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IMPROVING NK CELL THERAPY FOR OSTEOSARCOMA

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IMPROVING NK CELL THERAPY FOR OSTEOSARCOMA

A

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Presented to the Faculty of

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for the Degree of

DOCTOR OF PHILOSOPHY

by

Jennifer Ann Foltz, M.S.

Houston, Texas

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Improving NK Cell Therapy for Osteosarcoma

Jennifer Ann Foltz, M.S.

Advisory Professor: Dean Lee, M.D. Ph.D.

Osteosarcoma (OS) is the most common primary bone tumor. Despite new treatment options, 5-year survival for metastatic OS has remained at only 30% for the last 30 years. Adoptive transfer of Natural Killer (NK) cells holds promise for a new, non-toxic therapy for OS. NK cells are part of the innate immune system and readily kill metastatic and chemotherapy-resistant OS *in vitro* and in murine models. However, there is little data regarding their efficacy in animal models with an intact immune system. In addition, the OS tumor microenvironment is highly suppressive, producing TGF β which impedes NK cell killing of solid tumors. We set out to overcome these hurdles by characterizing NK cells in the canine model, which spontaneously develops OS, has a complete tumor microenvironment, an intact immune system, and is an established animal model with proven translatability to human OS. However, little is known about canine NK, preventing the testing of NK cell therapy in canine OS. We also set out to improve NK cell therapy by generating NK cells resistant to TGF β suppression. To this end, we generated a novel anti-canine NKp46 antibody and characterized canine NK cells as CD3⁻/NKp46⁺. Canine NK cells have a median 3-week expansion of 20,000-fold on K562 feeder cells expressing membrane-bound IL-21. Canine NK cells efficiently kill OS, secrete IFN γ and TNF α , and express genes for NK cell receptors. Using a novel non-genetic approach, we generated human NK with reduced sensitivity to TGF β - Resistant NK (ReNK). ReNK retain high cytolytic activity and produce remarkably more IL-6, IFN γ ,

and TNF α plus and minus TGF β treatment compared to Standard NK cells. We characterized the TGF β pathway in ReNK and found a near complete loss in SMAD3 protein and decreased TGFBR3 expression. ReNK also have enhanced STAT3 activation. In summary, we characterized canine NK cells, establishing the necessary tools to test NK cell therapy in canines to inform the use of NK cells in both human and canine OS. We also generated NK cells with enhanced anti-tumor activity that represent a promising therapeutic option for tumors with high TGF β production such as OS and brain tumors.

Table of Contents

Approval Page	i
Title Page.....	ii
Acknowledgements.....	iii
Improving NK Cell Therapy for Osteosarcoma	v
List of Illustrations	x
List of Tables	xiii
List of Abbreviations.....	xiv
Chapter 1: Background	1
1.1 Osteosarcoma	1
1.2 NK Cell Background	2
1.3 NK Cell Receptors	4
1.4 Clinical Evidence for NK Cell Impact on Cancer Outcomes.....	7
1.4.1 Adoptive Transfer of NK Cells	9
1.4.2 Expanded NK cells.	10
1.5 NK cells in Osteosarcoma	11
1.6 Tumor Avoidance of NK Cell Surveillance	11
1.7 Dog Models of Cancer	13
1.7.1 Canine Osteosarcoma as a Pre-clinical Model for Human Osteosarcoma	15
1.7.2 Canine NK Cells	16
Specific Aims.....	17
Specific Aim 1: To develop and validate a standard approach to characterizing canine NK cell phenotype and function.	17
Specific Aim 2: To improve NK cell expansion from healthy and OS-bearing canines for use in comparative oncology models.	18
Specific Aim 3: To assess the ability of NK cells expanded in TGF β to overcome TGF β mediated suppression of NK cell Anti-tumor Activity.	18
Chapter 2: Identification, Characterization, and Expansion of Canine NK Cells	19
2.1 Background	19
2.2 Results.....	22
2.2.1 Construction of an Anti-Canine NKp46 Antibody	22

2.2.2 NKp46 Expression on Canine Peripheral Blood Mononuclear Cells	22
2.2.3 Ex Vivo Expansion of Canine CD3 ⁻ /NKp46 ⁺ Cells	24
2.2.4 Function of CD3 ⁻ /NKp46 ⁺ Cells.....	30
2.2.5 NKp46 Identifies Distinct Subsets of NK Cells.....	32
2.2.6 NK Cells in Canine Osteosarcoma	40
2.2.7 Cross-Reacting Antibodies	41
2.3 Discussion	44
Chapter 3: Education of NK cells to be TGFβ Resistant	49
3.1 Background	49
3.2 Results.....	53
3.2.1 ReNK cells have similar proliferation compared to Standard NK cells.	53
3.2.2 ReNK cells retain high cytolytic capacity.	54
3.2.3 ReNK cells have increased cytokine secretion.....	62
3.2.4 ReNK cells have altered NK cell Phenotype.....	71
3.2.5 ReNK cells have changes in TGFβ Signaling.....	76
3.2.6 ReNK cells have increased STAT3 response.....	79
3.2.7 Persistence of ReNK Phenotype & Function.	80
3.3 Discussion	90
Chapter 5: General Discussion and Future Directions	99
5.1 Dissertation Summary	99
5.2 Future Directions	100
5.2.1 Pre-clinical Models for NK Cell Therapy	100
5.2.2 Inhibitory Receptors in Canine NK Cells.....	102
5.2.3 Canine NK Cells: Implications in Infectious Disease & Autoimmunity	104
5.2.4 Antibody Generation.....	105
5.2.5 TGFβ Resistance	106
5.2.6 Development of TGFβ Resistant T-cells.....	108
5.2.7 ReNK cells for Infectious Diseases	109
5.2.8 Conclusion.....	110
Chapter 6: Materials & Methods	110
6.1 Peripheral Blood Mononuclear Cell Isolation	111

6.2 Cell Culture	112
6.3 NK Cell Expansion	113
6.4 Sequencing	114
6.5 NKp46 Antibody Production	115
6.6 Flow Cytometry	116
6.7 Cell Sorting	117
6.8 Luminex	117
6.9 Cytotoxicity Assays	117
6.10 Intracellular Functional Flow Cytometry	118
6.11 Cytometric Bead Array (CBA)	118
6.12 RT-PCR/qPCR	119
6.13 Mass Cytometry	121
6.13.1 Clustering Analysis	122
6.14 Western Blotting	122
6.15 Statistical Analysis	122
Vita	180

List of Illustrations

Figure 1. Screening of a Novel Anti-Canine NKp46 Antibody.....	22
Figure 2. Expression of NKp46 on canine PBMC using a novel anti-canine NKp46 antibody.....	23
Figure 3. Expansion of CD3 ⁻ /NKp46 ⁺ cells on K562 mbIL-21 feeder cells with human versus canine IL-2.....	25
Figure 4. Phenotype of Canine CD3 ⁻ /NKp46 ⁺ cells Expanded on K562mbIL-21 feeder cells.....	26
Figure 5. Gene Expression of CD3 ⁻ /NKp46 ⁺ cells.....	27
Figure 6. Expansion of Canine CD3 ⁻ /NKp46 ⁺ cells on K562mbIL-21 feeder cells...	28
Figure 7. CD3 Depletion Improves NK Cell Purity.....	29
Figure 8. Canine CD3 ⁻ /NKp46 ⁺ cells kill without Prior Sensitization.....	30
Figure 9. Canine NK Anti-Tumor Cytotoxicity and Cytokine Secretion.....	31
Figure 10. Phenotype of CD3 ⁻ /CD21 ⁻ /CD14 ⁻ /NKp46 ⁻ (Null) cells in K562mbIL-21 Expanded Cells.....	34
Figure 11. Null Cells can be induced to express NKp46, and Null cells express other genes for other NK cell Receptors.....	36
Figure 12. Function of Null Cells.....	37
Figure 13. Phenotype of NKp46 ⁻ Human NK Cells.....	39

Figure 14. NK cell numbers, expansion, and function in canine osteosarcoma patients.....	40
Figure 15. Cross-Reacting DNAM-1 Expression on Canine CD3 ⁻ /NKp46 ⁺ cells.....	41
Figure 16. Proliferation of ReNK cells.....	54
Figure 17. ReNK cells have increased degranulation.....	56
Figure 18. ReNK cells have increased killing of DAOY.....	57
Figure 19. ReNK cells have variable cytotoxicity.....	59
Figure 20. Effect of Overnight TGFβ Treatment on Standard and ReNK cell Receptor Expression.....	61
Figure 21. Gene expression of TGFβ Antagonistic Cytokines.....	62
Figure 22. IL-6 Production of ReNK cells.....	63
Figure 23. ReNK cells have increased IFNγ production.....	66
Figure 24. ReNK cells have increased TNFα+ production.....	67
Figure 25. ReNK cells have increased IFNγ secretion.....	69
Figure 26. ReNK cells have increased TNFα secretion.....	70
Figure 27. ReNK cells have altered cell surface phenotype.....	73
Figure 28. Intracellular Protein Expression.....	74
Figure 29. Transcription factor profile of ReNK cells.....	75
Figure 30. ReNK cells have altered TGFβ Pathway.....	78,79

Figure 31. ReNK cells have increased STAT3 response.....	80
Figure 32. Cytokine Secretion of ReNK cells during and post-expansion.....	82
Figure 33. Cytotoxicity of ReNK cells during Expansion and post-Expansion.....	84
Figure 34. Cytotoxicity of ReNK cells after 21 and 33 days resting.....	85
Figure 35. Percent of ReNK and Standard NK expressing different receptors during, and post-expansion.....	88
Figure 36. Expression level of ReNK and Standard NK Receptors during and Post- expansion.....	89

List of Tables

Table 1. Major Human NK cell receptors for which corresponding ligands are known.....	4
Table 2. Screening of Mouse and Human Antibodies Recognizing NK Cell Proteins on Canine NK Cells.....	43
Appendix A. Screening of NKp46 Hybridomas with ELISA against L-cells expressing Canine NKp46.....	124
Appendix B. TGF β Pathway Genes Assessed on TaqMan Array.....	125

List of Abbreviations

ACVR1: Activin A Receptor Type 1

ACVR1B: Activin A Receptor Type 1B

ACVR1C: Activin A Receptor Type 1C

ACVR2A: Activin A Receptor Type 2A

ACVR2B: Activin A Receptor Type 2B

ADCC: Antibody-Dependent Cellular Cytotoxicity

AMH: Anti-Mullerian Hormone

AMHR2: Anti-Mullerian Hormone Type 2 Receptor

AML: Acute Myeloid Leukemia

BMP2: Bone Morphogenetic Protein 2

BMP3: Bone Morphogenetic Protein 3

BMP4: Bone Morphogenetic Protein 4

BMP5: Bone Morphogenetic Protein 5

BMP6: Bone Morphogenetic Protein 6

BMP7: Bone Morphogenetic Protein 7

BMP10: Bone Morphogenetic Protein 10

BMP15: Bone Morphogenetic Protein 15

BMPER: Bone Morphogenetic Protein-Binding Endothelial Regulator

BMPR1B: Bone Morphogenetic Protein Receptor, Type 1B

BMPR2: Bone Morphogenetic Protein Receptor Type 2

CDH1: Cadherin-1

CHRD: Chordin

CR: Complete Remission

CREBBP: cAMP Responsive Element Binding Protein-Binding Protein

CTAC: Canine Thyroid Adenocarcinoma

CUL1: Cullin 1

DCN: Decorin

E2F4: E2F Transcription Factor 4

E2F5: E2F Transcription Factor 5

EP300: E1A Binding Protein p300

FMOD: Fibromodulin

FNTA: Farnesyltransferase, CAAX box, alpha

FST: Follistatin

GDF2: Growth Differentiation Factor 2

GDF3: Growth Differentiation Factor 3

GDF5: Growth Differentiation Factor 5

GDF7: Growth Differentiation Factor 7

GDF9: Growth Differentiation Factor 9

GDF10: Growth Differentiation Factor 10

GDF11: Growth Differentiation Factor 11

GDF15: Growth Differentiation Factor 15

HLA: Human Leukocyte Antigen

HIPK2: Homeodomain Interacting Protein Kinase

HSCT: Hematopoietic Stem Cell Transplant

IDO: Indoleamine 2,3-dioxygenase

IFN γ : Interferon-gamma

IL-2: Interleukin-2

IL-6: Interleukin-6

IL-15: Interleukin-15

IL17F: Interleukin-17 F

IL-21: Interleukin-21

INHA: Inhibin Alpha Subunit

INHBA: Inhibin Beta A Subunit

INHBB: Inhibin Beta B Subunit

INHBC: Inhibin Beta C Subunit

INHBE: Inhibin Beta E Subunit

ITAM: Immunoreceptor Tyrosine-Based Activating Motif

ITIM: Immunoreceptor Tyrosine-Based Inhibitory Motif

KIR: Killer Immunoglobulin Receptor

LEFTY1: Left Right Determination Factor 1

LEFTY2: Left Right Determination Factor 2

L-MTP-PE: Liposomal-Muramyl-Triptide

LTBP1: Latent Transforming Growth Factor Beta Binding Protein 1

LTBP2: Latent Transforming Growth Factor Beta Binding Protein 2

LTBP3: Latent Transforming Growth Factor Beta Binding Protein 3

LTBP4: Latent Transforming Growth Factor Beta Binding Protein 4

MAP3K7/TAK1: Mitogen-Activated Protein Kinase Kinase Kinase 7, Tumor Growth
Factor-beta Activated Kinase 1

MAP3K7IP1/TAB1: Tumor Growth Factor-beta Activated Kinase 1 (MAP3K7)
Binding Protein 1

MAPK1: Mitogen-Activated Protein Kinase 1

MAPK3: Mitogen-Activated Protein Kinase 3

Mb: Membrane-Bound

MSTN: Myostatin

NCR: Natural Cytotoxicity Receptor

NK cells: Natural Killer Cells

NOG: Noggin

OS: Osteosarcoma

PBMC: Peripheral Blood Mononuclear Cells

PPP2CA: Protein Phosphatase 2 Catalytic Subunit Alpha

PPP2CB: Protein Phosphatase 2 Catalytic Subunit Beta

R/+ : ReNK cells, rested overnight in TGF β

R/- : ReNK cells, rested overnight in the absence of TGF β

RBL1: Retinoblastoma-like 1

RBL2: Retinoblastoma-like 2

RBX1: Ring-Box 1

rcIL-2: Recombinant Canine Interleukin-2

ReNK: Resistant Expanded Natural Killer Cells

rHIL-2: Recombinant Human Interleukin-2

RHOA: Ras Homolog Family Member A

ROCK1: Rho Associated Coiled-Coil Containing Protein Kinase 1

ROCK2: Rhoa Associated Coiled-Coil Containing Protein Kinase 2

S/+ : Standard NK cells, rested overnight with TGF β

S/- : Standard NK cells, rested overnight in the absence of TGF β

SKP1: S-Phase Kinase Associated Protein 1

SMURF1: SMAD Specific E3 Ubiquitin Protein Ligase 1

SMURF2: SMAD Specific E3 Ubiquitin Protein Ligase 2

STUB1: Stress Induced Phosphoprotein 1 Homology & U-box Containing Protein 1

TCR: T-cell Receptor

TFDP1: Transcription-Factor Dp-1

TGFA: Transforming Growth Factor-Alpha

TGF β : Transforming Growth Factor-Beta

TGFBR: Transforming Growth Factor Beta Receptor

TGFBRAP1: Transforming Growth Factor Beta Receptor Associated Protein 1

TNF(α): Tumor Necrosis Factor-alpha

TRAIL: Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand

TSC22D1: TSC22 Domain Family Member 1

ZFYVE9: Zinc Finger FYVE-type Containing 9

Chapter 1: Background

1.1 Osteosarcoma

Osteosarcoma (OS) is the most common primary bone cancer in children and adolescents, with a peak incidence just after the pubertal growth spurt. The standard of care is surgical resection and MAP chemotherapy (cisplatin, doxorubicin, methotrexate). Metastatic OS has a less than 30% 5-year survival rate. In addition, OS survival has not improved in over 30 years. Survivors of OS face life-long handicaps and limitations due to limb-salvage surgeries and amputations as well as long-term toxicities of chemotherapy (1-3).

Development of new therapies for OS has been hindered by the genetic heterogeneity and interpatient variability of OS. This has confounded the discovery of OS tumor-driving genes and the development of targeted therapies. Retinoblastoma-associated protein 1 (RB1) and p53 mutations, such as in Retinoblastoma and Li-Fraumeni syndrome, are associated with an increased frequency of OS development. Mutations are also frequently found within the mTOR and NOTCH pathways, and Erb family. Recent studies have also identified dysregulations in microRNA (miRNA) and long non-coding RNA expression, particularly in those that regulate cell cycle progression (3, 4).

Due to the lack of effective treatments for metastatic OS, there have been several new clinical trials testing new therapies. Recently, the European and American Osteosarcoma Study Group (EURAMOS) trial closed. In the EURAMOS trial, IFN $\alpha\beta$ or ifosfamide and etoposide were added to the standard MAP

chemotherapy regimen. However, this new treatment regime did not improve outcomes for OS, and instead increased treatment toxicity (5, 6). Another trial used metronomic chemotherapy, which involves the frequent administration of low-dose chemotherapy. Metronomic chemotherapy was chosen with the goal of inhibiting angiogenesis and stimulating an immune response. Unfortunately, this trial also failed to improve survival for both human OS patients (7, 8). A new approach towards OS treatment involves the use of natural killer (NK) cells, cells of the innate immune system that are highly effective at killing malignant cells, including chemotherapy resistant OS; yet, there is very little data on their clinical efficacy (9).

1.2 NK Cell Background

This chapter is based upon Foltz, J.A., J. S. Miller, D. A. Lee. In press. NK Cell Based Immunotherapy. In Immunotherapy in Translational Cancer Research. John Wiley & Sons, Inc., Hoboken, NJ, USA. (ISBN: 9781118123225) with permission from Claire Bonnett, Publisher at John Wiley & Sons.

NK cells were originally described in the 1970's as null lymphocytes with natural cytotoxicity against mouse tumor cell lines distinct from T-cell killing, which is antigen-specific and Class I major histocompatibility complex (Class I MHC) dependent (10-14). Decades later, we now understand that NK cells differ from T-cells by their expression of a repertoire of germline-encoded activating and inhibitory receptors that bind to conserved cell-surface protein ligands (**Table 1**), in contrast with the recombined receptors of T cells that bind to peptide antigens. The activating receptors identify elements of stress or danger, and are balanced by inhibitory receptors that recognize self. Through the balance of these activating and inhibitory

receptors, NK cells determine which cells to kill, such that the engagement of more activating than inhibitory receptors results in signal activation and subsequent effector responses- cytotoxicity and cytokine release.

Unlike T-cells in which recognition of peptide and Class I MHC leads to activation, NK cells are generally inhibited by recognition of Class I MHC. Thus, activation is more likely to occur against cells that lack MHC expression, termed “missing self” (14, 15). NK cells are highly important in the surveillance and destruction of virus and parasite-infected cells and tumor cells (16) because they over-express activating ligands and downregulate MHC.

Table 1. Major Human NK cell receptors for which corresponding ligands are known

<u>Receptor</u>	<u>Ligand</u>
<u>Activating Receptors</u>	
FcγRIII (CD16)	IgG1/IgG3 antibody
NKp30 (CD337)	BAG6, B7-H6
NKp44 (CD336)	PCNA
NKp46 (CD335)	viral hemagglutinin
NKp80	AICL
NKG2D (CD314)	MICA, MICB, ULBP1-6
NKG2C/E (CD159c, e)	HLA-E
DNAM-1 (CD226)	PVR, Nectin-1
2B4 (CD244)	SLAMF2 (CD48)

Inhibitory Receptors

KIR2DL1 (CD158a)	HLA-C group 2
KIR2DL2/DL3 (CD158b, b2)	HLA-C group 1
KIR3DL1/DL2 (CD158e, k)	HLA-Bw4 and some HLA-A
NKG2A (CD159a)	HLA-E

1.3 NK Cell Receptors

Phenotypically, NK cells in humans are defined by their lack of CD3 (e.g., lacking a T-cell receptor), and expression of CD56, CD16, or NKp46. NKp46 is part of a distinct family of activating receptors expressed by NK cells - the natural cytotoxicity receptors (NCRs) - which are implicated in NK cell mediated killing of tumors and also include NKp30 and NKp44. Higher expression levels of the NCRs are correlated with increased tumor killing (17, 18) and is often reduced in cancer patients compared to healthy individuals (19-22), thus predicting survival from some cancers (23). NKp46 is unique among the NK cell receptors because it is nearly exclusively expressed on NK cells (24-26). On the contrary, NKp44 expression is

limited to interleukin-2 (IL-2) activated NK cells and NKp30 is expressed only on a subset of NK cells. Despite the importance of the NCRs in NK cell killing of tumors, the majority of their ligands on tumors remain unknown (15, 18).

The primary activating receptors on NK cells that mediate recognition of cancer cells include NKG2D, DNAM-1, 2B4, NKp80, and CD16 (15). These activating receptors can act in synergy when the ligands for more than one activating receptor are present on the target cells (27).

NKG2D is a homodimeric lectin receptor that recognizes a variety of stress-induced ligands present on cancer cells. The ligands for NKG2D include MHC class I chain-related protein (MIC) A and B and UL16 binding protein 1–6 (28), which are expressed on virtually all tumor types including brain tumors, carcinomas, sarcomas, neuroectodermal cancers, lymphomas, and leukemias (29-35). 2B4 recognizes stress ligands of the SLAM family, and DNAM-1 recognizes the viral receptors PVR and Nectin (15).

CD16 is the low-affinity immunoglobulin receptor (Fc γ RIII) responsible for mediating antibody dependent cellular cytotoxicity (ADCC) in NK cells. ADCC augments NK cell killing through the binding of IgG antibodies to CD16 and a tumor cell ligand (15, 36). Two examples of exploiting ADCC for increasing NK cell killing are rituximab (anti-CD20) and dinutuximab (anti-GD2) antibodies. NK cell-dependent ADCC is a key mechanism of action for both rituximab and dinutuximab, and pre-clinical data support their combination with NK cell infusions. Rituximab is a chimeric monoclonal antibody (mAb) against CD20 expressed on B-cells. NK cell mediated ADCC is a major mechanism of action for rituximab therapeutic efficacy (37-39), and

loss of ADCC through decreased CD16 affinity for IgG is a recognized mechanism of loss of clinical benefit (40). GD2 is a disialoganglioside that is expressed on many solid tumor types. *In vitro* and *in vivo* mouse studies with both primary NK cells and activated, expanded NK cells demonstrate that the addition of anti-GD2 mAb improves NK cell cytotoxicity against neuroblastoma and survival of mice with neuroblastoma xenografts (32, 41). Efficacy of anti-GD2 mAb in clinical trials is associated with NK cell function (42, 43).

NK cells possess several inhibitory receptors, which are used to distinguish between healthy cells and malignant or infected cells. The inhibitory receptors are largely made up of a family of receptors called killer immunoglobulin receptors (KIR) and NKG2A/CD94 receptor. However, some KIRs and the related NKG2C receptor are activating. The ligands for inhibitory KIR are classical Class I MHC (mostly human leukocyte antigen (HLA)-B, C) and are grouped into specific receptor-ligand interaction groups (KIR2DL1 for HLA-C1, KIR2DL2/3 for HLA-C2, and KIR3DL1 for Bw4). While KIR2DS1 binds HLA-C2, resolving the ligands for other activating KIR ligands continues to evolve. The ligand for NKG2A and NKG2C is the non-classical HLA-E. Inhibitory receptor engagement results in an inhibitory signal indicating that the target cell is healthy self. Many cancers downregulate HLA expression making them more susceptible to NK-mediated killing (44). Through HLA typing, adoptive cellular immunotherapy and hematopoietic stem cell transplantation (HSCT) can exploit the presence or absence of specific KIR-ligand groups - mimicking missing-self - to evade inhibition and improve outcome. Termed KIR mismatch, this requires identification of a donor who has an inhibitory KIR ligand (HLA group) that is not

expressed in the recipient. This results in the donor's NK cells having less potential for inhibition in the recipient, and therefore increased killing of the recipient's cancer cells (45, 46).

The 15 KIR genes and 2 KIR pseudogenes can be inherited in many possible variations of gene content, and these are broadly grouped into KIR genotypes A and B, where type B individuals possess a greater number of activating KIRs. KIR type B is significantly associated with better clinical outcomes that will be discussed in more detail later in this chapter (15, 27, 36).

NK cells have several mechanisms by which they can kill tumor cells. These include granzyme B and perforin, Fas, and tumor necrosis factor-related apoptosis inducing ligand-mediated killing (TRAIL). In addition, NK cells have indirect anti-tumor effects by stimulating other cells of the immune system through the release of pro-inflammatory cytokines, most notably interferon gamma (IFN γ) and tumor necrosis factor-alpha (TNF α) (16). These methods of killing have varying importance dependent on the tumor cell, with some tumors being resistant to one or more methods of killing (16, 47-51). Immune-modulating drugs such as bortezomib and lenalidomide can overcome this resistance and sensitize cancer cells to NK cell killing in pre-clinical models, and are currently being tested in clinical trials.

1.4 Clinical Evidence for NK Cell Impact on Cancer Outcomes

Low NK cell numbers and deficient NK function are correlated with cancer risk (52, 53) and can be highly predictive of treatment outcome (53-56). In patients with acute myeloid leukemia (AML), NK cells have reduced NKp30 and NKp46 expression compared to healthy individuals, and this reduced expression correlates

with decreased cytotoxicity against leukemia cells. Expression of NKp46 and NKp30 is significantly increased in patients with AML upon achieving complete remission (CR) as compared to expression at the time of diagnosis, and increased expression correlated with overall survival (19, 20, 23). In addition to phenotypic differences, NK cells from leukemia patients are deficient in their cytokine production, and cytokine production and cytolytic activity against autologous leukemic blasts are positively associated with relapse-free survival and CR, respectively. NK cell cytotoxicity against autologous leukemia cells above 12% significantly correlated with a 100% disease-free survival of greater than 8 years. Thus, NK cells play a significant role in the host anti-leukemic response (57, 58).

Similarly, in solid tumors, NK cell function and phenotype are implicated in disease prognosis and progression. In osteosarcoma, increased lymphocyte recovery following chemotherapy is significantly associated with improved 5-year overall survival (24). In both breast and prostate cancer, the number of activated NK cells in peripheral blood correlated with improved survival (56, 59). In patients with melanoma, NK cells have lower expression of NK activating receptors and reduced NK functionality (cytotoxicity, cytokine secretion, and proliferation) compared to healthy controls. This dysfunction may be an immune-escape mechanism induced by cell-to-cell contact, as co-culture of NK cells with melanoma results in decreased activating receptor expression and decreased cytotoxicity (21, 22, 60). These dysfunctional changes are also seen in lung cancer, and can be reversed *in vitro* with TIM-3 blockade (61, 62).

1.4.1 Adoptive Transfer of NK Cells

The combination of *in vitro* findings of cancer sensitivity to NK cell lysis, *in vivo* evidence of reduced NK cell number and function in patients with cancer, and high sensitivity of NK cells to chemotherapy and radiation that further decreases NK cell numbers in these patients, results in NK cell adoptive transfer having the potential to restore NK cell function and improve cancer treatment. To this end, several methods have been developed for producing an NK cell product for infusion. A lymphodepleting pre-conditioning regimen is typically included with the goal of making physiological “space” in the recipient, which in turn promotes NK cell expansion *in vivo* through increased availability of the homeostatic cytokine, IL-15 (63). After the pre-conditioning regimen and NK cell infusion, IL-2 (aldesleukin) (or more recently, IL-15) administration can be given to further promote NK cell persistence and expansion *in vivo*.

The majority of completed studies have used primary NK cells derived from peripheral blood apheresis of a normal donor followed by depletion of T cells (CD3 depletion) to prevent GvHD, and in some cases B-cells (CD19 depletion) to prevent passenger lymphocyte syndrome or reactivation of Epstein Barr virus (63, 64). Other studies seeking to infuse a NK cell product of increased purity have utilized CD3 depletion followed by CD56-positive selection to isolate NK cells. However, this method reported reduced NK cell yield, which may be an issue especially in adult patients. After selection or depletion, the NK cell product can be administered immediately, activated with cytokines, or expanded *ex vivo* for generating greater NK cell doses.

1.4.2 Expanded NK cells.

Since collection of primary NK cells by steady-state apheresis is not able to achieve large NK cell doses, *ex vivo* expansion of NK cells has been explored to enable higher NK cell numbers per infusion and for repeated NK infusions. Expanded NK cells are also more activated than primary NK cells against tumor targets (65, 66). In addition, NK cells in patients with cancer are often functionally impaired and infusions of autologous NK cells in early trials were safe but showed no clear benefit. *Ex vivo* activated and expanded NK cells may correct that dysfunction.

There have been several methods developed for *ex vivo* expansion of NK cells. Approaches have generally centered on either cytokine-induced proliferation or feeder cell lines engineered to stimulate NK cell growth. For cytokine-induced proliferation, IL-2 in combination with IL-12, IL-15, or IL-18 are usually used. Cytokines have been effective at generating memory-like NK cells with enhanced function; however, proliferation with cytokines alone is limited by a low yield of NK cells (67).

On the other hand, feeder cell lines such as the K562 leukemia cell line, which is highly sensitive to NK cell killing, have been much more successful at generating large-fold expansion of NK cells (65, 66, 68). This K562 cell line was modified to express 41BBL and either membrane-bound IL-15 (K562 mbIL-15) or IL-21 (K562 mbIL-21). The Lee Lab compared NK expansion on K562 mbIL-15 to K562 mbIL-21 and found that K562 mbIL-21 promotes increased proliferation (median: 31, 747-fold vs. 325-fold in 3 weeks) and increased secretion of IFN γ and TNF α

compared to mbIL-15, while maintaining potent anti-tumor cytotoxicity (66). EBV-transformed cells have also been used as feeder cells for stimulating NK cell proliferation (68, 69). An EBV-transformed cell line with IL-2 plus IL-21 generated NK expansion of 10^{11} in 6 weeks; however, the authors noted that the expanded NK cells lost their anti-tumor cytotoxicity after injection into mice (70). Finally, NK cells have been expanded using irradiated PBMCs as a feeder cell with the addition of IL-2 and OKT3 (71-73).

1.5 NK cells in Osteosarcoma

Despite the clinical trials underway for NK cells in other malignancies, to date no NK cell clinical trial specifically focused on OS has opened. Human OS patients have decreased numbers of circulating NK cells, suggesting that NK cell deficiencies may play a role in the development of OS (74). However, upon *ex vivo* activation with IL-15, patient-derived NK cells become highly cytotoxic, able to kill both chemotherapy-resistant allogeneic cell lines and autologous tumor biopsies (9). Murine models of adoptively transferred K562 mbIL-21 expanded NK cells have potent anti-tumor activity in murine xenograft models of metastatic OS. This anti-tumor effect can be augmented by the addition of aerosolized IL-2 (75, 76). In addition, NK cells can target OS cancer stem cells (77). OS expresses GD2 and EGFR, and the administration of antibodies to these antigens, (dinutuximab & cetuximab, respectively) can augment NK cell killing of OS through ADCC (78).

1.6 Tumor Avoidance of NK Cell Surveillance

Despite the *in vitro* and *in vivo* activity of NK cells, immune therapies have been largely ineffective at eradicating minimal residual disease in patients. This is

believed to be due to the immune suppressive environment of the tumor caused by tumor cells themselves, myeloid derived suppressor cells, T-cells, and the stroma. Tumors can evade NK cell surveillance through several mechanisms. These include release of soluble NKG2D and NKp30 ligands, which then bind to the corresponding receptor on the NK cell, blocking engagement of the NK cell with the tumor, and preventing tumor cell lysis (79, 80). In addition, L-kynurenine which is generated from the breakdown of tryptophan by indoleamine-2,3-dioxygenase (IDO) induces selective downregulation of NKp46 and NKG2D in NK cells, leading to decreased killing of cell lines that are killed through NKp46 and NKG2D receptor engagement (81). IDO derived L-kynurenine can also inhibit NK cell proliferation (82). Another method of immune evasion is the hypoxic tumor microenvironment. Hypoxia inhibits cytokine-induced upregulation of the NCRs and NKG2D, but not CD16. This corresponds with decreased anti-tumor activity but maintenance of ADCC (83, 84). Tumor-associated fibroblasts also negatively modulate NK cell anti-tumor function through both cell-cell contact and secretion of PGE2 (85).

Another important method of suppression is the release of tumor growth factor- beta ($\text{TGF}\beta$). $\text{TGF}\beta$ is a potent inhibitor of NK cell anti-tumor activity and is believed to be an underlying cause for the inability of cell-based therapies to eradicate minimal residual disease (86). $\text{TGF}\beta$ is released from numerous cell types within the tumor microenvironment including NK and T-cells themselves, tumor associated fibroblasts and macrophages, myeloid derived suppressor cells, tumor cells, and regulatory T-cells (Tregs) (87, 88). Cancer patients, particularly in brain and solid tumors, have elevated serum $\text{TGF}\beta$ levels that correlate with disease

severity (89-93). Both human and canine OS patients have elevated serum TGF β levels (94, 95). TGF β inhibits NK cell IL-2 induced proliferation and decreases expression of several activating receptors including NKG2D, NKp30, and CD16 (87, 96). Through phosphorylation of SMAD3 by TGF β , TGF β inhibits NK cell secretion of IFN γ , TNF α , and chemokines (97-100). TGF β inhibits NK cell cytotoxicity by impairing the release of granzymes and perforin (43, 101).

However, TGF β plays an important role in homeostasis of immune cell development and activity such as in the prevention of autoimmunity (102). TGF β promotes the development of T-cells in the thymus by upregulating IL-7R α and promoting the survival of Tregs (103). Tregs, in turn, support peripheral tolerance by suppressing inflammatory Th1 cells, CD8+ T-cells, and NK cell activity, inhibiting anti-self reactions (104). TGF β decreases B cell and T-cell function and number by inhibiting proliferation and T-cell production of IL-2 (105-108). TGF β also induces the release of other immune suppressive proteins, such as IDO in dendritic cells (109). However, TGF β 's ability to suppress T-cell and dendritic cell function can be abrogated by ligation of CD28 or CD40, respectively, demonstrating that TGF β 's effect on the immune system is context dependent (110).

1.7 Dog Models of Cancer

Canines are an established spontaneous animal model for cancer. Canines naturally develop many cancers with high homology to human cancer including OS, melanoma, lymphoma, glioma, prostate, and mammary cancer. Many of these cancers have similar tumor driving genes. In addition, canines as household pets, share the same environment with humans allowing for study on the effect of

environmental exposures on tumor development. Of particular interest to immune-based therapies, canines have a fully intact immune system and complete tumor microenvironment (111, 112). Since the lifespan of canines is much shorter than humans, cancer progresses at a significantly faster rate allowing for rapid testing of therapeutics. This can be used to assess the efficacy of new therapies and to prioritize the translation of promising therapies from canines to humans (111).

Canine models have been effective in generating pre-clinical data for several new therapies which had successful translation to humans. For example, canines were used to develop bone marrow transplant protocols for subsequent use in humans (113, 114). Ibrutinib, an inhibitor of Bruton's tyrosine kinase, was tested in canines with non-Hodgkin's lymphoma before being approved for human use, where it has been highly effective in the treatment of lymphomas (115, 116). In OS, canines were used to optimize limb-sparing surgical removal of the primary OS tumor (117). Recently, the success seen in a pre-clinical trial in canine glioma using EGFR-mini cells loaded with doxorubicin facilitated the opening of a clinical trial in human glioblastoma (NCT02766699) (118). In breast cancer, a p62 DNA vaccine demonstrated efficacy in a pre-clinical trial in canines, and the recent completion of a Phase 1 trial in human breast cancer reported the same finding where patients receiving the vaccine had delayed disease progression (119, 120).

Several immune based therapies have also recently been tested in canines. These include *ex vivo* expanded T-cells engineered to express a CD20-CAR for lymphoma or a HER2-CAR for OS (121-123). In human patients, T-cells expressing a CD19 CAR, similar to the canine counterpart of CD20, have been effective in

treating lymphoma (124, 125). A phase 1 clinical trial in human OS recently closed and demonstrated that HER2-CAR T-cells are well-tolerated, and 4/17 patients had stable disease following T-cell infusion (126, 127).

Canines have also been used for retrospective evaluation of why drugs failed in human clinical trials. For example, iniparib which first demonstrated preliminary efficacy in human breast cancer trials, later did not improve survival. This culminated in lack of FDA approval. In response to these findings, a trial opened up in canines with melanoma, soft tissue sarcoma, and squamous cell carcinoma with the goal of better understanding why iniparib failed in humans. Similar to the findings in humans, iniparib did not improve survival in canine cancers despite administering doses significantly higher than used in human trials (up to 70 mg/kg in canine versus 5.6 mg/kg), and it was found that iniparib did not have substantial infiltration into tumors. Thus, the canine model can also be used to provide answers to why treatments fail in human clinical trials (128, 129).

1.7.1 Canine Osteosarcoma as a Pre-clinical Model for Human Osteosarcoma

Canine OS occurs with much higher frequency than human OS facilitating the pre-clinical testing of new therapies in this model. Of the over 1 million dogs diagnosed with cancer every year, over 8,000 are diagnosed with OS. Canine OS has a similar standard of care as human OS, but has extremely poor prognosis with only a 50% 1-year survival rate (111, 130).

At the genetic level, human and canine OS share all the same mutations and cellular signaling, highlighting the usefulness of testing novel therapies in canines that will provide benefit to both species (112). Canines with OS also develop

pulmonary metastasis. The most recent therapy to improve OS survival, Liposome-muramyl tripeptide phosphatidylethanolamine (L-MTP-PE; mifamurtide) was first tested in canine OS where it improved survival (131). Following this study, a large phase III study was conducted with L-MTP-PE in conjunction with chemotherapy. L-MTP-PE improved event-free and overall survival of human OS and is now approved for treatment for OS in Europe (132, 133).

1.7.2 Canine NK Cells

Despite the promising *in vitro* and *in vivo* murine data for NK cells in OS, much less is known about canine NK cells in OS. In addition, very little is known about the phenotype or function of canine NK cells entirely. This has largely been attributed to a lack of available reagents for identifying canine NK cells. Identification of canine NK cells has instead relied upon either negative selection using antibodies specific to other immune lineage markers or identifying NK cells by their functional capacity to kill canine thyroid adenocarcinoma (CTAC) without prior sensitization.

However, the use of these methods of identification of canine NK cells has led to conflicting results as to what constitutes NK cells in canines. For example, T-cell receptor (TCR) positive, TCR⁻, and CD3⁺/TCR⁻ cells can kill the CTAC cell line. In addition, these TCR⁺ cells have a NK-like genotype including NK cell receptors such as NKG2D, NKp46, and CD16. Further adding to the confusion, these experiments have been done on mixed cell populations including both TCR⁺ and TCR⁻ cells. Both CD3⁺ and CD3⁻ NK-like cells have expanded on K562 cells transduced to express human 41BBL and membrane-bound IL-15 (134-139). In order to further

comparative NK cell biology and to test NK cell therapy in spontaneous canine OS, there is a great need to clarify the phenotype of canine NK cells.

Specific Aims.

The goal of this dissertation was to improve NK cell therapy for OS, with the ultimate goal of providing the necessary tools to improve OS survival. To accomplish this goal, this project aimed to characterize NK cells in canines, which spontaneously get OS with over 8,000 new cases per year; yet, very little is known about the phenotype of canine NK cells. Second, to improve expansion of canine NK cells for adoptive transfer of NK cells in canine OS. Finally, to develop NK cells resistant to suppression by TGF β through non-genetic education, ultimately having increased anti-tumor activity.

Specific Aim 1: *To develop and validate a standard approach to characterizing canine NK cell phenotype and function.* In order to study NK cells in canine OS, characterization of canine NK cell phenotype and function is needed, which has thus far been done using mixed cell populations since no antibody to positively select for canine NK cells has been available. We **hypothesized** that canine NK cells would be identified by their expression of NKp46, the putative species-wide marker of NK cells, and would kill tumor targets without prior sensitization. Using our novel anti-canine NKp46 antibody, we characterized the phenotype of CD3⁻/NKp46⁺ cells by flow cytometry and PCR. We assessed the function of pure, sorted CD3⁻/NKp46⁺ cells by comparing to donor-matched T-cells to determine if CD3⁻/NKp46⁺ cells can kill without prior sensitization, and then

determined the cytotoxicity of CD3⁺/NKp46⁺ cells against primary and metastatic OS, melanoma, and thyroid adenocarcinoma canine cell lines. We determined the cytokine profile of CD3⁺/NKp46⁺ cells using Luminex. Finally, we identified a Lineage negative, NKp46⁺ cell population (Null cells) with phenotypic and functional similarity to canine NK cells (CD3⁺/NKp46⁺), and compared this subset to human NKp46⁺ and NKp46⁺ NK cells.

Specific Aim 2: *To improve NK cell expansion from healthy and OS-bearing canines for use in comparative oncology models.* In order to test NK cell therapy in canines, the development of a NK cell expansion platform capable of generating clinically relevant numbers of NK cells was necessary. Due to the potent anti-tumor activity of mbIL-21 expanded human NK cells, we desired to translate this NK cell expansion method from humans to canines. We **hypothesized** that canine NK cells would have robust proliferation on K562 mbIL-21 feeder cells. To this end, we optimized expansion of canine NK cells on mbIL-21 for both healthy and OS-bearing canines.

Specific Aim 3: *To assess the ability of NK cells expanded in TGFβ to overcome TGFβ mediated suppression of NK cell Anti-tumor Activity.* Both human and canine OS patients have elevated levels of TGFβ, which potently suppresses NK cell killing. Although TGFβ pathway inhibitors and dominant-negative receptors have demonstrated some improvement in immune therapy effectiveness, these methods have potential for off-target side effects. We *hypothesized* that expansion of NK cells in the presence of TGFβ would select for NK cells with reduced sensitivity to TGFβ suppression. To this end, we expanded human NK cells with

mbIL-21 feeder cells and TGF β and compared their anti-tumor function to NK cells expanded without TGF β by CD107a expression, cytotoxicity assays, and cytokine secretion. We also measured their cell surface and intracellular phenotype using flow cytometry, qPCR, and western blots.

Chapter 2: Identification, Characterization, and Expansion of Canine NK Cells

2.1 Background

This chapter is based upon Foltz, J. A., S. S. Somanchi, Y. Yang, A. Aquino-Lopez, E. E. Bishop, and D. A. Lee. 2016. NCR1 Expression Identifies Canine Natural Killer Cell Subsets with Phenotypic Similarity to Human Natural Killer Cells. Frontiers in Immunology 7. with permission from NK and Innate Lymphoid Cell Biology- Frontiers in Immunology under the terms of the CC BY license.

Canines are a large animal model with spontaneous development of many cancers, including osteosarcoma, leukemia, lymphoma, glioblastoma, prostate cancer, and mammary cancer. Canines provide an outbred, immune competent disease model with genetic heterogeneity and a shared environment with humans (111, 130, 140, 141). Testing of novel therapies in the canine model has educated the protocols for bone marrow transplants in humans, and more recently has been used for the testing of immune therapies such as adoptive transfer of T-cells, HER2-Listeria vaccine, and Liposomal-muramyl tripeptide (L-MTP-PE; mifamurtide) (113, 114, 123, 131, 142-145).

Despite the advantages of the canine model, NK cells are less well-characterized in canines than in mice and humans. The sequencing of the canine

genome in the early 2000s revealed that like humans, canines have all of the natural cytotoxicity receptors along with NKp80 in their genome. The primary inhibitory receptors that mediate licensing of NK cells are the Ly49 and KIR families of receptors, both of which recognize self through binding to MHC Class I. Mice have 16 Ly49 genes but only 2 KIR, whereas humans have 16 KIR genes but only a pseudogene of the Ly49 family (146). The sequenced canine genomes have no KIR and only one Ly49 gene, which has a predicted ITIM sequence suggesting that it functions as an inhibitory receptor (146-148); however, there are some reports of canines having KIR sequences but the data supporting this claim is not publicly available (148).

The identification of NK cells in canines has been met with seemingly conflicting results with some studies reporting CD3⁻ cell populations with NK cell properties while others report CD3⁺ cell populations with NK cell properties (134, 136, 138, 139). Recently, Grondahl-Rosado et al. provided more clarity on the phenotype of canine NK cells using a cross-reacting anti-bovine antibody to NCR1 (NKp46), the putative species-wide marker of NK cells in mammals (26, 149-155). Using this antibody, they identified a CD3⁻/NKp46⁺ cell population in most canines that were also positive for Granzyme B. Further, they confirmed that NKp46 is an activating receptor in canine. They also proposed that a CD3⁻/NKp46⁻/Granzyme B⁺ cell subset may be a subset of canine NK cells (155, 156). However, this anti-bovine NKp46 antibody is reported by the authors to not be suitable for sorting of CD3⁻/NKp46⁺ cells, limiting the ability to further characterize the receptor expression and function of CD3⁻/NKp46⁺ cells and this NKp46⁻ cell population (155, 156).

Additionally, expansion of canine NK-like cells, while more successful than *ex vivo* expansion of mouse NK cells, has been significantly less than reported in humans with expansions reported of up to 233-fold on average in 2-3 weeks (134-139, 157).

We sought to further characterize canine NK cells for use in osteosarcoma, where survival for metastatic human OS patients has largely remained stagnant at only 30% 5-year survival rate for the last 30 years (1, 2, 5, 6). Canine OS is highly prevalent, with over 8,000 new diagnoses per year, and an average survival rate of only 1 year, allowing for the rapid testing of new therapeutics. While mouse models have provided important discoveries in OS pathogenesis and treatment, the spontaneous canine model of OS has been well-characterized and is used as an additional important animal model of OS (111, 112, 130, 158).

To this end, the goal was to develop and validate a standard approach towards characterizing canine NK cells and to improve *ex vivo* expansion of canine NK cells. Since NKp46 has identified NK cells in all species studied to date and has been proposed as the species-wide marker of NK cells due to its' conservation across species which is unlike CD56, we hypothesized that NK cells in canine could be identified by their expression of NKp46. Extending our findings in human NK cells of increased NK cell proliferation with IL-21 expressing K562, we also hypothesized that expansion of canine NK cells would be improved compared to the literature with K562 mbIL-21 feeder cells.

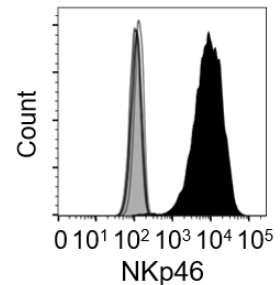
2.2 Results

2.2.1 Construction of an Anti-Canine NKp46 Antibody

Hybridomas obtained by immunization with L-cells expressing the NKp46 fusion protein (NKp46:L-cells) were screened via ELISA (Appendix A) and flow cytometry against NKp46:L-cells and Empty Vector:L-cells. Clone 48A had strong staining on NKp46:L-cells and no non-specific staining on Empty Vector:L-cells. Clone 48A was identified as a mouse IgG2a isotype and was selected for all future experiments (**Figure 1**). Clone 48A did not identify NKp46 by western blot or immunoprecipitation (data not shown).

Figure 1. Screening of a Novel Anti-Canine NKp46

Antibody. Clone 48A is specific for L-cells expressing NKp46 (open histograms: secondary antibody only, gray filled: vector only, black filled: NKp46:L-cells). Mouse immunization and screening of hybridomas against L-cells was done by MD Anderson Monoclonal Antibody Core. Screening of hybridomas against canine cells was done by Jennifer Foltz.



2.2.2 NKp46 Expression on Canine Peripheral Blood Mononuclear Cells

Next, we identified CD3⁻/NKp46⁺ cells in lymphocytes as largely negative for expression of CD4 (median = 0%, IQR = 0, 1.0, n = 7), with low expression of CD5 (median = 10.6%, IQR = 6.4, 25, n = 7) and CD8 (median = 9.3%, IQR = 4.4, 16.0, n = 10). NKp46 was not coexpressed with macrophage (CD14, data not shown) or B-cell lineage markers (CD21, **Figure 2**). On average, 2.3% (median = 1.2%, IQR =

0.6, 3.7, n = 12) of lymphocytes from healthy canines were CD3⁻/NKp46⁺, consistent with an NK cell expression pattern (**Figure 2**). NKp46 was also expressed on a subset of CD3⁺/TCR⁺ cells.

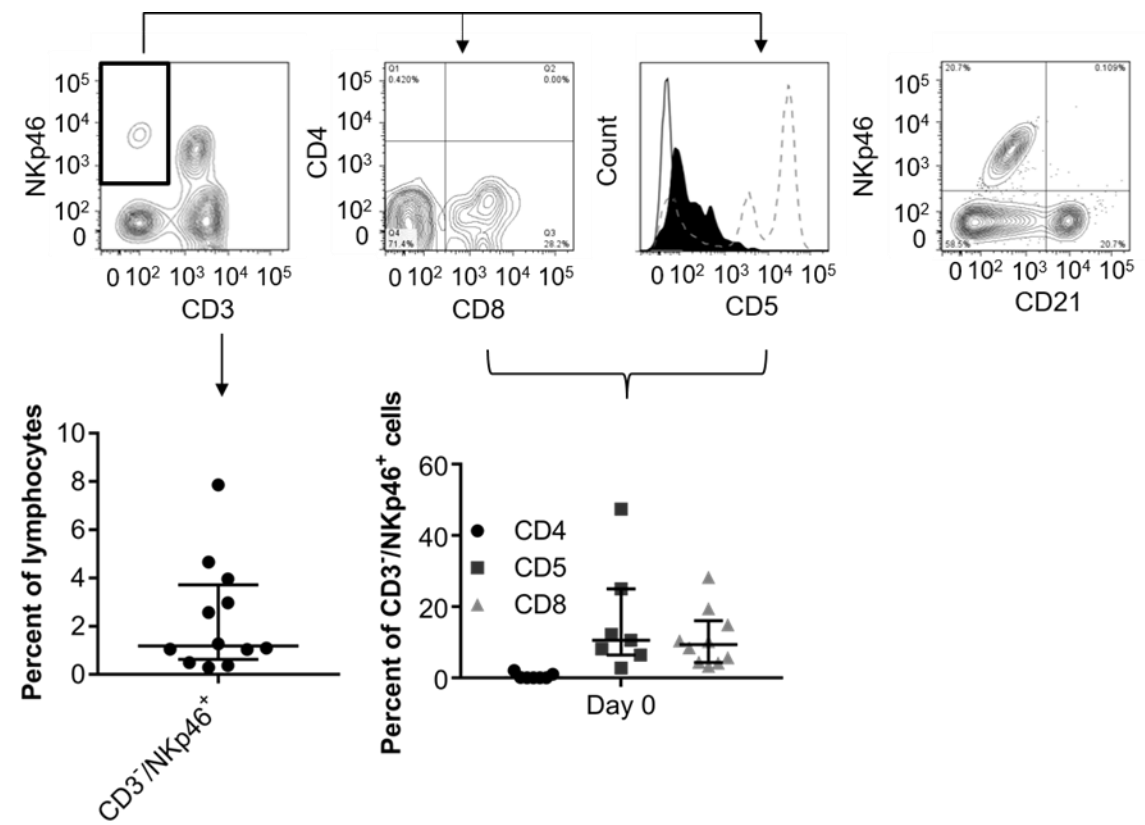


Figure 2. Expression of NKp46 on canine PBMC using a novel anti-canine

NKp46 antibody. NKp46 is expressed on CD3⁻ and CD3⁺ lymphocytes.

CD3⁻/NKp46⁺ cells make up on average 2.3% of lymphocytes. Shown is median \pm IQR (n = 12). Representative flow plot is shown. CD3⁻/NKp46⁺ cells are largely negative for CD4 (median = 0%), and a subset express CD8 α (median = 9.3%), and CD5^{low/-} (median = 10.6%) (Solid line: negative control, Black filled: CD3⁻/NKp46⁺, Dotted line: CD5 expression on total lymphocytes showing two populations of CD5⁺ cells). Shown is median \pm IQR for CD4 (n = 7), CD5 (n = 7), and CD8 (n = 10). NKp46 is not expressed on CD21⁺ B-cells.

2.2.3 Ex Vivo Expansion of Canine CD3⁻/NKp46⁺ Cells

Next, we expanded canine CD3⁻/NKp46⁺ cells from whole PBMC using K562 Clone9.mbIL-21 feeder cells in a 21-day culture, since we previously demonstrated significantly improved expansion of human NK cells with K562 Clone9.mbIL-21 feeder cells compared to K562 mbIL-15 feeder cells (36). The percentage of CD3⁻/NKp46⁺ cells in the culture was significantly increased at the end of expansion when using canine IL-2 (rcIL-2) compared to human IL-2 (p = 0.03, Human IL-2: median = 58.02, IQR = 10.05, 65.16; Canine IL-2: median = 72.9, IQR = 32.25, 84.35; n = 6) (**Figure 3**). There was significantly increased yield of CD3⁻/NKp46⁺ cells (p = 0.03) at the end of expansion with rcIL-2 (**Figure 3**) (Human IL-2: median = 7.07×10^7 , IQR = 2.9×10^7 , 1.67×10^8 ; canine IL-2: median = 1.71×10^8 , IQR = 9.58×10^7 , 5.65×10^8 , n = 6). For all future expansions reported, rcIL-2 was used.

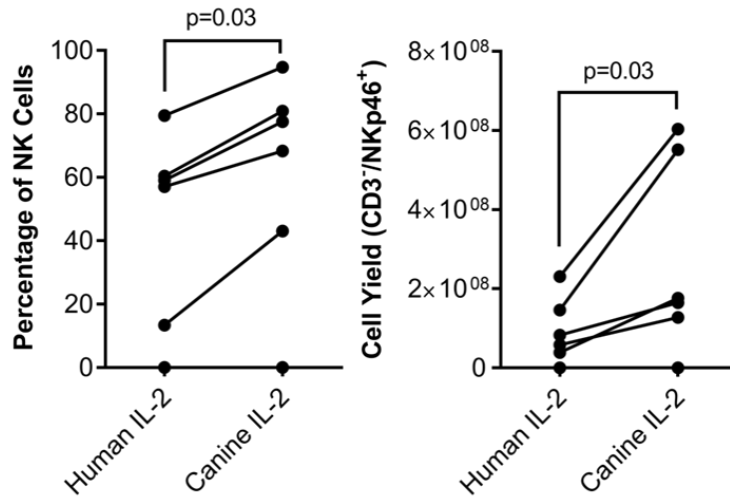


Figure 3. Expansion of CD3⁻/NKp46⁺ cells on K562 mbIL-21 feeder cells with human versus canine IL-2. Canine CD3⁻/NKp46⁺ cells expanded on K562 Clone 9.mbIL-21 feeder cells for 3 weeks with canine IL-2 have significantly increased purity and yield of CD3⁻/NKp46⁺ cells compared to expansion with human IL-2 (n = 6) Canine donors are depicted by connecting lines.

When PBMC was cultured for 21 days on K562 Clone9.mbIL21 with rcIL-2, CD3⁻/NKp46⁺ cells made up a median 62.31% (IQR = 45.7, 83.7, n = 16) and CD3⁺ T cells comprised a minor portion (median = 23.85%, IQR = 6.9%, 47.7%, n = 16) of the total expanded cell product (**Figure 4**), with the exception of two outliers. The phenotype of the CD3⁻/NKp46⁺ cells significantly changed during expansion, with CD5 decreasing (p = 0.037, Median = day 0: 10.6, IQR = 6.42, 25; day 21: 0.35, IQR = 0.1, 1.02) and CD8α increasing (p = 0.005, Median = day 0: 9.33, IQR = 4.35, 16.03; day 21: 35, IQR = 22.7, 48.1) (**Figure 4**).

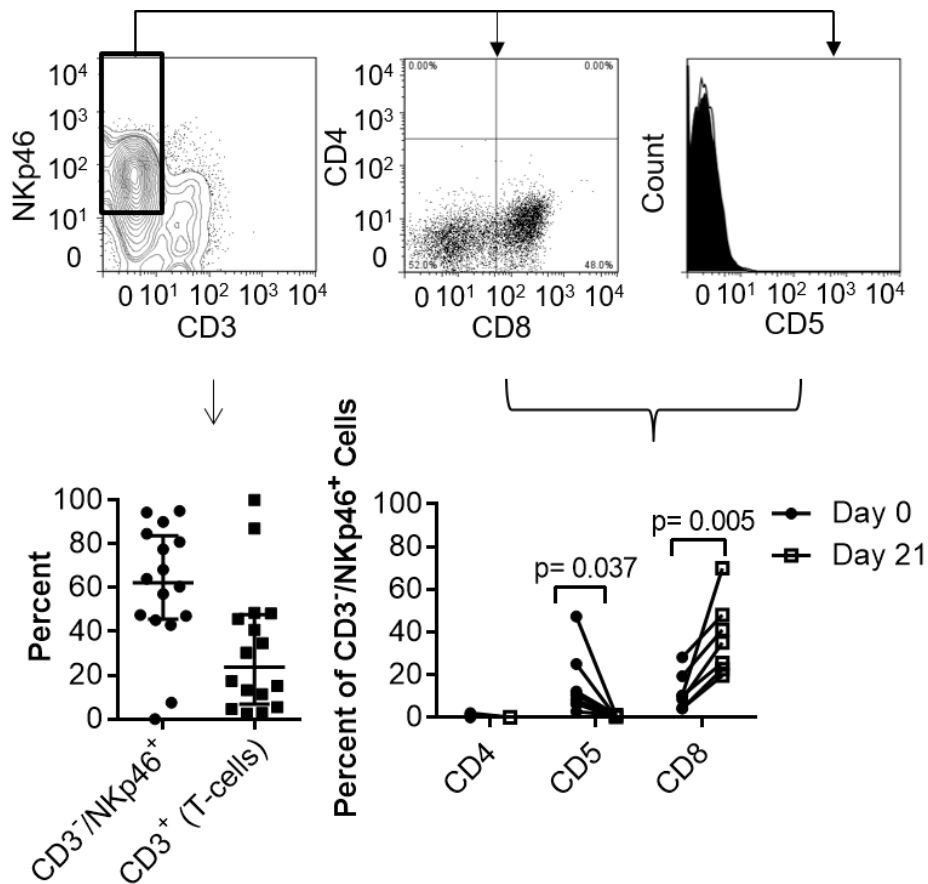


Figure 4. Phenotype of Canine CD3⁻/NKp46⁺ cells Expanded on K562mbIL-21 feeder cells. CD3⁻/NKp46⁺ cells expand on K562mbIL-21 feeder cells to make up on average 60.2% of total cells, with contaminating T-cells on average 31.8% (n = 16, median ± IQR shown). Expanded CD3⁻/NKp46⁺ cells are CD4⁻ (median = 0.08%) and have significantly decreased CD5 expression (median = 0.4%, p = 0.037) (open histogram = negative control, black filled = CD3⁻/NKp46⁺) and increased CD8 (median = 35%, p = 0.005, n = 7; median ± IQR shown).

To further characterize the expanded CD3⁻/NKp46⁺ cells, for which antibodies to other NK cell receptors are not available, semi-quantitative RT-PCR was performed on sorted CD3⁻/NKp46⁺ cells from 5 different donors for several known

human or mouse NK cell-associated genes for which canines are predicted to have homologous genes. Expanded NK cells from all donors expressed mRNA for NKp46, NKp30, NKp44, NKp80, NKG2D, DNAM-1, CD16, Ly49, Granzyme B, and Perforin, confirming that canine CD3⁻/NKp46⁺ cells express typical NK cell-associated genes (**Figure 5**).

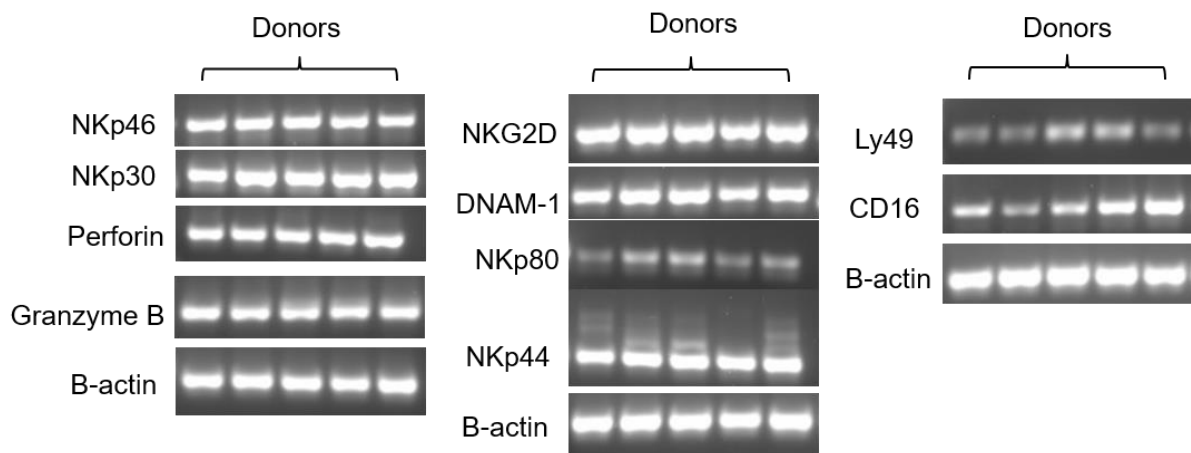


Figure 5. Gene Expression of CD3⁻/NKp46⁺ cells. CD3⁻/NKp46⁺ cells express mRNA for NKp46, NKp30, Perforin, Granzyme B, NKG2D, DNAM-1, NKp80, NKp44, Ly49, and CD16 (n = 5, all PCR reactions were conducted concurrently).

Expansion of CD3⁻/NKp46⁺ cells with K562 Clone9.mbIL-21 feeder cells for 21 days yielded a median 1.7×10^8 CD3⁻/NKp46⁺ cells (mean = 4.5×10^8 , IQR = 1.1×10^8 , 7.5×10^8) from a starting product of 7,000 (median) CD3⁻/NKp46⁺ cells (IQR = 2,375, 25,750, n = 10) (**Figure 6**) representing a median 20,283-fold expansion (mean = 89,151, IQR = 5,655, 108,749, n = 10; 2 week median fold-expansion: 4,622, 2 week mean fold expansion: 12,319 mean, n = 10). Expansion was

successful in 9/10 donors tested. The one donor where expansion was not successful had the lowest percentage of CD3⁻/NKp46⁺ in PBMC of all donors tested.

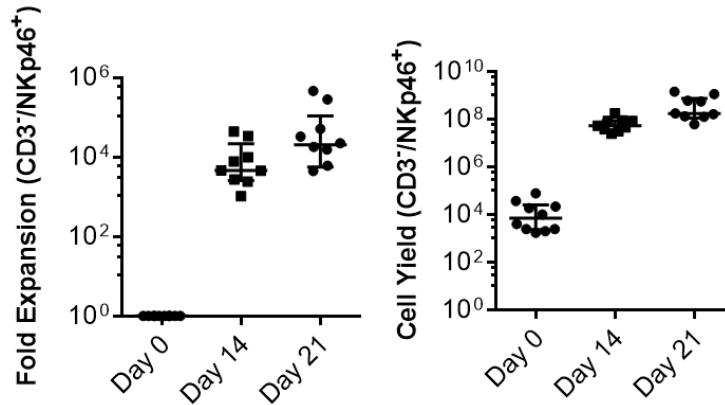


Figure 6. Expansion of Canine CD3⁻/NKp46⁺ cells on K562mbIL-21 feeder cells. On average, 4.5×10^8 (median = 1.7×10^8) CD3⁻/NKp46⁺ cells are produced at the end of 3 weeks from an average 17,774 CD3⁻/NKp46⁺ (median = 7,000) cells at day 0 (shown is median \pm IQR) representing a median 20,283-fold expansion.

Since expansion of CD3⁻/NKp46⁺ cells from whole PBMC resulted in donor-dependent T-cell contamination that would be detrimental in an allogeneic setting, we depleted T-cells from PBMC using FACS-sorting, leaving B-cells and macrophages/monocytes in the culture. We previously observed with human NK cell expansion that these cells provide support for NK cell expansion without hindering cell purity (36). Using this approach, the purity of CD3⁻/NKp46⁺ cells significantly increased (median = 96.5 versus 40.7%; $p = 0.01$) and fold expansion was not affected with 2/3 donors demonstrating increased fold expansion with T-cell depletion (**Figure 7**).

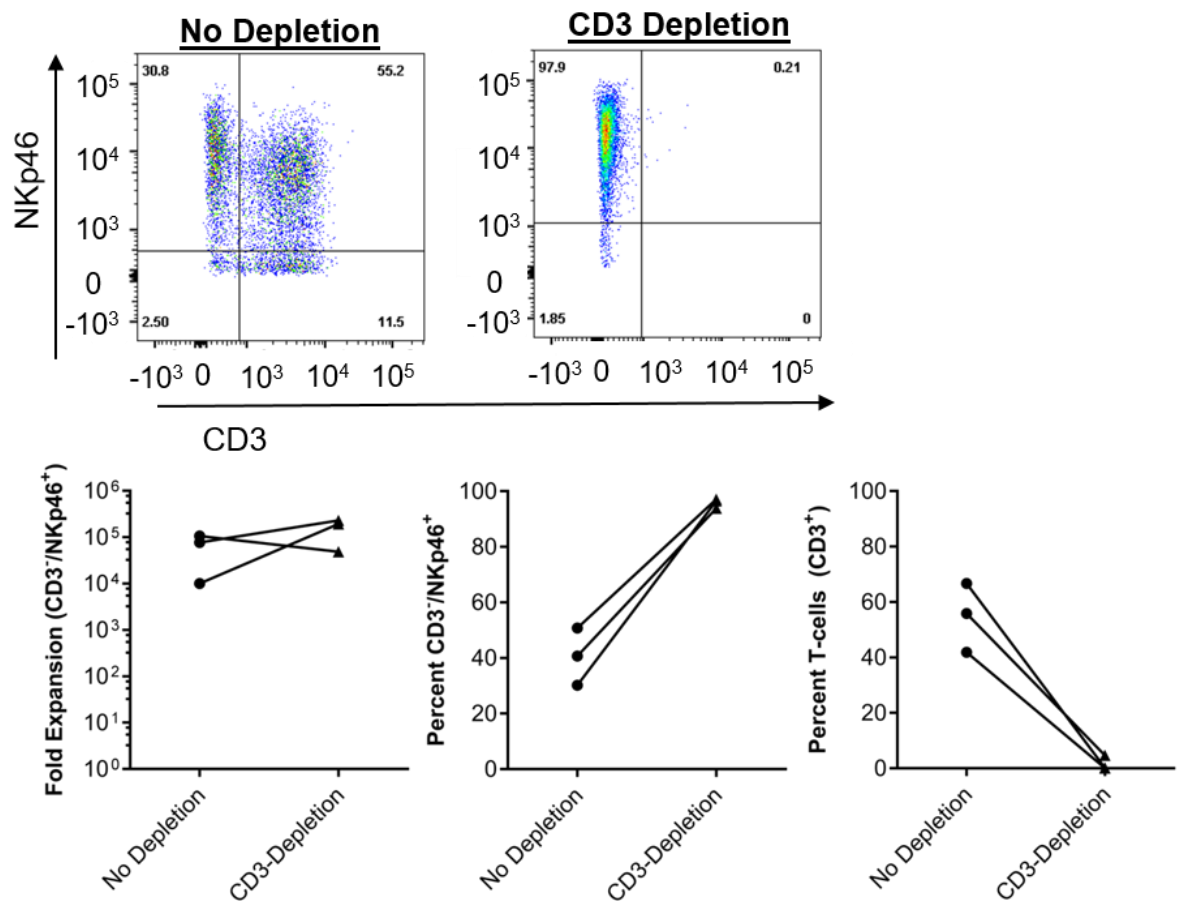


Figure 7. CD3 Depletion Improves NK Cell Purity. Depletion of T-cells at day 0 significantly increases the purity of CD3⁻/NKp46⁺ cells at day 21 (95.8 versus 40.6%) and does not hinder fold expansion (157,281 versus 64,686, n = 3). Connecting lines identify matched donor.

2.2.4 Function of CD3⁻/NKp46⁺ Cells

To determine whether CD3⁻/NKp46⁺ cells are able to kill spontaneously, a hallmark of NK cell function, cytotoxicity of expanded pure CD3⁻/NKp46⁺ cells and CD3⁺ T-cells from the same donor was assessed against the canine osteosarcoma cell line, Gray. CD3⁻/NKp46⁺ cells had significantly increased cytotoxicity than T-cells [$p = 0.009$, Median: NK = 30.92, IQR = 19.1, 48.4; T-cells = 5.36, IQR = 2.3, 13.06; 2.5 Effector:Target (E:T) Ratio], and this increase was consistent across three different E:T ratios (**Figure 8**).

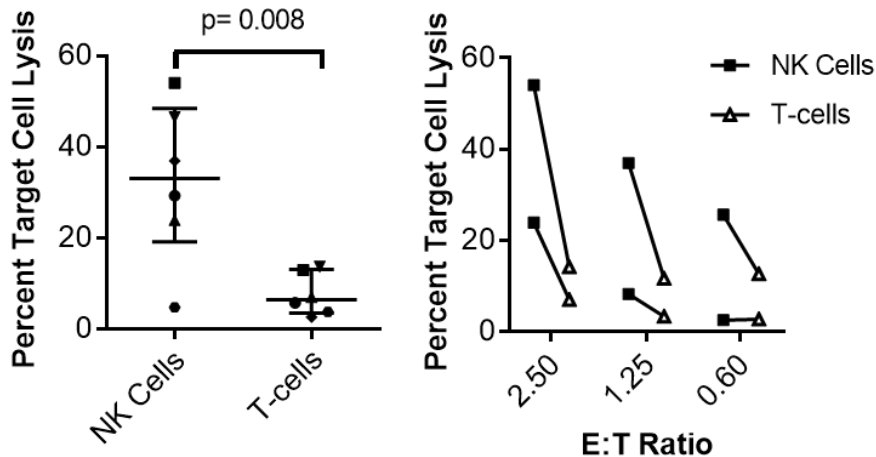


Figure 8. Canine CD3⁻/NKp46⁺ cells kill without Prior Sensitization. Sorted CD3⁻/NKp46⁺ cells have increased cytotoxicity against the canine osteosarcoma (Gray) cell line when compared to CD3⁺ T-cells (n = 6).

Next, we assessed the cytotoxicity of CD3⁻/NKp46⁺ cells against 3 osteosarcoma cell lines and one thyroid adenocarcinoma line. CD3⁻/NKp46⁺ cells displayed titratable, donor-dependent cytotoxicity against all of the 4 lines including metastatic (Gray) osteosarcoma and the thyroid adenocarcinoma line, CTAC. (**Figure 9**). Thus, CD3⁻/NKp46⁺ cells appear to possess NK cell function and

CD3⁻/NKp46⁺ will be used interchangeably with NK cells for the rest of the dissertation. To determine cytokine secretion capabilities of canine NK cells, NK cells were compared to CD3⁺ T-cells from the same donor after 6 hours coculture with K562 Clone 9.mbIL-21 feeder cells, since 6 hour co-culture is optimal for human NK cell cytokine secretion against K562 targets (159). Canine NK cells secreted GM-CSF, IFN γ , KC-like, IL-8, IL-10, and TNF α in response to K562 mbIL-21 stimulation. Cytokine secretion was similar to T-cells for all (**Figure 9**). There was no detectable secretion of IL-2, IL-6, IL-7, IL-15, IL-18, IP-10, or MCP-1 at baseline or after coculture with K562 Clone 9.mbIL-21 cells in either CD3⁻/NKp46⁺ cells or T-cells (not shown).

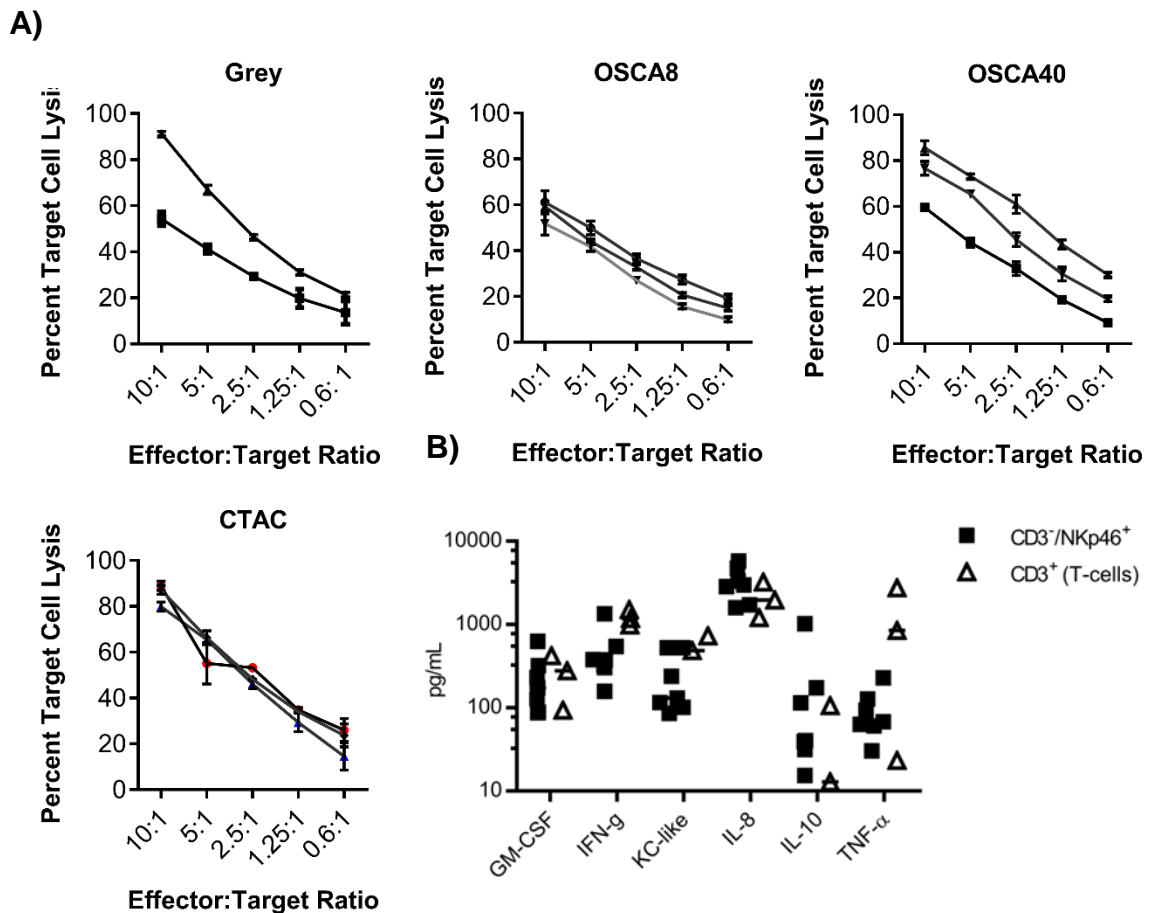


Figure 9. Canine NK Anti-Tumor Cytotoxicity and Cytokine Secretion. Canine

A) CD3⁻/NKp46⁺ cells have titratable, donor dependent cytotoxicity against four different canine osteosarcoma cell lines: Gray (n = 2), OSCA8 (n = 3), and OSCA40 (n = 3), and the canine thyroid adenocarcinoma cell line (CTAC, n = 3) (mean ± SD for each donor depicted). Unique symbols identify different donors.

B) Expanded CD3⁻/NKp46⁺ cells secrete GM-CSF, IFN-γ, KC-like, IL-8, IL-10, and TNF-α upon coculture with K562 Clone9.mbIL-21 feeder cells (n = 7, median depicted). When compared to CD3⁺ T-cells (n = 3) and also expanded on K562 mbIL-21, CD3⁻/NKp46⁺ cells secrete the same cytokines at similar concentrations.

2.2.5 NKp46 Identifies Distinct Subsets of NK Cells

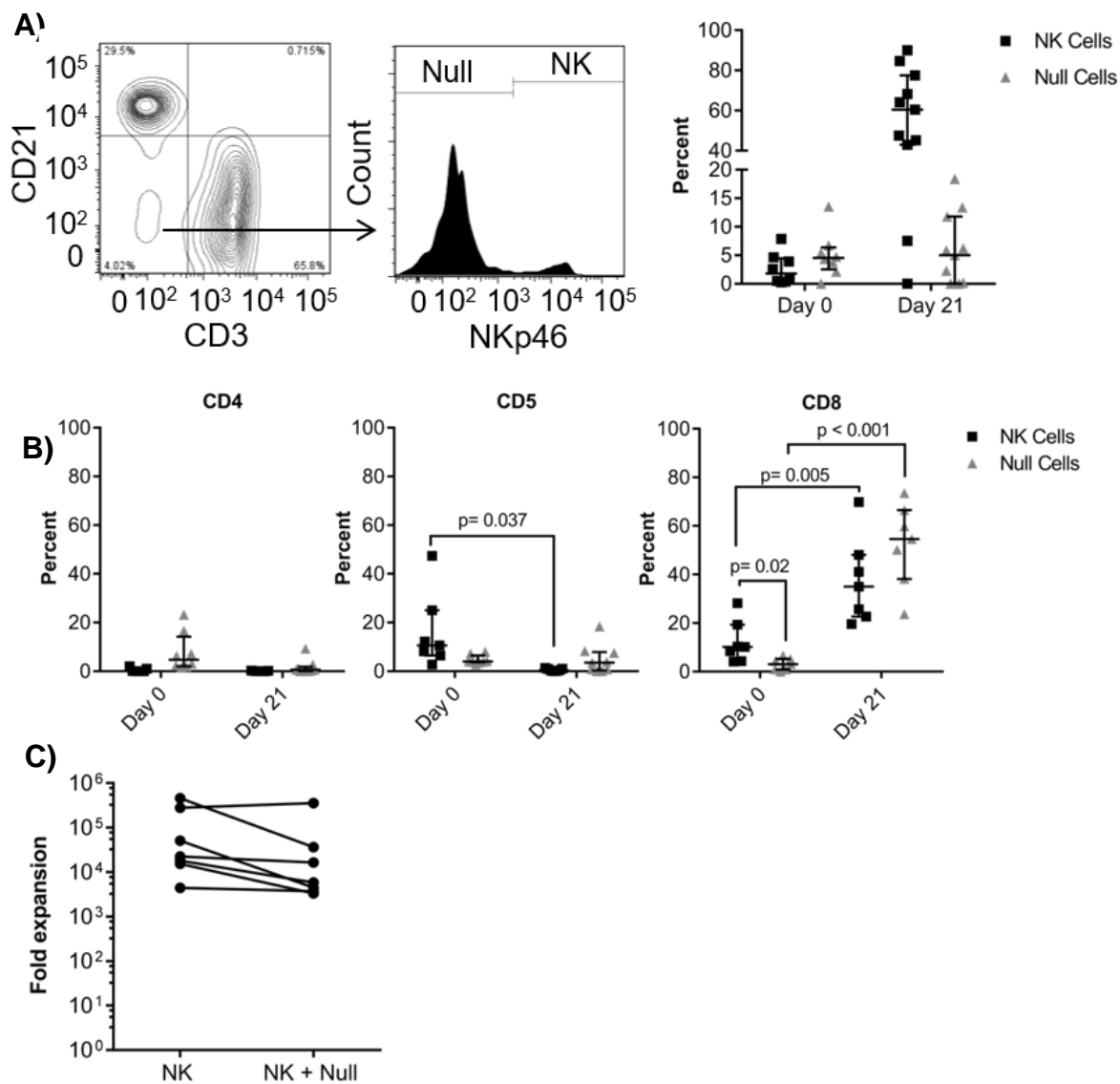
We observed that NKp46 was not expressed on all lineage negative (CD3⁻/CD21⁻/CD14⁻) cells in both PBMC (**Figure 10a**) and expanded cells (**Figure 4**), with donor-dependent variation in the percent of null cells (CD3⁻/CD21⁻/CD14⁻/NKp46⁻) observed. Null cells made up 0–13.52% of lymphocytes at day 0 (median = 4.5%, IQR = 2.54, 6.40), and 0–18.4% of the final expanded cell product (median = 5.0%, IQR = 0, 11.8) (**Figure 10a**).

Next, we determined the phenotype of these Null cells. Null cells had similar expression of CD4 (PBMC: median = 4.7%, IQR = 2.01, 14.2, n = 8; day 21: 0.6%, IQR = 0.03, 1.91, n = 9) and CD5 (PBMC: median = 4.07%, IQR = 3.72, 6.45, n = 8; day 21: 3.5%, IQR = 0.38, 7.89, n = 9) compared to CD3⁻/NKp46⁺ (NK) cells, but reduced expression of CD8 at day 0 (p = 0.02, Median = 3.1%, IQR = 0.9, 5.33, n =

7). However, similar to NK cells, CD8 expression in the null cells was significantly increased at day 21 compared to day 0 ($p < 0.001$, day 21 = 54.6%) (**Figure 10b**).

A significant negative correlation between percent of CD3⁻/NKp46⁺ cells at day 0 and CD3⁻/NKp46⁺ fold expansion was observed ($r = -0.81$, $p = 0.022$, not shown), which led us to question whether Null cells were significantly contributing to the yield of NK cells at day 21. When null cells were included in the fold expansion calculations, the mean fold expansion was 50% less than when using NKp46⁺ cells only, representing at most a two-fold over-estimation of expansion rates if all of the Null cells represented an NK population (**Figure 10c**).

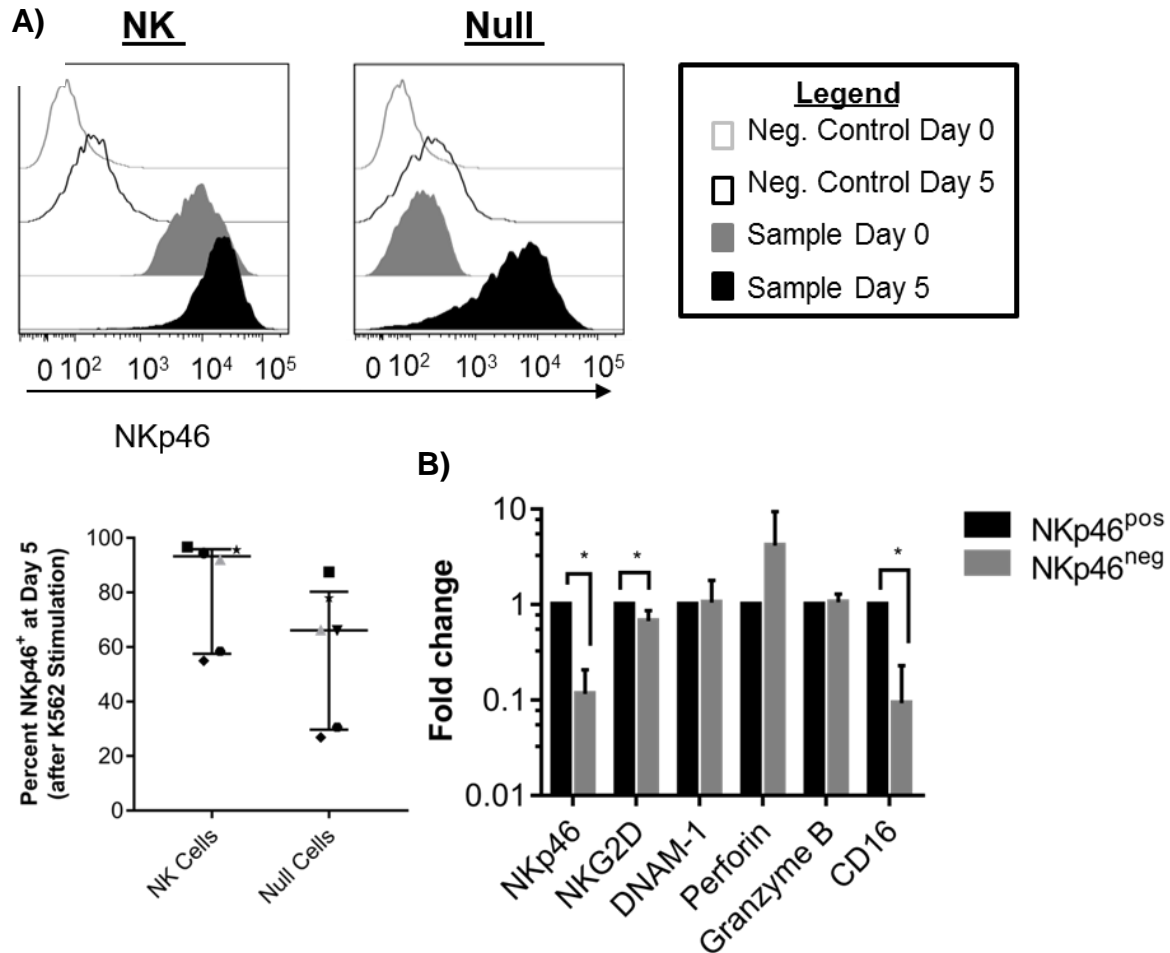
Figure 10. Phenotype of CD3⁻/CD21⁻/CD14⁻/NKp46⁻ (Null) cells in K562mbIL-21 Expanded Cells. CD3⁻/CD21⁻/CD14⁻/NKp46⁻ (Null) cells are present in canine PBMC **A)** as described for expanded cells in Figure 3A. Null cells consist of on average 5.1% of lymphocytes in PBMC, and 5.7% of expanded cells (median \pm IQR shown, day 0 $n = 8$, day 21 $n = 12$). **B)** Null cells have negligible expression of CD4, CD5, and CD8 in PBMC, but increase CD8 expression with expansion similar to CD3⁻/NKp46⁺ cells (3.1–52.4%, $n = 7$, median \pm IQR shown). **C)** The addition of null cells to fold expansion calculation decreases mean fold expansion by 50% ($n = 8$, median \pm IQR shown).



We questioned whether NKp46 might be inducible in these null cells as has been described in porcine NKp46⁺ cells (27). To address this question, we sorted the expanded cells into pure NK and Null cell populations and restimulated them on K562 Clone 9.mblL-21 feeder cells for five additional days. At the end of 5 days, NKp46 was expressed in Null cells (Null: median = 66.15, IQR = 29.69, 80.33; NK: 93.32, IQR = 57.57, 96.7) (**Figure 11a**).

Since NKp46 could be induced in formerly Null cells, we then determined if the Null cells express other NK cell-related genes. Since antibodies to other NK cell receptors are not available, we assessed the expression of several key NK cell receptors by qPCR. Confirming our flow cytometry data, NKp46 mRNA was significantly decreased in Null cells ($p = 0.004$). Null cells express Granzyme B ($n = 3$), Perforin ($n = 4$), and DNAM-1 ($n = 4$) at similar levels compared to NK cells. However, NKG2D ($p = 0.04$) and CD16 ($p = 0.009$, all $n = 4$) were significantly decreased compared to NK cells (**Figure 11b**).

Figure 11. Null Cells can be induced to express NKp46, and Null cells express other genes for other NK cell Receptors. A) NKp46 can be induced in Null cells after 5 days coculture with K562 Clone 9.mblL-21 feeder cells, while NKp46 expression is constant in sorted CD3⁺/NKp46⁺ cells ($n = 7$). **B)** Expanded Null cells express similar levels of DNAM-1, Perforin, and Granzyme B, but decreased expression of NKp46, NKG2D, and CD16, compared to CD3⁺/NKp46⁺ cells (mean \pm SD shown).



Since Null cells are capable of expressing NKp46 along with other NK cell genes, we wanted to determine if Null cells have NK cell function. Expanded NK (CD3⁻/NKp46⁺), Null (CD3⁻/NKp46⁻), and T-cells (CD3⁺) were sorted from the same donor. Null cells had significantly decreased cytotoxicity compared to NK cells ($p = 0.008$), but increased cytotoxicity compared to T-cells ($p = 0.01$) (Median: NK = 27.1%, IQR = 17.3, 39.3; Null = 15.7%, IQR = 4.4, 27.5; T-cell = 3.7%, IQR = 2.07, 6.24) (**Figure 12a**). Null cells had similar cytokine secretion capacity compared to NK cells (**Figure 12b**).

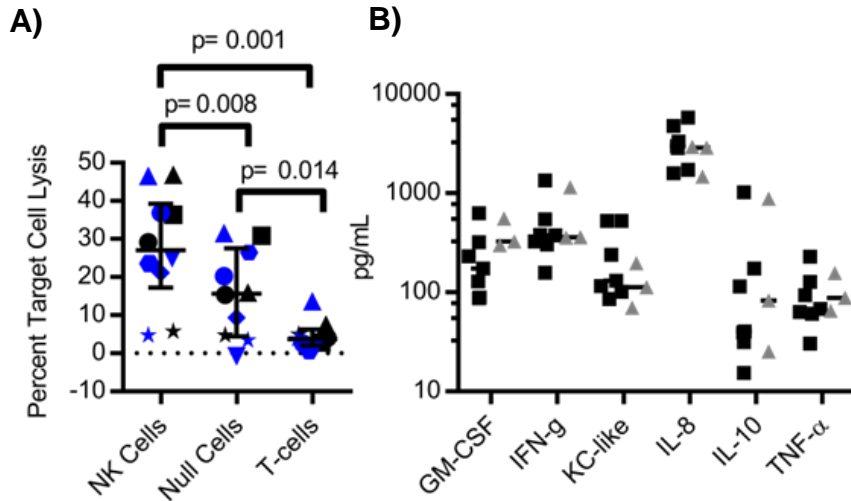
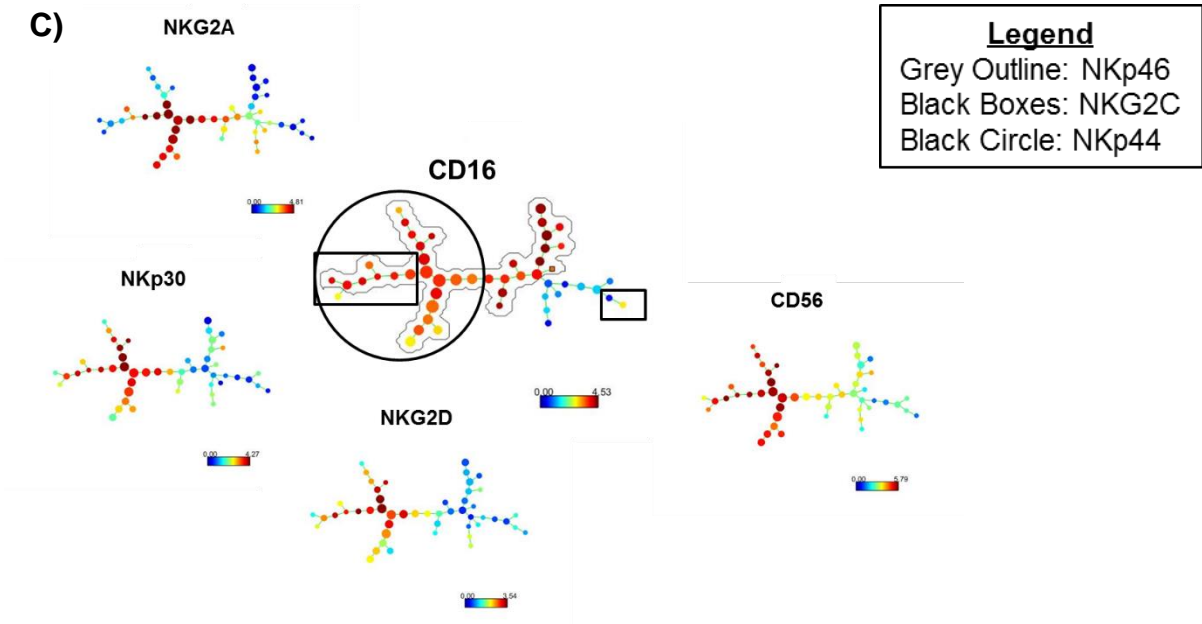
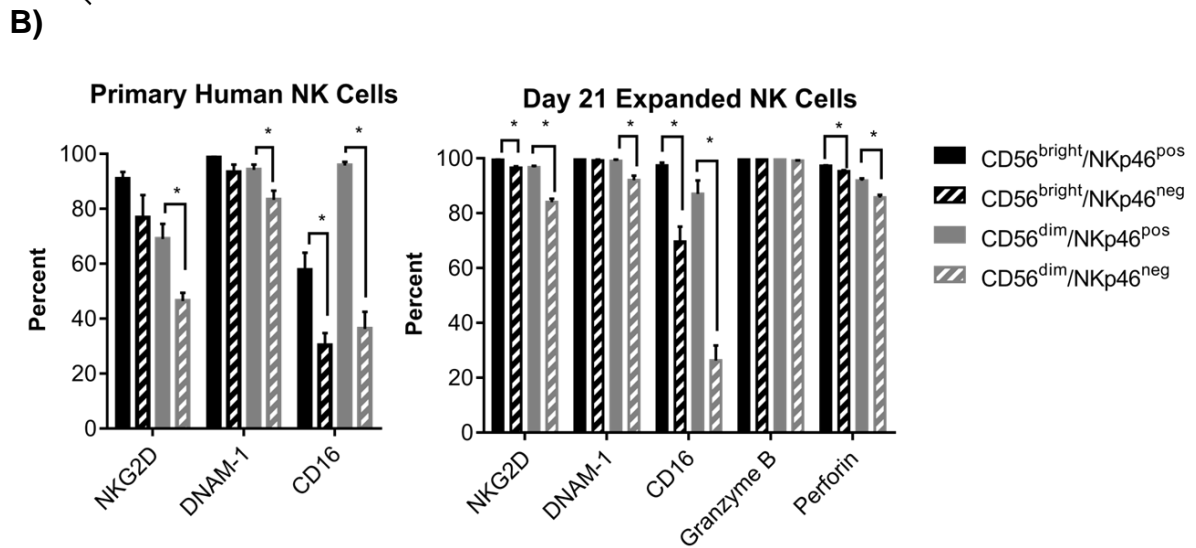
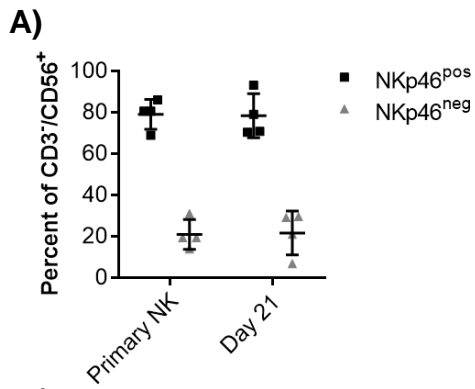


Figure 12. Function of Null Cells. **A)** Expanded Null cells have decreased cytotoxicity against Gray and melanoma-12 [$n = 9$, mel-12 (in black) = 4, Gray = 6 (in blue), individual donors designated by unique symbols, median \pm IQR shown], **B)** but similar cytokine secretion ($n = 3$) compared to $CD3^-/NKp46^+$ cells ($n = 7$, median depicted).

Finally, we sought to determine if human NK cells have similar subsets of NKp46 expressing cells. Using mass cytometry data of human $CD3^-/CD56^+$ primary NK cells and K562 Clone9.mbIL-21-expanded NK cells, we found several striking similarities between human and canine NK cells. $NKp46^-/CD3^-/CD56^+$ make up 21% of primary NK cells and 21.7% of mbIL-21 expanded NK cells (**Figure 13a**). $NKp46^-$ NK cells are primarily found within the $CD56^{dim}$ NK cell subset (**Figure 13c**). Primary and expanded human $NKp46^-$ NK cells had reduced expression of NKG2D (Primary – $CD56^{dim}$: $p = 0.02$; Expanded – $CD56^{bright}$: $p = 0.02$, $CD56^{dim}$: $p = 0.002$), and CD16 (Primary – $CD56^{bright}$: $p = 0.0035$, $CD56^{dim}$: $p = 0.0025$; Expanded – $CD56^{bright}$: $p = 0.02$, $CD56^{dim}$: 0.0012) (**Figure 13b**). In contrast to canine Null cells, $NKp46^-$ primary and expanded human NK cells had reduced expression of DNAM-1 (Primary

– CD56^{dim}: $p = 0.01$, Expanded – CD56^{dim}: $p = 0.01$) and Perforin (Expanded-CD56^{bright}: 0.04, CD56^{dim}: 0.01). Next, we used Spade to determine if NKp46⁺ NK cells are a distinct population of human NK cells. We clustered for CD56, NKp46, NKp30, NKp44, NKG2A, NKG2C, and NKG2D on both primary and mbIL-21 expanded human NK cells and found that NKp46 expression clusters into distinct NKp46⁺ and NKp46[−] NK populations and NKp46 expression overlaps with CD16 expression (**Figure 13c**).

Figure 13. Phenotype of NKp46[−] Human NK Cells. **A)** NKp46[−] NK cells are present in primary (mean = 21%) and K562 Clone 9.mbIL-21-expanded (mean = 21.7%) human NK cells ($n = 4$, median \pm IQR shown). **B)** NKp46[−] NK cells have decreased expression of CD16, DNAM-1, Perforin, and NKG2D ($n = 4$, mean \pm SD depicted). **C)** Spade clustering analysis of primary and mbIL-21 expanded human NK cells for CD56, NKp46, NKp30, NKp44, NKG2C, NKG2D, and NKG2A revealed that NKp46 and CD16 cluster together (outlining delineates cells that stain positive for the indicated marker). Mass Cytometry samples were prepared by Arianexys Aquino-Lopez and data was analyzed by Jennifer Foltz.



2.2.6 NK Cells in Canine Osteosarcoma

We obtained PBMC from canines that were not currently undergoing chemotherapy and found no difference in the percent of NK cells in OS-bearing canines compared to healthy controls (**Figure 14a**). Additionally, NK cells from OS patients had similar proliferation on K562 Clone 9.mbIL-21 feeder cells and cytotoxicity against three canine OS cell lines compared to healthy canines (**Figures 14b,c**).

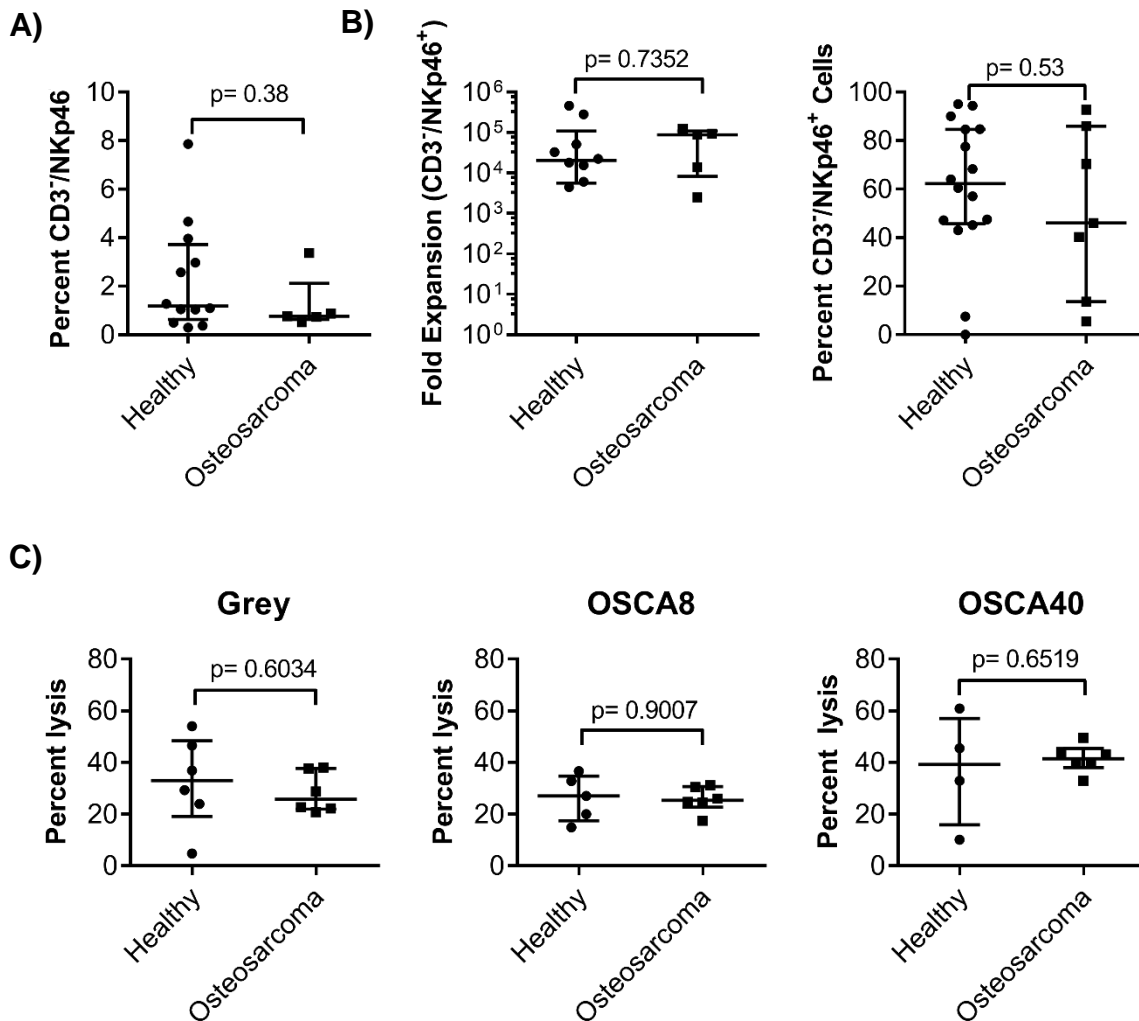


Figure 14. NK cell numbers, expansion, and function in canine

osteosarcoma patients. Canine osteosarcoma patients have **A)** similar percentages of NK cells in lymphocytes, **B)** retain proliferative capacity in response to K562 Clone9.mblL-21 feeder cells, and **C)** cytotoxicity against canine osteosarcoma cell lines when compared to healthy canines (median \pm IQR for all).

2.2.7 Cross-Reacting Antibodies

To determine if commercially available antibodies to human and mouse NK cell proteins would cross-react with canine NK cells, we screened twenty-four different antibodies against expanded canine NK cells (Table 2). Of these, only 1 antibody, anti-human DNAM-1 (clone DX11) was successful (**Figure 15**). Titration of this DNAM-1 antibody demonstrated that this antibody must be used at higher concentrations than specified by the manufacturer for adequate staining of canine NK cells.

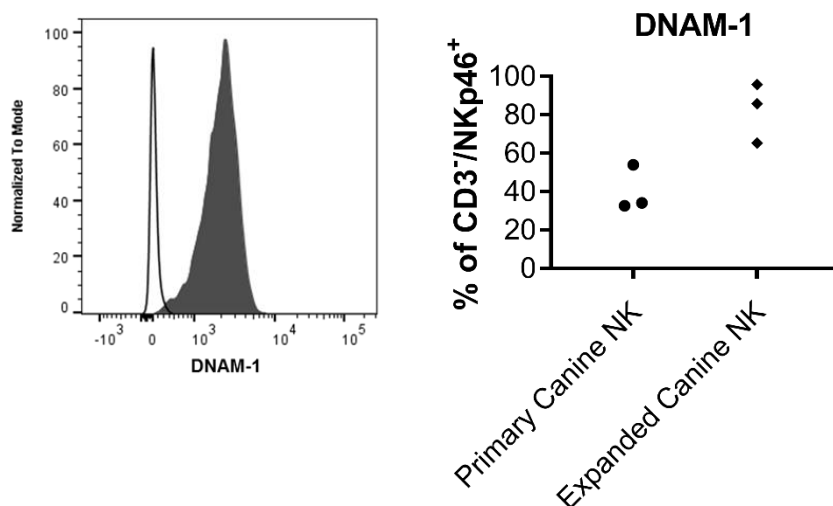


Figure 15. Cross-Reacting DNAM-1 Expression on Canine CD3⁻/NKp46⁺ cells. Representative histogram of DNAM-1 expression on expanded CD3⁻/NKp46⁺ cells is depicted. Percent of DNAM-1⁺ NK cells at Day 0 and in pure, expanded NK is shown (n=3).

Table 2. Screening of Mouse and Human Antibodies Recognizing NK Cell Proteins on Canine NK Cells.

<i>Protein</i>	<i>Clone</i>	<i>Species Specificity</i>	<i>Cross-Reactive?</i>
NKp30	P30-15	Human	No
NKG2A/C/E	20D5	Mouse	No
NKG2D	1D11	Human	No
CD11a	G43-25B	Human	No
CD11b	44/ICRF44	Human	No
CD16	3G8	Human	No
CD16	B73.1	Human	No
CD94	131412	Human	No
Fas	DX2	Human	No
CD62L	DREG-56	Human	No
CD57	NK-1	Human	No
DNAM-1	DX11	Human	Yes
FasL	NOK-1	Human	No
CD244	2-69	Human	No
CCR6	11A9	Human	No
CCR7	150503	Human	No
CX3CR1	2A9-1	Human	Not clear staining
NKp80	4A4.D10	Human	No
NKp80	239127	Human	No
NKp44	P44-8.1	Human	No
NKG2A	FAB1059P	Human	No
NKG2C	FAB138P	Human	No
Ly49C/I	5E6	Mouse	No
NKG2D	CX5	Human	No

2.3 Discussion

We sought to characterize canine NK cells and optimize their expansion for use as a comparative oncology model of NK immunotherapy of OS. To this end, we developed a monoclonal antibody specific to canine NKp46 and demonstrated that this antibody recognizes CD3⁻/NKp46⁺ cells that have striking phenotypic and functional similarity to human NK cells – expressing all of the NCRs and secreting IFN γ and TNF α . These CD3⁻/NKp46⁺ cells from both healthy and OS-bearing canines exhibit robust expansion with K562 Clone9.mbIL21 and canine IL-2 and are highly cytotoxic against OS. In addition, we identified a small population of NKp46⁻ NK cells that have reduced cytotoxicity but similar cytokine secretion and can be induced to express NKp46.

This antibody also identifies a subset of T-cells that are NKp46⁺. In humans, NKp46⁺ T-cells are rare, but NKp46 can be acquired upon activation in $\gamma\delta$ T-cells (160, 161). In bovines, NKp46⁺ T-cells have functional similarity to bovine NK cells as they were able to kill a tumor cell line spontaneously unlike T-cells that did not express NKp46 (162). We observed very little killing of the spontaneous OS lung metastasis cell line, Grey, by CD3⁺ canine T-cells, but it will be of interest to more fully characterize the phenotype and function of NKp46⁺ canine T-cells. Despite the low cytotoxicity of the T-cells against the OS cell line, it remains unknown whether the contaminating T-cells in K562 Clone9.mbIL-21 expanded cell cultures are capable of mediating Graft-versus host disease. Substantial work in humans suggests that contaminating T-cells might mediate GVHD and be detrimental in an allogeneic transplant setting (163, 164). Thus to reduce this potential adverse effect

by contaminating T-cells, we demonstrate that CD3-depleted PBMC can give rise to greater than 96% pure CD3⁻/NKp46⁺ expanded NK cells.

Spontaneous animal models of cancer for the study of NK cell therapies in a syngeneic setting have to date remained elusive due to difficulty in expanding NK cells in other species outside of human and the high cost of primate research. Instead, pre-clinical models of adoptive NK cell therapy have relied largely on infusing human NK cells into xenogeneic mouse models which lack a complete tumor microenvironment and an intact immune system that may influence the effectiveness of immune-based therapies (165, 166). We report here an improved expansion platform for canine NK cells on K562 Clone 9.mbIL-21 feeder cells obtaining a median fold expansion of over 20,000 in 3 weeks when supplemented with canine IL-2. To the best of our knowledge, this is the largest fold expansion of NK cells from any mammal outside of human, and is significantly greater than previously reported expansions of NK-like cells in canine (136, 139). Previous expansions of canine CD3⁻ NK-like cells have reported an average fold expansion of 140-fold in 3 weeks using cytokine alone (IL-2 and IL-15), 233-fold expansion in 2 weeks with EL08-1D2 feeder cells following CD5 depletion, and 90-fold expansion with K562 feeder cells and soluble IL-2, IL-15, and IL-21 in 3 weeks (136, 138). It will be of future interest to determine if the improved expansion of canine NK cells with mbIL-21 over mbIL-15 is also due to an increase in telomere length as we have previously demonstrated for human NK cells (165).

Similar to Grondahl-Rosado et al., we found that NKp46 was not constitutively expressed on all CD3⁻/Granzyme B⁺ cells (156). In the present study, we added to

this knowledge, by finding that these NKp46⁻, Null cells could be induced to express NKp46 and have a distinct function- displaying reduced cytotoxicity compared to NKp46⁺ NK cells. In contrast to Grondahl-Rosado et al., we found here that NKp46⁻ cells produce similar amounts of IFN- γ among all other cytokines tested. These differences may be explained by different cell stimulation as Grondahl-Rosado et al. measured IFN- γ stimulation after culturing NKp46⁺ and NKp46⁻ cells together with human IL-2, IL-12, and IL-15 while, in this paper, we stimulated NKp46⁺ and NKp46⁻ cells after sorting with canine IL-2 and K562 expressing membrane-bound IL-21 (**Figure 12b**) (156).

NKp46⁻ NK cells are not unique to canines, but have been previously reported in both porcine and humans, where NKp46 expression also discriminates human NK cells with reduced cytotoxicity. In contrast to our data in canine, porcine NKp46 discriminates NK cells with different IFN- γ secretion but not cytotoxicity (17, 152). Similar to porcine NKp46⁻ NK cells, NKp46 could be induced in canine NKp46⁻ cells; however, NKp46 has not been inducible in human NKp46⁻ cells (152). This may be due to differences in the regulation of NKp46 expression across species, where it is inducible in some species' (porcine and canine) NK cells but not in human, or NKp46 expression may require specific signals that have not been described yet (17).

We found phenotypic similarity between canine NKp46⁻ null cells and both primary and K562 Clone9.mbIL-21-expanded NKp46⁻ human NK cells (CD3⁻/CD56⁺). In humans, NKp46⁻ NK cells are predominantly CD56^{dim}. Expanded NKp46⁻ cells in both species have reduced expression of NKG2D and CD16. Thus,

we speculate that NKp46⁻ canine and human NK cells may represent similar subsets of NK cells in both species; however, additional functional and phenotypic characterizations of NKp46⁻ NK cells are necessary to elucidate this cell type to determine if they are homologous. Primary human NK cells also have reduced expression of CD16, suggesting that this population of NKp46⁻ NK cells is not an artifact of ex vivo expansion. There are several possible explanations for this cell type in humans that will be of interest to explore in future studies.

CD56^{dim}/CD16⁻/NKp46⁻ cells may represent an intermediate step in NK cell maturation between CD56^{bright}/CD16^{low/-}/NKp46⁺ and CD56^{dim}/CD16⁺/NKp46^{dim} NK cells, or CD56^{dim}/NKp46⁻/CD16⁻ cells may be an exhausted NK cell population that were formerly CD56^{dim}/CD16⁺/NKp46⁺ cells and have downregulated CD16 and NKp46 because of senescence.

Contrary to human OS patients, we found no significant difference in circulating NK cells in canine OS compared to healthy canines (74). It will be of future interest to determine if these primary NK cells are functionally impaired, which would be facilitating tumor growth and has been found in several human cancers (167, 168). However, our data demonstrates that expanded NK cells from canine OS patients are active against OS, both primary and metastatic cell lines. This parallels findings in human OS where cytokine-activated NK cells from human OS patients have similar cytotoxicity against both OS cell lines and autologous patient-derived biopsies compared to healthy controls (9). Based on these findings, autologous NK cells may be effective in canine OS.

The human leukemia cell line, K562, has proven to be immune-stimulatory for canine T-cells, B-cells, and NK cells (123, 137-139, 169). Interestingly, similar to what we observed for canine NK cells having optimal proliferation with canine IL-2 over human IL-2, canine B-cells also required species-specific IL-4 for their expansion (169). It is beneficial to the translation of adoptive immune therapies from canine to human that the same cell line used for the generation of human based immune therapies can be used for similar canine therapies with very slight modifications.

In summary, our study furthers the understanding of the canine NK cell phenotype and function. Furthermore, we describe the largest expansion of NK cells from another mammal besides humans, using K562 Clone 9.mbIL-21 feeder cells and canine IL-2. These mbIL-21 expanded NK cells are highly cytotoxic against OS cell lines, suggesting potential for immunotherapy of dogs with OS and potential for therapeutic use in other canine cancers such as lymphoma, leukemia, melanoma, and glioblastoma. This approach will allow for the study of NK cell therapy in an immune-intact, outbred, and spontaneous animal model. Many of these cancers progress rapidly in canines, allowing for rapid testing of NK cell therapies, which is of particular interest in rare and pediatric cancers such as OS where trial accrual is slow. These results support the development of canine NK cell trials to help prioritize the most promising treatment regimens for human clinical trials. Of particular interest is a trial of intratumoral injection of canine NK-like cells expanded on K562 Clone9.mbIL-21 feeder cells after radiation therapy in canine OS that is currently underway (UC Davis, Michael Kent & Bob Canter). The effectiveness of canine NK

cells in combination with other agents, such as cytokines, chemotherapy, and antibodies, will be of particular interest.

Chapter 3: Education of NK cells to be TGF β Resistant

3.1 Background

Immune therapy holds great promise for improving the outcome of cancers such as OS, where survival has not improved over the last 30 years. OS, including chemotherapy-resistant OS, is readily killed *in vitro* by both autologous and allogeneic activated NK cells. However, the tumor microenvironment in OS, and other solid tumors, has elevated levels of the highly immunosuppressive cytokine, transforming growth factor-beta 1 (TGF β) (94, 95). In fact, OS cells secrete TGF β and TGF β levels are elevated in the presence of metastatic disease (91, 95, 170). The addition of a blocking antibody to TGF β improved the efficacy of a dendritic cell vaccine in OS (171), providing proof of principle that TGF β is actively inhibiting immune therapies in OS.

TGF β is part of a family of ligands and receptors that also include the bone morphogenic proteins (BMP), activins, and inhibins. TGF β 's suppression of NK cell function is multi-faceted. TGF β modulates the development of NK cells and subsequently negatively affects their function upon reaching maturation. TGF β promotes an immature NK cell lineage, by preventing the progression of NK cells into CD16⁺ NK cells. CD16 identifies mature, cytolytic NK cells. TGF β can also induce formerly CD16⁺ NK cells to become CD16⁻ (172). In addition, mice expressing

a dominant negative TGF β R on NK cells have increased numbers of mature NK cells compared to wild-type mice (173-175).

In mature NK cells, TGF β inhibits anti-tumor activity through multiple mechanisms. TGF β decreases IL-2 and IL-15 induced NK cell proliferation (87, 176), and IL-15 induced mTOR activation (174). TGF β also inhibits IFN γ secretion, which is important for stimulating the adaptive immune system and can sensitive tumors to NK cell lysis. TGF β inhibits IFN γ both directly and indirectly. SMAD3 directly binds to the IFN γ promoter and can also inhibit IFN γ indirectly by decreasing expression of the IFN γ -promoting transcription factors, T-bet and E4BP4 (99, 100, 177). However, TGF β mediated inhibition of IFN γ secretion can be partially alleviated by pre-incubation of NK cells with IL-12, IL-15, or IL-18 (100). Furthermore, TGF β also inhibits TNF α and GM-CSF secretion and modulates chemokine receptor expression to promote the retention of NK cells within the bone marrow (97).

Specifically, TGF β mediates its' inhibition of NK cell cytotoxicity by decreasing Granzyme and Perforin secretion, and expression of the following activating receptors: NKG2D, NKp30, KIRs, DNAM-1, NKp44, TRAIL, and CD16. This therein inhibits NK cell recognition of malignant cells expressing their cognate ligands. Cell-surface expression of NKG2D, NKp30, and NKp44 is inhibited by downregulation of DAP10 and DAP12 adaptor proteins required for cell-surface expression (87, 96, 101, 172, 176, 178-181).

In NK cell biology, the roles of the other TGF β family ligands and receptors have been less extensively studied, but have distinct characteristics from TGF β . The activin family is suppressive like TGF β but activin only suppresses NK cell cytokine

secretion and proliferation; and not cytotoxicity (182-184). In contrast to TGF β and activin, BMP ligands enhance NK cell cytotoxicity and cytokine production (185).

TGF β signaling pathways are grouped into canonical signaling which is SMAD-dependent and non-canonical signaling pathways which are SMAD-independent. For both canonical and non-canonical signaling, TGF β homodimers bind to TGF β RI and TGF β RII, causing phosphorylation of TGF β R1 by TGF β RII. TGF β R1 subsequently phosphorylates SMAD2 and SMAD3 at serine residues thereby facilitating their binding to SMAD4. Complexes of SMAD2 or SMAD3 with SMAD4 translocate to the nucleus where they act on their target genes, such as IFN γ in NK cells. SMAD2 and SMAD3 are highly structurally similar, and often have redundant functions. However, SMAD3 can directly bind to DNA without SMAD4, whereas SMAD2 requires complex formation with SMAD4 to mediate DNA-binding (99, 100, 102, 110). In addition, in Th17 cells, SMAD2 and SMAD3 have strikingly different functions with SMAD2 promoting STAT3 activation, which is necessary for Th17 cell development, while SMAD3 inhibits STAT3 activation (186). Non-canonical TGF β signaling includes several pathways such as Rhoa, PI3K/AKT, Erk, and MAPK pathways (102).

TGF β signaling can be inhibited by SMAD6 and SMAD7. SMAD6 inhibits SMAD1 which is activated in response to BMP signaling. SMAD6 also inhibits the non-canonical TGF β pathway. SMAD7 promotes SMURF mediated ubiquitination of TGF β Rs, leading to degradation. SMAD7 can also inhibit BMP signaling. IFN γ and TNF α activate SMAD7 to antagonize TGF β (187-189).

There have been several approaches towards generating NK and T-cells resistant to TGF β . These include dominant negative TGF β RII expressing cells and combination therapies using TGF β small molecule inhibitors with immune-based therapies. Importantly, all of these methods have demonstrated increased *in vitro* and *in vivo* efficacy of NK and T-cell therapies (101, 190-197). However, broad spectrum inhibition of TGF β has potential for adverse side effects; since TGF β signaling is context dependent and can have both tumor-promoting and tumor-suppressive effects. For example, the inhibition of TGF β in murine models increased the number of circulating tumor cells (102), and broad spectrum inhibition of TGF β causes profound autoimmune disease (198). Therefore, inhibition of TGF β should be done only with caution and after determining the tumor's responsiveness to TGF β . Thus, non-systemic methods of generating innate TGF β resistance are a promising alternative to avoid the adverse effects that systemic TGF β inhibition may have.

The Lee Lab has developed a platform for expanding human NK cells using K562 feeder cells expressing 41BBL and membrane-bound IL-21. These NK cells expand on average 30,000-fold in 3 weeks and are highly cytotoxic against tumors of all types, including OS. However, they remain sensitive to suppression by TGF β , and thus, the effectiveness of adoptive NK cell therapy remains suboptimal.

Thus, the goal of this aim was to develop an approach towards generating NK cells that have intrinsic resistance to TGF β suppression of NK anti-tumor function. To this end, we **hypothesized** that expanding NK cells in the presence of TGF β with K562 feeder cells expressing membrane-bound IL-21 would inhibit the growth of NK cells that are sensitive to TGF β suppression and allow for NK cells with reduced

sensitivity to TGF β to grow, since NK cells have a range of sensitivity to TGF β (175). We hypothesized that this would result in less NK cells post-expansion than our Standard approach (without TGF β), but that the TGF β expanded NK cells- Resistant NK cells (ReNK), would have improved anti-tumor function and reduced sensitivity to TGF β .

3.2 Results

3.2.1 ReNK cells have similar proliferation compared to Standard NK cells.

Surprisingly, ReNK cells had similar overall 2 week proliferation compared to Standard NK cells. However, ReNK cells had significantly decreased proliferation during the first week ($p=0.01$). We also observed that during the 2nd week of expansion ReNK cells had a trend towards increased proliferation compared to Standard NK cells ($p=0.08$) (**Figure 16**).

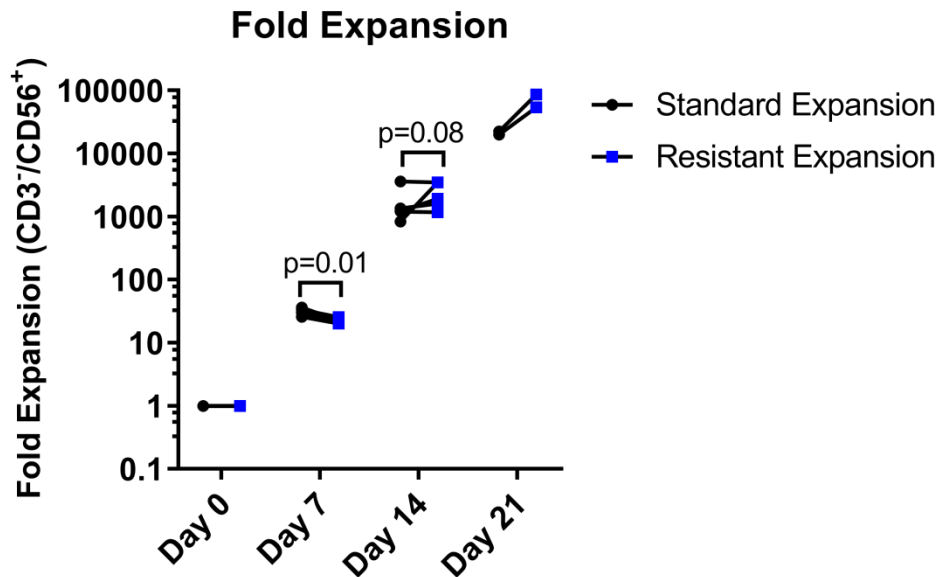


Figure 16. Proliferation of ReNK cells. ReNK cells have decreased proliferation in the 1st week of expansion, but a trend towards increased proliferation in the 2nd week of expansion.

3.2.2 ReNK cells retain high cytolytic capacity.

Next, we wanted to determine the anti-tumor function of ReNK cells. ReNK cell anti-tumor response was measured by a 3-hour degranulation assay for CD107a. To this end, NK cells were incubated with tumor cells with CD107a antibody and monensin to block degradation of CD107a. ReNK cells had significantly increased degranulation both in the presence (R/+) and absence (R/-) of TGF β against DAOY and MG63, but no significant difference in degranulation against HOS. Interestingly, degranulation was only inhibited in Standard NK cells in the presence of TGF β against HOS (S/+), but not DAOY or MG63 cells (Standard Expansion/No TGF β = S/-) (**Figure 17**) (Mean \pm SD, MG63: S/- = 11.3 ± 3.5 , S/+ = 9.0 ± 3.1 , R/- = 21.9 ± 9.8 , R/+ = 20.5 ± 9.7 ; HOS: S/- = 28.1 ± 16.1 , S/+ = 24.3 ± 13.7 , R/-

= 32.8 ± 12.2 , R/+ = 31.2 ± 9.9 ; DAOY: S/- = 14.0 ± 7.8 , S/+ = 11.0 ± 6.4 , R/- = 37.2 ± 17.3 , R/+ = 37.5 ± 16.3).

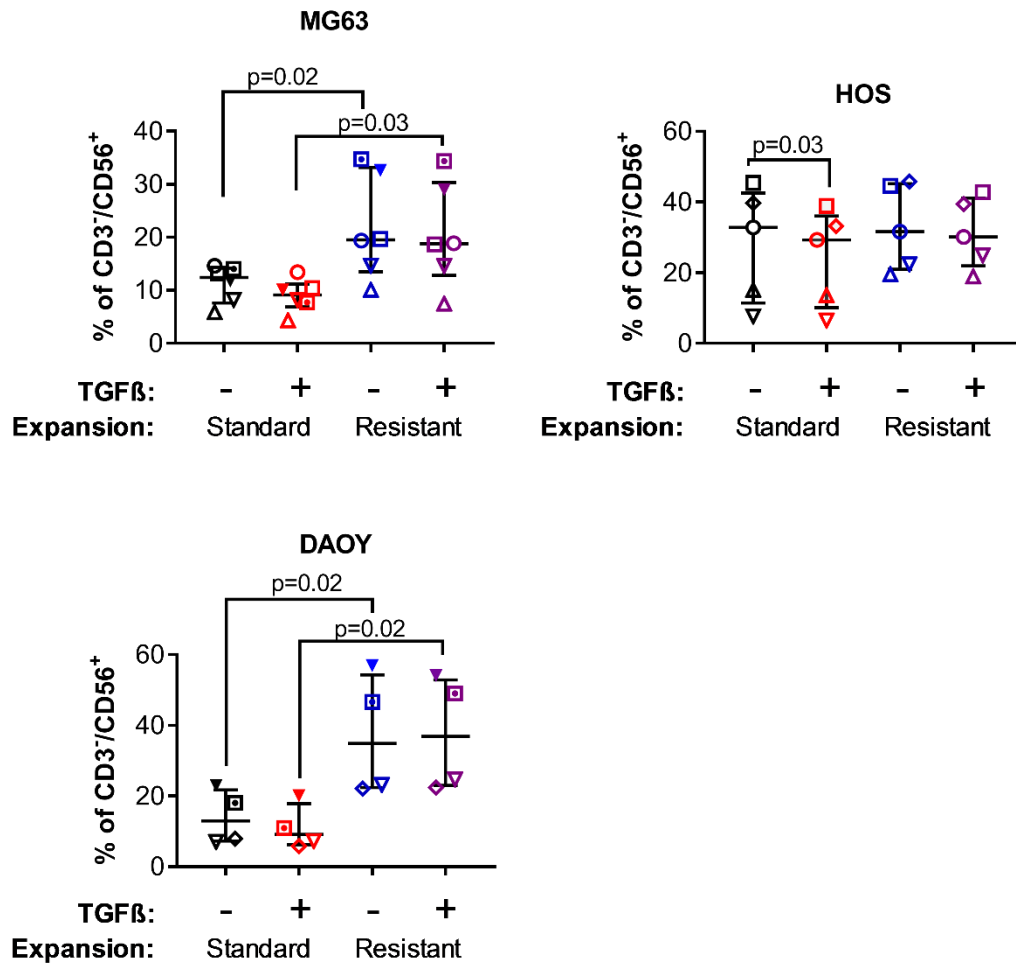


Figure 17. ReNK cells have increased degranulation. Day 14

Expanded NK cells were rested overnight with or without 10 ng/mL TGFβ, and then cultured with tumor cells for 3 hours to assess degranulation.

ReNK cells have increased degranulation against DAOY and MG63 plus and minus TGFβ treatment. Degranulation is not significantly different between Standard and ReNK cells against HOS. Median ± IQR depicted (n=4-6 donors).

Since degranulation does not always correlate with tumor cell death, we next assessed tumor cell lysis by ReNK and Standard NK cells. To this end, ReNK and Standard NK cells were rested overnight in the presence or absence of 10 ng/mL of TGF β and then co-cultured with calcein-AM labeled tumors for a 4-hour cytotoxicity assay under the same conditions as the degranulation assay. Preliminary data in the lab found that ReNK cells had increased cytotoxicity in both the presence and absence of TGF β against the medulloblastoma cell line, DAOY. ReNK had less inhibition by TGF β (**Figure 18a**), and increased cytotoxicity (**Figure 18b**).

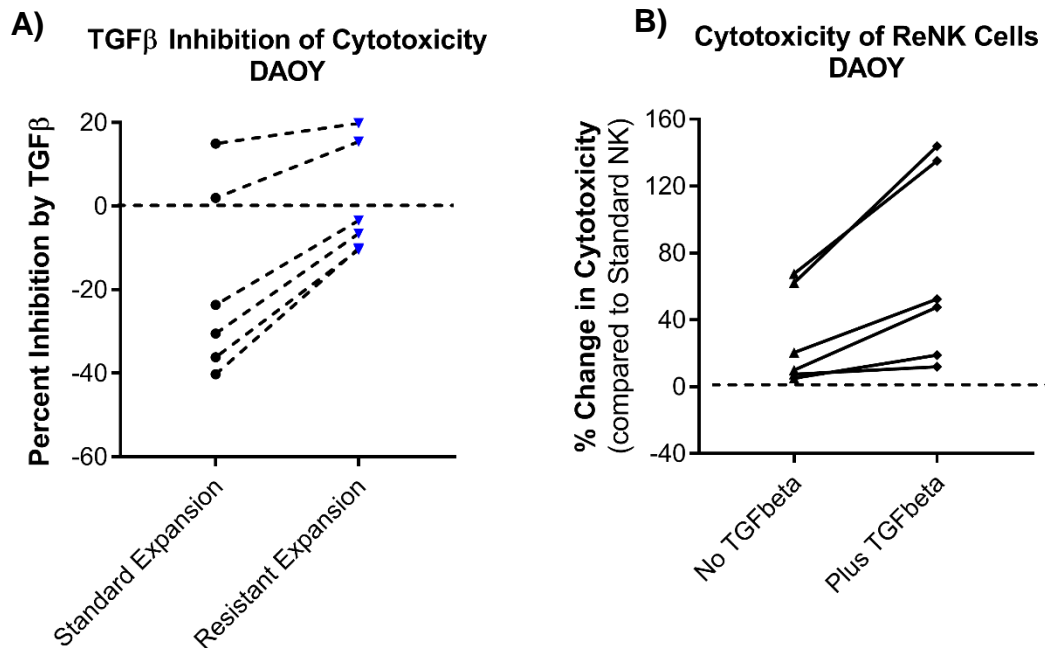
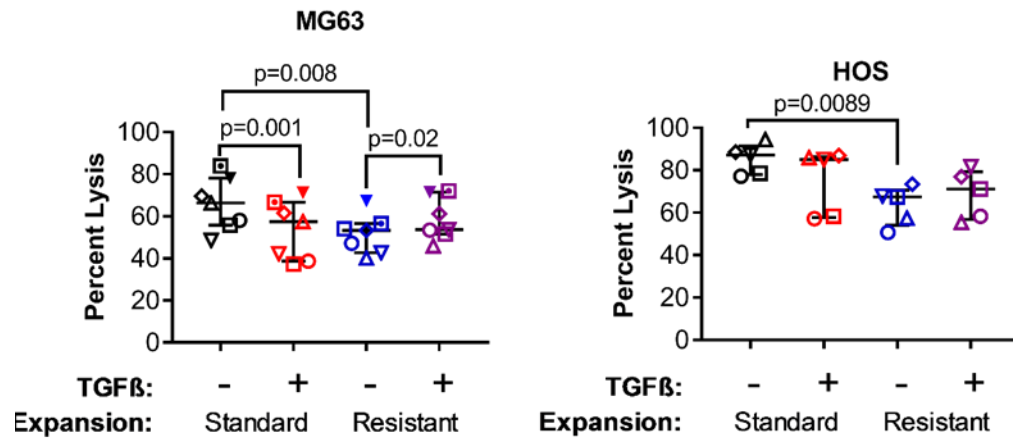


Figure 18. ReNK cells have increased killing of DAOY. Expanded NK cells were rested overnight plus or minus TGF β and **A)** ReNK cells were found to have decreased sensitivity to TGF β and to **B)** kill better than Standard NK cells in the presence and absence of TGF β (n=6). 4/6 donors were used with permission from Geoffrey Kannan.

We wanted to extend these findings to OS. Against OS cell lines MG63 and HOS, ReNK cells had decreased cytotoxicity without TGF β in the media compared to Standard NK cells (**Figure 14a**) (MG63: S/- = 65.8 ± 12.6 S/+ = 53.6 ± 14 , R/- = 51.6 ± 9.2 , R/+ = 58.5 ± 10.2 ; HOS: S/- = 85.2 ± 7.2 S/+ = 74.7 ± 15.5 , R/- = 63.4 ± 9.1 , R/+ = 68.7 ± 11.5). There was no decrease in killing in the presence of TGF β for ReNK. Unexpectedly, ReNK cells killed MG63 significantly better in the presence of TGF β than without TGF β .

To extend these findings to other tumors, we tested ReNK cells against the medulloblastoma cell lines, D283 and D425. Against these cell lines, cytotoxicity was variable with ReNK cells having similar cytotoxicity in all conditions against D425, but reduced cytotoxicity against D283 cells (**Figure 19**) (D283: S/- = 61.1 ± 11.9 , S/+ = 52.9 ± 6.7 , R/- = 17.8 ± 6.4 , R/+ = 30.1 ± 6.5 ; D425: S/- = 60.1 ± 12.8 S/+ = 51.0 ± 5.2 , R/- = 42.4 ± 4.7 , R/+ = 40.4 ± 11.1). Interestingly, Standard NK cell cytotoxicity in the presence of TGF β was only significantly inhibited against MG63 and DAOY, with HOS, D283, and D425 exhibiting a trend towards decreased cytotoxicity. As with MG63, ReNK cells killed D283 significantly better in the presence of TGF β than without.

A)



B)

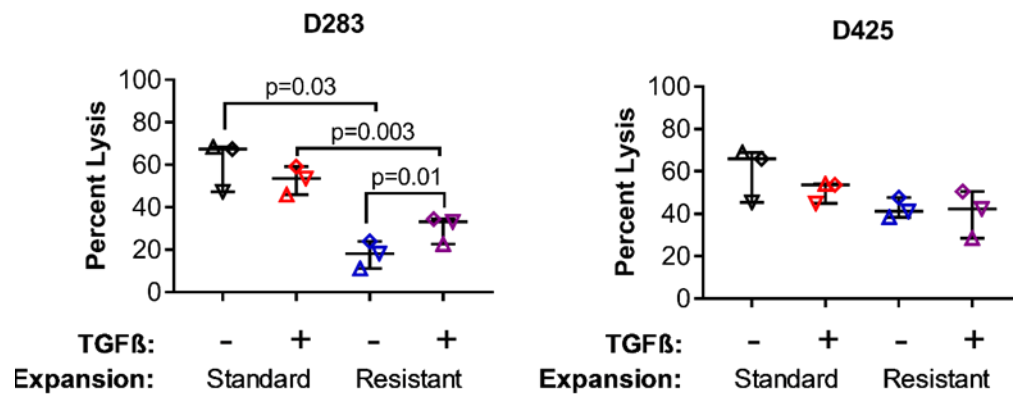


Figure 19. ReNK cells have variable cytotoxicity. Day 14 Expanded ReNK cells have decreased killing in the absence of TGFβ against MG63, HOS, and D283. ReNK cells have decreased cytotoxicity in the presence of TGFβ against D283, but similar killing against all other cell lines in the presence of TGFβ. Median ± IQR shown (n=3-7 donors).

Since ReNK cells killed MG63 and D283 more efficiently in TGF β than without TGF β , we hypothesized that receptor expression may be changed in the overnight treatment of the Standard and ReNK cells. To this end, we assessed the cell surface expression of TRAIL, NKG2D, and FasL; and the intracellular expression of Granzyme A, Granzyme B, and Perforin. We observed no significant differences in Standard NK cell expression of TRAIL, FasL, Granzyme A, Granzyme B, or Perforin with overnight treatment with TGF β . However, we did observe a trend towards decreased NKG2D expression level (MFI) in Standard NK cells rested overnight in TGF β that did not reach significance, likely because of the low sample number (**Figure 20a**) (% Positive- TRAIL= S/-: 28.3 ± 11.6 , S/+ 29.7 ± 11.0 , NKG2D= S/-: 99.8 ± 0.1 , S/+ 99.4 ± 0.3 , FasL= S/-: 20.4 ± 5.0 , S/+ 19.8 ± 7.5 , Granzyme A= S/-: 100 ± 0 , S/+ 100 ± 0 , Granzyme B= S/-: 57.3 ± 10.3 , S/+ 58.6 ± 12.5 , Perforin= S/-: 99.8 ± 0 , S/+ 99.3 ± 0.4 ; MFI- TRAIL= S/-: 557 ± 285 , S/+ 566 ± 297 , NKG2D= S/-: 11545 ± 3406 , S/+ 6022 ± 1019 , FasL= S/-: 231.6 ± 33 , S/+ 216.8 ± 73 , Granzyme A= S/-: 8433 ± 1058 , S/+ 7217 ± 919 , Granzyme B= S/-: 547.4 ± 120.4 , S/+ 561 ± 127 , Perforin= S/-: 5172 ± 737 , S/+ 3927 ± 922).

In ReNK cells, we observed a slight statistically significant decrease in the percentage of NK cells expressing cell surface FasL with TGF β treatment. We did not observe any differences in expression of other proteins in ReNK cells rested overnight in TGF β (Figure 15) (% Positive- TRAIL= R/-: 50.8 ± 14.1 , R/+ 53.9 ± 12.1 , NKG2D= R/-: 99.9 ± 0.1 , R/+ 99.7 ± 0.4 , FasL= R/-: 64.1 ± 12.0 , R/+ 60.3 ± 11.9 , Granzyme A= R/-: 99.9 ± 0 , R/+ 99.8 ± 0 , Granzyme B= R/-: 49.0 ± 14.7 , R/+ 50.4 ± 20.2 , Perforin= R/-: 97.1 ± 1.6 , R/+ 94 ± 1.1 ; MFI- TRAIL= R/-: 50.8 ± 14.1 , R/+ 53.9

± 12.1 , NKG2D= R/-: 99.9 ± 0.1 , R/+ 99.7 ± 0.4 , FasL= R/-: 64.1 ± 12.0 , R/+ 60.3 ± 11.9 , Granzyme A= R/-: 99.9 ± 0 , R/+ 99.8 ± 0 , Granzyme B= R/-: 49.0 ± 14.7 , R/+ 50.4 ± 20.2 , Perforin= R/-: 97.1 ± 1.6 , R/+ 94 ± 1.1 ; MFI- TRAIL= R/-: 1045 ± 459 , R/+ 1141 ± 420 , NKG2D= R/-: 10671 ± 1267 , R/+ 8148 ± 2634 , FasL= R/-: 685 ± 147 , R/+ 639 ± 138 , Granzyme A= R/-: 5864 ± 196 , R/+ 5561 ± 288 , Granzyme B= R/-: 480 ± 157 , R/+ 505 ± 221 , Perforin= R/-: 1727 ± 226 , R/+ 1331 ± 141) (**Figure 20b**).

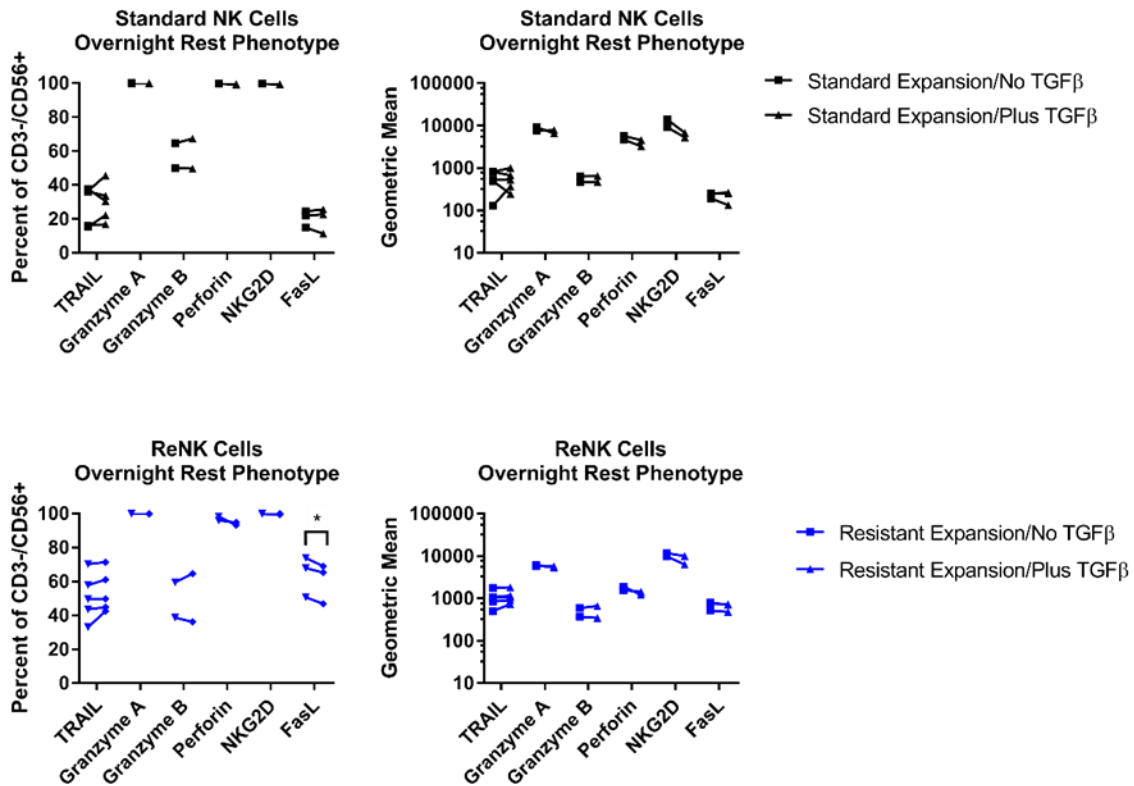


Figure 20. Effect of Overnight TGF β Treatment on Standard and ReNK cell Receptor Expression. Overnight TGF β treatment decreases MFI for Perforin and NKG2D in both Standard and ReNK. In ReNK cells, TGF β treatment has a slight but significant decrease in FasL+ NK cells (n=2-3).

3.2.3 ReNK cells have increased cytokine secretion.

IL-6, IFN γ , and TNF α are three cytokines that can inhibit TGF β signaling (199). In turn, TGF β is also able to inhibit NK cell IFN γ and TNF α secretion. We first assessed mRNA levels of IFN γ , TNF α , and IL-6 at Day 14 of expansion in Standard and ReNK cells using qPCR. No significant difference was observed (**Figure 21**) (Fold Change of ReNK= IFN γ : 2.1 ± 2.5 , TNF α : 0.7 ± 0.3 , IL-6: 1.1 ± 1.0 , n=5 for IFN γ , n=4 for IL-6 & TNF α).

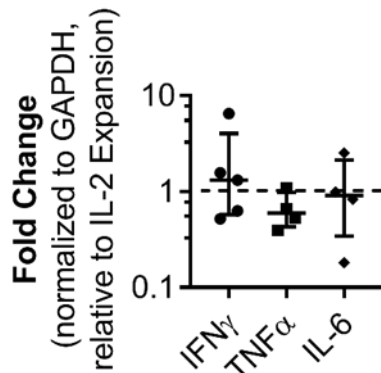


Figure 21. Gene expression of TGF β

Antagonistic Cytokines. Day 14 Expanded ReNK cells have similar gene expression for IFN γ , TNF α , and IL-6 as Standard NK cells. Median \pm IQR depicted (n=4-5 donors).

Next, we wanted to determine if ReNK cells produce more IL-6, IFN γ , and TNF α producing NK cells upon co-culture with tumor cells using intracellular flow cytometry. After 3 hours, we found that basal IL-6 production (no tumor target) was significantly elevated in ReNK cells in both the presence and absence of TGF β (**Figure 22a**) (S/- = 19.4 ± 5.1 , S/+ = 18.8 ± 6.0 , R/- = 24.8 ± 8.3 , R/+ = 24.1 ± 8.7). For HOS, IL-6 anti-tumor production was not significantly different in any of the groups; however, against MG63, IL-6 was significantly elevated in ReNK cells both with and without TGF β (**Figure 22b**) (HOS: S/- = 12.2 ± 6.6 , S/+ = 11.1 ± 5.8 , R/- = 13.1 ± 6.7 , R/+ = 13.5 ± 7.2 ; MG63: S/- = 8.4 ± 1.2 , S/+ = 8.5 ± 1.8 , R/- = 10.8 ± 1.1 , R/+ = $10.7 \pm$

1.3). Surprisingly, co-culture of both Standard and ReNK cells with either HOS or MG63 decreased the percentage of NK cells producing IL-6 compared to basal IL-6 production.

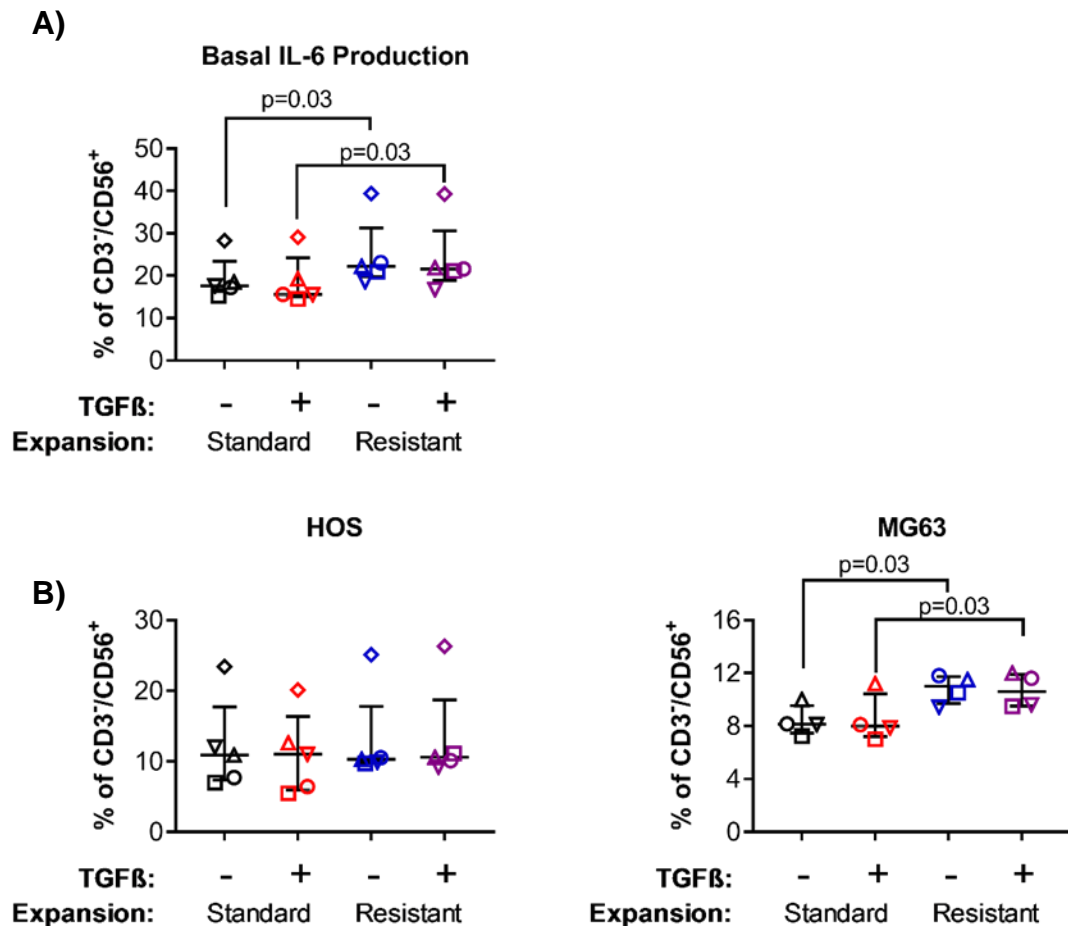


Figure 22. IL-6 Production of ReNK cells. A) ReNK cells have production of IL-6 without stimulation, **B)** and increased anti-tumor IL-6 production against MG63, but not HOS. Median \pm IQR (n= 4-5 donors).

For IFN γ and TNF α , there was negligible production at baseline (no tumor target); therefore, for IFN γ and TNF α , the percentage of NK cells producing cytokines in response to tumor was normalized to the basal percentage of NK cells

producing the cytokine without target (Anti-tumor cytokine production= % NK producing cytokine with target minus % NK producing cytokine with no target). We found a significant increase in the percentage of IFN γ and TNF α producing NK in ReNK cells without TGF β for MG63, HOS, and DAOY. In addition, there was significantly increased IFN γ and TNF α producing NK cells in the presence and absence of TGF β in ReNK cells compared to Standard NK cells against MG63 and HOS. We also observed a slight, but significant decrease in IFN γ producing ReNK cells with TGF β compared to no TGF β for MG63. DAOY had significantly increased TNF α producing ReNK cells in the presence of TGF β , and a trend towards increased IFN γ producing ReNK cells with TGF β (**Figure 23a, 24a**). (IFN γ - MG63: S/- = 7.5 ± 2.9 , S/+ = 6.6 ± 2.1 , R/- = 19.1 ± 5.4 , R/+ = 17.3 ± 6.6 ; HOS: S/- = 20.1 ± 11 , S/+ = 18.2 ± 9.8 , R/- = 41.6 ± 14.7 , R/+ = 38.2 ± 13.7 ; DAOY: S/- = 5.4 ± 4.5 , S/+ = 5.7 ± 5.3 , R/- = 26.8 ± 19.2 , R/+ = 28 ± 19.5) (TNF α - MG63: S/- = 12.7 ± 5.6 , S/+ = 12.6 ± 4.7 , R/- = 20.2 ± 6.4 , R/+ = 19.2 ± 6.6 ; HOS: S/- = 27.8 ± 16.9 , S/+ = 28.6 ± 15.7 , R/- = 38.1 ± 15.6 , R/+ = 36.9 ± 15.5 ; DAOY: S/- = 8.9 ± 4.8 , S/+ = 12.6 ± 8.7 , R/- = 27.3 ± 14.5 , R/+ = 28.9 ± 14.2).

Next, we wanted to determine if IFN γ + and TNF α + ReNK cells had higher production of IFN γ and TNF α than Standard NK cells. ReNK cells had increased cytokine production as measured by MFI in both IFN γ + or TNF α + NK cells both plus and minus TGF β compared to Standard NK cells against MG63 and HOS. For DAOY, ReNK cells produced more TNF α both plus and minus TGF β and more IFN γ in the absence of TGF β than Standard NK cells (**Figure 23b, 24b**). This suggests that ReNK cells not only have more cytokine producing NK cells but also produce

more cytokines per cell. (IFN γ - MG63: S/- = 1835 ± 524.8 , S/+ = 1851 ± 532.8 , R/- = 2657 ± 859.4 , R/+ = 2597 ± 819.2 ; HOS: S/- = 2352 ± 420 , S/+ = 2349 ± 436.7 , R/- = 3771 ± 1107 , R/+ = 3633 ± 1017 ; DAOY: S/- = 1584 ± 704.5 , S/+ = 1636 ± 696.6 , R/- = 2256 ± 586.9 , R/+ = 2241 ± 508.4) (TNF α - MG63: S/- = 1637 ± 444.5 , S/+ = 1672 ± 451.4 , R/- = 2479 ± 391.4 , R/+ = 2456 ± 408.7 ; HOS: S/- = 2279 ± 626.7 , S/+ = 2332 ± 680.6 , R/- = 3438 ± 819.5 , R/+ = 3428 ± 751.1 ; DAOY: S/- = 835.9 ± 100.1 , S/+ = 883.5 ± 147 , R/- = 1471 ± 431.5 , R/+ = $1432. \pm 402.2$).

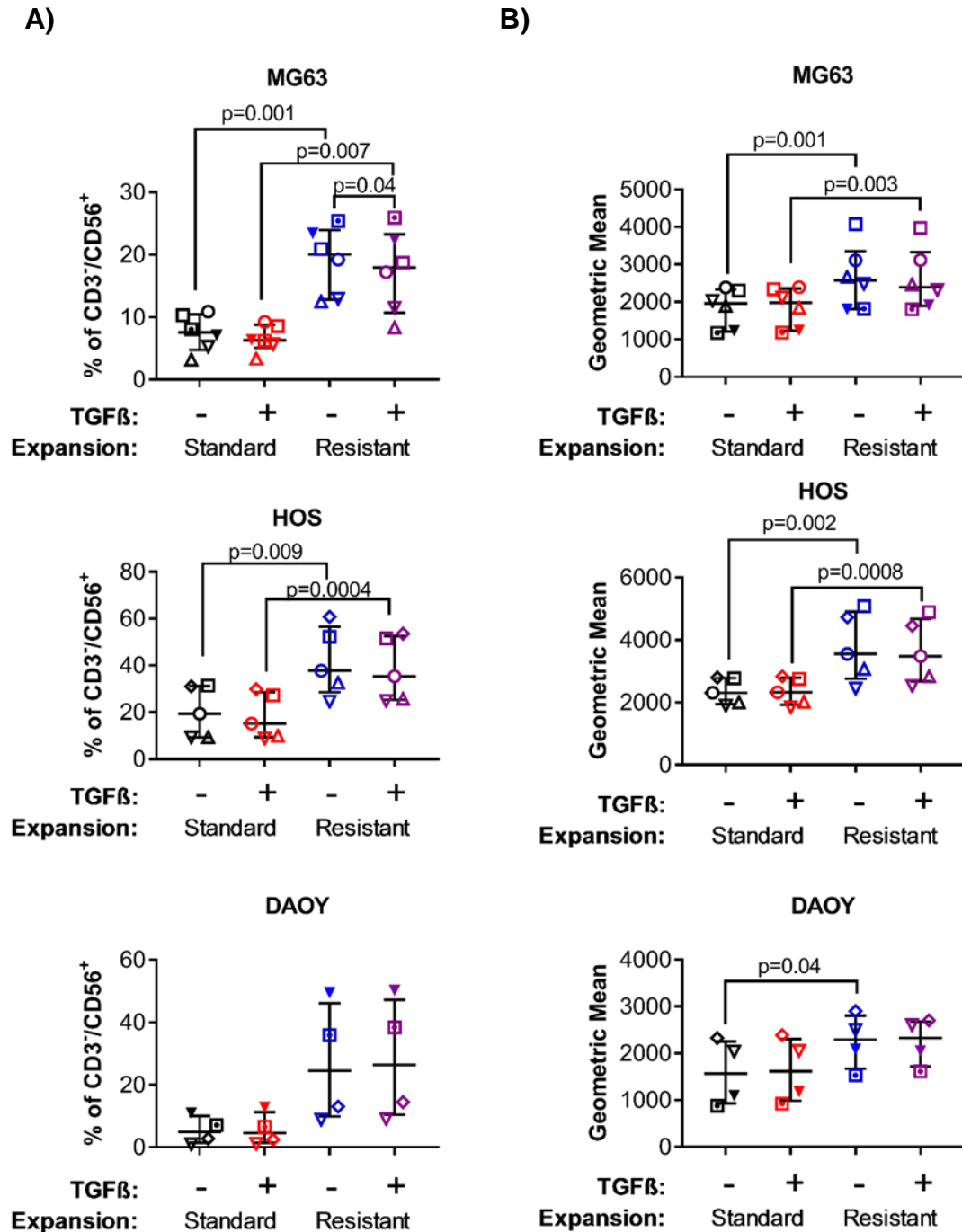


Figure 23. ReNK cells have increased IFN γ production. A) ReNK cells have significantly increased IFN γ + NK cells and **B)** IFN γ + ReNK cells have increased expression of IFN γ than Standard NK cells both plus and minus TGF β . Median \pm IQR depicted (n=4-6 donors).

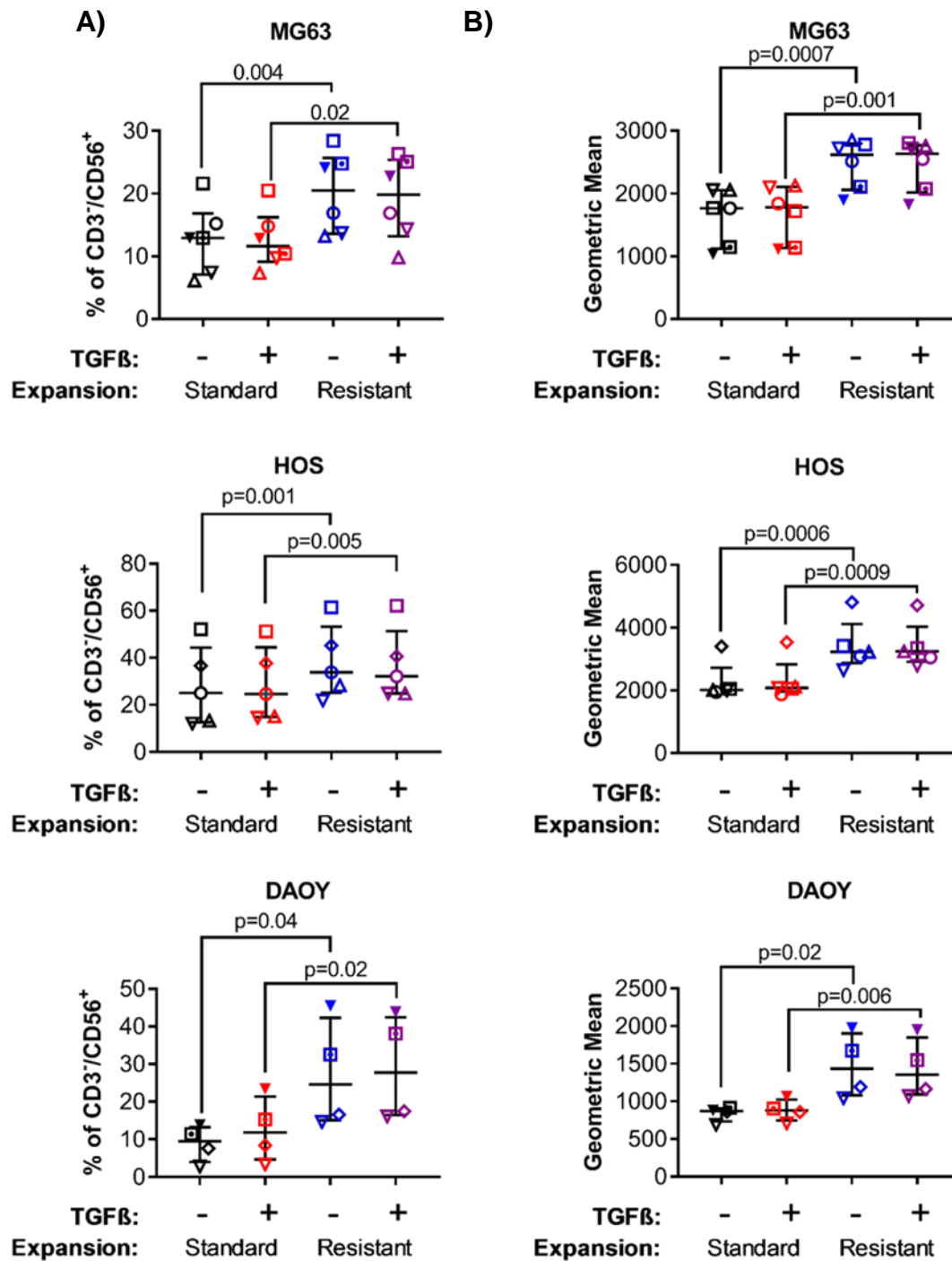


Figure 24. ReNK cells have increased TNFα⁺ production. A) ReNK cells have significantly increased TNFα⁺ NK cells and **B)** increased expression of TNFα compared to Standard NK cells both plus and minus TNFα. Median ± IQR (n=4-6 donors).

However, since TGF β has been reported to inhibit secretion but not production of Granzymes, we next wanted to confirm that ReNK cells are actually secreting more IFN γ and TNF α . To this end, Standard and ReNK cells were incubated with tumor targets as described above and supernatants were collected to measure IFN γ and TNF α secretion with Cytometric Bead Array. For secretion, TGF β did exhibit the expected inhibition of IFN γ secretion in the Standard expanded NK cells for MG63 but not for HOS and DAOY, nor did TGF β significantly inhibit Standard or ReNK cell secretion of TNF α for any cell line. In ReNK cells, TGF β inhibited ReNK cell secretion of IFN γ against MG63 and HOS. However, similar to the IFN γ and TNF α production data, we found a significant increase in IFN γ (HOS, MG63) secretion in both the presence and absence of TGF β compared to Standard expanded NK cells (**Figure 25, 26**). (IFN γ - MG63: S/- = 133.3 ± 109.9 , S/+ = 75.8 ± 73.4 , R/- = 862.6 ± 420.7 , R/+ = 684.4 ± 392.4 ; HOS: S/- = 209.9 ± 267.5 , S/+ = 97.9 ± 129.6 , R/- = 1020 ± 562.9 , R/+ = 863.6 ± 498.1 ; DAOY: S/- = 57.4 ± 68.2 , S/+ = 35.1 ± 57.7 , R/- = 1197 ± 953.4 , R/+ = 1002 ± 903.3).

TNF α was significantly increased in ReNK cells without TGF β in MG63 and HOS, and significantly increased in the presence of TGF β for MG63. ReNK cells had a trend towards increased IFN γ or TNF α secretion against DAOY (**Figure 25, 26**) (TNF α - MG63: S/- = 226.3 ± 60.9 , S/+ = 182.3 ± 32.7 , R/- = 666.5 ± 178.8 , R/+ = 632.2 ± 121.9 ; HOS: S/- = 249.9 ± 126.1 , S/+ = 243.1 ± 46.3 , R/- = 771.2 ± 139.3 , R/+ = 836.8 ± 121.6 ; DAOY: S/- = 57.6 ± 49.9 , S/+ = 53.4 ± 51.5 , R/- = 665.9 ± 513.1 , R/+ = 567.3 ± 512.3). Thus, TGF β in combination with IL-21 leads to NK cells with

increased anti-tumor IFN γ and TNF α secretion compared to Standard NK cells, expanded with IL-21 only.

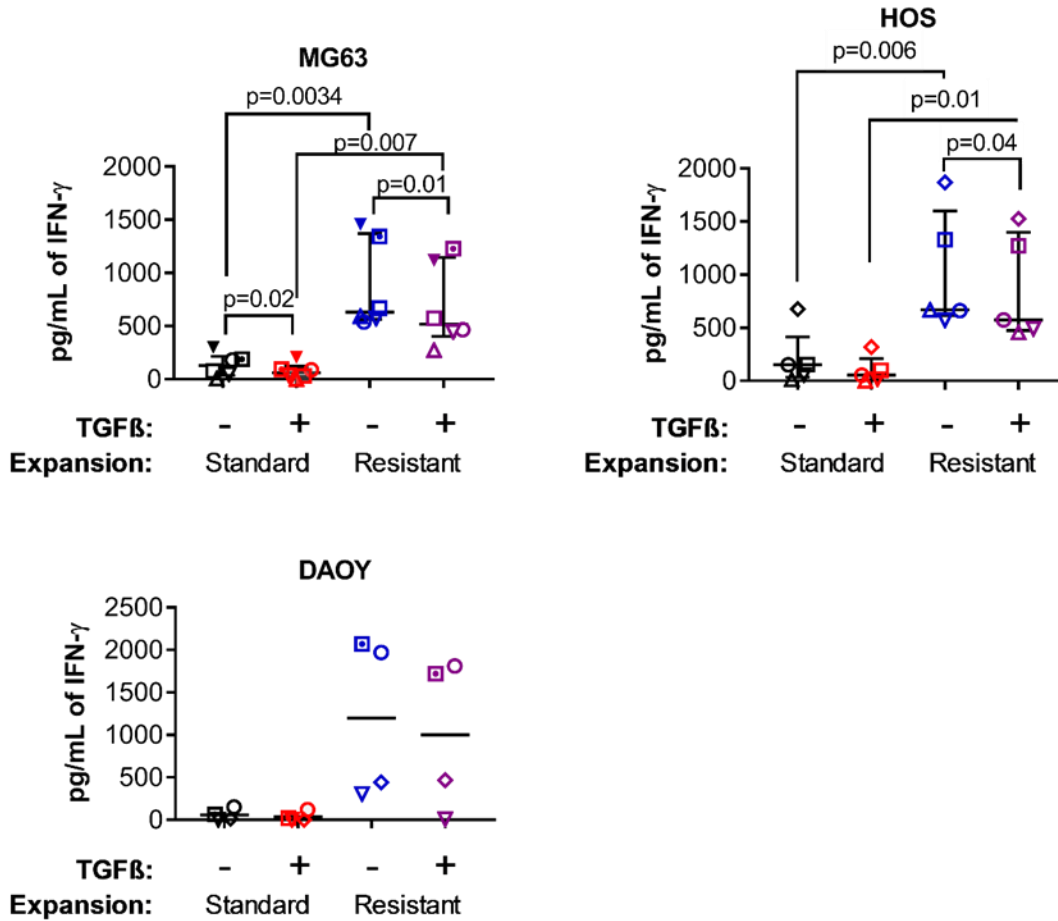


Figure 25. ReNK cells have increased IFN γ secretion. ReNK cells have increased IFN γ secretion compared to Standard NK cells, and have slight inhibition of IFN γ secretion with TGF β treatment against MG63 and HOS. Median \pm IQR (n=4-6 donors).

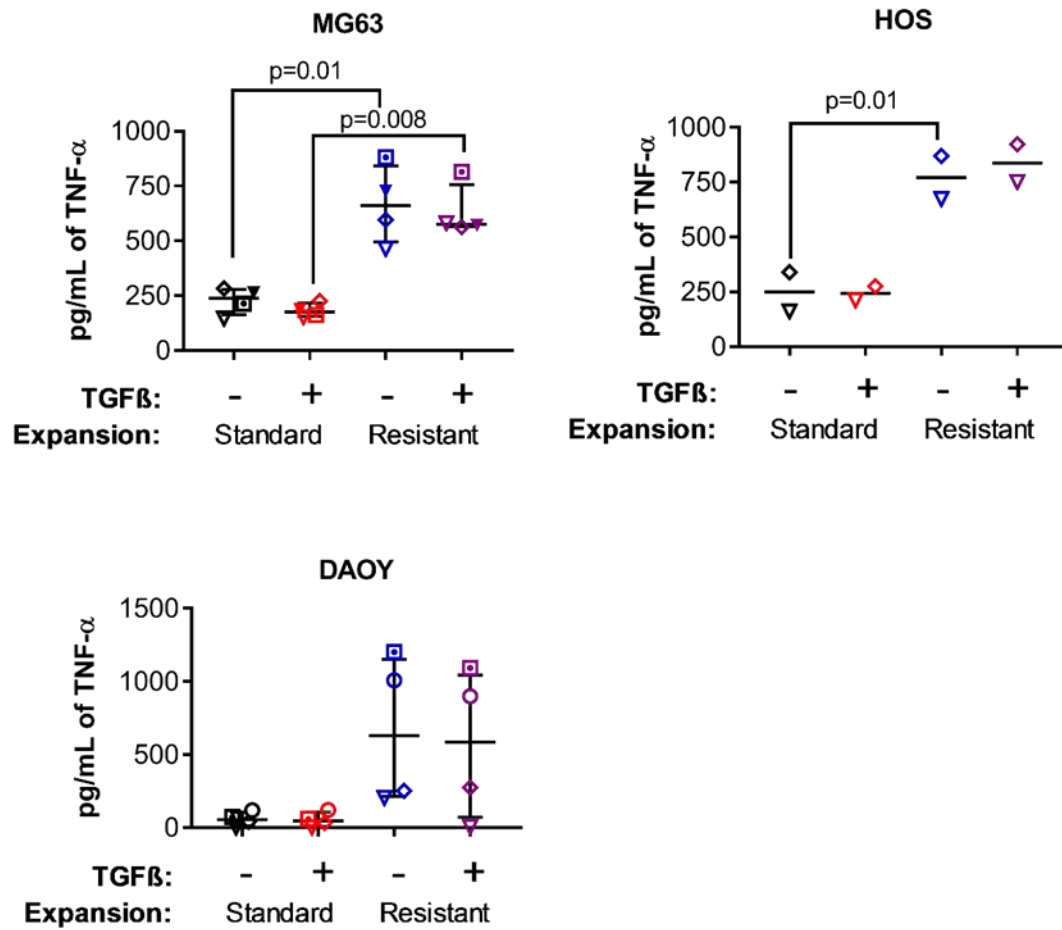


Figure 26. ReNK cells have increased TNF α secretion. ReNK cells have significantly increased TNF α secretion with no TGF β against MG63 and HOS and significantly increased TNF α secretion against MG63 in the presence of TGF β . Median \pm IQR (n=2-4 donors).

3.2.4 ReNK cells have altered NK cell Phenotype.

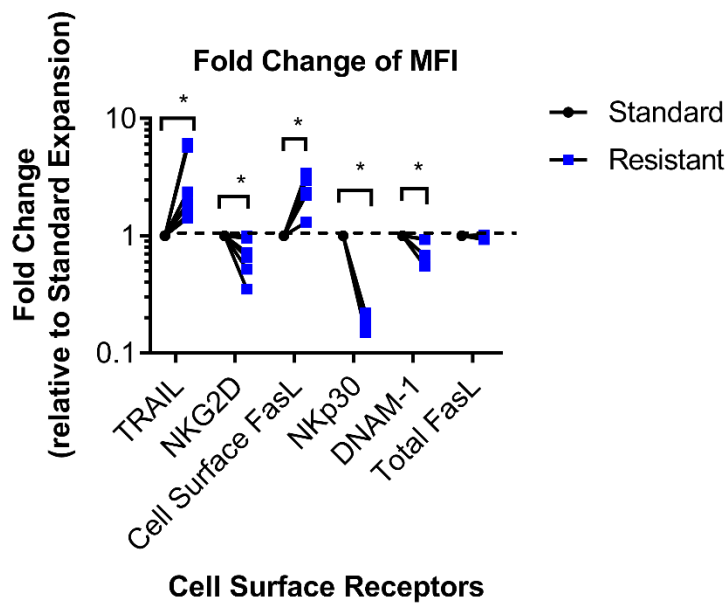
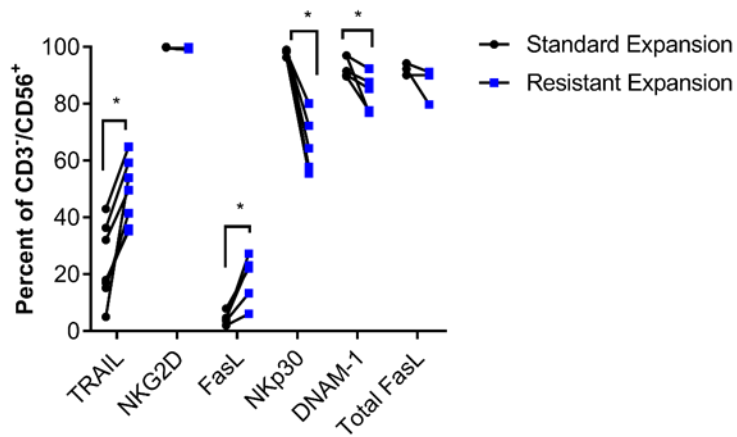
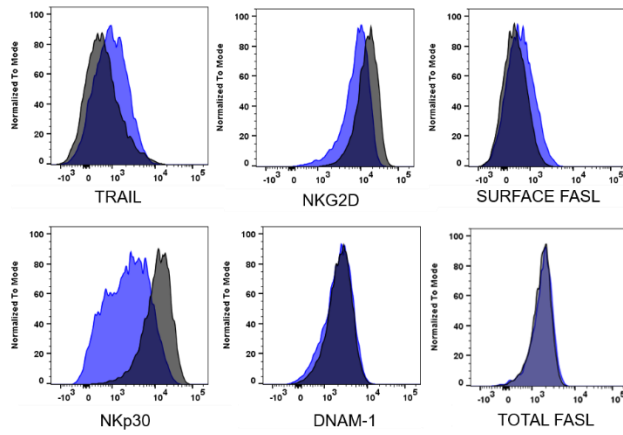
Since TGF β has been reported to decrease NK cell expression of NKG2D, NKp30, and DNAM-1, we assessed the expression of NK receptors on Day 14 expanded NK cells using flow cytometry. As expected, ReNK cells had a significant decrease in the percent of NK cells expressing NKp30 and DNAM-1 (**Figure 27**) (% Positive= NKp30: S: 98.1 ± 1.0 R: 66.0 ± 10.2 ; DNAM-1: S: 93.1 ± 3.6 R: 84.0 ± 6.7 ; Fold Change of ReNK= NKp30: 0.19 ± 0.03 , DNAM-1: 0.76 ± 0.17), and a significant decrease in fold change of expression level of NKp30 and DNAM-1 as measured by MFI (**Figure 28**). For NKG2D, no difference in the percent of NK cells expressing NKG2D was observed; however, there was a significant fold decrease in NKG2D MFI compared to Standard NK cells (% Positive= NKG2D: S: 99.9 ± 0.2 R: 99.5 ± 0.3 ; ReNK Fold Change: 0.70 ± 0.23). Surprisingly, ReNK cells had an increase in TRAIL and cell surface FasL positive NK cells and increased expression level of both TRAIL and cell surface FasL. However, there was no difference in expression of total FasL (cell surface + intracellular FasL) (**Figure 27, 28**) (% Positive= TRAIL: S: 23.8 ± 13.6 R: 48.6 ± 11.5 ; Cell Surface FasL: S: 4.5 ± 2.2 R: 18.4 ± 8.5 ; Total FasL: S: 92.2 ± 2.1 R: 87.0 ± 6.3 ; Fold Change of ReNK= TRAIL: 3.0 ± 2.0 , Cell Surface FasL: 2.5 ± 0.8 , Total FasL: 1.0 ± 0).

Next, the expression of intracellular proteins important for NK cell cytotoxicity was measured. No significant difference in percent positive or expression level of Granzyme A or Granzyme B was observed; however, ReNK cells had decreased expression level of Perforin but not in the percent of Perforin+ NK cells (**Figure 27, 28**) (% Positive= Granzyme A: S: 99.8 ± 0.4 R: 99.9 ± 0.1 ; Granzyme B: S: $61.9 \pm$

28.0 R: 62.1 ± 25.0 , Perforin: S: 98.9 ± 2.3 R: 96.6 ± 4.5 ; Fold Change of ReNK= Granzyme A: 0.8 ± 0.4 , Granzyme B: 1.1 ± 0.4 , Perforin: 0.5 ± 0.4).

TGF β has been reported to inhibit the expression of T-bet and GATA3 transcription factors. In addition, RUNX3, GATA3, and T-bet can modulate IFN γ secretion; therefore, we next determined the transcription factor profile of ReNK cells at Day 14 of expansion. No difference was observed in percent positive or MFI for GATA3. For T-bet, no difference in percent of T-bet+ NK cells was observed; however, ReNK had decreased expression level of T-bet as measured by MFI ($p < 0.001$). RUNX3 MFI had a trend towards increased MFI in ReNK, but no change in percent of RUNX3+ NK cells (**Figure 29**) (% Positive= RUNX3: S: 90.4 ± 4.7 R: 82.9 ± 12.5 , T-bet: S: 97 ± 2.3 R: 90.1 ± 8.8 , GATA3: S: 97.1 ± 3.1 R: 92.7 ± 7.6 ; MFI= RUNX3: S: 18106 ± 4441 R: 22080 ± 6124 , T-bet: S: 14787 ± 7173 R: 8806 ± 4419 , GATA3: S: 6400 ± 2070 R: 6527 ± 1758).

Figure 27. ReNK cells have altered cell surface phenotype. ReNK cells have increased expression of the death receptors, TRAIL and FasL, but decreased expression of NKp30 and DNAM-1. ReNK cells have similar numbers of NKG2D+ cells, but decreased level of NKG2D expression. Shown is representative histograms from 1 donor of 5-7 independent donors (Blue: ReNK, Black: Standard).



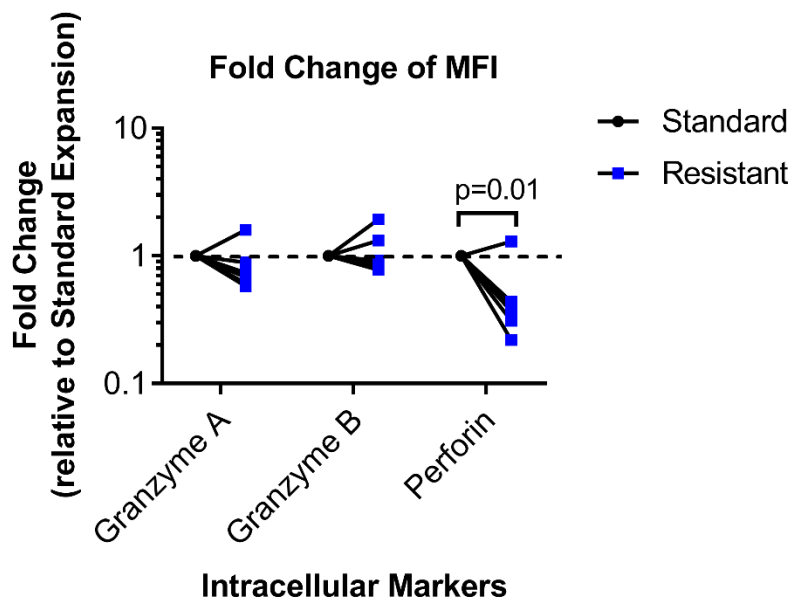
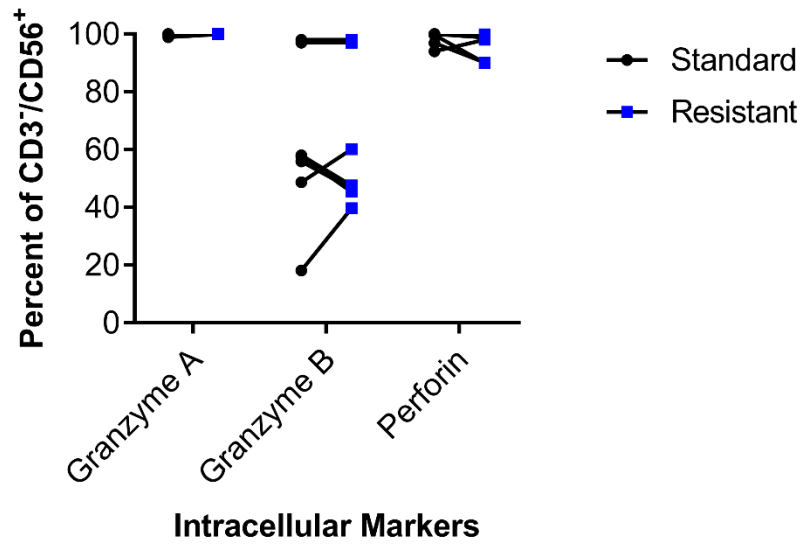
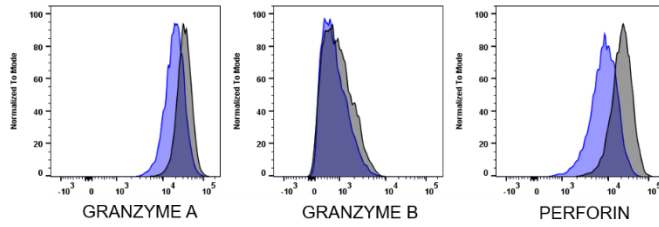


Figure 28. Intracellular Protein Expression. ReNK cells have similar percentages of Granzyme A, Granzyme B, and Perforin expressing NK cells, but have decreased level of Perforin expression. Representative histogram from one donor of 7 independent donors is shown.

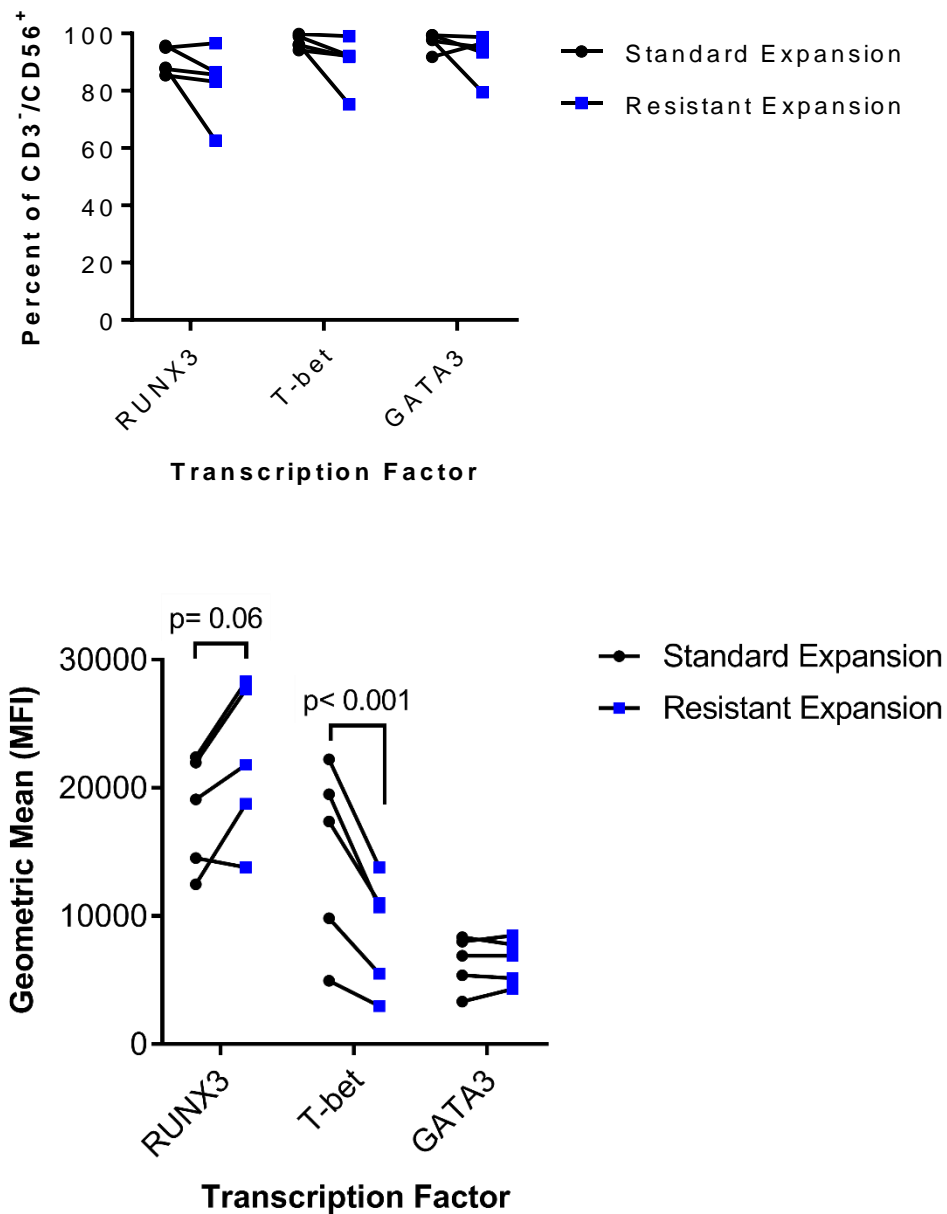


Figure 29. Transcription factor profile of ReNK cells. ReNK cells have decreased expression of T-bet compared to Standard NK cells, and a trend towards increased Runx3 expression.

3.2.5 ReNK cells have changes in TGF β Signaling.

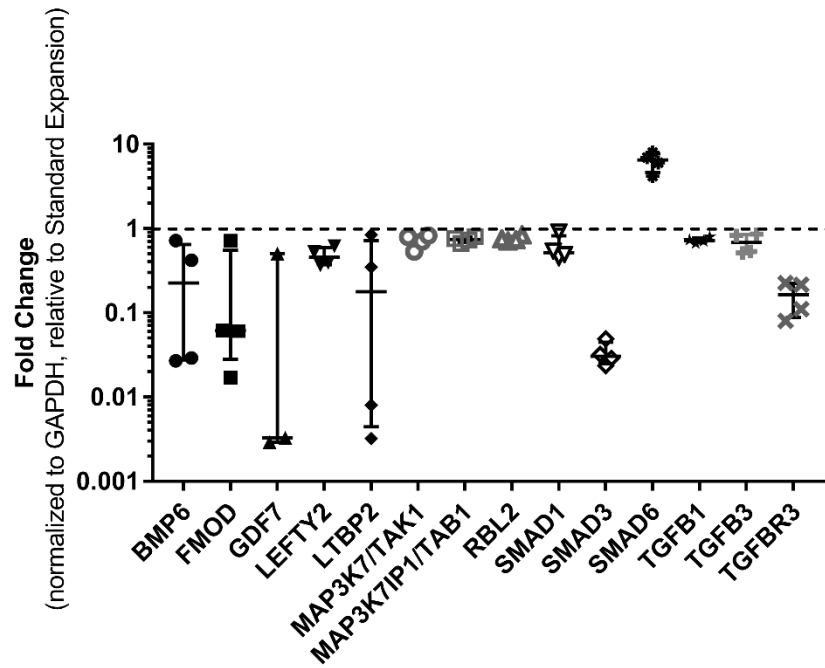
To determine if expansion with TGF β and the development of relative TGF β resistance was associated with alterations in the TGF β pathway, Day 14 expanded NK cells were assessed for the mRNA levels of 92 genes in the canonical and non-canonical TGF β pathway (Appendix B). Of these 92 genes, 12 were found to be significantly decreased in all 4 donors: SMAD3, TGFBR3, TGFB1, RBL2, MAP3K7IP1/TAB1, LEFTY2, FMOD, MAP3K7/TAK1, BMP6, SMAD1, LTBP2, and TGFB3 (see Appendix B for all of the genes & p-values) (Fold change relative to Standard Expansion, all Mean \pm SD; SMAD3= 0.03 \pm 0.01, TGFBR3= 0.16 \pm 0.07, TGFB1= 0.73 \pm 0.07, RBL2= 0.76 \pm 0.06, BMP6= 0.30 \pm 0.34, FMOD= 0.21 \pm 0.34, LEFTY2= 0.48 \pm 0.12, LTBP2= 0.30 \pm 0.40, MAP3K7/TAK1= 0.72 \pm 0.13, MAP3K7IP1/TAB1= 0.74 \pm 0.06, SMAD1= 0.60 \pm 0.21, TGFB3= 0.68 \pm 0.18),. GDF7 was decreased in 3 donors, but not detectable in the 4th donor (GDF7= 0.17 \pm 0.29). Only SMAD6 was significantly increased (**Figure 30a**) (SMAD6= 6.30 \pm 1.63). Of the significantly different genes, the highest expressed in Standard NK cells and with the most significant p-value were SMAD3 (p<0.0001) and TGFBR3 (p=0.0002) (**Figure 30b**).

The function of SMAD3 as a suppressor of NK cell anti-tumor function has been very clear. SMAD3 binds directly to the IFN γ promoter to inhibit IFN γ

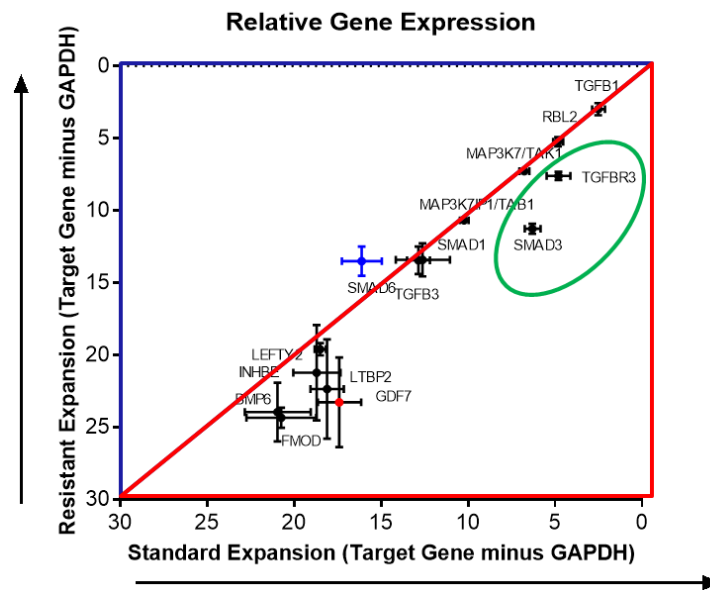
expression and SMAD3^{-/-} mice have enhanced NK cell function and decreased tumor growth. Thus, we wanted to determine if SMAD3 is also decreased at the protein level. Using western blot, we found that ReNK cells have no detectable SMAD3 protein in contrast to Standard NK cells where SMAD3 was readily detectable. On the contrary, both Standard and ReNK cells have similar levels of SMAD2 protein (**Figure 30c**). This is in agreement with other studies where SMAD3 is particularly important in TGFβ inhibition of NK cell function.

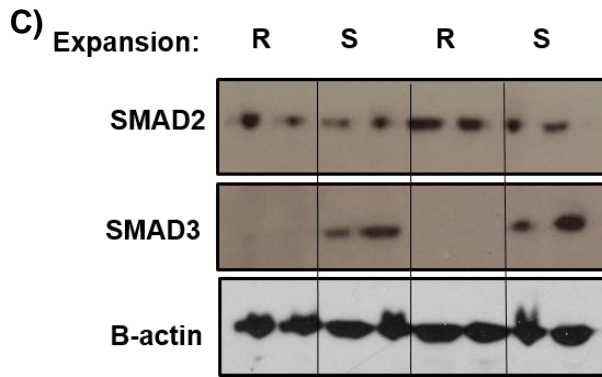
Figure 30. ReNK cells have altered TGFβ Pathway. A) ReNK cells had significantly different expression of 13 genes in the TGFβ pathway. **B)** Relative expression of the 13 genes was determined by subtracting the Ct value of the target gene from the Ct value of GAPDH to determine how highly expressed each of the target genes were in NK cells. Of the 13 genes, SMAD3 and TGFBR3 (circled in green) were the highest expressed and with the greatest difference between ReNK and Standard NK cells. **C)** ReNK (R) cells also have decreased SMAD3 protein compared to Standard (S) NK while they maintain SMAD2 protein. WB were run by Nitin Chakravarti, samples prepared by Jennifer Foltz.

A)



B)

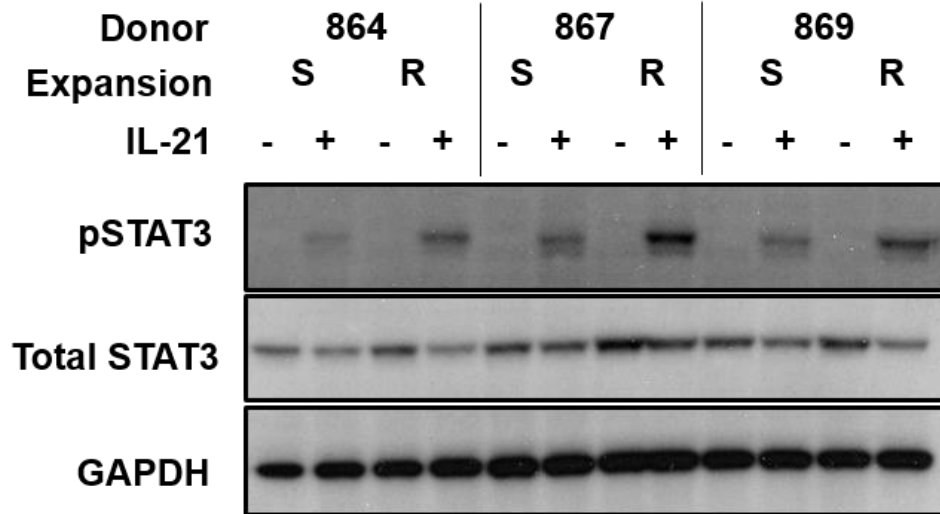




3.2.6 ReNK cells have increased STAT3 response.

Previous data from our lab demonstrated that STAT3 signaling promotes NK cell proliferation, NKG2D expression, and enhances anti-tumor cytokine secretion, when compared to IL-15/STAT5 signaling (66, 200). TGF β cooperates with IL-21 to drive development of Th17 cells by increasing STAT3 activity. Therefore, we wanted to determine if IL-21 and TGF β was increasing STAT3 signaling in ReNK cells. STAT3 activity was measured by treating NK cells plus or minus 20 ng/mL IL-21, a dose we previously reported to lead to phosphorylation of STAT3 (Y705). We found that ReNK cells have significantly increased STAT3 phosphorylation compared to Standard expanded NK cells ($p=0.0095$), but similar levels of total STAT3 protein (**Figure 31**).

A)



B)

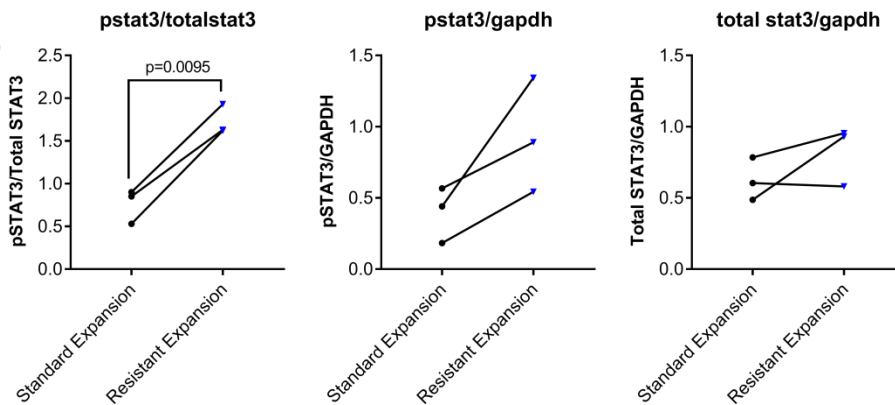


Figure 31. ReNK cells have increased STAT3 response. A) NK cells were treated plus or minus 20 ng/mL IL-21 for 30 minutes (S: Standard, R: Resistant Expansion) **B)** ReNK (R) cells have increased phosphorylation of STAT3 (Y705), but similar levels of total STAT3. WB were run by Adam Studebaker, samples prepared by Jennifer Foltz.

3.2.7 Persistence of ReNK Phenotype & Function.

Next, we wanted to determine the persistence of ReNK cell phenotype and function. To this end, ReNK and donor-matched Standard NK cells were rested in low-dose IL-2 alone (e.g. ReNK were removed from TGF β) after completion of 2

weeks of expansion. ReNK cells were assessed for secretion of IFN γ and TNF α and cytotoxicity against DAOY and MG63 every 7 days during expansion and at 7 days, 21 days, and 33 days (DAOY only) post-expansion. At 7 days of expansion, ReNK cells had significantly decreased TNF α secretion against MG63; but, after 14 days of expansion, ReNK cells had increased cytokine secretion, consistent with what we described earlier. In addition, we found that for DAOY, IFN γ and TNF α secretion increased from Day 7 to Day 14 for ReNK cells only, while Standard expanded NK cells trended towards decreased cytokine secretion, although this did not reach statistical significance most likely due to only 2 donors. After expansion, ReNK cells maintained their increase in IFN γ and TNF α secretion at both baseline and with TGF β treatment at least 33 days post-expansion. Slight sensitivity to TGF β for IFN γ secretion became evident at 33 days post-expansion against DAOY (**Figure 32**).

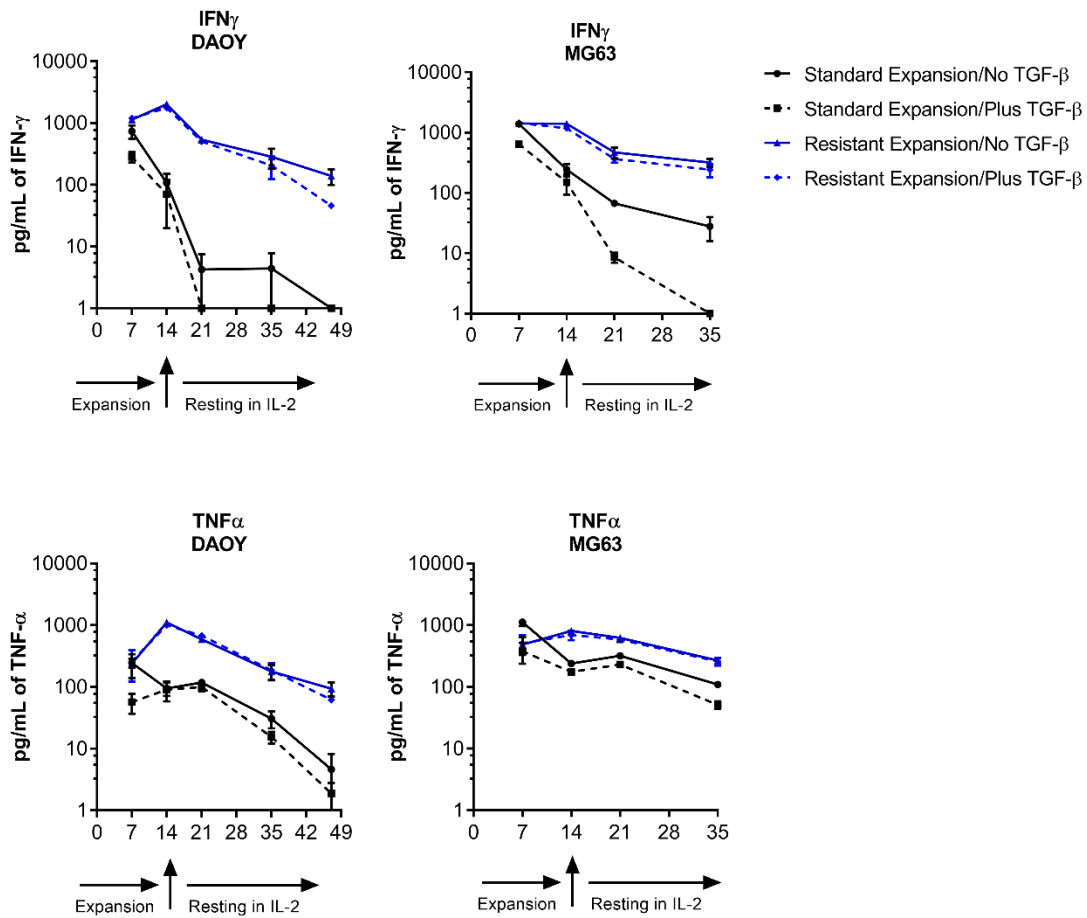


Figure 32. Cytokine Secretion of ReNK cells during and post-expansion. ReNK cells maintain increased IFN γ and TNF α secretion at least 33 days post-expansion, but display slight suppression of IFN γ secretion with overnight TGF β treatment at 33 days post-expansion. Median \pm range is depicted. (n=2).

For cytotoxicity, ReNK cells had increased cytotoxicity against DAOY in both donors in the presence of TGF β at Day 14 of expansion, and this effect persisted at least 33 days post-expansion. ReNK cell cytotoxicity against DAOY without TGF β treatment was increased in both donors at 21 and 33 days post-expansion, with variable cytotoxicity at the other time points. However, ReNK cell increased cytotoxicity against DAOY was more evident at 2.5 E:T ratio compared to 5:1 E:T ratio. Against DAOY, ReNK cells lost their resistance to TGF β at 33 days of resting; however, they remained more cytotoxic than Standard NK cells (**Figure 33a**).

As the NK cells from both the Standard and Resistant expansion had a decrease in cytolytic activity after expansion, we were unable to assess cytotoxicity for MG63 past 21 days of resting for Donor 22, and past 7 days of resting for Donor 25 due to loss of ability to kill MG63. Cytotoxicity against MG63 was variable, with one donor demonstrating increased killing by ReNK cells, while the other donor did not (**Figure 33b**).

Next, we wanted to determine if ReNK cell lysis of a previously resistant cell line, D283, was altered upon removal from TGF β (**Figure 19b**). To this end, we found that D283 was killed significantly better 33 days after resting by ReNK cells in the absence of TGF β . However, while ReNK cells still killed DAOY more efficiently in the presence of TGF β , ReNK cells were suppressed by TGF β to equivalent levels of killing as Standard NK cells despite higher baseline cytotoxicity in D283 at 33 days post-expansion (**Figure 34**).

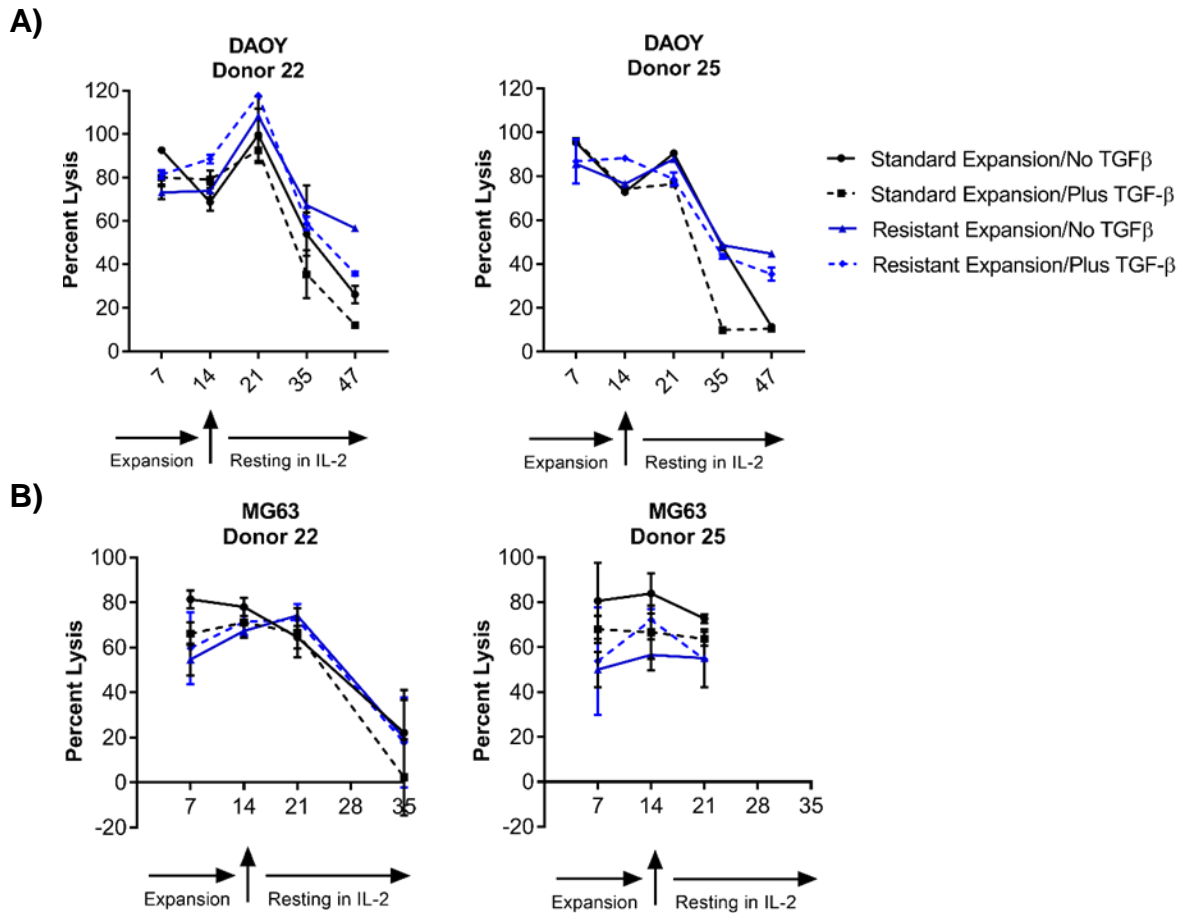


Figure 33. Cytotoxicity of ReNK cells during Expansion and post-Expansion.

A) ReNK cells have increased cytotoxicity against DAOY at 14 days of expansion and after expansion. ReNK cells lose their resistance to TGF β suppression of cytotoxicity at Day 47. **B)** ReNK cells have decreased cytotoxicity without TGF β against MG63, and both Standard and ReNK cells lose their ability to kill MG63 quickly after expansion. Mean \pm SD depicted for each individual donor.

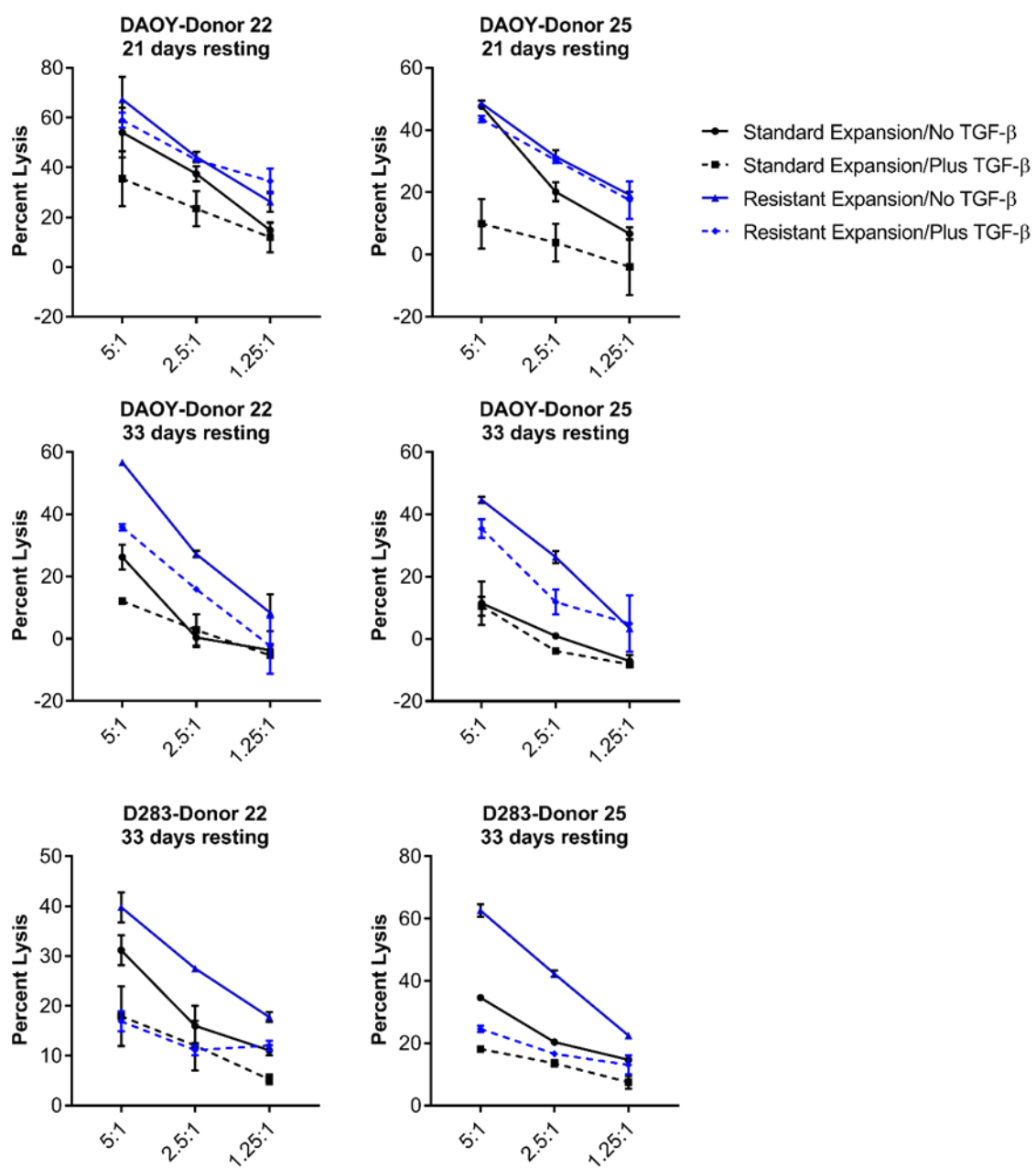


Figure 34. Cytotoxicity of ReNK cells after 21 and 33 days resting.

ReNK cells kill DAOY with increased efficiency compared to Standard NK cells at both 21 and 33 days resting; however, ReNK cells become suppressed by overnight TGF β treatment after 21 days. ReNK cells kill D283 with increased efficiency, which was previously resistant, at 33 days post-expansion; however, ReNK cells are sensitive to TGF β treatment against D283 targets. Mean \pm SD depicted for each individual

Next, we wanted to determine how the ReNK cell phenotype changed after removal from TGF β , and if the receptor expression correlated with the changes in cell lines sensitivity to ReNK cell lysis post-expansion. To this end, we measured expression of NKG2D, NKp30, DNAM-1, FasL, TRAIL, Granzyme A, Granzyme B, and Perforin using flow cytometry. Both the percentage of NK cells expressing TRAIL and cell surface FasL and the fold change of expression level decreased in Standard and ReNK cells after expansion. Notably, the increase in death receptor expression observed in ReNK cells during expansion was no longer present after expansion. In fact, cell Surface FasL expression was barely detectable 7 days after expansion (**Figure 35, 36**).

The expression of DNAM-1 was increased in ReNK after removal from expansion conditions. The percentage of NK cells positive for NKp30 increased to the level of Standard NK cells; however, NKp30 was still expressed at lower density than in Standard NK cells (MFI). On the contrary, for NKG2D which was previously expressed at a lower level in ReNK cells, was expressed at a higher level than in

Standard NK cells after ReNK removal from TGF β . For intracellular proteins, we found that ReNK cells had reduced Granzyme A, Granzyme B, and Perforin (MFI) despite removal from TGF β for 3 weeks. Unexpectedly, the percentage of NK cells expressing Granzyme B decreased after expansion in both groups; and this decrease was much larger in the ReNK cells compared to the Standard NK cells (**Figure 35, 36**).

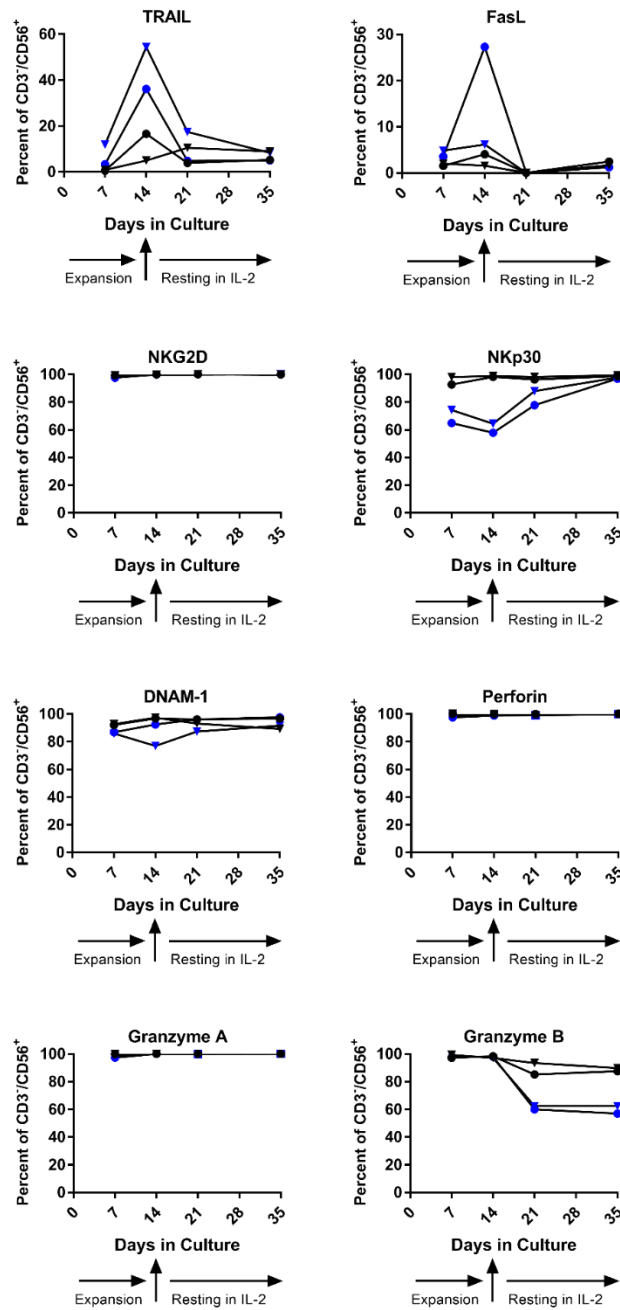


Figure 35. Percent of ReNK and Standard NK expressing different receptors during, and post-expansion. NKp30 expression in ReNK increases post-expansion, but Granzyme B and death receptor expression decreases after expansion. ReNK are shown in Blue, Standard NK in black, each line represents an individual donor.

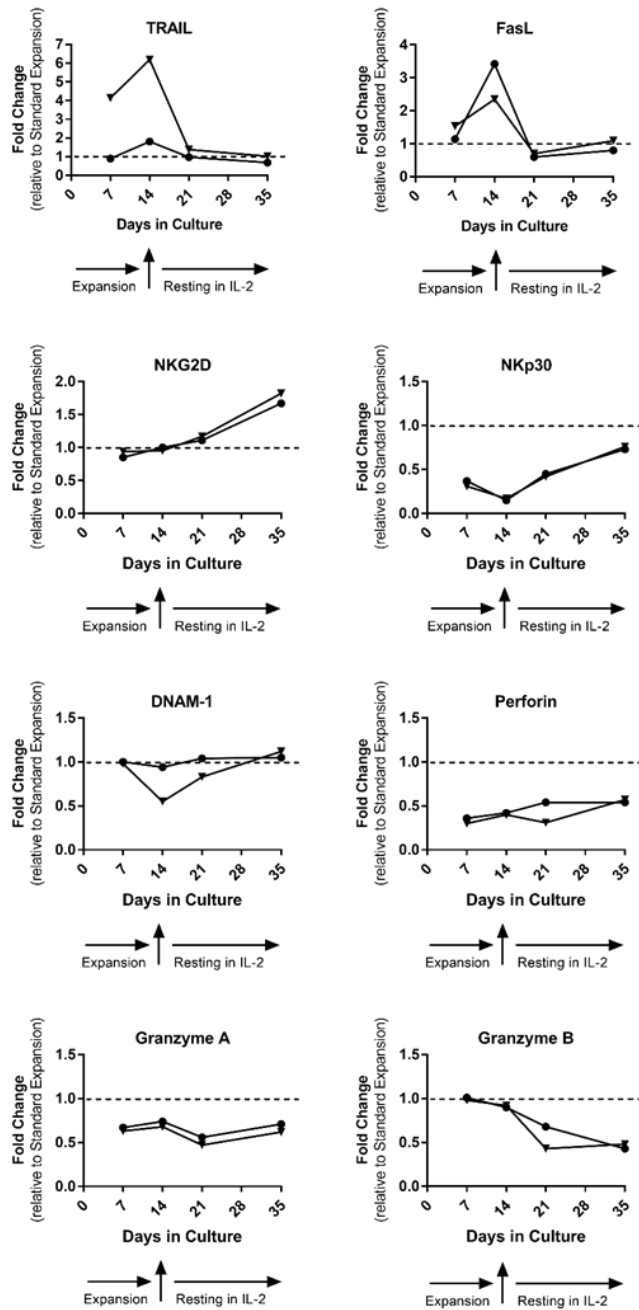


Figure 36. Expression level of ReNK and Standard NK Receptors during and Post-expansion. ReNK cells have increased NKG2D after expansion, but decreased NKp30, Perforin, Granzyme A, and Granzyme B. Depicted is fold change of Geometric Mean normalized to donor-matched Standard NK.

3.3 Discussion

In summary, human NK cells expanded with K562 mbIL-21 feeder cells plus TGF β (ReNK) have reduced sensitivity to TGF β , most likely through loss of one of the key TGF β signaling proteins- SMAD3. ReNK cells have remarkably increased IFN γ and TNF α secretion against OS and medulloblastoma cell lines. In addition, Day 14 Expanded ReNK cells have increased cytotoxicity against DAOY, while other cell lines are resistant to ReNK cell lysis. However, this can be overcome by resting ReNK cells in IL-2, which causes ReNK cells to be able to kill previously resistant tumors with greater effectiveness than Standard NK. Finally, ReNK cells retain TGF β desensitization at least 3 weeks post-expansion.

TGF β signaling in the immune system is context dependent. Other signals received by a cell in combination with TGF β dictate the effect that TGF β has on the cell's development and function. For example, in T-cells, TGF β promotes the development of anti-inflammatory regulatory T-cells (Tregs). However, TGF β in combination with either IL-6 or IL-21, leads to the development of pro-inflammatory Th17 cells instead of anti-inflammatory Tregs. In addition, CD28 on T-cells or CD40 on dendritic cells can inhibit TGF β suppression (110). There is some evidence of this in NK cells where pre-treatment of NK cells with pro-inflammatory cytokines such as IL-12, IL-15, and IL-18, reduces TGF β suppression of NK cells (100). It is not yet known if engagement of a cell surface receptor on NK cells can block TGF β inhibition. In light of our previous work on STAT3's enhancement of NK cell function and enhanced STAT3 response in ReNK cells, we propose that ligation of IL-21 expressed on the K562 feeder cells when presented simultaneously with TGF β may

block TGF β mediated suppression. For example, in Th17 cell development, IL-21 presented with TGF β prolongs STAT3 activation, and STAT3 inhibits SMAD3 (186, 201, 202). Conversely, SMAD2 promotes STAT3 activation (186). Importantly, STAT3 is required for Th17 cell differentiation, while STAT5 inhibits Th17 cell differentiation (203). Similar to Th17 cells, a CD8 T-cell subset termed Tc17 cells has been discovered that also requires IL-21 plus TGF β . Tc17 and STAT3 signaling. Tc17 cells produce IL-21 and IL-17. However, in contrast to ReNK cells, both Th17 and Tc17 cells have very low production of IFN γ and Tc17 cells have low cytotoxic activity (204). If STAT3 is necessary for the development of ReNK cells, expansion of NK cells with IL-15 will not be capable of generating ReNK (203).

Another alternative pathway to the development of ReNK cells is TGF β mediated augmentation of IL-21 signaling. In salivary gland innate lymphoid cells, TGF β upregulates IL-21 receptor expression (205), and in CD4+ T-cells, TGF β plus IL-21 stimulates increased production of IL-21 from T-cells than when either cytokine is administered alone (206). Thus, IL-21 in combination with TGF β may be increasing autocrine IL-21 signaling, stimulating ReNK cells to secrete their own IL-21 which subsequently binds to the increased levels of IL-21R on ReNK cell surface, thereby promoting STAT3 activation. STAT3 activation may then subsequently mediate epigenetic or microRNA changes that lead to enhanced intracellular response upon tumor contact. It is of future great interest to determine if enhanced IL-21/STAT3 signaling is causing ReNK cell development.

Surprisingly, and in contrast to previous papers, TGF β did not inhibit the overall 2 week proliferation of ReNK cells (87). However, we did observe

significantly reduced proliferation in the 1st week of expansion, suggesting that, TGF β selected for NK cells with decreased sensitivity to TGF β . The stimulation conditions used in previous papers for inducing proliferation (IL-2 and IL-15) and the time points may be key to the differences observed in proliferation. Previous measurements on TGF β 's effect on NK cell proliferation examined short-term proliferation, and not 2 week proliferation (87, 174). We propose that TGF β plus IL-21 driven expansion selects for a specific subset of NK cells, potentially those that are SMAD3^{neg} to survive. These SMAD3^{neg} NK cells would be resistant to basal constitutive phosphorylation of SMAD3 from TGF β in the cell culture media, allowing for increased proliferation (207). It will be interesting to determine if SMAD3^{neg} NK cells are found naturally in PBMC. Sorting of SMAD3^{neg} and SMAD3^{pos} NK cells with SmartFlare mRNA probes would provide insight into whether SMAD3 dictates NK expansion kinetics in the presence of TGF β .

We observed cell-line dependent sensitivity to ReNK cell lysis. DAOY was the most sensitive to ReNK cell lysis displaying increased cytotoxicity in both the presence and absence of TGF β treatment. On the other hand, ReNK cells had decreased cytotoxicity against MG63, HOS, and D283 without TGF β treatment, but similar cytotoxicity with TGF β treatment compared to Standard NK cells. Tumor sensitivity to ReNK cell lysis may be explained by differences in the contribution of certain activating receptors in tumor cell lysis. DAOY is known to be sensitive to death receptor-mediated killing which are highly expressed on ReNK cells (208, 209). Interestingly, D283, which was killed significantly less in Day 14 expanded ReNK cells, was conversely killed significantly better by ReNK cells after removal

from TGF β for 33 days (Section 4.8). This increase in killing by ReNK cells correlated with increased expression of NKp30 and NKG2D after TGF β withdraw. Therefore, cell-line sensitivity to ReNK cell lysis may be predictable based upon the NK cell receptors required for tumor cell killing. Future experiments using receptor-Fc proteins (i.e. NKG2D-Fc) to determine if the receptor's ligand is expressed on the tumor and/or blocking the receptor on the NK cells will be beneficial to explain the differences in sensitivity to ReNK cell lysis, and to predict which tumor types would most benefit from ReNK cells. In addition, Anti-tumor function of ReNK cells was maintained after expansion. In contrast, Standard NK cells have a drastic reduction in cytotoxicity, IFN γ , and TNF α secretion. Another advantage of ReNK cells may be their maintenance of function after expansion.

The cytotoxicity assays presented here are limited to the measurement of NK cell killing within 4 hours, which is known to be biased towards performing/granzyme-mediated killing. Perforin/Granzyme-mediating cytotoxicity is observed very quickly since perforin release disrupts membrane integrity prior to triggering caspase activation. Loss of membrane integrity is necessary for measurement of cytotoxicity in the calcein-AM based method, because cytotoxicity is measured by the extent of dye that has released into the supernatant. On the contrary, loss of membrane integrity is a late event in death receptor mediated killing, occurring after caspase activation. In addition, death receptor mediated killing can take longer than 4 hours to be observed, due to several reasons. NK cells can induce Fas expression in previously Fas negative tumors. NK cells can also be induced to express cell-surface FasL upon ligation of their activating receptors, such

as NKG2D (210). Thus, it can take several hours for Fas to be expressed, and then Fas-mediated tumor lysis. Further, IFN γ can sensitize cells to death receptor mediated killing by upregulating caspase 8 expression in the target cells, a requisite for death receptor mediated lysis (211-215). Thus, longer-term cytotoxicity assays will be important for providing a more thorough evaluation of the ability of ReNK cells to mediate tumor lysis through multiple pathways.

Unexpectedly, we observed significantly increased killing by ReNK cells in the presence of TGF β compared to no TGF β against MG63 and D283. We did not observe significant changes in NKG2D, TRAIL, Granzyme A, Granzyme B, or Perforin in ReNK cells rested overnight plus or minus TGF β . therefore, it is unlikely that overnight TGF β treatment is increasing ReNK cell killing by directly affecting the NK cells. Alternatively, TGF β may be sensitizing the tumor cells to NK cell lysis. In fact, TGF β has been well-described to inhibit proliferation and to induce apoptosis in several cell types (216, 217). Since TGF β does not inhibit ReNK cell killing, this effect on tumor cell sensitization is evident in ReNK cell lysis but not in Standard NK cells.

Interestingly, TGF β retained its' ability to suppress NK cell IFN γ secretion against MG63 and HOS even when SMAD3 is not present. This suggests a SMAD3-independent mechanism of IFN γ suppression that could be mediated either through SMAD2 or by non-canonical TGF β signaling. This finding is consistent with previous studies in primary NK cells demonstrating that both SMAD2 and SMAD3 can separately inhibit T-bet expression. Inhibition of T-bet decreases IFN γ secretion

(100). Future studies should better understand SMAD2 versus SMAD3 function and regulation of their expression in NK cells.

Receptor expression was significantly altered after expansion in both Standard and ReNK cells. NKG2D, which was inhibited by TGF β during expansion, was significantly increased after expansion corresponding to removal from TGF β . This effect may represent a compensatory response from TGF β suppression of NKG2D, or be indicative of increased STAT3 activity, as STAT3 is important for optimal NKG2D expression (218). Surprisingly, Granzyme B expression decreased in both Standard and ReNK cells after expansion. However, Granzyme B was decreased to a much greater degree in ReNK cells with only 60% of ReNK cells expressing Granzyme B. Nonetheless, cytotoxicity of ReNK cells after expansion in the persistent culture was significantly increased compared to Standard NK cells even against previously resistant tumors (D283).

There are several possible explanations for increased cytotoxicity in the absence of Granzyme B. These include FasL, TRAIL, TNF α -mediated apoptosis, or IFN γ tumor sensitization. Despite little to no detectable FasL expression after expansion, FasL is readily stored in the granules in NK cells and CD8 $^{+}$ T-cells and released upon degranulation or secreted (68, 210, 219, 220). In $\gamma\delta$ T-cells, NKG2D ligation stimulates the release of soluble TRAIL, which was an important pathway of cytotoxicity against certain tumor targets (221). Another alternative is cytotoxicity through TNF α or IFN γ as ReNK cells have significantly increased cytokine production. Both membrane-bound and soluble TNF- α can mediate apoptosis-directly by binding to TNFRs on target cells (222, 223). Recently, our lab reported

that IFN γ can sensitize tumors to NK cell lysis by upregulating ICAM-1 on the tumor but this effect is cell-line dependent (224). IFN γ can also enhance death receptor mediated killing (211-214).

We choose to assess the ability of ReNK cells to produce IL-6, IFN γ , and TNF α , because these cytokines can both inhibit TGF β and conversely, TGF β can inhibit the production of IFN γ and TNF α . In a spontaneous canine model, IL-6 secretion neutralized TGF β , causing restoration of NK-like tumor killing (199). In addition, TGF β stimulates IL-6 production (225). We found a similar effect in ReNK cells, which had increased IL-6 production without stimulation and increased IL-6 anti-tumor production against MG63. IL-6 production has been reported to decrease membrane-bound TGF β through STAT3 and to stimulate NK cell production of IL-17 (226, 227). We found evidence for this effect in ReNK cells which had significantly decreased mRNA for TGF β 1 and a trend towards increased IL-17 mRNA (3 out of 4 donors expressed detectable IL-17). However, decreased TGF β 1 may represent a feedback mechanism to decrease TGF β production by NK cells already receiving exogenous TGF β . Both HOS and MG63 stimulated less IL-6 production than when the NK cells were unstimulated; therefore, it is unlikely that IL-6 is contributing to the enhanced anti-tumor function observed in ReNK cells. Decreased IL-6 production in the presence of tumor may be due to ligation of CD158b (KIR2DL2/3), with HLA-C on the tumor which inhibits IL-6 production (228).

Surprisingly, we found remarkably increased anti-tumor IFN γ and TNF α secretion in ReNK cells compared to Standard NK cells both with and without TGF β treatment. Previous studies have demonstrated that SMAD3 deletion increases

baseline IFN γ production, therefore, it is likely that a similar mechanism is occurring in ReNK cells which do not express SMAD3 (177). TGF β is reported to inhibit TNF α production in primary NK cells (87); however, we did not observe decreased TNF α secretion in either Standard or ReNK cells with TGF β treatment. Nonetheless, ReNK cells had significantly increased secretion of TNF α both plus and minus TGF β treatment compared to Standard NK cells. Regulation of TNF α production is less well-understood. TNF α may be positively regulated through combined IL-21 and TGF β signaling, such as through increased STAT3, since IL-21/STAT3 expanded NK cells have increased TNF α secretion compared to IL-15/STAT5 expanded NK cells (66). On the contrary, TNF α upregulation in ReNK cells may be indirectly promoted by IFN γ . IFN γ promotes IRF-1 and IRF-8 which, in turn, increase TNF α secretion (229). Yet another alternative is increased activation of TACE/ADAM17. In NK cells, TACE is responsible for cleaving CD16 and TNF α from the cell surface. In fact, inhibition of TACE, decreases TNF α secretion, and TGF β is reported to activate TACE. TGF β activated TACE also cleaves TGF β R1 from the cell surface (230-233). Given the decreased CD16 expression observed in TGF β treated NK cells, and the increased TNF α secretion in ReNK cells, it will be interesting to determine if TACE is contributing to ReNK cell function.

We observed increased expression of SMAD6, an inhibitory SMAD. SMAD6 inhibits BMP signaling (e.g. BMPR1A/ALK-3 & BMPR1B/ALK-6), and can also inhibit non-canonical TGF β signaling through TGF β induced TRAF6-TAK1-p38 MAPK/JNK (187, 188, 234). We observed a slight but significant decrease in mRNA levels of TAK1 and of TAB1, which acts downstream of TAK1. Paradoxically, TAK1 inhibition

decreases NK cell cytotoxicity and cytokine production (235), whereas ReNK cells have increased NK cell cytokine secretion. However, TAK1 inhibition in NK cells was studied in the context of NKG2D signaling, raising the possibility that the role of TAK1 as an enhancer or suppressor of NK cell activity is dependent upon the stimuli. TAK1 could be contributing to the decreased killing by ReNK cells against MG63, HOS, and D283. In tumor cells, TAK1 is necessary for TGF β to mediate growth inhibition, thus, decreased TAK1 levels may promote proliferation of ReNK cells in the presence of TGF β (188). In line with this observation, we also observed significantly decreased Rbl2 expression which also mediates TGF β -induced growth arrest. Thus, ReNK cells are able to downregulate TGF β growth inhibitory target genes (236). Future studies are needed to better understand the effect of the non-canonical TGF β pathways on NK cell function.

Taken together, a possible explanation for increased NK cell function in ReNK cells may be due to an upregulation of STAT3 activity and a downregulation of SMAD3 caused by four potential mechanisms- 1) increased IL-21 signaling due to upregulation of IL-21R expression 2) combined IL-21 and TGF β signaling promoting sustained STAT3 activity 3) increased STAT3 due to decreased SMAD3 and 4) promotion of STAT3 activity by SMAD2. Future experiments comparing NK cells from IL-15 (which signals primarily through STAT5) versus IL-21- mediated expansion would provide clarity on whether IL-21/STAT3 is in fact necessary for the generation of ReNK cell phenotype. Even though ReNK cells have decreased cytotoxicity at the end of expansion against some cell lines, they have great promise for improving NK cell therapy as murine models have demonstrated that SMAD3-/-

NK cells have enhanced in vivo anti-tumor function. Further, ReNK cells have increased retention of anti-tumor function after expansion.

Chapter 5: General Discussion and Future Directions

5.1 Dissertation Summary

In summary, this project improves adoptive NK cell therapy for OS in 2 different ways. First, through the development of a novel canine specific NKp46 antibody which enabled more extensive characterization of canine NK cells. The expansion of canine NK cells allows for the testing of NK cell therapy for OS in canines. To the best of our knowledge, this is the only large animal model with spontaneous cancer development in which syngeneic NK cells can be expanded to clinically relevant numbers. This model is likely to be important for the pre-clinical testing of genetically modified NK cells and of ReNK cells. Second, we also describe a novel non-genetic method for the *ex vivo* generation of human NK cells with reduced sensitivity to TGF β and improved anti-tumor function.

We identified canine NK cells as CD3⁻/NKp46⁺ cells that have phenotypic and functional homology to human NK cells. Phenotypically, they express all of the NCRs, and CD16, along with Granzyme B and Perforin. Like human NK cells, they can be subdivided into NKp46 positive and negative NK cell subsets that have altered CD16 expression. Functionally, canine NK cells kill without prior sensitization against several tumor targets- OS, melanoma, and thyroid adenocarcinoma; and secrete the prototypic proinflammatory NK cell cytokines- IFN γ and TNF α . Further, similar to human NK cells, they proliferate rapidly (median= 20,283-fold in 3 weeks)

on K562 mbIL-21 feeder cells, the same feeder cell used for human NK cell propagation (237).

We describe here that expansion with IL-21 and TGF β generates ReNK cells which display drastically enhanced anti-tumor IFN γ and TNF α secretion in both the presence and absence of TGF β treatment. This increase in cytokine production persists for at least 33 days after expansion. In addition, ReNK cells have increased cytotoxicity after expansion compared to Standard NK cells. Recently, Romee et al. reported that cytokine-induced memory NK cells, which secrete significantly more IFN γ than non-memory NK cells but do not have improved cytotoxicity, had superior anti-tumor activity in murine models and in a clinical trial (67). These results highlight the importance and potential clinical impact that ReNK cells could have.

5.2 Future Directions

5.2.1 Pre-clinical Models for NK Cell Therapy

The development of a canine NK cell expansion protocol that is nearly identical to the human NK expansion platform has implications for the study of cancers outside of OS. Companion animal trials can be used to test the efficacy of novel NK cell therapies quickly in canine cancers with high homology to the corresponding human cancer such as OS, melanoma, leukemia, and lymphoma. The canine model is especially well-suited for the testing of new NK cell therapies in pediatric cancers, since pediatric cancers are relatively rare leading to slow accrual in clinical trials. Further, canine cancers progress quicker than cancers in humans allowing for new therapies to be tested and treatment efficacy to be assessed in less

than a year instead of in 5-10 years in human trials. Studies such as these directly benefit both canine and humans by providing an opportunity to immediately improve survival in canines and to aid in the design of human clinical trials and selection of the most promising therapies.

The canine model is ideal for testing NK cell therapies with unknown risk profiles. These include genetically modified NK cells, NK cell injections into the brain, and ReNK cells. Although ReNK cells are not genetically modified, their relative resistance to TGF β combined with the importance of TGF β in preventing autoimmunity renders it particularly important to test ReNK in an animal model with an intact immune system prior to human clinical trials. However, neither SMAD3^{-/-} or TGFB2^{-/-} murine NK cells have caused autoimmune disease in murine models, and in fact, SMAD3^{-/-} NK cells have demonstrated superior tumor control (174, 177). Since we have not been successful in *ex vivo* mBL-21 expansion of murine NK cells, the canine model is even more valuable.

Other areas where the canine model can directly benefit NK cell therapy include the measurement of NK cell persistence and trafficking. To facilitate imaging and NK cell tracking, labeling agents such as 19 Fluorine can be used to label expanded NK cells before infusion into the canine patient. MRI scans can be added to assess whether the NK cells trafficked to the tumor site and their persistence in the recipient's blood. Due to the larger size of canines compared to mice, regular blood draws are feasible enabling the measurement of persistence and function of adoptively transferred NK cells. These findings can then be used to correlate with disease progression or treatment outcomes. In addition, due to the high mortality

rate of canine cancers, post-mortem tissues can be readily obtained and used to determine the localization of NK cells within the tumor microenvironment and the phenotype of tumor-infiltrating NK cells.

5.2.2 Inhibitory Receptors in Canine NK Cells

The development of the canine specific NKp46 antibody allows for further study of NK cell biology in canines, which has previously not been feasible. Specifically, it is not known how canine NK cells recognize non-malignant cells. Determination of non-malignant and/or self- cells is by binding of MHC to the KIR family of receptors in humans or Ly49 family in mice. The canine genome has only one copy of Ly49 (compared to several copies in mice) and there are several problems raised by the canine Ly49 sequence. First, the Ly49 family in mice includes both activating and inhibitory receptors, and it is not known if canine Ly49 is an inhibitory or an activating receptor. Second, canine Ly49 does not have a conserved cysteine residue, which is believed to be necessary for function (147). Thus, canine Ly49 may not code a functional protein. Our PCR data found Ly49 to be barely detectable, with *Ct* values on expanded canine NK cells around 39 cycles. This extremely low level of detection raises the possibility that the Ly49 transcript may not encode a protein of functional consequence in canine NK cells.

In addition, some reports state that canines have KIR, the inhibitory receptors in humans. However, no data is made available to substantiate this statement and no KIR sequence can be found in the publicly available canine genome (148). However, the published canine genome is limited to only the standard poodle and boxer, with evidence that this sequenced 99% of the genome (238, 239). Due to the

considerable heterogeneity within canines, there exists a significant possibility of breed-to-breed heterogeneity in canine NK cell receptors, such that other breeds may express more Ly49 copies and/or KIR that was not found in the published genomes.

Other NK inhibitory receptors in mice and humans include CD94 and NKG2A/B; however, the canine genome has no predicted transcript for NKG2A or NKG2B although there is a transcript for CD94. In human NK cells, CD94 must dimerize with a NKG2-family protein to transduce the signal, but without the transcript for NKG2-family proteins capable of dimerizing with CD94, there remains uncertainty as to what function canine CD94 serves (240, 241). We attempted to determine CD94 expression on canine PBMC and expanded cells with the commercially available antibody reported to cross-react with canine, but did not find any discrete staining (clone HP-3D9). PCR to determine the relative expression of CD94 in canine NK cells and the development of a CD94 blocking antibody could be beneficial in determining what the functional significance is of CD94.

To further understand inhibitory receptors in canine, sequencing of the genome from several different dog breeds would aid in determining if there are breed to breed differences in inhibitory receptor expression. In addition, RNA sequencing on isolated canine NK cells would provide an unbiased screening on what genes are expressed in canine NK cells and their relative abundance. Additionally, the development of blocking antibodies to canine MHC would enable the determination of the functional consequences of blocking inhibitory receptor signaling in canine NK cells. If as expected, the blocking of canine Dog Leukocyte

Antigen (DLA) (canine orthologue to MHC) increased canine NK cytotoxicity, one could infer that a yet undetermined inhibitory receptor is functioning to inhibit canine NK cell function against cells expressing self. Improved clarity on inhibitory receptor signaling in canine NK cells could improve donor selection for the testing of adoptive NK cell therapies by allowing for the selection of donors that are missing self-ligands.

5.2.3 Canine NK Cells: Implications in Infectious Disease & Autoimmunity

The identification and characterization of canine NK cells has implications for the study of NK cell biology in areas outside of cancer, such as in infectious disease and autoimmune diseases. Canines are susceptible to developing many of the same ailments as humans including influenza, allergies, diabetes, rabies, and autoimmune diseases such as muscular dystrophy, X-linked severe combined immunodeficiency, systemic lupus erythematosus among others (242-245). Using the canine NKp46 antibody, a better understanding of canine NK cell biology in these ailments can be assessed. Given the propensity of specific dog breeds to develop different ailments (i.e. Retrievers and Lupus), canines are a beneficial model for determining whether changes in NK cell biology are affecting the development of various disorders. For example, NK cell function and phenotype can be compared between Retrievers with Lupus and without, and then compared to other dog breeds that are not susceptible to Lupus. The decreased heterogeneity within individual dog breeds can be exploited to determine what may be causative factors in diseases. This could lead to determining the underlying genetic changes within a breed that make certain breeds more susceptible to development of a disease, and could be directly applicable to

both veterinary and human biology. In addition, canines are susceptible to several viral and parasitic diseases such as parvovirus and heartworm that are not common in humans, but can be deadly in dogs. Given that NK cells are also potent killers of virally-infected cells, it would be of great interest to veterinary practice to determine whether viral diseases such as parvovirus are killed by canine NK cells.

5.2.4 Antibody Generation

The development of the canine NKp46 antibody and subsequent identification of canine NK cells exemplifies the utility and ease of generating antibodies to study proteins of interest. The majority of antibody generation has focused on first identifying a novel cell type before constructing an antibody that identifies this new cell type. The approach described in this project takes the opposite approach of first identifying a protein of interest (e.g. canine NKp46), and then using the antibody to determine what cells are identified by the protein of interest. This approach has potential for facilitating quicker identification of novel cell types and better understanding of proteins. It can also be used in combination with genomic searching or RNA sequencing for highly expressed genes, and then generating antibodies to the proteins the gene encodes. Then, the new antibody can be used to determine both the cell subsets that express that protein and the function of that protein. For example, in studying canine NK cells, this approach could be applied to generate an antibody to canine Ly49 to better understand the cells that express Ly49 and its' function.

5.2.5 TGF β Resistance

The present project on ReNK cells raises several unanswered questions. First, the mechanism behind generation of TGF β resistance and enhanced anti-tumor function is unknown. Of particular interest is whether IL-21 is required for these effects. Since IL-21 and TGF β cooperate to generate Th17 cells through prolonged STAT3 activation, it is possible that a similar Th17 development pathway can occur in NK cells (201, 246). Other alternative mechanisms include whether long-term TGF β treatment alone downregulates SMAD3, causing TGF β desensitization. However, since NK cells in cancer patients who have elevated serum TGF β levels have reduced functionality, it is unlikely that TGF β alone can generate a hyperfunctional NK cell. Another alternative is that another protein expressed on K562 feeder cells is cooperating with TGF β to generate ReNK cells. The K562 feeder cell was engineered to express 41BBL along with IL-21, and K562 are highly sensitive to NK cell lysis suggesting that they constitutively express other activating ligands that could cooperate with TGF β to produce ReNK.

In addition, it is not known why ReNK cells lose their relative resistance to TGF β after 3 weeks of resting in low-dose IL-2. Given the striking loss of both SMAD3 RNA and protein in ReNK cells and SMAD3's well-defined role in suppressing NK cell anti-tumor activity, we hypothesize this is due to a gain of SMAD3 protein. This would suggest that ReNK cells have transiently downregulated SMAD3 through either methylation of the SMAD3 locus or miRNA inhibition.

It will be interesting to determine if ReNK cells are an artifact of *ex vivo* manipulation or if they are a subpopulation of NK cells that exist naturally and have

been selected through IL-21 and TGF β expansion. It is likely that ReNK cells do constitute a subset of natural NK cells, as it is feasible to imagine situations where NK cells would be exposed to IL-21 and TGF β concurrently such as in lymphoid tissues where NK cells would be exposed to IL-21 producing Th17 cells and follicular T-cells along with TGF β from either regulatory T-cells or NK cell intrinsic production of TGF β (247, 248).

TGF β is necessary for preventing autoimmune reactions, as TGF β or TGF β RI/II knockout mice die of autoimmune disease at a young age (249). Further, expression of a dominant negative TGF β RII in CD8 $^{+}$ T-cells causes the development of lymphoma or lymphoproliferative disorders (250). Therefore, it is possible that infusion of ReNK cells could exacerbate autoimmune disease. In addition, the substantially increased secretion of IFN γ and TNF α could cause cytokine release syndrome. Both IFN γ and TNF α antagonize wound healing and NK cells are known to be present in wounds; therefore, ReNK cells may hinder proper wound healing by promoting excess inflammation through their secretion of IFN γ and TNF α (251-254). Although ReNK cells do not secrete cytokines in the absence of tumors, it will be necessary to determine their safety profile against non-malignant cells and their *in vivo* safety particularly in settings of bulky disease where their potent anti-tumor response could promote the development of cytokine release syndrome.

The generation of NK cells with relative resistance to TGF β Resistant NK cells through combined long-term exposure to TGF β and IL-21 raises the possibility as to whether NK cells can be generated that are resistant to other immune

suppressors. Since ReNK cells are SMAD3⁻, it is likely that they will be less sensitive to suppression by Activin A, which like TGFβ mediates its' suppressive effects on NK cell cytokine production through SMAD2/3 (183). It would be interesting to determine if TGFβ resistant NK cells display selective resistance to TGFβ as would be expected, or if they are resistant to other suppressive pathways such as Tim-3, IDO, and hypoxia. The mechanism by which ReNK cells become resistant will help elucidate the likelihood that ReNK cells are also resistant to other means of suppression and if NK cells resistant to other suppressive proteins can be generated through a similar method. Specifically, if long-term exposure to TGFβ is sufficient to induce the development or selection of NK cells resistant to TGFβ's effects, than long-term exposure to Tim-3 or IDO would be hypothesized to also select for NK cells resistant to Tim-3 and IDO. If so, the expansion of NK cells on different immunosuppressive factors could hold great potential for selecting for resistant NK cells.

5.2.6 Development of TGFβ Resistant T-cells

The generation of ReNK cells raise the possibility that expanded T-cells could also be generated that are resistant to TGFβ, given that T-cells can also expand robustly on K562 feeder cells. In fact, the K562 feeder cell line used in the described studies was originally constructed to stimulate T-cell growth. In addition, IL-21 has a potent stimulatory effect on CD8⁺ T-cells (255). TGFβ is also highly suppressive to T-cells, causing upregulation of PD-1, decreased IL-2 and IFNγ production, decreased proliferation and cytotoxicity (105, 106, 198, 256). Given the similarities

between NK and CD8 T-cells, it is likely that CD8 T-cells could be made resistance to TGF β and represents a promising area for improving T-cell therapy.

Since both IL-21 and TGF β are highly implemented in the development of Th17 CD4 T-cells, the expansion of CD4 T-cells on K562 mbIL-21 feeder cells with TGF β is likely to preferentially lead to an outgrowth of Th17 cells (246). This could represent a method of generating clinically relevant numbers of Th17 cells for therapeutic utility. Expanded Th17 cells could be beneficial in HIV, where they are preferentially depleted due to their expression of CCR5, the coreceptor for HIV. Depletion of Th17 cells in HIV disturbs intestinal mucosal integrity, leading to microbial translocation, and sustained immune activation (257). In fact, recovery of Th17 cell numbers by the administration of IL-21 to SIV-infected macaques decreased microbial translocation and immune activation. Therefore, if Th17 cells can be expanded with K562 mbIL-21 plus TGF β , their adoptive transfer into HIV patients could be a beneficial new therapy (258).

5.2.7 ReNK cells for Infectious Diseases

ReNK cells may have therapeutic benefit in other malignancies outside of cancer. Specifically, ReNK cells are specially poised for use in control of the parasite- Trypanosoma Cruzi (*T. cruzi*) which causes Chagas disease. *T. cruzi* requires expression of the TGF β receptor and an intact TGF β signaling pathway for infection (259). Since ReNK cells are SMAD3^{neg}, they are likely resistant to infection by *T. cruzi*. NK cells have been demonstrated to be important in control of *T. cruzi*, infection, as depletion of NK cells renders mice more sensitive to *T. cruzi* infection. In addition, asymptomatic Chagas patients have increased NK cell numbers while

Chagas patients with cardiac myopathy have decreased NK cell numbers and expression of genes related to NK cell function such as Granzymes and Perforin (260, 261). Thus, adoptive transfer of ReNK cells could be ideal in replenishing the reduced NK cell numbers in Chagas and resisting cell death by infection.

5.2.8 Conclusion

In conclusion, we describe here two novel approaches towards improving treatment for OS patients, where survival has not improved in the last 30 years. The development of a novel anti-canine NKp46 antibody allows for improved characterization of NK cells and 20,000-fold expansion of canine NK cells on K562 mbIL-21 will allow for NK cell therapy to be tested in canine OS. In fact, a similar expansion of canine NK cells is currently being used to test the effectiveness of intratumoral injections of canine NK cells after radiation in OS (UC Davis, R. Canter & W. Murphy). Second, we developed a non-genetic approach to generate NK cells with relative resistance to TGF β , which is elevated in OS, and represents a mechanism of immune evasion by cancer. Most importantly, these ReNK cells have improved anti-tumor activity against OS. Together these two approaches hold promise for improving OS outcomes through NK cell therapy.

Chapter 6: Materials & Methods

This chapter is based upon Foltz, J. A., S. S. Somanchi, Y. Yang, A. Aquino-Lopez, E. E. Bishop, and D. A. Lee. 2016. NCR1 Expression Identifies Canine Natural Killer Cell Subsets with Phenotypic Similarity to Human Natural Killer Cells.

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6.1 Peripheral Blood Mononuclear Cell Isolation

Animal research was conducted with approval from the Institutional Animal Care and Use Committee at MD Anderson Cancer Center (00001532-RN00). Healthy canine blood was obtained from established animal colonies at the following locations: Animal Blood Resources International, Hemopet, and Texas A&M University (IACUC Protocol: 2014-0294). Healthy canine blood from UC Davis was exempted from IACUC approval. All blood from client-owned animals was obtained with informed consent and was consistent with the established guidelines for safe canine blood draws. Blood from canine patients with suspected osteosarcoma who had not received chemotherapy within 1 month was obtained with informed consent from UC Davis under Protocol 18315. Osteosarcoma diagnosis was confirmed with radiographs or biopsies. Two out of the seven canine patients presented with potential chondroblastic osteosarcoma, with one of the two patients presenting with disease with alternative diagnosis of chondrosarcoma. Four out of the seven patients were females with ages ranging from 2 to 13 years old. Osteosarcoma tumor sites were frontal bone (1), metatarsal (1), tibia (1), femur (1), radius (2), and ileal body (1). Canine blood was drawn into lithium heparin tubes and was diluted upon receipt 1:5 in HBSS before Ficoll separation and canine RBCs were lysed with RBC lysis buffer (Stem Cell Technologies, 07800) for 4 min on ice (36, 37).

Experiments using discarded buffy coats from normal human red blood cell (RBC) donations were conducted under MD Anderson Cancer Center IRB

exemption PA13-0978 and Nationwide Children's Hospital IRB Exemption. Both canine and human blood were processed using Ficoll Plus (GE Healthcare; 17-1440-02), as described previously. Human NK cells were purified with RosetteSep Human NK Cell Enrichment Cocktail (Stem Cell Technologies, 15065).

6.2 Cell Culture

The canine cell lines OSCA8 (OS of the humerus, Jaime Modiano, University of Minnesota) and OSCA40 (OS of the femur, Jaime Modiano), Gray (OS lung metastasis, University of Wisconsin, Greg MacEwen), Abrams (OS, Ryan Roberts, Research Institute at Nationwide Children's Hospital), OSCA-78 (femur OS, Kerafast, EMN004), and melanoma-12 (Carlos Rodriguez, UC Davis) were cultured in DMEM High Glucose (Hyclone, SH30243.FS) with 10% FBS (Hyclone, SH30071.03HI), Penicillin Streptomycin Amphotericin Antibiotics (Lonza, 17-745E), and HEPES (OSCA-78 only; Gibco, 15630-080). Canine thyroid adenocarcinoma (CTAC) (Ohio State University, George Krakowka) was cultured in RPMI 1640 (Hyclone, SH30096.FS) with 10% FBS, Glutamax (ThermoFisher Scientific, 35050061), and Antibiotics. Manin–Darby canine kidney cells (MDCK) (Sigma-Aldrich, 84121903-1VL) were cultured in Eagles Minimum Essential Media (Hyclone, SH30244.FS) with 10% FBS, PSA, Glutamax, and 1% Minimum Essential Amino Acids (Sigma, M7145). All cell lines reported in this study were identified as canine origin by species-specific PCR and were unique from each other by canine STR fingerprinting (CellCheck Canine, IDEXX BioResearch). Only MDCK had an established genetic profile with which to compare, which matched the results obtained for our MDCK cell line. The OSCA8 cell line was reported as having been

derived from a male, but fingerprinting of our OSCA8 cell line showed only chromosome X (262). This could be caused by a loss of chromosome Y during culture over time. Cells were dissociated for cytotoxicity assays using enzyme-free Cell Dissociation Buffer, Hank's Based (ThermoFisher Scientific, 13150016). NK cells were cultured in RPMI 1640 media plus Glutamax, 10% FBS, and antibiotics. All cells were routinely tested for Mycoplasma contamination using Lonza MycoAlert (Lonza, LT027-58) and found to be negative at all time points with the exception of melanoma-12. K562 feeder cells were derived by transducing K562, a chronic myelogenous leukemia cell line, with human 4-1BBL and membrane-bound human IL-21 (Clone9.mbIL21) as previously described (165).

6.3 NK Cell Expansion

Five million canine PBMC were cocultured with 106 K562 Clone9.mbIL-21 in at day 0 (1:2 ratio), and additional K562s were added at a 1:1 ratio at days 7 and 14. Medium was supplemented with 6.1 ng/mL recombinant canine IL-2 (rcIL-2) (R&D systems, 1815-CL), and fresh medium was added every 2–3 days. For human IL-2 comparison expansions, human IL-2 was added at 100 IU/mL in place of canine IL-2. The concentration of human IL-2 was based upon the dose of IL-2 used in previous publications on canine NK-like cells (138, 139, 263).

For CD3 depletion experiments, canine PBMC was stained with CD3-FITC antibody and sorted for all PBMC that were CD3-negative. Sodium azide in CD3 antibody was reduced using Amicon Ultra-0.5 mL Centrifugal Filter Units with Ultracel-50 Membrane (EMD Millipore, UFC505024). Expansion was done according to the previous protocol, comparing CD3-depleted PBMC to undepleted PBMC.

Fold expansion was calculated as the percentage of CD3⁺/NKp46⁺ cells within the lymphocyte gate of total PBMC. Null cell calculations were determined by subtracting the percentage of CD21 and CD14 expressing cells in the lymphocyte gate from the CD3⁺ cell percentage.

Purified primary human NK Cells were stimulated at Day 0 1:2 with K562 mbIL-21 and 1:1 at Day 7. Standard expanded NK cells were supplemented with 50 IU/mL recombinant human IL-2, and Resistant (ReNK) expanded NK cells received 50 IU/mL IL-2 plus 10 ng/mL TGF β (Biolegend, 580706). Fresh media and cytokines were added every 2-3 days. NK Cell Expansion was calculated based on the percentage of CD3⁺/CD56⁺ cells.

6.4 Sequencing

The sequence of canine NKp46 was verified with the following primers:
Forward: 5' ACTCACTGCCCTTCTCTTCC 3', Reverse: 5' CTAAGTGTGGCCAGCACATC 3' via RT-PCR with the following conditions Initial Denaturation: 94°C – 3 min, Denature: 94°C – 30 s, Annealing: 58°C – 30 s, Extension: 68°C – 1 min, Final Extension: 68°C – 5 min for 35 cycles using Platinum Taq DNA Polymerase High Fidelity (ThermoFisher Scientific, Cat: 11304-011) and dNTPs (ThermoFisher Scientific, Cat: 18427013). The PCR product was cloned into pCR2.1 Vector using TA cloning (ThermoFisher Scientific, K202020) and sequenced using Sanger-based sequencing on an ABI 3730 with M13 (-21) Forward and M13 Reverse Primers.

6.5 NKp46 Antibody Production

A mouse monoclonal antibody specific to canine NKp46 was constructed by first confirming the expression and sequence of NKp46 in expanded canine lineage negative (CD3⁻/TCR⁻/CD21⁻/CD14⁻) cells, using primers which covered 940/955 bp of canine NKp46 (NM_001284448.1). The sequence obtained matched the NCBI database sequence (data not shown). A chimeric protein composed of the signal sequence of human granulocyte-macrophage colony-stimulating factor (GM-CSF, NP_000749.2; aa: 1–17), the extracellular domain of canine NKp46 as determined using Phobius protein prediction software (NP_001271377.1; aa: 21–255), and murine CD8 α extracellular and transmembrane regions (NP_001074579.1; aa: 28–247) was constructed and synthesized using Life Technologies (custom order). This construct was ligated into the MigR1 vector (Provided by Patrick Zweidler-McKay), and virus was produced using GP2-293 packaging cells (Clontech, 631530) with 0.8 μ g pVSV-G packaging vector (Clontech), 10 μ L Lipofectamine 2000, and 4 μ g of the NKp46-MigR1 vector or Empty vector per well of a six-well plate. Supernatant was collected after 40 h, spun down, and added to L-cells (ATCC, CRL-2648) along with 8 μ g/mL polybrene. Cell surface expression of the chimeric NKp46 protein was verified through flow staining with anti-mouse CD8 α antibody (BD Biosciences; 561093) and GFP. BALB/C mice were immunized via footpad with L-cells expressing NKp46 fusion protein for 33 days (NKp46:L-cells) as previously described. (264)

6.6 Flow Cytometry

Blocking was performed in blocking buffer (50% FBS/PBS) with 0.1 mg of dog gamma globulin (Jackson ImmunoResearch, 0004-000-002) per 1,000,000 cells. Cells were stained with 200 ng/1,000,000 cells of mouse anti-canine NKp46 Clone 48A (isotype: IgG2a) or unconjugated TCR $\alpha\beta$ /TCR $\gamma\delta$ for 30 min, followed by goat anti-mouse IgG RPE (Jackson ImmunoResearch, 115-116-146) in 50% FBS/PBS, and finally with anti-canine TCR $\alpha\beta$ / $\gamma\delta$ (FITC/Alexa Fluor 488 when indicated; Peter Moore, UC Davis) and commercially available antibodies (Biorad) to canine CD3 (MCA1774F; clone CA17.2A12), CD8 α (MCA1039A700; clone YCATE55.9), CD4 (MCA1038PECY7; clone YKIX302.9), CD21 (MCA1781A647; clone CA2.1D6), CD5 (MCA1037APC; YKIX322.3), and anti-human CD14 (MCA1568A647, MCA1568A700; TÜK4). Cell events were acquired on a LSR Fortessa. Flow cytometry gating was determined using cells stained with secondary only and single color controls were analyzed using FlowJo 7.6.5/10.

Staining of human NK cells was conducted as described above but without dog gamma globulin. Transcription factors were stained using the Transcription Factor Buffer Kit (BD Biosciences, 562725). All other intracellular flow cytometry was done using the BD Cytofix/Cytoperm Fixation/Permeabilization Kit with GolgiStop (BD Biosciences, 554715). Antibodies for the following proteins were used to assess NK phenotype and function: CD3 PeCy7/APC-H7 (clone SK7), CD56 FITC, NKG2D Pe-CF594, TRAIL PE/APC, FasL PE, NKp30 PE/Alexa Fluor 647, Granzyme A APC, Granzyme B BV510, Perforin BV421, DNAM-1 BV711, CD107a BV510, IFN γ

APC, TNF α BV421, IL-6 PE, CD16 PE, T-bet APC, GATA3 BV421, RUNX3 PE, and Tonbo Ghost Dye 510/710.

6.7 Cell Sorting

Canine cells were sorted on a FACS Aria Sorter at the flow cytometry core facility (UT MD Anderson Cancer Center) using four-way purity with post-sort purity verification for expression of CD3 and NKp46 to generate $\geq 99.5\%$ pure populations of CD3⁻/NKp46⁺, CD3⁻/NKp46⁻, CD3⁺, and CD3⁻ cells when indicated.

6.8 Luminex

Luminex assays were done, as described previously, using tissue culture supernatant from 2×10^5 sorted, CD3⁻/NKp46⁺, CD3⁻/NKp46⁻, or CD3⁺ expanded cells cocultured for 6 h at 37°C with rIL-2 or rIL-2 plus 4×10^5 K562 Clone9.mbIL-21 irradiated feeder cells with the CCYTOMAG-90K Canine Cytokine Chemokine Panel (EMD Millipore, CCYTMG-90K-PX13) on a Bioplex-200 using Bioplex Manager 6.0 Software (Biorad, USA) (41). Cytokine secretion with coculture with K562 Clone9.mbIL-21 feeder cells was normalized by subtracting cytokine secretion in IL-2 only.

6.9 Cytotoxicity Assays

Canine CD3⁻/NKp46⁺ were sorted when necessary to be $\geq 95\%$ pure. Comparison of CD3⁻/NKp46⁺, CD3⁻/NKp46⁻, and CD3⁺ expanded cells were sorted as described, rested overnight in IL-2, and cytotoxicity assays with calcein-AM based method were conducted in at least duplicate using 4 μ g of calcein AM/1,000,000 target cells (265).

Calcein assays with human cell lines were done similarly with the exception of 3 µg calcein AM/mL/1,000,000 target cells in complete media. NK cells were prepared for cytotoxicity assays by resting overnight in either human IL-2 alone or IL-2 (50 IU/mL) with 10 ng/mL soluble TGFβ (Biolegend). Calcein assays were conducted in the same cytokines as the NK cells were rested in overnight.

6.10 Intracellular Functional Flow Cytometry

To determine degranulation by CD107a expression and intracellular cytokine production in response to tumors, 300,000 NK cells were co-cultured in a 96-well round-bottom plate with 60,000 tumor cells (5:1 E:T ratio) or no target for a control in 200 µl media as described for cytotoxicity assays. One µl of monensin was added to each sample along with CD107a at the beginning of the assay. Plates were spun down at 100 g x 2 minutes to promote cell-cell contact, and placed in a 37°C incubator for 3 hours. After 3 hours, media was removed and staining began for cell surface and intracellular proteins as detailed.

6.11 Cytometric Bead Array (CBA)

To determine NK cell release of IFNγ and TNFα, NK cells were cultured as described for intracellular functional flow cytometry with the exception of monensin and CD107a antibody. After 3 hours, supernatant were collected and frozen at -70°C until use. On the day of the CBA assay, supernatants were thawed and 50 µl of undiluted supernatant was used according to manufacturer's instructions for the BD CBA Soluble Protein Master Kit and IFNγ and TNFα Flex Set. The analytes had a limit of detection of 10 pg/mL. At least 300 events per analyte was acquired on a BD LSR Fortessa. Geometric mean for each analyte was determined in Flow Jo v. 10.1

and unknown samples were interpolated using a standard curve with $R^2 \geq 0.99$ from known standards. Negative cytokine measurements were graphed as 0 pg/mL.

6.12 RT-PCR/qPCR

RNA was isolated from canine expanded cells using RNAeasy Kit, QiaShredder Columns, and RNAase-Free DNase Set (all Qiagen, 74104, 79654, 79254), and cDNA was synthesized using Omniscript RT Kit (Qiagen, 20511) with RNase inhibitor (New England Biolabs, M0307S) and Oligo(dt)20 (ThermoFisher Scientific, 18418-020). RT-PCR and qPCR reactions were performed on Roche 480 (Roche, USA) using Power Up Sybr Green Master Mix (ThermoFisher; A25742) or TaqMan Universal PCR Mastermix for qPCR for CD16 (ThermoFisher Scientific, 4304437) for 40 cycles. All qPCR was done in duplicate with at least three different donors. Semi-quantitative RT-PCR reactions were run on five different donors with 100 ng cDNA per sample and 6× DNA loading dye, and products were verified on a 1.5% agarose gel with TAE buffer and GelRed Nucleic Acid Stain (Phenix, RGB-4103) and 100 bp DNA Ladder (New England Biolabs, N3238S). Gels were imaged on BioRad ChemiDoc Touch Imaging System (BioRad, USA) for Faint Bands and analyzed using Image Lab 5.2.1. The following enhancements were uniformly applied to all gels (High: 65535, Low: 34, Gamma: 0.43).

PCR cycling conditions were as followings: UDG activation: 50°C – 2 min, Polymerase Activation: 95°C – 2 min, Denature: 95°C – 15 s, B-actin, NKp46, NKp30, Granzyme B, and Perforin – Anneal/Extend: 62°C – 1 min; B-actin, Ly49, and CD16 – Anneal: 54°C – 15 s, Extension: 72°C – 1 min; B-actin, DNAM-1, NKp30, NKp44, NKG2D, and NKp80 at the same conditions as Ly49 and CD16 with

the exception of Annealing at 59°C. Primer sequences for NKp30, NKG2D, Perforin, and Granzyme B were published and verified previously (22). Primers for all other genes were made using Blast Primer software and Primer 3 with predicted specificity verified by Blast, as follows: Ly49: Forward-5' TGGAAATGGGTTGACACTGA 3', Reverse-5' CTTTGGAAGGAGGCAGACAA 3'; NKp46: Forward-5' CCGCATCCCAAAAATGACCC 3', Reverse-5' GGGCCTATGGGAAACTCCAC 3'; NKp80: Forward-5' TCCCTGTCCTTACTGGTTTCTC 3', Reverse-5' TATTTGAGCCATTCCGATG 3'; CD16: Forward-5' CAGTGGAGAGTACAGGTGCC 3', Reverse-5' CCCTGCAGAAGTAGGAGCC 3'; DNAM-1: Forward-5' AATGTCACGCTCACCTGTCA 3', Reverse-5' CGGTAAAGTCCCGAGTCAGA 3'; NKp44: Forward-5' GAAGGAGGAAGACTCGGGATA 3', Reverse-5' GAAGGGGTGATGGTGGTAGA 3'; and B-actin: Forward-5' CGAGCATCCCCAAAGTTCTA 3', Reverse-5' ATTCTCTTTCCCTCCCCTGT 3'.

Primer specificity was verified by Melt Curve Analysis and agarose gel electrophoresis. Primer product sequences for new primers were verified using Qiaquick Gel Extraction Kit (Qiagen, 28704) and sequencing, as described previously. Primers were synthesized by Sigma-Aldrich. For qPCR for CD16, TaqMan Universal PCR Master Mix was used with B-actin (ThermoFisher Scientific, Catalog #: 4453320, Assay ID: Cf03023880_g1) and CD16 probes (ThermoFisher Scientific, Catalog #: 4448892 Assay ID: Cf02645051_m1) for 40 cycles according to the TaqMan Universal PCR Master Mix protocol. qPCR was determined using the $2^{-\Delta\Delta Ct}$ method and normalized to B-actin, and relative to CD3⁺/NKp46⁺ cells from the same donor.

RNA from fresh, never frozen, Day 14 expanded human NK cells was done as described for canine. cDNA was made using the high throughput cDNA kit. PCR for the TGF β pathway was done using Taqman Fast PCR Mastermix and Human Fast 96-well TGF β Pathway Array (ThermoFisher, 4418742) on an Applied Biosystems 7900HT.

6.13 Mass Cytometry

Antibodies for mass cytometry were labeled with heavy metals using Maxpar-X8 labeling reagent kits (DVS Sciences) according to manufacturer's instructions and titrated for determination of optimal concentration. The antibodies and their respective heavy metal labeling include CD314/NKG2D-150Nd (Biolegend, 320802), CD3-151Eu (BioLegend, 300443), CD335/NKp46-154Sm (BioLegend, 331902), CD56-162Dy (BD, 559043), CD336/NKp44-163Dy (BioLegend, 325102), CD337/NKp30-164Dy (BioLegend, 325202) CD16-165Ho (DVS, 3165001B), CD159a/NKG2A-166Er (R&D, MAB1059), CD226/DNAM1-167Er (BioLegend, 337102), and CD159c/NKG2C-169Tm (R&D, MAB1381). Frozen primary and K562 Clone9.mblL-21 expanded human NK cells were thawed 1 day prior to staining to allow for NK cell recovery overnight. After overnight recovery, 1.5×10^6 cells were stained with 2.5 μ M cell ID cisplatin (Fluidigm, 201064) in serum-free RPMI for 1 min for identification of viable populations. Subsequently, CyTOF staining was performed, as described previously, with the following minor modification: staining was performed in 5% FBS/0.01% sodium azide buffer in PBS (266). Data were acquired on the CyTOF instrument (DVS Sciences).

6.13.1 Clustering Analysis

Clustering analysis of mass cytometry data was performed using spanning-tree progression analysis of density-normalized events (SPADE V3.0) software (267). Markers used for clustering of data were CD56, NKp46, NKp30, NKp44, NKG2A, NKG2C, and NKG2D.

6.14 Western Blotting

Day 14 expanded NK cells were lysed with RIPA buffer. Protein yield was quantified with a Bradford assay. 20 µg of protein was loaded onto a gel. All antibodies used were from Cell Signaling.

Quantification of STAT3 levels was done using ImageJ software. The ratio of pSTAT3 to total STAT3 was calculated using the IL-21 treated NK samples. Total STAT3 to GAPDH calculations were done using untreated NK cell samples, since total STAT3 levels were determined to be affected by the degree of pSTAT3.

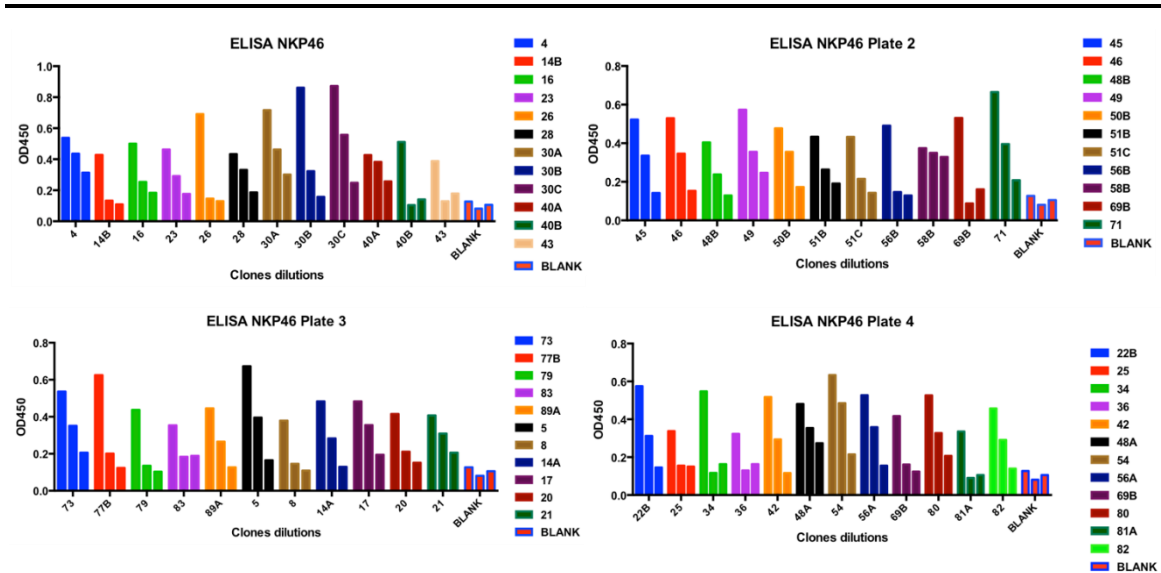
6.15 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6.0 or 7.0. Luminex assays were analyzed with unpaired t-tests and Holm–Sidak method for multiple comparisons. Paired t-tests were run for the following: cytotoxicity assays between NK and T-cells or Null Cells, qPCR, comparison of NKp46+ and NKp46– human NK cell phenotype, and all TGFβ analysis. Comparison between healthy and OS-bearing canines' percent NK cells and expansion used the Mann–Whitney test. All other statistical analyses were unpaired Student's t-tests (all two-tailed). Human versus canine IL-2 analysis used a one-tailed Wilcoxon-matched pairs test.

Correlations were done using the Spearman r test. p Values less than 0.05 were considered significant.

Appendix A. Screening of NKp46 Hybridomas with ELISA against L-cells expressing Canine NKp46. Dilutions are from L to R: Concentrate, 1:15, 1:225.

ELISA was done by MD Anderson Monoclonal Antibody Core.



Appendix B. TGF β Pathway Genes Assessed on TaqMan Array

Gene	p-value	Mean \pm SD
ACVR1	0.17	1.3 \pm 0.3
ACVR1B	0.17	2.0 \pm 1.1
ACVR1C	0.21	2.8 \pm 2.3
ACVR2A	0.73	1.2 \pm 0.8
ACVR2B	0.95	1.0 \pm 0.5
AMH	0.38	5.4 \pm 8.6
AMHR2	0.47	1.8 \pm 1.9
BMP2	0.79	0.8 \pm 1.2
BMP3	N/A	N/A
BMP4	0.07	0.6 \pm 0.3
BMP5	N/A	N/A
BMP6	0.03	0.3 \pm 0.3
BMP7	0.2	3 \pm 2.5
BMP10	N/A	N/A
BMP15	N/A	N/A
BMPER	0.37	7.7 \pm 12.8
BMPR1B	N/A	N/A
BMPR2	0.55	1.1 \pm 0.3
CDH1	NA/	N/A
CHRD	N/A	N/A
CREBBP	0.15	0.9 \pm 0.2
CUL1	0.45	1.9 \pm 2.1
DCN	0.54	1.5 \pm 1.3
E2F4	0.36	0.9 \pm 0.1
E2F5	0.57	1.2 \pm 0.7
EP300	0.05	0.8 \pm 0.1
FMOD	0.02	0.2 \pm 0.3
FNTA	0.48	1.2 \pm 0.5
FST	0.07	79.3 \pm 56.8
GDF2	N/A	N/A
GDF3	N/A	N/A
GDF5	0.23	8.7 \pm 7.9
GDF7	0.04	0.2 \pm 0.3
GDF9	0.51	1.4 \pm 1.0
GDF10	N/A	N/A
GDF11	0.3	1.3 \pm 0.5
GDF15	0.4	14.9 \pm 28.0
HIPK2	0.58	0.8 \pm 0.5
IFNG	0.38	2.1 \pm 2.5
IL17F	0.09	4.4 \pm 1.9
IL6	0.81	1.1 \pm 1.0

INHA	N/A	N/A
INHBA	0.12	0.6 ± 0.4
INHBB	0.41	7.7 ± 11.1
INHBC	0.98	1.0 ± 0.6
INHBE	0.02	0.3 ± 0.3
LEFTY1	0.46	0.7 ± 0.7
LEFTY2	0.0031	0.5 ± 0.1
LTBP1	0.54	1.3 ± 0.8
LTBP2	0.04	0.3 ± 0.4
LTBP3	0.05	0.9 ± 0.1
LTBP4	0.12	1.5 ± 0.5
MAP3K7/TAK1	0.02	0.7 ± 0.1
MAP3K7IP1/TAB1	0.003	0.7 ± 0.1
MAPK1	0.78	0.9 ± 0.4
MAPK3	0.41	1.8 ± 1.6
MSTN	0.19	8.1 ± 8.3
NODAL	N/A	N/A
NOG	0.1	0.5 ± 0.4
PPP2CA	0.47	1.2 ± 0.4
PPP2CB	0.81	1.0 ± 0.3
RBL1	0.57	1.4 ± 1.1
RBL2	0.0035	0.8 ± 0.1
RBX1	0.68	0.9 ± 0.3
RHOA	0.57	0.9 ± 0.3
ROCK1	0.94	1.0 ± 0.5
ROCK2	0.87	1.0 ± 0.5
SKP1	0.76	1.1 ± 0.6
SMAD1	0.03	0.6 ± 0.2
SMAD2	0.88	1.0 ± 0.4
SMAD3	<0.0001	0.03 ± 0.01
SMAD4	0.91	1.0 ± 0.3
SMAD5	0.12	1.2 ± 0.2
SMAD6	0.0074	6.3 ± 1.6
SMAD7	0.33	1.2 ± 0.4
SMAD9	0.24	10.7 ± 13.2
SMURF1	0.4	1.5 ± 0.9
SMURF2	0.06	1.7 ± 0.5
SP1	0.71	1.0 ± 0.2
STUB1	0.07	0.8 ± 0.2
TFDP1	0.37	1.8 ± 1.4
TGFA	0.35	26.6 ± 46.1
TGFB1	0.002	0.7 ± 0.1
TGFB2	0.48	2.0 ± 2.4
TGFB3	0.04	0.7 ± 0.2
TGFBR1	0.17	0.8 ± 0.3

TGFBR2	0.8	1.1 ± 0.5
TGFBR3	0.002	0.2 ± 0.1
TGFBRAP1	0.64	0.9 ± 0.3
TNF	0.11	0.7 ± 0.3
TSC22D1	0.84	1.1 ± 0.9
ZFYVE9	0.54	1.5 ± 1.4

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