HUMAN NK CELL ACTIVATION UPON STIMULATION WITH INTERLEUKINS

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By EVAN J LUSTY, B.Sc

A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree Master of Science

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Abstract

The WHO predicts that by the year 2035 the world will be facing a "cancer tidal wave". This has spurred on the development of many cancer immunotherapies. The adoptive transfer of *ex vivo* expanded NK cells is one such therapy that could have high efficacy and target specificity. However, the adoptive transfer of NK cells has some negative side effects. Fortunately, these are not due to the direct effects of the NK cells. Instead, toxicity arises from the systemic administration of IL-2 which supports NK cell function. To sidestep the need for IL-2 injections our project investigated the effect of stimulating NK cells with interleukin 12, 15, and 18 *in vitro*. Our hope is that one-day pre-stimulation of NK cells with these cytokines *in vitro* before their adoptive transfer will maintain NK cell activation and survival *in vivo*.

Our research has revealed that *ex vivo* expanded NK cells stimulated with IL-12 and IL-18 +/- IL-15 significantly upregulates the expression of IFN-γ, TNF-α, CXCL-8, CCL3L1, and LTA. Furthermore, production of these cytokines can continue up to 72 hours post stimulation *in vitro*. If the production of these cytokines continues after adoptive transfer of NK cells into cancer patients it could drastically alter the anti-inflammatory milieu of the cancer patient.

Our attention was then turned to elucidating the factors responsible for the long term activation of the NK cells in the IL-12 and IL-18 +/- IL-15 conditions. We have determined that the increase in production of proinflammatory cytokines is due to direct increases in IFN-γ transcription.

The results of these trials will direct the future use of NK cells in clinical trials. Specifically, there is great potential for this research to be used to predict potential negative side effects of using *ex vivo* expanded and stimulated NK cells as a cancer immunotherapy.

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I am so fortunate to have worked with all past and present members of the Ashkar lab and the McMaster Immunology Research Center. Your kindness, patience, friendship, knowledge, and individual virtues have made you all inspiring peers.

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This thesis is dedicated to my Aunt Linda. Your battle with cancer and the undying love you had for my brothers and I has been a constant inspiration for me to find a cure to the illness that ended our time together too soon.

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List of Abbreviations:

2-ME – 2-Mercaptoethanol

Abs – Antibodies

ADCC – Antibody-dependent cell-mediated cytotoxicity

AP-1 – Activator protein 1

BIM – Bcl-2-like protein

Brg1 – Brahma-related gene-1

BSA – Bovine Serum Albumin

cAMP – Cyclic adenosine monophosphate

CD – Cluster of Differentiation

CDKI – Cyclin-dependent kinase 1

CNS – Conserved Non-coding sequence

CpG – Cytosine-phosphate-Guanine

CTL – Cytotoxic Lymphocyte

DCs – Dendritic Cells

DNA – Deoxyribonucleic Acid

ELISA – Enzyme-linked Immunosorbent Assay

ERK1/2 – Extracellular signal-regulated protein kinase ½

ERK – Extracellular signal-regulated kinases

FBS – Fetal Bovine Serum

Foxo3a – Forkhead box O3

GATA3 – Trans-acting T-cell-specific transcription factor GATA3

HS – Hypersensitivity Site

ICAM-1 – Intercellular adhesion molecule 1

ICSBP – Interferon consensus sequence binding protein

IDO – Indolamine Dioxygenase

IFN – Interferon

IKK – Inhibitor of nuclear factor kappa-B phosphorylation

IκB – IκB kinase

IL – Interleukin

IP-10 – Interferon-gamma-inducible protein

IRAK – Interleukin receptor-associated kinase 1

IRF-1 – Interferon regulatory factor 1

I.V – intra venous

JAK – Janus Kinase

JNK – c-Jun N-terminal kinase

K562-mbIL-21 – K562-membrane bound interleukin-21

LFA-1 – Lymphocyte function-associated antigen 1

MAPK – Mitogen-activated protein kinases

MHC – Major Histocompatibility Complex

MIG – Monokine induced by gamma interferon

NK cell – Natural Killer Cell

NF-κB – Nuclear factor kappa-B

OCT-1 – Octamer-binding protein 1

PBS – Phosphate Buffered Saline

PFA – Paraformaldehyde

PI3K – Phosphoinositide 3-Kinase

PS – Post Stimulation

PW – Post Wash

RNA – Ribonucleic Acid

SMAD – Sma (*Caenorhabditis elegans* protein SMA) and Mad (mothers against decapentaplegic) related proteins

SNPs – Single Nucleotide Polymorphisms

STAT – Signal Transducer and Activator of Transcription

SWI/SNF – SWItch/sucrose non-fermentable

TGF – Transforming Growth Factor

TRAF6 – TNF receptor-associated factor 6

TRAIL – TNF-related apoptosis-inducing ligand

UTR – Untranslated Region

VCAM-1 – Vascular cell adhesion molecule 1

WT – Wild Type

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DECLARATION OF ACADEMIC ACHIEVEMENT

Evan J. Lusty performed all of the experiments described in this thesis with the exception of the following:

- The Illumina Human HT-12 v4 Expression BeadChip Kit Microarray was completed with the assistance of the Genetic and Molecular Epidemiology Laboratory (Director, Dr. Guillaume Paré) of the David Bradley Cardiac, Vascular and Stroke Research Institute
- The mouse experiments mentioned in discussion 4.5 were completed by other members of the Ashkar lab.

Chapter One

Introduction

1.1 NK cells, Cancer, and Cancer Immunotherapy

Natural Killer (NK) cells are innate immune lymphocytes classically described by their ability to destroy virally infected and cancerous cells without prior sensitization in healthy individuals[1–5]. Approximately, 90% of circulating NK cells are cytotoxic and are adept at destroying cancerous cells[6]. Therefore, a deficit in NK cell function or number heightens one's risk for developing cancer[7]. The combination of these facts gave birth to the NK cell immunotherapy field. Here, researchers hope to inject highly activated NK cells into cancer patients, or modulate endogenous NK cell activity in cancer patients to limit cancer metastasis and tumor growth. However, the activation of NK cell cytotoxicity is regulated by a delicate balance between the stimulation of activating and inhibitory receptors on the NK cell surface[8]. Classically, when NK cell receptors bind to both activating and inhibitory ligands suppression of NK cell cytotoxic activities results[9]. However, it is possible for stimulatory cytokines such as IL-12 and IL-18 in a combined stimulation to overcome suppression from the inhibitory ligands of the environment and induce NK cell activation in the form of pro-inflammatory cytokine and granzyme B production[9]. This suggests that cytokines have a powerful role in maintaining homeostasis in the body. The importance of cytokines for NK cell function is further highlighted by the fact that cytokines control the phenotype of NK cells at a fundamental level. For example, activating receptors are up-regulated on NK cells stimulated with IL-15, IL-2, IL-12, and IL-18[5]. Conversely, tumors, and tumor associated macrophage products such

as TGF-β and indoleamine-2,3-dioxygenase (IDO) down-regulate the activating receptor expression and up-regulate the expression of inhibitory receptors, limiting the cytotoxic capabilities of NK cells, in the absence of cytokines such as IL-12 and IL-18[5]. Therefore, cytokines can shift the nature of NK cell responses to various environmental stimuli.

Understanding the fluid nature of NK cell phenotypes and activation in response to cytokine stimulation is of critical importance upon consideration of the roles NK cells play in the field of cancer immunotherapy. NK cells have already been shown to limit the growth and metastasis of cancerous lesions in *in vivo* mouse experiments and human clinical trials[7,10,11]. Furthermore, current research trials are utilizing cytokines such as IL-2, IL-12, IL-15, IL-18, and IL-21 to promote the survival of NK cells *in vivo* and heighten their tumoricidal effects[7,12,13]. Therefore understanding how these cytokines affect NK cells is important to the field of NK cell immunotherapy. Consequently, my thesis and research has further elucidated the effects of the stimulatory cytokines IL-12, IL-15, and IL-18 on NK cells.

Due to the flexibility of NK cells and their dynamic responses to various stimulatory conditions attempts to utilize NK cells to treat cancer have been approached in many different ways. For instance, some researchers and clinicians have preferred to use *ex vivo* expanded allogeneic NK cells as a cancer immunotherapy[11]. This approach circumvents NK cell paralysis seen in some NK cell immunotherapies by ensuring that inhibitory receptors on NK cells cannot recognize self-major histocompatibility complexes (MHC) which classically inhibit the cytotoxic capabilities of the NK cells. This strategy has

proven effective in treating metastatic melanoma, renal cell carcinoma, poor prognosis AML, and advanced non-small cell lung cancer[7,10,11]. However, the allogeneic NK cells are eventually rejected by the host's immune system, limiting their long-term effectiveness[7,10,11].

Similarly, NK cell lines such as the NK-92 have had some success as an NK cell immunotherapeutic. Indeed, they are capable of short term cytotoxic and antitumor effects in malignant melanoma and renal cell carcinoma, however they too will be rejected by the host immune system[7,14–16]. Nevertheless, NK cell lines are amenable to genetic modification which could boost their effectiveness as directly anti-cancerous cells or as modulators of the immune system[7,14–16]. Previous attempts at this have increased the expression of activating receptors and cytokine production, decreased inhibitory receptor expression and inserted chimeric antigen receptors[7,15,16].

Other groups – including our own - are utilizing autologous NK cell transfer therapies as a means of reducing the cancer burden. The adoptive transfer of autologous NK cells is our preferred form of NK cell immunotherapy due to its theoretically high safety profile. This safety is partially attributed to the autologous NK cells' ability to recognize self MHC on healthy tissues[7]. This is an inhibitory signal for NK cells and should protect healthy tissues from destruction. Conversely, cancerous cells reduce MHC expression in order to evade activating tumoricidal T cells, this however weakens the cancer cells' ability to inhibit attack from NK cells[17]. Consequently, adoptively transferred autologous NK cells should only preferentially target cancer cells. However, supporting cytokines are

often delivered systemically in patients receiving NK cell immunotherapies in order to improve the survival and tumoricidal tendencies of the transferred NK cells[18]. Unfortunately, systemic toxicity in patients receiving these cytokines can arise[19]. To curtail these side effects we are stimulating NK cells with the cytokines IL-2, IL-12, IL-15, IL-18, and IL-21 before adoptive transfer into cancer patients. This should improve the anti-cancerous capabilities and survival of the adoptively transferred autologous NK cells.

The NK cells' ability to perform antibody dependent cell-mediated cytotoxicity (ADCC) has also been exploited by NK cell researchers in the hopes of improving the NK cells' ability to target and destroy cancer cells[20]. Utilizing cluster of differentiation (CD)-16 NK cells can bind to antibody Fc fragments resulting in targeted cell lysis[20]. Therefore, trials taking advantage of this phenomenon have used monoclonal antibodies such as: rituximab, trastuzumab, and herceptin with co-administration of: IL-12, IL-2, IL-21, the TLR agonist (CpG), and immunocytokines in order to direct NK cells' destructive potential[7,21]. It was found that these treatments resulted in higher cytotoxicity against Ab-coated target cells in non-Hodgkin's lymphoma, metastatic breast carcinoma, gastric carcinoma, melanoma, osteosarcoma, and soft-tissue sarcoma patients[7].

1.2 Mechanisms of NK Cell Killing:

Throughout evolution NK cells have developed and retained traits giving them the capacity to destroy cancerous tissues in many different ways. For instance, upon activation NK cells can release cytoplasmic granules containing both perforin and

granzyme[20]. Perforin disrupts the cell membrane creating a pore allowing granzymes to enter into the cell[20]. Subsequently, granzymes initiate cell death through both caspase-dependent and caspase-independent mechanisms[20]. Moreover, NK cells express a myriad of cell surface death inducing ligands which can bind specific receptors on target cells. For example, tumor necrosis factor (TNF) family proteins such as FasL and TNF-related apoptosis-inducing ligand (TRAIL) induce target cell death through receptor binding to FAS and TRAILR, on target tumor or unhealthy cells[10,22].

NK cells are also capable of mediating indirect target cell killing by activating both the innate and adaptive arms of the immune system. Various soluble effector molecules from NK cells such as: TNF-α and IFN-γ stimulate an adaptive immune response modulating CD8+ T cells to become cancer cell specific cytotoxic T lymphocytes (CTLs)[7,20]. IFN-γ also directs CD4+ T cell differentiation into a Th1 functional state, polarizing the immune system into a pro-inflammatory and cytotoxic state[7,20]. Moreover, tumor antigens from cancer cells killed by NK cells provide tumor antigens for dendritic cells (DCs), promoting their maturation and antigen presentation, this can further link the innate and adaptive immune systems[23]. Furthermore, cytokines produced by NK cells can result in B cell activation and the production of antitumor antibodies(Abs). These anti-tumour antibodies can then bind their targets on cancer cells enhancing NK cell killing[20]. In addition, cytokine production from activated NK cells can directly inhibit tumour angiogenesis and therefore limit the blood supply to a developing or advanced tumor[24].

Unfortunately, tumors develop ways to decrease their susceptibility to NK cell attack and disrupt normal NK cell function to improve their own chances of survival. For example, tumors decrease the expression of co-stimulatory ligands, ligands for activating receptors, secrete NK cell immunosuppressive agents such as: IL-10, TGF-β and indoleamine 2,3-dioxygenase (IDO) and resist perforin/granzyme mediated apoptosis[7],[25]. Due especially to immunosuppressive cytokine secretion an altered phenotype of NK cells in cancer patients is common. NK cells from cancer patients typically display a decreased expression of: activating receptors and an increased prominence of inhibitory receptors culminating in an inhibited cytotoxic profile of their NK cells[26]. This creates a downward spiral for the cancer patient. Consequently, our work and this thesis has looked at finding ways to counteract these phenomenon.

1.3 IL-2 and NK Cell Signaling:

IL-2 is important to NK cell development, homeostasis, and proliferation. These are traits shared by all the common gamma chain cytokines including: IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21[27,28]. IL-2 mediates its effects by binding to a high affinity receptor consisting of IL-2Rα, IL-2Rβ, and the common gamma chain used in the receptor complex for all the gamma chain cytokines[27]. The common gamma chain is associated with Janus tyrosine-kinases (JAK) which are essential for signal transduction of the common gamma chain cytokines[27,28]. These kinases transmit signals through the phosphorylation of multiple signal transducer and activators of transcription (STAT) molecules. Which STAT molecules are phosphorylated depends on the initial common

gamma chain cytokine receptor complex stimulated[28]. This specificity is mediated through tyrosine phosphorylation of amino acids in cytokine specific receptor molecules. These receptor specific phosphorylation events then attract unphosphorylated STAT monomers, which utilize their SH-2 domains to bind to the cytokine specific receptor complexes[27,28]. The STAT monomers are then phosphorylated, dimeriz, trafficked to the cell nucleus to bind to STAT-sensitive regulatory elements controlling gene transcription[28]. It is currently believed that IL-2 stimulation of cells results in phosphorylation of : STAT1, STAT3, STAT5a and STAT5b inducing NK cell proliferation, and a killer or cytotoxic phenotype[27–29]. Furthermore, it has been shown that PI3K and MAPK are also activated upon IL-2 engagement. This activation of MAPK is very important as the p38 MAPK has critical roles in stabilizing cytokine transcripts in activated NK cells[30]. Understanding this process is critical to determining how the stimulatory cytokines used in this thesis -which includes a maintenance dose of IL-2 - affects our *ex vivo* expanded NK cells.

Clinical trials have used infusion of *ex vivo* grown NK cells and IL-2 to treat cancer. Unfortunately, poor clinical outcomes were observed due to vascular leak syndrome induced by systemic IL-2[31,32]. Moreover, IL-2 injection into cancer patients following an adoptive NK cell transfer increases regulatory T-cell activity, which can then inhibit NK cell function[31,32]. Consequently, tumors can grow unchecked by the immune system. [31,32] Therefore, we are examining ways to activate NK cells before administering them to cancer patients so that side effects associated with concomitant IL-2 injection are avoided.

1.4 Roles of IL-12 on NK Cell Activation:

IL-12 is well characterized as an NK cell activator, and short-term IL-12 stimulation of NK cells induces IFN-γ production[33]. NK cells respond to IL-12 through a receptor complex consisting IL12Rβ1 and IL12Rβ2[33]. IL-12 binding to the aforementioned receptor complex results in Jak2 phosphorylation which induces tyk2 phosphorylation, which in turn mediates STAT4 phosphorylation causing IFN-γ and TNF-α production[33].

It is thought that IL-18 is needed to prime the NK cells' maximal response to IL-12[33]. This priming of NK cells by IL-18 results in increased IFN-γ production by NK cells following stimulation with IL-12[34]. Synergistic IFN-γ production has been attributed to IL-12 induced STAT-4 activation, stabilized by IL-18 induced activating protein-1 (AP-1) which also acts on the IFN-γ promotor[34].

1.5 Roles of IL-15 on NK Cell Activation:

Many functional similarities exist between IL-2 and IL-15. These redundancies have been attributed to the shared gamma chain receptor subunit in the IL-2 and IL-15 receptor complexes, but also to the promiscuous use of the IL-2Rβ chain in the IL-15 receptor[27,28]. However, the unique features of IL-15 signaling are continuously being elucidated. For instance, it is now well known that IL-15 can signal through both trans-presentation and cis-presentation and that both of these are important for NK cell activation[35,36]. In both of these presentation models IL-15 is presented bound to the IL-15Rα receptor subunit. However, for trans-presentation the IL-15 is presented by a

neighboring cell (trans-presentation), versus the individual NK cell to itself (cis-presentation)[34,35]. Therefore, IL-15 is most frequently presented to the IL-2Rβ, and common gamma chain receptor already bound to IL-15Rα[37]. Upon the complete formation of the receptor-cytokine complex, JAK1 and JAK 3 signal through STAT5/STAT1/STAT3, inducing changes in gene expression causing proliferation, survival, and activation of the cytotoxic abilities of the stimulated NK cells[28,36].

IL-15 plays several key roles in NK cell survival, activation, and proliferation. For instance, IL-15 and the specific IL-15Rα receptor subunit are both essential for the development of NK cell precursors into immature NK cells which eventually become mature NK cells[38–40]. This was highlighted by the finding that genetic ablation of IL-15 or IL-15Rα reduces the NK cell population to an almost non-existent level[38–40]. As avoiding cellular apoptosis is critical to survival it was suggested that IL-15 has anti-apoptotic effects. These effects of IL-15 are mediated through an inhibition of Bcl-2-like protein 11 (Bim) via extracellular signal-regulated kinases 1/2) (ERK1/2) phosophorylation[28,38–40]. However, the anti-apoptotic effects of IL-15 have also been shown to be mediated through the phosphatidylinositol-3-OH kinase (PI3K)-dependent inactivation of the transcription factor Foxo3a[28].

1.6 Roles of IL-18 on NK Cell Activation:

Because IL-18 is being used as part of NK cell immunotherapy trials investigating the complete effects of IL-18 on NK cells is a valuable endeavor. Investigations of the IL-18 signaling mechanisms have previously been completed by studying mouse MyD88

knockout models. Here it was shown that IL-18 signals through IL-18Rα and IL-18Rβ which are in close association with MyD88[41]. MyD88 continues signaling throughout the cell through interleukin receptor-associated kinase 1 (IRAK), TNF receptor-associated factor 6 (TRAF6), c-Jun N-terminal kinase (JNK), Inhibitor of nuclear factor kappa-B phosphorylation (IKK), Nuclear factor kappa-B (NF-κβ)[41]. These signaling cascades can contribute to the increased production of IFN-γ and TNF-α in response to IL-12 signaling due to stabilization of STAT4 activity by AP1 and stabilization of the cytokine transcripts by p38 MAPK[42,43].

1.7 Roles of IFNγ in the Clinic and the Tumour Microenvironment

IFN-γ was first approved as an antitumor and antiviral therapy in 1986 following extensive research highlighting the promising effects of IFN-γ in the treatment of cancer and viral infections in animals. The importance of IFN-γ in animal models of cancer was described by Robert Schreiber's laboratory. They showed that mice deficient in IFN-γ had a higher incidence of chemically induced or spontaneous tumors. Despite this observation, the results of human clinical trials using IFN-γ to treat cancer have been less promising. Nevertheless, IFN-γ can mediate antitumor activity through inhibition of angiogenesis, direct toxic effects, and the induction of potent innate and adaptive immune responses in the tumor microenvironment[44].

In order to establish a solid tumor *in vivo* new blood vessels must form in a process known as angiogenesis. Therefore, angiogenesis has been the focus of extensive research and a target of cancer treatments. Previous work has shown that angiogenesis is

strongly controlled by the immune milieu in the developing tumor. For instance, the recruitment of T cells producing IFN-γ to the tumor site was shown to be crucial to limiting angiogenesis in the tumor microenvironment[45]. However, before T cells are recruited to the tumor sites they must be primed with a tumor antigen, creating a delay period[45]. This delay in T cell maturation often means that the growth of tumors cannot be significantly inhibited before the T cell is prepared to destroy the cancer cell targets or produce anti-angiogenic IFN-γ[45]. However, IFN-γ can be produced in excess by NK cells which do not require priming in order to exert their anti-proliferative effects *in vivo*. Moreover, NK cells stimulated *ex vivo* to produce IFN-γ, transferred into a cancer patient could greatly limit the dissemination and growth of tumors upon infusion into cancer patients.

The virtues of IFN-γ production by NK cells goes far beyond its anti-angiogenic benefits. NK cells as the harbingers of IFN-γ production upon stimulation can bridge the initial innate response to a tumor with the specific activation of the adaptive immune system, including CD8 and Th1 cells[46]. Furthermore, IFN-γ from NK cells up-regulates MHC class II expression on macrophages[47]. Consequently, these activated macrophages provide stimulatory signals to T cells further involving the adaptive immune system in cancer immuno-editing. However, without the proper cell adhesion and chemoattractant molecules activated immune cells can do little to help cancer patients. Fortunately, IFN-γ induces the expression of adhesion molecules, including vascular cell adhesion molecule (VCAM)-1, lymphocyte function-associated antigen (LFA)-1, and intracellular adhesion molecule (ICAM)-1 on white blood cells and the vascular endothelium[45]. Furthermore,

IFN-γ induces the expression of MIG and IP-10, chemokines that assist in the recruitment of activated T cells to the tumor microenvironment[45].

IFNs also have direct anti-proliferative effects on cancer cells. For instance, STAT1, a component of the IFN-γ signaling cascade has been cited as a tumor suppressor gene because it can activate cyclin-dependent kinase inhibitors (CDKIs)[48]. Furthermore, continuous interaction of IFN-γ with its receptor can induced the sustained expression of p21 and interleukin regulatory factor 1 (IRF-1), both associated with the growth inhibition of cancer cells[49]. Moreover, while a number of tumor cells express Fas receptor most become resistant to Fas-mediated death[50,51]. However, IFN-γ can activate the FAS signaling pathway in tumor cells, rapidly inducing apoptosis[50,51]. This has been proposed to occur through caspase-1 activation and the induction of IFN-γ inducible gene IFN consensus sequence-binding protein (ICSBP)[50,51]. Moreover, the upregulation of FAS expression has been linked to the cleavage of the anti-apoptotic protein Bcl-2 in melanoma cells[52]. Similarly, IFN-γ can also restore TNF-related apoptosis inducing ligand (TRAIL)-induced apoptosis in resistant neuroblastoma cells by upregulating caspase-8 expression[53].

1.8 Controlling IFNγ production in NK cells

IFN-γ is induced by a plethora of signals including soluble mediators such as IL-2, IL-12, IL-15, and IL-18[25,45]. Furthermore, the crosslinking of cell surface receptors such as CD16 and LY49 can also induce IFN-γ expression[9]. Moreover, when multiple IFN-γ inducing signals are combined a synergistic induction of IFN-γ production is possible.

Several different theories have been put forward to explain this but the focus of our research will be on how a combination of IL-12 and IL-18 or IL-12, IL-15, and IL-18 can induce the synergistic production of IFN-γ in NK cells. For humans IFN-γ is located on chromosome 12 and typically has an invariant coding sequence[52]. Nevertheless, single nucleotide polymorphisms (SNPs) exist in the promotor, intron 1, and 3'- untranslated regions (UTRs)[52,54–56]. SNPs are thought to be particularly important for the synergistic production of IFN-γ following NK cell stimulation with IL-12 and IL-18. This is thought to be due to the SNPs involvement with the transcriptional control of IFN-γ via the SNPs AP-1 binding site[52,54–56]. Previously AP-1 has been shown to be induced by IL-18, and AP-1 has been shown to increase the transcriptional activity of STAT-4 by stabilizing STAT4 on the IFN-γ promotor and improving its transcriptional activity[42].

1.9 Transcriptional regulation of IFNγ production

The advent of whole genome sequencing has allowed for scientists to compare the exact nucleic acid composition of IFN-γ and its associated regions in humans and other animals. The study of the proximal promotor regions between -300 and -1 of the mouse and human IFN-γ genes has revealed approximately 80% sequence homology[52]. This suggests that this region is of utmost importance to the regulation of IFN-γ transcription and as such has triggered much research into this area. One of the most important findings of this research was the discovery of two highly evolutionarily conserved noncoding sequence (CNS) elements within the IFN-γ locus[57,58]. The first is located 5 kb upstream from the transcription start point and binds NFAT and T-bet[58]. These two transcription

factors are essential for IFN-γ expression and Th1 cell development[58]. The second CNS is located approximately 18 kb downstream of the initiation codon[59]. The functions of this site are still being elucidated, however, preliminary evidence suggests that T-bet-dependent histone modifications have been mapped to the functions of this region[59]. Furthermore, the proximal IFN-γ promotor contains binding sites for many trans-activating regulatory factors such as: T-bet, trans-acting T-cell-specific transcription factor GATA3 (GATA3), nuclear factor(NF)-kB, NF-AT, yin-yang (YY-1), STATs, Jun, AP-2, octamer binding-protein (OCT-1), peroxisome proliferator-activated receptor (PPAR)-y, and cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB)/activating transcription factor (ATF-2)[52]. With the exception of YY-1 and PPAR-y these factors all act as enhancers of IFN-γ transcription[52]. Importantly, YY-1 inhibits IFN-γ transcription by occupying regulatory sites typically utilized by the enhancer AP-1[60]. Therefore, if YY1 is bound to AP1 sites then STAT4 induced by IL-12 and stabilized by IL-18 induced AP-1 may not be capable of synergistic IFN-γ production. PPAR-y similarly inhibits the function of AP-1 by disrupting a subunit of AP-1, c-Jun's ability to bind to the IFN-γ proximal promotor[61]. This further exemplifies the importance of AP-1 in the production of IFN-γ.

The multitude of factors such as YY-1 and PPAR-y that alter STAT-4 functionality highlights the importance of STAT4 to the production of IFN-γ by NK cells. STAT4 is thought to be so important that research involving IFN-γ production is often done in STAT4 knockout mice. Research such as this has shown that STAT-4 also has a role in chromatin remodeling around the IFN-γ gene. Zhang and Boothby have shown

that the Switch(SWI)-sucrose nonfermenter (SNF) component Brahma-related gene 1 (Brg1) is recruited to the IFN-γ gene in developing Th1 cells in a STAT4-dependent way. Therefore, STAT4 has also been linked with the epigenetic alterations of the IFN-γ promotor.

T-bet is another transcription factor implicit in the regulation of IFN-γ expression. However, the method by which T-bet mediates its biological functions is the subject of much debate. This confusion is caused in part by T-bet's ability to bind multiple sites of the IFN-γ promotor. In separate studies T-bet half sites were shown to be bordered by NFAT and or ATF/AP-1/CEBP binding elements that function cooperatively in binding and trans-activating the IFN-γ promotor[62,63]. This could have several possible consequences. However, it has been shown that T-bet functions by binding the IFN-γ promotor and together with other DNA-binding proteins inhibits repressors of IFN-γ production such as mSin3a[62]. However, T-bet has also been shown to directly induce IFN-γ transcription[45]. Regardless of the mystery shrouding the mechanism of action of T-bet it is certainly required for optimal IFN-γ expression in NK cells.

While the roles of transcription factors which increase the production of IFN-γ are well documented the roles of transcription factors which inhibit IFN-γ expression are still poorly understood. However, a detailed study on how transforming growth factor (TGF)-b limits IFN-γ expression has shed some light on this. It was shown that TGF-b induced SMAD proteins can bind to the IFN-γ promotor directly and inhibit the activation of IFN-γ transcription[64]. The importance of this was shown by examining NK cells from

SMAD3-/- mice. Here, more IFN-γ was produced than NK cells from wild type (WT) mice. Therefore, IFN-γ production can be inhibited by interactions between proteins and the DNA.

However, the roles of IFN-γ transcription factors are not always so black and white. For example, GATA-3 is capable of suppressing IFN-γ expression in T helper cells but can enhance IFN-γ production in NK cells[37,65]. Furthermore, GATA-3 does not simply bind directly to the IFN-γ promotor, instead it exerts its control by inhibiting the activation of STAT-4 T helper cells[65]. However, the exact mechanism of GATA-3 mediated upregulation of IFN-γ in NK cells is still unknown.

1.10 Epigenetic Control of IFNγ Expression

Due to the plethora of effects IFN-γ has on the body it is activated for transcription only under the proper stimulatory conditions in healthy individuals. In order to maintain the expression of IFN-γ within appropriate boundaries the IFN-γ gene and bordering genomic data typically lies in a transcriptionally inactive state, due to extensive DNA methylation and chromatin hypoacetylation[45]. Moreover, studies have delineated that changes in the epigenetically controlled chromatin structure around the IFN-γ gene correlates with tissue selective expression of IFN-γ. This is due in part to the fact that highly condensed chromatin is not readily accessible to DNase I digestion[66]. However, DNase I cleavage will occur at hypersensitivity sites (HS), where the chromatin unwraps and the DNA becomes accessible for transcription[45]. New and permanent DNase I HSs develop in the IFN-γ gene upon the differentiation of naïve T cells into IFN-γ positive

Th1 cells[67]. It would be interesting to see whether or not epigenetic changes are responsible for "memory like NK cells". For this phenomenon, NK cells previously activated with cytokines have been shown to respond to a secondary stimulation with the same stimulus more rapidly than previously unstimulated NK cells[68].

DNA methylation occurs when the nucleotide cytosine is converted to 5-methylcytosine at CpG residues[45]. This results in silenced gene expression mediated directly by the blocking of protein-DNA interactions or indirectly by recruiting co-repressors such as the histone deacetylases. Conversely, removing methyl groups from the DNA will open the condensed portions of chromatin, improving regulatory proteins access to DNA and changing gene transcription[45]. For instance hypomethylation at a SnaB1 recognition site has been correlated with high levels of IFN-γ expression in newly activated primary T cells[69]. However, NK cells are capable of rapid transcriptional activation of IFN-γ transcription due to their constitutively demethylated IFN-γ locus[45]. Therefore, differences in the epigenetic control of IFN-γ production between T and NK cells identify significant differences between NK and T cells.

Histone acetylation is a dynamic regulatory tool that is reversible in nature and grants increased transcriptional opportunities by opening up the chromatin structure of targeted genes. While the data regarding histone acetylation with regards to NK cells remains scarce studies in T cells have elucidated the role of acetylation on the IFN-γ promotor. In naïve T cells there is little histone (H)3 and H4 acetylation at the IFN-γ promotors[70]. However, during the process of T cell differentiation there is a directed

increase in the acetylation levels of the IFN-γ promotors in Th1 cells[70]. However, this increased acetylation is abrogated in STAT4 deficient T cells[70]. As IL-12 is a potent inducer of STAT4 it must be recognized that IL-12 could have a role in controlling the epigenetic structure of the IFN-γ promotor.

1.11 Post transcriptional regulation of IFNγ expression

Studies on the effects of IL-12, IL-15, IL-18, and IL-2 on NK cells have shown that there is room for post transcriptional control of IFN-γ expression. Specifically, IFN-γ transcription is induced by IL-12, however co-stimulation with IL-12, IL-15, and IL-18 further triggers IFN-γ mRNA transcription, stabilization and storage in the nuclear compartment[45,71]. Subsequently, a secondary signal in the form of IL-2 causes the IFN-γ mRNA to traffic from the nucleus to the cytoplasm where translation occurs[71]. This has been suggested to occur to allow NK cells to rapidly respond to secondary activation signals from the environment.

IFN-γ mRNA has also been shown to regulate its own translation. Specifically, a pseudoknot in the 5'-UTR activates protein kinase PKR which phosphorylates the initiation factor eIF-2, resulting in the reduction of IFN-γ mRNA translation[72]. Furthermore, the Kaempfer lab has found that mutations decreasing the stability of this pseudoknot increases IFN-γ protein expression.

Combination studies with IL-18 and IL-12 have further elucidated the role of IFN-γ and mRNA stability on transcription. Several studies have shown that IL-12 and IL-18

signal through the mitogen-activated protein kinase (MAPK) p38 to stabilize IFN-γ produced by NK cells[73,74]. It is hypothesized that this post-transcriptional regulation is mediated by the sequence found in the 3'-UTR of the IFN-γ mRNA[45,73,74]. Therefore, the post-transcriptional control of IFN-γ expression requires the involvement of both the 5' and the 3'-UTRs.

1.12 IL-8 Multiple levels of Control

IL-8 is a highly inducible cytokine in NK cells and the founding member of the chemokine superfamily. IL-8 is expressed very little in healthy tissues but is induced by 10 to 100 fold in response to pro-inflammatory cytokines such as TNF-α or IL-1[75]. IL-8 has roles in angiogenesis, tumor progression, mitosis, and tissue remodeling and therefore its transcription is highly regulated at all times[75]. Obviously, if NK cells are producing it *in vivo* it could contribute to tumor progression.

Upon insult to the body maximal amounts of IL-8 are generated by a combination of three different mechanisms. Firstly, the gene promotor must be de-repressed, secondly the transcriptional activation of the IL-8 gene by NF-kB and JUN-N-terminal protein kinase pathways must occur, and thirdly stabilization of the IL-8 mRNA by the p38 MAPK pathway[75]. In all cell types used to study IL-8 production the promotor element of IL-8 contained an NF-kB binding site that was proven to be intrinsic to IL-8 production. NF-kB is a dimeric transcription factor, and is composed of a family of five subunits typically NF-kB1 (p50 and its precursor p105), NF-kB2 (p52 and its precursor p100), and c-Rel, REL A (p65), and REL B[75]. In non-active conditions these NF-kB precursors are

retained in the cellular cytoplasm by binding to inhibitory (IkB) proteins. However, under activated circumstances IkB is phosphorylated triggering ubiquitination and rapid degradation of IkBs by the proteasome. Consequently, NF-kB translocates to the nucleus and binds to the DNA. While this is critical to cytokine production, enhanced transcription is possible by phosphorylation of all the subunits and the binding of coactivators to NF-kB[76,77]. Interestingly, the core IL-8 promotor contains activating protein AP-1 and CAAT/enhancer binding protein (C/EBP)-binding sites. These sites have proven to be dispensable for IL-8 transcription[75,78–80]. However, they are required for maximal gene induction for both IL-8 and IFN-γ[75,78–80]. AP-1 is a homo or hetero-dimer composed of c-Jun, JUN D, JUN B, ATF-2, c-FOS, FRA-1, FRA-2, and other members[75]. Conveniently, these subunits are most often consistently bound to their respective DNA elements facilitating easy activation or enhancement of transcription[75]. AP-1 is activated by the MAPKs and three MAPK extracellular-regulated protein kinases (ERK), JUN-N-terminal protein kinase (JNK), and p38 MAPK[75]. Moreover, it was recently shown that the activation of p38 MAPK stabilizes the IL-8 mRNA[75,81,82]. Therefore, there appears to be overlap of the pathways responsible for increased IFN-γ and IL-8 production in the AP-1 and p38 MAPK pathways.

Chapter Two

Materials and Methods

2.1 *Ex vivo* Expansion of Natural Killer Cells from Peripheral Blood:

Using ficoll gradient density centrifugation lymphocytes were separated from the whole blood of healthy volunteers. These lymphocytes were counted and plated at a concentration of 200 000 cells/ml in RPMI-1640 media supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin, 1% L-glutamine, 1% sodium pyruvate, 50 mM 2- Mercaptoethanol (2-ME), 50units/ml of IL-2, and engineered K562 feeder cells expressing membrane bound IL-21 (K562-mbIL-21) at a 2:1 ratio. Every 48 hours co-culture media was replaced with fresh media at a volume equal to half of the total volume of co-culture media. Every 7 days of co-culture more irradiated K562-mbIL-21 feeder cells were added to the co-culture of expanding NK cells in order to maintain the proper 2:1 ratio. These culture conditions have been proven to promote rapid NK cell proliferation, with longer telomeres, and less senescence[83]. Incubator conditions were maintained at 37°C with an atmospheric CO_2 concentration of 5%. All experiments were completed 1 week after K562-mbIL-21 cells were added to the growing NK cell culture. This ensured minimal contamination of the co-culture with dead cell fragments. However, NK cells were isolated from co-culture contaminants using both CD56 and CD3 positive selection kits prior to conducting the human microarray.

2.2 *Ex vivo* Natural Killer Cell Stimulations:

NK cell stimulations always occurred at an NK cell concentration of $1x10^6$ cells/ml in RPMI-1640 media supplemented with 10% FBS, 1% Penicillin and streptomycin, 1% L-glutamine, 1% HEPES, 50 units/ml of IL-2, and 50 mM 2-ME. Recombinant human interleukin cytokines IL-2, IL-12, IL-15, and IL-18 were utilized at concentrations of 50 units/ml, 10ng/ml, 20 ng/ml, and 100 ng/ml, respectively, to stimulate NK cells. IL-18 was purchases from Medical and Biological Laboratories (MBL). IL-2, IL-12 and IL-15 were all purchased from Peprotech. Washes to remove stimulating cytokines were completed with sterile phosphate-buffered saline (PBS) and NK cells were always re-suspended in the aforementioned cell culture media to a concentration of $1x10^6$ cells/ml with a maintenance dose of IL-2 at 50 units/ml.

2.3 Cytokine Detection:

IFNγ and TNFα from stimulated cells were detected with R&D Duoset enzyme-linked immunosorbent assay (ELISA) kits. Endpoint absorbance was detected with a molecular devices spectramax i3.

2.4 NK Cell Intracellular and Extracellular Staining:

Following *ex vivo* NK cell stimulations with IL-12, IL-15, and IL-18 extracellular and intracellular staining for flow cytometry was completed by plating $1x10^6$ cells/well in 2% bovine serum albumin (BSA) in PBS. Staining was completed using the following antibodies: CD3 –APC-h7, CD56 – PE, CD16 – Alexa fluor 700, CD25 – PerCP-Cy5.5, and IFNγ – APC. All flow cytometry antibodies were purchased from BD biosciences.

Golgi Stop from BD biosciences was added to the stimulated NK cells 4 hours before samples were harvested. IFNγ-APC was utilized at a 1:400 dilution in BD perm/wash, CD3 - APC-h7 at 1:20, CD56 – PE at 1:20, CD25 – PerCP-Cy5.5 at 1:80, and CD16 at 1:25. The cells were fixed in 1% Paraformaldehyde (PFA) and run on a BD biosciences FACSCanto. FlowJo software was utilized for data analysis.

2.5 Immuno Blot Analysis:

NK cells were lysed with RIPA lysis buffer supplemented with complete protease inhibitor cocktail tablets(Roche) and phosphatase inhibitor cocktail 2 & 3(Cell Signaling), according to the manufacturer's instructions. Samples were quantified by Bradford protein assay and samples run on an 8% polyacrylamide resolving gel. Antibodies utilized were anti-pSTAT1, 3, 4, and 5 as well as tSTAT1, 3, 4, and 5, they were utilized at a 1:1000, 1:2000, 1-800, 1-700, 1:1000, 1:1000, 1:1000, and 1:1000, respectively. All antibodies were received from New England Biolabs. Image studio lite version 4.0 was utilized to assess the density of the bands.

2.6 Nucleic Acid Extraction:

mRNA was extracted from cultured NK cells with Qiagen RNeasy mini kits according to the manufacturer's instructions. Sample concentrations and quality were determined by optical density analysis on a general electric nanovue. Samples were utilized for experimentation if their 260/280 nm ratio was +/-0.1 from 2.0 as this would indicate pure RNA samples.

2.7 Microarray:

NK cells were isolated using human CD56 positive and human CD3 positive selection kits from stem cell technologies. RNA was isolated using Qiagen RNeasy mini kits. RNA quality was assessed with the general electric nanovue and bioananlyzer 2100 Bioanalyzer instrument by Agilent Technologies. RNA was quantitated using Quant-iT™ RiboGreen® RNA Assay (LifeTechnologies) and normalized prior to using the Illumina® TotalPrep™ RNA Amplification Kit (LifeTechnologies) for amplification and cRNA synthesis. Samples were then again quantitated by RiboGreen (LifeTechnologies) and normalized prior to testing using Illumina's Direct Hybridization Whole-Genome Expression method and HumanHT-12 v4 Expression BeadChip Kit with scanning on Illumina's iScan microarray scanner. Data analysis was completed using GenomeStudio (Illumina) and R commander.

2.8 Microarray Analysis:

The following comparisons in gene expression were performed: IL12 versus unstimulated, IL-15 versus unstimulated, IL18 versus unstimulated, IL12 and IL-18 versus unstimulated, IL-12, IL-15, and IL-18 versus unstimulated, and IL12 and IL-18 versus IL12 and IL12 and IL-18 versus IL18. The analysis was performed by using 'limma' package in R[84]. P-values were corrected with FDR correction for multiple hypothesis testing[85]. Differentially expressed genes from each analysis were used in the following two scenarios: (1) genes found in the comparisons vs unstimulated samples were used to build functional networks, using Cytoscape software and then were

annotated for Pathway Enrichment using Reactome FI plugin[86,87]. (2)Genes found in the comparisons vs IL12 and IL-18 samples were used to find genes that were differentially expressed in all 3 analyses. Then, similarly to scenario (1), the obtained genes were used to build functional networks, which were also examined for Pathway Enrichment. Only pathways and processes found by using both scenarios, were investigated further.

2.9 cDNA synthesis:

Prior to cDNA synthesis DNA removal utilizing DNase was completed using Qiagen RNeasy mini kits. Less than 5 ug of RNA was diluted in ddH2O with 10x DNase Buffer (2.5 ul) and 1 uL rDNase I up to a volume of 21.5 uL. These samples were then incubated at 37 ^{o}C for 25 minutes. 2.5 uL of DNase inactivation reagent was added to the samples prior to a 2 minute incubation at room temperature and centrifugation at 2000xg for 5 minutes was completed before a transfer of the samples to a new 96 well plate. Following the DNase treatment first-strand synthesis using Superscript II (Invitrogen by Life Technologies) was completed. 1-500 ng of mRNA was added to 250 ng random hexamer primers, and 10mM dNTP, and the samples were heated to 65 ^{o}C for 5 minutes and chilled quickly on ice. Subsequently, 4 uL of 5x first-strand buffer, 2 uL 0.1 M DTT and 1 uL RNaseOUT (40 units/uL) were added to the reaction mixture. Samples were then incubated at 25 ^{o}C for 2 minutes. 200 units of superscript II RT and ddH2O was added to the samples to a final volume of 20 uL. The samples were then incubated at 25 ^{o}C for 10 minutes and 42 ^{o}C for 50 minutes. Inactivation of the reaction was completed by incubating the samples at 70 ^{o}C for 15 minutes.

2.10 Quantitative Real Time Polymerase Chain Reaction:

Concentration of the cDNA for all samples was adjusted to 100 ng/ul with ddH_20 prior to running any reactions. cDNA was then placed in a reaction mixture containing: 200 ng of both forward and reverse primers (table 1), 100 ng of cDNA, 7.5 uL of SYBR mix, and 5.5 ul of ddH20. The reactions were then sealed and spun at 1000xg for 1 minute, and kept on ice and in the dark until the Quantitative PCR was carried out using the ABI 7700 of Applied Biosystems, sample reactions are detailed in table 2. Primers for target gene human IFN-γ and for the endogenous control human HSP90α real-time PCR were purchased from Mobix at McMaster and are detailed in table 1.

2.9.1 Table 1: Primers for gene amplification.

#	Name	Primer sequence in 5'-3' orientation	Amplicon size
1	*Homo sapiens* IFN-γ forward	TCGGTAACTGACTTGAATGTCCA	93
2	*Homo sapiens* IFN-γ reverse	TCGCTTCCCTGTTTTAGCTGC	93
3	*Homo sapiens* HSP90α forward	AGAAATTGCCCAACTCATGTCC	75
4	*Homo sapiens* HSP90α reverse	ATCAACTCCCGAAGGAAAATCTC	75

2.9.2 Table 2: polymerase chain reaction setup.

PCR product	template	5' Primer	3' Primer	RNA dye	# Cycles	Denaturing step	Annealing step	Elongation step
IFN-γ	cDNA	1 IFN-γ Forward	2 IFN-γ Reverse	SYBR master mix (life technologies)	40	95 ºC	61 ºC	72 ºC
HSP90α	cDNA	3 HSP90a Forward	4 HSP90a Reverse	SYBR master mix (life technologies)	40	95 ºC	61 ºC	72 ºC

Chapter Three

Results

3.1 Cytokine production and phenotype of *ex vivo* expanded human NK cells following stimulation with IL-12, IL-15, and IL-18

NK cells are capable of responding rapidly to pro-inflammatory signals from the environment and can release several factors to further coordinate the innate and adaptive arms of the immune system. However, the response of *ex vivo* expanded NK cells from human beings to stimulations with cytokines such as IL-12, IL-15, and IL-18 is still an area of interest. Understanding the behavior of *ex vivo* expanded NK cells in response to stimulation with IL-12 and IL-18, or IL-12, IL-15, and IL-18 is of critical importance to determining how safely these cytokine stimulations can be used prior to the adoptive transfer of the stimulated NK cells into cancer patients.

3.1.1 Cytokine production by *ex vivo* expanded NK cells following stimulation with IL-12, IL-15, and IL-18

The immunosuppressive environment of both the cancer patient's body and the tumor microenvironment requires that a careful approach be taken to developing cancer immunotherapies. NK cell researchers must find ways to ensure that NK cells remain cytotoxic in these environments to guarantee that the NK cells decrease tumor metastasis and limit the growth of primary tumors, instead of promoting tumor growth. Therefore, in attempts to find a way of activating NK cells for a prolonged period of time and to improve the therapeutic potential of *ex vivo* expanded NK cells as a cancer therapy I have examined the effects of IL-12, IL-15 and IL-18 on *ex vivo* expanded NK cells. The effects

of IL-12, IL-15 and IL-18 were tested on *ex vivo* expanded NK cells both individually and in every possible permutation of the aforementioned cytokines. Activation was measured by examining the production of the inflammatory cytokines IFN-γ and TNF-α after 24 hours of stimulation. Stimulation of *ex vivo* expanded NK cells with a combination of IL-12, and IL-18 or IL-12, IL-15, and IL-18 resulted in a significant production of IFN-γ and TNF-α after the 24 hour stimulatory period compared to the unstimulated control and IL-12, IL-15, and IL-18 alone stimulated NK cells (figures 1a and 2a). The cells were then washed three times with PBS and re-suspended in fresh culture media supplemented with the supporting cytokine IL-2. The supernatants were then collected every 24 hours after this up to 72 hours after the first washes. After each collection the cells were rinsed three times with PBS and re-suspended with fresh culture media supplemented with the supporting cytokine IL-2. IFN-γ levels were still significantly upregulated in IL-12 and IL-18 or IL-12, IL-15, and IL-18 stimulated NK cells after 24 hour post wash (figure 1b), 48 hours post wash (figure 1c), and 72 hours post wash periods(figure 1d). While IFN-γ levels remained high even in the absence of stimulatory cytokines in the 24 hour post wash, 48 hour post wash, and 72 hour post wash periods this was not the case for TNF-α production. Indeed, even in IL-12 and IL-18 or the IL-12, IL-15, and IL-18 stimulated NK cells TNF-α production was only significantly upregulated 24 hours post stimulation and there was non-significant amounts of TNF-α at the 24 hour post wash period (figures 2a and 2b).

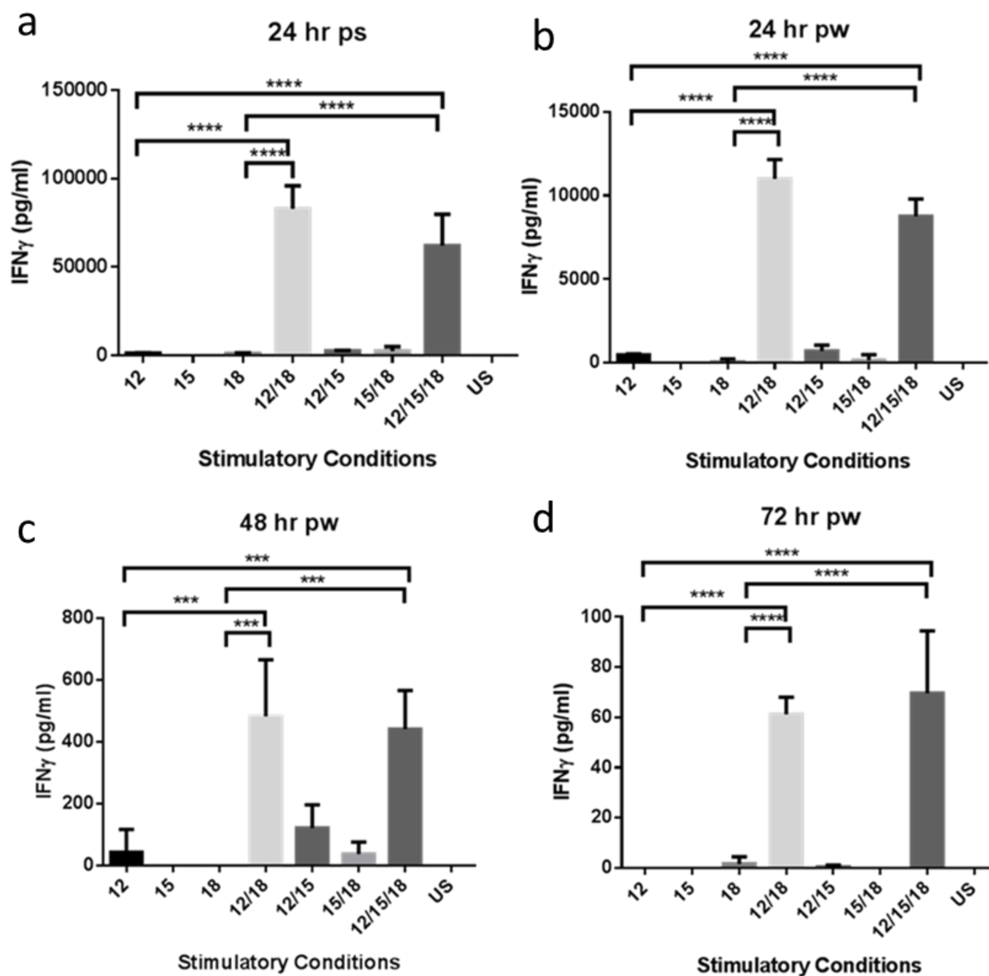

Figure 1 IFNγ production by *ex vivo* expanded NK cells stimulated with IL-12, IL-15, and IL-18:

Joint stimulation of NK cells with IL-12 and IL-18, (+/-) IL-15 results in profound IFN-γ secretion that is maintained even after stimulatory cytokines are removed from culture. Figure 1a shows the increase in IFN-γ production 24 hours post stimulation. After the 24 hour stimulation the stimulated NK cells were washed with PBS and re-plated in fresh cell culture media. Supernatants were collected again after incubating for another 24 hours without stimulatory cytokines. Figures 1b, 1c, and 1d show prolonged production of IFN-γ at the 24, 48, and 72 hours post wash periods, respectively. After each of these time points the NK cells were washed with PBS and re-plated in fresh cell culture media. Statistical significance (****) was found via one way ANOVA with p-value<0.0001. Data presented is representative of 6 independent trials. Cytokines were used at concentrations of 50 units/ml, 10 ng/ml, 20 ng/ml, and 100 ng/ml for IL-2/US control, IL-12, IL-15, and IL-18 respectively. Every treatment including US had supporting IL-2.

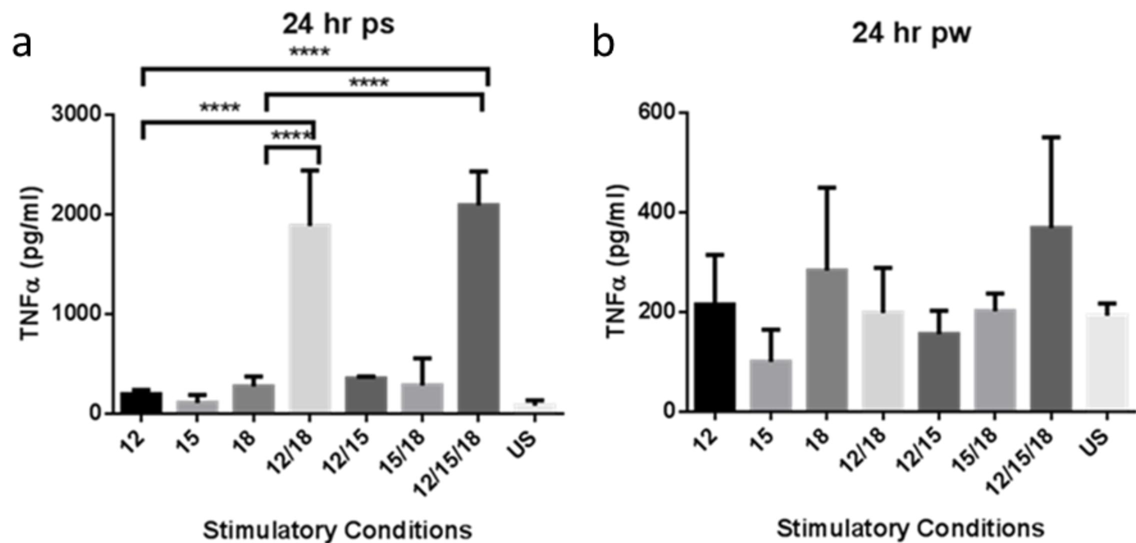

Figure 2 TNFα production by *ex vivo* expanded NK cells stimulated with IL-12, IL-15, and IL-18:

Joint stimulation of NK cells with IL-12 and IL-18, +/- IL-15 results in profound TNFα secretion 24 hours post stimulation, however unlike IFN-γ secretion TNFα secretion was not maintained at the 24, 48, and 72 hour post wash periods. Fig 2a shows the increase in TNFα production 24 hours post stimulation. After the 24 hour stimulation the stimulated NK cells were washed with PBS and re-plated in fresh cell culture media. Supernatants were collected again after incubating for another 24 hours without stimulatory cytokines. Figure 2b, reveals that TNFα production is not significantly up-regulated in the IL-12 and IL-18 or IL-12, IL-15, and IL-18 stimulated conditions at the 24 hour post wash period and therefore TNF-α production does not follow the same trend as IFNγ production following stimulation with IL-12/18 and IL-12/15/18. Statistical significance (****) was found via one way ANOVA with pvalue<0.0001. Data presented is representative of 6 independent trials. Cytokines were used at concentrations of 50 units/ml, 10 ng/ml, 20 ng/ml, and 100 ng/ml for IL-2/US control, IL-12, IL-15, and IL-18 respectively. Every treatment including US had supporting IL-2.

3.1.2 The cellular source of IFN-γ from the co-culture system is NK cells, with little CD16 and high CD25 expression on their cell surface.

To confirm that the IFN-γ producing cells in our co-culture system were indeed NK cells intracellular flow cytometry was performed. We discovered 94.7% of the co-cultured cells stimulated with IL-12, IL-15, and IL-18 produced IFN-γ and that 93.2% of the IFN-γ producing cells were NK cells (figure 3a). The unstimulated control for this study which only received supporting IL-2 treatment had only 13.2% of the total population producing IFN-γ, however 93.1% of the unstimulated IFN-γ positive cells were NK cells (figure 3b).

It was also found that the IL-12, IL-15, and IL-18 stimulated IFN-γ+ NK cells showed a lower expression of CD16 at 3.69% compared to the unstimulated IFN-γ+ NK cells at 59.6% (figures 3a and 3b). Conversely, IL-12, IL-15, and IL-18 stimulated IFN-γ+ NK cells had a much higher expression of CD25/IL-2Ra at 94.9%, compared to unstimulated IFN-γ+ NK cells which had approximately 5.38% expression of CD25/IL-2Ra (figures 3a and 3b). This upregulation of CD25 has been shown to result in the formation of a functional high-affinity IL-2 receptor, capable of being ligated by picomolar concentrations of IL-2[88,89]. Lastly, it was observed that when the stimulatory cytokines were removed by PBS wash after a 24 hour stimulation and re-plated in fresh IL-2 containing media the CD25/IL-2Ra expression had decreased by the 48 and 96 hour post wash periods (figure 3c).

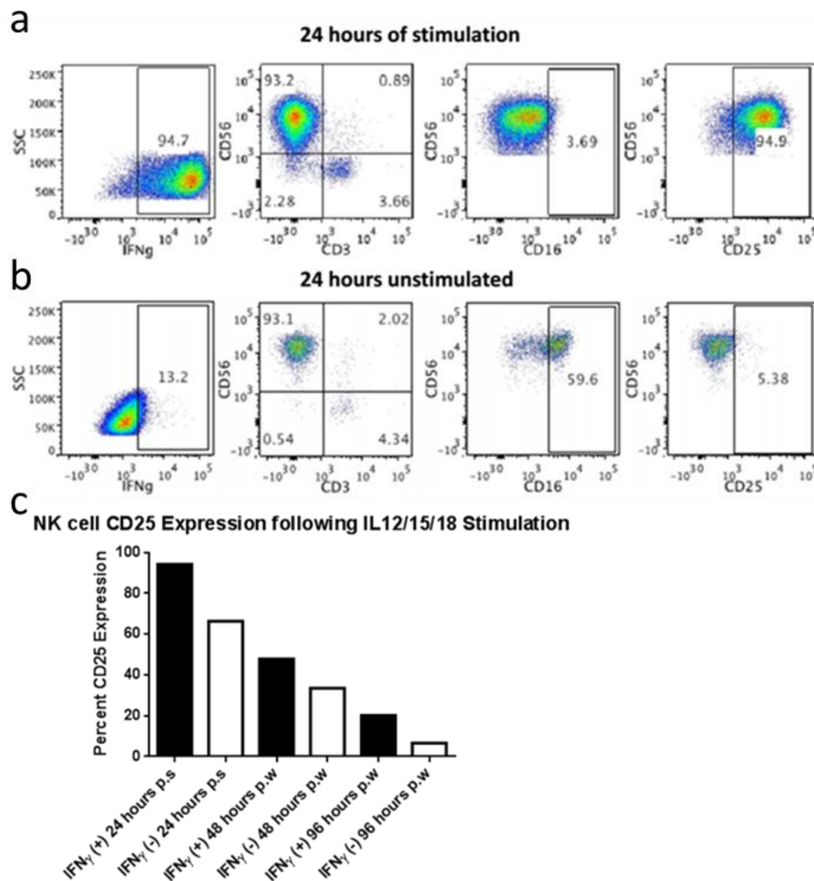

Figure 3 The source of IFNγ from the co-culture system is NK cells stimulated with IL-12, IL-15, and IL-18, with little CD16 and high CD25 expression.

Intracellular flow cytometry revealed that IFN-γ production occurs in NK cells low in CD16 expression and high in CD25. Figure 3a shows that 94.7% of cells stimulated with IL-12/15/18 within our coculture system produce IFN-γ, and that 93.2% of the IFN-γ producing cells are NK cells, and that these NK cells have low expression of CD16 and high expression of CD25. Conversely, figure 3b shows that, unstimulated cells have 13.2% expression of IFN-γ with 93.1% of IFN-γ positive cells being NK cells, which are higher than IL-12/15/18 stimulated cells in CD16 expression but have lower expression of CD25. Figure 3c, shows that the upregulation of CD25 on IL-12, IL-15, and IL-18 stimulated NK cells decreases over time as the stimulatory cytokines are removed from the co-culture system. Cytokines were used at concentrations of 50 units/ml, 10 ng/ml, 20 ng/ml, and 100 ng/ml for IL-2/US control, IL-12, IL-15, and IL-18 respectively. Every treatment including US had supporting IL-2. Data is representative of two separate trials from different NK cell donors.

3.2 STAT4 alone is not solely responsible for the increased production of IFNγ and TNFα in the case of IL-12 and IL-18 or IL-12, IL-15, and IL-18 stimulated NK cells.

Recognizing the sustained cytokine production in the absence of stimulatory cytokines we were interested in examining the potential mechanisms responsible for this phenomenon. As our stimulatory cytokines IL-12, IL-15, IL-21 and IL-18 are all well known to induce the phosphorylation of STATs 1, 3, and 4 we examined the phosphorylation status of these transcription factors. Our interest was focused especially on STAT4 as it is well known to induce the production of IFN-γ[90].

3.2.1 Phosphorylation status of STATs 1, 3, and 4 after 10 and 40 minutes of stimulation with IL-12, IL-15, and IL-18

Ex vivo expanded NK cells stimulated with IL-12, IL-15, and IL-18 or combinations of these cytokines had the phosphorylation status of STATs 1, 3, and 4 examined after 10 and 40 minutes of stimulation with IL-12, IL-15, and IL-18. Interestingly, STAT4 phosphorylation was induced within 10 minutes of stimulation and was maintained after 40 minutes of stimulation in *ex vivo* expanded NK cells that were stimulated with IL-12, IL-12 and IL-18, or IL-12, IL-15, and IL-18 (figure 4a and figure 4b). STAT 4 phosphorylation is well known to induce the transcription of IFN-γ in NK cells[90].

We examined STAT1 activation as it has been proven that STAT4 mediated IFN-γ production can be inhibited by pSTAT1 as pSTAT1 can interrupt the pSTAT4

transcriptional machinery[90]. This does not seem to be important in the case of our *ex vivo* expanded and stimulated NK cells as phosphorylation of pSTAT1 occurs at the 10 and 40 minute time points in the IL-12 and IL-18 and IL- 12, IL-15, and IL-18 stimulated *ex vivo* expanded NK cells (figure 4a and 4c). However, in spite of this IFN-γ production likely mediated to some degree by STAT4 activation is still quite high (figure 1). However, these phosphorylation events occur quite early. To explore if STAT4 is responsible for the prolonged production of IFNγ it was imperative to look at STAT4 activation after longer stimulations and in the absence of stimulatory cytokines.

IL-15 appears to play a role in STAT1 and STAT3 activation. However, activation of STAT3 does not appear to play a major role in NK cell mediated production of IFN-γ. This is because the stimulatory conditions IL-12 and IL-18 and IL-12, IL-15, and IL-18, have very similar IFN-γ production levels despite having pSTAT3 activated in IL-12, IL-15, and IL-18 stimulated cells (figures 1a, 4a, and 4d). However, STAT3 was also investigated to determine the extent of the effect our K562-mbIL-21 cells were having on our co-cultured NK cells during the stimulation. It is well known that IL-21 should activate STAT3 leading to its phosphorylation[91]. Therefore, as little pSTAT3 was seen in any of the stimulatory conditions except the conditions with IL-15 we can assume that the K562-mbIL-21 has little effect on the *ex vivo* expanded NK cells when they were utilized for experiments 1 week after replenishment (figure 4a and 4d).

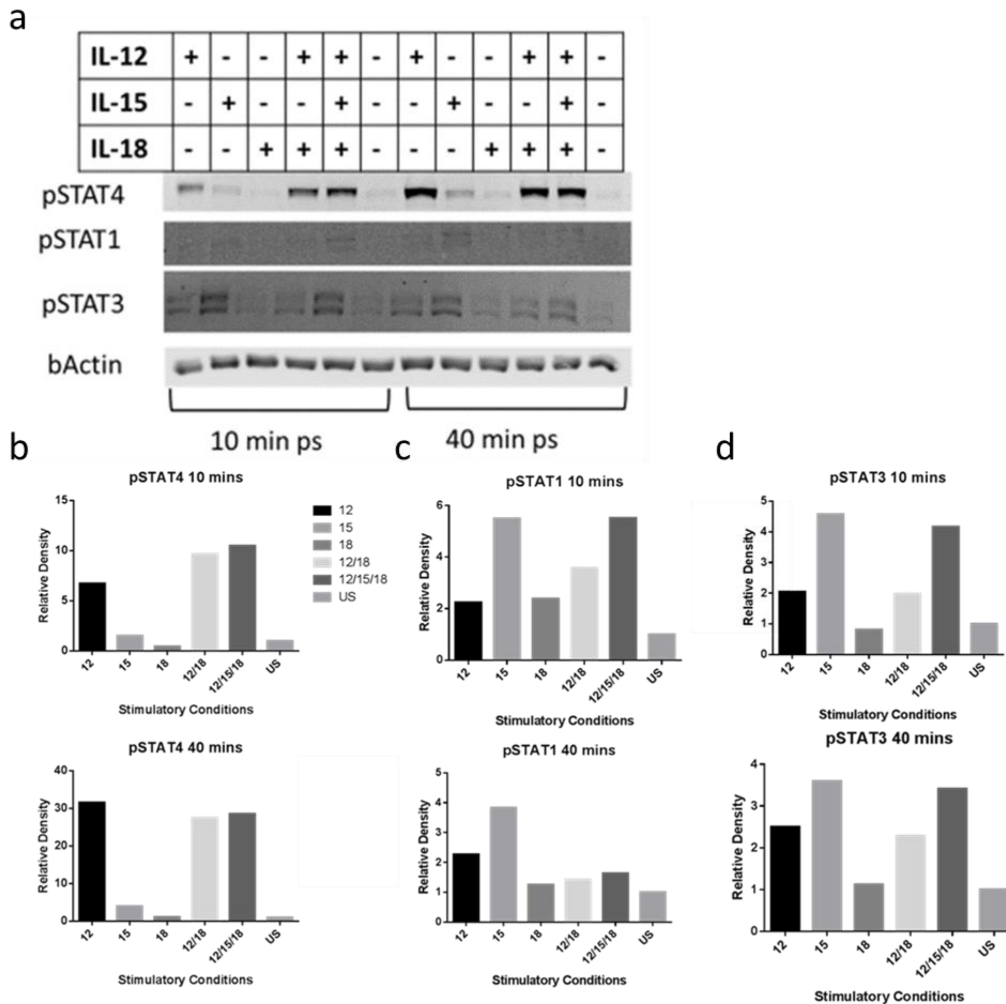

Figure 4 Western blot analysis of STAT 1, 3, and 4 activation following stimulation with IL-12, IL-15, and IL-18 for 10 and 40 minutes.

Figure 4a shows representative blots for pSTAT4, pSTAT1, and pSTAT3. Figure 4a and 4b shows the up-regulation of pSTAT4 at the 10 and 40 minute post stimulation time points by relative density of pSTAT4 bands versus the loading control actin. Figure 4a and 4c shows the up-regulation of pSTAT1 at the 10 and 40 minute post stimulation time points by relative density of pSTAT1 bands versus the loading control actin. Figure 4a and 4d depicts the up-regulation of pSTAT3 at the 10 and 40 minute post stimulation time points by relative density of pSTAT3 bands versus the loading control actin. Cytokines were used at concentrations of 50 units/ml, 10 ng/ml, 20 ng/ml, and 100 ng/ml for IL-2/US control, IL-12, IL-15, and IL-18 respectively. Every treatment including US had supporting IL-2. Data is representative of two separate trials from different NK cell donors.

3.2.2 Phosphorylation status of STATs 1, 3, 4, and 5 at 24 hours post stimulation with IL-12, IL-15, and IL-18, and 24 hours post wash after stimulation with IL-12, IL-15, and IL-18

While the observed level of STAT4 phosphorylation at the 10 and 40 minute time points may explain the initial up-regulation of IFN-γ transcription, we were interested in examining the potential mediators responsible for long term production of IFN-γ even after stimulatory cytokines had been removed from the co-culture system as seen in figures(1a-d). We therefore ran western blots for pSTAT1, 3, 4, and 5 as well as tSTAT1, 3, 4, and 5 on samples from *ex vivo* expanded NK cells stimulated for 24 hours with IL-12, IL-15, and IL-18. pSTAT5 and tSTAT5 were added to the experimental panel of STAT1, 3, and 4 because evidence had shown that IL-2 stimulation can post-transcriptionally control IFN-γ mRNA translation, and IL-2 is known to signal through STAT5[71]. Specifically, it was shown that IL-2 initiates the trafficking of the IFN-γ mRNA from the nucleus to the cytoplasm allowing for translation to occur[71]. We then thought that IL-12 and IL-18 or IL-12, IL-15, and IL-18 stimulated NK cells may just stockpile more IFN-γ mRNA than IL-12 stimulated cells following the initial 24 hour stimulation despite similar STAT4 activation between the groups. Subsequently, this stockpiled IFN-γ mRNA could be released to the cytoplasm in the post wash periods in response to IL-2. However, pSTAT5 phosphorylation was upregulated in IL-15 only stimulated cells (fig 5a and 5b) and these NK cells did not produce a large amount of IFN-γ at the 24 hour post wash period(fig1b). Consequently, it would seem that the

phenomenon of IL-2 induced posttranscriptional nucleocytoplasmic shuttling is not occurring via IL-2 and STAT5 in the NK cells under our stimulatory conditions.

Importantly, STAT4 was still phosphorylated after a 24 hour stimulation and remained phosphorylated for at least 24 hours after the stimulatory cytokines were washed away (figure 5a and 5b), suggesting that pSTAT4 could be responsible for the prolonged production of IFN-γ in the IL-12 and IL-18 and IL-12, IL-15, and IL-18 stimulated NK cells. This also suggests that these cells may continue to actively transcribe IFN-γ mRNA in the absence of stimulatory cytokines. However, levels of pSTAT4 are higher in IL-12 stimulated NK cells versus IL-12 and IL-18 and IL- 12, IL-15, and IL-18 stimulated NK cells at the 24 hours post wash point (figure 5a and 5b). Furthermore, IFN-γ protein secretion levels are significantly lower for IL-12 stimulated cells versus IL-12 and IL-18 and IL-12, IL-15, and IL-18 stimulated cells (figure 1a-d). Consequently, we presume that there must be some other mechanism responsible for the synergistic and prolonged production of IFN-γ in *ex vivo* expanded NK cells stimulated with IL-12 and IL-18 or IL-12, IL-15, and IL-18 and it is not mediated by prolonged STAT4 activation alone. Finally, long term production of IFN-γ likely has little to do with STAT 1, 3, and 5 phosphorylation as levels of phosphorylation are negligible at the 24 hour post wash stages (figure 5a, c and d). Due to these results we decided to look deeper into the transcriptional and translational machinery that could mediate the differences in IFN-γ production observed despite having similar levels of activation of STAT4.

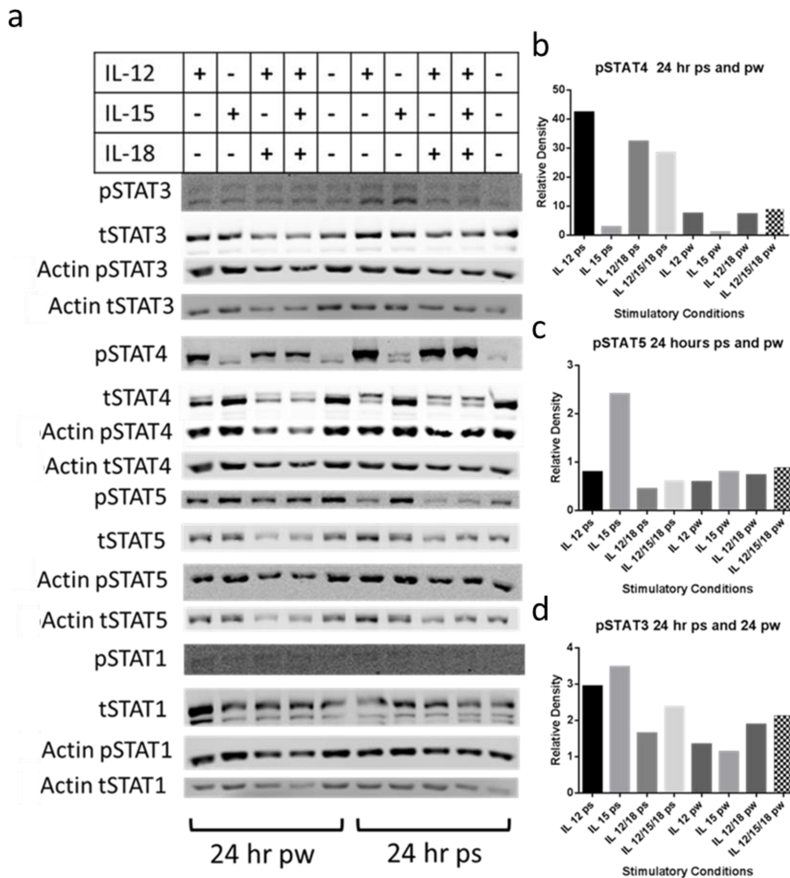

Figure 5 Western blot analysis of STAT 1, 3, 4, and 5 activation following stimulation with IL-12, IL-15, and IL-18 for 24 hours.

Western blot analysis revealed prolonged phosphorylation of STAT4 in the absence of the stimulatory cytokines IL-12, IL-12 and IL-18, and IL-12, IL-15, and IL-18. Figure 5a shows representative western blots of pSTAT3, 4, 5, and 1 as well as representative western blots of tSTAT3, 4, 5, and 1 at 24 hour post stimulation and 24 hour post wash time points. Figure 5b shows the prolonged phosphorylation of STAT4 at the 24 hours post wash point, the relative density of the pSTAT4 band was compared to total STAT and the loading control actin. Figure 5c shows the initial phosphorylation of STAT5 at the 24 hour post stimulation point and the relative density of pSTAT5 versus tSTAT5 and the actin loading control. Figure 5d shows the relative density of pSTAT3 versus tSTAT3 and the loading control actin. Cytokines were used at concentrations of 50 units/ml, 10 ng/ml, 20 ng/ml, and 100 ng/ml for IL-2/US control, IL-12, IL-15, and IL-18 respectively. Every treatment including US had supporting IL-2.

3.3 Increased IFNγ production is unlikely to be due to increased stability of the IFNγ mRNA mediated by p38 MAPK

As it was found that STAT4 phosphorylation was approximately equal among the IL-12, IL-12 and IL-18 and the IL-12, IL-15, and IL-18 stimulated groups but IFN-γ and TNF-α production was significantly different we desired to examine what was responsible for this. As we had found that IL-2 nucleocytoplasmic shuttling of mRNA was not responsible, we questioned whether or not transcription was identical across the aforementioned groups and that differing stability of the IFN-γ mRNA was responsible for the observable difference in IFN-γ production.

3.3.1 Increased IFN-γ production in IL-12 and IL-18 or IL-12, IL-15, and IL-18 stimulated NK cells is mediated by measurable differences in IFN-γ transcription

Research similar to our own has shown that stimulation of NK cells with IL-12, and IL-18 results in a synergistic production of IFN-γ compared to stimulation of NK cells with either IL-12 or IL-18 alone[42]. It was shown this can be mediated by an increase in mRNA stability caused by activation of the p38 MAPK pathway[43]. In order to assess if the increase in IFN-γ in our system was due to increased mRNA stabilization we conducted qPCR analysis for IFN-γ at the 6 and 24 hour post stimulation periods. We then washed the cells stimulated for 24 hours 3 times with PBS and re-suspended the cells in fresh media supplemented with IL-2. After 24 hours with only maintenance levels of IL-2 IFN-γ mRNA was again measured by qPCR analysis. As is shown in (figure 6a-c) IFN-γ transcript levels are the same in IL-12 and IL-18 or IL-12, IL-15, and IL-18

stimulated NK cells. However, the IFN-γ mRNA transcript levels are much lower for IL-12 stimulated NK cells compared to IL-12 and IL-18 or IL-12, IL-15, and IL-18 stimulated NK cells even at the 6 hour post stimulation period, suggesting that mRNA stabilization is not the sole force driving the increased production of IFN-γ and that increased transcription is likely a key driver of the increased production of IFN-γ and TNF-α in IL-12 and IL-18, or IL-12, IL-15, and IL-18 stimulated NK cells.

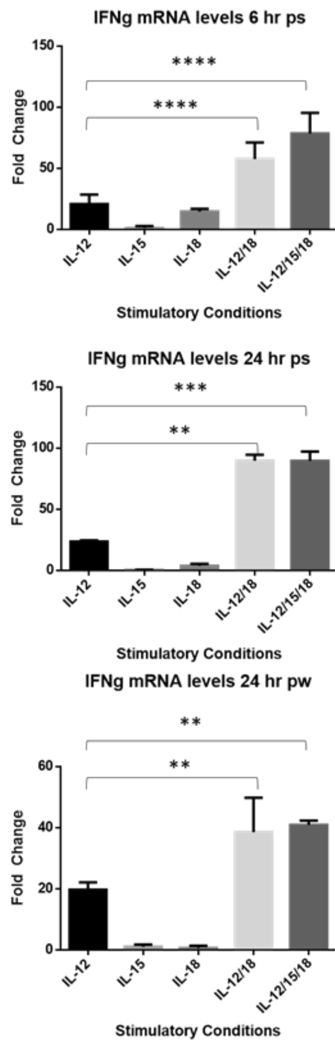

Figure 6 Increased IFNγ production is due to increased transcription of IFNγ.

qPCR analysis revealed increased IFN-γ transcript levels at the 6, and 24 hour post stimulation periods, respectively (figure 6a and 6b). Further analysis of the IFN-γ transcript levels at the 24 hour post wash period showed that IFN-γ transcription was still upregulated in the IL12 and IL-18, or the IL-12, IL-15, and IL-18 stimulated conditions (figure 6c). Cytokines were used at concentrations of 50 units/ml, 10 ng/ml, 20 ng/ml, and 100 ng/ml for IL-2/US control, IL-12, IL-15, and IL-18 respectively. Every treatment including US had supporting IL-2. Data is representative of three separate trials from different NK cell donors. Fold change in gene expression was measured relative to a housekeeping gene HSP90a. Statistical significance (****) was found via one way ANOVA with pvalue<0.0001.

3.4 NK cell stimulation with IL-12, IL-15, IL-18, IL-12 and IL-18, and IL-12, IL-15, and IL-18 results in dramatic changes in the gene expression of NK cells in each stimulatory group

In order to determine the complete response of *ex vivo* expanded NK cells to the cytokines IL-12, IL-15, and IL-18 and the combinations of IL-12 and IL-18 and IL-12, IL-15, and IL-18 we ran a human microarray. We found that a multitude of pro-inflammatory cytokines were up-regulated in IL-12 and IL-18 or IL-12, IL-15, and IL-18 stimulated conditions NK cells including IFN-γ and TNF-α.

3.4.1 IL-12 and IL-18 stimulated NK cells uniquely up-regulate gene sets associated with amino acid utilization and bioenergetics compared to both IL-12 and IL-18 stimulated NK cells.

In order to analyze the unique effects of the IL-12 and IL-18 combined stimulation on NK cells we compared the effects of this combination to the individual stimulations of NK cells with IL-12 and IL-18. This would aid us in determining if changes in gene expression induced by IL-12 and IL-18 stimulation could potentiate the IFN-γ production we observed. Therefore, to focus on the genes uniquely up or down-regulated in the IL-12 and IL-18 stimulated cells we removed from the up and down regulated gene lists the genes that were also up or down-regulated by any degree in the IL-12 only and IL-18 only stimulated groups. Figure 7a is a rudimentary representation of our analysis selection protocol. We then separated all the genes either up or down-regulated from the IL-12 and IL-18 stimulated conditions into two groups. Group 1 was

the up-regulated group and group 2 the down-regulated group. The data from group 1 and 2 were then sorted into functional clusters of genes known as gene sets. Genes within each gene set are known to have interrelated functions that drive an aspect of cell function such as biosynthesis of amino acids. The complete list of up or down-regulated genes and their position in specific gene clusters are outlined in appendices tables 3 and 4, respectively. For perspective, the total number of genes from the gene set and the number of genes from IL-12 and IL-18 stimulated NK cells that are up or downregulated in each gene set are given in the tables as well.

16 gene processes/sets are uniquely upregulated in our IL-12 and IL-18 stimulated cells. From within the 16 gene processes that are upregulated 224 genes from a possible 1276 were uniquely upregulated. Interestingly, many of the processes upregulated are related to eukaryotic ribosome biogenesis, eukaryotic translational elongation and termination. This suggests NK cells stimulated with IL-12 and IL-18 are effective at producing IFN-γ because they have changed the expression of a multitude of genes to produce and secrete more protein.

Conversely, several gene processes/sets potentially critical to the success of NK cell adoptive therapy were downregulated. For example, IL-2 signaling events mediated by PI3K, inflammation mediated by chemokine and cytokine signaling pathways, natural killer cell mediated cytotoxicity, and the NF-kB signaling pathways were all down regulated. Moreover, the down-regulation of JAK1 from the IL-2 signaling events mediated by PI3K or the IL-2 mediated signaling events mediated by STAT5 as seen in

appendix table 4 could explain why our increase in IL-2Ra (figure 3b and 3c) expression does not increase phosphorylation of STAT5 as seen in figure 5a and c. However, this information allows us to further rule IL-2 post-transcriptional control of IFN-γ mRNA translation out as the factor controlling the increase in IFN-γ expression in IL-12 and IL-18 or IL-12, IL-15, and IL-18 stimulated cells.

Interestingly, a down-stream effector of JAK1 and JAK3, LCK, is also downregulated[92]. LCK is well known to activate c-FOS (part of the heterodimeric AP-1 transcription factor) that enhances IFN-γ transcription[93–97]. This suggests that the up-regulation of IFN-γ production in IL-12 and IL-18 stimulated cells may not be solely due to IL-18 induced AP-1 stabilizing STAT4 on the IFN-γ promotor.

Many killer immunoglobulin-like receptors are also downregulated in the IL-12 and IL-18 stimulated NK cells, suggesting that these cells may have an improved cytotoxic profile against target cancer cells. Conversely, while the NK cells may receive less inhibitory receptor signals they also have a reduction in the expression of naturally cytotoxic receptors such as NCR2/NKp44 suggesting these NK cells may have diminished ability to lyse target cancer cells. Furthermore, ICAM2, was also downregulated which is involved with cellular adhesion and important in NK cell mediated clearance and circulation[98].

a

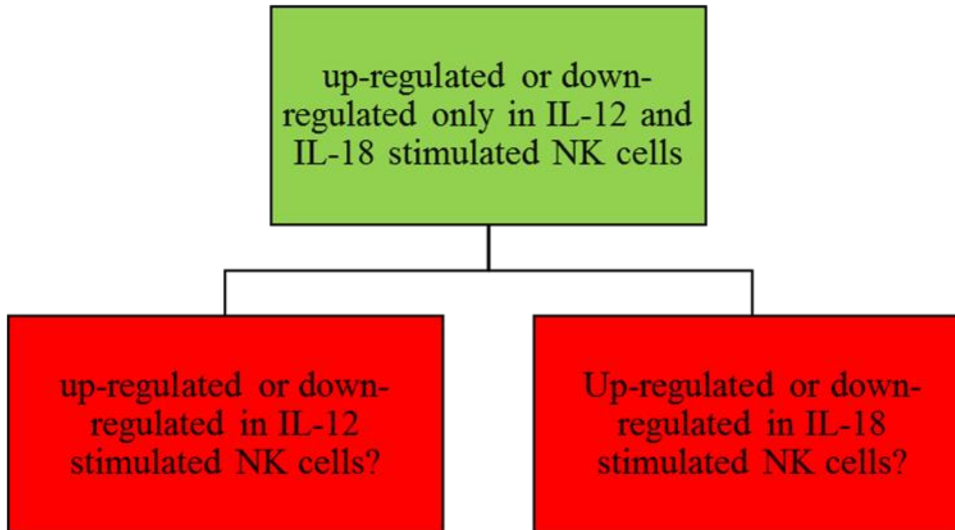

Figure 7 Hierarchical selection of genes uniquely up or down-regulated in IL-12 and IL-18 combined stimulated NK cells versus IL-12 or IL-18 stimulation alone.

Represented here is a diagram of our selection protocol for the genes represented in appendix tables 3 and 4. Any gene that was either up or downregulated in IL-12 or IL-18 stimulated NK cells was excluded from the results highlighted in appendix tables 3 and 4(red). This allowed for us to uniquely represent genes up or downregulated in the *ex vivo* expanded IL-12 and IL-18 stimulated NK cells(green).

3.4.2 The up-regulation of pro-inflammatory cytokine mRNA and cell adhesion molecules was abundant in NK cells stimulated with IL-12 and IL-18 or IL-12, IL-15, and IL-18.

The comparison of the IL-12 and IL-18 stimulated cells to the IL-12 or IL-18 only stimulated NK cells revealed that the changes in NK cell gene expression when NK cells become synergistically activated are numerous and complex. Therefore, we narrowed our search to look at the fold change in gene expression of over 47 000 probes from IL-12, IL-15, IL-18, IL-12 and IL-18 and IL-12, IL-15, and IL-18 stimulated NK cells compared to an unstimulated control. The top significantly up and down-regulated genes from each stimulatory group are represented in figure 8a-h. In both the IL-12 and IL-18 and IL-12, IL-15, and IL-18 stimulated conditions the majority of the most highly upregulated genes were pro-inflammatory cytokines such as: IFN-γ, IL-8, CCL3L1, LTA (figure 8f and 8h). The NK cells in these conditions also had increased expression of receptors essential to NK cell function such as CTLA4 and IL-2Ra (figure 8f and 8h). Conversely, these NK cells also downregulated genes such as S100A4, KRT86, RARRES3 and LTB (figure 8e and 8g). Downregulation of LTB may limit the expression of the heterotrimer of LTB and LTA limiting the effectiveness of increased LTA expression in recruiting inflammatory cells. However, LTA can be secreted as a homotrimer to exert its immunological roles[99].

The IL-18 stimulated NK cells also showed an up-regulation of pro-inflammatory cytokines including CCL1, a potent chemokine[100], LTA, IL-8, and IFN-γ (figure 8d). Similarly, IL-15 stimulated NK cells up-regulated the expression of pro-inflammatory

cytokines such as: LTA, CCL3, and CCL1 (figure 8c). Neither the IL-15 or IL-18 genes had any significantly down-regulated genes in comparison to unstimulated NK cells. Interestingly, IL-12 stimulated cells had S100A4 and KRT86 as the two most down-regulated genes (figure 8a). These are the same 2 genes that were highly down-regulated in IL-12 and IL-18, and IL-12, IL-15, and IL-18 stimulated NK cells (figures 8e and 8g). Furthermore, the IL-12 stimulated NK cells up-regulated a similar gene profile as the IL-12 and IL-18, and IL-12, IL-15, and IL-18 stimulated NK cells (figure 8b). Therefore, it would seem that adding IL-18 to the IL-12 stimulation means that many of the genes induced by IL-12 alone are simply up-regulated to a greater degree when IL-18 is present and vice versa. For instance, when IL-12 was combined with IL-18 the IL-18 induced IL-8 expression increased markedly (figure 8f). Knowing that IL-8 is a potent inducer of angiogenesis, we measured secretion of IL-8 from NK cells stimulated with IL-12, IL-15, IL-18, IL-12 and IL-18, and IL-12, IL-15, and IL-18[75]. Indeed IL-8 is secreted significantly from the IL-12 and IL-18, and IL-12, IL-15, and IL-18 stimulated NK cells (data not shown). Therefore, further studies into the angiogenic effects of *ex vivo* expanded and stimulated NK cells should be conducted before utilizing them as a cancer therapeutic.

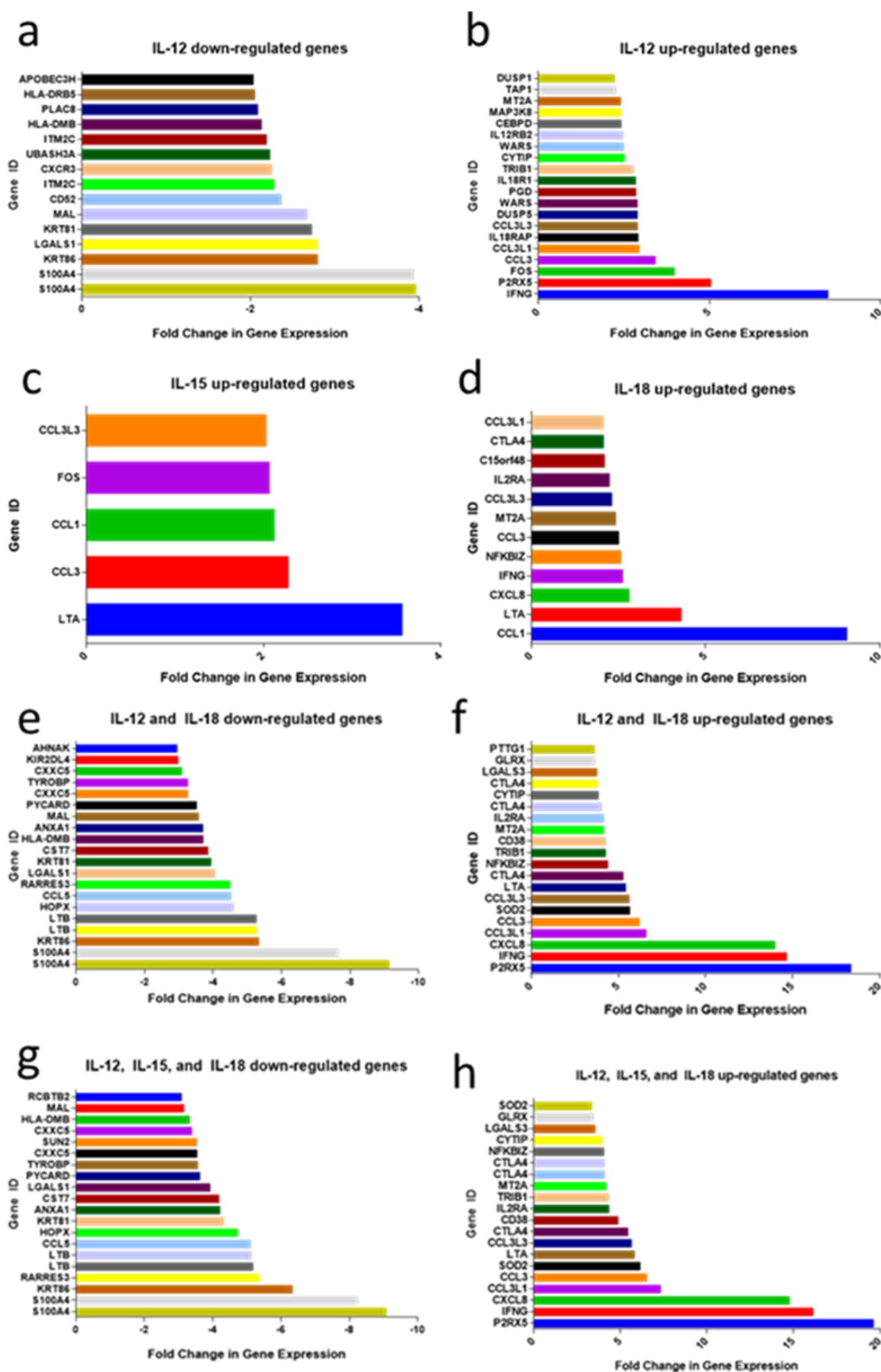
a
IL-12 down-regulated genes
b
IL-12 up-regulated genes
c
IL-15 up-regulated genes
d
IL-18 up-regulated genes
e
IL-12 and IL-18 down-regulated genes
f
IL-12 and IL-18 up-regulated genes
g
IL-12, IL-15, and IL-18 down-regulated genes
h
IL-12, IL-15, and IL-18 up-regulated genes
Gene ID
Fold Change in Gene Expression

Figure 8 Genes up and downregulated in *ex vivo* expanded NK cells stimulated with IL-12, IL-15, or IL-18, or in combinations of IL-12 and IL-18, and IL-12, IL-15, and IL-18. The fold change in gene expression from each group of cells and their respective stimulatory condition have been normalized to the unstimulated control. Figure 8a, e, and g represent the genes most downregulated in IL-12, IL-12 and IL-18, and IL-12, IL-15, and IL-18 stimulated NK cells, respetively. Figure 8b, c, d, f, and h represent the genes upregulated in IL-12, IL-15, IL-18, IL-12 and IL-18, and IL-12, IL-15, and IL-18 stimulated NK cells, respectively.

Chapter Four

Discussion

4.1 Do *ex vivo* expanded NK cells produce pro-inflammatory cytokines in response to stimulation with a combination of IL-12 and IL-18 or IL-12, IL-15, and IL-18?

Our lab previously showed that stimulation of mouse NK cells with a combination of IL-12 and IL-18 or IL-12, IL-15, and IL-18 resulted in a synergistic production of IFN-γ and TNF-α after 24 hours of stimulation. However, because my project utilized *ex vivo* expanded NK cells from humans we were interested in seeing if these NK cells stimulated with IL-12 and IL-18 or IL-12, IL-15, and IL-18 maintained their synergistic production of IFN-γ and TNF-α. We found that the synergistic production of IFN-γ was maintained following stimulation with IL-12 and IL-18 or IL-12, IL-15, and IL-18 to at least the 72 hour post wash period. However, the synergistic production of TNF-α was not maintained to even the 24 hour post wash period. Instead, TNF-α production was synergistic only in the 24 hour post stimulation period.

Our lab is interested in the long term production of proinflammatory cytokines by NK cells as it is possible that these cytokines may counteract the immunosuppresive environment of the tumor and aid the immune system in inhibiting metastatic events and primary tumour growth. For example, TNF-α and IFN-γ assist with CD8+ T cells becoming cancer cell specific cytotoxic T lymphocytes (CTLs)[7,20]. Moreover, IFN-γ skews CD4+ T cell differentiation into a Th1 functional state polarizing the immune system into a pro-inflammatory and cytotoxic state[7,20]. Moreover knowing the kinetics of cytokine production may help future scientists and clinicians predict how patients will

respond to treatment with stimulated NK cell immunotherapies and whether they will assist a patients recovery or exacerbate their illness. Moreover, IFN-γ from NK cells up-regulates MHC class II expression on macrophages[47]. Therefore, macrophages in cancer patients may be able to provide stimulatory signals to T cells. Furthermore, IFN-γ induces the expression of adhesion molecules, including vascular cell adhesion molecule (VCAM)-1, lymphocyte function-associated antigen (LFA)-1, and intracellular adhesion molecule (ICAM)-1 on white blood cells and the vascular endothelium[45]. Furthermore, IFN-γ induces the expression of MIG and IP-10, chemokines that assist in the recruitment of activated T cells to the tumor microenvironment[45]. Therefore, the activated cells would be able to traffic to the cancer site.

IFN-γ production in the tumor microenvironment has been shown to be highly capable of limiting tumor angiogenesis, a necessity for a developing tumor. Specifically, the recruitment of T cells producing IFN-γ to the tumor site was shown to be crucial to limiting angiogenesis[45]. However, before T cells are recruited to the tumor sites they must be primed with a tumor antigen, creating a delay period. However, IFN-γ can be produced in excess by our stimulated NK cells which do not require priming *in vivo* to exert their anti-proliferative effects.

IFN-γ could also have direct anti-proliferative effects on cancer cells. For instance, signaling molecules activated by IFN-γ such as STAT1 have been cited as a tumor suppressor gene because it can activate cyclin-dependent kinase inhibitors (CDKIs)[48]. Furthermore, continuous interaction of IFN-γ with its receptor can induced the sustained expression of p21 and IRF-1, both associated with the growth inhibition of

cancer cells[49]. Furthermore, through caspase-1 activation and the induction of IFN-γ inducible gene IFN consensus sequence-binding protein (ICSBP) IFN-γ can activate the FAS signaling pathway in tumor cells, rapidly inducing apoptosis[50,51]. Moreover, the upregulation of FAS expression has been linked to the cleavage of the anti-apoptotic protein Bcl-2 in melanoma cells[52]. Similarly, IFN-γ can also restore TNF-related apoptosis inducing ligand (TRAIL)-induced apoptosis in resistant neuroblastoma cells by upregulating caspase-8 expression[53].

As our NK cells were grown in an NK cell coculture system with an irradiated feeder cell line in media supplemented with IL-2 we were aware that minor T cell contamination could occur. Therefore, we stimulated *ex vivo* expanded NK cells with IL-12, IL-15, and IL-18 and conducted intracellular flow cytometry for IFN-γ. We discovered that approximately 93.2% of the IFN-γ producing cells were NK cells and that of the total IFN-γ positive cells T cells composed only 3.66% of the population. Interestingly of the IFN-γ positive NK cells in the IL-12, IL-15, IL-18 stimulated group only 3.69% expressed CD16 versus 59.6% in the IFN-γ positive unstimulated control. This suggests that the presence of IL-12, IL-15, and IL-18 somehow suppresses CD16 expression. The low expression of CD16 could have important clinical ramification as it is possible these NK cells may have an inferior ability to mediate ADCC compared to their unstimulated counterparts. However, how important this reduced expression in CD16 is must be weighed against the potential benefits of IFN-γ production in the tumor microenvironment and cancer patient. Furthermore, as IL-2 systemic injections are being used in conjunction with NK cell adoptive transfer immunotherapies our lab was also

interested in investigating the expression of CD25 also known as IL-2Ra. As IL-2 injections are associated with several detrimental side effects but can assist the survival of NK cells we were interested in finding ways to decrease the amount of IL-2 needed by increasing the NK cells sensitivity to IL-2. We found that NK cells stimulated with IL-12, IL-15, and IL-18 positive for IFN-γ had a surface expression of CD25 at approximately 94.9%. Conversely, IFN-γ+ cells from unstimulated conditions had an expression level of approximately 5.38% for CD25. Therefore, if IL-2 is continued to be used *in vivo* to support NK cell survival pre-treatment with IL-12, IL-15, and IL-18 may increase sensitivity to IL-2. Therefore, less IL-2 could be injected to the patients receiving IL-12, IL-15, and IL-18 stimulated NK cells meaning that the NK cells could receive the support they need but function in a person suffering from fewer side effects.

4.2 What other cytokines are upregulated in NK cells stimulated with IL-12 and IL-18 or IL-12, IL-15, and IL-18?

Due to the benefits proinflammatory cytokines have on limiting cancer growth and the level of cytokine production our lab observed upon stimulating *ex vivo* expanded NK cells with IL-12 and IL-18 or IL-12, IL-15, and IL-18 we became interested in exploring the full range of cytokines upregulated in our studies. Furthermore, in recognition of the fact that IL-12 and IL-18 or IL-12, IL-15, and IL-18 stimulated NK cells are moving towards clinical trial it is prudent to examine other cytokines that are upregulated. This is necessary as we must consider the full range of outcomes that these stimulated NK cells could have in a cancer patients healthy and tumour tissues. In order to determine all cytokines up-regulated we ran a microarray. In addition to IFN-γ and

TNF-α upregulation in IL-12 and IL-18, or IL-12, IL-15, and IL-18 stimulated cells we found that CXCL-8, CCL3L1, LTA, and CCL3 were also upregulated. IFN-γ and LTA are well known to have a multitude of pro-inflammatory and anti-viral effects. CCL3L1 can attract lymphocytes to sites of infection or damage. Similarly, CCL3 also known as MIP1a is a member of the chemokine family and attracts polymorphonuclear leukocytes.

It is possible that these cytokines are significantly upregulated for a prolonged period of time similar to the profile of IFN-γ production we observed. Moreover, as the cytokine production could be so drastic and prolonged it is worthwhile to consider how these cytokines will affect a cancer patient and the tumor microenvironment. Therefore, further studies of *ex vivo* expanded NK cells stimulated in this way for use as a cancer immunotherapeutic must not only consider the effects on the NK cells IFN-γ production alone. Rather the studies and clinical trials must examine for the complete products of NK cell stimulation and for the role, presence, and products of other immune cells such as neutrophils, macrophages, T cells, eosinophils, and basophils. A proposed starting point for investigation is to elucidate the role of IL-8 production by IL-12 and IL-18 or IL-12, IL-15, and IL-18 stimulated NK cells. This is a functional starting point as IL-8 is a potent chemoattractant for neutrophils, a cell type that has been wrongfully assumed to have negligible roles in tumour immunology. However, neutrophils can exert either pro or anti-tumor effects depending on the tumour specific signals received. Moreover, the IL-8 is known to assist with tumor growth due to its angiogenic nature. Researching these questions would allow scientists to get either one step closer to curing cancer or giving up on a therapy likely to exacerbate disease severity.

4.3 The upregulation of IFN-γ is not due to prolonged phosphorylation of STAT4 or stabalization of the IFN-γ mRNA but is likely due to increased transcriptional activity.

After finding that *ex vivo* expanded NK cells produce pro-inflammatory cytokines synergistically in response to stimulation with IL-12 and IL-18 or IL-12, IL-15, and IL-18 we examined the mechanisms of activation responsible for this. Initially we examined the phosphorylation status of STATs 1, 3, and 4 after 10 and 40 minutes of stimulation with IL-12, IL-15, and IL-18. STAT4 phosphorylation was induced within 10 minutes of stimulation and was maintained after 40 minutes of stimulation in cells that were stimulated with IL-12, IL-12 and IL-18, and IL-12, IL-15, and IL-18. It is highly likely that this STAT4 activation is responsible, to some degree, for NK cell production of IFN-γ as this has been demonstrated before.

It has been proven that STAT4 mediated IFN-γ production can be inhibited by pSTAT1 as pSTAT1 can interrupt the pSTAT4 transcriptional machinery[101]. This does not seem to be important in the case our *ex vivo* expanded and stimulated NK cells as phosphorylation of pSTAT1 occurs at the 10 and 40 minute time points in the IL-12 and IL-18 and IL- 12, IL-15, and IL-18 stimulated *ex vivo* expanded NK cells. However, in spite of this IFN-γ production and STAT4 phosphorylation is still quite high. However, these phosphorylation events occur quite early, and therefore may not explain the prolonged production of IFN-γ we observed unless the phosphorylation of these proteins is also prolonged.

The activation status of STAT3 was examined because IL-15 is known to have a role in its activation. However, activation of STAT3 does not appear to play a major role

in NK cell mediated production of pro-inflammatory cytokines. This can be infered as the stimulatory conditions IL-12 and IL-18 and IL-12, IL-15, and IL-18, have very similar IFN-γ production levels despite having pSTAT3 activated in IL-12, IL-15, and IL-18 stimulated cells. However, STAT3 was also investigated to determine the extent of the effect the K562-mbIL-21 cells were having on our co-cultured NK cells during the stimulation. It is well known that IL-21 should activate STAT3 leading to its phosphorylation. Therefore, as little pSTAT3 was seen in any of the stimulatory conditions except the conditions with IL-15 we can assume that the K562-mbIL-21 cells have little effect on the *ex vivo* expanded NK cells when they were utilized for experiments one week after replenishment.

To further elucidate the signaling patterns responsible for prolonged pro-inflammatory cytokine production we examined the activation status of STATs 1, 3, 4, and 5 in samples from *ex vivo* expanded NK cells stimulated for 24 hours with IL-12, IL-15, and IL-18. pSTAT5 and tSTAT5 were added to the experimental panel of STAT1, 3, and 4 from earlier experiments because evidence has shown that IL-2 signaling can post-transcriptionally control IFN-γ mRNA translation. Specifically, it has been shown that IL-2 can initiate the trafficking of IFN-γ mRNA from the nucleus to the cytoplasm allowing for translation to occur. We thought, therefore that IL-12 and IL-18 or IL-12, IL-15, and IL-18 stimulated NK cells may just stockpile more IFN-γ mRNA than IL-12 stimulated cells from the initial 24 hour stimulation. This stockpiled IFN-γ mRNA could then be released to the cytoplasm in the post wash system in response to IL-2. However, pSTAT5 phosphorylation was only upregulated in IL-15 only stimulated cells and these NK cells

do not produce a large amount of IFN-γ at the 24 hour post wash period. Furthermore, qPCR analysis revealed that the transcript levels of IFNγ at the 6 and 24 hour post stimulation as well as the 24 hour post wash periods are drastically different. Consequently, it would seem that IL-2 induced posttranscriptional nucleocytoplasmic shuttling is not occurring via IL-2 and STAT5 in the NK cells under our stimulatory conditions.

Importantly, STAT4 was still phosphorylated after a 24 hour stimulation and remained phosphorylated for at least 24 hours after stimulatory cytokines were washed away, suggesting that pSTAT4 could be responsible for the prolonged production of IFN-γ in the IL-12 and IL-18 and IL-12, IL-15, and IL-18 groups. Furthermore, this suggests that these cell may continue to actively transcribe IFN-γ mRNA in the absence of stimulatory cytokines. However, levels of pSTAT4 are higher in IL-12 stimulated NK cells versus IL-12 and IL-18 and IL- 12, IL-15, and IL-18 stimulated NK cells at 24 hours post wash. Furthermore, IFN-γ protein secretion levels are significantly lower for IL-12 stimulated cells versus IL-12 and IL-18 and IL-12, IL-15, and IL-18 stimulated cells. Consequently, we presume that there must be some other mechanism responsible for the synergistic production of IFN-γ in *ex vivo* expanded NK cells stimulated with either IL-12 and IL-18 or IL-12, IL-15, and IL-18, and it is not mediated by STAT4 activation alone. Finally, long term production of IFN-γ likely has little to do with STAT 1, 3, and 5 phosphorylation as levels of phosphorylation for these STATs are negligible at the 24 hour post wash stages.

4.4 IL-8 and IFNγ Gene Expression from The Same NK Cells

In the course of our studies it was discovered that NK cells stimulated with a combination of IL-12 and IL-18 or IL-12, IL-15, and IL-18 produce significant amounts of the pro-inflammatory cytokines IL-8 and IFN-γ. Production of both of these cytokines from the same NK cells could be due to the significant overlap in the signaling pathways responsible for their production. For instance, both the NF-kB and STAT4 transcription factors seem to be involved in the IL-12 mediated increase of IL-8 and IFN-γ release, respectively. Interestingly, JAK2 is involved in the tyrosine phosphorylation of STAT4 and also in the activation of NF-kB. Therefore, it is possible that JAK-2 activates NF-kB and STAT4 to induce the expression of IL-8 and IFN-γ, respectively. Moreover, it is well known that there is an intronic enhancer region (C3) of the IFN-γ gene that binds the NF-kB protein c-Rel, which could upregulated NF-kB activation. Furthermore, an NF-kB site (IFN-γ kB) has been identified within the promotor region of IFN-γ. Therefore, JAK2 could also activate NF-kB to generate IFN-γ transcription.

Another layer of overlap between the IL-12 and IL-18 induction of IFN-γ and the IL-12 and IL-18 induction of IL-8 is the presence of AP-1 enhancer regions in both the IFN-γ and IL-8 promotors. The importance of this is further highlighted upon consideration of the fact that AP-1 activation can amplify the transcription of both IFN-γ and IL-8. Importantly, for IFN-γ production there is overlap of the mRNA stabilization phenomenon via p38 MAPK and the activation of AP-1. P38 MAPK is known to stabilize IFN-γ mRNA upon STAT4 induction of IFN-γ, but can also activate AP-1. Furthermore, p38 MAPK can also increase IL-8 production via activation of AP-1. Therefore, activation of P38 could induce increased production of both IL-8 and IFN-γ.

Therefore, the combination of IL-12 and IL-18 could upregulate the expression of IFN-γ and IL-8 by several mechanisms. These mechanisms include 1) increased mRNA stability of IFN-γ via p38 MAPK activation, which could lead to 2) MAPK activation of AP1 to enhancing the transcription of both IFN-γ and IL-8, and 3) NF-kB induced expression of both IFN-γ and IL-8 mRNA. Importantly, studies have shown that while AP-1 activation is not necessary for IL-8 and IFN-γ production it is needed for maximal IFN-γ and IL-8 production.

4.5 Future use of NK cells as a cancer immunotherapy

A critical factor that will determine whether or not any NK cell immunotherapy moves from bench to bedside will be how active the NK cells stay once they are in a cancer patient or the tumor microenvironment. Particular attempts must be made to understand what these activated cells will do in the body. In attempts to find a way to activate *ex vivo* expanded NK cells for long periods of time our lab has looked at stimulating NK cells with IL-12, IL-15 and IL-18. It was noted that stimulating the *ex vivo* expanded NK cells with IL-12 and IL-18 or IL-12, IL-15, and IL-18 upregulated the expression of many immunomodulatory genes such as IFN-γ, CXCL-8, CCL3L1, LTA, and CCL3 all of which could have significant effects on the immune system and the outcome of the cancer therapy. IFN-γ, and LTA are well known to have a multitude of pro-inflammatory and anti-viral effects[99]. CCL3L1 can attract lymphocytes to sites of infection or damage. Similarly, CCL3 also known as MIP1a is a member of the chemokine family and attracts polymorphonuclear leukocytes[102]. However, high levels of

plasma CCL3 has been associated with an increased risk of disease progression in chronic lymphocytic leukemia[102].

This thesis has outlined the most significantly up-regulated genes from IL-12, IL-15, and IL-18 stimulated NK cells including the combined effects of IL-12 and IL-18 and IL-12, IL-15, and IL-18. However, we have not addressed the complete role that this change in gene expression will have on cancer patients or the tumor microenvironment. In attempts to study the effects of the cytokine production from IL-12, IL-15, and IL-18 stimulated NK cells in an *in vivo* model our lab injected IL-12, IL-15, and IL-18 stimulated NK cells into NRG mice. We could not detect IFN-γ in the serum of these mice after 24 hours. As a follow up we injected media from NK cells stimulated for 24 hours with IL-12, IL-15, and IL-18. This media had significant amounts of IFN-γ present as detected by ELISA but could not be detected in the mouse model. Therefore, we conclude that something in the mouse serum is binding the human IFN-γ and limiting our ability to measure its production *in vivo*.

As well as having limited animal models available to study the complete effects of IL-12 and IL-18, and IL-12, IL-15, and IL-18 stimulated *ex vivo* expanded NK cells as a cancer therapy another potential problem has arisen. While NK cells express both activating and inhibitory receptors on their surface in the presence of the sufficient inflammatory conditions NK cells can become non-specifically activated. John Ortaldo and Howard Young discovered that murine NK cells bound to both activing Ly49D ligands and inhibitory Ly49G2 ligands can have the inhibitory response abrogated by stimulation with the cytokines IL-12 and IL-18. If this were to occur in humans it could

lead to widespread destruction of both tumour and healthy tissues in cancer patients. Therefore, one of the highlights of NK cell immunotherapy, the NK cells ability to tell healthy from unhealthy tissues, could be lost. However, this study does not consider the other families of NK cell activating and inhibitory receptors and how these might function on NK cells stimulated with IL-12 and IL-18. Nevertheless, such issues must be addressed to give due diligence to cancer patients safety.

4.6 Global and national burden of cancer and need for effective therapies

The findings of this thesis and how it relates to cancer come at a critical time. The WHO predicts that by 2035 there will be 24 million people living with cancer. In 2015 alone Canada will experience an estimated 196 000 new cases of cancer and 78 000 deaths due to cancer[103]. These numbers are quite conservative considering they do not include 78 300 cases of new non-melanoma skin cancer[103]. Therefore, on average 539 Canadians will be diagnosed with cancer and 214 Canadians will die from cancer everyday in 2015. This is therefore a major financial burden for the Canadian healthcare system as well as the leading cause of death in Canada.

There is also a large population globally that will have either limited access to therapeutic opportunities or that will be faced with a poor panel of treatment options as cancers are often unresponsive to classical: chemical, hormonal, and radiation therapies. Furthermore, these therapies lack specificity translating into detrimental side effects bedside. However cancer immunotherapies such as adoptive transfer NK cell therapies may offer new hope as a high level of endogenous NK cells has been recognized as a positive prognostic indicator[104]. Nevertheless, more research is necessary to determine

how NK cells activated with IL-12 and IL-18 or IL-12, IL-15 and IL-18 will alter neutrophil, macrophages and lymphocyte activity in cancer patients via the cytokines that these stimulated NK cells produce. Depending on the behaviour of the injected NK cells, their trafficking to the tumor site and the attraction they have on other immune cells either a pro or anti-tumor environment could be created.

4.7 Future directions

The harsh landscape of cancer therapies is further worsened by the classical approach of cancer researchers. Many focus on their small field alone, not considering the true intricacies of the body. Moreover, non-surgical interventions have focused on treating one cell type in a heterogeneous tumour landscape, promoting cancer cell resistance[105]. Therefore, researchers have combined surgical and non-surgical approaches, however these classical intercessions suppress the immune system, specifically NK and T cells, leading to tumour desemination, growth and angiogenesis[106–108]. Specifically, classical treatments and surgery have been linked to IL-6 and IL-10 production induced post-operatively which can promote healing of surgically damaged tissues but also contribute to tumor growth[109,110]. Consequently, research has focused on boosting the proinflammatory activation of the immune system both pre and peri-operatively. One approach to this has been the peri-operative use of oncolytic viruses. This field thrives because IFN and IFN responsive genes are known to respond to viral infection by inducing apoptosis, reducing angiogenesis, arresting cell growth and modulating the innate and adaptive immune systems[111]. These things are all contrary to the survival interests of cancer cells. Consequently, cancer cells suppress IFN responses, revealing an

Achilles heel, which is an increased susceptibility to viral infection. This weakness can be exploited to activate the immune system. For instance pre-operative OV Vaccinia Virus (VV) treatment has been shown to prevent postoperative NK cell dysfunction and attenuate tumoral dissemination[107]. This is due in part to the direct oncolytic effects of VV but also VVs immuno-stimulatory role on NK cells. The cytokine profile responsible for NK cell activation induced upon OV VV infection in cancer cells in a real patient would be impossible to replicate with recombinant cytokine injections or prestimulation, but manages to still muster NK cell cytotoxicity. Therefore, OV VV administration preoperatively or at any time before NK cell injection could abrogate the necessity for systemic recombinant cytokine injection intravenously (iv) or prestimulation of NK cells. To further improve the therapeutic value of OVs they are continuously being "weaponized". For instance, a VV expressing GM-CSF (JX-594) has been shown to induce necrosis in cancer cells, and cause immune cell infiltration into tumor vasculature[112,113]. Other OV vectors have utilized IL-12, IL-15, and IL-18 cytokine transgenes to promote endogenous NK cell activity[34,114]. However, this thesis points to the fact that these cytokines may activate NK cells best together. Therefore, a combination of IL-12 and IL-18 or IL-12, IL-15 and IL-18 in a single VV vector delivered intravenously (iv) could serve to activate endogenous NK cells classically suppressed by the surgical intervention or maintain the activation of adoptively transferred NK cells. Furthermore, if a VV-IL-12/18 or VV-IL-12/15/18 was utilized pre-operatively or before NK cell treatment it could direct *ex vivo* expanded NK cells to the cancer sites.

Furthermore, activating signals for the immune system would come in the form of the virally infected cancer cells, cytokines elicited by the viral infection, damage associated molecular patterns (DAMPs), and cytokines secreted by the transgenic OV and activated NK cells responding to the transgenic IL-12, IL-15, and IL-18[115]. This could help improve the safety profile of adoptively transferred NK cells *in vivo* as it could direct their functions directly to the tumor environment. If used properly this could further enhance the use of the OV and NK cell therapeutic fields and limit the metastasis and size of primary tumours.

This thesis highlights the pro-inflammatory cytokine production of *ex vivo* expanded NK cells stimulated with IL-12, IL-15, and IL-18 and the combinations of these cytokines on NK cells *in vitro*. This stimulatory protocol was adopted by our lab to stimulate NK cells prior to injection into cancer patients, so they could remain highly active *in vivo*. Previously researchers have used iv injection of supporting cytokines to maintain NK cell functions. Unfortunately, concomitant injections of IL-2 and NK cells increases incidences of vascular leak syndrome and can expand T regulatory cells which can inhibit NK cell tumoricidal activities[31,32]. While we have certainly found prolonged NK cell activation we do not fully know yet how these highly pro-inflammatory NK cells will respond to the complex physiology of the intact human body. For instance, the change in sugar and protein concentrations presented by human plasma could shock the activated NK cells metabolism leading to a change in cytokine transcription and translational activity. In order to truly understand how these NK cells will behave in a cancer patient *in vivo* we must test them in an immunologically and physiologically

relevant manner. Ideally humanized mouse models using syngeneic tumour tissues and *ex vivo* expanded NK cells could provide us with valuable data about how these cells will likely respond *in vivo*. However, it may be more feasible to test the behaviour of these NK cells in a syngeneic mouse model, with expanded mouse NK cells.

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Appendices

Table 3: Gene processes/sets and individual genes upregulated by IL-12/18 stimulation of NK cells when compared to IL-12 or IL-18 stimulated NK cells. This table represents genes that are uniquely upregulated in IL-12 and IL-18 stimulated NK cells any genes that were upregulated in IL-12 or IL-18 stimulated NK cells alone were excluded from the table.

GeneSet	Number Of Protein In Gene Set	Protein From Network	Nodes
Biosynthesis of amino acids(K)	73	10	PKM,PSAT1,GOT2,GOT1,ALDOA,PHGDH,PGK1,CTH,GAPDH,ENO1
Coregulation of Androgen receptor activity(N)	60	9	PTK2B,RPS6KA3,PA2G4,PRDX1,CDK6,CARM1,FKBP4,SRF,PELP1
Cysteine and methionine metabolism(K)	34	6	LDHA,GOT2,GOT1,SRM,CTH,SMS
Detoxification of Reactive Oxygen Species(R)	25	6	TXN,PRDX3,PRDX1,TXNRD1,SOD1,SOD2
erythropoietin mediated neuroprotection through nf-kb(B)	12	4	JUN,HIF1A,CDKN1A,SOD2
Eukaryotic Translation Elongation(R)	89	21	RPL18,RPL14,RPLP0,RPS15,RPS24,RPS7,RPL38,RPL34,RPL24,RPL29, RPL36A,EEF2,UBA52,RPL6,RPL9,RPL8,RPL5,RPL4,RPS2,RPS4X,EEF1B2
Eukaryotic Translation Termination(R)	84	20	RPL18,RPL14,RPLP0,RPS15,RPS24,RPS7,RPL38,RPL34,RPL24,ETF1, RPL29,RPL36A,UBA52,RPL6,RPL9,RPL8,RPL5,RPL4,RPS2,RPS4X
ISG15 antiviral mechanism(R)	71	11	EIF4A1,UBA52,NUP50,SEH1L,STAT1,NUPL2,NUPL1,NUP188,KPNB1, EIF4G1,EIF4G3
Metabolism of non-coding RNA(R)	49	9	NCBP2,PRMT5,NUP50,SEH1L,NUPL2,NUPL1,SNRPF,SNRPG,NUP188
Nonsense-Mediated Decay(R)	106	24	RPL18,RPL14,RPLP0,RPS15,RPS24,RPS7,RPL38,RPL34,PPP2CA,RPL24, ETF1,RPL29,NCBP2,RPL36A,UBA52,RPL6,RPL9,RPL8,RPL5,RPL4,RPS2, RPS4X,PPP2R2A,EIF4G1
Processing of Capped Intron-Containing Pre-mRNA(R)	138	19	PTBP1,NXF1,SRSF9,YBX1,PHF5A,NCBP2,SNRPB2,HNRNPD,HNRNPC, NUP50,SEH1L,POLR2H,EFTUD2,NUPL2,NUPL1,SNRPF,SNRPG,CSTF2, NUP188
Regulation of Cholesterol Biosynthesis by SREBP (SREBF)(R)	50	8	SAR1B,SEC24C,SEC24D,NCOA1,CARM1,RAN,FASN,KPNB1
Ribosome biogenesis in eukaryotes(K)	85	18	EMG1,NAT10,NXF1,GAR1,IMP4,DKC1,FBL,GTPBP4,RAN,RRP7A, NOP10,UTP14A,NHP2,NOP56,GNL2,GNL3,CIRH1A,NOL6
Ribosome(K)	135	26	RPL18,RPL14,RPLP0,RPS15,RPS24,RPS7,MRPL4,MRPL3,RPL38,RPL34, RPL24,RPL29,RPL36A,UBA52,RPL10L,RPL6,RPL9,RPL8,RPL5,RPL4, RPS2,RPS4X,MRPL12,MRPL24,MRPS12,MRPS2
RNA Polymerase II Transcription(R)	101	12	SRSF9,TCEB1,NCBP2,GTF2E2,SUPT5H,CCNH,GTF2B,POLR2H,SNRPF, SNRPG,CSTF2,NELFE
RNA transport(K)	164	21	EIF2S1,NXF1,EIF4A1,NCBP2,XPO5,XPOT,PRMT5,EIF2B3,RAN,NUP50, SEH1L,EIF5B,NUPL2,NUPL1,EIF3D,EIF3B,EIF3E,NUP188,KPNB1,EIF4G1, EIF4G3

Table 4: Gene processes and individual genes downregulated by IL-12/18 stimulation of NK cells when compared to IL-12 or IL-18 stimulated NK cells. This table represents genes that are uniquely down-regulated in IL-12 and IL-18 stimulated NK cells any genes that were down-regulated in IL-12 and IL-18 stimulated NK cells alone were excluded from the table.

GeneSet	Number Of Protein In Gene Set	Protein From Network	Nodes
5HT3 type receptor mediated signaling pathway(P)	10	3	STX3,VAMP8,VAMP1
5HT4 type receptor mediated signaling pathway(P)	23	6	GNGT2,STX3,GNB1,VAMP8,VAMP1,ADCY7
Adherens junction(K)	73	12	CTNNA1,MAPK1,MAPK3,RAC2,RAC1,TGF-BR2,IQGAP1,ACTN4,CDC42,RHOA, SMAD3,WASF2
ALK1 signaling events(N)	26	6	MAPK1,PPP1CA,MAPK3,TGF-BR2,BMPR2,SMAD7
Alpha4 beta1 integrin signaling events(N)	33	6	RAC1,PRKAR1A,YWHAZ,AMICA1,ITGB1,PTPRA
Arf6 downstream pathway(N)	15	4	MAPK1,MAPK3,RAC1,RHOA
Axon guidance(K)	127	18	ROCK1,MAPK1,MAPK3,GNAI2,RAC2,RAC1,NCK2,SRGAP3,PPP3CA,CDC42, RHOA,LIMK1,ABLIM1,NFATC3,PLXNA1,ITGB1,CXCR4,CFL1
Beta1 adrenergic receptor signaling pathway(P)	27	6	GNGT2,STX3,GNB1,VAMP8,VAMP1,ADCY7
Beta2 adrenergic receptor signaling pathway(P)	21	5	GNGT2,STX3,GNB1,VAMP8,VAMP1
Beta2 integrin cell surface interactions(N)	29	6	ICAM2,ICAM3,ITGB2,ITGAM,ITGAX,ITGAD
Beta3 adrenergic receptor signaling pathway(P)	21	5	GNGT2,STX3,GNB1,VAMP8,VAMP1
CDC42 signaling events(N)	70	16	CTNNA1,MAPK1,MAPK3,RAC1,TNK2,ARHGEF6,IQGAP1,ARPC1B,ARPC4, CDC42,LIMK1,ACTR2,SEPT2,PARD6A,CFL1,PIK3R1
Circadian entrainment(K)	97	12	MAPK1,MAPK3,GNAI2,GNGT2,GNB1,GNAS,ADCY3,ADCY7,CALM1,PLCB2, PRKCA,CAMK2G
control of skeletal myogenesis by hdac and calcium/calmodulin-dependent kinase (camk)(B)	17	4	MEF2D,CABIN1,YWHAH,KAT2B

Cortocotropin releasing factor receptor signaling pathway(P)	25	5	GNGT2,STX3,GNB1,VAMP8,VAMP1
DAG and IP3 signaling(R)	31	6	PRKAR1A,ADCY3,ADCY7,ITPR2,CALM1,PRKCA
DAP12 interactions(R)	171	30	KLRC2,KLRD1,SYK,MAPK1,MAPK3,KIR3DS1,RAC1,KIR2DS5,KIR2DS4,SOS1, LAT,CDKN1B,PRKAR1A,LCK,LCP2,ADCY3,ADCY7,PDGFRB,TYROBP, KIT,YWHAB, ITPR2,CALM1,FOXO3,FOXO4,PLCG2,AKT2,PRKCA,NCR2,PIK3R1
Dopaminergic synapse(K)	131	16	PPP2R5A,PPP2R5C,PPP1CA,GNAI2,GNGT2,GNB1,GNAS,PPP3CA,ATF6B,ITPR2, MAPK13,CALM1,PLCB2,AKT2,PRKCA,CAMK2G
E-cadherin signaling in the nascent adherens junction(N)	36	9	CTNNA1,RAC1,IQGAP1,CDC42,RHOA,WASF2,ITGB7,CYFIP2,PIK3R1
Endocytosis(K)	203	28	VPS4B,TGF-BR2,ARRB1,PDCD6IP,RAB7A,EPS15,CDC42,RHOA,VPS36,VPS28, MVB12B,ACAP1,SMAD7,SMAD3,CBLB,GRK5,RAB5B,RAB5C,AP2B1,SH3KBP1, KIT,CHMP4B,PARD6A,CXCR4,SH3GLB2,IL2RB,ARAP3,ARAP1
Endometrial cancer(K)	52	8	ILK,CTNNA1,MAPK1,MAPK3,SOS1,FOXO3,AKT2,PIK3R1
EPHB forward signaling(N)	38	6	ROCK1,MAPK1,MAPK3,RAC1,CDC42,PIK3R1
Ephrin B reverse signaling(N)	24	5	RAC1,NCK2,FGR,LCK,PIK3R1
EPO signaling pathway(N)	33	6	SOS1,SH2B3,INPP5D,BCL2,PLCG2,PIK3R1
Gastrin-CREB signalling pathway via PKC and MAPK(R)	207	21	LTB4R,MAPK1,MAPK3,GNGT2,RGS2,NMUR1,GNB1,SOS1,DGKQ,P2RY10,XCL1, ANXA1,LPAR6,GRK5,YWHAB,ITPR2,PLCB2,PRKCA,PRKCH,PIK3R1,RGS19
GP1b-IX-V activation signalling(R)	10	3	FLNA,YWHAZ,PIK3R1
Hippo signaling pathway(K)	154	15	CTNNA1,PPP1CA,TGF-BR2,RASSF1,BMPR2,SMAD7,SMAD3,YWHAZ,YWHAB, BIRC5,YWHAH,ITGB2,PARD6A,BBC3,WNT11
how does salmonella hijack a cell(B)	11	5	RAC1,ARPC1B,ARPC4,CDC42,ACTR2
Insulin-mediated glucose transport(N)	27	5	YWHAZ,YWHAB,YWHAH,CALM1,AKT2
Insulin Pathway(N)	45	9	NCK2,SOS1,EXOC4,INPP5D,FOXO3,PARD6A,AKT2,PIK3R1,PTPRA

Insulin signaling pathway(K)	141	14	MAPK1,PPP1CA,MAPK3,PHKG2,SOS1,PRKAR1A,IKBKB,CBLB,PRKAA1,CALM1, PRKAB1,PRKAG1,AKT2,PIK3R1
Integration of energy metabolism(R)	105	13	GNAI2,GNGT2,GNB1,GNAS,IQGAP1,STK11,PRKAR1A,ADCY3,ADCY7,STX1A, ITPR2,PLCB2,PRKCA
Integrin-linked kinase signaling(N)	45	8	ILK,RAC1,ARHGEF6,NCK2,IQGAP1,TACC3,CDC42,DIAPH1
Integrins in angiogenesis(N)	47	9	ILK,ROCK1,MAPK1,MAPK3,RAC1,TGF-BR2,CDKN1B,RHOA,PIK3R1
Internalization of ErbB1(N)	35	6	SOS1,EPS15,SPRY2,CDC42,CBLB,SH3KBP1
Lissencephaly gene (LIS1) in neuronal migration and development(N)	28	6	RAC1,IQGAP1,CDC42,RHOA,PAFAH1B1,CALM1
Lysosome(K)	122	15	AP3S1,AP1S1,AP1S2,AP1G2,LAMP1,TCIRG1,SMPD1,GGA2,CD63,GNS,LAPTM5, CTSZ,CTSO,CTSC,CTSB
Metabolism of porphyrins(R)	14	4	ALAD,UROD,UROS,PPOX
N-cadherin signaling events(N)	33	8	ROCK1,CTNNA1,RAC1,CDC42,RHOA,CALM1,CAMK2G,PIK3R1
Nectin adhesion pathway(N)	30	6	CTNNA1,RAC1,IQGAP1,CDC42,PDGFRB,PIK3R1
Opioid Signalling(R)	80	13	MAPK1,PPP1CA,GNAI2,GNGT2,GNB1,PPP3CA,PRKAR1A,ADCY3,ADCY7, ITPR2,CALM1,PLCB2,PRKCA
Oxytocin receptor mediated signaling pathway(P)	21	5	GNGT2,STX3,GNB1,VAMP8,VAMP1
p53 pathway(N)	57	8	ATM,DAXX,RASSF1,YY1,CHEK2,PIN1,CSNK1G2,KAT2B
PAR4-mediated thrombin signaling events(N)	15	5	ROCK1,GNB1,GNA13,RHOA,PLCB2
Parkinson disease(P)	61	8	MAPK1,MAPK3,UBE2L6,YWHAZ,YWHAB,YWHAH,SEPT2,SEPT1
Phosphatidylinositol signaling system(K)	81	10	DGKQ,INPP5E,INPP5D,INPP5B,ITPR2,CALM1,PLCG2,PLCB2,PRKCA,PIK3R1
Platelet homeostasis(R)	74	9	PPP2R5A,PPP2R5C,GNGT2,GNB1,GNAS,FGR,ATP2A3,ITPR2,PECAM1
Posttranslational regulation of adherens junction stability and dissassembly(N)	48	8	CTNNA1,RAC1,GNA13,IQGAP1,RAB7A,CDC42,NTRK2,ZBTB33

Primary immunodeficiency(K)	36	8	ZAP70,LCK,CD3D,CD3E,AIRE,RFX5,CD8A,PTPRC
RAC1 signaling pathway(N)	54	13	CTNNA1,RAC1,IQGAP1,ARPC1B,ARPC4,LIMK1,CYBA,WASF2,ACTR2, CYFIP2,PLCB2,CFL1,RACGAP1
Regulation of RAC1 activity(N)	38	10	RAC1,ARHGEF6,SOS1,PREX1,DEF6,RAP1GDS1,CHN2,ARHGAP9,RAL BP1, RACGAP1
Response to elevated platelet cytosolic Ca2+(R)	82	11	FLNA,ACTN4,TIMP1,TUBA4A,CD63,CAP1,CALM1,WDR1,PRKCA,CFL 1,PECAM1
Retrograde endocannabinoid signaling(K)	103	12	MAPK1,MAPK3,GNAI2,GNGT2,GNB1,ADCY3,ADCY7,ITPR2,MAPK13, GABRA6, PLCB2,PRKCA
SHP2 signaling(N)	51	8	SOS1,RHOA,LCK,JAK1,PDGFRB,PAG1,PIK3R1,IL2RB
Signaling by ERBB2(R)	155	19	MAPK1,MAPK3,SOS1,MATK,CDKN1B,PRKAR1A,LCK,ADCY3,ADCY7, PDGFRB, KIT,YWHAB,ITPR2,CALM1,FOXO3,FOXO4,AKT2,PRKCA,PIK3R1
Signaling by FGFR(R)	154	19	MAPK1,MAPK3,SOS1,CDKN1B,PRKAR1A,SPRY2,LCK,ADCY3,ADCY7, PDGFRB, KIT,YWHAB,ITPR2,CALM1,FOXO3,FOXO4,AKT2,PRKCA,PIK3R1
Signaling by Insulin receptor(R)	109	13	MAPK1,MAPK3,EEF2K,SOS1,STK11,TCIRG1,ATP6V1G1,PRKAA1,YW HAB, PRKAB1,PRKAG1,AKT2,PIK3R1
Signaling by PDGF(R)	176	22	MAPK1,MAPK3,NCK2,SOS1,CDKN1B,PRKAR1A,LCK,COL9A2,ADCY3, ADCY7, PDGFRB,KIT,STAT6,YWHAB,ITPR2,CALM1,FOXO3,FOXO4,PDGFD,A KT2,PRKCA,PIK3R1
Signaling by Robo receptor(R)	32	7	RAC1,NCK2,SRGAP3,SOS1,CDC42,CAP1,EVL
Signaling by Type 1 Insulin-like Growth Factor 1 Receptor (IGF1R)(R)	86	11	MAPK1,MAPK3,EEF2K,SOS1,STK11,PRKAA1,YWHAB,PRKAB1,PRKA G1,AKT2, PIK3R1
Signaling events mediated by focal adhesion kinase(N)	62	8	MAPK1,RAC1,NCK2,SOS1,ACTN4,RHOA,ITGB1,PIK3R1
Signaling events regulated by Ret tyrosine kinase(N)	39	7	MAPK1,MAPK3,RAC1,SOS1,RHOA,PRKCA,PIK3R1
Signaling mediated by p38-gamma and p38-delta(N)	11	4	EEF2K,STMN1,PKN1,MAPK13

SNARE interactions in vesicular transport(K)	36	7	STX3,STX2,VAMP8,VAMP1,STX8,STX16,STX1A
Thrombin signalling through proteinase activated receptors (PARs)(R)	32	6	MAPK1,MAPK3,GNGT2,GNB1,GNA13,ARRB1
TRAIL signaling pathway(N)	22	5	MAPK1,MAPK3,TNFSF10,IKBKB,SMPD1
ucalpain and friends in cell spread(B)	15	4	RAC1,ACTN4,RHOA,ITGB1
Urokinase-type plasminogen activator (uPA) and uPAR-mediated signaling(N)	42	7	RAC1,PDGFRB,MMP9,PDGFD,ITGB2,ITGB1,ITGAM
VEGFR1 specific signals(N)	27	5	MAPK1,MAPK3,CALM1,PRKCA,PIK3R1
VEGFR3 signaling in lymphatic endothelium(N)	25	5	MAPK1,MAPK3,SOS1,ITGB1,PIK3R1
y branching of actin filaments(B)	16	6	RAC1,ARPC1B,ARPC4,CDC42,WASF2,ACTR2