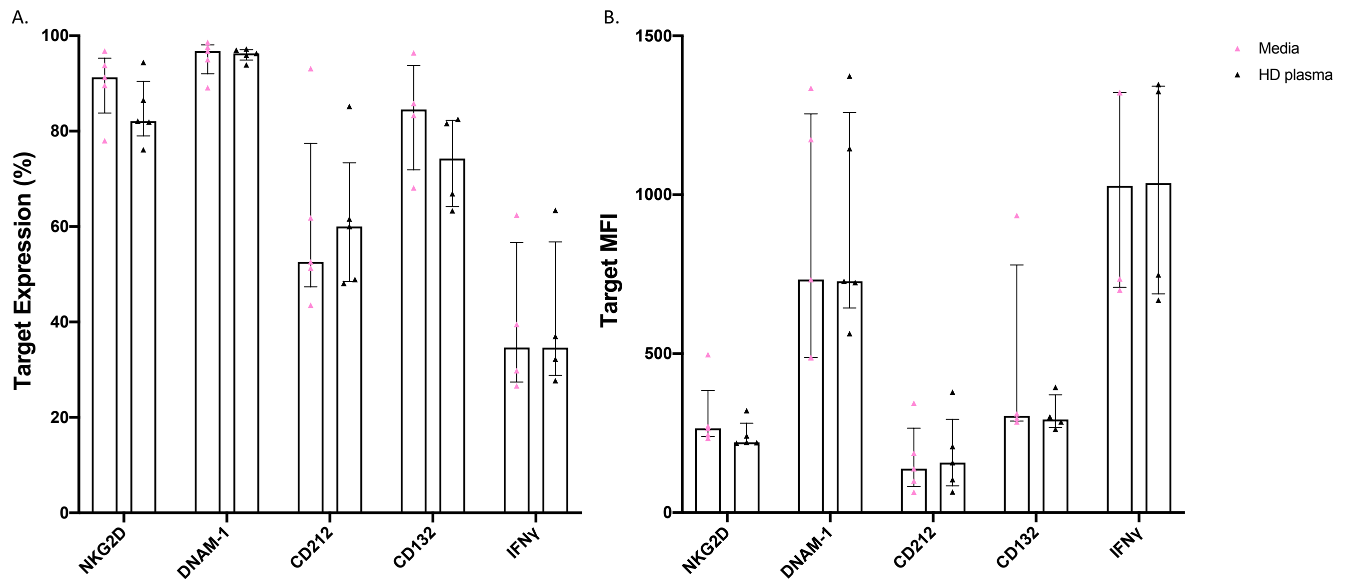

Figure 28. Culture of healthy NK cells with heterologous combined healthy plasma does not alter NK cell phenotype or function.
$CD56^+$ cells were isolated from healthy donors and cultured in the presence of 2% CRPMI + low-dose rIL-2 in the absence/presence of 25% combined heterologous healthy plasma. At 24 hours cells were either stained for extracellular receptor expression or stained for intracellular IFNγ after stimulation with rIL-2/12. (A/B) No difference was observed between media only and healthy donor plasma for NKG2D (n=5), DNAM-1 (n=5), CD212 (n=5), or CD132 (n=4) or in the production of IFNγ (n=4) (p=ns). The Wilcoxon matched-pairs signed rank test was used to assess statistical significance. Shown are the median values ± IQR.

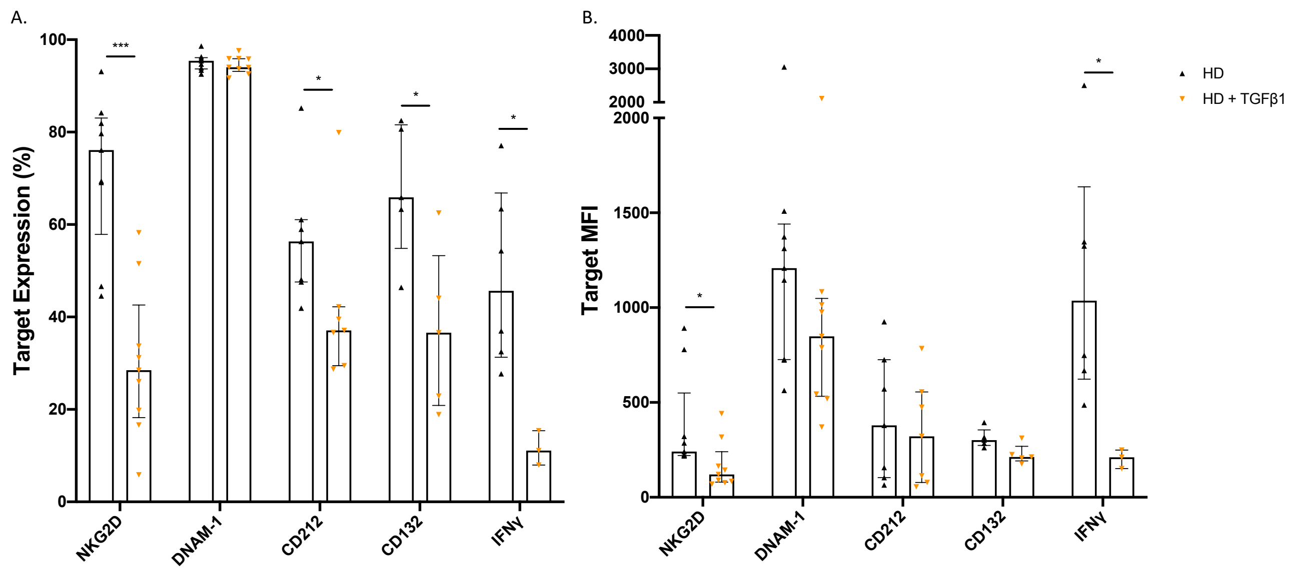

Figure 29. Healthy NK cells cultured with rTGFβ1 have reduced receptor expression and IFNγ production.

CD56^{+} cells were isolated from healthy donors and cultured with 25% combined healthy plasma +/- rTGFβ1. At 24 hours cells were either stained for extracellular receptor expression or stained for intracellular IFNγ after stimulation with rIL-2/12. (A/B) A significant reduction in receptor expression (NKG2D % and MFI, CD212 %, and CD132 %) and intracellular IFNγ (% and MFI) was observed in healthy NK cells incubated with rTGFβ1. The Wilcoxon matched-pairs signed rank test was used to assess statistical significance. $p \leq 0.05$ (*p≤0.05, **p≤0.005, ***p≤0.0005, ****p≤0.00005). Shown are the median values ± IQR.

4.14 Healthy NK cells cultured with POD1 plasma show reduced receptor expression and IFNγ production

I developed the plasma culture assay in order to assess the effects of plasma from POD1 cancer patients on NK cell receptor expression and IFNγ production, as compared to plasma from healthy donors and baseline cancer patients. Baseline and POD1 plasma were collected from cancer surgery patients (n=25) and cryopreserved before being used in plasma culture experiments (**Table 6**). Healthy isolated $CD56^+$ cells were incubated for 24 hours in the presence of low-dose rIL-2 and baseline or POD1 plasma. Cells were then either stained for receptor expression (NKG2D, DNAM-1, CD132, and CD212) or stimulated with rIL-2/12 (400 U/ 20 ng/well) prior to assessment of intracellular IFNγ production. Target protein expression was quantified in $CD56^+CD3^-$ cells via flow cytometry. Receptor expression was reduced in the presence of POD1 plasma, with reductions in NKG2D and CD212 achieving statistical significance (**Figure 30**) ($p<0.05$). In addition, NK cells were profoundly suppressed in their ability to produce IFNγ in the presence of POD1 plasma (**Figure 30**). Thus, these results suggest that a soluble factor(s) present in POD1 patient plasma is able to induce NK cell dysfunction in healthy NK cells, thereby replicating the suppressed phenotype observed in POD1 NK cells. I hypothesize that the main soluble factor is TGFβ.

4.15 TGFβ-specific therapeutics can prevent dysfunction in healthy NK cells incubated with rTGFβ1 or POD1 plasma

The plasma culture assay used for the above experiments was also employed to test whether TGFβ-specific therapeutics could prevent the dysfunction observed in the presence of rTGFβ1 or POD1 plasma. For the first therapeutic, combined plasma was incubated with an anti-TGFβ mouse monoclonal antibody prior to being added to healthy $CD56^+$ cells in culture. The mAb was titrated against rTGFβ1 using healthy $CD56^+$ isolated cells to determine the optimal concentration (100 μg/mL) (**Appendix E**). For the second therapeutic, $CD56^+$ cells were incubated with the small molecule TGFβ RI inhibitor SB525334 prior to the addition of combined plasma. Cells were then

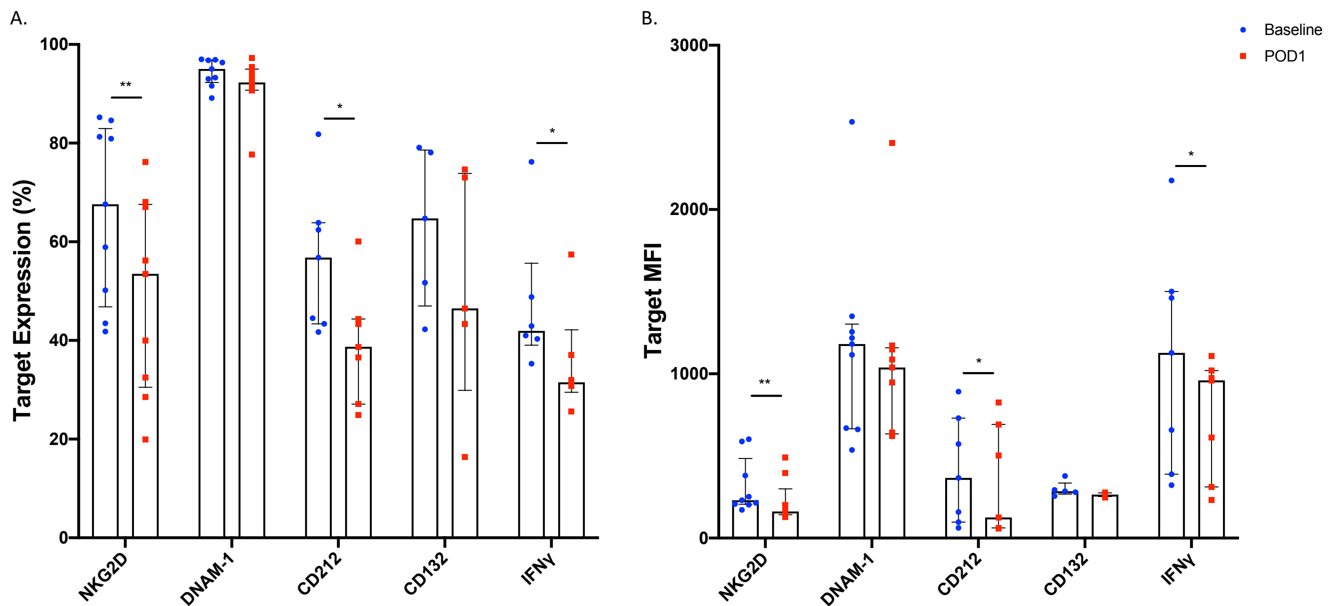

Figure 30. Healthy NK cells cultured with POD1 plasma show reduced receptor expression and IFNγ production.

CD56^{+} cells were isolated from healthy donors and cultured with 25% combined baseline or POD1 plasma. At 24 hours cells were either stained for extracellular receptor expression or stained for intracellular IFNγ after stimulation with rIL-2/12. (A/B) Significant reductions in receptor expression (NKG2D % and MFI and CD212 % and MFI) and IFNγ production (% and MFI) were observed in healthy NK cells cultured with POD1 plasma. The Wilcoxon matched-pairs signed rank test was used to assess statistical significance. $p \leq 0.05$ (*p≤0.05, **p≤0.005, ***p≤0.0005, ****p≤0.00005). Shown are the median values ± IQR.

either stained for receptor expression (NKG2D, DNAM-1, CD132, and CD212) or stimulated with rIL-2/12 (400 U/ 20 ng/well) prior to assessment of intracellular IFNγ production. Target protein expression was quantified in $CD56^{+}CD3^{-}$ cells via flow cytometry. Targets assessed in the presence of the anti-TGFβ mAb included: NKG2D (n=5), DNAM-1 (n=5), CD212 (n=4), CD132 (n=4), and IFNγ (n=3). Targets assessed in the presence of the TGFβ RI inhibitor included: NKG2D (n=2), DNAM-1 (n=2), CD212 (n=2), CD132 (n=2). Healthy $CD56^{+}$ cells incubated with healthy combined plasma showed a partial recovery of receptor expression upon incubation with either the TGFβ mAb or the TGFβ RI smi in the presence of rTGFβ1, with the TGFβ mAb achieving a greater recovery. Incubation with the TGFβ mAb also showed a partial recovery of IFNγ production in the presence of rTGFβ1 (**Figure 31A/B**). This suggests that these therapies are specifically acting against rTGFβ1 and that they have the potential to recover NK cell function. More importantly, a near full recovery of healthy NK cell phenotype and function was observed upon incubation with the TGFβ mAb in the presence of POD1 combined plasma. Incubation with the TGFβ RI smi in the presence of POD1 plasma achieved a near full recovery NK cell receptor expression (**Figure 31C/D**). To ensure that these results were not due to non-specific effects of the mAb or smi in culture, healthy $CD56^{+}$ cells incubated with either healthy, baseline, or POD1 combined plasma were incubated with the mAb or smi. If TGFβ is the primary soluble factor present in POD1 plasma, I should not see a significant change in NKG2D or CD212 receptor expression in healthy cells incubated with healthy or baseline plasma. Indeed, the maintenance of receptor expression was observed only in healthy cells incubated with POD1 plasma (**Appendix F**). This indicates that the effect of the TGFβ-specific therapeutics seen with POD1 plasma is a direct result of TGFβ being present in POD1 plasma. These results strongly support a role for soluble TGFβ as the postoperative factor primarily responsible for the induction of NK cell dysfunction (reduced receptor expression and IFNγ production). Furthermore, these results suggest that by blocking TGFβ in plasma or by inhibiting TGFβ signaling, NK cell function can be preserved.

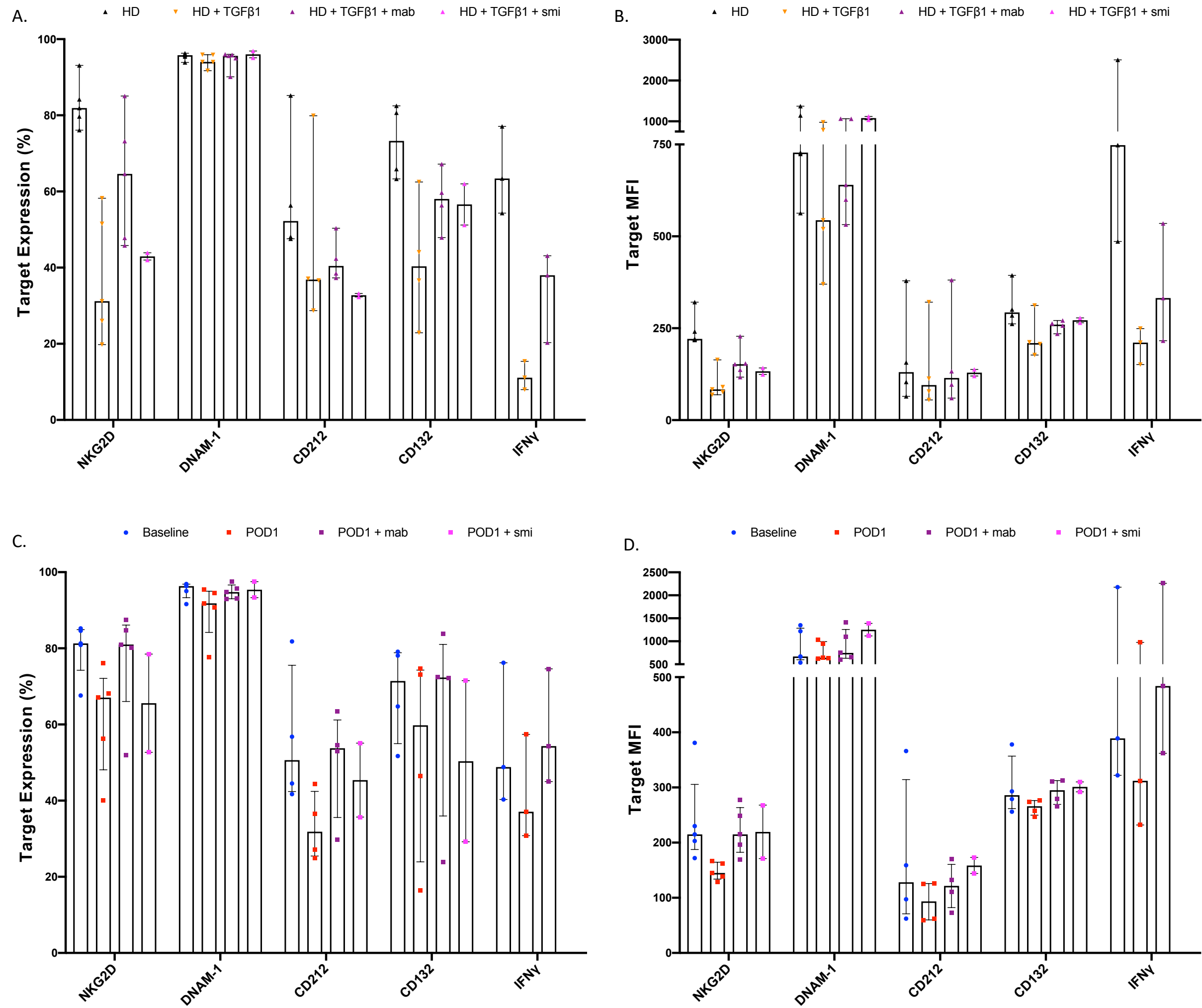

Figure 31. A TGFβ mAb and TGFβ RI small molecule inhibitor prevents a dysfunctional phenotype in healthy NK cells incubated with rTGFβ1 or POD1 plasma.

CD56^{+} cells were isolated from healthy donors and cultured with 25% combined healthy plasma +/- rTGFβ1 (A/B) or baseline/POD1 plasma (C/D). Prior to incubation, plasma was incubated +/- TGFβ mAb or NK cells were incubated +/- TGFβ RI smi. At 24 hours cells were either stained for extracellular receptor expression or stained for intracellular IFNγ after stimulation with rIL-2/12. (A/B) Incubation with a TGFβ mAb or TGFβ RI smi was able to partially prevent receptor reduction and impaired IFNγ production in NK cells cultured with rTGFβ1. (C/D) Incubation with a TGFβ mAb or TGFβ RI smi was able to completely prevent receptor reduction and IFNγ impairment in NK cells cultured with POD1 plasma.

4.16 NKG2D expression and IFNγ production show a strong correlation *in vitro*

Extracellular receptor expression and IFNγ production were assessed in the same healthy donor samples using the plasma culture assay protocol. This allowed for the investigation of a correlation between receptor expression and NK cell activity. I observed a moderate correlation between NKG2D expression and intracellular IFNγ (R^2=0.5806) (**Figure 32**), suggesting that NKG2D expression is strongly linked to NK cell activity and that NKG2D may be used as a proxy for the functional status of NK cells. A weak correlation was observed between DNAM-1 (R^2=0.2468), CD212 (R^2=0.3590), and CD132 (R^2=0.3661) expression and IFNγ production (**Figure 32**).

4.17 TGFβ mediates NK cell dysfunction through mTOR

Single-cell RNA-sequencing was performed on cryopreserved PBMCs from six colorectal cancer surgery patients recruited to the PERIOP-02 clinical trial. All 6 patients were male, 3/6 patients were 60-69 years old, and 4/6 patients had stage 2 CRC (**Table 7).** I observed a reduction in the percentage of $CD3^+$ cells and an increase in the $CD14^+$ population on POD1 ($p<0.05$). Changes in NK cell levels were variable and did not reach statistical significance. Cell viability was above 80% in 9/12 samples (**Figure 33A**). I was able to visualize clean immune cell populations in baseline and POD1 samples (overlaid in **Figure 33B**). In addition, I was able to visualize changes in the gene expression profiles of immune cell types (**Figure 33C**). Based on our previous findings highlighting the dysfunction of postoperative NK cells, we focused our analysis on only the NK cell population and explored the expression changes associated with POD1 samples (**Figure 34**). We found 163 significantly downregulated genes and 218 significantly upregulated genes on POD1 as compared to baseline ($p<0.03$). I identified significant postoperative decreases in genes associated with canonical mTORC1 functions including mRNA translation, rRNA metabolic processes, oxidative phosphorylation, and cellular respiration (**Figure 35**). It has prev-

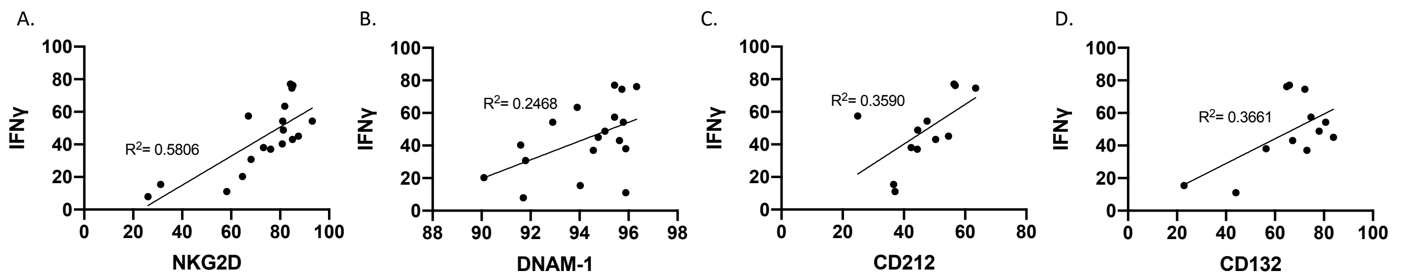

Figure 32. NKG2D expression and IFNγ production show a moderate correlation *in vitro*. In combined plasma culture experiments, healthy NK cells were treated with healthy plasma +/- rTGFβ1, baseline plasma or POD1 plasma +/- anti-TGFβ mAb. NK cells were stained extracellularly for NKG2D, DNAM-1, CD212, and CD132, and intracellularly for IFNγ. The relationship between receptor expression and IFNγ production was assessed by linear regression, which revealed a moderate correlation ($R^2 = 0.5806$) between NKG2D and IFNγ.

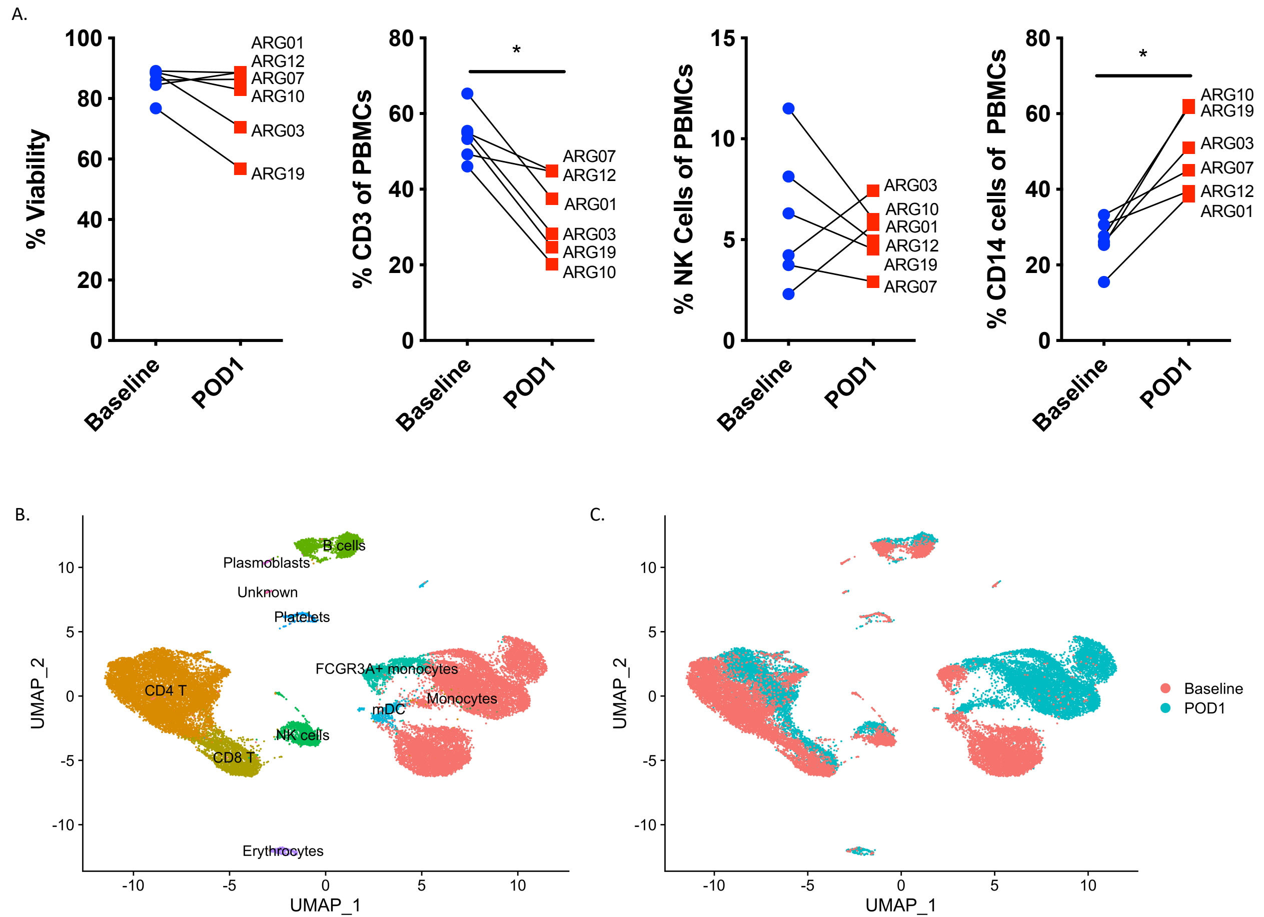

Figure 33. Single-cell RNA-sequencing reveals transcriptomic differences between baseline and POD1 populations.

Blood was drawn from six CRC surgery patient at baseline and on POD1. (A) PBMCs were processed and cryopreserved prior to thawing and immunophenotyping. Cell viability was > 80% in 9/12 patients. The proportion of $CD3^+$ cells was significantly reduced on POD1. No significant change was observed in $CD56^+$ $CD3^-$ levels. The proportion of $CD14^+$ cells was significantly increased on POD1. The Wilcoxon matched-pairs signed rank test was used to assess statistical significance. $p \leq 0.05$ (*p≤0.05, **p≤0.005, ***p≤0.0005, ****p≤0.00005). (B/C) UMAP plots of scRNA-seq data from baseline and POD1 PBMC samples. Each point corresponds to an individual cell (Baseline: 15108 cells, POD1: 15665 cells) and points are coloured based on their cluster/cell type (B) or their experimental group (C).

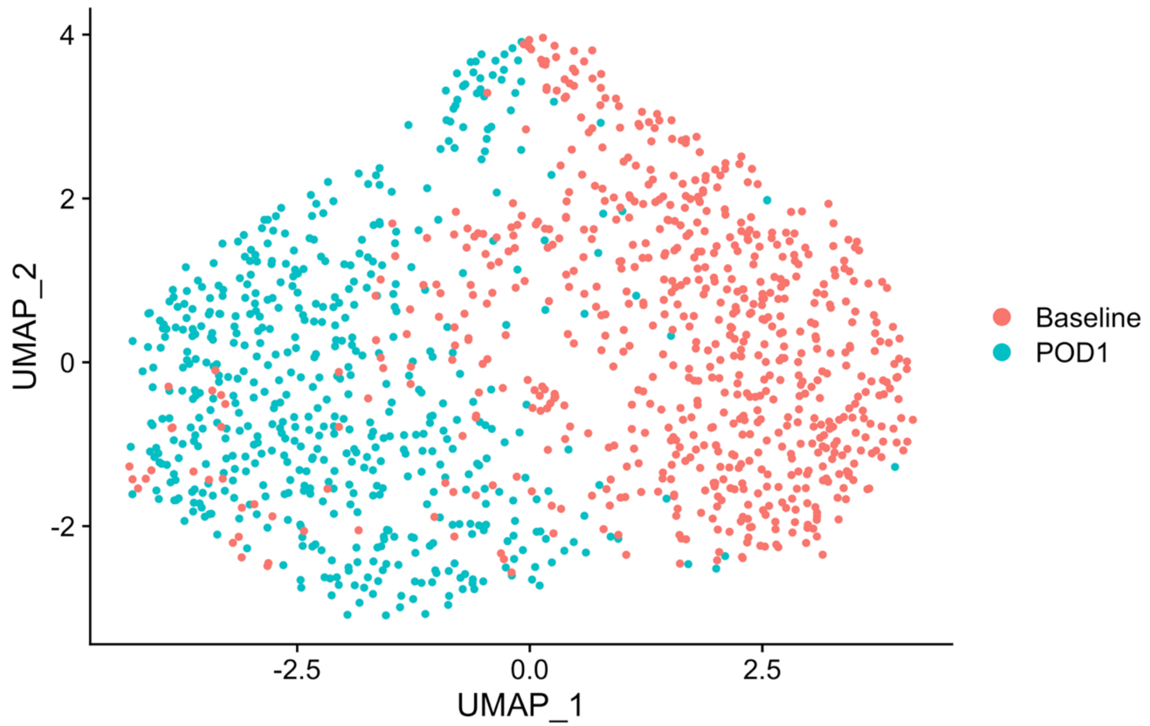

Figure 34. Postoperative NK cells display transcriptomic shift.
UMAP plots of NK cells from scRNA-seq data. Each point corresponds to an individual cell (Baseline: 1434 cells, POD1: 906 cells) and points are coloured based on their experimental group.

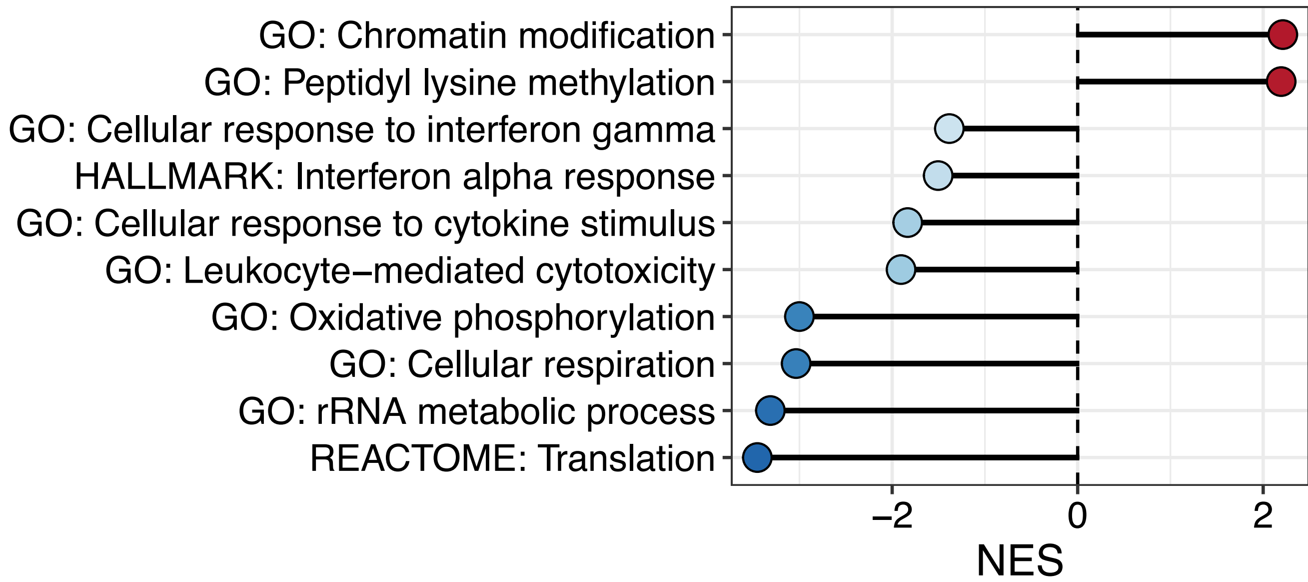

Figure 35. POD1 NK cells are associated with reduced mTOR activity.
Normalized enrichment scores (NES) from gene set enrichment analysis of genes ranked by their fold change between baseline and POD1 NK cells. Negative values correspond to gene sets that are enriched in genes that are downregulated on POD1.

iously been reported that NK cell IFNγ production requires mTORC1 signaling, mediated via phosphorylation of S6 in response to stimulation with activating cytokines (IL2/12/15/18) or engagement of activating receptors (NKG2D)[324–326]. mTORC1 is a critical signaling molecule responsible for controlling cellular growth and proliferation through mRNA translation, ribosomal biogenesis, and autophagy[96]. S6 is phosphorylated directly downstream of mTORC1 and for this reason has been used as a functional readout for mTORC1 activity[47,78,327,328]. I have observed a significant reduction in S6 phosphorylation (pS235/236) in postoperative NK cells in response to rIL-2/12 stimulation, which is associated with reductions in critical NK cell receptors (IL-2R, IL-12R, NKG2D, DNAM-1). Furthermore, phosphorylation of S6 was also impaired on POD1 in the absence of stimulation (**Figure 36**). In fact, I also assessed Akt phosphorylation at PI3K (T308) and mTORC2 (S473) phosphorylation sites in unstimulated NK cells at baseline or on POD1 and found impaired phosphorylation (**Figure 37**). Together this suggests an impairment in the PI3K pathway and mTORC1 activity in postoperative NK cells. Viel et al. recently showed that mTORC1 is a major target of TGFβ in NK cells[78]. mTORC1 deletion, rapamycin, and TGFβ had similar and overlapping effects on NK cells resulting in mTORC1-dependent inhibition of metabolic activities, cell surface expression of activating and cytokine receptors, and NK cell cytotoxicity and IFNγ secretion in response to activating cytokines[78]. I have also shown that TGFβ present in POD1 plasma can induce NK cell dysfunction in healthy NK cells and that this dysfunction can be prevented by blocking TGFβ signaling. To further characterize the mechanism of TGFβ-induced NK cell dysfunction, I assessed S6 phosphorylation in healthy NK cells in the presence of rTGFβ or POD1 plasma using the plasma culture assay protocol. I observed a significant reduction in pS6 MFI in the presence of rTGFβ1 (n=4) (**Figure 38A**) and POD1 plasma (n=4) **(Figure 38B**). Moreover, the addition of a TGFβ mAb (n=5) or TGFβ RI smi (n=2) was able to prevent pS6 impairment and mirrored the effect of these therapeutics on NK cell receptor expression and IFNγ production (**Figure 39**). Taken together, these results suggest that plasma derived TGFβ induces

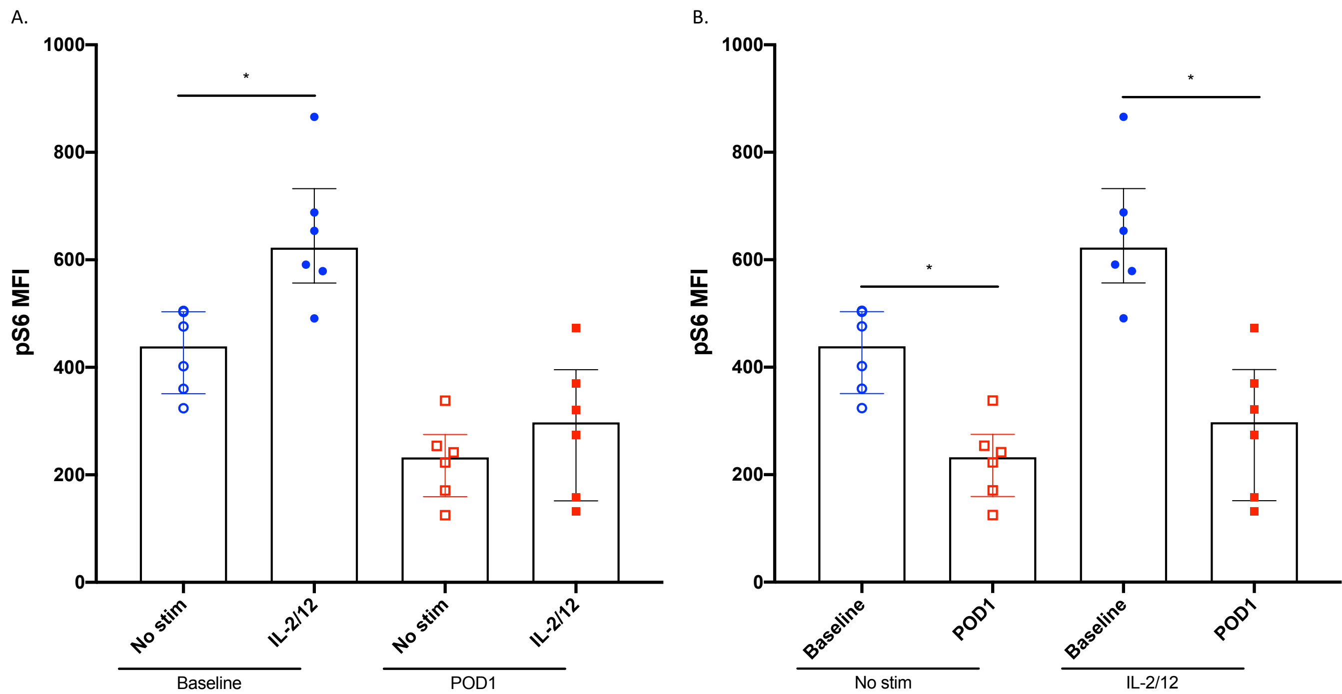

Figure 36. pS6 is reduced in the absence of stimulation on POD1.
Whole blood from cancer surgery patients (n=6) was collected at baseline and POD1 and incubated in the presence/absence of rIL-2/12 for 20 minutes. (A) A significant increase was observed between non-stimulated and rIL-2/12 stimulated baseline blood, however S6 phosphorylation in POD1 cells was not significantly increased in response to stimulation. (B) There is a significant difference in the level of S6 phosphorylation between baseline and POD1 whole blood samples, in the absence and presence of rIL-2/12 stimulation. The Wilcoxon matched-pairs signed rank test was used to assess statistical significance. $p \leq 0.05$ (*$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$, ****$p \leq 0.00005$). Shown are the median values ± IQR.

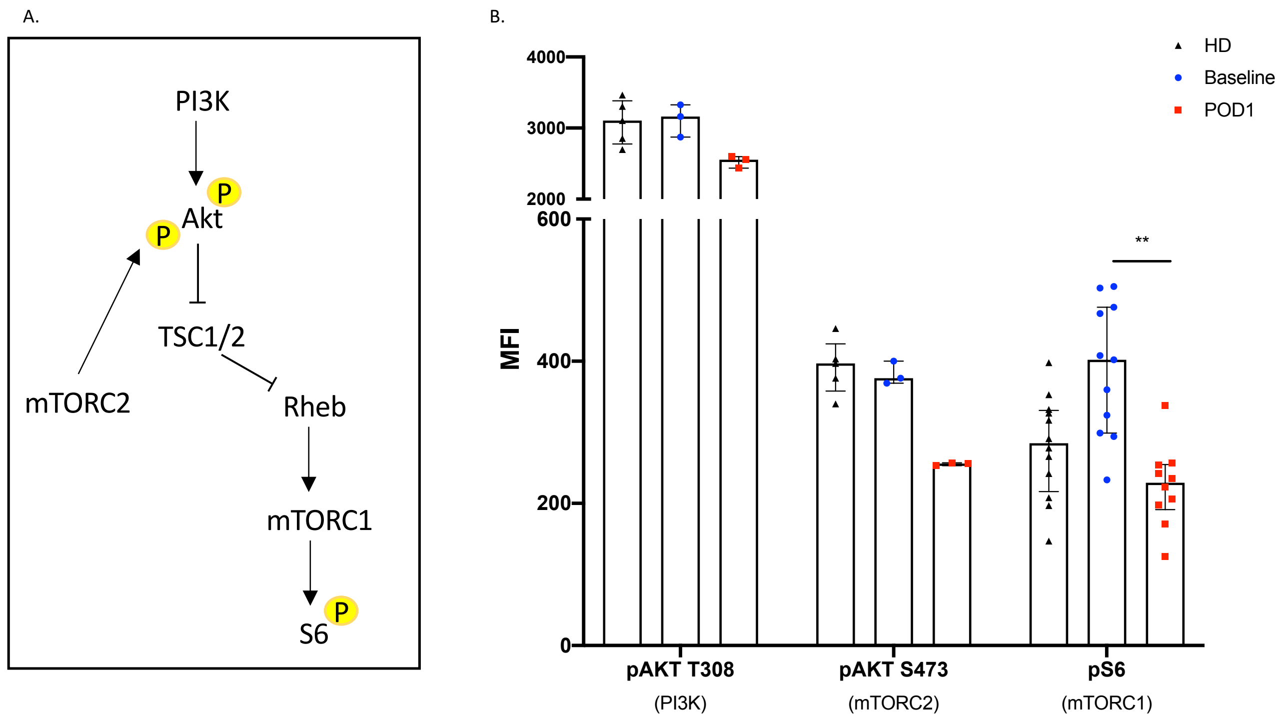

Figure 37. Baseline phosphorylation of Akt and S6 is reduced in whole blood on POD1. (A) Activation of the PI3K pathway leads to the phosphorylation and activation of Akt at the T308 residue, which inhibits the tuberous sclerosis complex (TSC1/2), a physiologic inhibitor of mTOR complex (mTORC) 1. mTORC1 phosphorylates downstream targets, including S6, and mTORC2 phosphorylates Akt at the S473 residue. (B) pAkt and pS6 were assessed in unstimulated whole blood from healthy donors (n=5 and n=12, respectively) and cancer surgery patients (n=3 and n=11, respectively). NK cells from unstimulated POD1 whole blood had significantly impaired phosphorylation of S6 and reduced pAkt (p=ns) as compared to baseline samples. Wilcoxon matched-pairs signed rank test was used to assess statistical significance. $p \leq 0.05$ (*p≤0.05, **p≤0.005, ***p≤0.0005, ****p≤0.00005). Shown are the median values ± IQR.

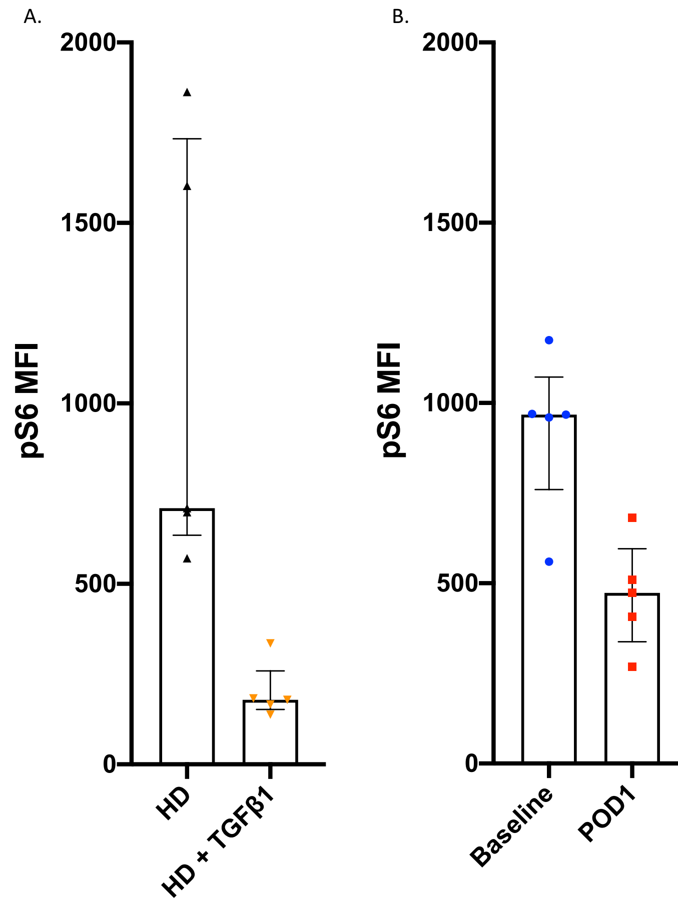

Figure 38. pS6 is reduced in healthy NK cells in the presence of rTGFβ1 or POD1 plasma. CD56^{+} cells were isolated from healthy donors and cultured with 25% combined healthy plasma +/- rTGFβ1 (A) or baseline/POD1 plasma (B). At 24 hours cells were stained for intracellular pS6. (A) Healthy NK cells incubated with rTGFβ1 showed reduced pS6. (B) Healthy NK cells incubated with POD1 show reduced pS6 compared to incubation with baseline plasma. Shown are the median values ± IQR.

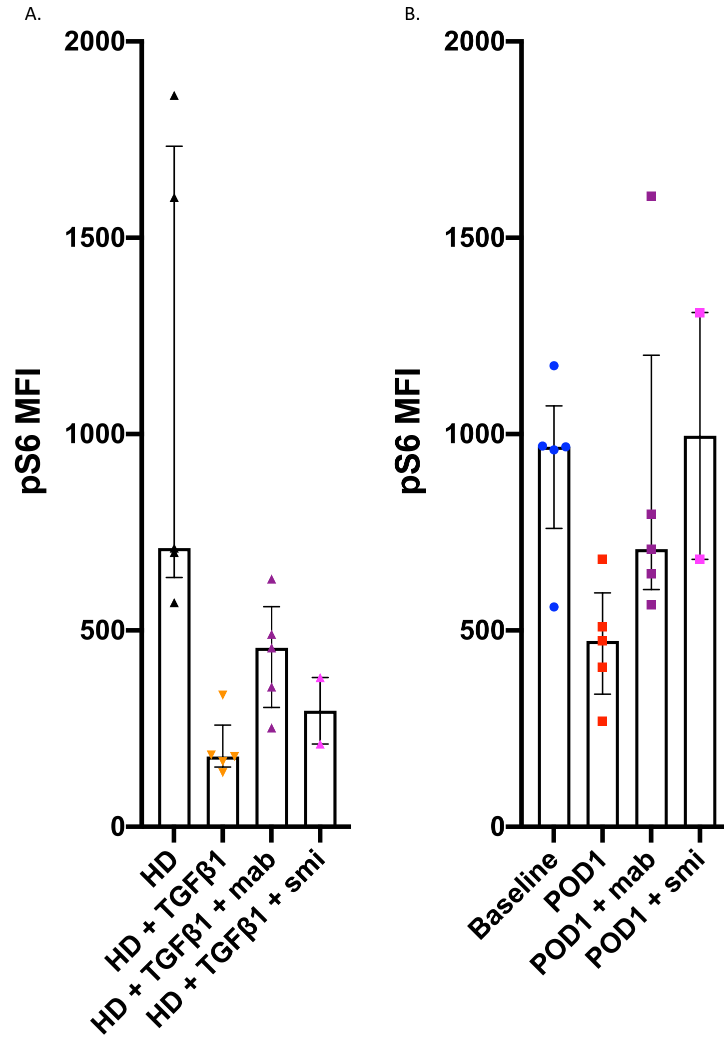

Figure 39. TGFβ-specific therapeutics can maintain S6 phosphorylation in the presence of rTGFβ1 or POD1 plasma.

CD56^{+} cells were isolated from healthy donors and cultured with 25% combined healthy plasma +/- rTGFβ1 (A) or baseline/POD1 plasma (B). Prior to culture, plasma was incubated +/- TGFβ mAb or NK cells were incubated +/- TGFβ RI smi. At 24 hours cells were stained for intracellular pS6. (A) Incubation with TGFβ mAb or TGFβ RI smi was able to partially prevent the impairment in S6 phosphorylation in NK cells cultured with rTGFβ1. (B) Incubation with TGFβ mAb or TGFβ RI smi was able to almost fully prevent the impairment in S6 phosphorylation in NK cells cultured with POD1 plasma.

NK cell dysfunction via the inhibition of mTORC1 activity and that blocking of TGFβ signaling is alone sufficient to restore mTORC1 activity.

4.18 Co-culture with sxMDSCs can induce healthy NK cell dysfunction

Our lab and others have previously shown that surgical stress results in a dynamic environment characterized by the expansion of highly immunosuppressive populations, including regulatory T cells and MDSCs. MDSCs are a heterologous population of immature myeloid cells with suppressive capabilities and expand rapidly in response to surgical stress[175]. MDSCs are known to suppress NK cell function and are therefore excellent candidates with the potential to contribute to postoperative NK cell dysfunction. To investigate MDSCs in this context I optimized a co-culture assay whereby isolated healthy $CD56^+$ cell receptor expression and function (IFNγ) were assessed in the presence/absence of isolated POD1 $CD33^+$ cells (sxMDSCs) at a 2:1 (sxMDSC: NK cell) ratio. Cells were either co-cultured for 24 hours before undergoing staining for extracellular receptors NKG2D (n=5), DNAM-1 (n=4), CD132 (n=3), and CD212 (n=4), or were stimulated using rIL-2/12 (400U/20ng) for an additional 24 hours before undergoing intracellular staining for IFNγ (n=6) (**Figure 40A**). Isolated $CD56^+$ and $CD33^+$ cells were stained for immunophenotyping immediately post-isolation (**Figure 40B/C**). For isolated $CD56^+$ cells viability was > 80% and purity ($CD56^+$) was > 90%; for isolated $CD33^+$ cells viability was > 80% and purity ($CD33^+CD14^+$) > 80%. $CD33^+$ POD1 cells were isolated from patients with a variety of cancer types (**Table 8**). Incubation in the presence of $CD33^+$ cells resulted in reduced expression of NKG2D (mean 79.4% s.d. 13.5 to 43.8% s.d. 19.2, 582.8 s.d. 138.6 to 236.2 s.d. 77.1 MFI), DNAM-1 (mean 93.1% s.d. 2.1 to 85.1% s.d. 10.9, 1550.8 s.d. 1030.6 to 1145.3 s.d. 885.9 MFI), CD212 (mean 61.1% s.d. 4.5 to 58.1% s.d. 24.3, 469 s.d. 113.4 to 458.8 s.d. 249.0 MFI), and CD132 (mean 96.64% s.d. 4.5 to 86.2% s.d. 35.3, 672 s.d. 261.5 to 333.7 s.d. 220.0 MFI) and impaired IFNγ production (mean 56.3% s.d. 21.5 to 12.3% s.d. 22.9, 1835.7 s.d. 605.5 to 857.2 s.d. 430.8 MFI) in isolated healthy NK cells (**Figure 41**). $CD33^+$ cells isolated from POD1 patients are

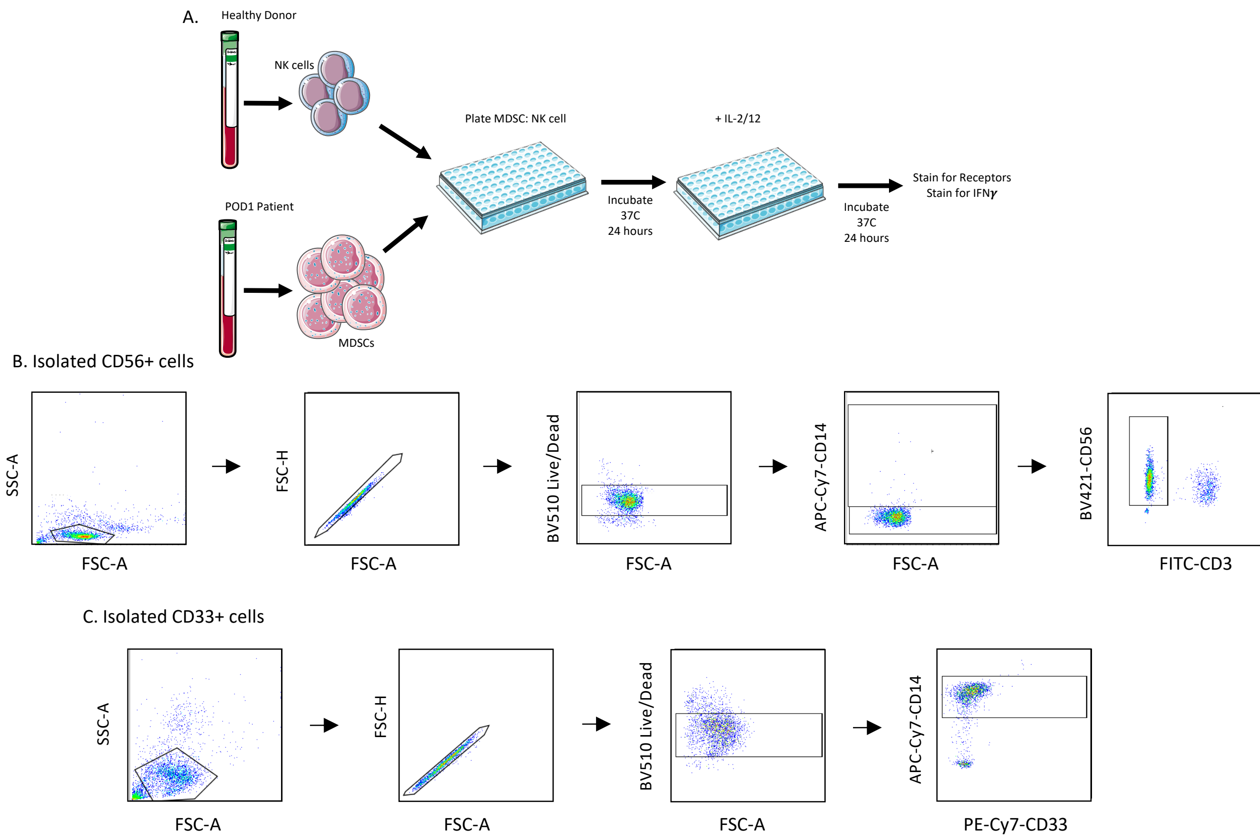

Figure 40. sxMDSC: NK cell co-culture schematic and gating strategy.
(A) $CD56^+$ cells were isolated from healthy donors and $CD33^+$ cells were isolated from POD1 cancer surgery patients. Cells were incubated at 2:1 ($CD33^+$:$CD56^+$) for 24 hours and either stained for extracellular surface receptors or stimulated for an addition 24 hours with rIL-2/12 for quantify intracellular IFNγ. (B) Cells were gated on to exclude debris, doublets, dead cells, and $CD14^+$ cells prior to gating on $CD56^+CD3^-$ cells. (C) Debris, doubles, and dead cells were excluded prior to gating on $CD14^+$ cells to assess purity.

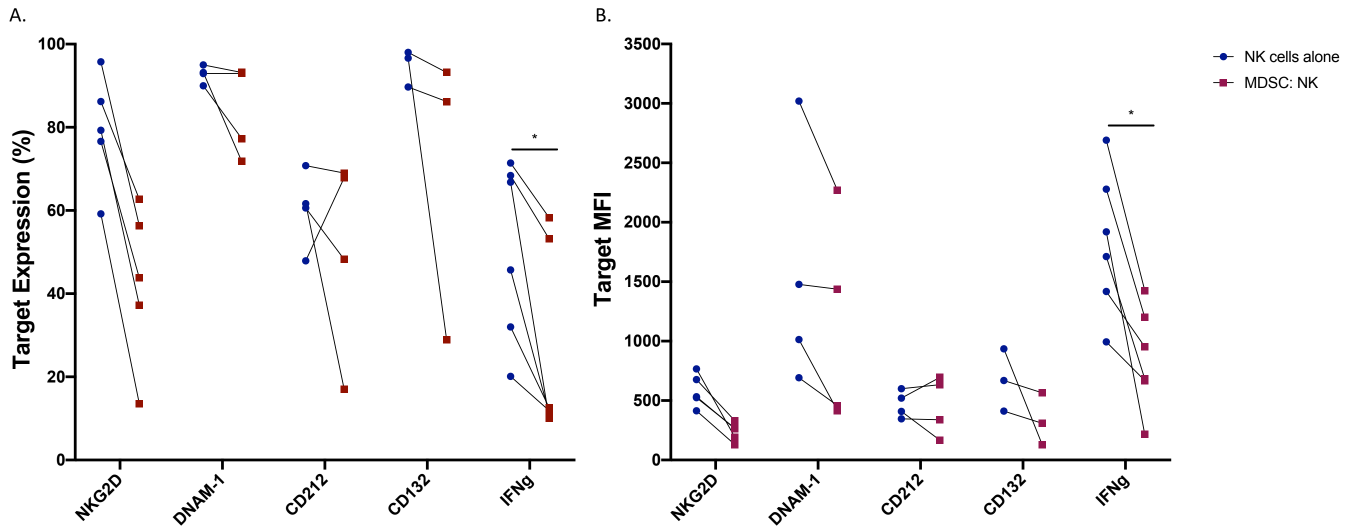

Figure 41. Co-culture with sxMDSCs can induce NK cell dysfunction.
CD56^{+} cells were isolated from healthy donors and CD33^{+} cells were isolated from POD1 cancer surgery patients and cells were incubated at 2:1 (CD33^{+}:CD56^{+}) for 24 hours. (A/B) Healthy NK cells cultured with CD33^{+} cells (sxMDSCs) for 24 hours showed reductions in receptor expression (% and MFI, p=ns) (NKG2D (n=5), DNAM-1 (n=4), CD212 (n=4), and CD132 (n=3)) and significantly impaired IFNγ production (n=6) (% and MFI) upon stimulation with rIL-2/12. Wilcoxon matched-pairs signed rank test was used to assess statistical significance. $p \leq 0.05$ (*p≤0.05, **p≤0.005, ***p≤0.0005, ****p≤0.00005). Shown are the median values ± IQR.

thus able to reproduce the dysfunctional IFNγ phenotype observed in NK cells from postoperative patient whole blood. In addition, this effect on NK cells appears to be titratable in response to increasing ratios of sxMDSCs: NK cells. Isolated $CD56^+$ cells were cultured in a similar way in the absence or presence of POD1 $CD33^+$ cells at 8:1, 4:1, and 2:1 (sxMDSC: NK cell). NKG2D and intracellular IFNγ were then assessed by flow cytometry. NKG2D expression (% and MFI) and IFNγ production (% and MFI) both decreased proportionally with increasing levels of $CD33^+$ cells, thus supporting that the dysfunctional NK cell phenotype is induced by the presence of $CD33^+$ POD1 cells (**Figure 42**).

While there are numerous potential sources of TGFβ, including release by activated platelets[329], TGFβ is both expressed on the surface of MDSCs[330] and can be secreted by MDSCs[331–334]. Indeed, when isolated POD1 $CD33^+CD14^+$ cells were stained for extracellular LAP-TGFβ1, an average of 40.2% of cells expressed LAP-TGFβ1 with an average MFI of 202.3 (n=3) (**Figure 43**). Additionally, in the context of cancer, MDSC-derived TGFβ has been shown to suppress NK cell function in both *in vivo* murine models of cancer[330,331] and *in vitro* assays using MDSCs isolated from cancer patients[333]. Furthermore, Viel et al. showed that suppression of TGFβ signaling in NK cells reduced metastases in two murine models of cancer[76]. In addition to expressing TGFβ on their cell surface, sxMDSCs also produce TGFβ, as quantified by ELISA from sxMDSC culture supernatants (**Figure 44**). Finally, pS6 MFI was also reduced in healthy $CD56^+$ cells cultured with POD1 $CD33^+$ cells (n=2) (**Figure 45**), suggesting that mTORC1 activity is inhibited in the presence of sxMDSCs. These results suggest a role for sxMDSC-derived TGFβ in the suppression of NK cell function.

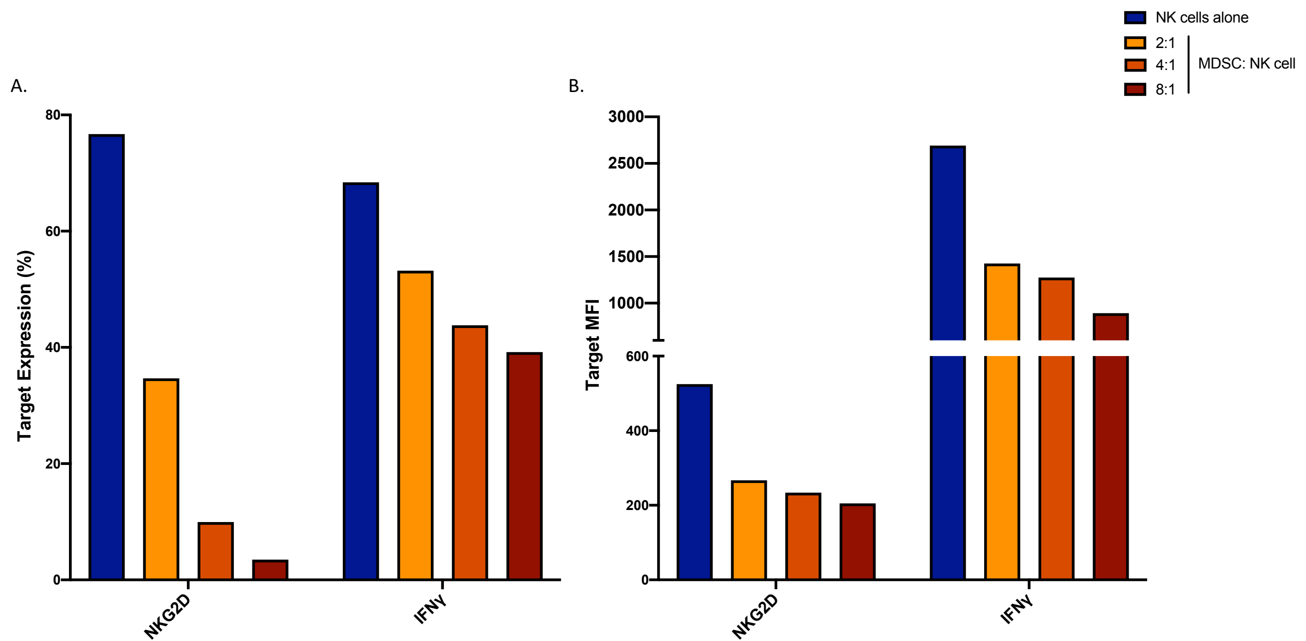

Figure 42. The effect of sxMDSCs on healthy NK cells is titratable.

The inhibitory effect of sxMDSCs on NK cells is titratable in response to increasing concentrations of sxMDSCs. Isolated $CD56^+$ cells were cultured in the absence or presence of POD1 $CD33^+$ cells at 8:1, 4:1, and 2:1 (sxMDSC: NK cell) (n=1). NKG2D was assessed at 24 hours and intracellular IFNγ was assessed after an additional 24 hours in the presence of rIL-2/12. (A/B) NKG2D expression (% and MFI) and IFNγ production (% and MFI) both decreased proportionally with increasing levels of $CD33^+$ cells, thus supporting that the dysfunctional NK cell phenotype can be induced by the presence of sxMDSCs cells.

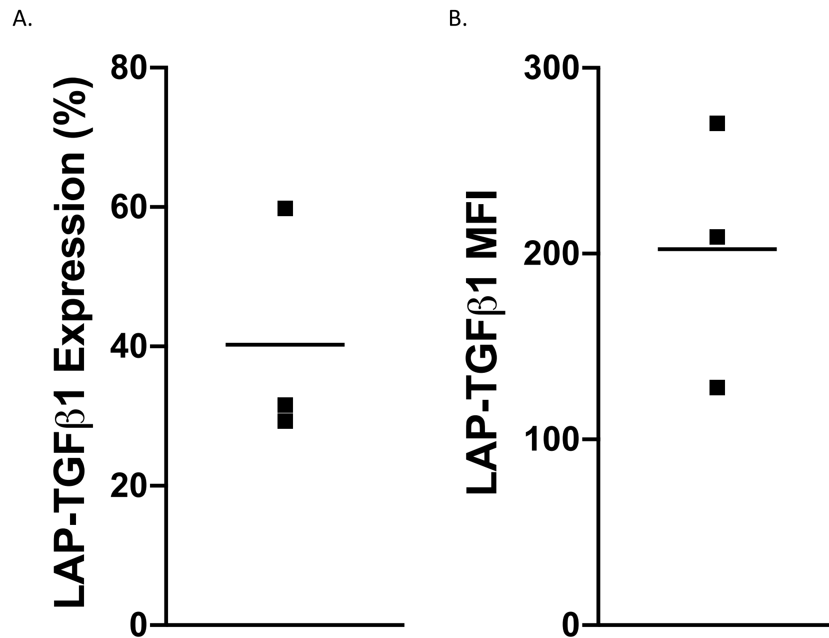

Figure 43. sxMDSCs express LAP-TGFβ1 on their cell surface.
CD33$^+$ cells were isolated from POD1 whole blood (n=3) and stained for extracellular LAP-TGFβ1. One out of three patients was female, with an average age of 58 years. Two of three patients had lung cancer and one patient had bladder cancer. CD33$^+$CD14$^+$ cells were gated on and LAP-TGFβ1 gating was set based on an isotype control. (A/B) An average of 40.2% of POD1 CD33$^+$ cells expressed LAP-TGFβ1 on their cell surface with an average MFI of 202.3.

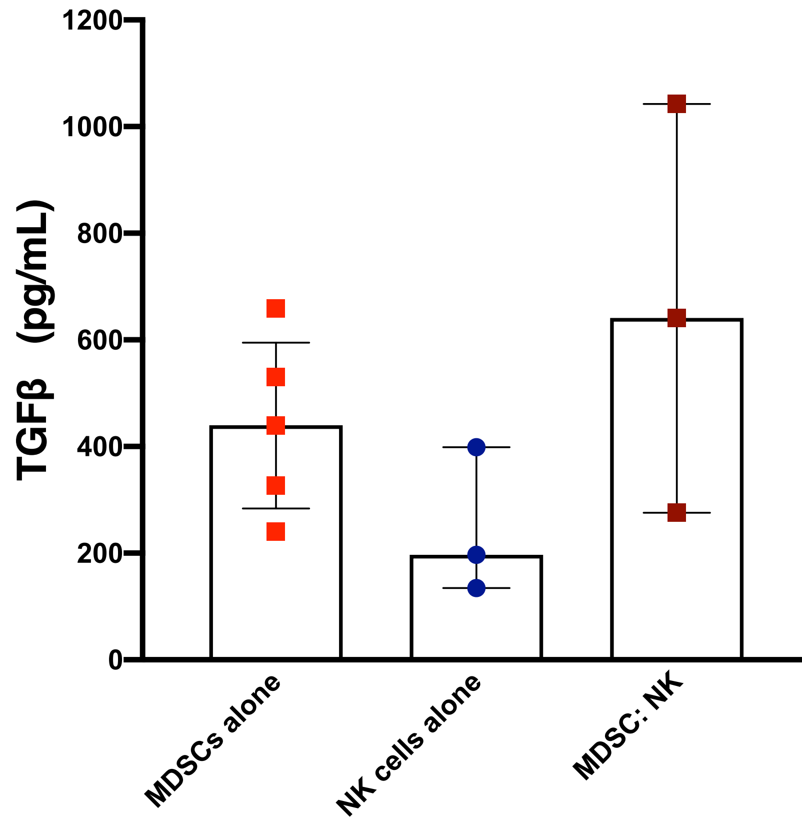

Figure 44. sxMDSCs produce TGFβ in culture.

sxMDSCs (isolated $CD33^+$ POD1 cells) and NK cells (isolated healthy $CD56^+$ cells) were cultured separately or together at 37°C overnight and culture supernatant was collected at 24 hours and stored at -80°C. TGFβ was quantified from frozen culture supernatant by ELISA (n=5 sxMDSCs alone, n=3 NK cells alone/co-culture). Shown are the median values ± IQR.

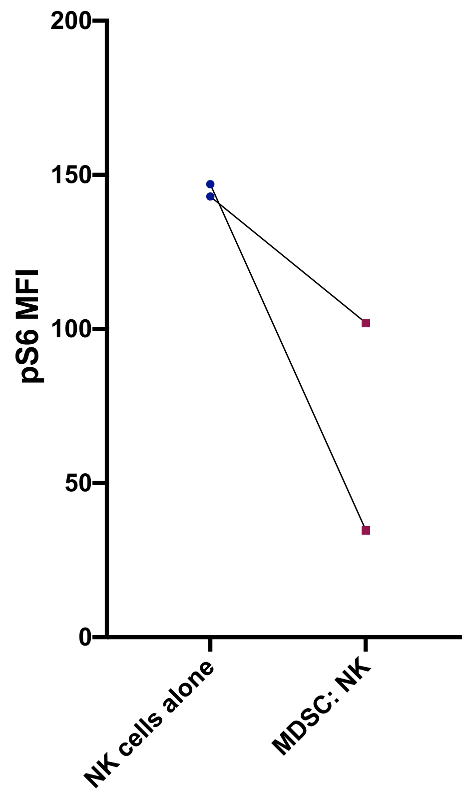

Figure 45. Phosphorylation of S6 is reduced in the presence of sxMDSCs.
CD56^{+} cells were isolated from healthy donors and CD33^{+} cells were isolated from POD1 cancer surgery patients and cells were incubated at 2:1 (CD33^{+}:CD56^{+}) for 24 hours. Healthy NK cells cultured with CD33^{+} cells (sxMDSCs) had reduced S6 phosphorylation at 24 hours compared to isolated NK cells cultured alone (n=2).

Chapter 5 Discussion

5.1 Soluble TGFβ acts by inhibiting mTORC1 to reduce receptor expression and suppress IFNγ production on POD1

Our lab and others have shown that NK cells are dysfunctional as a result of surgical stress and that this dysfunction is associated with increased metastases and cancer recurrence[224,284–286]. One of the main functions of NK cells is cytokine secretion, with IFNγ playing a critical role in the anti-cancer response. For these reasons, this project sought to characterize the pathway responsible for IFNγ production to further elucidate the mechanisms behind postoperative NK cell suppression. NK cells from patients on POD1 had a profoundly suppressed IFNγ response when stimulated with the non-specific activator PMA-ionomycin and the activating cytokines rIL-2 and rIL-12. This phenotype was observed in NK cells from both cryopreserved PBMCs and whole blood samples. While investigating pathways upstream of IFNγ production, we encountered logistical challenges that led to the development of whole blood assays which allowed for the assessment of extracellular receptors, downstream phospho-signaling activity, and cytokine production (IFNγ)[310]. There was a significant reduction in CD132, the common γ subunit of the IL-2 receptor, and CD212, the β1 subunit of the IL-12 receptor, in addition to a profound and consistent reduction in the expression of the activating receptors NKG2D and DNAM-1. Furthermore, a significant impairment in the phosphorylation of signaling proteins STAT5 (downstream of IL-2), STAT4 (downstream of IL-12), p38 MAPK (IL-12), and S6 (downstream of IL-2, NKG2D, and DNAM-1) were observed in POD1 samples. In addition, the predominantly cytokine secreting CD56^{Bright} population and the predominantly cytotoxic CD56^{Dim} population were assessed separately and significant differences in baseline expression of extracellular receptors, signaling protein phosphorylation, and IFNγ production as well as in the degree of postoperative reduction were observed, although both subpopulations displayed a suppressed phenotype. The postoperative period is also characterized

by the secretion of anti-inflammatory factors, including TGFβ, which has been shown to induce NK cell receptor downregulation[30,85,87,88,117] and suppress NK cell function *in vivo* murine models and in cancer patients[330,331,333]. With this knowledge, I developed an assay to assess whether POD1 plasma contained some soluble factor(s) that could induce NK cell dysfunction. Upon incubation of healthy NK cells in the presence of combined plasma I was able to once again reproduce the suppressed postoperative phenotype, but only in the presence of POD1 plasma and not heterologous healthy or baseline plasma. TGFβ is a pleotropic cytokine involved in both suppressive and inflammatory immune responses[346]. Due to its pleiotropic effects, blocking agents have been developed and used by other groups to inhibit TGFβ signaling in murine models of cancer[78,347–349]. Two categories of such TGFβ-specific therapeutics include blocking mAbs and small molecule inhibitors. In an attempt to prevent the dysfunction caused by POD1 plasma in healthy NK cells, cells were incubated with the smi or plasma was incubated with the mAb prior to conducting the assay, which resulted in a near-full recovery of NK cell receptor expression and IFNγ production. Finally, TGFβ has been shown to inhibit NK cell activity via inhibition of mTOR[78], a master regulator critical for mediating cell growth, metabolism, proliferation, and survival[350]. TGFβ was associated with poor NK cell-dependent suppression of metastasis, inhibition of granzyme B and perforin degranulation and IFNγ secretion, and reduced activation of S6 as well as other mTORC1 substrates including 4EBP1 and Akt[78]. To investigate whether this is the mechanism by which TGFβ is acting in postoperative NK cells I assessed S6 phosphorylation as a proxy for mTORC1 function. S6 phosphorylation was significantly reduced in unstimulated POD1 cells, as compared to healthy or paired baseline samples; in addition, POD1 cells could not be stimulated to phosphorylate S6 in response to rIL-2/12. Furthermore, incubation with POD1 plasma inhibited S6 phosphorylation. Finally, phosphorylation was recovered in the presence of the TGFβ mAb and TGFβ RI smi, suggesting that this is the primary mechanism by which TGFβ signals and induces a suppressed phenotype in postoperative NK cells. A suppressive population of myeloid cells,

MDSCs, expand rapidly in the postoperative period and have been shown to suppress NK cell function in the context of cancer[330,331,333]. For these reasons, I investigated the potential of sxMDSCs to induce a dysfunctional phenotype in isolated healthy $CD56^{+}CD3^{-}$ cells. I found that upon incubation with sxMDSCs, healthy NK cells acquired a hyporesponsive phenotype similar to the POD1 NK cells characterized in whole blood assays. MDSCs secrete many soluble factors, one of them being TGFβ, which they also express on their cell surface[330–334]. Taken together, this suggests that sxMDSC-derived TGFβ is acting through mTOR inhibition to alter NK cell phenotype and function, contributing to postoperative immune suppression and metastases.

5.2 Limitations

5.2.1 Whole blood assays

A limitation of whole blood assays includes having to process patient samples immediately and therefore the number of samples that can be tested at once is limited by access to whole blood collection, which could lead to greater inter-assay variability. However, technical expertise, appropriate controls (unstained, isotype, fluorescence minus one, "healthy donors"), and validated standard operating procedures can be implemented to help mitigate this limitation. In addition, patients may be more likely to consent to blood draws for research that requires minimal blood volumes.

5.2.2 Working with human specimens

5.2.2.1 Patient heterogeneity

Mice have long served as models of human biology and disease due to their phylogenetic relationship to humans, the ease of maintenance, housing and breeding, and the wide variety of available murine strains. In addition, the development of transgenic, knockout, and knockin mice allowed for murine models to be used as powerful tools for the study of human disease[351]. However, the limitations of using animal models has become apparent in that many findings have not proved

to be clinically translatable — most notably in the fields of immunology and oncology[351–355]. While there are many factors that may impact the capacity for the translation of murine studies, one major factor may be a lack of genetic variability; one of their greatest strengths is also one of their greatest weakness[351].

There has been a pressing need to improve upon our knowledge of the human immune system. A method by which this can be achieved is through "systems immunology". Systems immunology mirrors systems biology in that the purpose is to identify and measure the major components of a system in response to external stimuli[355–357]. The major components of the immune system are the immune cells and cytokines, all of which are present in whole blood[358]. This kind of approach can prove useful in assessing the nuances of the human immune system. While healthy adults show very stable immune systems in the absence of perturbation there is inherent heterogeneity and variability between patient samples[359–361]. Inter-patient variability is a consequence of both heritable factors including host genetics and sex, and non-heritable factors including the microbiota, bacterial dysbiosis, viral history, and other environmental factors[358]. Juxtaposed with murine models that tout "unity in biology" through syngeneity, the inter-patient heterogeneity of humans may seem challenging for data interpretation. Moreover, human systems do not allow for the same level of manipulation as murine models. However, the significant advantage over murine models is that studies may be more clinically relevant.

5.2.2.2 Sample size

Some of the experiments described are underpowered due to a small sample size. Investigations using human specimens are reliant upon individuals who fit the inclusion criteria; specifically, cancer patients undergoing surgery who agree to participate and have successful pre- and postoperative blood draws. Moreover, the use of "fresh" whole blood may limit the number of samples that can be assessed in a given experimental run. However, the immediate processing of whole blood samples reduces cell manipulation, and this may prove to be more informative and

accurate in determining the mechanism behind NK cell dysfunction in the postoperative period. Despite these benefits, small sample sizes make it challenging to perform statistical analyses. However, there has been a recent push against relying on statistical significance to determine usefulness of results[362,363] and instead focusing on biological significance when interpreting results. Due to the limited sample sizes of some of these experiments the change in target expression on POD1 did not quite reach statistical significance, although the more important question is whether these differences are relevant biologically. Ergo, the continuation of this research should expand upon some of the data sets present here to increase sample size for statistical analyses and interpretation.

5.2.3 COVID-19 Pandemic

Of note, the smaller sample sizes for the latter experiments that involved the use of TGFβ-specific therapeutics (particularly testing the small molecule inhibitor) are due to the lack of opportunity to continue experimenting in light of the COVID-19 pandemic, which shut down all non-essential laboratory work in March of 2020.

5.3 Areas of further investigation

With these limitations in mind, future work should focus on (1) characterizing the timeline of NK cell dysfunction postoperatively and (2) further elucidating the pathway of TGFβ-induced NK cell suppression.

5.3.1 The timeline of NK cell dysfunction in the postoperative period

NK cell cytotoxicity and production of IFNγ are most profoundly suppressed on POD1[147–150], and for this reason this study has focused on baseline and POD1 as the relevant timepoints. However, understanding the kinetics of this suppression may be important for determining the timeline of therapeutic delivery.

My work suggests that TGFβ is the inciting agent for NK cell dysfunction, although baseline to POD1 changes in plasma TGFβ levels did not reach significance. While partly due to sample size, it is entirely possible that TGFβ levels peak earlier near the induction of surgical stress. The timeline of TGFβ production in response to surgical stress is as of yet incompletely characterized. This could be investigated using whole blood drawn from patients preoperatively and at multiple timepoints during and after surgery. A similar study was conducted by Kato et al., which reported that IL-10 levels peaked at 4 hours during surgery and IL-6/IL-8 levels peaked at the end of surgery[220]. In addition, my preliminary experiment assessing NKG2D expression suggests that a short exposure to this inhibitory cytokine may have long-lasting consequences for NK cell function. This is supported by work by Viel and colleagues who described an early mTOR-dependent inhibition of NK cells that may contribute later to inhibition of proliferation[78]. Our current PHBSP protocol does not allow for blood draws during surgery. Amending this protocol to allow for the collection of blood from patients intraoperatively and immediately postoperatively would facilitate a more detailed investigation of TGFβ in the postoperative period and would allow for the assessment of NK cell phenotype and function via the whole blood assays described here.

Angka et al. showed that the impairment in NK cell IFNγ secretion lasted until POD56 in one third of CRC surgery patients[148]. While not characterized here, whole blood collected at timepoints extending beyond POD1 could be analyzed using the described assays. Knowledge of whether reduced receptor expression and signaling protein phosphorylation parallel the timeline of dysfunctional IFNγ production could provide insight into the mechanism behind the prolonged suppression in response to surgery.

Finally, it is unknown whether NK cell dysfunction is transient and cells are able to recover their effector functions postoperatively, or if this dysfunction is permanent and cells are instead replaced by functional NK cells from the bone marrow. The half-life of a human NK cell is reported to be < 10 days[364]. It will be critical for the development of perioperative therapeutics to know if

phenotypic and functional changes in hyporesponsive postoperative NK cells are reversible. This could be investigated using an adoptive transfer system whereby NK cells from surgically stressed (laparotomy and heminephrectomy[151,197]) or control mice are transferred into Ncr1 (NKp46)-Cre NK-deficient mice[367]. NK cell function could then be monitored (via *ex vivo* rIL-2/12-stimulated IFNγ quantification) at various postoperative time points to assess whether dysfunctional surgically stressed NK cells could regain functionality with time.

5.3.2 Elucidating the postoperative NKT cell phenotype

With their expression of an invariant TCR α-chain and restricted β-chain repertoire and NK cell-like effector functions, iNKT cells make up a unique lymphoid cell population. This population can be divided into five functional subsets: T_h1-like, T_h2-like, T_h17-like, T_{reg}-like, and T_{FH}-like, characterized by their cytokine profile[368]. T_h1-like iNKT cells produce pro-inflammatory cytokines such as IFNγ and TNFα upon stimulation, while T_h2-like iNKT cells secrete IL-4 and IL-13 upon stimulation and are associated with regulatory functions[368]. Recently, iNKT cells have been shown to undergo a phenotypic switch where overstimulated T_h1-like cells switch to an immunosuppressive T_h2/T_{reg}-like phenotype. Specifically, this has been shown to occur during the process of tumourigenesis[368]. Lee et al. sought to delineate the T_h1-like/T_h2-like phenotypes using human peripheral blood lymphocytes[369]. They showed that T_h1-like iNKT cells were $CD4^-CD8^-$ (double negative), while T_h2-like iNKT cells were $CD4^+$. In addition, T_h1-like iNKT cells expressed NK cell lineage-specific markers including 2B4, CD94, and NKG2A. Interestingly, CD25 was expressed exclusively on $CD4^+$ T_h2-like iNKT cells[369].

Whole blood assays were used to assess NK cell phenotype and function postoperatively but also allowed for the assessment of NKT phenotype and function. Postoperative NKT cells ($CD56^+CD3^+$) exhibited reduced IFNγ production in response to rIL-2/12 stimulation (p=ns), significantly altered receptor expression (reduced NKG2D, DNAM-1, PD-1 and increased NKG2A, TIGIT, and CD25), and significantly impaired signaling protein phosphorylation. These

results suggest that pre-operative T_h1-like iNKT cells may become hyporesponsive as a result of surgical stress or may be undergoing a phenotypic switch to become pro-tumourigenic T_h2-like. It is difficult to discriminate between these mechanisms because I did not stain cells for CD4 or CD8 surface markers. There is currently a paucity of research into NKT cell function in the postoperative period, although NKT cells do appear to play an important anti-tumour role[126]. Thus, the whole blood assays presented here could be used to further investigate the NKT cell postoperative phenotype(s) and possible mechanisms of dysfunction. However, despite their role as tumour-suppressors, NKT cell frequencies are extremely variable in humans and make up only 0.01-0.1% of peripheral blood lymphocytes[119–121,126]. In fact, NKT cell-specific immunotherapies may only be beneficial for patients with high NKT cell frequencies[126]. Thus, effective perioperative treatments would likely encompass combined NKT and NK cell-targeted therapeutics.

5.3.3 Investigating the TGFβ-mTOR relationship

The molecular pathways by which TGFβ signals in NK cells remain largely uncharacterized. TGFβ has been shown to override IL-2[85] and IL-15[78] signaling in culture and induce the downregulation of critical activating receptors[30,85–88] while subsequently inhibiting the release of soluble effectors like IFNγ[78,89,370]. This is likely accomplished through both Smad-dependent and Smad-independent pathways (**Figure 46**). It was previously proposed that NK and T cell IFNγ secretion is inhibited via direct binding of Smads to the Tbx21 promoter, thus preventing T-bet expression[78,89,370]. In addition, Viel et al. showed that the inhibition of mTOR by TGFβ was rapid and concomitant with Smad2/3 phosphorylation. This suggests that Smad2/3 inhibition of mTOR may be a proximal event, although it is unknown whether this interaction is direct or occurs through an intermediary protein[78]. Independent of Smad signaling, TGFβRI constitutively binds to FKBP12[371], which is released upon TGFβ signaling. The binding of FKBP12 with its endogenous binding partners or rapamycin inhibits mTOR activity[372], and therefore could contribute to TGFβ inhibition of mTOR in the postoperative period. Finally, TGFβ upregulates the

expression of and activates protein tyrosine phosphatase (PTP) Src homology region 2 domain-containing phosphatase-1 (Shp-1), which inhibits the activity of signaling kinases in T cells[373,374]. Moreover, Shp-1 activity has been shown to mediate mTOR function in NK cells via intracellular propagation of inhibitor receptor signals[97,375]. Although, to date there are no reports of Shp-1 activity downstream of TGFβ signaling in NK cells.

In keeping with the literature, I have shown here that the addition of rTGFβ1 to healthy NK cells in culture induced a reduction in receptor expression and impaired signaling protein activity and IFNγ production in response to rIL-2/12. Moreover, this phenotype can be reproduced in the presence of POD1 plasma and can be prevented with pre-incubation of TGFβ blocking therapies. In both the sxMDSC-NK cell co-culture and plasma culture experiments, healthy NK cells were exposed to sxMDSCs, POD1 plasma, or rTGFβ1 for 24 hours prior to washing and resus pension in a rIL-2/12 stimulation media. Thus, despite the removal of rTGFβ1 from culture for the second 24 hours period, NK cells were still impaired in their ability to produce IFNγ. In addition, a 20-minute exposure to rTGFβ1 induced a reduction in NKG2D expression in healthy NK cells in culture at 24 hours. This supports the hypothesis that TGFβ may have early and late pathways by which NK dysfunction is mediated. Early pathways may include the activation of phosphatases[373], inhibition of relevant kinase activity[76], or post-transcriptional modification of miRNAs[376], while late pathways may include altered gene expression or epigenetic changes[377].

Further elucidating these pathways will fill a knowledge gap from a molecular biology perspective and could contribute to therapeutic development. A next step would be to assess the relative contributions of Smad-dependent and -independent pathways. This could be achieved by treating healthy $CD56^+$ cells with SIS3, a small molecule Smad 3 inhibitor, or PD169316, a small molecule Smad2/3 inhibitor in the presence of rTGFβ1[378–380]. Additionally, the sxMDSC:NK cell

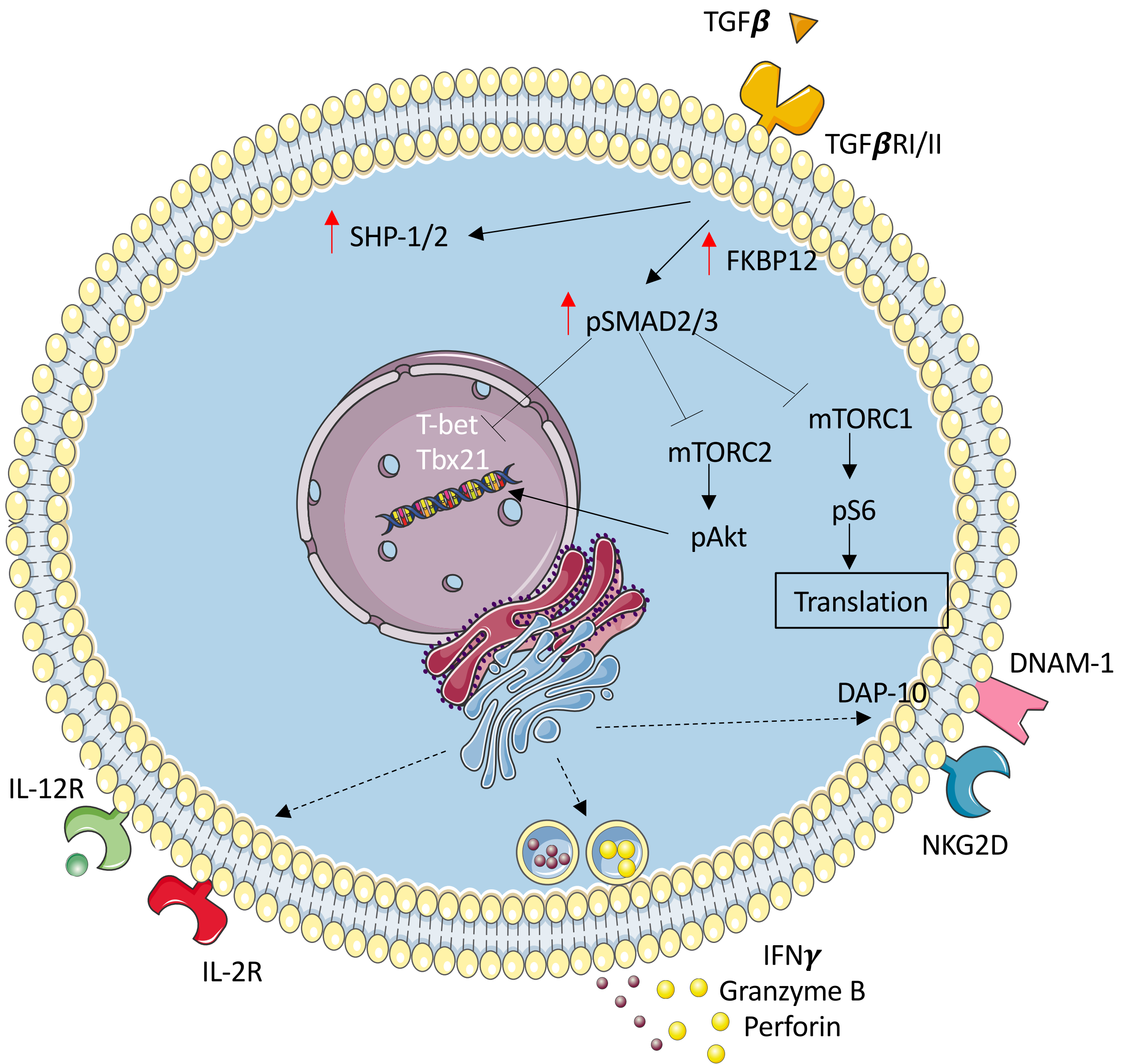

Figure 46. Hypothesized TGFβ signaling pathways in postoperative NK cells.
Upon binding to its receptor, TGFβ may signal through SMAD2/3 to directly inhibit the expression of the transcription factor T-bet (*Tbx21*) or to inhibit mTOR functions via unknown intermediary protein(s). Smad-independent mechanisms may include the production and activation of SHP-1 phosphatase or the release of FKB12, which may inhibit mTORC1 activity. The pathways may result in early responses (near-immediate inhibition of mTOR) and late responses (downregulation of important cell surface receptors and inhibition of proliferation).

co-culture or plasma culture assays could be used to elucidate these pathways in the context of surgical stress.

5.3.4 Assessing the impact TGFβ blockade *in vivo*

Characterizing the phenotype and potential pathways of dysfunction in postoperative NK cells is an essential step in the development of therapeutics to prevent postoperative immune suppression and metastasis. However, the safety and efficacy of such therapeutics must be assessed prior to administration in humans. It is here that murine models provide an advantage over the use of human specimens.

The TGFβ RII subunit could be deleted in NK cells by crossing Ncr1Cre mice[381] with Tgfbr2fl/fl mice[382] to generate NK-Tgfbr2$^{-/-}$ animals. NK cells from these mice are unresponsive to TGFβ and display reduced mTOR signaling. Moreover, these animals have less lung metastases following tail vein injection of B16F10 melanoma cells[78]. The contribution of TGFβ to postoperative metastasis formation could be assessed using the previously described murine model of surgical stress whereby NK-Tgfbr2$^{-/-}$ mice and littermate controls receive a tail vein injection of B16F10 melanoma cells followed by a laparotomy (abdominal incision) and left kidney resection, and pulmonary metastases are quantified[146,151,197,261,383]. Additionally, the contribution of postoperative TGFβ to NK cell dysfunction could be assessed in the same model by quantifying mTORC1 signaling (pS6 and p4EBP1), expression of activating (NKG2D, DNAM-1) and cytokine (CD212, CD132) receptors and IFNγ production in response to cytokine stimulation. If the proposed mechanisms hold true in this model, NK cells should not display reduced receptor expression or be hyporesponsive postoperatively.

This same model of surgical stress[151,197] could then be used to assess the safety and efficacy of TGFβ-specific therapeutics, including the anti-TGFβ mAb and TGFβ RI small molecule inhibitors (SB525334 or SB431542[384,385]), to assess viability of these therapeutics for human patients.

5.4 Clinical applications

5.4.1 Tracking NKG2D expression as a diagnostic tool

NKG2D is involved in both the innate and adaptive immune responses and specifically in NK cells, NKG2D signaling has been shown to override inhibitory signals allowing NKG2D to act as a "master switch" for NK cell activation[386]. In fact, the activating potency of NKG2D in conjunction with the selective expression of NKG2D stress-induced ligands on tumour cells has made the NKG2D-NKG2DL pathway a promising target for cancer immunotherapy[345]. TGFβ has been reported to regulate NKG2D expression, resulting in impaired cytotoxicity and IFNγ production[30,85,89]. In the context of cancer, NKG2D expression is inversely correlated with disease severity[344,345] and elevated TGFβ has been reported to play a role in its downregulation in a myriad of malignancies including: glioblastoma, lung cancer, CRC, Hodgkin's lymphoma, gastric cancer, and head and neck squamous cell carcinoma[30,87,387–390]. Impaired NK cell effector functions and TGFβ-mediated NKG2D downregulation have also been reported in the context of viral infections[88,391]. Lazarova et al. even went so far as to suggest that NKG2D expression could serve as a biomarker specifically for TGFβ-mediated NK cell dysfunction[30]. Evidence suggests that surgery results in increased metastases and cancer recurrence and that this is due primarily to NK cell dysfunction. I have identified a profound reduction in NKG2D expression in dysfunctional postoperative NK cells. Furthermore, I have shown a strong correlation between NKG2D expression and IFNγ production in NK cells exposed to POD1 plasma. With the knowledge that NKG2D expression and NK cell function are directly related to cancer severity/progression, NKG2D should be investigated as a proxy for NK cell function in a diagnostic context. NKG2D may have the potential to be used as a biomarker to predict cancer progression and to track patient recovery or predict cancer recurrence in cancer surgery patients. Future work could focus on a prospective assessment of NKG2D expression at various time points following surgery using whole

blood assays to investigate the relationship between the proportion of NKG2D^{+} NK cells and cancer recurrence.

5.4.2 Targeting surgery-induced MDSCs and TGFβ

Myeloid-derived suppressor cells are a suppressive population which expands rapidly following surgical stress[260,262,265,291,293]. There are many mechanisms by which MDSCs induce immunosuppression, one of which is the production of TGFβ[30,259,330,392]. My results suggest that TGFβ in the plasma of postoperative cancer patients may be produced by sxMDSCs. Future research should focus on investigating this relationship further. Prior to the COVID-19 pandemic I was optimizing the use of TGFβ-specific therapeutics (mAb and smi) in the sxMDSC: NK co-culture assay. If TGFβ is being produced primarily by sxMDSCs, healthy NK cells should exhibit a functional phenotype upon blocking TGFβ in culture.

In the context of cancer, MDSC expansion and activation has been correlated with tumour progression and recurrence and thus MDSCs have become an important immunotherapeutic target. Specifically, MDSC modulation is associated with significant therapeutic benefit in preclinical models and early phase trials (reviewed in 2018 by Fleming et al.) and has been achieved by: (1) inhibiting MDSC immunosuppressive activity, (2) blocking MDSC migration to the TME, and (3) depletion of MDSCs[259]. The activity of iNOS, ARG1, and COX-2 can be inhibited using phosphodiesterase-5 inhibitors (sildenafil and tadalafil)[259,261] and the class I histone deacetylase inhibitor entinostat alone or in combination with anti-PD-1 antibodies. The transcription factor STAT3 is involved in immunosuppressive pathways and can be blocked using siRNA or decoy oligonucleotides in combination with immune checkpoint inhibitors or CpG oligonucleotides (TLR9 agonists). In addition, pharmacological inhibition of fatty acid oxidation has been shown to decrease the immunosuppressive capacity of MDSCs. MDSC migration can be impaired by blocking chemokine signaling using small molecule inhibitors or chemotherapeutic drugs[259,393]. The MDSC population can be depleted by inhibiting the conversion of immature myeloid cells into

MDSCs using dimethyl amiloride or omeprazole to reduce extracellular vesicles in serum. In addition, MDSCs can be induced to differentiate into mature DCs or macrophages using the vitamin A derivative aTRA or chemotherapeutic agents like docetaxel. Finally, tyrosine kinase inhibitors like sunitinib have been reported to reduce circulating MDSCs, chemotherapeutics like gemcitabine and 5-fluorouracil can induce selective apoptosis of MDSCs, and mAbs such as anti-Gr1 mAb have been used to deplete MDSCs in murine models[259,394]. However, while widely used, the efficacy of anti-Gr1 depletion on both MDSC subtypes is controversial, as M-MDSCs express less Gr1[395,396]. Although these therapeutics have been tested in the context of cancer, they could also be applied to target sxMDSCs and could be tested for safety and efficacy in our murine model of surgical stress. It is important to note, however, that sxMDSCs may also be playing a role in homeostasis in response to postoperative inflammation, as they have recently been reported to be beneficial in the context of pregnancy, obesity, diabetes, and autoimmune disorders[397].

Finally, directly blocking the activity of TGFβ in the plasma of postoperative patients may be an option to prevent postoperative NK cell dysfunction. Viel et al. showed that the addition of an anti-TGFβ mAb enhanced IL-15-induced mTOR activity in murine splenocytes[78]. Mariathasan and colleagues showed that co-administration of anti-TGFβ and anti-PD-L1 mAbs in an EMT6 murine model of mammary carcinoma facilitated T cell-mediated anti-tumour immunity and tumour regression[349]. Nam et al. assessed the efficacy of an anti-TGFβ mAb in a transplantable 4T1 model of metastatic breast cancer and reported increased infiltration of T and NK cells, increased expression of NKG2D and cytotoxic granules, and enhanced $CD8^+$ T cell-mediated anti-tumour responses[348]. Alvarez and colleagues also investigated the effects of an anti-TGFβ mAb in combination with rIL-2 and found significant increases in the numbers and functionality of NK cells and $CD8^+$ T cells. In addition, this combination therapy significantly increased survival of mice injected with 3LL Lewis lung carcinoma cells in a $CD8^+$ T cell- and NK cell-dependent manner. Despite these promising results in preclinical cancer models, targeting TGFβ in the

postoperative period may have adverse effects related to wound healing, as TGFβ is known to play a major role[223]. However, this role is complex as TGFβ can both stimulate and protract wound re-epithelialization. One study investigated the temporal effects of TGFβ in a rabbit dermal ulcer model and reported the administration of an anti-TGFβ mAb early after injury resulted in delayed wound healing, while administration of the mAb at 7 days post-injury did not affect wound healing but reduced hypertrophic scar formation[398]. This is likely due to TGFβ-mediated fibrosis[398,399].

5.4.3 The making of an antibody-drug conjugate

In 1909, the German physician and scientist Paul Ehrlich postulated that the host immune system could suppress cancer development[400]. Immunotherapy, the fourth pillar of cancer treatment, has developed from this very principle. Among the first approved immunotherapeutics were the cytokines IFNα and IL-2 for the treatment of melanoma and renal cell carcinoma in 1986 and 1992 respectively, and vesicular Bacillus Calmette Guerin for the treatment of superficial bladder cancer in 1990[401]. Ehrlich also predicted the use of antibodies as therapeutics describing them as target-specific "magic bullets" and foresaw improving their therapeutic specificity by the conjugation of toxins[402]. Subsequently, the first use of antibodies as drug carriers was tested pre-clinically against L1210 cells by Mathé et al. in 1958[403] and clinically in malignant melanoma by Ghose et al. in 1972[404]. Antibody-drug conjugates (ADCs) are currently one of the fastest growing classes of anti-cancer therapeutics and have traditionally consisted of mAbs attached via a chemical linker to a cytotoxic payload directed towards a cancer-specific antigen[405]. A consequence of the specificity of ADCs is reduced systemic toxicity, which affords ADCs an incredible advantage over traditional cancer treatments like chemotherapy and radiotherapy. However, successful ADC development is complex and is dependent upon appropriate target selection, linker design, attachment chemistry, and payload potency/MOA[406]. ADCs are administered intravenously to avoid degradation of the mAb. Upon attachment to its target, the ADC-antigen complex is internalized via receptor-mediated endocytosis resulting in early followed by late endosome

formation. The late endosome is coupled to lysosomes, which facilitate the cleavage of the ADC and release of the cytotoxic payload into the cytoplasm[405]. Four ADCs are currently approved for the treatment of various cancers and a myriad of novel ADCs are being tested preclinically[405]. Only recently has the potential for ADCs been explored beyond anti-cancer therapeutics for inflammatory/autoimmune diseases like arthritis, systemic sclerosis, and multiple sclerosis, and infectious pathogens like *S. auerus*[406,407]. Current non-oncology ADC payload classes include glucocorticoid receptor modulators, antibiotics, kinase inhibitors, liver X receptor (LXR) agonists, PDE4 inhibitors, bisphosphonate, and microtubule inhibitors[406]. Clinically, Abbvie's anti-TNF glucocorticoid receptor modulator, ABBV-3373, is being investigated to treat rheumatoid arthritis (NCT03823391), and Bolt Biotherapeutics' TLR-targeted Immune Stimulating Antibody Conjugate (ISAC), BDC-1001, is being investigated for localized immune-stimulation at the tumour site (NCT04278144). The latter trial is the only known trial to adapt the ADC for immune stimulating purposes.

My results suggest that blocking TGFβ signaling via a TGFβ RI small molecule inhibitor (SB525334) could prevent postoperative NK cell dysfunction. Since this dysfunction has been linked to increased metastatic burden and cancer recurrence, the effective delivery of a small molecule inhibitor to prevent TGFβ signaling may prove to be an excellent perioperative therapeutic. The activating receptor NKp46 (NCR1, CD335) is selectively expressed on NK cells and a subset of NKT cells and is reported to be the most specific marker of NK cells in mammals[408,409]. An NKp46-targeted mAb could therefore be conjugated to a TGFβ RI smi (SB525334 or SB431542[384,385]). The efficacy and safety of this ADC could be investigated using the *in vitro* assays described here (sxMDSC: NK cell co-culture or plasma culture) and *in vivo* using our previously described murine model of surgical stress.

Significance

The immunosuppressive effects of surgery present an opportunity for cancer cells to form metastases as well as a therapeutic window in which to intervene. Unfortunately, there are currently no therapeutics targeted to the perioperative period. We and others have shown that NK cells are functionally suppressed in the postoperative period and that this suppression strongly influences metastatic formation postoperatively. Specifically, IFNγ production by NK cells is profoundly suppressed postoperatively and this is associated with phenotypic perturbations in receptor expression and critical signaling molecules. Knowledge of the postoperative NK cell phenotype and mechanisms responsible for the induction of immune dysfunction is critical for the development of perioperative immunotherapies. Sixty-five thousand Canadians undergo surgical resection of their cancer each year. Since ancient times we have known that despite the complete removal of the a cancerous tumour, the disease may return again. The prevention of immune suppression is vital to the prevention of cancer recurrence not only among Canadians, but globally. The work I have completed presents sxMDSC-derived TGFβ as a potential target and lays the foundation for the development of a novel perioperative therapeutic. Continued pre-clinical investigations are necessary and the prevention of postoperative immune suppression will likely be multi-modal and require combination immunotherapy. The results of my research have allowed us to recently apply for funding from the Cancer Research Society (CRS) for preclinical studies to engineer an antibody drug conjugate that could deliver a TGFβR small molecule inhibitor directly to NK cells, thereby preventing postoperative NK cell dysfunction and subsequent metastases. A successful perioperative therapy has enormous potential to reduce the burden of cancer worldwide.

Contribution of Collaborators

In their capacity as Clinical Coordinators Ahwon Jeong, Marlena Scaffidi, and Juliana Ng identified and consented patients as part of the PHBSP protocol for use in the experiments presented here.

Gayashan Tennakoon was a TMM and Honours Thesis student who I trained and mentored from 2017-2019. Gayashan was involved in optimizing the whole blood phosphorylation protocol and was involved in staining and flow cytometry for the whole blood activating/inhibitory receptor data. In his capacity as a laboratory technician he collected patient plasma and ran some iterations of the plasma culture experiments.

Single-cell RNA-sequencing was done in conjunction with Leonard Angka and David Cook. Patient samples were used from patients recruited to the PERIOP-02 study by Leonard Angka. Leonard also helped thaw and assess the viability of the baseline and POD1 samples and performed immunophenotyping staining and flow cytometry on these samples. David Cook barcoded and pooled the samples and facilitated communication with the StemCore facility. David also analyzed the scRNA-seq results and created Figures 30-32.

Leonard Angka optimized the NK-92 cytotoxicity assay (Section 3.8).

Tables

Table 1. FACS monoclonal antibodies.

Antibody	Vendor	Cat #	Clone
CD3 FITC (mouse)	Invitrogen	11-0039-41	HIT3a
CD56 BV421 (mouse)	BD biosciences	562751	NCAM16.2
CD16 BV650 (mouse)	BD biosciences	563692	3G8
CD14 APC-Cy7 (mouse)	BD biosciences	557831	MϕP9
CD45 AF700 (mouse)	BD biosciences	560566	HI30
Fixable viability dye BV510	BD biosciences	564406	-
IFNγ APC (mouse)	Invitrogen	17-7319-82	4S.B3
CD25 PE-Cy7 (mouse)	BD biosciences	557741	M-A251
CD122 PE (mouse)	BD biosciences	554522	Mik-β2
CD132 APC (rat)	Biolegend	338607	TUGh4
p-STAT5 PE-Cy7 (pY694) (mouse)	BD biosciences	560117	47/Stat5
CD212 BV786 (mouse)	BD biosciences	744207	2.4E6
Mouse BV786 IgG1	BD biosciences	563330	X40
p-STAT4 PE (pY693) (mouse)	BD biosciences	558249	38/p-Stat4
NKG2D BV650 (mouse)	BD biosciences	563408	1D11
NKG2A PE (mouse)	R&D Systems	FAB1059P-025	131411
BV786 TIM-3 (mouse)	BD biosciences	742857	7D3
PE-Cy7 DNAM-1 (mouse)	BioLegend	338315	11A8
APC TIGIT (mouse)	BioLegend	372705	A15153G
PD-1 PerCP-Cy5.5 (mouse)	BioLegend	329913	EH12.2H7
S6 PE (pS235/236) (mouse)	BD biosciences	560433	NF-548
p38 MAPK APC (pThr180, Tyr 182) (mouse)	Invitrogen	17-9078-42	4NIT4KK
Akt PE-CF594 (pS473) (mouse)	BD biosciences	562465	M89-61
Akt PE (pT308) (mouse)	BD biosciences	558275	J1 223.371
LAP-TGFβ1 PE (mouse)	Biolegend	349603	TW4-2F8
Mouse PE IgG2a	BioLegend	400214	MOPC-173
Mouse APC IgG2A	BioLegend	400219	MOPC-173
Mouse PerCP-Cy5.5 IgG1	BioLegend	400149	MOPC-21
Mouse PE IgG1	Biolegend	400113	MOPC-21
Mouse APC IgG1	Biolegend	400119	MOPC-21
Mouse PE-Cy7 IgG1	BD biosciences	557872	MOPC-21
Mouse PE IgG2b	Invitrogen	12-4732-41	eBMG2b
Mouse BV650 IgG1	BD biosciences	563231	X40
Rat APC IgG2b	Biolegend	400611	RTK4530

Table 2. Reagents.

Reagent	Vendor	Cat #
Phosphate-buffered saline (PBS)	Hyclone	SH3025601
Ficoll- Plaque	GE Healthcare	17144003
Fetal bovine serum (FBS)	GE Healthcare	SH3039603
Dimethyl sulfoxide (DMSO)	Fisher Scientific	17144003
RPMI-1640 medium	Sigma-Aldrich	R1780-500ML
Human Fc block (Trustain)	Biolegend	422302
4% Paraformaldehyde	Thermo Scientific	AAJ19943K2
Recombinant IL-2	Tecin™ Teceleukin	-
Recombinant IL-12	R&D Systems	219-IL-005
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich	P1585-1MG
Ionomycin	Sigma-Aldrich	I9657-1MG
Golgiplug™ (Brefeldin A)	BD biosciences	555029
FACS lyse/fix buffer	BD biosciences	558049
Bovine serum albumin (BSA)	Sigma-Aldrich	A8412-100ML
0.5 M Ethylenediaminetetraacetic acid (EDTA)	Invitrogen	AM9260G
Perm III buffer	BD biosciences	558050
Intracellular (IC) fixation buffer	Invitrogen	00-8222-49
Cytofix buffer	BD biosciences	554655
Ethidium Bromide homodimer (EthD)	Thermo Fisher Scientific	E1169
Miltenyi BioTec CD33+ microbeads	Miltenyi	130-045-501
Miltenyi BioTec CD56+ microbeads	Miltenyi	130-090-875
Quantikine ELISA Human IFNγ Immunoassay	R&D Systems	DIF50
Quantikine ELISA Human TGFβ Immunoassay	R&D Systems	DB100B
Recombinant human TGFβ1	Abcam Biochemicals	Ab50036
Recombinant human IL-10	R&D Systems	217-IL-005
Anti-TGFβ mouse mAb	Bio X Cell	BE0057
TGFβ RI inhibitor SB525334	Selleck Chemicals	S1476
Cytochrome P450 Vivid™ green fluorescent standard	Thermo Fisher Scientific	P2875

Table 3. Patient information for cryopreserved samples used to characterized cytokine and activating/inhibitory receptors and intracellular/extracellular IFNγ.

Category	Subcategory	Healthy Donors	Patients
Total (n)		12	32
Sex	Male	4	24
	Female	8	8
Patient Age	< 60 years	12	9
	60-69 years	0	15
	> 70 years	0	8
Cancer Type	Prostate	-	13
	Lung	-	10
	Renal	-	3
	Colorectal	-	3
	Endometrial	-	1
	Pancreatic	-	1
	Esophageal	-	1
Staging	I	-	10
	II	-	5
	III	-	12
	IV	-	4
	Unknown	-	1

Table 4. Patient information for whole blood samples used to characterized cytokine and activating/inhibitory receptors, phospho-signaling proteins, and intracellular/extracellular IFNγ.

Category	Subcategory	Healthy Donors	Patients
Total (n)		42	39
Sex	Male	16	21
	Female	26	18
Patient Age	< 60 years	26	11
	60-69 years	9	19
	> 70 years	7	9
Cancer Type	Prostate	-	11
	Lung	-	7
	Colorectal	-	6
	Renal	-	3
	Ovarian	-	3
	Sarcoma	-	3
	Pancreatic	-	1
	Parathyroid	-	1
	Neuroendocrine	-	1
	Endometrial	-	1
	Duodenal	-	1
	Uterine	-	1
Staging	I	-	14
	II	-	7
	III	-	15
	IV	-	3
	Unknown	-	0

Table 5. Patient information for TGFβ ELISA.

Category	**Subcategory**	**Healthy Donors**	**Patients**
Total (n)		3	11
Sex	Male	1	7
	Female	2	4
Patient Age	< 60 years	1	1
	60-69 years	1	5
	> 70 years	1	5
Cancer Type	Lung	-	4
	Sarcoma	-	2
	Prostate	-	2
	Renal	-	1
	Liver	-	1
	Thymoma	-	1
	Non-cancer	3	-
	Inconclusive pathology	-	-
Staging	I	-	4
	II	-	2
	III	-	3
	IV	-	-
	Unknown	-	2

Table 6. Demographics for healthy CD56+ donors and healthy/cancer patient plasma used in plasma culture experiments.

Category	Subcategory	Healthy NK cell donors	Healthy plasma	Cancer patient plasma
Total (n)		10	19	25
Sex	Male	1	6	14
	Female	9	13	11
Patient Age	< 60 years	8	10	5
	60-69 years	2	4	10
	> 70 years	0	5	10
Cancer Type	Lung	-	-	6
	Sarcoma	-	-	4
	Prostate	-	-	3
	Renal	-	-	2
	Endometrial	-	-	1
	Uterine	-	-	1
	Pancreatic	-	-	1
	Thymoma	-	-	1
	Hepatic	-	-	1
	Non-cancer	10	19	3
	Inconclusive pathology	-	-	0
Staging	I	-	-	7
	II	-	-	5
	III	-	-	7
	IV	-	-	0
	Unknown	-	-	3

Table 7. Patient information for cryopreserved samples used in single-cell RNA-sequencing.

Category	**Subcategory**	**Cancer patients**
Total (n)		6
Sex	Male	6
	Female	0
Patient Age	< 60 years	2
	60-69 years	3
	> 70 years	1
Cancer Type	Colorectal	6
Staging	I	1
	II	4
	III	1
	IV	0

Table 8. Demographics for healthy CD56+ donors and POD1 CD33+ patients used in sxMDSC: NK cell co-culture assays.

Category	Subcategory	NK cell donors	sxMDSC donors
Total (n)		6	6
Sex	Male	0	4
	Female	6	2
Patient Age	< 60 years	3	5
	60-69 years	3	0
	> 70 years	0	1
Cancer Type	Colorectal	-	1
	Bladder	-	1
	Pancreatic	-	1
	Non-cancer	6	2
	Inconclusive pathology	-	1
Staging	I	-	0
	II	-	1
	III	-	1
	IV	-	1
	Unknown	-	0

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406. McPherson MJ, Hobson AD. Pushing the Envelope: Advancement of ADCs Outside of Oncology. In: *Methods in Molecular Biology*. ; 2020. doi:10.1007/978-1-4939-9929-3_2
407. Gingrich J. How the Next Generation Antibody Drug Conjugates Expands Beyond Cytotoxic Payloads for Cancer Therapy. ADC Review.
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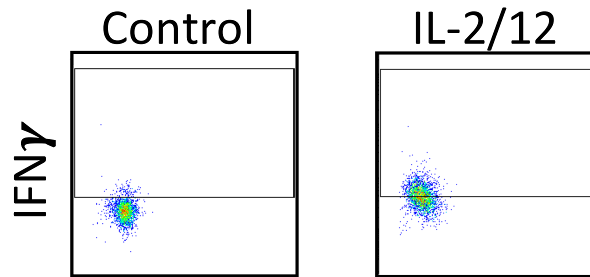
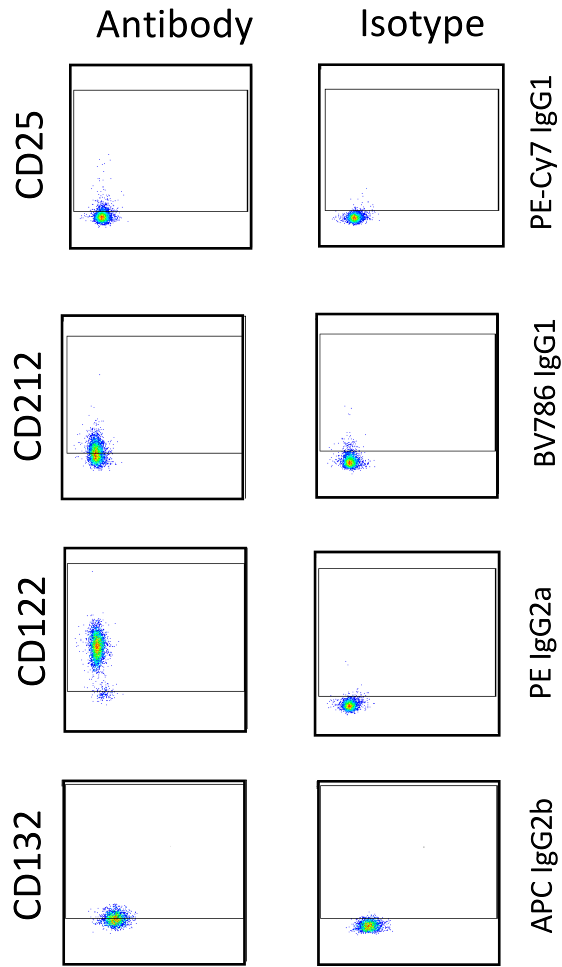
409. Walzer T, Bléry M, Chaix J, et al. Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46. *Proc Natl Acad Sci U S A*. 2007. doi:10.1073/pnas.0609692104

Appendix A



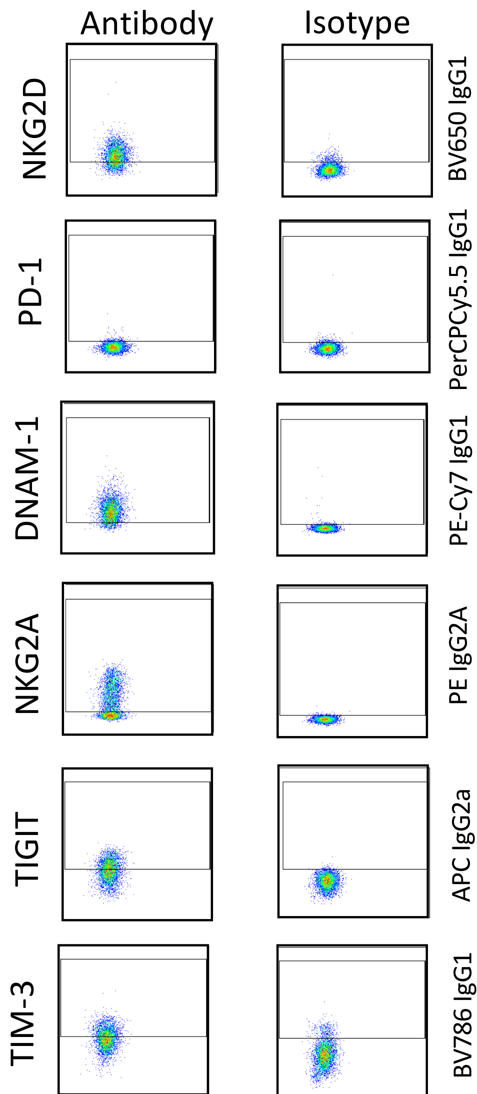
Gating for intracellular IFNγ staining was set based on paired unstimulated control samples.

Appendix B



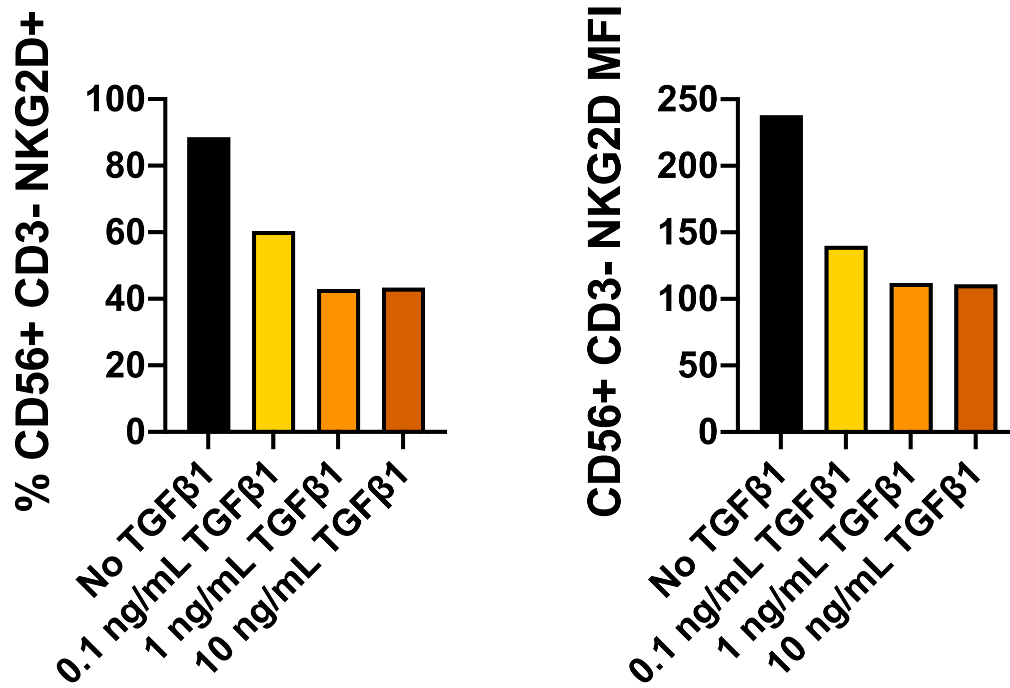
Matched antibody isotype controls were used to set the gates for quantification of NK cell cytokine receptors CD25, CD212, CD122, and CD132.

Appendix C



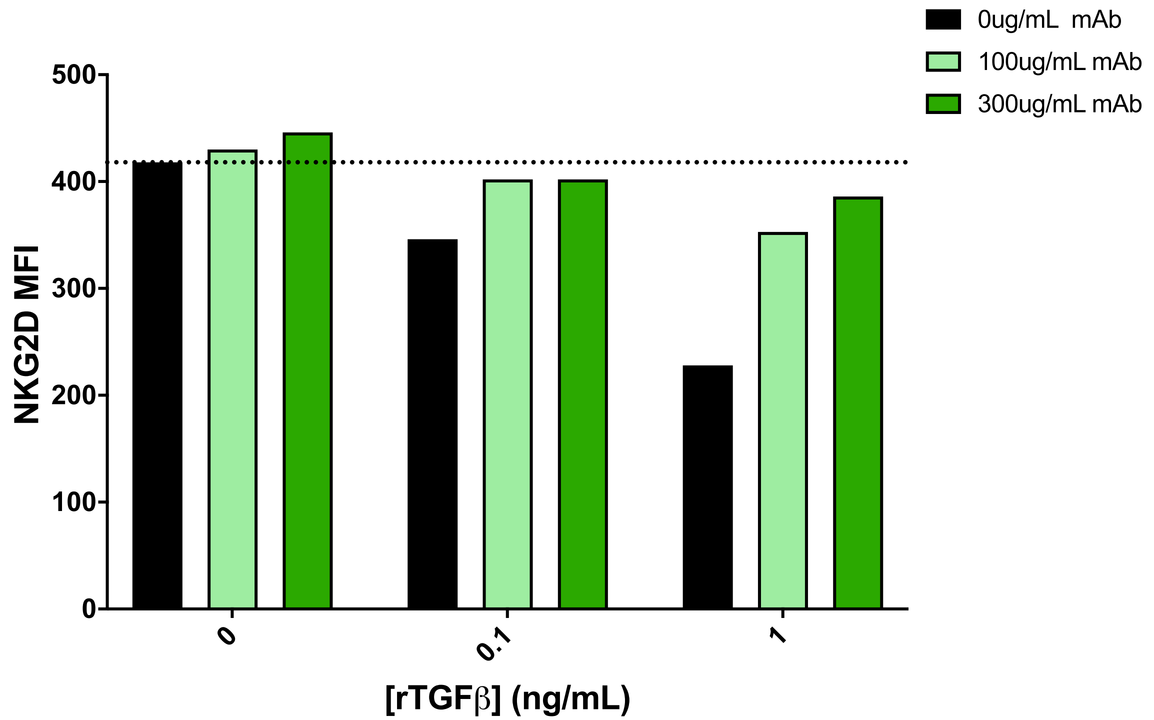
Matched antibody isotype controls were used to set the gates for quantification of NK cell activating/inhibitory receptors NKG2D, PD-1, DNAM-1, NKG2A, TIGIT, and TIM-3.

Appendix D



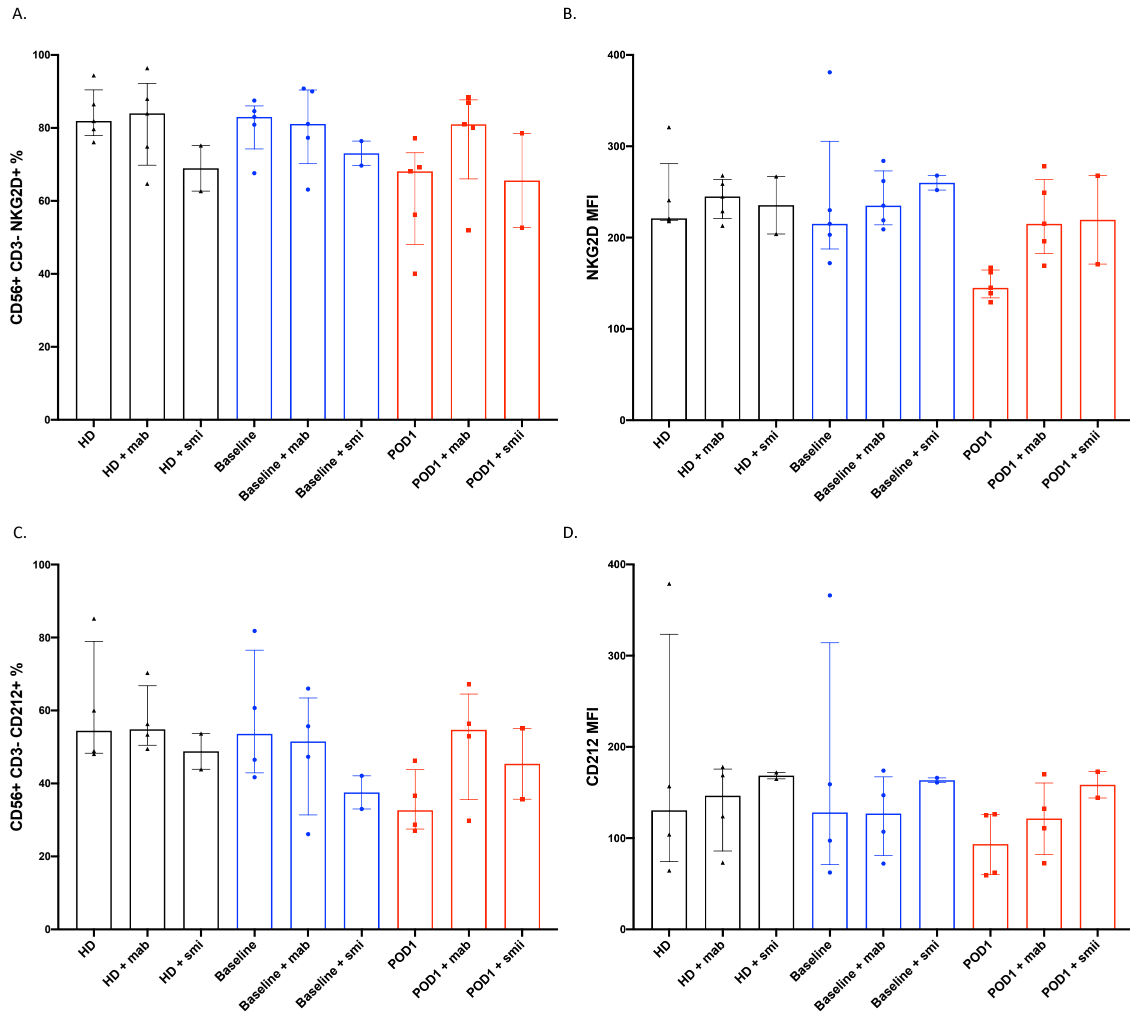
The concentration of rTGFβ1 to be used in combined plasma culture assays was determined by a titration assay. Isolated healthy $CD56^+$ cells were incubated overnight in the presence of 0.1 ng/mL, 1 ng/mL, or 10 ng/mL rTGFβ1 and stained for extracellular NKG2D expression at 24 hours (n=1; male, < 60 years old). A titratable effect was observed and both 1 ng/mL and 10 ng/mL rTGFβ1 achieved similar NKG2D suppression.

Appendix E



Combined plasma was incubated with an anti-TGFβ mouse monoclonal antibody prior to being added to healthy $CD56^{+}$ cells in culture (n=1; female, < 60 years old). The mAb was titrated (0 μg/mL, 100 μg/mL, and 300 μg/mL) against rTGFβ1 (0 ng/mL, 0.1 ng/mL, and 1 ng/mL). At 24 hours incubation cells were stained for extracellular NKG2D.

Appendix F



Healthy CD56^{+} cells incubated with either healthy, baseline, or POD1 combined plasma were incubated with a TGFβ mAb or TGFβ RI smi. (A/B) NKG2D and (C/D) CD212 expression was increased only in healthy cells incubated with POD1 plasma, suggesting that TGFβ is not present/not acting to impair NK cells in healthy or baseline plasma. Shown are the median values ± IQR.

Appendix G

Curriculum Vitae

Education

University of Ottawa 2015-Present
Combined MD/PhD Program
Ottawa, ON

PhD project title: *TGFβ Potentiates Postoperative Natural Killer Cell Paralysis Through Reduced Receptor Expression*
Supervisor: Dr. Rebecca Auer

University of Windsor 2011- 2015
Bachelor of Biological Sciences, Honours with Thesis
Windsor, ON
with Great Distinction

Assumption College Catholic High School 2007- 2011
International Baccalaureate Diploma
Windsor, ON
Ontario Secondary School Diploma

Academic Employment

University of Ottawa February 2020-Present
Research Assistant (Dr. Claudia Malic)
Ottawa, ON
Plastic Surgery

University of Ottawa September 2016- Present
Research Assistant (Dr. Kevin Cheung)
Ottawa, ON
Plastic Surgery

University of Windsor January 2014- May 2014
Research Assistant (Dr. Cavallo-Medved)
Windsor, ON
Pedagogical research assistant

University of Windsor September-December 2013/2014
Teaching Assistant (Dr. Cavallo-Medved)
Windsor, ON
Biology-141: Cellular Biology
Biology-140: Biological Diversity

University of Windsor — May-August 2013, 2014, 2015
NSERC USRA Undergraduate Researcher (Dr. Lisa Porter)
Windsor, ON
Cell and molecular biology research assistant

Academic Tutor — 2008- 2015
English, Reading, French, Math (Grades 1-9)
Windsor, ON

Awards/ Achievements

- **Syed Sattar Student PhD Award** — May 2020
  Outstanding contribution to research
  University of Ottawa
- **Dr. Sidney Medine Essex County Medical Student Scholarship,** — October 2019
  University of Ottawa
- **Queen Elizabeth II Scholarship in Science and Technology,** — September 2019
  University of Ottawa
- Graduate Excellence Scholarship, University of Ottawa — September 2019
- **First Prize- Short Abstracts Session** — May 2019
  Canadian Society of Surgical Oncology Annual Meeting
- 3rd Place Best Poster PhD category — May 2019
  BMI Seminar Day
- **Medical Student Research Award** — April 2019
  The Ottawa Hospital General Surgery Research Day
- Best Poster WCRG 4th Biennial Cancer Research Conference — November 2018
- Best Poster University of Ottawa Flow Cytometry & Virology Symposium — October 2018
- **Ontario Graduate Scholarship (OGS),** — May 2018
  University of Ottawa
- Graduate Excellence Scholarship, University of Ottawa — September 2018
- **Audrey J. Boyce MD/PhD Fellowship,** — January 2018
  University of Ottawa
- **CIHR Frederick Banting and Charles Best CGS**, — May 2017
  University of Ottawa
- Graduate Excellence Scholarship, University of Ottawa — September 2017
- **Dr. James J. Wiley CHEO Research Institute Endowment Fund**, — April 2017
  University of Ottawa
- Undergraduate Research Opportunity Program, University of Ottawa — October 2016
- **Surgical Exploration and Discovery (S.E.A.D.) Program participant**, — June 2016
  University of Ottawa
- **Outstanding Undergraduate Research Award**, University of Windsor — February 2016
- Medical Education Bursary Ottawa, University of Ottawa — November 2016
- MD/PhD Program Scholarship, University of Ottawa — September 2015
- **Holder-Franklin Honours Project Award 1st Place**, — March 2015
  University of Windsor
- **Corning Award for Best Overall Presentation** — March 2015

Ontario Biology Day, Carleton University

- **Freed-Orman Foundation Scholarship**, University of Windsor — December 2014
- Alexander, Felixa and Dr. Walter Kindiak Fund Scholarship, University of Windsor — March 2014
- **NSERC USRA** (Summer), University of Windsor — 2013, 2014, 2015
- UWSA Scholarship, University of Windsor — 2013, 2014, 2015
- Dean's Honour Roll, University of Windsor — 2011-2015
- President's Honour Roll, University of Windsor — 2011-2015
- Renewable Entrance Scholarship, University of Windsor — 2011-2015
- **The Bill Eansor Award**, University of Windsor — 2011-2015
- Outstanding Scholar, University of Windsor — 2011-2012
- Gold Medal University of Windsor Admissions Scholarship — April 2011
- Windsor Regional Science Fair Health Sciences Gold Medal — April 2011

Refereed Publications

Original Research:

1. **M Market**, M Bhatt, A Agarwal, and K Cheung. "*Pediatric Hand Injuries Requiring Repeat Reduction at a Tertiary Care Centre*" HAND **IF: 2.09**; Epub ahead of print (2019). doi:10.1177/1558944719850635

2. **M Market**, F Battaglia, J Shin, E Langlois. *"Using Self-Reported Measures of Confidence and Anxiety to Determine the Efficacy of the Surgical Exploration And Discovery (SEAD) Program"* Journal of Surgical Education **IF: 2.209**; Epub ahead of print (2020). doi:10.1016/j.jsurg.2020.03.010

3. **M Market,** G Tennakoon, J Ng, M Scaffidi, M A Kennedy, C Tanese de Souza, R C Auer. "*A Method of Assessment of Human Natural Killer Cell Phenotype and Function in Whole Blood*" Frontiers Immunology **IF: 5.085**; 11: 963 (2020). doi:10.3389/fimmu.2020.00963 (Biorxiv: https://doi.org/10.1101/2020.02.03.932640)

4. **M Market,** J Zhu-Pawlowsky, M Bhatt, and K Cheung. **"***Hands-On Workshops Improve Emergency Department Physicians' Self-Reported Understanding of Pediatric Hand Injuries"* (accepted to Pediatric Emergency Care **IF: 1.170**)

5. **M Market,** J Zhu-Pawlowsky, M Bhatt, and K Cheung. "*Assessing Hands-On Interventions for Quality Improvement of Pediatric Hand Fractures"* (in preparation for Pediatrics IF: 5.417)

6. **M Market**, G Tennakoon, J Ng, M Scaffidi, M A Kennedy, C Tanese de Souza, R C Auer. "*Postoperative Natural Killer Cell paralysis is associated with reduced receptor expression and is potentiated by soluble TGFβ*" (in preparation for Cancer Immunology Research IF: 8.619)

Review Articles:

1. **M Market**, K E Baxter, L Angka, M A Kennedy, R C Auer. "*The Potential for Cancer Immunotherapy in Targeting Surgery-Induced Natural Killer Cell Dysfunction*" Cancers **IF: 6.162**; 11(1); 2 (2018). doi:10.3390/cancers11010002

2. **M Market**† and L Angka†, A B Martel, D Bastin, O Olanubi, G Tennakoon D Boucher, J Ng, M Ardolino‡ and R C Auer‡. "*Flattening the COVID-19 Curve with NK based immunotherapies*" Frontiers in Immunology **IF: 5.085**; 11: 1512 (2020). doi:10.3389/fimmu.2020.01512

3. **M Market**, G Tennakoon, Michael A Kennedy, Rebecca C Auer. *"Natural Killer Cell Dysfunction and Mechanisms of Metastasis in the Postoperative Period: A Review"* (in preparation for submission to Journal of Innate Immunology)

Systematic Reviews:
1. D D Pereira, N S Cormier, **M Market**, S G Frank. "*Assessing the quality of reporting on quality improvement initiatives in breast reconstruction – a systematic review*" (in preparation for Plastics and Reconstructive Surgery IF: 3.946)

Commentaries:
1. L Angka†, **M Market**†, M Ardolino‡ and R C Auer‡. "*Is Innate Immunity the key to "flattening the curve"?*" Journal of Clinical Investigation **IF: 12.282;** Epub ahead of print (2020). doi:10.1172/JCI140530

Book Chapters:
1. **M Market**, K Scott, K Diemer, M Woghiren, A Hegazi, M Crawford, et al. "*Epigentics in Society"* Windsor: University of Windsor, 2015. **ISBN: 9780920233726**

> **M Market**, K Scott, L Chaker, R Hetzel. (2015). Dietary Epigenetics: Are you what your parents ate? Epigenetics in Society. 1(1): 67-124.
> **M Market** and S Zakaria. (2015). Cancer: An example of epigenetic peril and promise. Epigenetics in Society. 1(1): 49-66.
> **M Market**. (2015). Developmental epigenetics and reproductive medicine: Apparently always a parent. Epigenetics in Society. 1(1): 157-206

Non-refereed Contributions
Dissertations:
1. **M Market** (2015) *"Visualizing the Tuberin-Cyclin B1 Interaction using Live Cell Microscopy"* BSc Honours Thesis. University of Windsor.

2. **M Market** (2020) "*TGFβ Causes Postoperative Natural Killer Cell Paralysis Through mTOR Inhibition*" PhD Thesis, Microbiology and Immunology. University of Ottawa.

Protocols:
1. **M Market,** G Tennakoon, J Ng, M Scaffidi, M A Kennedy, C Tanese de Souza, R C Auer. "*A Comprehensive Assessment of Human Natural Killer Cell Phenotype and Function in Whole Blood*" Canadian Natural Killer Cell Consortium (CanNKC) https://www.cannkc.com/in-vivo-protocols

Oral and Poster Presentation

1. **M Market*,** G Tennakoon, L Angka, A Jeong, M Scaffidi, J Ng, C Tanese de Souza, M A Kennedy, R C Auer. "*TGFβ1 potentiates postoperative Natural Killer Cell paralysis through reduced receptor expression*" General Surgery Research Day (April 2020) Ottawa, ON. Oral Presentation.

2. **M Market*,** G Tennakoon, L Angka, A Jeong, M Scaffidi, J Ng, C Tanese de Souza, M A Kennedy, R C Auer. "*Postoperative Natural Killer cell paralysis is associated with reduced levels of IL-2 /IL-12 cytokine receptors and signaling*" Society of Surgical Oncology (SSO) Annual Meeting. (March 2020) Boston, MA, USA. Global Partner Poster Presentation, Canada representative (invited). * Postponed due to COVID-19 pandemic

3. **M Market***, G Tennakoon, A Jeong, M Scaffidi, J Ng, C Tanese de Souza, M A Kennedy, R C Auer. (2019) **"***Postoperative Factors Cause Dysfunctional NK Cell Phenotype*" Annual OHRI Research Day. Ottawa, ON. Oral Presentation.

4. **M Market***, G Tennakoon, A Jeong, M Scaffidi, J Ng, C Tanese de Souza, M A Kennedy, R C Auer. (2019) "*Downregulating Activating Receptors and Suppressed Signaling Characterize Postoperative Natural Killer Cell Phenotype*" BioCanRx Summit for Cancer Immunotherapy. Victoria, BC. Poster Presentation.

5. **M Market***, G Tennakoon, L Angka, J Ng, M Scaffidi, C Tanese de Souza, M A Kennedy, R C Auer. (2019) "*Characterizing the Dysfunctional Natural Killer Cell in the Postoperative Period*" Canadian Society of Surgical Oncology (CSSO) Annual Meeting. Toronto, ON. Brief Oral Presentation.

6. **M Market***, G Tennakoon, L Angka, J Ng, M Scaffidi, C Tanese de Souza, M Kennedy, R C Auer. (2019) "*Receptor Expression: The Key to Understanding Postoperative Natural Killer Cell Dysfunction?*" Biochemistry, Microbiology and Immunology Graduate Symposium. Ottawa, ON. Poster Presentation.

7. F Battaglia†, E Langlois†, **M Market**†*, J Shin†*. (2019) *"Determining Efficacy of the Surgical Exploration And Discovery (SEAD) Program in Reducing Anxiety and Increasing Confidence in Performing Procedural Skills".* Association for Surgical Education Conference. Chicago, IL, U.S.A. Poster Presentation.

8. **M Market***, G Tennakoon, L Angka, J Ng, M Scaffidi, C Tanese de Souza, M Kenney, R C Auer. (2019) "*Receptor Expression: The Key to Understanding Postoperative Natural Killer Cell Dysfunction?*" General Surgery Research Day (The Ottawa Hospital). Ottawa, ON. Oral Presentation.

9. **M Market*,** A Jeong, M Scaffidi, G Tennakoon, C Tanese de Souza, M Kennedy, R Auer. (2019) "*Characterizing Intracellular Mechanisms of Surgical Stress-Induced Natural Killer Cell Suppression*". WCRG 4th Biennial Cancer Research Conference. Windsor, ON. Poster Presentation.

10. **M Market*,** A Jeong, M Scaffidi, G Tennakoon, C Tanese de Souza, M Kennedy, R Auer. (2018) "*Characterizing Intracellular Mechanisms of Surgical Stress-Induced Natural Killer Cell Suppression*". Ottawa Hospital Research Institute (OHRI) Annual Research Day. Ottawa, ON. Poster Presentation.

11. **M Market*,** L Angka, A Jeong, M Scaffidi, C Tanese de Souza, M Kennedy, R Auer. (2018). *"Understanding Natural Killer Cell Dysfunction in the Post-Operative Period".* General Surgery Research Day (The Ottawa Hospital). Ottawa, ON. Oral Presentation.

12. **M Market*,** A Jeong, M Scaffidi, C Tanese de Souza, M Kennedy, R Auer. (2018) "*Natural Killer Cell Functional Suppression Following Surgical Stress*". Flow Cytometry Symposium. Ottawa, ON. Poster Presentation.

13. **M Market*,** A Jeong, M Scaffidi, C Tanese de Souza, M Kennedy, R Auer. "*Natural Killer* (2018) *Cell Functional Suppression Following Surgical Stress*". Biochemistry, Microbiology and Immunology Graduate Symposium. Ottawa, ON. Poster Presentation.

14. L Angka, A B Martel, A Jeong, M Sadiq, M Kilgour, **M Market***, L Baker, C Tanese de Souza, M Kennedy, R Auer. (2017). "*Periopertive Surgical Stress Suppresses Natural Killer Cell IFNγ Release in Colorectal Cancer Patients*". Ottawa Hospital Research Institute (OHRI) Annual Research Day. Ottawa, ON. Poster Presentation.

15. **M Market***, M Bhatt, and K Cheung (2017) "*Assessment of Pediatric Hand Injuries Requiring Repeat Reduction*" Canadian Society of Plastic Surgeons 71st Annual Meeting. Winnipeg, MB. Oral Presentation.

16. **M Market***, M Bhatt, and K Cheung (2017) "*Assessment of Pediatric Hand Fractures Requiring Repeat Reduction*" Undergraduate Research Opportunity Program Symposium. Ottawa, ON. Poster Presentation.

17. **M Market***, D Cavallo-Medved*, and H Hammoud (2015) *"Applying a virtual experience to complement first year undergraduate biology teaching labs"* Ninth Annual Conference on Teaching and Learning. Windsor, ON. Oral Presentation.

18. **M Market***, S Botsford, E Fidalgo da Silva, and LA Porter (2015) *"Visualizing the Tuberin-Cyclin B1 Interaction using Live Cell Microscopy"* University of Windsor Honours Undergraduate Colloquium. Windsor, ON Oral Presentation.

19. **M Market***, S Botsford, E Fidalgo da Silva, and LA Porter (2015) *"Visualizing the Tuberin-Cyclin B1 Interaction using Live Cell Microscopy"* UWill Discover Undergraduate Research Conference. Windsor, ON. Oral Presentation.

20. **M Market***, D Cavallo-Medved*, and H Hammoud (2015) *"Blended Learning Enhances Practical Experiences and Promotes Student Leadership"* UWill Discover Undergraduate Research Conference. Windsor, ON. Oral Presentation.

21. **M Market***, S Botsford, E Fidalgo da Silva, and LA Porter (2015) *"Visualizing the Tuberin-Cyclin B1 Interaction using Live Cell Microscopy"* Ontario Biology Day. Ottawa, ON. Oral Presentation.

22. **M Market***, J Dare-Shih*, S Botsford, E Fidlago da Silva, and LA Porter (2014) *"Bimolecular Fluorescence as a Tool to Monitor the Effects of Growth Factors on Cell Proliferation"* WCRG 2nd Biennial Cancer Research Conference. Windsor, ON. Poster Presentation.

23. **M Market***, D Cavallo-Medved*, and H Hammoud* (2014) *"Applying a virtual experience to complement first year undergraduate biology teaching labs"* Eighth Annual Conference on Teaching and Learning. Rochester, MI, USA. Poster Presentation.

Certifications

1**. Basic Life Support**, Heart & Stroke Foundation. April 2019.

2. **Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans Course on Research Ethics** (TCPS 2: CORE). July 2018.

3. **National Institutional Animal User Training (NIAUT) Program**. Canadian Council on Animal Care, University of Ottawa. July 2017.

4. **Health Canada Division 5 – Drugs for Clinical Trials Involving Human Subjects – Stage 1**. July 2017- July 2027.

5. **Canada GCP – Stage 1 Basic**. Ottawa Hospital Research Institute/ CITI Program. July 2017- July 2020.

6. **Radiation Safety Training**. Ottawa Hospital Research Institute. July 2017.

Leadership Roles/Memberships

2020-2021

- **Medical Students for Choice University of Ottawa chapter representative**
- **End FGM Canada Network member – Healthcare working group, Education and Research working group**
- Federation of Medical Women of Canada member

2019- 2020

- **End FGM Canada Network member – Healthcare working group, Education and Research working group**
- Women's Health Interest Group (WHIG) member
- Federation of Medical Women of Canada member

2018-2019

- **UOJM (University of Ottawa Journal of Medicine) Senior Publication Director**
- Federation of Medical Women of Canada member

2017-2018

- **Surgery Interest Group and S.E.A.D. Co-director**
- **UOJM (University of Ottawa Journal of Medicine) Junior Publication Director**
- Global Health Interest Group (GHIG) member

2016-2017

- **UOJM (University of Ottawa Journal of Medicine) reviewer**
- Surgery Interest Group member
- Anatomy Interest Group member
- **Global Health Interest Group (GHIG) member**
- French as a Second Language course: 2811-H Winter 2017

2015-2016
- **UOJM (University of Ottawa Journal of Medicine) reviewer**
- **Surgery Interest Group member**
- **Anatomy Interest Group member**
- French as a Second Language course: FLS 2511-X Fall 2016
- Ontario Medical Student Weekend (OMSW) 2015 University of Ottawa representative

2014-2015
- WCRG Lead Volunteer
- Windsor Cancer Research Group (WCRG) Conference Committee member
- Voted in as University of Windsor Students' Association (UWSA) Science Representative
- Golden Key Honorary Members Liaison
- Free the Children UWindsor Chapter member
- Mental Health Awareness Club member

2013-2014
- WCRG Lead Volunteer
- WCRG Conference Committee member
- UWSA Science Representative
- Golden Key Community Co-Chair
- Free the Children UWindsor Chapter member
- Mental Health Awareness Club member

2012-2013
- UWSA Lance Oversight Board
- Member of the Lance Oversight Board hiring committee for Editor-In-Chief of The Lance
- Golden Key Honorary Members Liaison
- Free the Children UWindsor Chapter member
- Mental Health Awareness Club member

2011-2012
- UWSA First Year Representative
- UWindsor Lancer Cheerleader
- Residence Life Food Services Committee member and UWSA Representative
- Free the Children UWindsor Chapter member
- VP Administration Interfaculty Society
- Bachelor of Arts and Science First Year Representative