THE CRITICAL ROLE OF PI3K-AKT-mTOR PATHWAY FOR IL-15 INDUCED NK CELL EFFECTOR RESPONSES

BY
NEETHI NANDAGOPAL

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF SCIENCE

In the Department of Microbiology and Immunology

Faculty of Medicine, University of Ottawa

ABSTRACT

Natural killer (NK) cells were so named for their uniqueness in killing certain tumor and virus-infected cells without prior sensitization unlike T lymphocytes. NK cells possess a myriad of activation receptors and cytokine receptors that allow them to recognize stress ligands on infected/tumor cells and respond to the cytokines produced during the inflammatory process. Upon activation, NK cells produce pro-inflammatory cytokines, cytotoxic granules and chemokines to recruit other cells which ultimately result in killing of target cells. These functions of NK cells are modulated *in vivo* by several immune mediators; IL-15 being the most potent in enabling NK cell homeostasis, maturation and activation. Indeed, IL-15 knockout mice have no detectable NK cells.

During microbial infections, NK cells stimulated with IL-15 display enhanced cytokine responses. This priming effect has previously been shown with respect to increased IFN-γ production in NK cells upon IL-12 and IL-15/IL-2 co-stimulation. In this study, I explored if this effect of IL-15 priming can be extended to other cytokines and observed enhanced NK cell responses to stimulation with IFN-α, IL-21, IL-2 and IL-4 in addition to IL-12. Notably, we also observed elevated IFN-γ production in primed NK cells upon stimulation through the Ly49H activation receptor. IL-15 treatments induced NK cell proliferation, enhanced NK cell responses to activating stimuli and equipped them with cytotoxic granules thereby "readying" them for battle against infections and tumors. Here, we try to understand the signaling mechanisms underlying IL-15 treatments that activate NK cells. Currently, the fundamental processes required for priming and whether these signaling pathways work collaboratively or independently for NK cell functions are poorly understood.

To identify the key signaling events, we examined IL-15 priming on NK cells in which the pathways emanating from IL-15 receptor activation were blocked with specific inhibitors. Our results demonstrate that the PI3K-AKT-mTOR pathway is indispensable for cytokine responses in IL-15 primed NK cells. Furthermore, this pathway is also implicated in a broad range of IL-15 induced NK cell effector functions such as proliferation and cytotoxicity. Given that NK cells are critical for control of viral infections like murine cytomegalovirus (MCMV), we decided to analyze the consequences of blocking the PI3K-AKT-mTOR pathway in NK cells on its anti-viral responses. Likewise, NK cells from mice treated with rapamycin to block the mTOR pathway displayed defects in proliferation, IFN-γ and granzyme B production resulting in elevated viral burdens upon MCMV infection. Taken together, our data demonstrates the requirement of PI3K-mTOR pathway for enhanced NK cell functions by IL-15. It also shows that IL-15 primes NK cell responses to several cytokines and to Ly49H activation receptor stimulation. To our knowledge this is first report to demonstrate the requirement of mTOR activity in NK cells for efficient control of acute MCMV infections; thereby coupling the metabolic sensor mTOR to NK cell anti-viral responses.

ACKNOWLEDGEMENTS

I would like to begin by thanking my parents and my sister for their support and encouragement to let me follow my passion and continue my graduate studies in Canada. Most importantly, I must thank Dr. Seung Hwan Lee for his patience and guidance. I appreciate his advice and the countless hours invested in making me improve the quality of everything I did. Thank you!

I would like to thank my TAC committee members –Drs. Earl Brown and Marc-Andre Langlois for their guidance and the valuable feedbacks on my research projects. I also thank Drs. Lionel Fillion and Vera Tang for their motivational talks and assistance with Flow cytometry-related work. I thank the Makrigiannis laboratory members for assisting us with the reagents required for performing NK cell killing assay. Special thanks to Dr. Munir Rahim for his patience and ever-available assistance. It was of great help to me! I would also like to thank our past technicians – Yuxia Bo and Jee Yeon Lee for technical support including the Animal Care Staff who have helped me with animal handling trainings, setting up new experiments and assisting with the smooth maintenance and daily care of my mice.

I appreciate my laboratory members- Amandeep Kaur Komal and Alaa Kassim Ali. Alaa and Amandeep, as my co-authors have helped me with the project. I thank Alaa for making me laugh and staying up until daybreak to finish our experiments. Most importantly, I am extremely grateful to Amandeep Komal who has been very supportive for the past two years, keeping my spirits high and lending me her ear whenever I needed a friend.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ADCC	Antibody Dependent Cellular Cytotoxicity
APC	Antigen Presenting Cells
BrdU	Bromodeoxyuridine
CBA	Cytometric Bead Assay
CC	Chemokines
CMV	Cytomegalovirus
cDCs	classical Dendritic Cells
DMSO	Dimethyl Sulfoxide
dsRNA	double stranded Ribonucleic acid
dsDNA	double stranded Deoxyribonucleic acid
ERK	Extracellular signal-Regulated Kinase
GM-CSF	Granulocyte/Monocyte Colony Stimulating Factor
JAK	Janus Activated Kinase
HA	Haemagglutinin
HCMV	Human Cytomegalovirus
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
IFN	Interferon
IL-	Interleukin-
IRF	Interferon Regulatory Factor
ITAM	Immuno-receptor Tyrosine-based Activation Motifs
ITIM	Immuno-receptor Tyrosine-based Inhibition Motifs
KIR	Killer Ig-like Receptor
LCMV	Lymphocytic Choriomeningitis Virus
MAPK	Mitogen Activated Protein Kinase
MCMV	Murine Cytomegalovirus

MEK	MAPK/ERK Kinase
MHC	Major Histocompatibility Complex
MIP	Macrophage inflammatory protein
MIC A/B	MHC class I polypeptide-related sequence A/B
ml	millilitre
mTOR	mammalian Target of Rapamycin
mTORC	mammalian Target of Rapamycin Complex
MyD88	Myeloid differentiation primary response gene 88
ng	nanograms
NCR	Natural Cytotoxicity triggering Receptor
NF-κB	Nuclear Factor Kappa-light-chain- enhancer of activated B cells
NK	Natural Killer
NKC	NK gene Complex
PAK	p21 Activated Kinases
PAMPs	Pathogen Associated Molecular Patterns
pDCs	Plasmacytoid Dendritic Cells
PDK-1	Phosphoinositide-Dependent Kinase-1
PFU	Plaque Forming Units
PIP2	Phosphatidylinositol Biphosphate
PIP3	Phosphatidylinositol Triphosphate
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
AKT/PKB	Protein Kinase B
PH	Pleckstrin homology
p.i.	post infection
pg	picograms
PRAS40	Proline Rrich Akt Substrate of 40 kDa
PTK	Protein Tyrosine Kinases

PLC-γ	Phospholipase C-γ
Syk	Spleen tyrosine kinase
PRRs	Pattern Recognition Receptors
pS6K	phosphorylated ribosomal protein S6 Kinase
pSTATs	phosphorylated Signal Transducer and Activator of Transcription
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
RAE-1	Retinoic Acid Early transcript-1
SH-2	Src homology-2
ssRNA	single stranded Ribonucleic Acid
STAT	Signal Transducer and Activator of Transcription
TLRs	Toll- Like Receptors
TNF-α	Tumor Necrosis Factor-α
TSC2	Tuberous Sclerosis Complex 2
ZAP70	Zeta-chain-associated protein kinase 70
μM	micromolar
4E-BP	Eukaryotic 4E Binding Protein

LIST OF FIGURES AND TABLES

1. INTRODUCTION

In this section, I shall review the cells of the innate immune system and their roles in early protection against pathogens. I shall focus on NK cells, the key players of anti-viral and anti-tumor immunity and the role of dendritic cells (DC) in NK cell activation. With respect to NK cells, I have talked about various activation and cytokine receptors on their surfaces, and the importance of these receptors in shaping NK cell effector responses. I shall also focus on MCMV infections, which is a good murine model to study NK cell functions. My project primarily deals with the role of IL-15 cytokine in "priming" and activating NK cell functions. Therefore, I have introduced the importance of IL-15 in NK cell biology with special focus on the pathways emanating from IL-15 receptor activation. Finally, the role of PI3K-mTOR (Phosphatidylinositol-4,5-bisphosphate 3-kinase-mammalian Target of Rapamycin) pathway has been reviewed, which has been extensively studied in T cells but requires a more in-depth understanding in NK cells.

1.1. INNATE IMMUNITY OVERVIEW

The immune system protects us from small scars to dangerous pathogens like viruses and bacteria. Immune responses are broadly classified as innate and adaptive that comprises of variety of cells in each category. Innate immunity ranges from simple barriers against pathogens to recognition of stressed cells like virus-infected or cancers by several cellular mechanisms. The cells of the innate immune system comprises of antigen presenting cells (APCs) like DCs, macrophages, including NK cells and granulocytes that are ready for immediate responses to all pathogens (Beutler, 2004).

At its most basic level is the first line of defense against infections that comprises of a myriad of pathogen recognition receptors (PRRs) for recognition of unique pathogen associated molecular patterns (PAMPs) like viral nucleic acids and lipopolysaccardies (LPS) of bacteria. Toll-Like receptors (TLRs) are most well studied of the PRR families, present on the APCs both on their cell-surfaces and in the intracellular compartments like endoplasmic reticulum (Mogensen, 2009; Randall and Goodbourn, 2008). Recognition of pathogens by TLRs mounts an inflammatory response that involves transcription of genes required for induction of chemokines and cytokines thereby recruiting immune cells, production of anti-microbial peptides and up-regulation of adhesion molecules for leukocyte arrests in the areas of infection etc. With recognition of viral infections, especially by plasmacytoid DCs and macrophages, most TLRs signal intracellularly through a MyD88-dependent pathway (e.g. TLR7 and TLR9 that recognize ssRNA and DNA respectively) while some TLRs use a TRIF-dependent pathway (e.g. TLR3 that recognizes dsRNA). This results in production of NF-κB and mitogen activated protein kinase (MAPK) genes which results in transcription of several other inflammatory genes. In parallel, type I interferon production occurs through regulation of interferon regulatory factors (IRF3, -5, -7, -9) thereby initiating an inflammatory cascade (Randall and Goodbourn, 2008; Tabeta et al., 2004). The importance of TLR3 in anti-viral response is suggested by the fact that TLR3-/- mice are susceptible to MCMV most likely due to reduced interferon production (Tabeta et al., 2004). Alternatively, TLRs are also expressed on NK cells and can activate them directly or indirectly through interaction of NK cells with macrophages and DCs (McCartney et al., 2009).

While innate immune responses have the potential of delivering immediate effects upon microbial infections, they also exert immunoregulatory roles in order to shape the adaptive arm of immunity mainly through activation of T lymphocytes by the peptide-major histocompatibility complex (MHC) complexes on APCs (Beutler, 2004; Biron et al., 1999). Innate Immune cells like neutrophils and macrophages are among the first to take up the pathogen or damaged apoptotic cells by phagocytic processes. Coating of damaged or infected cells with antigen-specific antibodies promotes opsonization due to recognition of the Fc portion of antibody by the Fc receptors on phagocytes. APCs like DCs digest and process the antigenic peptides which are presented on MHC complexes to lysis-inducing T cells. Simultaneously, cytokines and chemokines released by phagocytes result in activation and recruitment of other immune cells such as NK cells and T cells to sites of inflammation. Cytotoxic cells like NK and CD8 expressing T cells induce target cell death through directed release of proteins such as perforin and granzyme that activate cell death pathways. Such cells can also express receptors to recognize tumor-necrosis-factor related apoptosis inducing ligand (TRAIL) and Fas-ligand (FasL) and can directly induce apoptosis of target cells (Biron et al., 1999; Biron et al., 1996; Smyth et al., 2003). Therefore upon receiving "danger" signals, several mechanisms persist within the innate arm of immunity, leading to increase in metabolic activity and proliferation of immune cells and culminating in an inflammatory cascade that helps in the fight against the invading pathogen.

1.2. NATURAL KILLER CELLS AND THEIR ROLE IN MCMV INFECTION

Natural Killer (NK) cells are innate immune cells derived from a common lymphoid precursor during hematopoiesis in the bone marrow (BM) and are the third largest of the lymphocyte populations after B cells and T cells. In 1975, they were first discovered as a unique leukocyte population in BM, spleen and blood, had large granular appearance and possessed spontaneous "natural" anti-tumor properties. They were so named for their propensity to kill target cells without the need for antigenic stimulation (Herberman et al., 1975; Kiessling et al., 1976; Trinchieri, 1989). In C57BL/6 mice model most commonly used for studying NK cells, they are classified by expression of the cell surface markers-CD161c (NK1.1) or CD335 (NKp46) in addition to the lack of expression of T and B cell antigen receptors (Yokoyama et al., 2004).

NK cells are important for early defenses against pathogens before T cells come into the picture. Their lytic functions are mediated by contact-dependent cytotoxicity and cytokine production. In response to inflammatory signals and upon recognition of tumor or virus-infected cells, NK cells become metabolically active and rapidly proliferate. *In vivo* exposure to type I interferons generated early during inflammation can induce proliferation and blastogenesis in NK cells through IL-2/IL-15 dependent manner (Biron et al., 1999; Biron and Welsh, 1982; Nguyen et al., 2002). Simultaneously, the signals also induce production of pro-inflammatory cytokines such as IFN-γ and TNF-α (Biron et al., 1999). IFN-γ interferes with viral replication and thus helps to control infections (Lucin et al., 1994). In addition, signals through activating receptors stimulate NK cells to release pre-existing cytotoxic granules such as perforin and granzymes thereby

destroying infected and cancerous cells. Perforin induces a transient Ca^{2+} flux in the target, which triggers a damaged cell membrane repair process and delivers apoptosis-inducing granzymes to the cytosol of target cells (Thiery et al., 2011). Additionally, NK cells can secrete chemokines like macrophage inflammatory protein 1α (MIP-1α), macrophage inflammatory protein 1β (MIP-1β), including regulated on activation—normal T cell expressed and secreted (RANTES) to attract other immune cells to sites of infection (Biron et al., 1999). Therefore NK cells are important components of the host's protective mechanisms towards pathogens and cancers.

One of the best models to study the innate defense mechanisms of NK cells is MCMV infections. MCMV is the mouse counterpart of Human Cytomegalovirus (HCMV) and belongs to the Beta-herpesviridiae virus subgroup (Virology, 4th Edition). They have similar virion structures, pathogenesis and cell biology (Smith et al., 2008). MCMV infection is characterized by cytomegaly of infected cells with liver and spleen being the major sites of infection (Ho, 1991). Different species of CMV induce host specific immune responses; MCMV genome is stable and laboratory strains do not undergo mutations further making them a good tool to study CMV pathogenesis (Cheng et al., 2010). HCMV is generally asymptomatic but is detrimental in immunocompromised hosts like HIV patients and new born infants (Weller, 1971). Infection is controlled by a combination of innate and adaptive immune functions, after which latent infection continues for life. Primary infection, lifelong latency, and intermittent shedding resulting from reactivation of virus commonly occurs unnoticed suggesting the unique relationship between CMV and its host.

Several genetically inherited host resistance mechanisms mediated by NK cells have been identified towards MCMV. For example, the *Cmv1* gene locus was mapped to the NK gene complex (NKC) region of chromosome 6 in mice which also contains several NK activation receptors like the Ly49 family. This locus was identified in certain inbred mouse strains and confers resistance to and mediated survival upon MCMV infections (Brown et al., 2001; Daniels et al., 2001; Lee et al., 2001). This resistance was further attributed to the recognition of m157, a MCMV-encoded homologue of MHC class I by Ly49H activation receptor present in roughly 50% of NK cells in C57BL/6 strains. This recognition results in NK cell mediated viral control mechanisms. On the other hand absence of Ly49H resulted in uncontrolled viral replication and death of MCMV-infected mice.

Viral immunity in MCMV-resistant mice to some extent is conferred by secretion of IFN-γ by NK cells. NK cells can produce IFN-γ during MCMV infection and this production is induced by IL-12. Depletion of NK cells *in vivo* affected the production of IFN-γ during MCMV infection and depletion of anti-IFN-γ drastically increased viral titers and MCMV-induced liver pathology compared to control mice (Orange and Biron, 1996). The importance of NK cells in controlling MCMV is apparent in mice models where NK cells are depleted prior to viral infections. NK-depleted mice had drastically more titers of vaccinia virus, mouse hepatitis virus and MCMV. They displayed hepatitis of livers, characteristic of severe MCMV infections due to lack of NK cell anti-viral lytic functions. Whereas NK cell-depleted and control mice had similar amounts of LCMV titers further indicating the importance of NK cell-mediated specific resistance

mechanisms to MCMV (Bukowski et al., 1984). In humans, the most famous case of NK cell deficiency was a female adolescent with no other immunological impairment. She along with another patient lacking CD56+NK cells were both highly susceptible to herpesvirus infections further demonstrating the importance of NK cells in keeping these infections under control (Biron et al., 1989; Orange, 2002; Wendland et al., 2000).

1.2.1. NATURAL KILLER CELL RECEPTORS

NK cell effector responses are often determined by the integration of signal transduction pathways from multiple activating and inhibitory receptors (Lanier, 2008; Lee et al., 2007). NK cell receptor families are germ-line coded and include: human killer Ig- like receptor (KIR) family, mouse Ly49 family and NKG2 lectin like receptor family in both humans and mice which are involved in the recognition of viral/tumor ligands or MHC class I molecules (Lanier, 1998). All nucleated cells in our body express MHC class I protein which can bind antigenic peptides that are recognized by T cell receptors eliciting a cytotoxic response from T lymphocytes (Kuby 4th Edition). However, certain tumor cells and virus-infected cells have evolved strategies to escape immune recognition by down-modulating MHC expression. This is where NK cells come in, by "missing-self hypothesis" inhibitory receptors on NK cells can recognize self-MHC I molecules and suppress activation of NK cells thereby sparing normal cells (Ljunggren and Karre, 1990). Therefore, aberrant cells (deficient in or lacking MHC) do not result in such inhibitory signals, so signaling through activation receptors result in destruction of target cells **(Figure 1A)**. In this way, MCMV can downregulate MHC I expression by various means to avoid CD8 T cell recognition (del Val et al., 1992). For example, m04, m152

and m06 MCMV-encoded genes have been shown to downmodulate MHC I expression (Holtappels et al., 2006).

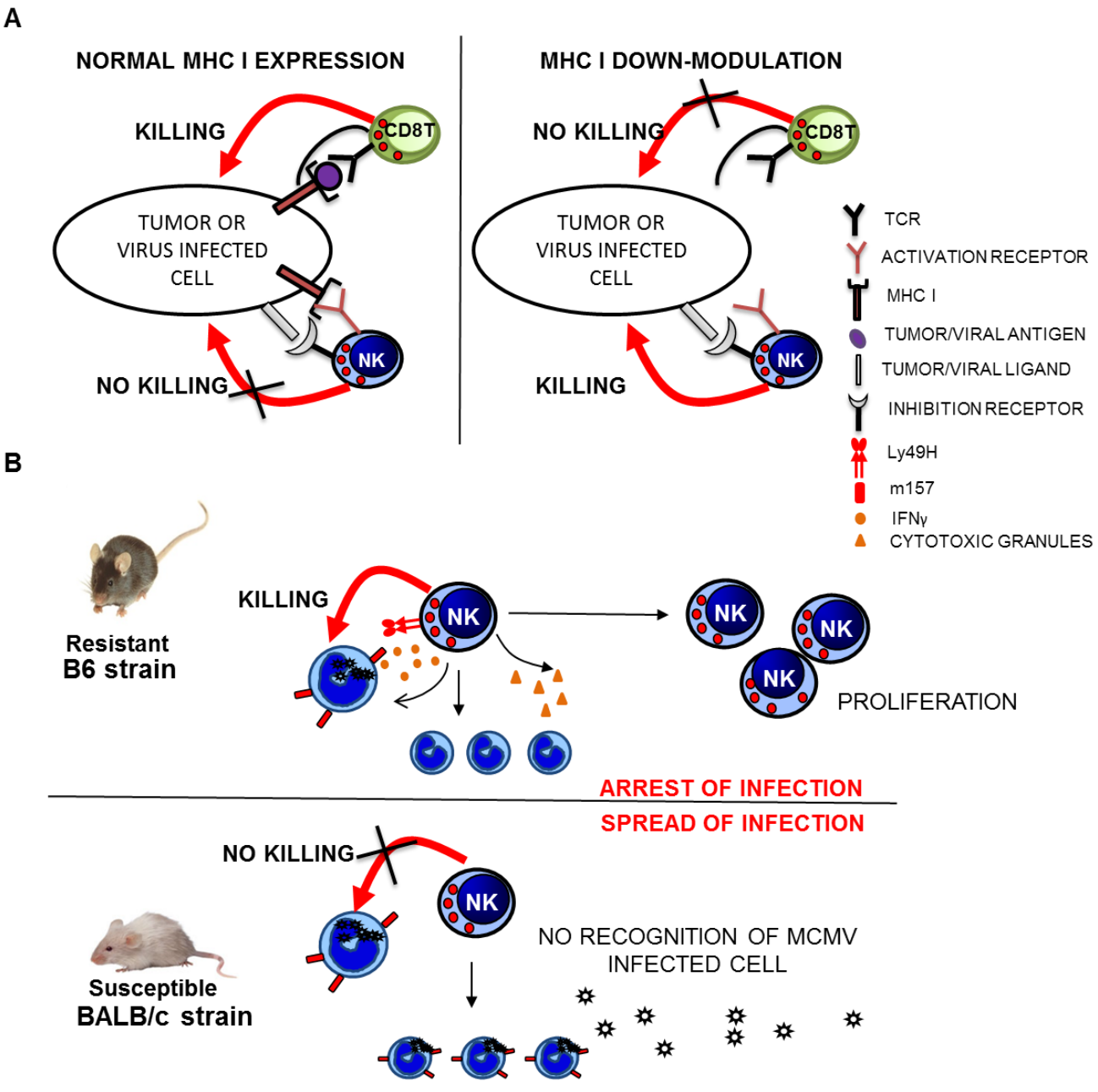
In mice, self-tolerance is achieved through critical signals received by NK cells from interactions between inhibitory Ly49 receptors and self-MHC class I (Thielens et al., 2012). The main function of inhibitory receptors is to regulate the activation of NK cells. While some inhibitory receptors are specific for MHC class I, others recognize ligands on cell surfaces such as tumor or viral specific proteins. Inhibitory receptors belong to two main families- type I glycoproteins like Killer and Immunoglobulin Inhibitory receptors (KIR/LILR) of the Immunoglobulins superfamily and lectin type CD94/NKG2A and Ly49 (-A, -C, -G, -I) families. Inhibitory receptors have a common signaling pathway and contain the immunoglobulin tyrosine based motif (ITIM). Engagement of inhibitory receptors lead to intracellular recruitment of SHP-1 and SHIP families of tyrosine phosphatases which dephosphorylate signals in the vicinity of activation receptors thereby dampening the proliferation, Ca2+ flux mediated de-granulation, and cytokine production of NK cells (Lanier, 2008).

As discussed previously, lack of inhibitory signal leads to NK cell lytic responses, but this also requires the presence of appropriate ligands on target cells for NK cell activation receptors. One of the best examples of recognition of the pathogen-derived ligands by activation receptors on NK cells is Ly49H recognition of MCMV infected cells. MCMV infection induces m157 expression on the surface of infected cells and this is specifically recognized by Ly49H activation receptor present on surface of NK cells (Arase et al., 2002; Dokun et al., 2001; Smith et al., 2002). In immunocompetent mice, roughly 50% of

NK cells express Ly49H and these cells are preferentially expanded for control of MCMV infection. On the other hand, BALB/C mice strains lack Ly49H expression and are susceptible to MCMV (Brown et al., 2001; Forbes et al., 1997; Lee et al., 2001) **(Figure 1B)**. Ly49H activation in response to MCMV infection is apparent at day 2 p.i. and is sustained during the initial stages of infection up until day 6 in the infected organs (Biron et al., 1999; Dokun et al., 2001). Recognition of ligands on virus-infected cells is not restricted to MCMV infection alone, NKp46 and NKp44 activation receptors are also capable of binding *in vitro* to haemagglutinin (HA) envelope proteins of influenza viruses (Arnon et al., 2001; Bar-On et al., 2014).

Figure 1. Role of NK cells in Innate Immunity

(A) Diagram depicts the missing-self hypothesis of NK cells for recognizing tumor or virus-infected cells. Balance of activation and inhibitory receptors on NK cells determines target cell fate. (B) Diagram depicts the specific recognition of m157 viral ligand on infected cells by Ly49H activation receptor on NK cells and consequent killing of target cells and control of MCMV replication.


A
NORMAL MHC I EXPRESSION
KILLING
CD8T
TUMOR OR VIRUS INFECTED CELL
NK
NO KILLING
MHC I DOWN-MODULATION
NO KILLING
CD8T
TUMOR OR VIRUS INFECTED CELL
NK
KILLING
TCR
ACTIVATION RECEPTOR
MHC I
TUMOR/VIRAL ANTIGEN
TUMOR/VIRAL LIGAND
INHIBITION RECEPTOR
Ly49H
m157
IFNγ
CYTOTOXIC GRANULES
B
Resistant
B6 strain
KILLING
NK
NK
NK
NK
PROLIFERATION
ARREST OF INFECTION
SPREAD OF INFECTION
NO KILLING
NK
NO RECOGNITION OF MCMV INFECTED CELL
Susceptible
BALB/c strain

T cells and B cells express a single dominant surface receptor that is responsible for their activation but NK cells possess a myriad of activation receptors. This complex of activation receptors are associated with several co-stimulatory transmembrane molecules like FcεRI-γ, CD3-ζ, DAP10 or DAP12, but they all contain a common immunoglobulin tyrosine based motif (ITAM) that mediate the downstream activation signals. Most activation receptors have shared downstream signaling events and result in recruitment of Src family of Protein Tyrosine Kinases (PTKs) including Lyn, Fyn and Lck and activation of SyK/ZAP70 molecules. Some other common downstream signaling molecules include phospholipase C-γ (PLC-γ), phosphatidylinositol 3-kinase (PI3K), extracellular signal-regulated kinase (ERKs) and Vav family of guanine nucleotide exchange factors.

Some examples of activation receptors that mediate their signaling through adaptors containing ITAM are discussed below. CD16 or FcγRIII is a low affinity receptor for IgG present on both human and mouse NK cells and triggers antibody-dependent cell cytotoxicity (ADCC). Immunoglobulins bound to antigen on target cells elicit cross-linking of CD16 receptors and mediate ADCC and IFN-γ production thereby destroying target cells. Natural cytotoxicity receptors (NCR) namely NKp46, NKp44, and NKp30 activate natural cytotoxicity of NK cells upon contact with tumor or infected cells. NKp46 is shared between mice and humans while Nkp30 is specific to humans (Colucci et al., 2002). Some other examples of activation receptors are KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5 and KIR3DS1 in humans which bind MHC I molecules, Ly49D and Ly49H belonging to the Ly49 gene family in mice.

NKG2D is a unique activation receptor that can override signals from inhibitory receptors. NKG2D can bind to certain tumor ligands like retinoic acid early transcript -1 (RAE-1) and histocompatibility antigen H-60 in mice. It can also recognize MCMV infected cells though interaction with the murine UL-16 binding protein like transcript -1 (MULT-1). In humans NKG2D can bind MHC class I chain-related proteins A and B (MICA/B) and recognizes HCMV encoded-UL16 binding proteins. These ligands are up-regulated on stressed, transformed or infected cells indicating the important role played by NKG2D in control of tumor and virus-infected cells (Raulet et al., 2001). Overexpressing these ligands leads to tumor rejection *in vivo* (Cerwenka et al., 2001; Diefenbach et al., 2001) and expression of these ligands in patients co-related with T cell infiltration in solid tumors (Vetter et al., 2002). NKG2D activation receptors can associate with both DAP12 and DAP10 adaptor molecules (Diefenbach et al., 2002). DAP10 signaling does not require Syk family of kinases unlike ITAM associated receptors; instead, they have a unique YINM motif which upon phosphorylation recruits the p85 subunit of phoshpoinositide-3-kinase (PI3K) and Grb2–Vav1. (Lanier, 2008). In conclusion, different specificities of activating and inhibitory receptors endows NK cells with multiple mechanisms to distinguish between self-cells and stressed cells.

1.2.2. NATURAL KILLER CELL RESPONSE TO CYTOKINES

Besides activation receptors, NK cells possess cytokine receptors that enable them to respond to a myriad of cytokines namely type I interferons and interleukins such as IL-12, IL-2, IL-18, IL-2, IL-15 etc. Innate cytokines like type I interferons and IL-12 are produced early in many viral infections by innate immune cells as a part of the

inflammatory process. It was initially discovered that type I interferons (IFN α/β) could potentiate NK cell cytotoxicity (Trinchieri, 1989) in addition to promoting proliferation indirectly by inducing IL-15 expression (Biron et al., 1999; Nguyen et al., 2002). With respect to MCMV infections, survival and lytic activity of NK cells was regulated by production of IL-15 in IFN α/β-STAT1 dependent manner, while production of pro-inflammatory cytokine IFN-γ was controlled by IL-12 (Biron et al., 1999; Nguyen et al., 2002). IL-12 stimulates IFN-γ production in STAT4 dependent manner (Kaplan et al., 1996). NK cell derived IFN-γ is observed during various viral infections, like, MCMV (Orange and Biron, 1996) and influenza virus (Monteiro et al., 1998). NK cell derived IFN-γ influences macrophages to secrete IL-12, IFN-α and IL-18 further regulating NK cell activity; suggesting a positive feedback and cross-talk between macrophages and NK cells (Siren et al., 2004). In fact, IL-12 and IL-18 deficient mice had defects in NK activity (Takeda et al., 1998). In cultures, human $CD56^{bright}$ NK cells produced negligible IFN-γ with IL-12, IL-18 or IL-15 alone. But IL-12 and IL-18 co-stimulation produced significantly more IFN-γ and this synergy in IFN-γ production is also observed with IL-15 and IL-12 co-stimulation (Lauwerys et al., 2000). This provides an example of how NK cells can respond to several cytokines produced in the inflammatory milieu and the combination of cytokines elicits maximal effector responses in NK cells.

Studies have shown that IL-21 is important for terminal differentiation and activation of NK cells. IL-21 treatment induces maturation of NK cells, increase in perforin mediated cytolytic activity and up-regulates expression of activation markers both *in vivo* and *in vitro* (Brady et al., 2004). IL-21 also induced production of pro-inflammatory IFN-γ and anti-inflammatory IL-10 in NK cells. Interesting developments have been made recently

with regards to using IL-21 as co-stimulus for NK cell expansion *ex vivo*. Given the therapeutic use of NK cells, both IL-2 and IL-15 treatment are used to expand NK cells *ex vivo*, but this is limited to few generations as they undergo senescence due to telomere shortening. This was shown to be rescued by IL-21 treatment which increases telomerase expression in pSTAT3 dependent manner in NK cells (Denman et al., 2012).

As mentioned above, IL-2 is routinely used to expand mice NK cells and generate lymphokine activated killers (LAK) cells and administration of this cytokine to humans results in expansion of NK cells (Caligiuri et al., 1993; Shiloni et al., 1987). But perhaps, the most potent among the cytokines in inducing NK cell activation is IL-15. IL-15 is a pleiotropic cytokine secreted by several cells like DCs, macrophages, fibroblasts and keratinocytes (Fehniger and Caligiuri, 2001; Grabstein et al., 1994). It was initially discovered as a T-cell growth factor and belongs to the four α- helix superfamily along with IL-4, IL-7, GM-CSF and IL-2. IL-2 and IL-15 possess structural and functional similarities (Ma et al., 2006). IL-15 receptor (IL-15R) is heterotrimeric consisting of a unique α chain, a shared β subunit with IL-2 and a common γ subunit with several cytokines. IL-2 and IL-15 have similar roles *in vivo* such as activation and proliferation of immune cells and generation of T-cell dependent antibodies which were attributed to the common receptor subunits (Carson et al., 1994; Ma et al., 2006).

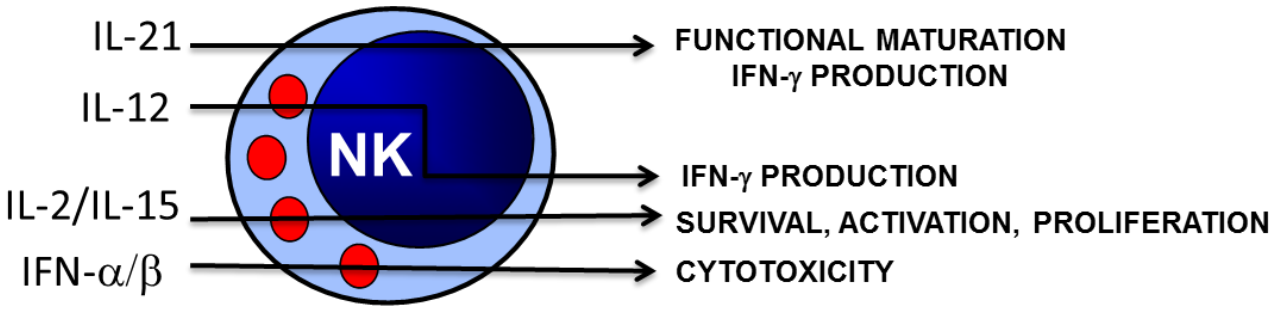
Notably, IL-15 is required for survival and homeostasis of CD8-T, NK and NK-T cells as demonstrated by IL-15-/- and IL-15Rα-/- knockout studies in mice which possess severe defects in these populations (Fehniger and Caligiuri, 2001; Lodolce et al., 1998; Ma et al., 2006). On the other hand, humans with rare genetic deficiencies in and knockout mice

of IL-2 and IL-2Rα have intact NK cell numbers and activity further indicating the specific role of IL-15 signaling for NK cell survival (DiSanto et al., 1994; Kundig et al., 1993). Importantly, IL-15 and IL-15Rα subunits are present in several non-lymphocyte populations further hinting at the importance of IL-15 in regulating innate immune responses (Fehniger and Caligiuri, 2001). Studies have demonstrated that IL-15 can promote differentiation of CD34+ human hematopoietic progenitor cells to $CD56^{bright}$ NK cells which have large granular morphology, are cytotoxic and capable of producing inflammatory cytokines (Mrozek et al., 1996). Parallel studies in mice have shown that IL-15 or low IL-2 is required for CD122+ NK progenitor cells to differentiate to mature NK cells (Rosmaraki et al., 2001).

Even though NK cells were originally recognized as "ready to act" cells that can immediately cope with virus-infected or transformed cells (Herberman et al., 1975; Kiessling et al., 1975), NK cells derived from laboratory strains of mice housed in specific pathogen free (SPF) environments show minimal effector functions (Bryceson et al., 2006; Fehniger et al., 2007; Lucas et al., 2007). Unlike human NK cells, naïve mouse NK cells are devoid of perforin and granzyme B cytotoxic granules and require additional stimulations by IL-2 or IL-15 to induce rapid translation of their pre-existing mRNAs in order to be fully equipped for action (Fehniger et al., 2007). Interestingly, their data also shows that IL-15 among other cytokines was the most potent activator for synthesis of cytotoxic granules. Thus murine NK cells require short term IL-15 exposure to become large granular activated cells. Taken together, production of the cytokines vary depending on the context and infection in question and regulates NK cell functions like cytokine

production, proliferation and/or cytotoxicity at different stages of inflammation **(Figure 2)**.

Figure 2. NK cell responses to various cytokines
NK cells possess several cytokine receptors in addition to activation and inhibition receptors. Diagram summarizes the effect of different innate and adaptive immune cytokines on NK cells functions.


IL-21
IL-12
IL-2/IL-15
IFN-α/β
NK
FUNCTIONAL MATURATION
IFN-γ PRODUCTION
IFN-γ PRODUCTION
SURVIVAL, ACTIVATION, PROLIFERATION
CYTOTOXICITY

1.3. ROLE OF DENDRITIC CELLS IN ACTIVATION AND PRIMING OF NATURAL KILLER CELLS

DCs are messengers that link the adaptive and innate arms of the immune system and were first described in 1973 (Steinman and Cohn, 1973). These so called classical DCs (cDCs) are present in several non-lymphoid organs and can respond to environmental and pathogen cues, migrate towards T cell rich areas where they process and present antigens as peptide-MHC complexes to activate T cells. DCs are also important for formation of T cell dependent antibodies and for initiating transplantation rejection reactions (Steinman, 1991). Another population distinct from cDCs were called plasmacytoid DCs capable of producing innate cytokines like IL-12 and type I interferons (IFN α/β) upon antigenic stimulation (Steinman and Hemmi, 2006). IFN α/β produced by DCs regulate induction of adaptive immune responses thereby limiting viral replication (Dalod et al., 2003).

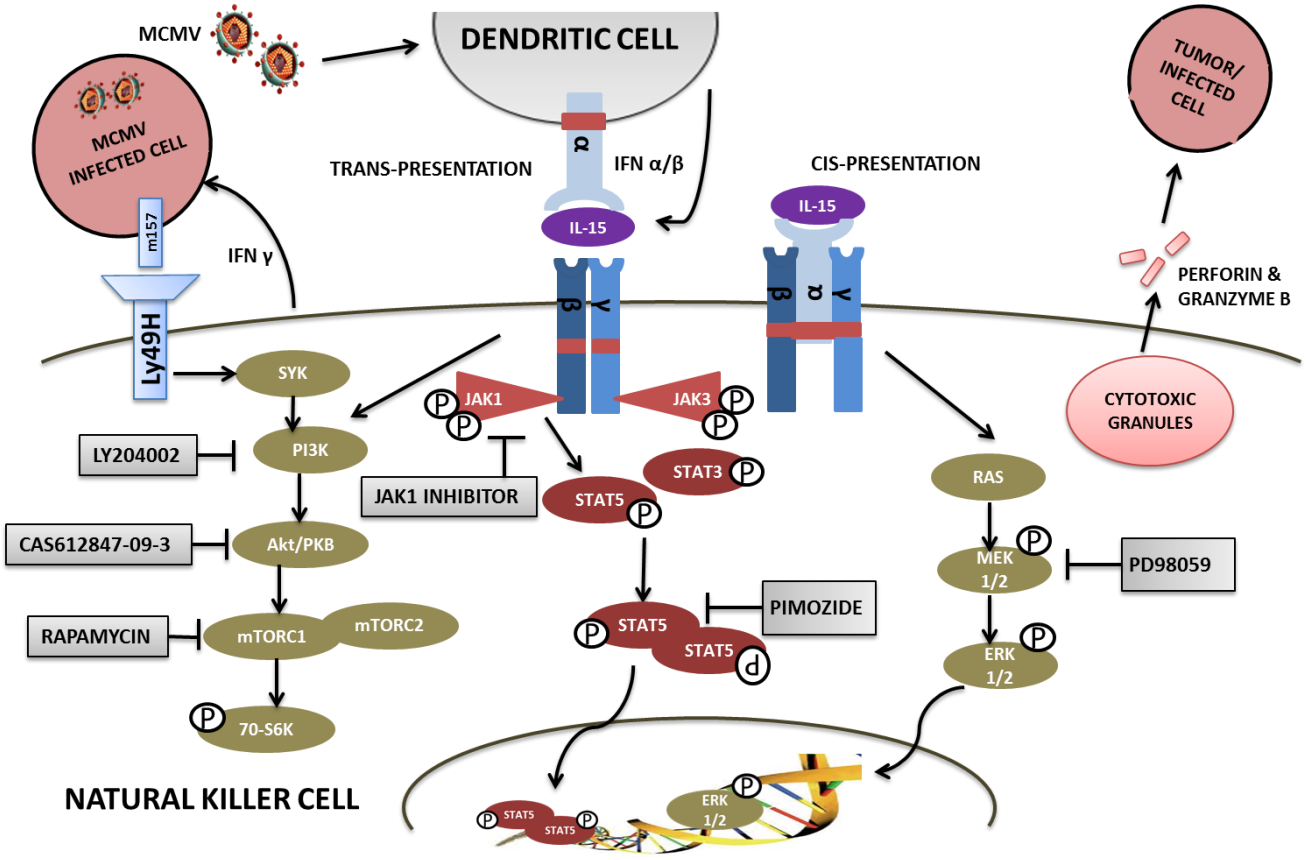
Originally NK cells were assumed to be "ready to act" cells that can kill transformed cells, but NK cells from naïve mice are minimally cytotoxic and show barely detectable cytokine production even when activated through their surface receptors. This suggets that additional cues are required for NK cells to become active (Bryceson et al., 2006). Several studies show that TLR ligands or interferon treatment in mice or IL-2 and IL-15 treatments *ex vivo* are required for activation of NK cells (Bryceson et al., 2006; Herberman et al., 1979). Additionally, it was shown that bone-marrow derived DCs can activate NK cells in cultures (Andoniou et al., 2005). Importantly, in the co-culture experiments with NK cells and DCs, it was shown that IFN-γ production by NK cells is diminished in the absence of IL-12 and that cytotoxic functions of NK cells are dependent on type 1 IFN production. Both of these innate cytokines- IL-12 and type I

interferons are produced by DCs. Interestingly, DCs also possess ligands for the NKG2D receptor which could potentially contribute to further activation of NK cells. It was also shown by another group that IL-15 which as discussed previously is an important factor for NK cell survival can be produced by DCs in co-culture experiments (Ferlazzo et al., 2004).

Several theories exist for how the NK cells and DCs cross talk; possibly through recruitment at inflammed sites (Moretta, 2002), or exposure at secondary lymphoid organs (Ferlazzo et al., 2004). Notably, it was shown that NK cells are recruited to draining lymph nodes after injecting tumor or TLR-stimulated DCs (Martin-Fontecha et al., 2004). However, recent evidences of IL-15 trans-presentation (discussed below) by dendritic cells to NK cells have conclusively proved the importance of DCs in activating naïve NK cells (Koka et al., 2004; Lucas et al., 2007). Inducible ablation of $CD11c^{high}$ DCs abrogated NK cell mediated IFN-γ production and anti-tumor cytolytic activity. These NK cells also failed to produce granzyme B cytotoxic granules and had increased pathogen loads after infection with Listeria monocytogenes (Lucas et al., 2007). The same group also demostrated that NK cell migration to the lymph nodes p.i. is a pre-requisite for priming by dendritic cells and type I interferon signaling in non-autonomous cells are required for NK cell priming. Indeed, type I interferon exposed DCs can produce IL-15 (Lucas et al., 2007), which can bind to IL-15Rα in non-autonomous fashion on presenting cells. In this way IL-15 is trans-presented to the intermediate IL15R β/γ subunits on nearby NK cells **(Figure 3)**.

Figure 3: IL-15 trans-presentation and activation of NK cells

IL-15 binds to its α subunit of IL-15 receptor with high affinity either in cis or in trans. In the latter case, IL-15 bound to its α subunit on a nearby cell is presented to the β/γ subunit on NK cells. Binding of IL-15 to its receptor subunits results in activation of at least three parallel pathways downstream of JAK signaling as indicated in the figure. The following inhibitors were used to study the importance of these signaling pathways for IL-15 induced NK cell activation; JAK Inhibitor I, STAT5 Inhibitor III Pimozide, PI3K Inhibitor LY294002, mTOR inhibitor rapamycin, AKT Inhibitor VIII AKTi-1/2, and MEK Inhibitor PD98059.


MCMV
DENDRITIC CELL
TUMOR/ INFECTED CELL
MCMV INFECTED CELL
m157
Ly49H
IFN γ
TRANS-PRESENTATION
IFN α/β
IL-15
CIS-PRESENTATION
IL-15
PERFORIN & GRANZYME B
CYTOTOXIC GRANULES
SYK
PI3K
LY204002
Akt/PKB
CAS612847-09-3
mTORC1
mTORC2
RAPAMYCIN
70-S6K
JAK1
JAK3
JAK1 INHIBITOR
STAT5
STAT3
STAT5
STAT5
PIMOZIDE
RAS
MEK 1/2
PD98059
ERK 1/2
NATURAL KILLER CELL
STAT5
STAT5
ERK 1/2

IL-15 can bind to their α subunit with high affinity either on same cell (cis) or on a neighbouring cell (trans). In the latter case, IL-15 bound to IL-15 receptor α subunit is presented to the β/γ subunits of IL-15 receptor on NK cells. Trans-presentation is a common mechanism by which IL-15 produced by neighboring cells activates and primes NK cells. As previously discussed, during inflammation NK cells are recruited to lymph nodes where they are activated by trans-presentation of IL-15 by IL-15Rα expressed on DCs (Lucas et al., 2007). A pivotal role of DC-expressed IL-15 receptor α chain (IL-15Rα) for trans-presenting IL-15 to NK cells has been demonstrated (Dubois et al., 2002; Koka et al., 2004). Studies on monocytes have revealed that IL-15/IL-15Rα complex can be recycled and trans-presented several times (Dubois et al., 2002).

Koka and colleagues showed that IL-15Rα expression on NK cells is dispensable for their activation. They also showed that IL-12 or IL-15/IL-15Rα individually only modestly produced IFN-γ, but trans-presentation of IL-15 primes NK cells and synergizes with IL-12 co-stimulation for maximal IFN-γ production. Interestingly, it has been elegantly demonstrated that IL-15 activates murine NK cells to become equipped with cytotoxic granules and sensitize them to secondary stimuli. This "priming" has been previously demonstrated with respect to IL-12 and IL-15 co-stimulation, which induces an exaggerated IFN-γ response in NK cells (Carson et al., 1994; Fehniger et al., 1999; Lucas et al., 2007). Recently, it was shown that DAP10 knockout mice could not respond to IL-15 and that DAP10 could be phosphorylated by IL-15 induced Jak3 kinase (Horng et al., 2007). Interestingly, it was shown that the ability of cytotoxic T cells to efficiently kill through NKG2D activation receptor required prior exposure to IL-2 or IL-15 (Meresse et

al., 2004; Verneris et al., 2004). This suggests existence of cross-talk between IL-15 cytokine receptor and NKG2D activation receptors for priming of NK cells. "Priming" is possibly required by NK cells to enable some of their activation receptors to function effectively against target cells. However, it is largely unknown which pathways are responsible for NK cell priming or if it is achieved by a collaborative effort of multiple pathways.

1.4. IL-15 RECEPTOR SIGNALING

Despite differences in humans and mice NK cells, the intracellular signaling components are fairly similar. The signaling cascades induced by the γ_c chains of cytokine receptors are well characterized and includes at least three parallel pathways: Ras-Raf-MAPK, PI3K-AKT-mTOR, and JAK- STAT (Kovanen and Leonard, 2004; Ma et al., 2006) **(Figure 3)**

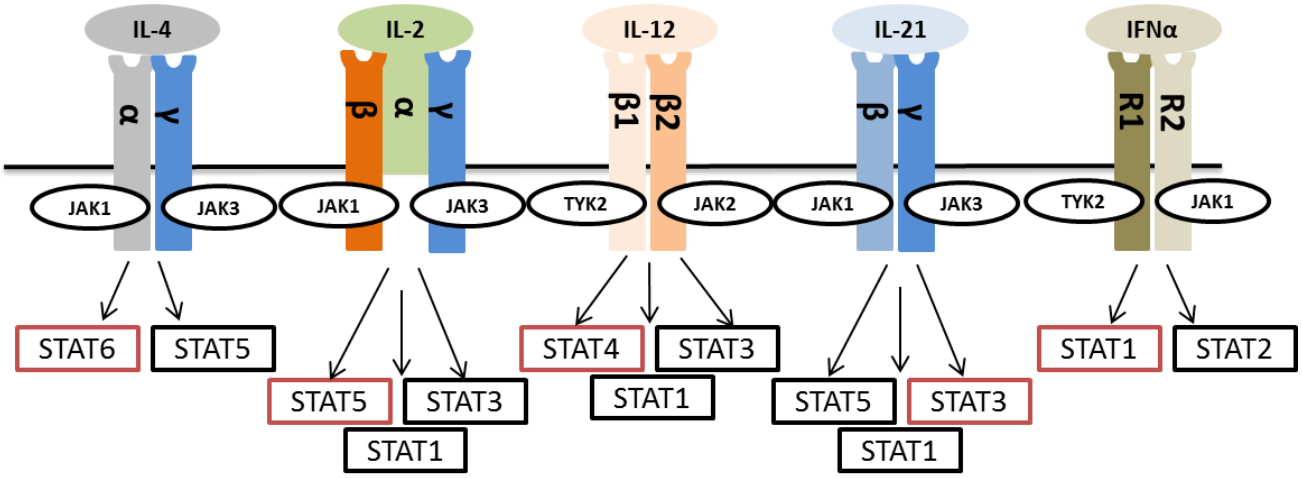
1.4.1. IL-15-JAK-STAT PATHWAY

Engagement of IL-15R causes association of the β and γ subunits on NK cells leading to auto-phosphorylation and activation of Janus Kinases (JAK1 and JAK3), which in turn activates signal transduction and activation of transcription proteins (STAT5 and STAT3). JAKs are tyrosine kinases found in association with cytokine receptors; IL-2Rβ, IL-4Rα, IL-7Rα, IL-9R, and IL-21R associate with Jak1, whereas common γ chain associates with Jak3 (Leonard and O'Shea, 1998). Mutations in Jak3 cause severe combined immunodeficiency in humans (Buckley, 2004) and Jak3 knockout mice do not have NK cells (Park et al., 1995).

Different cytokines employ different STAT proteins downstream of JAK activation. There are seven mammalian STAT proteins in total (Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6) **(Figure 4)**. They are latent cytoplasmic transcription factors until recruited by their SH2 domains to cytokine receptor docking sites for activation by JAKs. This results in homo/heterodimerization of STAT proteins and translocation to nucleus where they associate with nuclear factors to induce the transcription of cytokine responsive genes. Stat5a and Stat5b are the most commonly associated with the γ_c cytokines. Data from NK cell-specific STAT5 ablated mice showed that NK cells are absent in peripheral lymphoid organs (Eckelhart et al., 2011). Interestingly, there have also been reports of a 16year old girl with a rare mutation in STAT5B that resulted in low NK numbers combined with immunodeficiency (Bernasconi et al., 2006), suggesting a critical role of IL-15-STAT5 pathway in NK cell development.

Figure 4: Different STAT proteins are activated by different cytokine families

NK cells possess several cytokine receptors in addition to activation and inhibitory receptors. Diagram depicts how various cytokines employ different STAT molecules and these cytokines were chosen for this study. Predominant STAT protein employed by particular cytokines is indicated in red.


IL-4
IL-2
IL-12
IL-21
IFNα
α
γ
β
α
γ
β1
β2
β
γ
R1
R2
JAK1
JAK3
JAK1
JAK3
TYK2
JAK2
JAK1
JAK3
TYK2
JAK1
STAT6
STAT5
STAT5
STAT3
STAT1
STAT4
STAT3
STAT1
STAT5
STAT3
STAT1
STAT1
STAT2

1.4.2. IL-15-PI3K-AKT PATHWAY

Given the pro-survival and proliferative effects of IL-15 on NK and CD8 T cells, IL-15 can activate PI3K-AKT pathway in lymphocytes (Barata et al., 2004; Hand et al., 2010). Generally, RTKs activate PI3K which phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 interacts with pleckstrin homology (PH) domain-containing target proteins such as AKT and phosphoinositide-dependent protein kinase (PDK1). AKT phosphorylation of two negative regulators, tuberous sclerosis complex 2 (TSC2) and proline rich Akt substrate of 40 kDa (PRAS40), leads to activation of the TOR complex-mTORC1. mTORC1 activation in turn controls protein synthesis, cell growth and metabolism (Laplante and Sabatini, 2012).

PI3K and AKT have been shown to be involved in proliferation and survival (through Bcl-2 induction) in B and T cells (Ahmed et al., 1997; Nelms et al., 1999). This pathway is also implicated in T cell differentiation (at the double negative stage) and in effector CD8 T cell generation (Araki et al., 2009; Kim and Suresh, 2013; Macintyre et al., 2011). With respect to NK cell biology, PI3K has been shown to play a major role in ADCC; CD160 and NKG2D activation receptors' signaling in NK cells is also PI3K dependent (Loza and Perussia, 2001; Rabot et al., 2007). Indeed, pharmacological inhibition of PI3K blocks NKG2D mediated tumor lysis by NK cells.

Previous studies using human NK cell lines have shown that blocking PI3K pathway inhibited cytotoxic function of NK cells by inhibiting the movement of cytotoxic granules towards targets. This was shown to be regulated upstream by NK activation receptor

associated Syk/ZAP70 kinases, that in-turn leads to the phosphorylation of PI3K and to further activation of downstream Rac1-PAK1-MEK-ERK molecules (Jiang et al., 2000; Jiang et al., 2002). In another study, PI3K-AKT inhibition affected cytotoxic functions and pro-inflammatory cytokine production in human peripheral blood NK cells and this was also regulated upstream by CD160 associated Syk activation receptor (Rabot et al., 2007). Taken together, PI3K pathway is closely associated with activation and downstream signaling events of IL-15 receptor and several other receptors like CD160 and NKG2D further implicating its critical role in activating NK cell functions. But these studies have been limited to freshly isolated blood human NK cells or human NK cell lines and these are different from inbred mice models. Therefore mice models are good tools for analyzing the IL-15 receptor associated pathways and their roles in NK cell activation, whose NK cells are truly naïve without the appropriate cytokine signaling.

1.4.3. IL-15-MEK-ERK-MAPK PATHWAY

Adaptor protein Shc is known to mediate IL-2, IL-15 and IL-7 dependent growth and survival by activating PI3K-AKT pathway but it can also activate the Ras-Raf-MEK-MAPK pathway in parallel; the importance of the latter in T cell development is known. T cell receptor (TCR) activation recruits Grb-2 and Sos adaptor molecules that in turn activate MAPK-ERK through the Ras-Raf pathway (Franklin et al., 1994; Qian and Weiss, 1997). Ras inactivation did not affect NK cell cytotoxic activity and surprisingly MAPK-ERK activation was found to be Ras independent but PI3K dependent in human NK cell lines (Jiang et al., 2000; Jiang et al., 2002). These studies have shown that MEK-MAPK specifically controls the mobilization of cytotoxic granules towards target cells

and killing of target tumor cells, however proliferation of NK cells was not altered with MEK inhibition (Wei et al., 1998).

This cross talk between PI3K and MEK pathway for NK cell functions was also reported by another group which demonstrated that the production of pro-inflammatory cytokines like IFN-γ, IL-6 and TNF-α were reduced by MEK blockade in human peripheral blood NK cells. Similar to the former study, MEK was regulated upstream by the activation of PI3K pathway in NK cells (Rabot et al., 2007). Contradictory results have been obtained with respect to the type of NK sensitive-tumor cell lines used to analyze NK cell killing functions. While it was shown that ADCC and granule exocytosis were blocked by PI3K inhibitor (Wortmannin), lytic activity of NK cells towards target tumors (K562) was unaffected in the presence of PI3K inhibitor (Bonnema et al., 1994). Therefore the exact role of MEK-ERK in NK cell functions and whether it is always under the control of PI3K for NK cell functions still needs further assessment in murine NK cells.

1.5. RECENT ADVANCES IN THE mTOR PATHWAY AND DISEASES

The serine/threonine kinase mTOR is a well-studied regulator of cell growth, proliferation and metabolism. It acts downstream of the PI3K-AKT pathway and is activated by growth factors, nutrients and various other signals controlling several cellular functions (Laplante and Sabatini, 2012; Xu et al., 2012). mTOR functions through mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2); Akt activates mTORC1 which leads to the phosphorylation of several downstream targets such as ribosomal protein S6 kinase (S6K) and eukaryotic 4E binding protein (4E-BP) and promotes protein translation. This kinase activity of mTORC1 is specifically blocked by

the drug rapamycin (Mamane et al., 2006). Rapamycin is a small molecule that is derived from the bacteria *Streptomyces hygroscopicus* and binds to the FK506 binding protein (FKBP12). This drug-FKBP12 complex further binds to and inhibits the mTORC1 complex, however prolonged exposures to rapamycin can also inhibits mTORC2 complex (Abraham and Wiederrecht, 1996).

mTOR deregulation is seen in many human diseases like neurodegenerative disorders, cancers and diabetes. Many tumor cells over activate PI3K-mTOR pathway and this supports their proliferation, growth and survival. Several mTOR inhibitors are being tested in cancer therapy but their effectiveness is marred by the fact that several feedback loops are activated in cancers upon mTOR inhibition (Blagosklonny, 2011; Laplante and Sabatini, 2012; Xu et al., 2012). Active mTORC1 also down-regulates autophagy, promotes lipid biosynthesis and mitochondrial biogenesis and therefore is implicated in several neurodegenerative disorders associated with aging due to impaired mitochondrial activity and protein degradation mechanisms (Rubinsztein, 2006). Recently accumulated evidence demonstrates that mTOR pathway can regulate functions of immune cells by modulating their metabolism. In fact, loss of mTOR activity due to rapamycin treatments inhibits T cell proliferation and therefore rapamycin is used clinically as immunosuppressive agent for the prevention of allograft rejection (Halloran, 2004). Additionally, mTOR-null CD4 T cells were unable to differentiate to Th1, Th2 and Tth17 subsets due to impaired STAT phosphorylation while differentiation to Tregs was preferentially activated (Delgoffe and Powell, 2009).

While high doses of rapamycin are immunosuppressive, low dose rapamycin seem to have immunostimulatory effects. Since mTOR controls metabolism, reduced mTOR activity mimics dietary restriction and this is of great interest in attempts to improve life span and quality of life. Several animal models display increased life span due to low mTOR activity, but interesting results have been obtained with increased longevity in rapamycin-treated, genetically heterogeneous older male and female mice (Harrison et al., 2009). The most important and clinically relevant finding with respect to reduced mTOR activity is that treating rapamycin at low doses improves quality and quantity of memory CD8 T cells *in vivo* (Araki et al.). The observed increases in CD8 T memory responses in rapamycin-treated mice also co-related with enhanced survival and delayed tumor growth in mice (Li et al., 2011). B cell response to infections involves a series of steps including migration of cells, formation of germinal centers, growth/proliferation, all of which are controlled by mTOR (Blagosklonny, 2011; Laplante and Sabatini, 2012; Xu et al., 2012).

Recently, it was shown that low dose rapamycin administration during primary infection with influenza subsequently protected against secondary infections with different influenza subtypes. This "cross-protective" immunity was mediated by inhibiting class switch of B cells thereby enabling a unique repertoire of antibodies against conserved epitopes (Keating et al., 2013). In conclusion, these immune-stimulatory roles probably co-relate with the established anti-aging properties of reduced mTOR activity and increased life-span observed in rapamycin-treated animals. Given the importance of

mTOR pathway in disease and immune regulation, analyzing its role in NK cell functions during viral infections is of great interest.

1.6. SPECIFIC OBJECTIVES OF THE PROJECT:

The main interest of this project was to study the role of cytokine IL-15 in priming NK cells. Previous work on IL-15 has demonstrated its importance in the survival and maturation of NK cells (Fehniger and Caligiuri, 2001; Lodolce et al., 1998; Ma et al., 2006). It was also shown that IL-15 signals are required for murine NK cells to produce cytotoxic granule like granzyme B and proliferation (Fehniger et al., 2007). Research thus far shows that IL-15 receptor α subunit on dendritic cells is indispensable for trans-presenting IL-15 and priming NK cells (Koka et al., 2004; Lucas et al., 2007). With respect to cytokine production in NK cells, it is known that co-stimulation of IL-15 and IL-12 produces exaggerated IFN-γ production in NK cells than IL-12 stimulation alone (Carson et al., 1994; Fehniger et al., 1999; Lucas et al., 2007), suggesting IL-15 signaling primes NK cells for enhanced responses to IL-12 stimulation. These findings have laid the foundation for my thesis and have led to the following specific aims.

AIM 1: To analyze NK cell responses to various cytokines and the Ly49H activation receptor upon treatment of IL-15/IL-15Rα complex.

IL-15/IL-15Rα complex mimics trans-presentation mechanism by DCs thereby providing a good tool to study the effects of IL-15 activation on NK cell functions and to compare this with IL-15 priming *in vivo*.

1. Can “priming” of NK cell responses by IL-15 be extended to cytokines other than IL-12?

2. In addition, do IL-15 treated NK cells respond better to activation receptor stimulations?

AIM 2: To analyze the role of the three parallel pathways downstream of IL-15 receptor on NK cell activation mediated by IL-15.

This was done by blocking the pathways with reversible cell-permeable inhibitors in NK cells prior to analyzing the effects of IL-15 treatment.

1. What are the signaling mechanisms required for IL-15 mediated priming of NK cell functions?
2. Is there a major pathway involved, or is it a collaborative effort of several pathways downstream of IL-15 receptor activation?
3. How does inhibiting these pathways affect IL-15 induced effector functions of NK cells such as cytokine responses, proliferation and cytotoxicity?

AIM 3: **To analyze the role of PI3K-mTOR pathway in NK cell anti-viral functions**

Given that PI3K-AKT-mTOR pathway is required for IL-15 induced NK effector functions *in vitro*, I analyzed the importance of this pathway in IL-15 induced NK cell responses *in vivo*. IL-15 is induced early in the inflammatory milieu following acute infections and NK cells are exposed to it by way of trans-presentation by dendritic cells (Lucas et al., 2007). This process generates activated NK cells in the periphery. For this study, rapamycin was used to inhibit mTOR and MCMV was chosen as the model to analyze the importance of mTOR activity in NK cell anti-viral functions.

1. Does *in vivo* blocking of this pathway affect IL-15 priming of NK cell effector functions?
2. Are NK cells from rapamycin-treated mice capable of controlling MCMV infections in immunocompetent B6 mice, in which the requirement of NK cell anti-viral roles is well established?

2. MATERIALS AND METHODS

2.1. MICE

WT C57BL/6 and B6.SJL (C57BL/6 congenic mice with CD45.1 allotype marker) mice were purchased from Charles River and housed in specific pathogen-free environments. Mice were used for experiments at 7-12 weeks of age. All procedures were approved by and conducted in accordance with the institution's animal guidelines of the University of Ottawa.

2.2. MCMV STOCK PREPARATION

The Smith strain MCMV (ATCC® VR-1399™) was originally purchased from the American Type Culture Collection (ATCC, Rockville, MD). MCMV stocks were prepared in our laboratory as per the following procedure. Four week old BALB/c mice were infected with 5×10^4 Plaque Forming Units (PFU) of MCMV. Mice were sacrificed 21 days p.i., their salivary glands harvested and pooled in 50ml of Media 199 (Gibco). The mixture was then completely homogenized by using a Polytron homogenizer (Model: PT 1600E) followed by centrifugation at 2500rpm for 15 minutes at 4^0C. Supernatants were stored in 1.5ml volumes in liquid nitrogen. Three days later, working stocks were prepared by aliquoting the 1.5ml master stocks to 30µl volumes at -80^0C. For determining the concentration of my viral stocks, three vials from working stocks were thawed, serially diluted in chilled 2% DMEM (DMEM containing 2%FBS) and PFU's of these dilutions were determined in triplicates by standard plaque assays (described in later section).

2.3. RAPAMYCIN AND MCMV INJECTIONS

Rapamycin (Calcibiochem), an mTOR inhibitor was dissolved in DMSO to make 20mg/ml stocks. Body weights of the mice were measured and the amount of rapamycin to be injected was determined. Dilutions were made in sterile 1xPBS which was used as injection buffers. Rapamycin (3mg/kg/day) or DMSO as vehicle control was administered through intraperitoneal injections once per day until sacrificed on 1.5 or 2.5 days.

MCMV viral stocks were appropriately diluted in sterile cold MCMV injection buffer (1xMEM containing 2% FCS). WT C57BL/6 mice were injected with 5,000PFU of MCMV in 200µl of injection buffer intraperitoneally. MCMV infections were performed 4 hrs after the first rapamycin injection.

2.4. PREPARATION OF MOUSE EMBRYONIC FIBROBLASTS (MEF)

Embryos were obtained from BALB/c mice at 14-15 days of gestation and placed in sterile 80mm petridish containing Phosphate Buffer Saline (PBS). Organs such as liver, heart and blood tissues were teased apart and the remaining embryonic tissue minced to tiny pieces. This was then washed with PBS and trypsinized with 0.05% Trypsin-EDTA (Wisent Inc.) at 4^0C overnight. Next day, excess trypsin was carefully removed and the tissue containing residual trypsin was further incubated at 37^0C for 15 mins. Warm 10% DMEM (Dulbecco Modified Eagle's Medium) (DMEM, 10% FCS, 10mM HEPES, 1×Penicillin/Streptomycin, 1% L-Glutamine) was added and single cell suspension was prepared by using cell dissociation sieve tissue grinder kit (Sigma CD1-1KT). The mixture was then centrifuged at 500g for 10 mins and cells resuspended in 2ml of 10%

DMEM per embryo. About 5×10^5 cells/ml were seeded and cultured in the tissue culture plate until they reached confluency. Cells were then recovered, centrifuged and resuspended in sterile ice-chilled DMEM containing 20% FBS and 10% DMSO. Working socks were prepared at a cell concentration of 2×10^6 cells/ml and stored in liquid nitrogen.

2.5. VIRAL QUANTIFICATION ASSAY

Frozen MEF cells were thawed and cultured with 10% DMEM until they reached confluency. A day prior to performing plaque assays, monolayers of cells were prepared using 1.6×10^5cells/ml in 24 well culture plates. After incubation at 37^0C for 24 hrs in 5% CO_2 incubator, the monolayer was washed with 2% DMEM (DMEM containing 2% FBS). For measuring viral burdens in organs, spleens and livers from infected mice were isolated and weighed into centrifuge tubes containing SS grinding 5 mm balls (Fisher Scientific). They were homogenized using Magnalyser (Roche Applied Science) with the following conditions- 7000rpm for 5 s for spleen, 7000rpm for 10 s for liver. The lysates were appropriately diluted and overlaid on mouse embryonic fibroblasts cells in duplicates. The virus was allowed to adsorb to cells for 1 h at 37^0C in 5% CO_2 incubator following which they were overlaid with DMEM containing low melting (LM) agar. The media containing LM agar was prepared by dissolving 12.5 ml 2% LM in DMEM and 37.5 ml DMEM containing 13.5% FBS. After three days, cells were fixed with 10% formalin for 10 min and stained with 0.1% crystal violet (in 70% ethanol) to determine the number of plaques; plaques were represented as log PFU/organ.

2.6. REAGENTS AND ANTIBODIES

The following monoclonal antibodies were used: α-CD16/32 (clone 2.4G2) from Bioexpress, α-human/mouse Granzyme B (clone GB12) and Fixable Far Red Live/Dead from Invitrogen. α-Ly49H (clone 3D10), α-TCR-β (clone H57-597), α-NK1.1 (clone PK136), α-CD49b (clone DX5), α-CD8a (clone 53-6.7), and α-IFN-γ (clone XMG1.2) from eBiosciences, α-BrdU (clone B44), α-CD4 (clone RM4-5) and mouse Isotype IgG-κ from BD Biosciences. For detection of phosphorylated signals, BD PhosFlow antibodies against pSTAT1 (clone 49), pSTAT3 (clone 4), pSTAT4 (clone 38), pSTAT5 (clone 47) and pSTAT6 (clone 18) were used except α-pS6 ribosomal protein (Ser235/236) (clone D57.2.2E) from Cell Signaling. Cytokines, recombinant murine (rm) IL-2, rmIL-4, rmIL-12, rmIL-15/IL-15Rα complex, and rmIL-21, are from eBiosciences except rmIFN-α from Miltenyi Biotec. To physiologically mimic trans-presentation of IL-15 to NK cells by DCs *ex vivo*, we decided to use rmIL-15/IL-15Rα complex to enable IL-15 stimulation of NK cells. The following inhibitors were purchased from Calbiochem and used at the indicated concentrations; JAK Inhibitor I (0.4μM), STAT5 Inhibitor III Pimozide (10μM), PI3K Inhibitor LY294002 (4μM), mTOR inhibitor rapamycin (1.6μM *in vitro*; 3mg/kg *in vivo*), AKT Inhibitor VIII AKTi-1/2 (0.8μM), and MEK Inhibitor PD98059 (20μM).

2.7. SPLEEN LEUKOCYTE ISOLATION

The spleen was harvested under sterile conditions and transferred to 6-well plate containing 5ml RPMI-1640 media/well (HyClone). The single cell suspension was prepared by grinding spleen on 70 μm mesh using end of 3cc syringe plunger. The cells

were lysed with red blood lysis buffer, suspended in RPMI-1640 media and centrifuged at 1200 rpm for 10 min at 4^0C.

2.8. CELL STIMULATIONS

Cells were suspended in RP10 media (RPMI-1640, 10% FCS, 10mM HEPES, 1×Penicillin/Streptomycin, 1% L-Glutamine, 50μM β-Mercaptoethanol). To block signaling molecules, 1×10^6 splenic leukocytes were incubated in 96 well plates in triplicates with the inhibitors for 1 hr prior to stimulation with rmIL-15/IL-15Rα complex (10ng/ml) at 37°C and 5% CO_2. The dose of IL-15 was chosen to induce maximum proliferative response in NK cells without affecting CD8 T cells.

For evaluation of STAT activation, cells were harvested 24 hrs later, washed and rested for 4 hrs in RP10 media at 37°C and 5% CO_2 (without IL-15) to reverse the effect of cell-permeable inhibitors and remove the residual effects of IL-15 on STAT activation. This was followed by stimulation with rmIL-21 (100ng/ml), rmIL-12 (10ng/ml), rmIFN-α (1,000U/ml), rmIL-4 (100ng/ml) or rmIL-2 (50ng/ml) for 30 mins at 37°C and 5% CO_2 before harvesting and measuring pSTAT expression for flow analyses. The cytokine concentrations were determined from prior titration experiments that induced maximal experiments on NK cells without affecting T cells.

To measure IFN-γ production, IL-15/IL-15Rα complex-treated cells were added to 96 well plates pre-coated with plate bound α-Ly49H or control IgG antibody (both 10μg/ml). Alternatively, IL-15/IL-15Rα complex-treated cells were added to 96 well plates with either media alone or rmIL-12 (50ng/ml). Cells were stimulated for 5 hrs at 37°C and 5%

CO_2, last 4 hrs in the presence of 5μg/ml brefeldin A, harvested and stained for intracellular IFN-γ expression.

2.9. FLOW CYTOMETRY

For Live/Dead surface stainings, cells were recovered after 24 hrs of IL-15 treatment and surface stained for NK and T cells along with the Live/Dead viability marker to measure the extent of cell toxicity after inhibitor treatment. For detection of phosphorylated STATs, cells were blocked with 2.4G2 and surface stained for NK and T cells with monoclonal antibodies specific for NK1.1 and TCR-β. Following fixation with BD Cytofix/Cytoperm buffer and permabilization with pre-chilled 100% methanol, cells were stained for respective intracellular pSTATs, as previously reported (Miyagi et al., 2010). Similar procedure was followed for the analysis of phosphorylated S6 Kinase (pS6K) expression, which is blocked with rapamycin treatments (or mTOR inhibition).

Similarly, cells were blocked with 2.4G2, surface stained for NK and T cell proportions followed by staining for intracellular Granzyme B expression or intracellular IFN-γ measurements *in vitro* immediately after 24 hrs of IL-15 stimulation. NK cell proliferation was measured at 42 hrs (*ex vivo*) post treatment with 10, 50 or 100ng/ml of IL-15/IL-15Rα complex or 2.5 days (*in vivo*) post- MCMV infection. BrdU (200μM) was added to cultures (*ex vivo*) at 37°C or given as i.p. injections *(in vivo)* 2 hrs prior to isolating cells. Cells were blocked with 2.4G2, surface stained for NK and T cell proportions followed by intracellular staining for BrdU incorporation with anti-BrdU antibody.

For intracellular IFN-γ measurements *in vivo*, splenic leukocytes were harvested p.i. at D1.5 and incubated in media containing brefeldin A in addition to DMSO or rapamycin (1.6μM) for 4 hrs at 37°C and 5% CO_2. This was followed by staining for intracellular IFN-γ. In all conditions, freshly isolated untreated naïve total splenic leukocytes were used as controls. All intracellular procedures were carried out using BD Cytofix/Cytoperm protocols. Cells were suspended in staining buffer (1×PBS, 2% FCS and 0.09% sodium azide), acquired using FACS Cyan ADP and analyzed with Kaluza software v2 (Beckman Coulter).

2.10. CYTOKINE QUANTIFICATION ASSAYS

Blood serum and lysates of homogenized spleen and livers from mice were appropriately diluted and analyzed for the production of mouse IFN-γ using Cytometric Bead Array kit (BD Biosciences). Samples were prepared according to manufactures instructions, acquired on FACS Cyan ADP (Beckman Coulter) and analyzed using the BD FCAP Array Software (BD Biosciences. Cytokine production was presented as pg/ml for serum and pg/10^6 cells for organs.

2.11. NATURAL KILLER CELL CYTOTOXICITY ASSAY

NK cell cytotoxicity was measured by analyzing the anti-tumor activity of NK cells against YAC-1 target cells. Splenic leukocytes were isolated from infected and control mice treated with either DMSO or rapamycin. To ensure that equal numbers of NK cells were used for assays and to calculate the effector cell (NK cell) to target ratio, spleen populations were surface stained for NK and T cells to ascertain NK cell proportions and each sample adjusted to appropriate concentrations in sterile NK media (RPMI-1640,

10% FCS, 10mM HEPES, 1×Penicillin/Streptomycin, 1% L-Glutamine, 50μM β-Mercaptoethanol, 1% Non-Essential amino acids, 1mM Sodium Pyruvate) containing either DMSO or 1.6μM rapamycin. YAC-1 cells were cultured in sterile RP-10 media and labeled with 100μCi of ^{51}Cr for 1 hr at 37°C, washed three times with PBS, and made to a concentration of 5×10^4 cells/ ml in NK medium. In V-bottom 96-well plates, 100ul of YAC-1 cells were added in triplicates and incubated for 4 hrs with 100ul of effector cells with NK cell: target ratios ranging from 0.3:1 to 10:1. Supernatants were then used to quantify the amount of ^{51}Cr released due to specific lysis of YAC-1 targets by NK cells, and counted using 2470 WIZARD^2 Automatic Gamma Counter (Perkin Elmer). NK cytotoxicity was calculated according to the formula: % specific ^{51}Cr release= [(experimental release - minimum release)/ (maximum release - minimum release)] ×100.

2.12. RNA EXTRACTION AND QUANTITATIVE PCR

Total RNA was extracted from splenic leukocytes using TRIZOL reagent (GIBCO BRL) according to manufacturer's instructions. cDNA was reverse transcribed from 500ng of total RNA in a 20μl reaction using First strand cDNA synthesis kit (Thermo Scientific). For quantification of β-actin and IL-15 genes by real-time PCR, one tenth volume of cDNA was added to a 15μl reaction of FastStart Universal Probe Master Kit (Roche) and amplified using ViiA 7 Dx Real-Time PCR Instrument (Applied Biosystems). Expression of IL-15 in all the samples was normalized to β-actin levels. The primer sequences for

β-actin forward: 5'CCAACCGTGAAAAGATGAC3'

reverse: 5'GTACGACCAGAGGCATACAG3',

IL-15 forward: 5'ACATCCATCTCGTGCTACTTGT3'

reverse: 5'GCCTCTGTTTTAGGGAGACCT3' (Zhou et al., 2006).

2.13. STATISTICS

Significance of results was determined by two-tailed unpaired student *t* tests (*$*p \leq 0.05$;**$p \leq 0.01$; *** $p \leq 0.001$*) and graphed using Graph Pad Prism 5 software.

3. RESULTS

This section is divided into three parts corresponding to the three specific aims of my project as outlined previously. The first section demonstrates how IL-15 treatments sensitize NK cells for enhanced responses to several cytokines in addition to the Ly49H activation receptor. The second section deals with analyzing which pathway is responsible for this broad range "priming" of NK cells by IL-15 to various receptors. The main finding was that PI3K-AKT-mTOR pathway is required for IL-15 induced effector functions and priming *in vitro*. Finally the last section concludes with the importance of mTOR activity for infection-induced NK anti-viral responses is demonstrated *in vivo*.

3.1. IL-15 PRIMES NATURAL KILLER CELLS TO A MYRIAD OF CYTOKINE RECEPTORS AND Ly49H ACTIVATION RECEPTOR

In addition to inhibitory and activating receptors, NK cells express receptors for various cytokines. Because the priming effect has only been shown with respect to enhanced responsiveness to IL-12 when co-stimulated with IL-2 or IL-15 (Carson et al., 1994; Fehniger et al., 1999; Grant et al., 2008; Lauwerys et al., 2000; Lucas et al., 2007; Wang et al., 2000), we hypothesized that IL-15 "priming" of NK cell responses can be extended to a broader range of cytokines that transmit their signal by employing the JAK-STAT pathway.

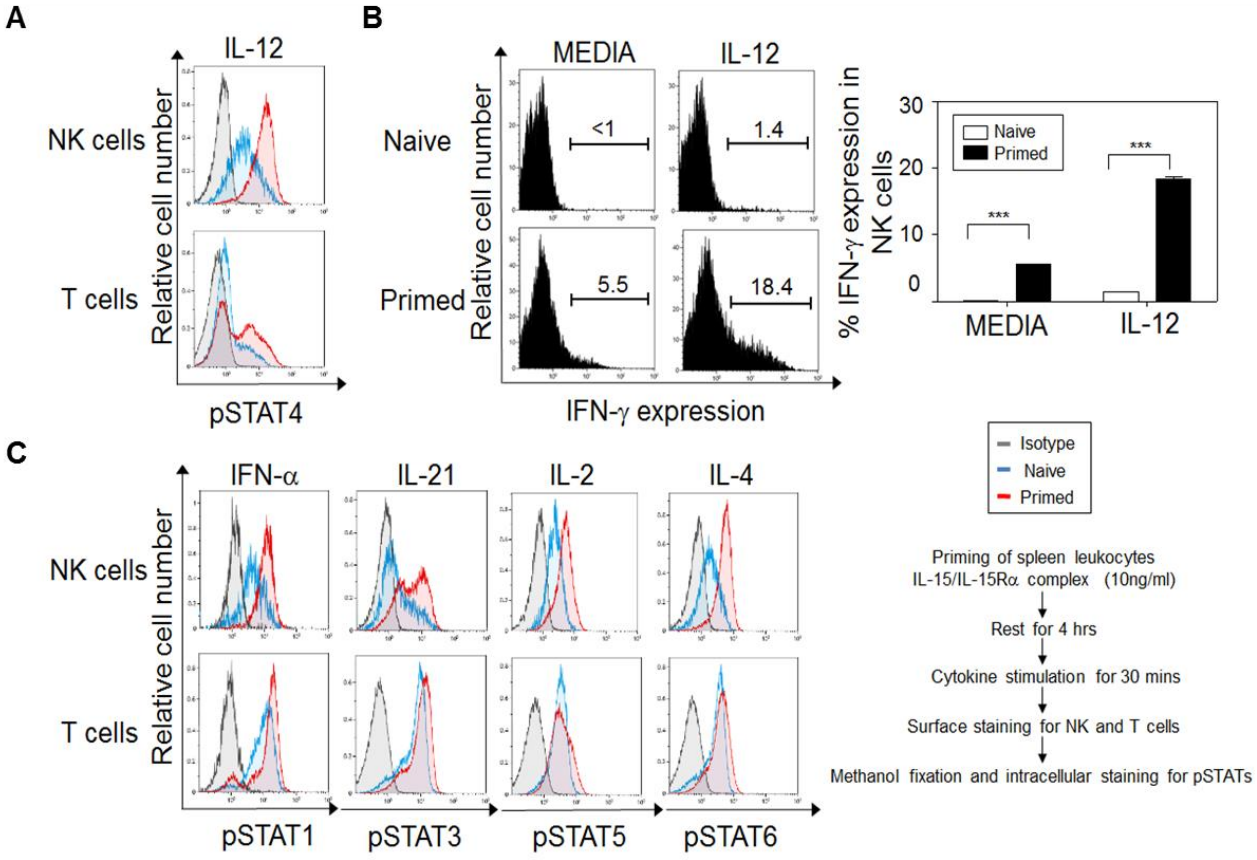
Initially, I wanted to confirm the effects of NK cell priming with respect to IFN-γ production in our system. Therefore, I analyzed the sequential responses to IL-12 stimulation (rather than co-stimulation with IL-15 and IL-12) in NK cells that have been

previously exposed to IL-15. IFN-γ production in NK cells is regulated by IL-12 in a STAT4-dependent manner (Kaplan et al., 1996; Nguyen et al., 2002). Indeed, our data indicates that NK cells pre-stimulated with IL-15 display enhanced phosphorylation of STAT4 and consequently increased IFN-γ production upon IL-12 stimulation compared to naïve cells (**Figure 5A, B**).

Signal transduction following cytokine stimulation involves multiple pathways, making it difficult to determine whether functional consequences result from direct or indirect effects. Therefore, to measure direct responses to cytokine stimulation, we decided to evaluate the level of phosphorylated STAT (pSTATs), an activation event occurring proximal to cytokine receptors. To test whether IL-15 can prime NK cells for an extensive list of cytokines in addition to IL-12, we have stimulated naïve and IL-15/IL-15Rα complex-primed spleen leukocytes with five representative cytokines (i.e. Type I IFN, IL-21, IL-12, IL-2, and IL-4), which transmit their signaling through STAT1, STAT3 STAT4, STAT5 and STAT6 molecules respectively **(Figure 4)** (Leonard and O'Shea, 1998). Notably, our results demonstrated that in addition to previously described enhanced effects upon IL-12 stimulation, IL-15 can prime NK cells to stimulations with all cytokines tested based on enhanced phosphorylated STAT expression (**Figure 5C**).

Figure 5. IL-15 primes NK cells through a broad range of cytokine receptors

(A) Naïve and IL-15 primed splenic leukocytes (for 24 hrs) were stimulated with IL-12 for 30 mins followed by intracellular staining for phosphorylated STAT4 protein. Histograms depict the expression of phosphorylated STAT4 protein in NK and T cells. Naïve cells are depicted in blue, IL-15 treated cells in red and isotype antibody (negative controls for intracellular staining) in grey. (B) Naïve and IL-15 primed splenic leukocytes were stimulated with media or IL-12 for 5 hrs; Brefeldin A was added during the last 4 hrs. Cells were then stained for intracellular IFN-γ. Histograms depict IFN-γ expression upon IL-12 stimulation in NK cells and summarized in graphs where each bar indicates an average of duplicate samples. (C) Naïve and IL-15 primed splenic leukocytes (for 24 hrs) were stimulated with the indicated cytokines. After 30 mins, cells were stained for intracellular expression of phosphorylated STAT proteins. Histograms depict the phosphorylation of respective STAT proteins in NK and T cells. NK cells were defined as NK1.1+TCRβ- populations and T cells as NK1.1-TCRβ+ populations. Figures are representative of three independent experiments. Numbers on histograms indicate proportions of IFN-γ expressing cells. Significance of results was determined between naïve and primed cells. *$**p \leq 0.01$;*** $p \leq 0.001$*

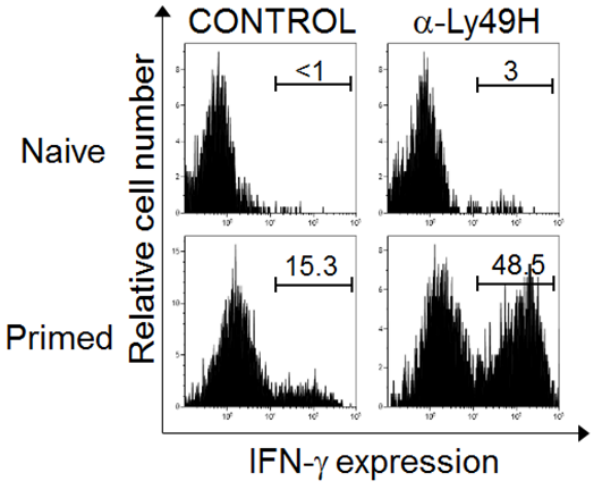

A
IL-12
NK cells
T cells
Relative cell number
pSTAT4
B
MEDIA
IL-12
Naive
Primed
<1
1.4
5.5
18.4
Relative cell number
IFN-γ expression
% IFN-γ expression in NK cells
30
20
10
0
Naive
Primed
***
***
MEDIA
IL-12
C
IFN-α
IL-21
IL-2
IL-4
NK cells
T cells
Relative cell number
pSTAT1
pSTAT3
pSTAT5
pSTAT6
Isotype
Naive
Primed
Priming of spleen leukocytes
IL-15/IL-15Rα complex (10ng/ml)
Rest for 4 hrs
Cytokine stimulation for 30 mins
Surface staining for NK and T cells
Methanol fixation and intracellular staining for pSTATs

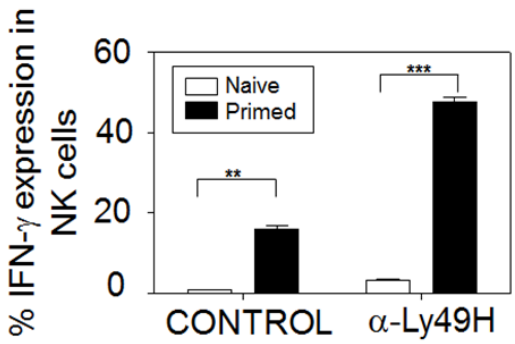
We next analyzed whether this increased responsiveness in primed NK cells can be extended to stimulation through activation receptors. It is known that stimulation through both IL-12-STAT4 and Ly49H-Syk/ZAP70 receptors induces IFN-γ production in activated NK cells (Biron et al., 1999; Lanier, 2008; Lee et al., 2007). We therefore analyzed the IFN-γ expression in primed NK cells after stimulation through Ly49H activation receptor. Our results indicate that primed NK cells display significantly elevated IFN-γ expression in NK cells when stimulated though their Ly49H receptor compared to naïve cells (**Figure 6**). Even though stimulations induced basal IFN-γ production in naïve NK cells, IL-15-primed NK cells produced dramatically enhanced levels of IFN-γ.

Taken together, priming with even low doses of IL-15/IL-15Rα complex sensitizes NK cells to stimulations through cytokine receptors and Ly49H activation receptor. IL-15 pre-exposed NK cells are more activated and produce significantly more IFN-γ than naïve cells. It is worth noting that this priming effect was predominantly observed in NK cells compared to T cells, indicating the unique effect IL-15 has on NK cell activation *in vivo*.

Figure 6. IL-15 primes NK cells through Ly49H activation receptor

Naïve and IL-15 primed splenic leukocytes were stimulated on α-Ly49H or control antibody coated plates for 5 hrs; Brefeldin A was added during the last 4 hrs. Cells were then stained for intracellular IFN-γ. Histograms depict IFN-γ expression upon Ly49H or control IgG1 stimulation in NK1.1+TCRβ- cells and summarized in graphs where each bar indicates an average of duplicate samples. Figures are representative of three independent experiments. NK cells were defined as NK1.1+TCRβ- populations. Numbers on histograms indicate proportions of IFN-γ expressing cells. Significance of results was determined between naïve and primed cells. *$**p \leq 0.01$;*** $p \leq 0.001$*


CONTROL
α-Ly49H
Naive
Primed
Relative cell number
<1
3
15.3
48.5
IFN-γ expression


% IFN-γ expression in NK cells
60
40
20
0
Naive
Primed
**
***
CONTROL
α-Ly49H

3.2. SIGNALING MECHANIMS REQUIRED FOR IL-15 PRIMING

My preliminary data shows IL-15 broadly primes NK cells to stimulations through a variety of cytokine receptors and Ly49H activation receptor. We hypothesized that this IL-15 priming of NK cell functions like cytokine responses, cytotoxicity and proliferation can be attributed to a common signaling mechanism. IL-15 binding to its receptor initiates signaling through activation of Janus kinases (JAK) 1/3, which induces at least three parallel cascades: Ras-Raf-MEK, PI3K-AKT-mTOR, and STAT5 (Kovanen and Leonard, 2004; Ma et al., 2006).

In order to identify which signaling pathway downstream of IL-15-JAK activation is predominately responsible for priming, NK cells were prepared by blocking each pathway by pretreatment with specific cell permeable inhibitors. The pathways were blocked prior to priming with IL-15/IL-15Rα complex. We used PD98059 for blocking ERK 1/2, LY294002 for blocking PI3K, and Pimozide (STAT5 inhibitor III) for blocking STAT5. In addition, AKT-1/2 inhibitor (CAS 612847-09-3) and Rapamycin (mTOR inhibitor) were used to investigate the signaling events downstream of PI3K pathway.

3.2.1. DETERMINATION OF CELL TOXICITY OF INHIBITORS

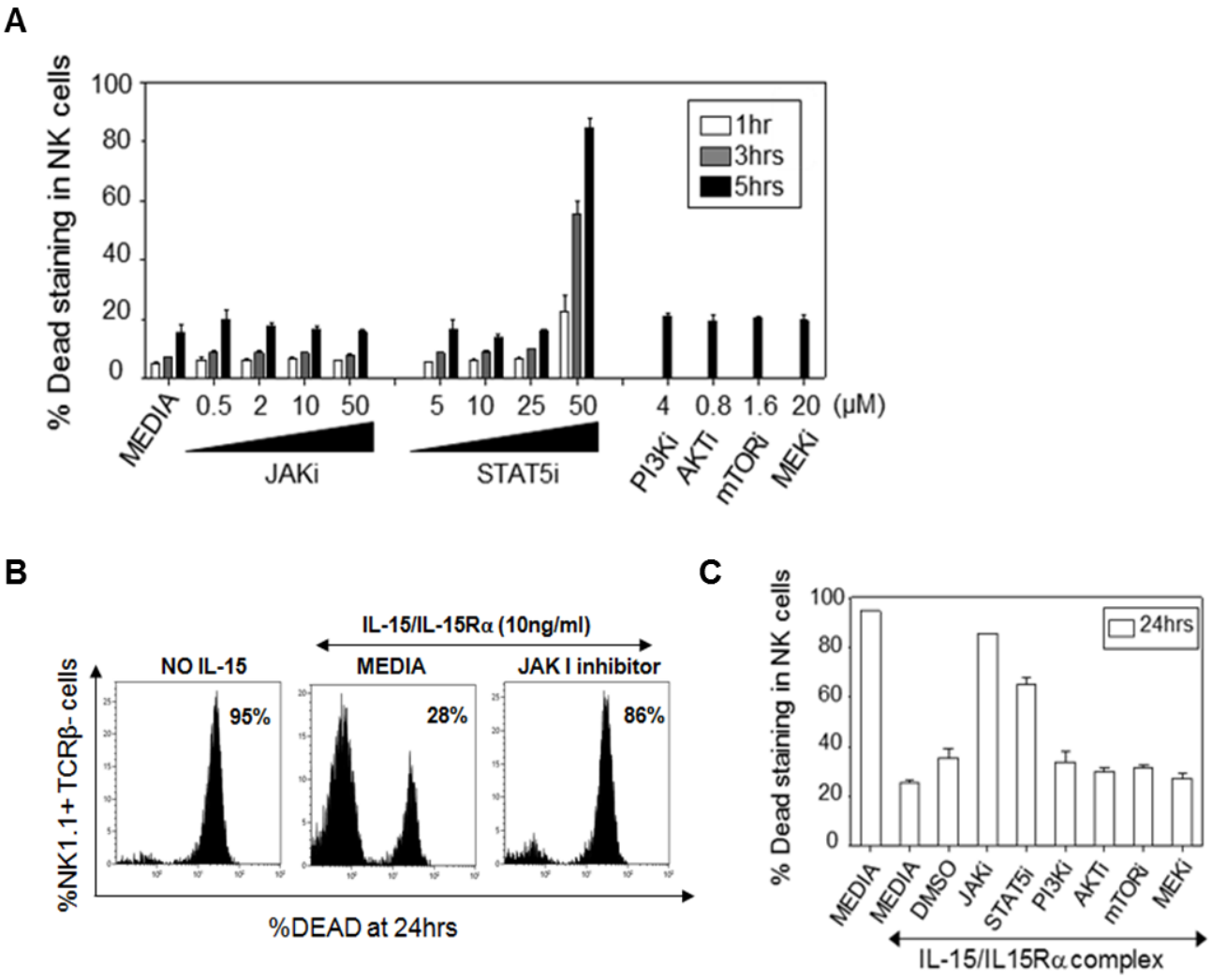
To identify the working concentrations of inhibitors that did not affect viability of cells, spleen leukocytes were treated with three different concentrations of drugs – high, low and medium in the presence of IL-15 for 24 and 48 hrs. The samples were then recovered and surface stained for NK and T cells along with a viability stain to determine the percentage of live and dead cells. Only the dose that induced NK cell viability compared to controls (IL-15 untreated cells) were chosen as working concentrations for further

experiments. Having established the working concentrations, their toxicity on naïve cells were determined. In other words, the toxicity of working concentrations of the inhibitors on naïve NK cells that were not cultured with IL-15 was analyzed within a few hours of treatment. Notably, I observed that naïve NK cell deaths in samples with working concentrations of inhibitors were comparable to controls (without any inhibitor treatment), suggesting no direct toxicity contributed by inhibitors (**Figure 7A**).

Next I wanted to re-confirm if the chosen concentrations maintained cell viability over a 24 hr culture period with IL-15. Therefore, NK cells were analyzed for cell deaths after 24 hrs of IL-15 stimulation. Cells were treated with inhibitors for 1 hr followed by IL-15/IL-15Rα complex treatment. Since IL-15 is essential for NK cell survival, cells deprived of IL-15 exhibit drastic cell deaths (**Figure 7B**). JAK1/3-STAT5 has been known for mediating pro-survival effects of IL-15 in the cell (Ma et al., 2006; Park et al., 1995; Yao et al., 2006). As expected, blocking JAK mimicked the dramatic cell death observed with cells cultured in the absence of IL-15. The results are summarized in a graph (**Figure 7C**) which indicates that blocking STAT5 pathway also resulted in dramatic cell deaths. This further confirms the requirement of IL-15 for NK cell survival and correlates with the observed NK cell deficiency in IL-15 and IL-15R-/- mice. Notably, all other inhibitors tested maintained NK cell viability compared to IL-15 treated controls (cells without any inhibitor treatment), suggesting that blocking of PI3K and MEK pathways at the indicated inhibitor concentrations did not affect NK cell survival.

Figure 7. Effect of various inhibitors on NK cell viability

(A) Naïve splenic leukocytes were incubated with indicated concentrations of JAK and STAT5 inhibitors for 1, 3 and 5 hrs, in addition to media alone or working concentrations of the other inhibitors for a total of 5 hrs. Toxicity of the inhibitors was analyzed by staining for proportions of Live/dead cells among various populations. Graph depicts percentages of NK cells that stain positive for dead staining. (B) Spleen leukocytes were incubated with working concentrations of JAK inhibitor for 1 hr, following which cells were cultured for 24 hrs in media with or without IL-15/IL-15Rα. Numbers on histograms depict percentages of NK cells that stain positive for dead staining. (C) Similarly, splenic leukocytes were incubated with working concentrations of the inhibitors or DMSO controls for 1 hr, following which cells were cultured for 24 hrs in media with or without IL-15/IL-15Rα. Graph depicts percentages of IL-15 treated NK cells that stain positive for dead staining. NK cells were defined as NK1.1+TCRβ- populations. Figures are representative of at least three independent experiments. Each bar indicates an average of duplicate samples.


A
% Dead staining in NK cells
100
80
60
40
20
0
1hr
3hrs
5hrs
MEDIA
0.5
2
10
50
5
10
25
50
4
0.8
1.6
20
(μM)
JAKi
STAT5i
PI3Ki
AKTi
mTORi
MEKi
B
IL-15/IL-15Rα (10ng/ml)
NO IL-15
MEDIA
JAK I inhibitor
95%
28%
86%
%NK1.1+ TCRβ- cells
%DEAD at 24hrs
C
% Dead staining in NK cells
100
80
60
40
20
0
24hrs
MEDIA
MEDIA
DMSO
JAKi
STAT5i
PI3Ki
AKTi
mTORi
MEKi
IL-15/IL15Rα complex

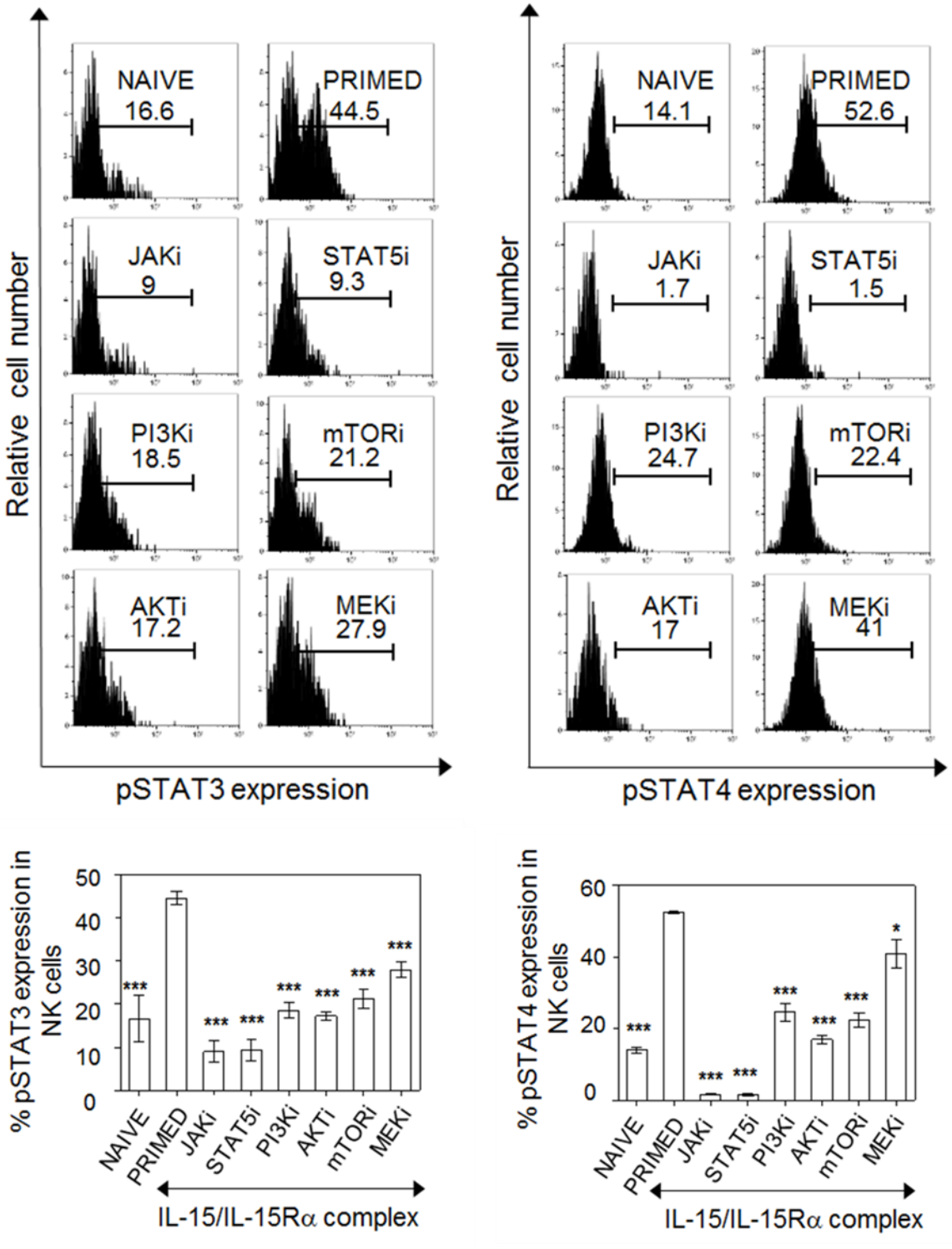
3.2.2. PI3K-mTOR PATHWAY IS REQUIRED FOR CYTOKINE RESPONSIVENESS OF IL-15 PRIMED NATURAL KILLER CELLS

Having established the optimal concentration of inhibitors, we investigated the major pathway responsible for cytokine responsiveness of IL-15 primed NK cells. IL-15 induced pathways were blocked in naïve splenic leukocytes by adding inhibitors 1hr before IL-15/IL-15α complex stimulation for a total of 24 hrs. Treated cells were washed and rested for 4 hrs to remove residual effects of IL-15 and reverse the effects of inhibitors. They were then evaluated for phosphorylated STAT3 and STAT4 upon the stimulation with IL-21 and IL-12 respectively. Notably, treatment with PI3K inhibitor abrogated the priming effect, with phosphorylated STAT levels similar to that of naïve cells. Such inhibition was also observed with blocking mTOR and AKT, downstream signaling components of PI3K pathway, suggesting that PI3K-AKT-mTOR pathway is critical for optimal responses of "primed" NK cells to IL-21 (**Figure 8A**) and IL-12 (**Figure 8B**) stimulations.

Treatment of naïve cells with the inhibitors did not show any reduction in their cytokine responsiveness compared to controls (data not shown), indicating that the pathway is specifically implicated in IL-15 primed NK cells. In addition, blocking MEK reduced phosphorylated STAT levels significantly with IL-21 and modestly with IL-12 stimulations. Therefore, IL-15 treatment enhances NK cell responsiveness to cytokine stimulations and the effects of priming is abrogated by blocking PI3K-AKT-mTOR pathway during IL-15 treatment.

Figure 8. PI3K-AKT-mTOR pathway is critical for responsiveness of primed NK cells to cytokine stimulations

Splenic leukocytes were incubated with working concentrations of the inhibitors for 1 hr; followed by culturing of cells for 24 hrs with IL-15/IL-15Rα. Naïve and inhibitor treated-primed spleen leukocytes were washed and rested for 4 hrs followed by stimulation with the indicated cytokines. After 30 mins of cytokine stimulation, cells were stained for intracellular phosphorylated STAT proteins. Histograms depict expression of phosphorylated STAT3 and STAT4 proteins in primed NK cells upon stimulation with (A) IL-21 and (B) IL-12 respectively and the effect of various inhibitors on the same. Results are summarized in graphs where each bar indicates an average of triplicate samples. Figures are representative of at least three independent experiments. Numbers on histograms indicate percentages of pSTAT expression in NK cells. NK cells were defined as NK1.1+TCRβ- populations. Significance of results was determined with IL-15 primed cells without any inhibitor treatment. *$*p \leq 0.05$;*** $p \leq 0.001$*


NAIVE
16.6
PRIMED
44.5
JAKi
9
STAT5i
9.3
PI3Ki
18.5
mTORi
21.2
AKTi
17.2
MEKi
27.9
Relative cell number
pSTAT3 expression
NAIVE
14.1
PRIMED
52.6
JAKi
1.7
STAT5i
1.5
PI3Ki
24.7
mTORi
22.4
AKTi
17
MEKi
41
Relative cell number
pSTAT4 expression
% pSTAT3 expression in NK cells
50
40
30
20
10
0
***
NAIVE
PRIMED
JAKi
STAT5i
PI3Ki
AKTi
mTORi
MEKi
IL-15/IL-15Rα complex
% pSTAT4 expression in NK cells
60
40
20
0
*
IL-15/IL-15Rα complex

3.2.3. PI3K-mTOR PATHWAY IS IMPLICATED IN IL-15 INDUCED EFFECTOR FUNCTIONS OF NATURAL KILLER CELLS

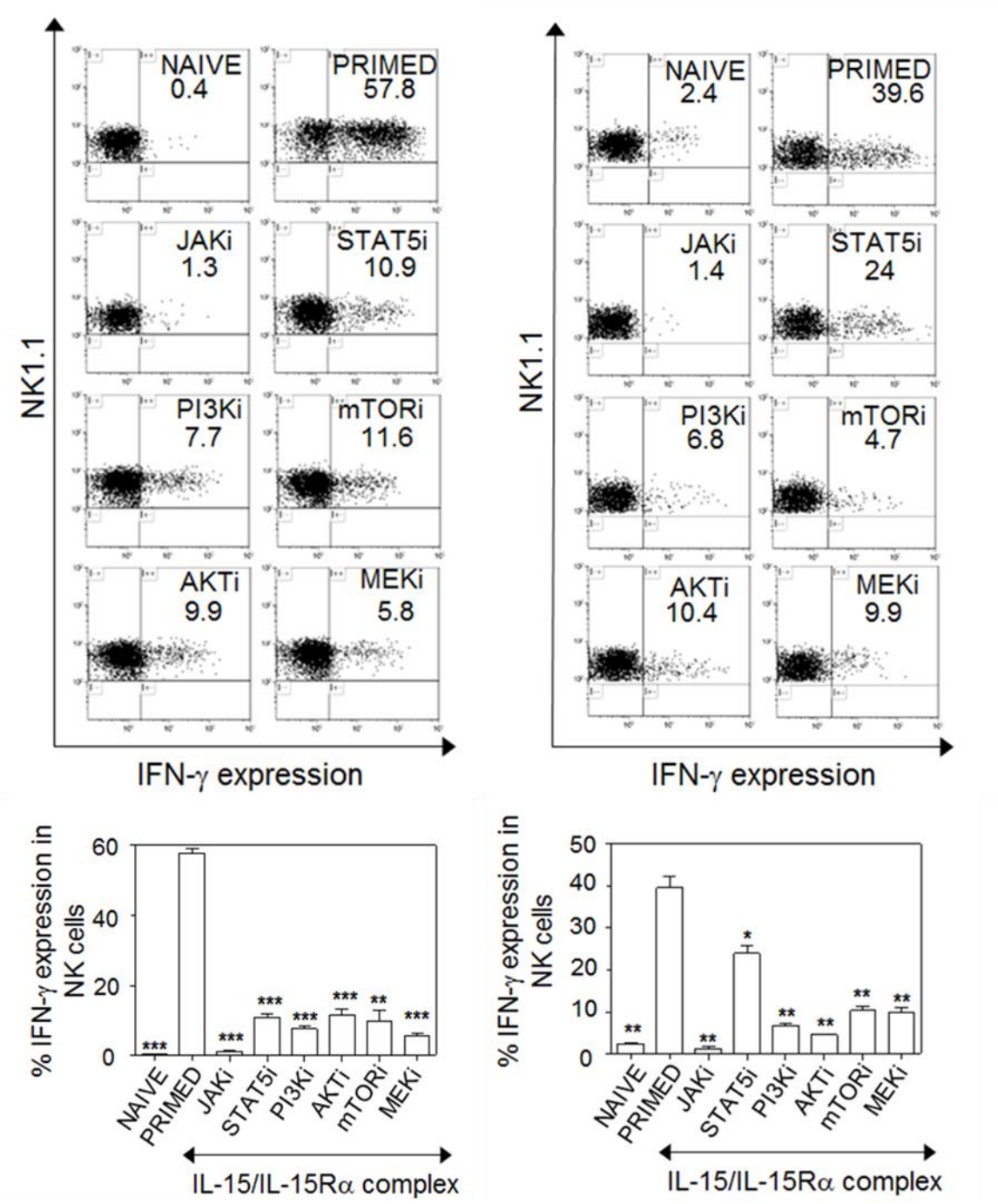
3.2.3.1. IFN-γ RESPONSE OF PRIMED NATURAL KILER CELLS

During inflammation, NK cells are exposed to IL-15 at early stages in lymph nodes and become activated as a result of recruitment to sites of inflammation in the periphery (Lucas et al., 2007). Activation of NK cells produce pro-inflammatory cytokines like IFN-γ and kill target cells via perforin/granzyme-mediated cytotoxicity. Therefore, we decided to evaluate if PI3K-AKT-mTOR pathway is also required for NK cell effector functions induced by IL-15. Consistent with previous data, IL-15 primed NK cells produced significantly more IFN-γ than naïve cells upon IL-12 (**Figure 9A**) and α-Ly49H (**Figure 9B**) stimulations. Interestingly, IFN-γ production in both stimulations was severely reduced by PI3K-AKT-mTOR inhibition.

Blocking JAK1/3 and STAT5 abolished IL-15 induced IFN-γ production upon IL-12 stimulation of NK cells, but STAT5 inhibitor did not dramatically affect IFN-γ production in NK cells with α-Ly49H stimulation. This suggests that unlike in IL-12 stimulation, blocking STAT5 may not be detrimental for primed NK cell responses to α-Ly49H stimulation. In addition, MEK inhibition in NK cells reduced IL-15 induced elevation in IFN-γ production with both cytokine and activation receptor stimulation.

Figure 9. PI3K-AKT-mTOR pathway is required for optimal IFN-γ production in primed NK cells

Splenic leukocytes were incubated with working concentrations of the inhibitors for 1 hr; followed by culturing of cells for 24 hrs with IL-15/IL-15Rα. Naïve and inhibitor treated-primed spleen leukocytes were stimulated with IL-12 or in α-Ly49H coated wells for 5 hrs; Brefeldin A was added in the last 4 hrs. Cells were then stained for intracellular expression of IFN-γ. Histograms depict IFN-γ expression in NK cells upon stimulation of (A) IL-12 receptor and (B) Ly49H receptor and the effect of various inhibitors on the same. Results are summarized in graphs where each bar indicates an average of duplicate samples. Figures are representative of two independent experiments. Numbers on histograms indicate proportions of IFN-γ expressing NK cells. NK cells were defined as NK1.1+TCRβ- populations. Significance of results on each bar was determined with primed cells without any inhibitor treatment. *** $p \leq 0.01$; **** $p \leq 0.001$*


NAIVE
0.4
PRIMED
57.8
JAKi
1.3
STAT5i
10.9
PI3Ki
7.7
mTORi
11.6
AKTi
9.9
MEKi
5.8
NK1.1
IFN-γ expression
NAIVE
2.4
PRIMED
39.6
JAKi
1.4
STAT5i
24
PI3Ki
6.8
mTORi
4.7
AKTi
10.4
MEKi
9.9
NK1.1
IFN-γ expression
% IFN-γ expression in NK cells
60
40
20
0
***
***
***
***
***
**
***
NAIVE
PRIMED
JAKi
STAT5i
PI3Ki
AKTi
mTORi
MEKi
IL-15/IL-15Rα complex
% IFN-γ expression in NK cells
50
40
30
20
10
0
**
**
*
**
**
**
**
NAIVE
PRIMED
JAKi
STAT5i
PI3Ki
AKTi
mTORi
MEKi
IL-15/IL-15Rα complex

3.2.3.2. CYTOTOXIC AND PROLIFERATIVE RESPONSES OF PRIMED NATURAL KILLER CELLS

Unlike human NK cells, NK cells from mouse housed in specific pathogen free vivarium are devoid of perforin/granzyme B expression, but can translate these cytotoxic granules from pre-existing mRNA pools upon the activation by IL-15 (Fehniger et al., 2007). As discussed previously, IL-15 treatment results in proliferation and expansion of NK cells (Biron and Welsh, 1982; Nguyen et al., 2002). IL-15 treated cells, but not naïve NK cells, expressed significantly more granzyme B and BrdU incorporation suggesting proliferation (**Figure 10**). The production of cytotoxic granules and proliferation in NK cells were dose dependent and increased with increasing concentrations of IL-15/IL-15Rα complex.

Next I analyzed the effect of inhibiting the various pathways on IL-15 induced granzyme B synthesis. Blocking PI3K-AKT-mTOR pathway drastically reduced granzyme B expression in IL-15 activated NK cells. Interestingly, MEK inhibition did not affect the ability of primed NK cells to express granzyme B **(Figure 11)**. Since the PI3K-mTOR pathway is implicated in NK cell cytotoxic granule production, the effect of this pathway in proliferation of NK cells was determined. Splenic leukocytes were stimulated with three different concentrations (10, 50 and 100 ng/ml) of IL-15/IL-15Rα complex and pulsed with BrdU 2 hrs prior to analysis for BrdU incorporation at 42hrs. Consistent with their negative effect on survival, blocking either JAK or STAT5 completely abrogated NK cell proliferation (**Figure 12**).

Figure 10. Effect of IL-15 on proliferation and cytotoxic granule production in NK cells

Splenic leukocytes were incubated with indicated concentrations of IL-15/IL-15Rα for 24 hrs. Naïve and IL-15 primed cells were harvested; surface stained for NK and T cells and stained intracellular granzyme B and BrdU incorporation. Graph depicts the percentage of NK cells expressing intracellular granzyme B or BrdU. NK cells were defined as NK1.1+TCRβ- populations. Figures are representative of at least three independent experiments.

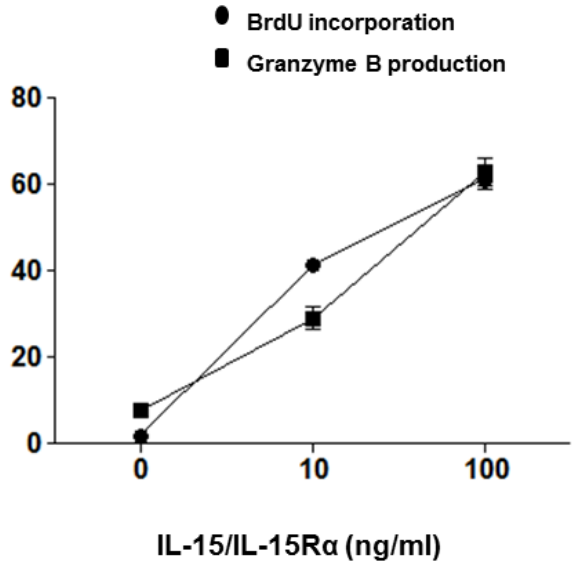
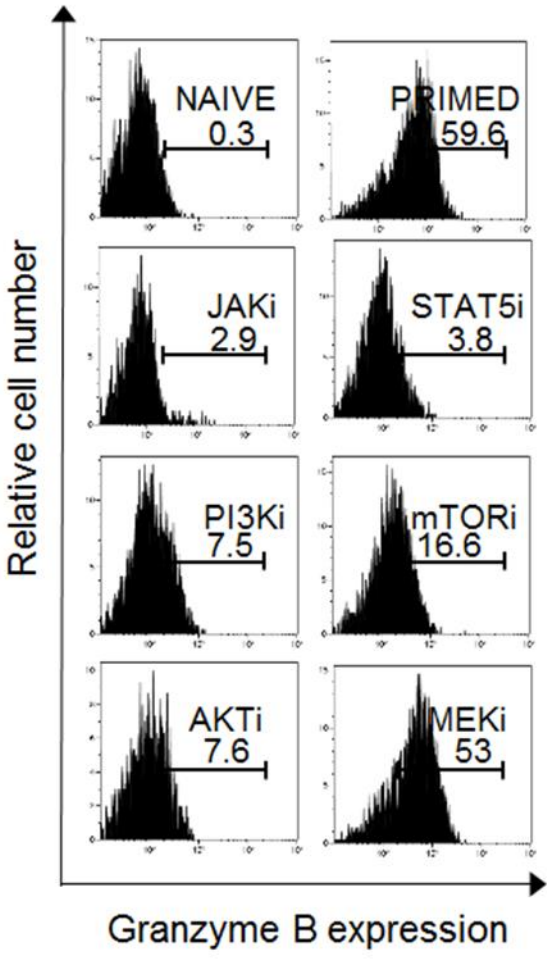

BrdU incorporation
Granzyme B production
80
60
40
20
0
0
10
100
IL-15/IL-15Rα (ng/ml)

Figure 11. PI3K-AKT-mTOR pathway is required for granzyme B production in IL-15 treated NK cells

Splenic leukocytes were incubated with working concentrations of the inhibitors for 1 hr; followed by culturing of cells for 24 hrs with IL-15/IL-15Rα. Naïve and primed cells were stained for intracellular granzyme B. Histograms depict intracellular granzyme B expression in NK cells and the effect of inhibitors on the expression. Results are summarized in graphs where each bar indicates an average of triplicate samples. Figures are representative of at least three independent experiments. Numbers on histograms indicate proportions of cells expressing granzyme B. NK cells were defined as NK1.1+TCRβ- populations. Significance of results on each bar was determined with primed cells without any inhibitor treatment. *** $p \leq 0.001$


NAIVE
0.3
PRIMED
59.6
JAKi
2.9
STAT5i
3.8
PI3Ki
7.5
mTORi
16.6
AKTi
7.6
MEKi
53
Relative cell number
Granzyme B expression

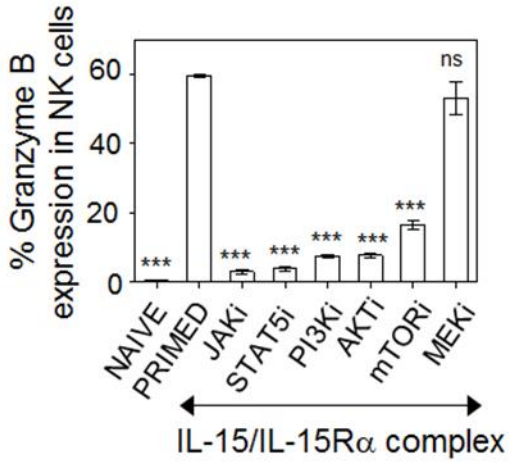
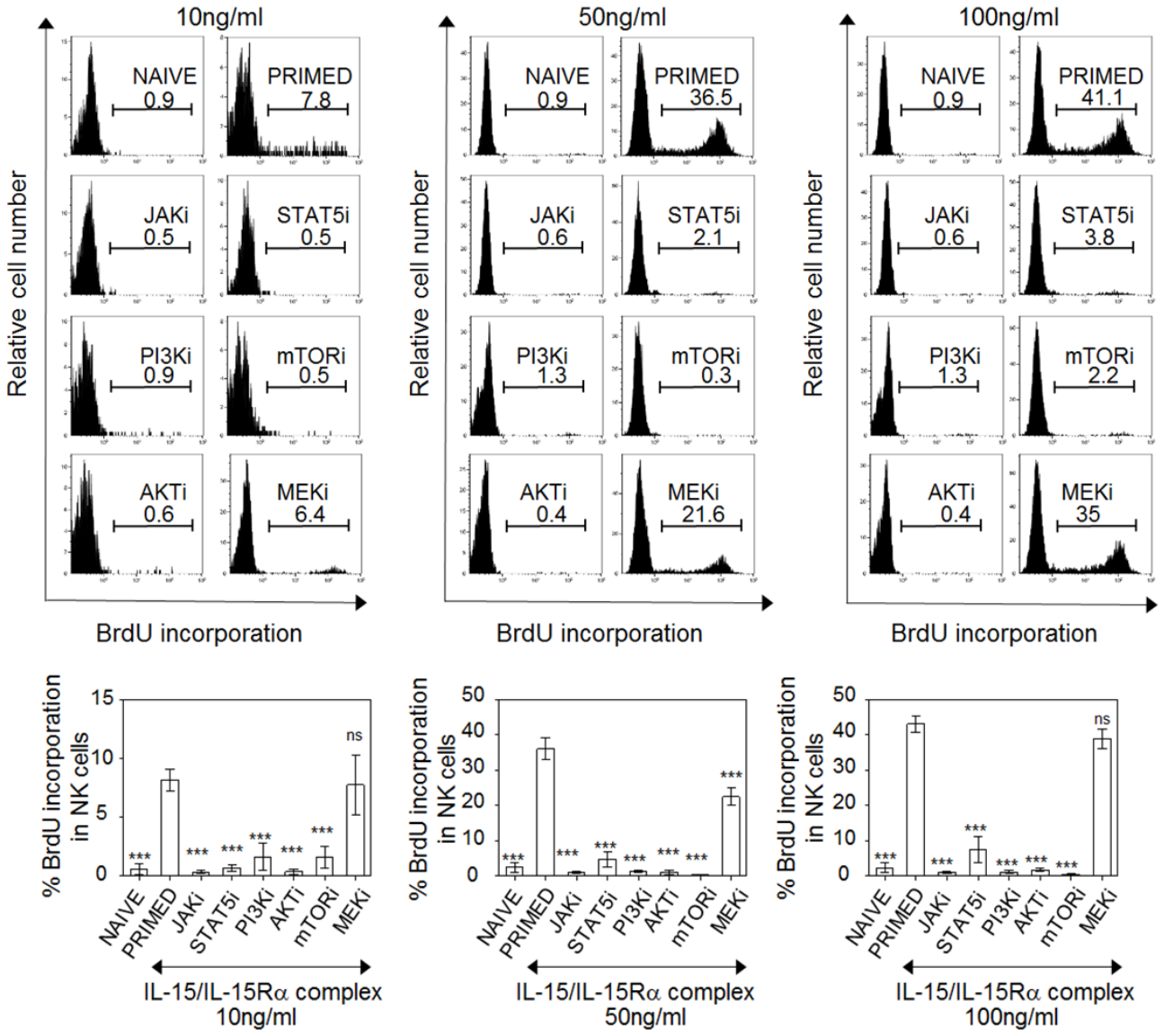

% Granzyme B expression in NK cells
60
40
20
0
***
ns
NAIVE
PRIMED
JAKi
STAT5i
PI3Ki
AKTi
mTORi
MEKi
IL-15/IL-15Rα complex

Figure 12. PI3K-AKT-mTOR pathway is required for proliferation of IL-15 treated NK cells

Splenic leukocytes were incubated with working concentrations of the inhibitors for 1 hr; followed by culturing of cells for 24 hrs with IL-15/IL-15Rα at the indicated concentrations. BrdU was added *in vitro* (one tenth volume of a 96-well) to the cells, 2 hrs prior to intracellular staining for BrdU. Histograms depict BrdU incorporation in naïve and primed NK cells and the effect of inhibitors on NK cell proliferation at different concentrations of IL-15/IL-15Rα. Results are summarized in graphs where each bar indicates an average of 6 samples pooled from two independent experiments. Figures are representative of at least three independent experiments. Numbers on histograms indicate percentages of BrdU incorporation in NK cells. NK cells were defined as NK1.1+TCRβ- populations. Significance of results on each bar was determined with primed cells without any inhibitor treatment. *** $p \leq 0.001$


10ng/ml
50ng/ml
100ng/ml
NAIVE
PRIMED
JAKi
STAT5i
PI3Ki
mTORi
AKTi
MEKi
0.9
7.8
0.5
0.5
0.9
0.5
0.6
6.4
0.9
36.5
0.6
2.1
1.3
0.3
0.4
21.6
0.9
41.1
0.6
3.8
1.3
2.2
0.4
35
Relative cell number
BrdU incorporation
% BrdU incorporation in NK cells
ns
***
IL-15/IL-15Rα complex
10ng/ml
IL-15/IL-15Rα complex
50ng/ml
IL-15/IL-15Rα complex
100ng/ml

Notably, NK cell proliferation was severely affected with PI3K-AKT-mTOR inhibition and this was reproducible in NK cells treated with higher doses of IL-15/IL-15Rα complex. When the effect of MEK inhibition was analyzed at different IL-15 doses, blocking MEK did not abrogate NK cell proliferation with 10 and 100ng/ml of IL-15/IL-15Rα complex. However, there was decrease in proliferation of NK cells primed with 50ng/ml of IL-15/IL-15Rα complex, but this was not as dramatic as the effects seen with inhibition of the PI3K-AKT-mTOR pathway.

Taken together, our data suggests that PI3K-AKT-mTOR pathway is central for IL-15 mediated NK cell effector responses including granzyme B expression, proliferation and IFN-γ production. MEK is important for cytokine responses and IFN-γ production but is not critical for granzyme B expression and proliferation; suggesting differential regulation of MEK for effector functions of NK cells upon IL-15 stimulation.

3.3. mTOR ACTIVITY IS REQUIRED FOR INFECTION-INDUCED NATURAL KILLER CELL EFFECTOR FUNCTIONS *IN VIVO*

To further extend my findings of the importance of PI3K-AKT-mTOR pathway for IL-15 induced NK cell functions *in vivo*, the effects of blocking mTOR pathway by administering rapamycin were analyzed during MCMV infections where critical role of NK cell for early control of virus replication is well known (Biron et al., 1999; Lee et al., 2007). As previously mentioned, NK cells are recruited to secondary lymph nodes in response to infections, where they are activated by IL-15 trans-presentation by DCs and this releases activated NK cells in the periphery (Lucas et al., 2007).

Wild Type (WT) B6 mice were given mTOR inhibitor rapamycin or DMSO by intraperitoneal injections daily and either infected with 5,000PFU MCMV or left uninfected as controls. To confirm inhibition of the mTOR pathway by rapamycin treatment *in vivo*, we evaluated levels of phosphorylated S6 ribosomal protein which is a target of S6 kinase downstream of mTOR pathway (Mamane et al., 2006). Significantly low phosphorylation levels of S6 ribosomal protein were found in rapamycin-treated mice compared to controls, indicating efficient blocking of mTOR pathway by rapamycin (**Figure 13A**).

Next, we evaluated the effects of rapamycin treatment on IL-15 production. IL-15 not only plays a key role in survival, induction of cytotoxicity and proliferation of NK cells; but also functions as a central mediator of inflammation by amplifying the inflammatory response in various immune cells (Ma et al., 2006). To confirm if rapamycin treatment does not hamper IL-15 production thereby enabling NK cell priming *in vivo*, we quantified IL-15 mRNA levels in splenic leukocytes on day 1.5 by real-time PCR. IL-15 was induced upon MCMV infection and no significant differences in expression levels between rapamycin-treated and control samples were observed (**Figure 13B**).

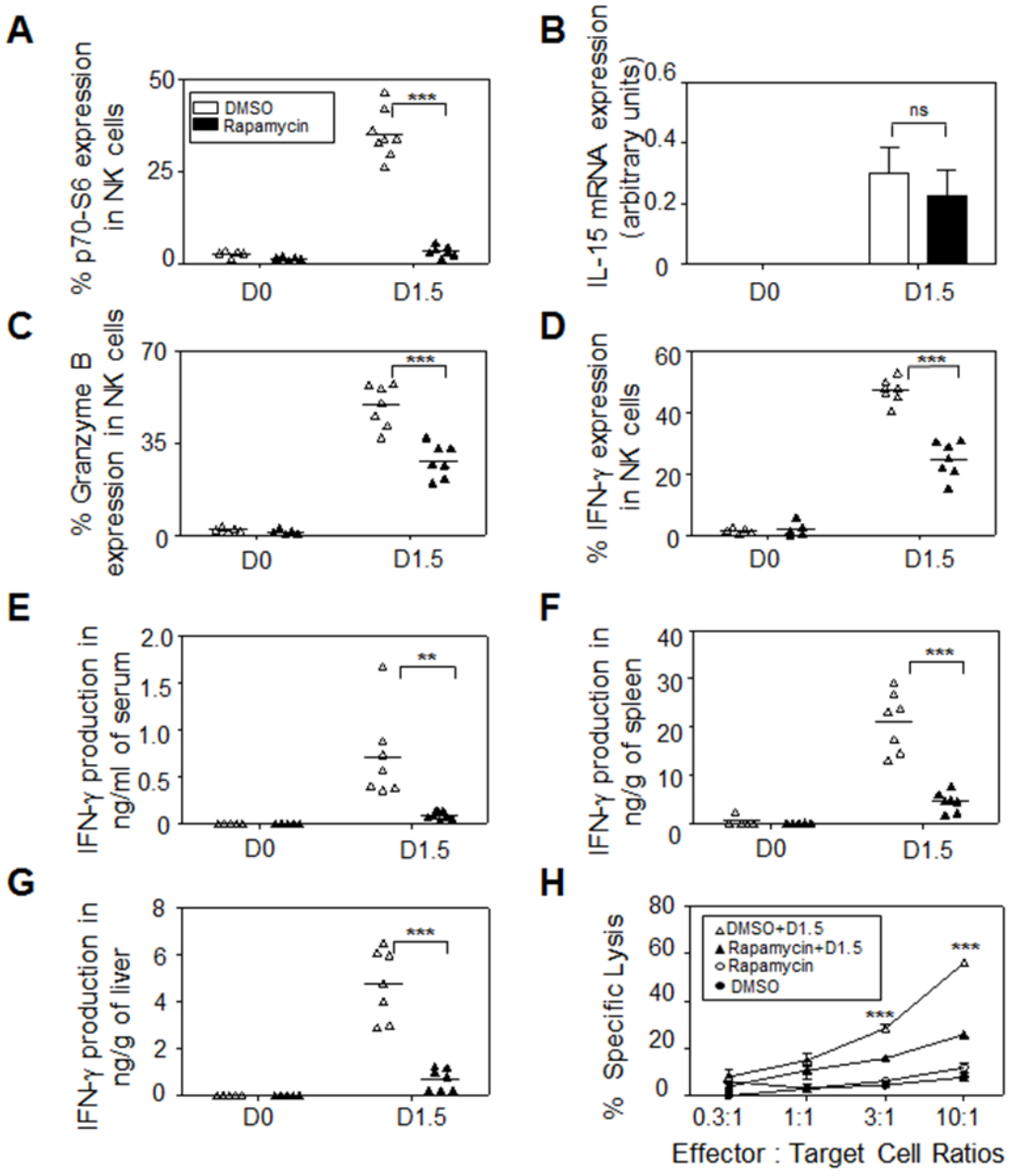
This was followed by evaluation of NK cell effector functions during MCMV infections such as intracellular IFN-γ and granzyme B production in rapamycin treated mice on day 1.5. Both granzyme B (**Figure 13C**) and IFN-γ (**Figure 13D**) expression levels were induced after MCMV infection in NK cells. However, their productions were diminished

in rapamycin-treated mice compared to controls. At this timepoint, MCMV infection induced a well-defined innate cytokine peak response of IFN-γ in serum, which was dramatically reduced (>75%), indicating that cytokine response of NK cells is compromised in the rapamycin-treated mice (**Figure 13E**). Similar patterns were observed in the organs; rapamycin-treated mice displayed reduced IFN-γ production in the infected spleens (**Figure 13F**) and livers (**Figure 13G**).

Additionally, cytotoxic responses were studied *ex vivo* by measuring the ability of NK cells from rapamycin or DMSO treated- control and infected mice to kill the NK cell sensitive YAC-1 cells. NK cells from infected mice were able to efficiently lyse target cells while naive cells displayed poor killing activity. Notably, splenic NK cells from rapamycin-treated MCMV-infected mice on day 1.5 had attenuated lytic activities towards target tumor cells (**Figure 13H**).

Figure 13. *In vivo* inhibition of mTOR affects NK cell cytokine production and cytotoxic responses

Rapamycin or DMSO treated mice were either uninfected or given 5000PFU MCMV *i.p.* and sacrificed on day 1.5. Splenic leukocytes were isolated and used for the following analyses: (A) Graph depicts intracellular expression of phosphorylated S6 ribosomal protein in splenic NK cells. (B) Total RNA was extracted and quantified for IL-15 production by real-time PCR. Graph depicts IL-15 mRNA expression in controls and infected samples normalized to β-actin levels. Graphs depict percentages of intracellular granzyme B (C) and IFN-γ (D) expressions in NK cells. Infected and control organs were isolated, homogenized and the lysates used for analysis of cytokine production along with blood serum by cytometric bead assays. Graphs depict the amount of IFN-γ levels in serum (E), spleens (F), and livers (G). (H) To measure NK cytotoxic activity, infected and control spleen leukocytes were incubated *ex vivo* with their target YAC-1 tumor cells at the indicated effector (NK cells): target cell ratios for 4 hrs in media containing DMSO or rapamycin. Graph shows percentages of YAC-1 cells lysed by splenic NK cells. For intracellular stainings, n=5-7 mice pooled from two independent experiments that were matched for sex and age of mice. NK cells were defined as NK1.1+TCRβ- populations. Figures are representative of at least three independent experiments. Significance of results was determined between DMSO and rapamycin treated samples. ** $p \leq 0.01$; *** $p \leq 0.001$

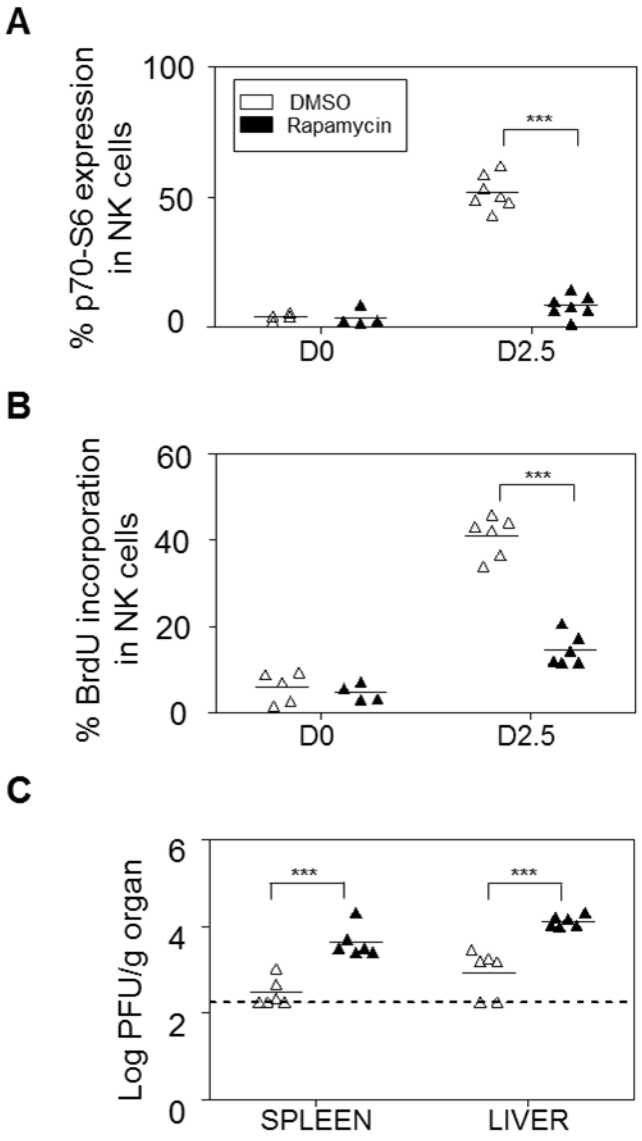

A
% p70-S6 expression in NK cells
50
25
0
DMSO
Rapamycin
***
D0
D1.5
B
IL-15 mRNA expression (arbitrary units)
0.6
0.4
0.2
0
ns
D0
D1.5
C
% Granzyme B expression in NK cells
70
35
0
***
D0
D1.5
D
% IFN-γ expression in NK cells
60
40
20
0
***
D0
D1.5
E
IFN-γ production in ng/ml of serum
2.0
1.5
1.0
0.5
0
**
D0
D1.5
F
IFN-γ production in ng/g of spleen
40
30
20
10
0
***
D0
D1.5
G
IFN-γ production in ng/g of liver
8
6
4
2
0
***
D0
D1.5
H
% Specific Lysis
80
60
40
20
0
DMSO+D1.5
Rapamycin+D1.5
Rapamycin
DMSO
***
***
0.3:1
1:1
3:1
10:1
Effector : Target Cell Ratios

As previously mentioned, NK cell anti-viral defenses are critical at early stages for the control of MCMV replication. Therefore, I investigated the consequences of blocking mTOR pathway in NK cells by treating mice with rapamycin and evaluated NK cell responses to MCMV infections on day 2.5; as specific proliferation of NK cells with respect to MCMV begins at day 2. Significant reductions in the phosphorylation of S6 ribosomal protein were observed in MCMV-infected rapamycin treated mice compared to controls, indicating efficient blocking of the mTOR pathway (**Figure 14A**).

The proportions of NK cells were comparable to controls and unaffected by rapamycin treatment (data not shown). However consistent with the *ex vivo* data, proliferation of NK cells as measured by BrdU incorporation was severely affected in rapamycin-treated mice during infections (**Figure 14B**). Consequently, when virus loads in organs of mice treated with rapamycin was measured at this timepoint, significantly higher viral burdens were found in spleens and livers compared to those that received DMSO (**Figure 14C**). Taken together, similar to *ex vivo* results blocking mTOR pathway *in vivo* affects IL-15 induced cytokine production, proliferative and cytotoxic responses of NK cells during infections, thereby resulting in elevated viral burdens upon MCMV infection.

Figure 14. *In vivo* inhibition of mTOR affects NK cell proliferative and anti-viral responses

Rapamycin or DMSO treated mice were either uninfected or given 5000PFU MCMV *i.p.* and sacrificed on day 2.5 post-infection for the following analyses: (A) Splenic leukocytes were isolated from infected and control organs and analyzed for the effects of rapamycin treatment on mTOR pathway. Graph depicts intracellular expression of phosphorylated S6 ribosomal protein in NK cells. (B) BrdU was injected intraperitoneally (2 hrs prior to sacrifice) following which splenic leukocytes were isolated from infected and control organs and analyzed for NK cell proliferation. Graph depicts proportion of BrdU incorporation in NK cells. (C) Infected and control organs were isolated, homogenized and the lysates used for viral quantification by plaque assays. Graph indicates viral titers in the infected spleens and livers of DMSO or rapamycin treated mice. For all graphs, n=4-7 mice pooled from two independent experiments that were matched for sex and age of mice. Figures are representative of at least three independent experiments. Viral titers for organs with no detectable plaques were arbitrarily set at the level of detection for statistical calculation and graphical representation. NK cells were defined as NK1.1+TCRβ- populations. Figures are representative of at least three independent experiments. Significance of results was determined between DMSO and rapamycin treated samples. *$p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$*


A
% p70-S6 expression in NK cells
100
50
0
DMSO
Rapamycin
***
D0
D2.5
B
% BrdU incorporation in NK cells
60
40
20
0
***
D0
D2.5
C
Log PFU/g organ
6
4
2
0
***
***
SPLEEN
LIVER

4. DISCUSSION

4.1. SUMMARY OF RESULTS

Priming of NK cells was generally understood as increased response to IL-12 when NK cells were co-stimulated with IL-15. Here, my data demonstrate that pre-treatment of NK cells to IL-15 sensitizes NK cell responses not only to IL-12 but also to subsequent stimulations with other cytokine families that employ the JAK-STAT pathway. In addition, I observed increased responses of IL-15 pre-treated NK cells to Ly49H receptor stimulations. My data also supports the notion that IL-15 treatment is required for induction of major NK cell effector functions. It is known that during inflammation NK cells are recruited to lymph nodes where they are "primed" by trans-presentation of IL-15 by DCs. This priming is particularly important as murine NK cells are minimally cytotoxic and require appropriate signals to become activated. Indeed, my data shows that IL-15 treatment is required for induction of granzyme B production and proliferation in a dose dependent manner. In addition, I have demonstrated that IL-15 "priming" increases IFN-γ production in NK cells through Ly49H activation receptor stimulation. Overview of IL-15 trans-presentation to NK cell is demonstrated in a model by which activated NK cells are generated in the periphery during infections **(Figure 15)**.

Analyses of the signaling pathways downstream of IL-15 receptor activation demonstrated that inhibiting JAK and STAT5 abolished the pro-survival effect of IL-15 on NK cells. Importantly, my data implicates PI3K-AKT-mTOR pathway for IL-15 "priming" of NK cell responses to cytokine stimulations. In addition, this pathway is critical for IL-15 induced NK cell proliferation, IFN-γ and granzyme B production.

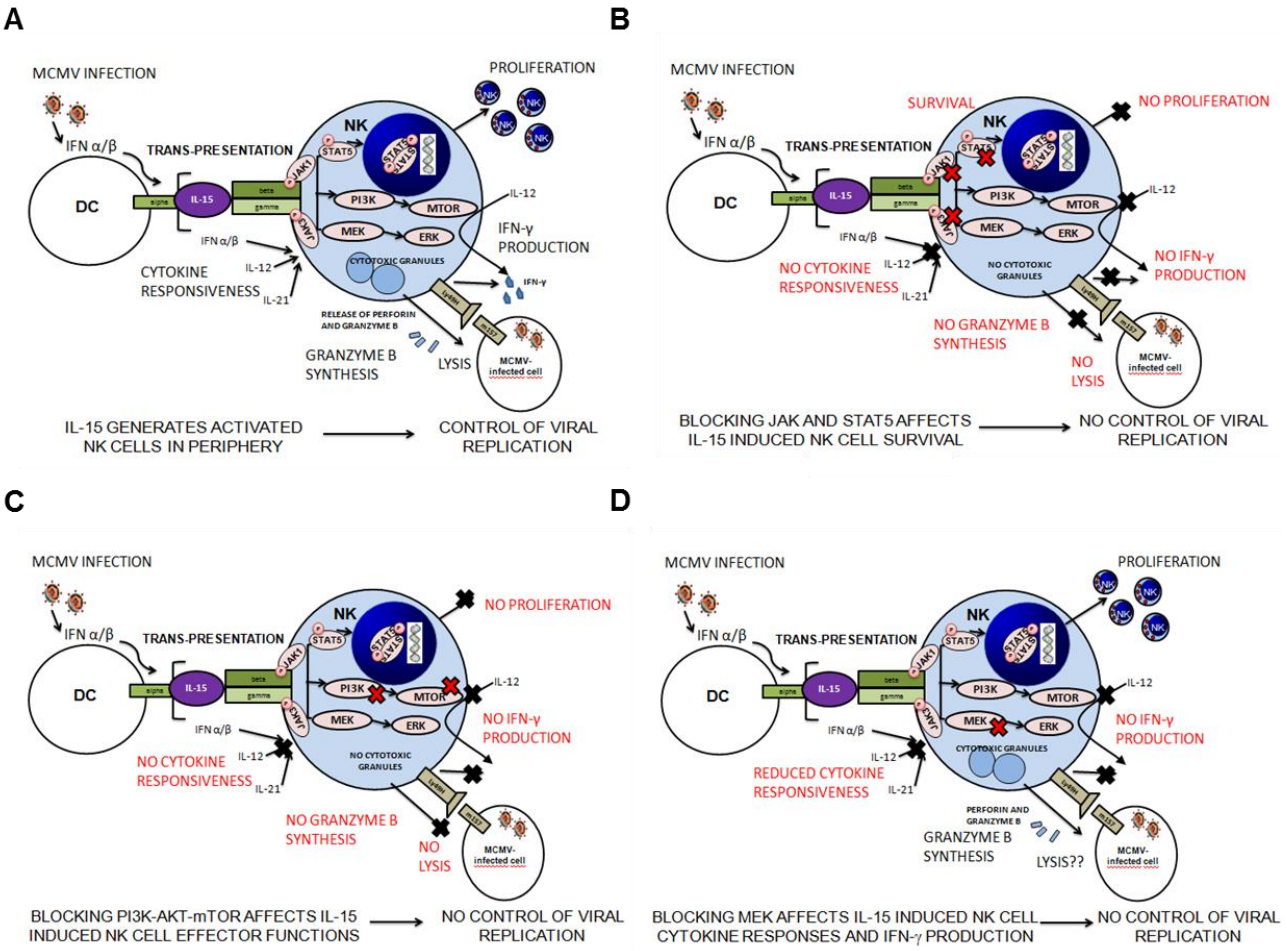
Therefore blocking PI3K, AKT and mTOR *ex vivo* affected all the major functions of NK cells. These results were confirmed *in vivo* where blocking mTOR pathway affected NK cell effector functions including proliferation, IFN-γ and granzyme B production. Consequently due to a lack of NK cell anti-viral mechanisms, rapamycin-treated mice were unable to efficiently control viral replication upon MCMV infections conclusively demonstrating the role of PI3K-mTOR pathway for IL-15 induced NK cell functions.

4.2. IL-15 PRIMING OF NATURAL KILLER CELL FUNCTIONS

The role of NK cells in killing infected and tumor cells are well studied over the years. They share cytotoxic functions with T cells but unlike them, NK cells do not require prior antigen stimulation. They are capable of specific and non-specific recognition of stressed cells and are therefore important for both innate and adaptive immune responses (Biron et al., 1999; Trinchieri, 1989). NK cell effector responses are often determined by the integration of signal transduction pathways from multiple activating and inhibitory receptors (Lanier, 2008; Lee et al., 2007). NK cells also possess a myriad of cytokine receptors that allow them to respond to the various cytokines produced in the inflammatory milieu of innate and adaptive immune responses.

Figure 15. Effect of blocking different signaling pathways on IL-15 induced NK cell effector functions

(A) IL-15 trans-presentation by DCs in secondary lymph nodes generates activated NK cells in the periphery which can efficiently mount anti-viral functions. (B) Inhibition of JAK and STAT5 molecules downstream of IL-15 receptor activation affects survival of NK cells. By extension this results in an absence of activated NK cells in the periphery and inefficient control of viral replication. (C) Inhibition of PI3K-AKT-mTOR pathway affects IL-15 induced NK cell effector functions and results in increased viral tires during MCMV infections due to a lack of activated NK cells in the periphery. (D) Inhibition of MEK pathway affects IL-15 induced increased cytokine responsiveness and IFN-γ production but is less critical for proliferation and cytotoxic granule production in activated NK cells. NK cells produced IFN-γ is necessary for control of MCMV viral titers.


A
MCMV INFECTION
IFN α/β
TRANS-PRESENTATION
DC
IL-15
alpha
beta
gamma
JAK1
JAK3
STAT5
NK
PI3K
MTOR
MEK
ERK
CYTOTOXIC GRANULES
IL-12
IL-21
IFN α/β
CYTOKINE RESPONSIVENESS
PROLIFERATION
IFN-γ PRODUCTION
IFN-γ
RELEASE OF PERFORIN AND GRANZYME B
GRANZYME B SYNTHESIS
LYSIS
MCMV-infected cell
IL-15 GENERATES ACTIVATED NK CELLS IN PERIPHERY
CONTROL OF VIRAL REPLICATION
B
MCMV INFECTION
SURVIVAL
NO PROLIFERATION
NO IFN-γ PRODUCTION
NO CYTOKINE RESPONSIVENESS
NO GRANZYME B SYNTHESIS
NO LYSIS
BLOCKING JAK AND STAT5 AFFECTS IL-15 INDUCED NK CELL SURVIVAL
NO CONTROL OF VIRAL REPLICATION
C
MCMV INFECTION
NO PROLIFERATION
NO IFN-γ PRODUCTION
NO CYTOKINE RESPONSIVENESS
NO GRANZYME B SYNTHESIS
NO LYSIS
BLOCKING PI3K-AKT-mTOR AFFECTS IL-15 INDUCED NK CELL EFFECTOR FUNCTIONS
NO CONTROL OF VIRAL REPLICATION
D
MCMV INFECTION
PROLIFERATION
REDUCED CYTOKINE RESPONSIVENESS
PERFORIN AND GRANZYME B
GRANZYME B SYNTHESIS
LYSIS??
BLOCKING MEK AFFECTS IL-15 INDUCED NK CELL CYTOKINE RESPONSES AND IFN-γ PRODUCTION
NO CONTROL OF VIRAL REPLICATION

IL-15 is most potent in inducing NK activation; IL-15 is unique in that it is required for survival, maturation and homeostasis of NK cells. Indeed, IL-15-/- and IL-15Rα-/- knockout mice have no detectable CD8 T, NK and NK T cell populations (Fehniger and Caligiuri, 2001; Lodolce et al., 1998; Ma et al., 2006). IL-15mRNA is expressed in a wide variety of hematopoietic and non-hematopoietic cells but not by T cells, IL-15 is difficult to detect *in vivo* and predominantly occurs as a membrane bound form on many accessory cells like macrophages, DCs and monocytes (Fehniger and Caligiuri, 2001; Stoklasek et al., 2006). The IL-15 receptor consists of high affinity α subunit, and the lower-intermediate affinity β (CD122) and γ subunits (CD132), the latter two subunits are shared between IL-15 with IL-2 (Carson et al., 1994; Giri et al., 1994). Unlike IL-2Rα, which exhibits low affinity for IL-2 in the absence of CD122 and CD132, IL-15 can bind to the high affinity α subunit on same cell or in a non-autonomous cell fashion. Studies have shown that IL15Rα on NK cells are dispensable for their activation and survival (Koka et al., 2004). During infections, activated NK cells in the periphery are generated by recruiting naïve NK cells to secondary lymph nodes where innate immune cells like dendritic cells trans-present IL-15 to NK cells (Koka et al., 2004; Lucas et al., 2007; Ma et al., 2006). Activation of NK cells releases pre-existing cytotoxic granules such as perforin and granzymes. Simultaneously, the signals also induce production of inflammatory cytokines such as IFN-γ and TNF-α (Biron et al., 1999). Both of these functions are required for killing tumor cells and controlling pathogen replication. It was shown IFN-γ production by NK cells during viral infections is due to virus induced IL-12 (Biron et al., 1999). NK cell priming by IL-15 is generally understood along the lines of IL-12 or IL-15 stimulations. Such stimulations individually produced modest IFN-γ in

NK cells but IL-15 co-stimulation with IL-12 results in exaggerated IFN-γ production (Carson et al., 1994; Fehniger et al., 1999; Lucas et al., 2007).

In this report, I have demonstrated that **prior exposure** to IL-15/IL-15Rα primes NK cell responses to further stimulations through a myriad of cytokine receptors and the Ly49H activation receptor. IL-15/IL-15Rα (10ng/ml) was chosen to mimic trans-presentation mechanisms by DCs. IL-15 is linked to IL-15Rα at the sushi domain along with extracellular region of IL-15Rα subunit and this complex considerably enhances IL-15 binding to the CD122 and CD132 chains on the nearby cells. As complex increases the binding of IL-15 to the CD122 and CD132 subunits by several fold, reduced concentrations of IL-15 complex (i.e. 10ng/ml) are sufficient to activate NK cells. This bypasses the requirement of high doses of IL-15 (in the absence of the IL-15Rα subunit) which is capable of inducing proliferation of NK cells in addition to activation. CD122 is also expressed in high amounts on memory CD8 T and CD4 regulatory T cells in addition to NK cells. However, our data shows that 10ng/ml of IL-15 induced proliferation and enhanced cytokine responses specifically in NK cells and not in T cells. My data demonstrate that IL-15 priming of NK cell to cytokines is somewhat universal and enhanced responses was observed to stimulations with cytokines employing different STATs downstream of their receptor activation. As previously mentioned, IFN-γ production produced by NK cells is required for their anti-viral control mechanisms (Lucin et al., 1994). Therefore, it was interesting to see that IL-15 primed NK cells display enhanced IFN-γ production through both IL-12 cytokine receptor and Ly49H activation receptor stimulations. In addition, IL-15 is also critical for effector functions of NK cells such as proliferation and granzyme B production. There was a dose-dependent

increase in proliferation and granzyme B production with IL-15/IL-15R complex treatments. Notably the effects of IL-15 observed in our model were specific to NK cells and not observed in T cells, re-iterating the importance of IL-15 trans-presentation for NK cell priming.

4.3. PI3K-AKT-mTOR PATHWAY IS REQUIRED FOR IL-15 INDUCED NK CELL PRIMING AND ACTIVATION

Given that IL-15 primes NK cell cytokine and effector responses, we hypothesized that there is a common signaling mechanism underlying IL-15's effects on NK cells. Engagement of IL-15R on NK cells causes auto-phosphorylation and activation of Janus Kinases (JAK1 and JAK3), which induces at least three parallel signaling cascades: Ras-Raf-MAPK, PI3K-AKT-mTOR, and signal transduction and activation of transcription (STAT) 5 pathways (Kovanen and Leonard, 2004; Ma et al., 2006). However, it is largely unknown if one of three major signaling pathways is responsible for NK cell priming or it is achieved by a collaborative effort of multiple pathways. By using specific cell-permeable reversible inhibitors to enable efficient blocking of individual signaling pathways emanating from IL-15 receptor, I further demonstrated that PI3K-mTOR pathway is critical for IL-15-induced enhanced responses to cytokine stimulations. As expected, JAK and STAT5 inhibition mimicked the negative impact of lack of IL-15 on NK cell survival at 24 hrs of culture, indicating the importance of IL-15 in the homeostasis of NK cells. But whether STAT5 is required for activation and priming of NK cells independent of survival remains to be tested. As mentioned above, use of inhibitors *ex vivo* or NK cell specific- STAT5 knockout mice models greatly hamper NK

cell numbers. However recent advancements with tamoxifen inducible-Cre mice allows STAT5 to be conditionally knocked out in mature NK cells during infection and inducing the ablation of *STAT5*gene by injecting the mice with tamoxifen. This will overcome the NK survival issue while addressing the role of STAT5 in priming and cytotoxic responses *in vivo*.

PI3K-mTOR pathway is implicated in IFN-γ production by primed NK cells when stimulated through both IL-12 and Ly49H receptors. This is particularly relevant in our B6 mice model, as Ly49H recognition of the viral ligand m157 during MCMV infection triggers NK cell proliferation and enhances NK cell anti-viral responses (Arase et al., 2002; Dokun et al., 2001; Smith et al., 2002). However, Ly49H expression in B6 mice strains is restricted to roughly 50% of NK cells; therefore further investigation of NK cell priming to other NK cell activation receptors is warranted. Moreover, several receptors on NK and T cells using different adaptor molecules such as CD3, Syk/ZAP70, DAP10, DAP12 etc. could be employed upstream and possibly converge to a common PI3K-mTOR pathway for NK cell effector functions (Deshpande et al., 2013; Djeu et al., 2002; Horng et al., 2007). For example, it has already been demonstrated that PI3K is a critical component of NKG2D receptor signaling in NK cells, and IL-15 can enhance NKG2D signaling by augmenting the phosphorylation of DAP10 signaling molecule. This along with my data with Ly49H suggests a cross talk between activation receptors and IL-15 cytokine receptor for achieving maximum NK cell activation. NK cells could possibly employ a multitude of mechanisms such as activation by cytokines and engagement of activation receptor for maximal effector functions; but PI3K-AKT-mTOR pathway

induced by IL-15 is central for achieving maximal NK cell activation.

In addition, PI3K-mTOR pathway is required for IL-15 induced major NK cell functions including granzyme B expression and proliferation. Previous studies have shown that MEK inhibition affected NK cytotoxic activity by interfering with the mobilization of cytotoxic granules towards target cells in human NK cell lines, while proliferation of NK cells was unaffected (Wei et al., 1998). This was further shown to be regulated upstream by Syk/ZAP70 molecules associated with the NK activation receptors, that in-turn leads to the phosphorylation of PI3K and to the activation of downstream Rac1-PAK1-MEK-ERK molecules (Jiang et al., 2000; Jiang et al., 2002). The importance of the MEK pathway in NK cell cytokine responses was also reported by another group which demonstrated that the production of pro-inflammatory cytokines like IFN-γ, IL-6 and TNF-α were reduced by MEK blockade in human peripheral blood NK cells. Similarly in their system, MEK was regulated upstream by the activation of Syk-PI3K pathway upon CD160 receptor ligation on NK cells (Rabot et al., 2007).

Both these studies are consistent with my data that implicate MEK in responsiveness to cytokine stimulations and also in IFN-γ production upon IL-12 and α-Ly49H stimulations, while MEK is relatively less important for proliferation. In addition, my data show that the intracellular production of granzyme B was unaffected with MEK inhibition. However, this does not necessarily translate to efficient NK cytotoxic responses as NK cell activity is regulated at multiple steps. Therefore the mobilization of cytotoxic granules towards the target cells needs to be analyzed in our model to further understand the effect of MEK inhibition on NK cell lytic activity. As mentioned previously, IFN-γ production in NK cells during MCMV infection is necessary for

efficient control of viral titers; and increased viral replication was observed when this NK cell IFN-γ production was blocked. Interestingly, it was shown that ablation of IFN-γ did not affect the cytotoxic granule mediated lytic activity of NK cells (Orange et al., 1995). Therefore, it is likely that MEK inhibition will also result in uncontrolled viral titers during MCMV infections compared to control due to defects in IFN-γ production.

On the other hand, both *in vivo* and *ex vivo* results clearly show that PI3K-AKT-mTOR is required for granzyme B, proliferation and IFN-γ production in NK cells. It is also important to understand the differences in models used to study NK cell functions. All NK cells were initially thought of "ready to act cells" but unlike human NK cells which are constantly exposed to microbes and other activating stimulus, mice housed at SPF environments have long known to be poorly cytotoxic against tumor cells. This is attributed to lack of perforin/granzyme B expression, but can translate the cytotoxic granules from pre-existing mRNA pools upon the activation by IL-15 (Fehniger et al., 2007). Therefore, murine NK cells are excellent models to study- 1) IL-15 activation of naïve NK cells and 2) The effect of blocking PI3K-mTOR pathway in IL-15 induced NK cell functions, without the influence of pre-existing stimuli.

4.4. mTOR ACTIVITY IS REQUIRED FOR NK CELL ANTI-VIRAL FUNCTIONS DURING MCMV INFECTIONS

The serine/threonine kinase mTOR is a well-studied regulator of cell growth and metabolism. It acts downstream of the PI3K-AKT pathway and is activated by growth factors, nutrients and various other signals (Laplante and Sabatini, 2012; Xu et al., 2012).

Activation of mTOR complex 1 (mTORC1) leads to the phosphorylation of several downstream targets such as 70-S6 kinase (S6K); and this kinase activity of mTORC1 is blocked by rapamycin treatment (Mamane et al., 2006). Recently accumulated evidence demonstrates that mTOR pathway can regulate immune response by modulating metabolism of the individual immune cells. For example, rapamycin inhibits T cell proliferation and therefore is used clinically as immunosuppressive agent for the prevention of allograft rejection (Halloran, 2004). However, treating rapamycin at low doses improves naïve CD8 T cell differentiation to memory CD8 T cells *in vivo*, suggesting immunostimulatory roles in memory response (Mamane et al., 2006). mTOR-deficient CD4 T cells fail to differentiate into Th1, Th2, and Th17 cells and this defect was largely due to impaired phosphorylation of respective STAT molecules required for each lineage differentiation. This is similar to my *ex vivo* experiments where diminished levels of phosphorylated STATs in primed NK cells were observed with inhibition of mTOR (Powell and Delgoffe, 2010).

Since my *ex vivo* data implicated the importance of PI3K-AKT-mTOR pathway for major NK cell functions, I decided to extrapolate these results to *in vivo* MCMV models where the role of NK cells in controlling viral replication is well studied. My data showed that *in vivo* blocking of mTOR by rapamycin affects NK cell cytotoxic and proliferative responses during MCMV infection, culminating in elevated viral burdens compared to untreated controls. Our results show that the PI3K-mTOR pathway is indispensable for efficient NK cell activity and provides an example of how mTOR activity is linked to immune functions of NK cells. The effect of rapamycin on NK cell functions during MCMV infection was dramatic, showing severe defects in IFN-γ and granzyme B

productions in addition to lack of NK cell proliferation. It is well known that granzymes and IFN-γ are required for efficient NK cell lytic activity towards target cells (Loh et al., 2005; Orange et al., 1995; Tay and Welsh, 1997; van Dommelen et al., 2006). Consequently, mTOR inhibition affected NK cell cytotoxic responses towards target YAC-1 tumor cells and were unable to efficiently control viral replication in the infected organs. Presumably, the drastic effect of rapamycin on NK cells is due to the fact that they are the most metabolically active cell population during acute MCMV infection. During infections, NK cells undergo rapid blastogenesis and this expansion is dependent on IL-15 (Nguyen et al., 2002; Orange and Biron, 1996). Studies have shown that IL-15 trans-presentation is not likely to be affected by rapamycin treatments (Marcais et al., 2014). My data also suggests that mTOR pathway might be required for the efficient metabolic transformation of NK cells and the associated immune functions. However, rapamycin can also have considerable effects on other innate immune cells such as macrophages, which are reservoirs for MCMV infection and known to play important roles in innate immune defense mechanisms. It remains to be tested in my rapamycin-treated and infected mice models, if increased MCMV viral titers observed during infection are due to the immune-supressive functions of rapamycin on macrophages and dendritic cells.

4.5. EFFECT OF OTHER CYTOKINES IN NK CELL PRIMING AND ACTIVATION

My project focuses on the effect of IL-15 primed NK cell responses to five representative cytokines IL-2, IL-4, IL-12, IL-21 and IFN-α, with each cytokine employing different

STAT molecules downstream of the JAK pathway. But as indicated in **Table 1**, naïve NK cells possess several other receptors such as IL-3, IL-7, IL-10, IL-23, IL-27 etc. The response of NK cells to stimulations with the latter set of cytokines have not been tested in this study and it is likely that IL-15 exposure enhances NK cell responses to the above-mentioned cytokines. Therefore to further confirm if IL-15 priming of NK cell cytokine responses through the PI3K-AKT-mTOR pathway is a general phenomenon, the response of IL-15 treated NK cells to the extended list of cytokines in the table that have the corresponding receptors on NK cell surfaces needs to be analyzed. Furthermore, it could be possible that IL-15 treatment up-regulates the expression of those cytokine receptors on surfaces of NK cells that were absent in untreated naïve cells.

Table 1. Different cytokines, their receptors and the STAT molecules employed

The table displays the extensive list of cytokines that employ the JAK-STAT pathway. It also shows the presence or absence of the cytokine receptor expression on naïve NK cells. Only dominant STAT molecules that are employed by the particular cytokine are shown. The five representative cytokines that employ different STAT molecules downstream of JAK pathway and tested in this study are highlighted in yellow.

Cytokine	Receptors	Receptor on NK cells	STATs**
Type I IFN	IFNAR	Yes	STAT1, STAT2
IL-2	IL-2Rα, IL-2/15Rβ, common γ chain	Yes	STAT5
IL-3	IL-3Rα, common β chain	Yes	STAT5
IL-4	IL-4Rα, common γ chain	Yes	STAT6
IL-5	IL-5Rα, common β chain	No	STAT5
IL-6	IL-6Rα, gp130	Yes	STAT3
IL-7	IL-7Rα, common γ chain	Yes	STAT5
IL-9	IL-9Rα, common γ chain	Yes	STAT5
IL-10	IL-10Rα, IL-10Rβ	Yes	STAT3
IL-12	IL-12Rβ1, IL-12Rβ2	Yes	STAT4
IL-13	IL-13Rα, common γ chain	No	STAT6
IL-15	IL-15Rα, IL-2/15Rβ, common γ chain	Yes	STAT5
IL-20	IL-20Rα, IL-20Rβ	No	STAT3
IL-21	IL-21Rα, common γ chain	Yes	STAT3
IL-22	IL-22R1, IL-10R2	No	STAT3
IL-23	IL-12Rβ1, IL-23R	Yes	STAT3, STAT4
IL-24	IL-22R1, IL-20R2	No	STAT3
IL-26	IL-20R1, IL-10R2	No	STAT3
IL-27	IL-27Rα, gp130	Yes	STAT1, STAT3
IL-28	IFNLR1, IL-10R2	No	STAT1, STAT3

In this way, while naïve NK cells do not possess receptors for some cytokines such as IL-5 and IL-13, IL-15 treatments might induce the expression of the same on primed NK cells. Therefore, while it is true that IL-15 exposure enhances NK cells responses to certain cytokines, their responses to the extensive list of cytokines that employ the JAK-STAT pathway and the relative expression of cytokine receptors on their cell surfaces after IL-15 treatment needs further analysis.

As previously mentioned IL-15 is constantly required for survival of NK cells, while other cytokines like IL-2, IL-4, IL-7, IL-12, IL-18 and IL-21 either do not affect or have minor roles in NK cell development and homeostasis. These cytokines, however, have pronounced effects on NK cell functions and maturation. For example, IL-12 and IL-18 produced by innate immune cells is necessary for IFN-γ production and tumor clearance by NK cells (Takeda et al., 1998). It is well known that combination of IL-15 and IL-12 enhances IFN-γ production in NK cells, but such synergistic effects on NK cell cytokine production is also observed with other cytokines. In fact, IL-12 and IL-18 in combination elicited maximum cytokine responses in NK cells (Lauwerys et al., 2000). Besides IL-2 and IL-15, IL-21, IL-12 and IL-18 treatments also resulted in large granular morphology and maturation of NK cells *in vitro*. Interestingly IL-15 and IL-2 induced the lytic capacity of NK cells which was enhanced in presence of IL-21 *in vitro* (Brady et al., 2010).

Taken together this suggests that several cytokines can modulate different functions of NK cells and the cytokine milieu that NK cells are exposed to influences the functional subsets of NK cells *in vivo*. These results also indicate that other cytokines can activate certain functions of NK cells to a similar or lesser degree compared to IL-15. Since many

of these cytokines employ the JAK-STAT pathway, prior exposure of NK cells to these cytokines could possibly enhance NK cell responses to subsequent cytokine stimulations *in vivo*. Additionally, several of these cytokines which are induced during inflammatory responses *in vivo* are likely to activate the PI3K-mTOR pathway and therefore could "potentially prime" NK cells through enhanced activity of this pathway. This is especially relevant in mature NK cells exposed to IL-15 and IL-21 which significantly enhances their cytokine production and lytic activity (Brady et al., 2004). This suggests that IL-21 treatments can enhance NK cell functions and their responses to other cytokines similar to that of IL-15 "priming" of NK cells. Finally given that IL-2 and IL-15 possess functional similarities, it would be interesting to probe if IL-2 exposure can "prime" NK cell responses to other cytokines.

5. CONCLUDING REMARKS

To our knowledge, this is the first study to link the IL-15-induced mTOR pathway to NK cell functions *in vivo* during acute virus infections like MCMV. This project has demonstrated that the signaling axis of IL-15-PI3K-mTOR in NK cells is important for their cellular proliferation, responsiveness to cytokine stimulations and cytotoxic functions. IL-2/IL-15 share receptor subunits of IL-2/IL-15R-β and -γ chains and both are being widely used for *ex vivo* expansion of NK cells in immunotherapy. Their promising therapeutic capacity for a variety of human malignancies has stimulated an interest in using NK cells for anti-cancer treatments (Kalinski et al., 2005; Vivier et al., 2012). IL-2 is accompanied by severe toxicity; IL-15 might be better for NK expansion and activation

but warrants use of physiologically irrelevant high doses. My data shows low doses of IL-15/IL-15Rα complex sensitizes NK cells to several activating stimuli. Therefore, understanding the molecular mechanisms by which IL-15 primes and activates NK cells will allow manipulation of IL-15 signaling for improving NK cell-based therapeutic strategies against cancers and infectious diseases.

Given that IL-15Rα on dendritic cells are required for activation of NK cells, studying how IL-15 can enhance NK cell functions will help in designing cell based vaccines for tumor therapy similar to the recently approved Provenge^{TM} vaccine for prostate cancers. For example, treating patients DCs with IL-15 in addition to tumor ligands *ex vivo* can enhance antigen presentation to tumor infiltrating lymphocytes and simultaneously activate NK cell tumor activity *in vivo*. Alternatively, DCs can be stimulated with TLR ligands to promote IL-15 synthesis and presentation. In fact, transfecting cancer cells with IL-15Rα has shown positive outcome in mice with trans-presentation of IL-15 to NK cells and rejection of colon carcinoma cells in mice (Kobayashi et al., 2005). In conclusion, given that IL-15 is indispensable for their survival and maturation it is imperative to study the mechanisms of how IL-15 primes NK cell functions *in vivo.* Therefore the results of this study will provide insights into optimal activation of NK cells and can be applied to developing NK cell based immunotherapies for various diseases.

Abraham, R.T., and G.J. Wiederrecht. 1996. Immunopharmacology of rapamycin. *Annu Rev Immunol* 14:483-510.

Ahmed, N.N., H.L. Grimes, A. Bellacosa, T.O. Chan, and P.N. Tsichlis. 1997. Transduction of interleukin-2 antiapoptotic and proliferative signals via Akt protein kinase. *Proc Natl Acad Sci U S A* 94:3627-3632.

Andoniou, C.E., S.L. van Dommelen, V. Voigt, D.M. Andrews, G. Brizard, C. Asselin-Paturel, T. Delale, K.J. Stacey, G. Trinchieri, and M.A. Degli-Esposti. 2005. Interaction between conventional dendritic cells and natural killer cells is integral to the activation of effective antiviral immunity. *Nat Immunol* 6:1011-1019.

Araki, K., A.P. Turner, V.O. Shaffer, S. Gangappa, S.A. Keller, M.F. Bachmann, C.P. Larsen, and R. Ahmed. 2009. mTOR regulates memory CD8 T-cell differentiation. *Nature* 460:108-112.

Arase, H., E.S. Mocarski, A.E. Campbell, A.B. Hill, and L.L. Lanier. 2002. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science* 296:1323-1326.

Arnon, T.I., M. Lev, G. Katz, Y. Chernobrov, A. Porgador, and O. Mandelboim. 2001. Recognition of viral hemagglutinins by NKp44 but not by NKp30. *Eur J Immunol* 31:2680-2689.

Bar-On, Y., E. Seidel, P. Tsukerman, M. Mandelboim, and O. Mandelboim. 2014. Influenza Virus Uses Its Neuraminidase Protein to Evade the Recognition of Two Activating NK Cell Receptors. *J Infect Dis*

Barata, J.T., A. Silva, J.G. Brandao, L.M. Nadler, A.A. Cardoso, and V.A. Boussiotis. 2004. Activation of PI3K is indispensable for interleukin 7-mediated viability, proliferation, glucose use, and growth of T cell acute lymphoblastic leukemia cells. *J Exp Med* 200:659-669.

Bernasconi, A., R. Marino, A. Ribas, J. Rossi, M. Ciaccio, M. Oleastro, A. Ornani, R. Paz, M.A. Rivarola, M. Zelazko, and A. Belgorosky. 2006. Characterization of immunodeficiency in a patient with growth hormone insensitivity secondary to a novel STAT5b gene mutation. *Pediatrics* 118:e1584-1592.

Beutler, B. 2004. Innate immunity: an overview. *Mol Immunol* 40:845-859.

Biron, C.A., K.S. Byron, and J.L. Sullivan. 1989. Severe herpesvirus infections in an adolescent without natural killer cells. *N Engl J Med* 320:1731-1735.

Biron, C.A., K.B. Nguyen, G.C. Pien, L.P. Cousens, and T.P. Salazar-Mather. 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu Rev Immunol* 17:189.

Biron, C.A., H.C. Su, and J.S. Orange. 1996. Function and Regulation of Natural Killer (NK) Cells during Viral Infections: Characterization of Responses in Vivo. *Methods* 9:379-393.

Biron, C.A., and R.M. Welsh. 1982. Blastogenesis of natural killer cells during viral infection in vivo. *J Immunol* 129:2788-2795.

Blagosklonny, M.V. 2011. Cell cycle arrest is not senescence. *Aging (Albany NY)* 3:94-101.

Bonnema, J.D., L.M. Karnitz, R.A. Schoon, R.T. Abraham, and P.J. Leibson. 1994. Fc receptor stimulation of phosphatidylinositol 3-kinase in natural killer cells is associated with protein kinase C-independent granule release and cell-mediated cytotoxicity. *J Exp Med* 180:1427-1435.

Brady, J., S. Carotta, R.P. Thong, C.J. Chan, Y. Hayakawa, M.J. Smyth, and S.L. Nutt. 2010. The interactions of multiple cytokines control NK cell maturation. *J Immunol* 185:6679-6688.

Brady, J., Y. Hayakawa, M.J. Smyth, and S.L. Nutt. 2004. IL-21 induces the functional maturation of murine NK cells. *J Immunol* 172:2048-2058.

Brown, M.G., A.O. Dokun, J.W. Heusel, H.R. Smith, D.L. Beckman, E.A. Blattenberger, C.E. Dubbelde, L.R. Stone, A.A. Scalzo, and W.M. Yokoyama. 2001. Vital involvement of a natural killer cell activation receptor in resistance to viral infection. *Science* 292:934-937.

Bryceson, Y.T., M.E. March, H.G. Ljunggren, and E.O. Long. 2006. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood* 107:159-166.

Buckley, R.H. 2004. Molecular defects in human severe combined immunodeficiency and approaches to immune reconstitution. *Annu Rev Immunol* 22:625-655.

Bukowski, J.F., B.A. Woda, and R.M. Welsh. 1984. Pathogenesis of murine cytomegalovirus infection in natural killer cell-depleted mice. *J Virol* 52:119-128.

Caligiuri, M.A., C. Murray, M.J. Robertson, E. Wang, K. Cochran, C. Cameron, P. Schow, M.E. Ross, T.R. Klumpp, R.J. Soiffer, and et al. 1993. Selective modulation of human natural killer cells in vivo after prolonged infusion of low dose recombinant interleukin 2. *J Clin Invest* 91:123-132.

Carson, W.E., J.G. Giri, M.J. Lindemann, M.L. Linett, M. Ahdieh, R. Paxton, D. Anderson, J. Eisenmann, K. Grabstein, and M.A. Caligiuri. 1994. Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *J Exp Med* 180:1395-1403.

Cerwenka, A., J.L. Baron, and L.L. Lanier. 2001. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. *Proc Natl Acad Sci U S A* 98:11521-11526.

Cheng, T.P., M.C. Valentine, J. Gao, J.T. Pingel, and W.M. Yokoyama. 2010. Stability of murine cytomegalovirus genome after in vitro and in vivo passage. *J Virol* 84:2623-2628.

Colucci, F., J.P. Di Santo, and P.J. Leibson. 2002. Natural killer cell activation in mice and men: different triggers for similar weapons? *Nat Immunol* 3:807-813.

Dalod, M., T. Hamilton, R. Salomon, T.P. Salazar-Mather, S.C. Henry, J.D. Hamilton, and C.A. Biron. 2003. Dendritic cell responses to early murine cytomegalovirus infection: subset functional specialization and differential regulation by interferon alpha/beta. *J Exp Med* 197:885-898.

Daniels, K.A., G. Devora, W.C. Lai, C.L. O'Donnell, M. Bennett, and R.M. Welsh. 2001. Murine cytomegalovirus is regulated by a discrete subset of natural killer cells reactive with monoclonal antibody to Ly49H. *J Exp Med* 194:29-44.

del Val, M., H. Hengel, H. Hacker, U. Hartlaub, T. Ruppert, P. Lucin, and U.H. Koszinowski. 1992. Cytomegalovirus prevents antigen presentation by blocking the transport of peptide-loaded major histocompatibility complex class I molecules into the medial-Golgi compartment. *J Exp Med* 176:729-738.

Delgoffe, G.M., and J.D. Powell. 2009. mTOR: taking cues from the immune microenvironment. *Immunology* 127:459-465.

Denman, C.J., V.V. Senyukov, S.S. Somanchi, P.V. Phatarpekar, L.M. Kopp, J.L. Johnson, H. Singh, L. Hurton, S.N. Maiti, M.H. Huls, R.E. Champlin, L.J. Cooper, and D.A. Lee. 2012. Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. *PLoS One* 7:e30264.

Deshpande, P., M.M. Cavanagh, S. Le Saux, K. Singh, C.M. Weyand, and J.J. Goronzy. 2013. IL-7- and IL-15-mediated TCR sensitization enables T cell responses to self-antigens. *J Immunol* 190:1416-1423.

Diefenbach, A., E.R. Jensen, A.M. Jamieson, and D.H. Raulet. 2001. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature* 413:165-171.

Diefenbach, A., E. Tomasello, M. Lucas, A.M. Jamieson, J.K. Hsia, E. Vivier, and D.H. Raulet. 2002. Selective associations with signaling proteins determine stimulatory versus costimulatory activity of NKG2D. *Nat Immunol* 3:1142-1149.

DiSanto, J.P., F. Rieux-Laucat, A. Dautry-Varsat, A. Fischer, and G. de Saint Basile. 1994. Defective human interleukin 2 receptor gamma chain in an atypical X chromosome-linked severe combined immunodeficiency with peripheral T cells. *Proc Natl Acad Sci U S A* 91:9466-9470.

Djeu, J.Y., K. Jiang, and S. Wei. 2002. A view to a kill: signals triggering cytotoxicity. *Clinical cancer research : an official journal of the American Association for Cancer Research* 8:636-640.

Dokun, A.O., S. Kim, H.R. Smith, H.S. Kang, D.T. Chu, and W.M. Yokoyama. 2001. Specific and nonspecific NK cell activation during virus infection. *Nat Immunol* 2:951-956.

Dubois, S., J. Mariner, T.A. Waldmann, and Y. Tagaya. 2002. IL-15Ralpha recycles and presents IL-15 In trans to neighboring cells. *Immunity* 17:537-547.

Eckelhart, E., W. Warsch, E. Zebedin, O. Simma, D. Stoiber, T. Kolbe, T. Rulicke, M. Mueller, E. Casanova, and V. Sexl. 2011. A novel Ncr1-Cre mouse reveals the essential role of STAT5 for NK-cell survival and development. *Blood* 117:1565-1573.

Fehniger, T.A., S.F. Cai, X. Cao, A.J. Bredemeyer, R.M. Presti, A.R. French, and T.J. Ley. 2007. Acquisition of murine NK cell cytotoxicity requires the translation of a pre-existing pool of granzyme B and perforin mRNAs. *Immunity* 26:798-811.

Fehniger, T.A., and M.A. Caligiuri. 2001. Interleukin 15: biology and relevance to human disease. *Blood* 97:14-32.

Fehniger, T.A., M.H. Shah, M.J. Turner, J.B. VanDeusen, S.P. Whitman, M.A. Cooper, K. Suzuki, M. Wechser, F. Goodsaid, and M.A. Caligiuri. 1999. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. *J Immunol* 162:4511-4520.

Ferlazzo, G., M. Pack, D. Thomas, C. Paludan, D. Schmid, T. Strowig, G. Bougras, W.A. Muller, L. Moretta, and C. Munz. 2004. Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs. *Proc Natl Acad Sci U S A* 101:16606-16611.

Forbes, C.A., M.G. Brown, R. Cho, G.R. Shellam, W.M. Yokoyama, and A.A. Scalzo. 1997. The Cmv1 host resistance locus is closely linked to the Ly49 multigene family within the natural killer cell gene complex on mouse chromosome 6. *Genomics* 41:406-413.

Franklin, R.A., A. Tordai, H. Patel, A.M. Gardner, G.L. Johnson, and E.W. Gelfand. 1994. Ligation of the T cell receptor complex results in activation of the Ras/Raf-1/MEK/MAPK cascade in human T lymphocytes. *J Clin Invest* 93:2134-2140.

Giri, J.G., M. Ahdieh, J. Eisenman, K. Shanebeck, K. Grabstein, S. Kumaki, A. Namen, L.S. Park, D. Cosman, and D. Anderson. 1994. Utilization of the beta and gamma chains of the IL-2 receptor by the novel cytokine IL-15. *Embo J* 13:2822-2830.

Grabstein, K.H., J. Eisenman, K. Shanebeck, C. Rauch, S. Srinivasan, V. Fung, C. Beers, J. Richardson, M.A. Schoenborn, M. Ahdieh, and et al. 1994. Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. *Science* 264:965-968.

Grant, L.R., Z.J. Yao, C.M. Hedrich, F. Wang, A. Moorthy, K. Wilson, D. Ranatunga, and J.H. Bream. 2008. Stat4-dependent, T-bet-independent regulation of IL-10 in NK cells. *Genes and immunity* 9:316-327.

Halloran, P.F. 2004. Immunosuppressive drugs for kidney transplantation. *N Engl J Med* 351:2715-2729.

Hand, T.W., W. Cui, Y.W. Jung, E. Sefik, N.S. Joshi, A. Chandele, Y. Liu, and S.M. Kaech. 2010. Differential effects of STAT5 and PI3K/AKT signaling on effector and memory CD8 T-cell survival. *Proc Natl Acad Sci U S A* 107:16601-16606.

Harrison, D.E., R. Strong, Z.D. Sharp, J.F. Nelson, C.M. Astle, K. Flurkey, N.L. Nadon, J.E. Wilkinson, K. Frenkel, C.S. Carter, M. Pahor, M.A. Javors, E. Fernandez, and R.A. Miller. 2009. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature* 460:392-395.

Herberman, R.B., J. Djeu, H.D. Kay, J.R. Ortaldo, C. Riccardi, G.D. Bonnard, H.T. Holden, R. Fagnani, A. Santoni, and P. Puccetti. 1979. Natural killer cells: characteristics and regulation of activity. *Immunol Rev* 44:43-70.

Herberman, R.B., M.E. Nunn, and D.H. Lavrin. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity. *International journal of cancer. Journal international du cancer* 16:216-229.

Ho, M. 1991. Cytomegalovirus: Biology and Infection.

Holtappels, R., D. Gillert-Marien, D. Thomas, J. Podlech, P. Deegen, S. Herter, S.A. Oehrlein-Karpi, D. Strand, M. Wagner, and M.J. Reddehase. 2006. Cytomegalovirus encodes a positive regulator of antigen presentation. *J Virol* 80:7613-7624.

Horng, T., J.S. Bezbradica, and R. Medzhitov. 2007. NKG2D signaling is coupled to the interleukin 15 receptor signaling pathway. *Nat Immunol* 8:1345-1352.

Jiang, K., B. Zhong, D.L. Gilvary, B.C. Corliss, E. Hong-Geller, S. Wei, and J.Y. Djeu. 2000. Pivotal role of phosphoinositide-3 kinase in regulation of cytotoxicity in natural killer cells. *Nat Immunol* 1:419-425.

Jiang, K., B. Zhong, D.L. Gilvary, B.C. Corliss, E. Vivier, E. Hong-Geller, S. Wei, and J.Y. Djeu. 2002. Syk regulation of phosphoinositide 3-kinase-dependent NK cell function. *J Immunol* 168:3155-3164.

Kalinski, P., R.B. Mailliard, A. Giermasz, H.J. Zeh, P. Basse, D.L. Bartlett, J.M. Kirkwood, M.T. Lotze, and R.B. Herberman. 2005. Natural killer-dendritic cell cross-talk in cancer immunotherapy. *Expert opinion on biological therapy* 5:1303-1315.

Kaplan, M.H., Y.L. Sun, T. Hoey, and M.J. Grusby. 1996. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* 382:174-177.

Keating, R., T. Hertz, M. Wehenkel, T.L. Harris, B.A. Edwards, J.L. McClaren, S.A. Brown, S. Surman, Z.S. Wilson, P. Bradley, J. Hurwitz, H. Chi, P.C. Doherty, P.G. Thomas, and M.A. McGargill. 2013. The kinase mTOR modulates the antibody response to provide cross-protective immunity to lethal infection with influenza virus. *Nat Immunol* 14:1266-1276.

Kiessling, R., E. Klein, and H. Wigzell. 1975. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur J Immunol* 5:112-117.

Kiessling, R., G. Petranyi, K. Karre, M. Jondal, D. Tracey, and H. Wigzell. 1976. Killer cells: a functional comparison between natural, immune T-cell and antibody-dependent in vitro systems. *J Exp Med* 143:772-780.

Kim, E.H., and M. Suresh. 2013. Role of PI3K/Akt signaling in memory CD8 T cell differentiation. *Front Immunol* 4:20.

Kobayashi, H., S. Dubois, N. Sato, H. Sabzevari, Y. Sakai, T.A. Waldmann, and Y. Tagaya. 2005. Role of trans-cellular IL-15 presentation in the activation of NK cell-mediated killing, which leads to enhanced tumor immunosurveillance. *Blood* 105:721-727.

Koka, R., P. Burkett, M. Chien, S. Chai, D.L. Boone, and A. Ma. 2004. Cutting edge: murine dendritic cells require IL-15R alpha to prime NK cells. *J Immunol* 173:3594-3598.

Kovanen, P.E., and W.J. Leonard. 2004. Cytokines and immunodeficiency diseases: critical roles of the gamma(c)-dependent cytokines interleukins 2, 4, 7, 9, 15, and 21, and their signaling pathways. *Immunol Rev* 202:67-83.

Kundig, T.M., H. Schorle, M.F. Bachmann, H. Hengartner, R.M. Zinkernagel, and I. Horak. 1993. Immune responses in interleukin-2-deficient mice. *Science* 262:1059-1061.

Lanier, L.L. 1998. NK cell receptors. *Annu Rev Immunol* 16:359-393.

Lanier, L.L. 2008. Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol* 9:495-502.

Laplante, M., and D.M. Sabatini. 2012. mTOR signaling in growth control and disease. *Cell* 149:274-293.

Lauwerys, B.R., N. Garot, J.C. Renauld, and F.A. Houssiau. 2000. Cytokine production and killer activity of NK/T-NK cells derived with IL-2, IL-15, or the combination of IL-12 and IL-18. *J Immunol* 165:1847-1853.

Lee, S.H., S. Girard, D. Macina, M. Busa, A. Zafer, A. Belouchi, P. Gros, and S.M. Vidal. 2001. Susceptibility to mouse cytomegalovirus is associated with deletion of an activating natural killer cell receptor of the C-type lectin superfamily. *Nat Genet* 28:42-45.

Lee, S.H., T. Miyagi, and C.A. Biron. 2007. Keeping NK cells in highly regulated antiviral warfare. *Trends Immunol* 28:252-259.

Leonard, W.J., and J.J. O'Shea. 1998. Jaks and STATs: biological implications. *Annu Rev Immunol* 16:293-322.

Li, Q., R.R. Rao, K. Araki, K. Pollizzi, K. Odunsi, J.D. Powell, and P.A. Shrikant. 2011. A central role for mTOR kinase in homeostatic proliferation induced CD8+ T cell memory and tumor immunity. *Immunity* 34:541-553.

Ljunggren, H.G., and K. Karre. 1990. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunology today* 11:237-244.

Lodolce, J.P., D.L. Boone, S. Chai, R.E. Swain, T. Dassopoulos, S. Trettin, and A. Ma. 1998. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity* 9:669-676.

Loh, J., D.T. Chu, A.K. O'Guin, W.M. Yokoyama, and H.W.t. Virgin. 2005. Natural killer cells utilize both perforin and gamma interferon to regulate murine cytomegalovirus infection in the spleen and liver. *J Virol* 79:661-667.

Loza, M.J., and B. Perussia. 2001. Final steps of natural killer cell maturation: a model for type 1-type 2 differentiation? *Nat Immunol* 2:917-924.

Lucas, M., W. Schachterle, K. Oberle, P. Aichele, and A. Diefenbach. 2007. Dendritic cells prime natural killer cells by trans-presenting interleukin 15. *Immunity* 26:503-517.

Lucin, P., S. Jonjic, M. Messerle, B. Polic, H. Hengel, and U.H. Koszinowski. 1994. Late phase inhibition of murine cytomegalovirus replication by synergistic action of interferon-gamma and tumour necrosis factor. *J Gen Virol* 75 (Pt 1):101-110.

Ma, A., R. Koka, and P. Burkett. 2006. Diverse functions of IL-2, IL-15, and IL-7 in lymphoid homeostasis. *Annu Rev Immunol* 24:657-679.

Macintyre, A.N., D. Finlay, G. Preston, L.V. Sinclair, C.M. Waugh, P. Tamas, C. Feijoo, K. Okkenhaug, and D.A. Cantrell. 2011. Protein kinase B controls transcriptional programs that direct cytotoxic T cell fate but is dispensable for T cell metabolism. *Immunity* 34:224-236.

Mamane, Y., E. Petroulakis, O. LeBacquer, and N. Sonenberg. 2006. mTOR, translation initiation and cancer. *Oncogene* 25:6416-6422.

Marcais, A., J. Cherfils-Vicini, C. Viant, S. Degouve, S. Viel, A. Fenis, J. Rabilloud, K. Mayol, A. Tavares, J. Bienvenu, Y.G. Gangloff, E. Gilson, E. Vivier, and T. Walzer. 2014. The metabolic checkpoint kinase mTOR is essential for IL-15 signaling during the development and activation of NK cells. *Nat Immunol*

Martin-Fontecha, A., L.L. Thomsen, S. Brett, C. Gerard, M. Lipp, A. Lanzavecchia, and F. Sallusto. 2004. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. *Nat Immunol* 5:1260-1265.

McCartney, S., W. Vermi, S. Gilfillan, M. Cella, T.L. Murphy, R.D. Schreiber, K.M. Murphy, and M. Colonna. 2009. Distinct and complementary functions of MDA5 and TLR3 in poly(I:C)-mediated activation of mouse NK cells. *J Exp Med* 206:2967-2976.

Meresse, B., Z. Chen, C. Ciszewski, M. Tretiakova, G. Bhagat, T.N. Krausz, D.H. Raulet, L.L. Lanier, V. Groh, T. Spies, E.C. Ebert, P.H. Green, and B. Jabri. 2004. Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. *Immunity* 21:357-366.

Miyagi, T., S.H. Lee, and C.A. Biron. 2010. Intracellular staining for analysis of the expression and phosphorylation of signal transducers and activators of transcription (STATs) in NK cells. *Methods in molecular biology* 612:159-175.

Mogensen, T.H. 2009. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin Microbiol Rev* 22:240-273, Table of Contents.

Monteiro, J.M., C. Harvey, and G. Trinchieri. 1998. Role of interleukin-12 in primary influenza virus infection. *J Virol* 72:4825-4831.

Moretta, A. 2002. Natural killer cells and dendritic cells: rendezvous in abused tissues. *Nat Rev Immunol* 2:957-964.

Mrozek, E., P. Anderson, and M.A. Caligiuri. 1996. Role of interleukin-15 in the development of human CD56+ natural killer cells from CD34+ hematopoietic progenitor cells. *Blood* 87:2632-2640.

Nelms, K., A.D. Keegan, J. Zamorano, J.J. Ryan, and W.E. Paul. 1999. The IL-4 receptor: signaling mechanisms and biologic functions. *Annu Rev Immunol* 17:701-738.

Nguyen, K.B., T.P. Salazar-Mather, M.Y. Dalod, J.B. Van Deusen, X.Q. Wei, F.Y. Liew, M.A. Caligiuri, J.E. Durbin, and C.A. Biron. 2002. Coordinated and distinct roles for IFN-alpha beta, IL-12, and IL-15 regulation of NK cell responses to viral infection. *J Immunol* 169:4279-4287.

Orange, J.S. 2002. Human natural killer cell deficiencies and susceptibility to infection. *Microbes Infect* 4:1545-1558.

Orange, J.S., and C.A. Biron. 1996. Characterization of early IL-12, IFN-alphabeta, and TNF effects on antiviral state and NK cell responses during murine cytomegalovirus infection. *J Immunol* 156:4746-4756.

Orange, J.S., B. Wang, C. Terhorst, and C.A. Biron. 1995. Requirement for natural killer cell-produced interferon gamma in defense against murine cytomegalovirus infection and enhancement of this defense pathway by interleukin 12 administration. *J Exp Med* 182:1045-1056.

Park, S.Y., K. Saijo, T. Takahashi, M. Osawa, H. Arase, N. Hirayama, K. Miyake, H. Nakauchi, T. Shirasawa, and T. Saito. 1995. Developmental defects of lymphoid cells in Jak3 kinase-deficient mice. *Immunity* 3:771-782.

Powell, J.D., and G.M. Delgoffe. 2010. The mammalian target of rapamycin: linking T cell differentiation, function, and metabolism. *Immunity* 33:301-311.

Qian, D., and A. Weiss. 1997. T cell antigen receptor signal transduction. *Curr Opin Cell Biol* 9:205-212.

Rabot, M., H. El Costa, B. Polgar, A. Marie-Cardine, M. Aguerre-Girr, A. Barakonyi, S. Valitutti, A. Bensussan, and P. Le Bouteiller. 2007. CD160-activating NK cell effector functions depend on the phosphatidylinositol 3-kinase recruitment. *Int Immunol* 19:401-409.

Randall, R.E., and S. Goodbourn. 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. *J Gen Virol* 89:1-47.

Raulet, D.H., R.E. Vance, and C.W. McMahon. 2001. Regulation of the natural killer cell receptor repertoire. *Annu Rev Immunol* 19:291-330.

Rosmaraki, E.E., I. Douagi, C. Roth, F. Colucci, A. Cumano, and J.P. Di Santo. 2001. Identification of committed NK cell progenitors in adult murine bone marrow. *Eur J Immunol* 31:1900-1909.

Rubinsztein, D.C. 2006. The roles of intracellular protein-degradation pathways in neurodegeneration. *Nature* 443:780-786.

Shiloni, E., A. Eisenthal, D. Sachs, and S.A. Rosenberg. 1987. Antibody-dependent cellular cytotoxicity mediated by murine lymphocytes activated in recombinant interleukin 2. *J Immunol* 138:1992-1998.

Siren, J., T. Sareneva, J. Pirhonen, M. Strengell, V. Veckman, I. Julkunen, and S. Matikainen. 2004. Cytokine and contact-dependent activation of natural killer cells by influenza A or Sendai virus-infected macrophages. *J Gen Virol* 85:2357-2364.

Smith, H.R., J.W. Heusel, I.K. Mehta, S. Kim, B.G. Dorner, O.V. Naidenko, K. Iizuka, H. Furukawa, D.L. Beckman, J.T. Pingel, A.A. Scalzo, D.H. Fremont, and W.M. Yokoyama. 2002. Recognition of a virus-encoded ligand by a natural killer cell activation receptor. *Proc Natl Acad Sci U S A* 99:8826-8831.

Smith, L.M., A.R. McWhorter, L.L. Masters, G.R. Shellam, and A.J. Redwood. 2008. Laboratory strains of murine cytomegalovirus are genetically similar to but phenotypically distinct from wild strains of virus. *J Virol* 82:6689-6696.

Smyth, M.J., K. Takeda, Y. Hayakawa, J.J. Peschon, M.R. van den Brink, and H. Yagita. 2003. Nature's TRAIL--on a path to cancer immunotherapy. *Immunity* 18:1-6.

Steinman, R.M. 1991. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* 9:271-296.

Steinman, R.M., and Z.A. Cohn. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* 137:1142-1162.

Steinman, R.M., and H. Hemmi. 2006. Dendritic cells: translating innate to adaptive immunity. *Curr Top Microbiol Immunol* 311:17-58.

Stoklasek, T.A., K.S. Schluns, and L. Lefrancois. 2006. Combined IL-15/IL-15Ralpha immunotherapy maximizes IL-15 activity in vivo. *J Immunol* 177:6072-6080.

Tabeta, K., P. Georgel, E. Janssen, X. Du, K. Hoebe, K. Crozat, S. Mudd, L. Shamel, S. Sovath, J. Goode, L. Alexopoulou, R.A. Flavell, and B. Beutler. 2004. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proc Natl Acad Sci U S A* 101:3516-3521.

Takeda, K., H. Tsutsui, T. Yoshimoto, O. Adachi, N. Yoshida, T. Kishimoto, H. Okamura, K. Nakanishi, and S. Akira. 1998. Defective NK cell activity and Th1 response in IL-18-deficient mice. *Immunity* 8:383-390.

Tay, C.H., and R.M. Welsh. 1997. Distinct organ-dependent mechanisms for the control of murine cytomegalovirus infection by natural killer cells. *J Virol* 71:267-275.

Thielens, A., E. Vivier, and F. Romagne. 2012. NK cell MHC class I specific receptors (KIR): from biology to clinical intervention. *Curr Opin Immunol* 24:239-245.

Thiery, J., D. Keefe, S. Boulant, E. Boucrot, M. Walch, D. Martinvalet, I.S. Goping, R.C. Bleackley, T. Kirchhausen, and J. Lieberman. 2011. Perforin pores in the endosomal membrane trigger the release of endocytosed granzyme B into the cytosol of target cells. *Nat Immunol* 12:770-777.

Trinchieri, G. 1989. Biology of natural killer cells. *Adv Immunol* 47:187-376.

van Dommelen, S.L., N. Sumaria, R.D. Schreiber, A.A. Scalzo, M.J. Smyth, and M.A. Degli-Esposti. 2006. Perforin and granzymes have distinct roles in defensive immunity and immunopathology. *Immunity* 25:835-848.

Verneris, M.R., M. Karami, J. Baker, A. Jayaswal, and R.S. Negrin. 2004. Role of NKG2D signaling in the cytotoxicity of activated and expanded CD8+ T cells. *Blood* 103:3065-3072.

Vetter, C.S., V. Groh, P. thor Straten, T. Spies, E.B. Brocker, and J.C. Becker. 2002. Expression of stress-induced MHC class I related chain molecules on human melanoma. *J Invest Dermatol* 118:600-605.

Vivier, E., S. Ugolini, D. Blaise, C. Chabannon, and L. Brossay. 2012. Targeting natural killer cells and natural killer T cells in cancer. *Nat Rev Immunol* 12:239-252.

Wang, K.S., D.A. Frank, and J. Ritz. 2000. Interleukin-2 enhances the response of natural killer cells to interleukin-12 through up-regulation of the interleukin-12 receptor and STAT4. *Blood* 95:3183-3190.

Wei, S., A.M. Gamero, J.H. Liu, A.A. Daulton, N.I. Valkov, J.A. Trapani, A.C. Larner, M.J. Weber, and J.Y. Djeu. 1998. Control of lytic function by mitogen-activated protein kinase/extracellular regulatory kinase 2 (ERK2) in a human natural killer cell line: identification of perforin and granzyme B mobilization by functional ERK2. *J Exp Med* 187:1753-1765.

Weller, T.H. 1971. The cytomegaloviruses: ubiquitous agents with protean clinical manifestations. I. *N Engl J Med* 285:203-214.

Wendland, T., S. Herren, N. Yawalkar, A. Cerny, and W.J. Pichler. 2000. Strong alpha beta and gamma delta TCR response in a patient with disseminated Mycobacterium avium infection and lack of NK cells and monocytopenia. *Immunol Lett* 72:75-82.

Xu, X., L. Ye, K. Araki, and R. Ahmed. 2012. mTOR, linking metabolism and immunity. *Semin Immunol* 24:429-435.

Yao, Z., Y. Cui, W.T. Watford, J.H. Bream, K. Yamaoka, B.D. Hissong, D. Li, S.K. Durum, Q. Jiang, A. Bhandoola, L. Hennighausen, and J.J. O'Shea. 2006. Stat5a/b are essential for normal lymphoid development and differentiation. *Proc Natl Acad Sci U S A* 103:1000-1005.

Yokoyama, W.M., S. Kim, and A.R. French. 2004. The dynamic life of natural killer cells. *Annu Rev Immunol* 22:405-429.

Zhou, C., M.M. Tabb, E.L. Nelson, F. Grun, S. Verma, A. Sadatrafiei, M. Lin, S. Mallick, B.M. Forman, K.E. Thummel, and B. Blumberg. 2006. Mutual repression between steroid and xenobiotic receptor and NF-kappaB signaling pathways links xenobiotic metabolism and inflammation. *J Clin Invest* 116:2280-2289.

EDUCATIONAL QUALIFICATONS

DEGREE	SUBJECT	UNIVERSITY/SCHOOL	DURATION
Master of Science	Microbiology and Immunology	Faculty of Medicine, University of Ottawa, Canada	2012-2014
Master of Science	Molecular Biology and Human Genetics	Manipal Life Sciences Centre, Manipal University, India	2009-2011
Bachelor of Technology	Biotechnology	SASTRA University, India	2005-2009

PUBLICATIONS:

Nandagopal, N., et al., *The critical role of IL-15-PI3K-mTOR pathway in natural killer cell effector functions*. Frontiers in Immunology, April 2014. 5:187.

RESEARCH PROJECTS

- The critical role of IL-15-PI3K-mTOR pathway in natural killer cell effector functions, Department of Microbiology and Immunology, University of Ottawa, 2014
  Thesis supervisor: Dr. Seung-Hwan lee
- Effect of deletions and aminoacid substitutions on the phosphorylation patterns of Rotavirus Non Structural Protein 5 (NSP5), Department of Microbiology and Cell Biology**,** Indian Institute of Science, 2011
  Thesis supervisor: Dr. Durga Rao
- Formulation and stability studies on two IgG1 therapeutic antibody drugs, Analytical and protein formulations lab, R&D, Biocon Ltd , 2009
  Thesis supervisor: Dr. Karthik Ramani

SCHOLARSHIPS & AWARDS

- Full international admission scholarship, University of Ottawa, 2012-2014
- Tuition fee bursary, Employee financial aid fund, CUPE, University of Ottawa, 2014
- First in poster presentations, Annual poster day, "Effect of STAT6 deletions on anti-viral immune responses", Department of Microbiology, Immunology and Biochemistry, University of Ottawa, 2013
- Undergraduate merit scholarship (Top 10% of students), SASTRA University, India, 2007
- Second in contraptions, National technical fest at College of Engineering, Anna University, India, 2008
- Third in contraptions, International technical fest at National Institute of Technology, India, 2008
- Awarded School First in Chemistry, State level grade-12 examinations, St. Joseph's Higher Secondary School, India, 2005

\

TECHNICAL SKILLS

Molecular Biology: Basic and real-time PCR, basic electrophoresis techniques like SDS-PAGE, agarose gels and western blotting, *in vitro* kinase and chromatin immunoprecipitation assays

Genetic engineering: Cloning into *E.coli* strains (DH5α, BL21), bacterial transformation ($CaCl_2$ Method), Mini & Maxi plasmid preparations

Protein purification: Protein isolation and purification by Immobilized Affinity Chromatography and protein dialysis

Genetics: Routine genotyping of mouse tail and ear snips, chromosome karyotyping and banding techniques, Isolation of polytene chromosomes from Drosophila, Comet and MTT assays for genotoxicity studies

Cell isolation and culture: Isolation of liver-, spleen-, blood and bone marrow lymphocytes, isolation of peripheral blood mononuclear cells from infected human HCV samples, intracellular and surface staining of cells and analysis by flow cytometry, enrichment of natural killer and T cells from lymphocytes by magnetic beads followed by flow sorting, basic long-term culturing of primary mouse and mouse cell lines

Animal handling: Isolation of embryonic stem cells from mouse fetus, preparation of murine cytomegalovirus-viral stocks from BALB/C mice, intraperitoneal MCMV- and intranasal influenza infections, intravenous tail injections for adoptive cell transfers, saphenous vein bleeding and basic mice colony maintenance and breeding

PRESENTATIONS

- Poster on "The critical Role of PI3K-AKT-mTOR pathway in IL-15 mediated priming of natural killer cell functions" at the Canadian Society for Immunologists, Quebec city, Canada, 2014
- Poster on "Effect of STAT6 deletions on anti-viral immune responses", Department of Biochemistry, Microbiology and Immunology student poster presentations, University of Ottawa, 2013
- Poster on "The formulation and development of an automated agarose gel dispensing system – Gel X ", Bangalore Bio International expo, 2010

CERTIFICATE COURSES

- Teaching assistantship training workshop, Centre for University Teaching, University of Ottawa certificate course, 2012
- Training in animal care and handling, National Institutional Animal User training (NIAUT), certificate course, University of Ottawa, 2012
- Cell to cell communication, Centre for Cellular and Molecular Biology (CCMB), India, certificate course, SASTRA University, 2007
- Technical Writing in English, certificate course, SASTRA University, 2006

INTERSHIPS

- Research Trainee, Department of Microbiology and Cell biology, Indian Institute of Science, India 2010-2011
- Research Trainee, Analytical and protein formulations lab, R&D, Biocon Ltd, India 2008-09
- Summer student, Sangene Institute of Biosciences, India, 2008

ASSISTANTSHIPS

- Teaching Assistant for the undergraduate laboratory course on Introduction to Biochemistry, 2014
- Demonstrator for protein isolation and purification techniques at the Department of Biotechnology-teachers training course, Indian Institute of Science, 2011
- Teaching assistant for the undergraduate course on Genetics, Manipal University, 2010

ACADEMIC CONFERENCES & TECHNICAL SYMPOSIUMS

Organized

- Event coordinator, The 2nd National conference of Molecular Virology, Indian Institute of Science, Bangalore, 2011
- Student Event coordinator for the theme event, DAKSH 2007, National technical symposium of SASTRA University, India

Attended

- 27th Annual Canadian Society for Immunology Conference, Quebec City, Canada, 2014
- Genetics in Clinical Practice, Kasturba Medical College, Manipal and Manipal Life Science Centre, Manipal University in 2009
- International Technical Festival, National Institute of Technology, India, 2008
- National workshop on Biomaterials and Biosciences organized by Students' Chapter of Society for Biomaterials and Artificial Organs (India) , SASTRA University, 2005

MEMBERSHIPS

- Canadian Society for Immunology, CSI, 2013
- BIOTIKOS, a Biotechnology students association, SASTRA University, 2005-2009

EXTRA-CURRICULAR ACTIVITIES

- Volunteer for South Asian community festival of Canada, Ottawa 2013
- Taught Arts &Crafts at the Vananthurai Children's Community Center, Chennai, India, 2012

- Open day volunteer, an initiative by the scientific community of Indian Institute of Science to educate school students and public on on-going research activities, 2011
- Event volunteer for UTSAV, The International Cultural Festival of Manipal University, 2010
- Student committee for organizing activity-day for the children of Kendriya Vidhyalaya Orphanage, Manipal University, India, 2010
- Marketing head for NUCLEO, Student Biotechnology Magazine, SASTRA University, 2009
- Graded A+ for an undergraduate human resources project on "Assessing the basic amenities like Education, Employment and other issues like Health care in Udaiyalur village", India, SASTRA University, 2009
- Consecutively awarded first for interpreting issues like "Child marriage", "Women empowerment" and "War on terrorism" through Eastern group dances at university events, 2006-2010