Mechanisms of Natural Killer Cell Activation to Viral Infection

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

Natural killer (NK) cells are lymphocytes of the innate immune response with well-demonstrated activities against viral infections and tumors. Because of these abilities, we sought to glean insights into the mechanisms of NK cell activation so that they may be applied toward the design of new therapies.

NK cells are particularly critical for the control of poxviral infections. Vaccinia virus (VV) is the most-studied member of the poxviral family. It is robustly immunogenic and functions as the live vaccine responsible for the successful elimination of smallpox. VV infection provides a useful model for studying NK cell activation: NK cells play an important role in its clearance and the virus efficiently activates NK cells and recruits them to the site of infection. We had previously used this model to identify Toll-like receptor (TLR)-dependent and –independent mechanisms of NK cell activation to VV. One method of TLR-independent activation to VV requires the activation receptor NKG2D, which recognizes host ligands expressed upon viral infection by accessory cells such as dendritic cells (DCs) and macrophages.

In the first aim of this thesis, we sought to determine how the ligands for the NKG2D activation receptor become upregulated in the context of VV infection. Specifically, we asked whether interleukin-18 (IL-18), known to play a role in the innate immune response, could boost the expression of NKG2D ligands on DCs in response to

viral infection. Using an in vivo infection model with IL-18R-deficient mice, our results confirmed an important role for IL-18 in NK cell activation to VV and viral control. We then made use of an NK-DC co-culture to show that IL-18 signaling on DCs, in addition to NK cells, is necessary to achieve efficient NK cell activation to viral infection. We further demonstrated in a cell-transfer experiment that cell-extrinsic IL-18 signaling is critical for NK cell activation in vivo. DC ablation via a mouse model designed to specifically ablate $CD11c^+$ cells showed that DCs are also required for NK cell activation to VV in vivo. We finally showed how IL-18 can act on DCs in vivo and in vitro to boost the expression of Rae-1, an NKG2D ligand. Collectively, our data uncover a novel mechanism whereby NK cells become activated by IL-18 control of NKG2D ligand expression on DCs.

In the second aim of this project, we detailed how IL-18 signaling results in the upregulation of the NKG2D ligand Rae-1. Using an in vitro macrophage model, we showed how recombinant IL-18 was sufficient to upregulate Rae-1 expression. We compared IL-18 control of Rae-1 expression to LPS, a TLR ligand that also signals through the common adaptor MyD88 to govern Rae-1 expression. Using chemical inhibitors to cell signaling molecules, we then identified the importance of MyD88 signaling through PI3K. We then revealed that glycogen synthase kinase 3 (GSK-3) can act as a negative regulator of Rae-1 expression downstream of IL-18/TLR signaling. Specifically, we have shown that during inflammatory signaling, PI3K (acting

downstream of MyD88) can inhibit GSK-3 to relieve its tonic suppression of Rae-1 expression and upregulate the NKG2D ligand. Finally, we showed that PI3K and GSK-3 signaling are also important to Rae-1 expression on DCs - the accessory cell where IL-18 signals to control Rae-1 expression to boost NK cell activation against VV.

In its entirety, this work seeks to address how NK cells become activated in the context of VV infection in order to identify new ways NK cells may be harnessed therapeutically.

Dedication

For my family, who make it all possible – my wife, Kaitlin, my sister, Rachel, and my parents, Bonnie and Marty. Love you forever and always.

Contents

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List of Abbreviations

ANOVA	Analysis of variance
ANCOVA	Analysis of covariance
AP-1	Activator protein-1
APC	Antigen-presenting cell
BM	Bone marrow
BMDC	Bone marrow-derived dendritic cell
CD	Cluster of differentiation
cDC	Conventional dendritic cell
CEV	Cell-associated enveloped virus
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
EBV	Epstein Barr virus
EEV	Extracellular enveloped virus
ELISA	Enzyme-linked immunosorbant assay
ERK	Extracellular signal-regulated kinases
FACS	Fluorescence activated cell sorting

GRB	Granzyme B
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPCR	G protein-coupled receptor
GSK-3	Glycogen synthase kinase-3
H60	Histocompatibility 60
HA	Hemagglutinin
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSV	Herpes simplex virus
IEV	Intracellular enveloped virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-18R	Interleukin-18 receptor
IMV	Intracellular mature virus
ip	Intraperitoneal
IPS-1	IFNβ promoter stimulator-1
IRAK	Interleukin 1 receptor-associated kinase
IRF	Interferon regulatory factor

ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
iv	Intravenous
IV	Immature virus
JNK	c-Jun N-terminal kinase
Kbp	Kilobase pair
KIR	Killer immunoglobulin receptor
LCMV	Lymphocytic choriomeningitis virus
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MAPK	Mitogen-activated protein kinase
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complex
MICA	MHC class-I-chain-related protein A
MIP-1α	Macrophage inflammatory protein 1α
miRNA	microRNA
MKK	Mitogen-activated protein kinase kinase
MOI	Multiplicity of infection
Mult-1	Mouse UL16-binding protein-like transcript 1
MyD88	Myeloid differentiation primary response gene 88

NCR	Natural cytotoxicity receptor
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NKG2D	NK group 2 member D
PAMP	Pathogen-associated molecular pattern
pDC	Plasmacytoid dendritic cell
PI3K	Phosphatidylinositol 3-kinase
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptor
pAkt	Phosphorylated Akt
pGSK-3	Phosphorylated GSK-3
PCR	Polymerase chain reaction
pfu	Plaque-forming units
Rae-1	Retinoic acid early transcript 1
RIG-1	Retinoic acid-inducible gene I
rIL-18	Recombinant IL-18
RT-PCR	Reverse transcription PCR
sem	Standard error of the mean
STAT	Signal transducers and activators of transcription

TAK	Transforming growth factor-β-activated kinase I
TCR	T cell receptor
TGFβ	Transforming growth factor β
TGN	*trans*-Golgi network
TIR	Toll/interleukin 1 receptor-like domain
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TRAF	TNF receptor associated factor
TRAIL	Tumor-necrosis factor-related apoptosis-inducing ligand
ULBP	UL-16 binding proteins
VSV	Vesicular stomatitis virus
VV	Vaccinia virus
WNV	West Nile virus
WR	Western reserve
WT	Wild type

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1. Introduction

A modified version of some content in this chapter was published in the *Journal of Innate Immunity* (Brandstadter and Yang, 2011). Copyrighted material is used with permission from the journal.

1.1 NK Cells

1.1.1 NK Cells & the Innate Immune Response

The vertebrate immune system has long been divided into two responses: innate and adaptive. The adaptive immune response, of particular interest to early immunologists, is evolutionarily newer and named based on its ability to mount specific attacks against pathogenic antigens. Adaptive immunity's clonal specificity, which occurs after genetic recombination in a pool of T and B lymphocytes, can also be recalled upon repeat antigen exposure due to the persistence of certain long-lived memory lymphocytes. The specificity and memory provided in the adaptive immune response have become the cornerstones of modern vaccination and immunotherapeutic strategies.

However, over the last twenty-five years, immunologists have renewed their attention in the innate immune response (Janeway, 1989). Innate immunity is the first line of defense against invading pathogens, which it identifies by germline-encoded pattern recognition receptors (PRRs) capable of recognizing pathogen-associated molecular patterns (PAMPS) to discern "self" from "non-self." Innate immune cells, like neutrophils and macrophages, not only engulf pathogens by phagocytosis, but produce soluble cytokines capable of activating and recruiting other inflammatory cells of both

the innate and adaptive immune systems. Other innate immune cells, such as dendritic cells (DCs), are professional antigen presenting cells and focus on eliciting the adaptive immune response. Innate immunity plays critical roles in provoking and controlling the adaptive immune system.

Natural killer (NK) cells are a unique population of lymphocytes that - despite sharing morphology, a common hematopoietic progenitor, and cellular machinery with T and B cells - lack a clonally specific receptor and act as part of the innate immune response. They respond during the first few days of an infection and rely on an array of receptors capable of recognizing pathogens, host stress ligands, and peptide-loaded and unloaded class I major histocompatibility complex (MHC) molecules. NK cells have particularly well-demonstrated activities in antiviral immunity and tumor immune surveillance.

1.1.2 Markers for NK Cell Identification

Initial recognition of NK cells relied on histologic identification where they were distinguishable as larger and more granular lymphocytes. NK cells had often been referred to as "null cells" as they were believed to be completely devoid of cell surface markers (Lanier, 2007). While one hallmark for all NK cells remains the absence of CD3, the T cell receptor, NK cells express many cell surface molecules and a number of them are sufficiently sensitive and specific to be used to positively identify them. The markers used to discern NK cells, however, are different between humans and mice and even between mouse backgrounds.

In humans, NK cells are commonly defined as $CD56^{+}CD3^{-}$ cells – a population which accounts for 15 percent of all peripheral blood lymphocytes (Jost and Altfeld, 2013). However, CD56 expression varies during NK development as immature $CD56^{bright}$ cells differentiate into $CD56^{dim}$ cells, which express the activating CD16 receptor as well as killer immunoglobulin receptors (KIRs) (Chan et al., 2007; Cooper et al., 2001). There is also population of $CD56^{-}CD16^{+}$ NK cells present in increased numbers in HIV-1 seropositive patients (Mavilio et al., 2003). This population increases during ongoing viral replication with concomitant decreases in mature $CD56^{dim}CD16^{+}$ NK cells and may result from exhaustion or anergy due to chronic infection (Alter et al., 2005).

Murine NK cells, on the other hand, do not express CD56. They can be identified, in C57BL/6 mice, by the presence of NK1.1. However, this marker isn't expressed by NK cells from other mouse backgrounds like Balb/c or 129. CD49b (DX5), an integrin, can be used to distinguish NK cells across murine backgrounds. CD49b can be found on mature NK cells, and will exclude both immature and some populations of tissue-resident NK cells. CD49b is also weakly expressed on basophils.

NKp46, the natural cytotoxicity receptor, has recently become the favored marker for NK cells as it is both the most sensitive and specific identifying cell surface molecule available (Walzer et al., 2007c). NKp46 is found on both immature $CD49b^{-}$ and mature $CD49b^{+}$ NK cells and is absent on CD1d-restricted NKT cells. NKp46 is expressed on NK cells from a wide range of mouse backgrounds and can also be found on human NK cells (Walzer et al., 2007a) However, even this marker is absent on a small number of $CD56^{+}CD3^{-}$ cells in humans (Caligiuri, 2008).

1.2 Mechanisms of NK Cell Activation

NK cells were originally identified as a subset of lymphocytes capable of mediating cytolysis of cultured lymphoma cells without any prior sensitization. Based on this observation, NK cells were originally considered to activate and lyse cells nonspecifically.

Subsequent work revealed that NK cells had a predilection for targeting cells that had downregulated their class I MHC molecules due to viral infection or transformation to evade T cell recognition. This predicted the existence of inhibition receptors on NK cells – later identified – and led to the genesis of the 'missing self' hypothesis whereby NK cells default toward killing unless their inhibition receptors recognize intact class I MHC expression, indicating the cell is healthy (Karre et al., 1986).

However, NK cell are capable of targeting cells that maintain expression of class I MHC (Khakoo and Carrington, 2006; Stewart et al., 2005). In addition, many activation receptors capable of recognizing pathogens and host stress signals have been identified. NK cell activation is now best understood as the result of a balance between activation and inhibition signaling (Lanier, 2008).

1.2.1 Activation Receptors

The "missing self" hypothesis predicted the mechanism whereby NK cells destroy virally infected cells that have downregulated expression of MHC class I (Karre, 2008; Karre et al., 1986). However, in many circumstances NK cells can efficiently eliminate virus-infected cells that maintain expression of the inhibitory MHC class I (Khakoo and Carrington, 2006; Stewart et al., 2005). Recent advances have indicated that

NK cell activation and function are regulated by the interplay between the inhibitory and activating receptors (Bryceson et al., 2009; Lanier, 2008). Indeed, accumulating evidence has revealed the importance of NK activating receptors in antiviral defense.

Table 1: A summary of NK cell receptors during viral infections.

	Virus	Activation Receptors	Inhibition Receptors
mouse	MCMV	Ly49H, NKG2D	Ly49A, Ly49I
	VV	NKG2D, TLR2	
	WNV	NKp44	
	Influenza	NKp46	
human	HCMV	NKp46, NKp30, DNAM-1	KIR2DL1, NKG2A/CD94
	HCV	NKp30	KIR2DL3, NKG2A/CD94
	HIV-1	KIR3DS1	
	EBV	KIR2DS1	

Downstream of these activating receptors, ligand recognition triggers an intracellular kinase cascade to transmit the activation signal (Lanier, 2008). This cascade begins with Src tyrosine kinase phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs), which are found on adaptor subunits KARAP/DAP-12, FcεRIγ, and CD3ζ. The phosphorylated ITAMs then recruit tandem-SH2 tyrosine kinases Syk and ZAP70, which propagate a phosphorylation signaling cascade through transmembrane and cytosolic adaptors similar to T/B-cell receptor signaling in fellow lymphocytes. Ultimate downstream activation pathways include phosphatidylinositol 3-kinase (PI3K)/ERK, Ras/ERK, and PLCγ/DAG/IP_3.

1.2.1.1 Ly49 and KIR

The first NK cell activating receptor identified to be critical for viral control in vivo was Ly49H, which is necessary to clear MCMV infection (Brown et al., 2001). Ly49H, a C-type-lectin-like receptor, specifically recognizes the m157 open reading frame of MCMV. This ligand was identified by two independent groups using heterologous reporter cells exposed to MCMV-infected cells (Arase et al., 2002; Smith et al., 2002b). Activation of Ly49H by m157 is required for NK cell-mediated clearance in MCMV-resistant mice. Deletion of Ly49H's genetic locus, *Cmv1*^{r}, or use of Ly49H-blocking antibodies confers susceptibility to the virus (French and Yokoyama, 2003). Deletion of m157 also facilitates viral escape and persistence later in the infection.

Ly49H is a unique member of the Ly49 receptor family as it is the only one known to directly recognize a pathogen (Jost and Altfeld, 2013). All other Ly49 receptors, which can also be inhibitory, recognize specific allotypes of the H-2D and H-2K MHC class I molecules. Ly49D, for example, activates NK cells after an interaction with its ligands (H-2D^{d}, H-2D^{r}, and H-2D^{sp}) and can result in rejection of bone marrow allografts in vivo (George et al., 1999).

The Ly49 lectin-like receptors do not exist in humans. However, structurally different killer immunoglobulin-like receptors (KIR) function similarly and recognize MHC class I molecules. Specifically, KIRs recognize particular serotypes of classical human leukocyte antigen (HLA) -A, -B, and -C in addition to non-classical HLA- G. For example, KIRs can discern between the serological motifs HLA-Bw4 (which are recognized by KIR3DL) and HLA-Bw6 (which is not known to be recognized by any

KIR). KIR genes, like HLA genes, are a genomically diverse multi-gene family characterized by differences in gene content and allelic polymorphism (Hsu et al., 2002). A patient's genotype for KIRs and HLA molecules can determine susceptibility to viral infection (Bashirova et al., 2006). The presence of more than one activating KIR in donor allogeneic stem cell transplantation reduces the risk of CMV reactivation (Cook et al., 2006). KIR3DS1 interaction with HLA-B delays the progression to AIDS in HIV-1 infected patients (Martin et al., 2002). Homozygosity for KIR3DS1 can even apparently protect HIV-1-exposed individuals from chronic infection (Boulet et al., 2008). KIR2DS1 can activate NK cells by recognizing MHC class I molecules loaded with peptide during Epstein-Barr virus (EBV) infection (Stewart et al., 2005). There is also some preliminary evidence associating certain KIR genotypes with severity of influenza infection (La et al., 2011).

1.2.1.2 Natural Cytotoxicity Receptors

The natural cytotoxicity receptors (NCRs) represent another class of activating receptors that recognize viral-derived products (Mandelboim et al., 2001). In humans, there are three members of this receptor family: NKp30, NKp44 and NKp46.

Of these, NKp46 is the most prominent and is found on all NK cells. NKp46 recognizes hemagglutinin of influenza virus and hemagglutinin-neuraminidase of parainfluenza virus, suggesting that it may be involved in resistance to these viruses (Mandelboim et al., 2001). Indeed, mice deficient for NCR1, a murine homolog of NKp46, fail to protect against lethal influenza infection (Gazit et al., 2006). NKp46, and

another activating receptor, DNAM-1, are critical for activation in response to human CMV-infected myeloid dendritic cells, however, a cellular ligand for NKp46 remains to be identified (Magri et al., 2010).

NKp30 also appears to play a role combating hepatitis C virus (HCV) infection. It is downregulated upon exposure to HCV-infected hepatoma cells, a phenomenon that is unique to this activation receptor and corresponds with an inhibition of ex vivo responses (Holder et al., 2013). And HCV exposed, but uninfected patients tend to have higher expression levels of NKp30 on their NK cells than patients who became chronically infected (Golden-Mason et al., 2010). In vitro, human NKp30^{high} NK cells seem particularly effective at preventing HCV infection.

While there is less evidence for roles for NKp44 in viral infection, it has been associated with NK cell recognition of the envelope protein of two flaviviruses – dengue and West Nile virus (WNV) (Hershkovitz et al., 2009). Also, like NKp46, NKp44 appears capable of recognizing viral hemagglutinins (Arnon et al., 2001).

1.2.1.3 NKG2D

Beyond direct recognition of viral-derived products or viral peptide-loaded MHC molecules, NK cells can recognize autologous stress-induced ligands through the NKG2D activation receptor (Jamieson et al., 2002). NKG2D, a C-type-lectin-like receptor, stands apart from other NKG2 receptors as a homodimer that recognizes host stress proteins – Rae-1, Mult-1, and H60 in mice and the ULBP and MIC classes in humans – to activate the NK cell (Raulet et al., 2013). It therefore acts as a sentinel system capable of

provoking an immune response after recognizing host stress proteins induced upon viral infection, transformation, or other forms of cellular stress such as DNA damage (Gasser et al., 2005; Raulet, 2003). While the receptor is encoded in the NK gene complex and is found on all NK cells, it is also expressed on $CD8^+$ αβ T cells and γδ T cells (Raulet, 2003). In humans, it is found on all $CD8^+$ αβ T cells, but in mice $CD8^+$ αβ T cells only express NKG2D upon activation (Groh et al., 2001; Groh et al., 1998). Murine macrophages can also express NKG2D upon activation – although this is not true in humans (Diefenbach et al., 2000).

Stress ligands have been shown to play an important role in the control of human CMV and MCMV infections (Andoniou et al., 2005; Guma et al., 2006). The importance of NKG2D in viral defense is highlighted by the viral evasion mechanisms that attempt to escape NKG2D recognition (Orange et al., 2002). In both humans and mice, CMV infection leads to an upregulation of NKG2D ligand transcription, but that ligand expression is ultimately blunted by viral proteins. Specifically, in humans, CMV-encoded UL16 prevents the surface expression of several ULBPs and MICB (Welte et al., 2003; Wu et al., 2003). In mice, gp40, encoded by the viral *m152* gene, prevents the expression of Rae-1 (Krmpotic et al., 2002; Lodoen et al., 2003). Deletion of MCMV's *m152* results in lower viral loads, which can be rescued by blocking the NKG2D receptor. While the viral hampering of NKG2D ligand expression is imperfect, it is sufficient to boost early virulence in vivo as blocking the NKG2D receptor has no effect against the wild-type virus. NKG2D has also been shown to be critical in NK cell-mediated control of infection with VV (Martinez et al., 2010) and adenovirus (Zhu et al.,

2010). HIV-1 can both induce NKG2D ligands by activating the DNA damage response element ATR and suppress ligand expression via Vif-mediated decreases in the APOBEC3G antiviral protein (Norman et al., 2011; Ward et al., 2009).

Table 2: A summary of known NKG2D receptor ligands, their identified relevance to viral infection, and their mechanisms of regulation.

NKG2D Ligand	Role in viral infection	Mechanisms of regulation
Rae-1α,β,γ,δ,ε (Mouse)	MCMV, VV, adenovirus	DNA damage, TLR, PI3K, miRNA, RasV12D, c-Myc, E2F, STAT1, IL-18
Mult-1 (Mouse)	adenovirus	Heat shock, UV irradiation, ubiquitination
H60a,b,c (Mouse)	MCMV	?
MICA,B (Human)	HCMV	DNA damage, miRNA, heat shock stress, E2F, NF-κB, Sp1
ULBP1-6 (Human)	HCMV, HIV-1	DNA damage, E2F, p53, Sp1

The ligands for NKG2D are a remarkable example of convergent evolution – they share very little sequence homology, but are all structurally analogous to class I MHC molecules and engage NKG2D (Raulet, 2003). While NKG2D ligands have similar α1 and α2 domains to MHC class I molecules, they lack β2 microglobulin domain and have a closed peptide-binding groove (Li et al., 2002; Li et al., 1999). Functionally, they are all absent or expressed at only low levels on healthy adult cells, but become expressed during instances of cellular stress. However, they all have seemingly different

mechanisms of regulation and different affinities for the NKG2D receptor (Carayannopoulos et al., 2002).

Their different patterns of expression suggest that NKG2D ligands are functionally not redundant. Rae-1 in mice and MICA/B in humans are not transcribed in healthy cells while Mult-1 in mice and ULBP molecules in humans are transcribed efficiently but regulated to low levels of surface expression post-transcriptionally (Raulet, 2003). Mult-1 surface expression instead appears to be regulated by post-translational ubiquitination as Mult-1 in stressed cells is less ubiquitinated and has a longer half-life and greater surface expression (Nice et al., 2009; Nice et al., 2010). MCMV infection enhances the transcription of Rae-1, but not H60, in a manner dependent on PI3K signaling (Lodoen et al., 2003; Tokuyama et al., 2011). Inflammatory signaling, through TLRs and type I interferons, appears capable of upregulating Rae-1, but not Mult-1, expression (Fortin et al., 2013; Hamerman et al., 2004). There are also differences in NKG2D ligand regulation between species as the heat shock stress pathway controls MICA/B expression in humans but does not appear to effect murine NKG2D ligand transcription (Groh et al., 1996). In humans, there has also been growing evidence of a role for micro-RNAs in regulating NKG2D ligand expression in the context of viral infection and inflammatory signaling (Eissmann et al., 2010; Stern-Ginossar et al., 2007).

The expression of NKG2D ligands on accessory cells such as DCs and macrophages also mediates "crosstalk" with NK cells. MICA and MICB can be expressed on DCs, for example, in response to interferon-α (IFNα) signaling (Jinushi et

al., 2003). Similarly, in mice, cell-extrinsic STAT1 signaling can upregulate Rae-1 expression on DCs and boost NK cell activation in response to VV infection (Fortin et al., 2013). TLR stimulation on macrophages can also induce Rae-1 transcription and surface expression, resulting in engagement with the NKG2D receptor on NK cells (Hamerman et al., 2004).

Importantly, NKG2D signaling differs from the traditional ITAM activation pathway. In humans, NKG2D relies on DAP-10 to transmit its activation signal, unlike other activating receptors that signal through DAP-12. DAP-10 requires the PI3K signaling pathway, which it activates via phosphorylation cascades involving either PI3K's p85 subunit, Grb2 directly, or the Sos1-Vav1-Grb2 complex (Giurisato et al., 2007). Recent studies have shown that NKG2D signaling alone is not sufficient to activate NK cells and that efficient NK cell activation requires cooperation of other activating receptors acting synergistically (Bryceson et al., 2006). In mice, NKG2D can signal through both DAP-10 and DAP-12, leading to NK cell activation (Diefenbach et al., 2002; Gilfillan et al., 2002).

1.2.1.4 Toll-like Receptors

TLRs – the classic PRR – are found on a broad range of leukocytes where they play important roles in recognizing conserved patterns on pathogens (Takeda et al., 2003). Constitutively expressed, germline-coded, and non-clonal, TLRs provide a fundamental means by which the immune system distinguishes 'self' from 'nonself' and are capable of recognizing a wide range of components from viruses and other

pathogens. Originally found in *Drosophila melanogaster*, the Toll gene was at first identified as a transmembrane receptor necessary for dorso-ventral polarity in a developing embryo. Subsequently, at least 12 homologs have been discovered in mammals – TLRs – that are capable of recognizing PAMPS and provoking the production of pro-inflammatory cytokines and initiating an immune response (Kawai and Akira, 2011). TLRs recognize PAMPs via leucine-rich repeats on their N-terminal extracellular domain.

TLRs can recognize viruses in many ways. Viral genome sensing is perhaps the best-characterized. Viral RNA classically engages intracellular TLR3, TLR7 and TLR8. Similarly, viral DNA can be recognized by intracellular TLR9. However, viral structural proteins can also be sensed byTLR2 and TLR4, which are found on the cell surface.

While TLRs are far from unique to NK cells, direct TLR stimulation on NK cells has emerged to play an important role in NK cell activation. It has been shown that TLR3, TLR7, TLR8, and TLR9 are expressed on human NK cells and that ligands for these TLRs can activate human NK cells in vitro (Hart et al., 2005; Schmidt et al., 2004; Sivori et al., 2004). Intranasally administered CpG can in fact activate and recruit NK cells to the lung (Pesce et al., 2010). Recent studies have demonstrated that direct TLR2 stimulation on murine NK cells is critical for their activation and function in the control of VV infection in vivo (Martinez et al., 2010). Similarly, direct activation of TLR4 by a bacterial component fimbrial protein, FimH, appears to be important for the activation of both mouse and human NK cells (Mian et al., 2010).

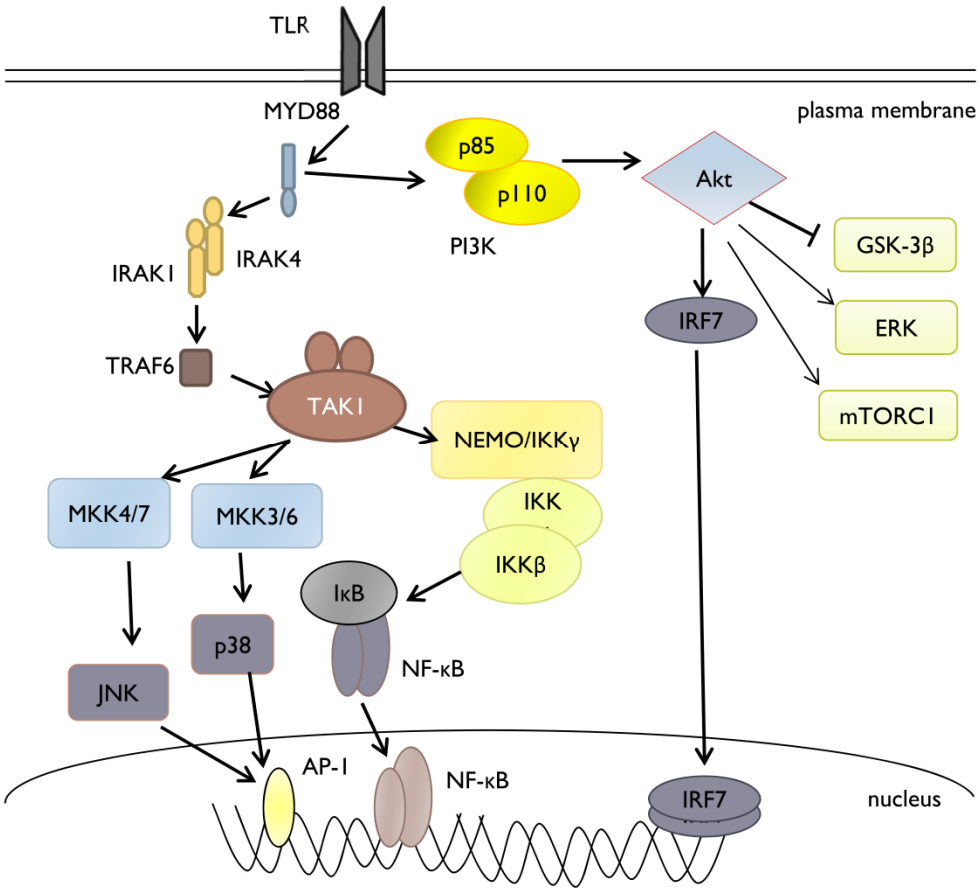

Figure 1: A summary of 'canonical' and 'non-canonical' TLR signaling.

TLRs, being evolutionarily older and quite distinct from other NK cell activation receptors, signal intracellularly in a very different fashion from other activation receptors described here (Akira et al., 2006). Upon recognition of their respective PAMPs, TLR intracellular Toll/interleukin-1 receptor (TIR) domains recruit adaptor molecules which contain TIR domains of their own to form homotypic interactions with the TLR. The two best-studied adaptor proteins include the myeloid differentiation primary-response gene 88 (MyD88) and TIR-domain-containing adaptor protein-inducing IFNβ (TRIF). MyD88 is the most common TLR adaptor protein and upon its recruitment it engages with IL1R-associated kinase 4 (IRAK4) – which complexes with

IRAK1 and IRAK2. IRAK1, in turn, activates TNFR-associated factor 6 (TRAF6), an E3 ubiquitin ligase, which can ubiquitinate both the IKK-γ/NF-κB essential modulator (NEMO) and the transforming growth factor-β-activated kinase I (TAK1). TAK-1 then phosphorylates and activates the members of the IKK complex - NEMO, mitogen-activated protein kinase kinase 6 (MKK6), and IKKβ – which then modify and degrade IκB to free NF-κB for translocation to the nucleus where it initiates the transcription of a wide panel of pro-inflammatory genes. TAK-1 also activates MAPK cascades that culminate in JNK and p38 translocation to the nucleus and AP-1-mediated transcription of pro-inflammatory cytokine genes.

MyD88 can also signal independent of IRAK4 through PI3K (Gelman et al., 2006). MyD88-PI3K signaling arises from a physical association between a Src-homology domain (SH2) binding motif in the MyD88 TIR domain and PI3K. In $CD4^+$ T cells, this association has proven critical for the phosphorylation of Akt and interleukin-2 (IL-2) production. In human plasmacytoid DCs (pDCs), MyD88-PI3K signaling is necessary for interferon regulatory factor 7 (IRF7) translocation to the nucleus and type I interferon (IFN) production (Guiducci et al., 2008). In NK cells, direct TLR2 stimulation in response to VV infection requires MyD88-PI3K-ERK signaling to activate NK cells to the virus (Martinez et al., 2010).

1.2.2 Inhibition Receptors

To control inappropriate activating signals, there is a repertoire of inhibitory receptors that repress NK cell activation (Long, 2008). These include lectin-like heterodimers such as CD94-NKG2A (Moretta et al., 1994), KIRs found in humans

(Wagtmann et al., 1995), or lectin-like Ly49 homodimers found in mice (Karlhofer et al., 1992). These inhibitory receptors survey MHC class I molecules and seem to protect healthy cells from inappropriate NK cell-mediated killing. While most inhibitory receptors engage MHC class I molecules, some recognize other ligands such as cadherins and collagen. The ability of different KIRs and Ly49 homodimers to be either activating or inhibitory while recognizing class I MHC molecules is dependent on the specific receptors' downstream signaling and the strength of interaction between the particular Ly49/KIR and its respective ligand (Jost and Altfeld, 2013). Weaker interactions between the receptor and its ligand, which can be influenced by the peptide loaded into the MHC class I molecule, tend to result in a receptor being activating whereas receptors that strongly bind their ligand tend to be inhibitory. Despite the structural differences between the murine lectin and human immunoglobulin receptors, these classes of receptors signal downstream similarly.

The expression profile of inhibitory KIRs can have a profound effect on the antiviral response. Patients homozygous for the inhibitory KIR2DL3 and HLA-C are much more likely to clear acute HCV rather than progress to chronic infection compared with other HCV-exposed patients (Khakoo and Carrington, 2006; Khakoo et al., 2004). There is also at least one patient who suffers from recurrent CMV infections and has been identified to have uniformly KIR2DL1^{+} NK cells, a possibly novel immunodeficiency (Gazit et al., 2004).

The balance between activating and inhibitory receptors is achieved within the cell, downstream of receptor-ligand binding (Long, 2008). Contradictory signaling

between the activating and inhibitory receptors depends on the ITAMs and immunoreceptor tyrosine-based inhibition motifs (ITIMs), respectively. Upon stimulation of inhibitory receptors, ITIMs on the cytosolic domains of inhibitory receptors become phosphorylated by Src kinases. Phosphorylated ITIMs activate phosphatases to counter the kinase cascade of the activating receptors. They can recruit SHP-1 and SHP-2 protein phosphatases and SHIP-1, a lipid phosphatase, which dephosphorylate many downstream molecules in the activation signaling pathway to dampen Ca^{2+} influx and effector function. Only Vav1, a guanine nucleotide exchange factor for Rac1, has emerged as a direct SHP-1 substrate from a "functional substrate trapping" screen that employed a catalytically inactive KIR2DL1-SHP-1 chimera (Stebbins et al., 2003). Dephosphorylation of Vav1 may prevent its promotion of Rac1 remodeling of the actin cytoskeleton for cell cytotoxicity.

The model for integration of inhibitory and activating signaling has progressed further with the identification of subsets of "unlicensed" NK cells in mice (Fernandez et al., 2005). These "unlicensed" cells lack any known inhibitory receptor, but are apparently self-tolerant and hyporesponsive upon ligation of activating receptors in vitro. This important finding followed work characterizing the hyporesponsiveness of NK cells from MHC class I-deficient mice. The hyporesponsiveness could be reversed only in NK cells expressing inhibitory receptors capable of binding the particular class I MHC allele reintroduced (Kim et al., 2005). However, a more recent study indicates that only "unlicensed" NK cells are capable of mediating MCMV control in vivo (Orr et al., 2010), suggesting that NK cells lacking inhibitory receptors may be critical for NK cell

control of certain infections. This also likely explains how homozygosity for the inhibitory KIR2DL3 and HLA-C promotes HCV clearance – NK cells in these patients may be more responsive due to the baseline presence of strong inhibition signals (Kim et al., 2008).

1.2.3 Activating Cytokines

In addition to direct stimulation through activating receptors, NK cells can be activated by cytokines during the initial stages of viral infection (Dokun et al., 2001). Cytokines can also enhance receptor-mediated NK activation. The four principal cytokines involved in NK cell activation are type I interferons (IFNs), IL-12, IL-15, and IL-18. These cytokines can be produced directly by infected cells or by activated DCs or macrophages (Nguyen et al., 2002).

Table 3: A summary of activating cytokines during viral infection.

	Virus	Activating Cytokines
mouse	MCMV	Type I IFN, IL-12, IL-18
	VV	Type I IFN, IL-12, IL-18
	LCMV	Type I IFN
	Influenza	Type I IFN, IL-15, IL-18
human	HSV	Type I IFN, IL-12
	HCV	IL-12, IL-18
	HIV-1	Type I IFN
	EBV	IL-18

1.2.3.1 Type I Interferons

Type I IFNs, which include over a dozen different subtypes of IFNα and one IFNβ, signal through IFN$\alpha\beta$R1 and IFN$\alpha\beta$R2 (Garcia-Sastre and Biron, 2006). They

directly activate NK cells to enhance perforin-mediated cytotoxicity (Lee et al., 2007). This role of type I IFNs has been well established in the setting of infection with MCMV, lymphocytic choriomeningitis virus (LCMV), and several other viruses. In the setting of MCMV infection, pDCs were identified as particularly strong producers of type I IFN (French and Yokoyama, 2003). In addition to its critical role in NK cell cytotoxicity, type I IFNs boost tumor-necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated killing. And type I IFNs can also induce secretion of IL-15, a cytokine capable of inducing NK cell proliferation. Furthermore, type I IFNs can act indirectly on NK cells by causing other cells to release other cytokines or chemokines critical for NK cell activation or recruitment. For example, type I IFNs are needed for the production of macrophage inflammatory protein 1α (MIP-1α), a chemokine critical for NK cell trafficking to the liver during MCMV infection (Salazar-Mather et al., 2002). Type I IFNs also appear to control NKG2D ligand expression in the setting of VV infection (Fortin et al., 2013).

Type I IFN secretion can occur from just about any infected cell or by accessory cells – such as DCs or macrophages – that become activated in response to PRR recognition (Garcia-Sastre and Biron, 2006). TLR3-TRIF signaling on cells in response to an infection can culminate in transcription from IRF3, NF-κB, and AP-1, which are capable of transcribing IFNβ for secretion. Similarly, TLR7/8/9-MyD88 signaling activates IRF6, NF-κB, and AP-1 to drive the transcription of IFNα. Other cytoplasmic sensors – such as retinoic acid-inducible gene I (RIG-1) – act as RNA helicases able to

recognize viral dsRNA and trigger IFNβ production via the mitochondrial IFNβ promoter stimulator (IPS-1), which activates IRF3, NF-κB, and AP-1.

1.2.3.2 IL-12

Unlike type I IFNs, IL-12 activates NK cells to augment production of IFN-γ (Orange and Biron, 1996). IL-12 is produced in response to MCMV infection and is capable of activating NK cells alone. In LCMV infection, the absence of high levels of IL-12 likely accounts for the weak NK cell response. Herpes simplex virus (HSV) provokes robust IL-12 and type I IFN responses in both mice and humans whereas patients with HIV show little IL-12 response, which correlates to the degree of NK cell activation, respectively (Lee et al., 2007). Receptors for IL-12 are found principally on NK cells and T cells and though it is mostly an inducer of IFN-γ, it can to a lesser degree boost NK cell-mediated cytotoxicity (Gately et al., 1998).

1.2.3.3 IL-15

IL-15 promotes NK cell survival and drives its proliferation upon co-culture with DCs (Ferlazzo et al., 2004). *Trans*-presentation of IL-15 by $CD11c^{high}$ DCs provides a necessary and sufficient priming signal in eliciting an NK cell response to a wide range of stimuli in vivo (Lucas et al., 2007). DCs must themselves be type I IFN-experienced in order to transmit the IL-15 priming signal to NK cells. This interaction takes place in the secondary lymphoid organs. Type I IFN-mediated NK cell accumulation also requires IL-15 (Nguyen et al., 2002). Another key effect of IL-15 is its promotion of the survival of

peripheral NK cells in vivo. It accomplishes this by maintaining the anti-apoptotic factor Bcl-2 (Ranson et al., 2003).

1.2.3.4 IL-18

Another cytokine, IL-18, can also induce IFN-γ production by NK cells (Gherardi et al., 2003). In fact, IL-18 was originally named the IFN-γ-inducing factor (IGIF). IL-18 has been shown in at least one setting to act synergistically with IL-12 in its ability to induce IFN-γ expression in NK cells. Both IL-12 and IL-18 appear to prime NK cells in response to viral infection. The mechanism of this priming is not entirely clear, but appears to involve increasing translation of IFN-γ mRNA (Chaix et al., 2008). IL-18, a member of the IL-1 superfamily, appears to act on a much broader range of cells than IL-12. In addition to NK and T cells, the receptor for IL-18 can be found on B cells, macrophages, neutrophils, and DCs (Arend et al., 2008; Gutzmer et al., 2003; Nakamura et al., 2000). Macrophages, DCs, Kupffer cells, keratinocytes, and osteoblasts all express the precursor form of IL-18, pro-IL-18, and are capable of secreting it in response to an inflammatory insult (Arend et al., 2008). The inactive precursor peptide of IL-18 is cleaved into its mature, secretable form by the inflammasome: a caspase-1 containing complex activated by PRRs. It is this post-translational modification that presents the key step at IL-18 regulation as the mRNA is stably expressed in non-inflammatory conditions. Importantly, IL-18 signaling can also be regulated after secretion via IL-18 binding protein (IL-18bp), which sequesters IL-18 away from its receptor. This binding protein is made both by the host to buffer IL-18 production and by viruses, notably

poxviruses, to suppress IL-18-provoked immunity. VV engineered to delete its IL-18bp is cleared more efficiently from the host in a fashion dependent on NK and T cells (Reading and Smith, 2003). On the other hand, engineering VV to express IL-12 and/or IL-18 itself can boost viral clearance synergistically when both cytokines are expressed together (Gherardi et al., 2003).

1.2.3.5 Cytokine Receptor Signaling Pathways

Cytokine receptors signal through the JAK-STAT pathway to induce NK cell activation (Garcia-Sastre and Biron, 2006). Seven members of the STAT family facilitate the differential effects of various cytokines. Type I IFNs, for example, induce phosphorylation of STAT1 and STAT2 for STAT1-STAT2 heterodimers and STAT1 homodimers to drive gene expression that enhances cell cytotoxicity (Nguyen et al., 2000). IL-15 also uses STAT1 to trigger NK cell proliferation. STAT1, on the other hand, impairs IL-12 sensitivity and IFN-γ production. Instead, STAT4 drives IFN-γ production in response to IL-12. The regulation of STAT molecules has clear consequences for cytokine signaling and loss of STAT molecules can increase viral sensitivity at early times after infection.

The IL-18R, however, has a unique intracellular signaling pathway distinct from all others mentioned here and most resembling its IL-1R family members: It signals through a MyD88 pathway similar to TLRs (Arend et al., 2008). Just like TLR signaling, IL-18R signaling starts by a homotypic interaction between the TIR domains of intracellular IL-18R and MyD88. And, like TLR signaling, it follows the familiar IRAK-

TRAF6-TAK1 pathway to culminate in activation and translocation of the pro-inflammatory transcription factors NF-κB, p38, and JNK.

1.2.4 NK-Accessory Cell Interactions

NK cells do not activate in a vacuum. Instead, they receive important signals from accessory cells – namely DCs and macrophages – that are critical for their activation against a variety of stimuli. At the same time, this 'crosstalk' is bidirectional as NK cells are capable of transmitting signals back to the accessory cell.

1.2.4.1 NK-DC Crosstalk

NK cells receive important soluble and contact-dependent activation signals from DCs (Walzer et al., 2005). Many of the cytokines already discussed are produced by DCs – and DCs are required for the cytokine-mediated effects on NK cells. While both NK cells and DCs can be recruited to the site of inflammation, they appear to interact in the secondary lymphoid organs with NK cells found in the paracortex of lymph nodes near DCs around the high endothelial venule (HEV). With regards to activating NK cells, there are at least two main types of DCs: $CD11c^{+}B220^{-}$ conventional DCs (cDCs) and $CD11c^{+}B220^{+}$ plasmacytoid DCs (pDCs). These DC subsets have both distinct and, at times, overlapping functions in NK cell activation.

IL-12, for example, is produced by cDCs and pDCs in different contexts in response to TLR ligand stimulation and MCMV infection (Dalod et al., 2003; Gerosa et al., 2005). *Trans*-presentation of IL-15 for NK cell priming appears to be mediated by cDCs carrying the IL-15Rα (Lucas et al., 2007). Type I IFNs can be produced by both

cDCs and pDCs in response to TLR ligands and viral infection, but pDCs appear specialized to secrete IFNs particularly well (Colonna et al., 2004).

Cell-cell contact is also important to NK-DC crosstalk. NK cells can form synapses with DCs via cytoskeletal rearrangement and lipid raft mobilization, which allows the polarized secretion of IL-12 (Borg et al., 2004). Importantly, DCs can expression the host stress ligands for the NKG2D activation receptor (Jinushi et al., 2003). DC upregulation of MICA/B in humans appears to be critical for NK cell activation in the setting of HCV.

But NK cells can influence DCs as well. NKp30 receptor engagement appears to trigger the release of tumor necrosis factor α (TNFα) and IFN-γ, which induce DC maturation (Piccioli et al., 2002; Walzer et al., 2005). During co-culture of human NK cells with immature DCs at low NK-DC ratios (1:5), NK cells release TNFα and IFN-γ to mature DCs and induce their expression of pro-inflammatory cytokines. However, when the ratios are reversed (5 NK cells to 1 DC), the NK cells will instead kill the immature DCs in the culture, sparing only mature DCs. NKp30 also appears capable of lysing immature DCs. The upregulation of class I MHC molecules during DC maturation appears to provide protection from NK cell lysis. In this way, NK cells may influence the adaptive response toward mounting a response and work against tolerance.

1.2.4.2 NK-Macrophage Crosstalk

Similarly, macrophages – which, like DCs, can play a role in antigen presentation to T cells – can act as accessory cells in the activation of NK cells. Macrophages, for

example, produce soluble factors, including IL-18, in response to viral infection by influenza or EBV (D'Addario et al., 1999; Pirhonen et al., 1999). Macrophages appear capable of polarized IL-12 secretion to NK cells as IL-12R is relocalized to the NK-macrophage interface (Lapaque et al., 2009). Macrophages also express contact-dependent signals in the form of ligands for the NK activating receptor 2B4.

Notably, human and murine macrophages express NKG2D ligands in response to TLR ligand stimulation (Eissmann et al., 2010; Hamerman et al., 2004; Nedvetzki et al., 2007). These ligands are capable of functionally engaging the NKG2D receptor on NK cells. If activated by particularly high doses of LPS, macrophages can be killed directly by NK cells.

1.3 NK Cell Responses to Viral Infection

NK cells were first discovered for their role against cancer in culture. Following this observation, NK cells were shown to exhibit important antitumor activities in both clinical studies and murine models of malignancy.

But NK cells also play essential roles in antiviral immunity. Patients with NK cell deficiencies suffer from severe, recurrent viral infections – particularly from members of the herpesvirus, poxvirus, and papillomavirus families (Biron et al., 1989; Orange, 2002). Mouse models provide additional evidence that NK cells give critical help to control viral infections, most notably murine cytomegalovirus (MCMV), poxviruses, and influenza (Biron et al., 1999; French and Yokoyama, 2003; Lee et al., 2007). Viral products can be directly recognized by NK cell activation receptors and many viral infections, to evade recognition by the adaptive immune system, downregulate class I MHC

molecules. Many viruses also encode strategies to evade NK cell-mediated clearance which are critical for viral persistence (Orange et al., 2002).

1.3.1 Effector Functions to Virally Infected Cells

Upon activation, NK cells employ three main strategies to kill virally infected cells: the production of cytokines, the secretion of cytolytic granules, and the use of death-receptor-mediated cytolysis (Lee et al., 2007). Production of IFN-γ is an important effector function of activated NK cells. IFN-γ exerts direct effects in making host cells less hospitable to the virus and can act distally to prevent infection in other cells. It also recruits and activates other effector leukocytes, including cytotoxic T lymphocytes (CTLs) and $CD4^+$ T helper type 1 cells (Th1) (Lee and Biron, 2010). NK cell production of IFN-γ helps to control MCMV. Mice with deficiencies in IFN-γ or IFN-γR are increasingly susceptible to VV, HSV, and other viral infections. Similar susceptibilities are seen in humans with identified IFN-γ or IFN-γR deficiencies (Novelli and Casanova, 2004).

NK cells, called "large granular lymphocytes" in early work, can also directly kill infected cells by mediating cytolysis through preformed granules (Lee et al., 2007; Natuk and Welsh, 1987). These granules, notably perforin and granzymes, are similar to those used by CTLs, and function after direct interaction between the NK and infected cells. Perforin, a membrane pore-forming molecule, can permeabilize the cell (Tay and Welsh, 1997). On the other hand, granzymes, a family of serine proteases, disrupt cell cycle progression, inflict DNA damage, and dissolve the nucleus upon entrance into the cell. Humans with identified deficiencies in perforin are susceptible to herpesvirus infections

and perforin knock-out mice have impaired clearance of MCMV, influenza, and several other viruses (Lee et al., 2007; Orange, 2002).

FasL and tumor-necrosis factor-related apoptosis-inducing ligand (TRAIL) can also be used in NK cell-mediated cytolysis of infected cells (Colucci et al., 2003). In these pathways, NK cells express ligands capable of activating death receptors on the target cell that can trigger the extrinsic pathway of apoptosis. TRAIL-mediated cytotoxicity is particularly important for immature NK cells, which cannot use perforin-dependent mechanisms (Colucci et al., 2003).

1.3.2 Vaccinia Virus

VV is the most-studied member of the poxvirus family and the live viral vaccine responsible for the successful eradication of smallpox. Other notable members of this family include variola virus (the disease-causing agent of smallpox), ectromelia virus, and mousepox virus. These viruses are all morphologically and antigenically similar – prior exposure to one confers a degree of protection against infection with another (Moss, 2011; Smith et al., 2002a). This has been the basis for using VV (and, earlier by Edward Jenner, cowpox virus) as the vehicle for vaccination against smallpox.

As a member of the poxvirus family, VV carries a large (~200kb), linear dsDNA genome. Its genome contains a central region of ~100kb which encodes the enzymes needed for transcription and DNA replication (Smith et al., 2013). The terminal regions of the genome encode virulence factors, immunomodulatory proteins, and factors that influence host range. VV replicates exclusively in the cytoplasm of the infected cells and produces two forms of infectious particles: the intracellular mature virus (IMV) and the

extracellular enveloped virus (EEV). The morphogenesis of VV starts upon the formation IMV from precursor viral crescents and immature virus (IV) (Smith et al., 2002a). These IMV, which represent the bulk of the infectious viral progeny and mostly remain within the host cell until lysis, can become transported by microtubules and wrapped in a double layer of intracellular membrane from the early endosomes or *trans*-Golgi network (TGN). When this happens, the enveloped IMV now constitutes the intracellular enveloped virus (IEV), which can then move to the cell surface via microtubules where its outer membrane fuses with the cell's plasma membrane. This results in the exposure of an enveloped virion on the cell surface called the cell-associated enveloped virus (CEV) and the release of infectious EEV. The attached CEV can recruit actin tails which promote cell-to-cell spread of the virus while EEV is the agent of longer range viral dissemination.

Interestingly, EEV appears essential for the formation of protective immunity in vaccination strategies as killed vaccines – comprised largely of IMV – have continually proven ineffective in creating long-term immunity to the live virus. Despite this, only one EEV protein – B5R – has so far been identified as an antigen for neutralizing antibodies (Galmiche et al., 1999). This creates a dilemma whereby a live viral vaccine is needed to elicit protective immunity though it carries a relatively high incidence of associated adverse events (Fulginiti et al., 2003).

1.3.2.1 NK Cell Responses to VV

NK cells are important in the control of VV. Antibody-mediated depletion of NK cells increases viral loads (Bukowski et al., 1983; Martinez et al., 2010). NK cells are efficiently activated by VV infection both in vitro and in vivo and they accumulate at the site of VV infection (Martinez et al., 2010; Natuk and Welsh, 1987).

The ability of VV to activate NK cells and recruit them to the site of infection – and the necessity of this activation for the virus's control – makes VV a compelling model for advancing our understanding of how NK cells activate.

So far, we know that VV downregulates the MHC class I molecules H2-K^k and H2-K^d in infected cells to evade $CD8^+$ T cell recognition, which renders them more vulnerable to NK cell-mediated lysis (Brutkiewicz et al., 1992). However, VV infection also produces a number of positive activation signals to NK cells as well. The innate immune response to VV – as measured by viral load and DC production of pro-inflammatory cytokines – depends upon TLR2-MyD88 signaling and TLR-independent IFN-β signaling (Zhu et al., 2007b).

In fact, direct TLR2-dependent and –independent signaling on the NK cells are required for efficient activation and viral clearance. Loss of direct TLR2-MyD88 signaling, type I IFN signaling, or NKG2D signaling is sufficient to impair NK cell activation to VV and increase viral susceptibility (Martinez et al., 2008; Martinez et al., 2010). TLR2-dependent activation of NK cells signals through PI3K and ERK, which have been shown to be important for NK cell activation and cytotoxicity (Jiang et al., 2000; Tassi et al., 2007). Notably, TLR2-MyD88 signaling appears necessary for $CD8^+$ T

cell expansion and memory formation (Quigley et al., 2009) although the viral protein capable of engaging TLR2 remains unknown.

There also appears to be a role for IL-18 in NK cell activation to VV as the viral C12 protein acts as an IL-18bp capable of sequestering the pro-inflammatory cytokine from its receptor. Deletion of the viral gene encoding this protein attenuates VV infection as does engineering the virus to express additional IL-18 (Gherardi et al., 2003; Reading and Smith, 2003).

1.3.3 Remaining Questions

The requirement for NKG2D signaling in NK cell activation to VV and viral clearance raises the question of how the ligands for this receptor are controlled during viral infection. The ligands for NKG2D are host cellular stress molecules, not viral proteins, so it is curious how these ligands become upregulated in setting of VV infection. An explanation about how NKG2D ligand expression is controlled during viral infection is important not only because of its critical role in NK cell activation to VV, but because NKG2D ligands provide powerful activating signals in other contexts of therapeutic interest. Specifically, in a wide range of murine models of malignancy, loss of NKG2D signaling translates into impaired immune surveillance and rapid tumor growth (Guerra et al., 2008). On the other hand, retroviral expression of NKG2D ligands in otherwise quickly lethal tumors can be sufficient to clear the tumor in an NK cell-dependent fashion (Diefenbach et al., 2001). Therefore, a better appreciation for how NKG2D ligands are controlled in this setting could be exploited in the development of

NK cell-based therapies capable of provoking NK cell responses against viral infections or tumors.

Furthermore, it remains unclear exactly what role IL-18 has in NK cell activation to VV. While others have shown the importance of the viral IL-18bp in viral persistence, the necessity for IL-18 in NK cell-mediated clearance of VV remains to be demonstrated. In addition, though IL-18 is capable of acting directly on NK cells ex vivo to boost IL-12-mediated production of IFN-γ, how IL-18 acts in a physiologic setting of VV infection has not been shown. Does IL-18 solely signal directly to the NK cell or, as its receptor is expressed by a wide variety of cells, does it also influence additional activation signals by accessory cells in NK cell activation?

1.4 Thesis Prospectus

NK cells exert powerful activities in the control of viral infections and tumors. These capabilities make NK cell-based treatments a promising new avenue in the development of cellular therapies. Insights into how NK cells activate could facilitate the development of these strategies.

VV provides a good model for studying NK cell activation as it activates NK cells robustly, recruits them to the site of the infection, and requires NK cells to be effectively cleared. While we have clarified roles for TLR-dependent and TLR-independent signaling in NK cell activation to VV, much remains poorly understood. One gap in our knowledge is the mechanism by which NKG2D ligands are controlled during VV infection. The regulation of these ligands is important not only to VV control, but in a broad range of NK cell responses. To address how NKG2D ligands are controlled during

VV infection, we proposed the following two aims of this thesis project, presented here as chapters:

Chapter 3

Does inflammatory signaling via IL-18 control NKG2D ligand expression on DCs to boost NK cell activation and viral clearance?

Chapter 4

What is the mechanism by which IL-18 signaling leads to the upregulation of NKG2D ligands on the surface of the accessory cell?

2. Materials and Methods

2.1 Mice

CD45.1^{+} and CD45.2^{+} C57BL/6 mice were obtained from the Frederick National Laboratory for Cancer Research at the National Cancer Institute (Frederick, MD). IL-18R$^{-/-}$ and CD11c-DTR mice on the C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). MyD88$^{-/-}$ mice were kindly provided by Shizuo Akira (Osaka University, Osaka, Japan). Groups of 8- to 12-week-old mice were selected for this study. All experiments involving the use of mice were done in accordance with protocols approved by the Institutional Animal Care and Use Committee of Duke University (Durham, NC).

2.2 Vaccinia virus

The Western Reserve (WR) strain of VV was purchased from American Type Culture Collection (ATCC, Manassas, VA). VV was grown in TK-143B cells (ATCC) and purified by a 35% sucrose cushion as described (Zhu et al., 2007a). The titer was determined by plaque assay on TK-143B cells and VV was stored at −80°C until use. For in vitro studies, VV was used at an MOI of 1. For in vivo studies, 5 x 10^6 pfu of live VV in 0.05 mL of 1 mM Tris pH 9.0 was injected intraperitoneally.

2.3 Antibodies and flow cytometry

PE-conjugated anti-CD49b (clone DX5), PE-Cy5-conjugated anti-CD3ε (clone 145-2C11), FITC-conjugated IFN-γ (clone XMG1.2), FITC-conjugated CD11c (clone HL3), FITC-conjugated CD11b (clone M1/70), APC-conjugated IFN-γ (clone XMG1.2), APC-

conjugated B220 (clone RA3-6B2), and Streptavidin-conjugated APC were purchased from BD Biosciences. PE-conjugated anti-NKp46 (clone 29A1.4), Biotin-conjugated CD45.1 (clone A20), FITC-conjugated GRB (clone NGZB), PE-Cy5-conjugated CD11b (clone M1/10), PE-conjugated Rae-1 (clone CX1), PE-conjugated rat IgG2b (clone eB149/10H5), PE-conjugated hamster IgG (clone eBio299Arm), and PE-conjugated rat IgG2a (clone eBR2a) were purchased from eBioscience. Alexafluor 546-conjugated goat anti-rabbit IgG was purchased from Invitrogen. APC-conjugated F4/80 (clone CI:A3-1) was purchased from Abd Serotec. AlexaFluor 647-conjugated pAkt (S473) and unconjugated pGSK-3β (S9) were purchased from Cell Signaling.

To assess intracellular production of IFN-γ, splenocytes were incubated with 25ng/mL PMA, 25ng/mL ionomycin, and 5μg/mL Brefeldin A containing Golgi-Plug (BD Biosciences) for 4 hours at 37°C. To assess intracellular production of GRB, splenocytes were cultured with 5μg/mL Brefeldin A containing Golgi-Plug alone for 4 hours at 37°C. Following incubation, cells were stained for surface molecules, permeablized using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions and stained for intracellular molecules. FACS Canto (BD Biosciences) was used for flow cytometry event collection, which was analyzed using FlowJo software 9.5.3 (Tree Star, Ashland, OR).

2.4 NK cell cytotoxicity assay

NK cell cytotoxicity was performed by a standard 4-hour chromium-51 release assay as described (Martinez et al., 2008). Splenocytes were enriched for $DX5^+$ NK cells by positive selection with PE-conjugated anti-DX5 and anti-PE microbeads (Miltenyi

Biotec). YAC-1 target cells (ATCC), which are susceptible to NK cell-mediated cytotoxicity, were labeled with chromium-51 and then incubated with $DX5^+$ cells at different effector:target ratios for 4 hours at 37°C. The specific release of chromium-51 into the supernatant, measured by counts per minute, assessed target cell lysis with no effector (spontaneous lysis) wells and 1% SDS (maximum lysis) wells used as controls. Specific release was calculated as (experimental – spontaneous)/(maximum-spontaneous) x100.

2.5 Measurement of viral load

Viral load in the ovaries was measured by plaque-forming assay as described (Martinez et al., 2008). Two days post-infection with 5×10^6 pfu VV, ovaries were harvested into 10 mM Tris pH9.0 and stored at -80°C until use. Ovarian viral load was then measured by plaque-forming assay. Ovaries were first homogenized and freeze-thawed three times. Serial dilutions of the lysate were added to confluent TK-143B cells and the number of plaques was counted using crystal violet staining after two days of culture at 37°C.

2.6 NK cell-DC co-culture

NK-DC co-culture was performed as described (Martinez et al., 2010). Briefly, splenocytes were enriched for NK cells by positive selection with PE-conjugated anti-DX5 and anti-PE microbeads (Miltenyi Biotec). DCs were generated from bone-marrow cells as described after five days of culture with GM-CSF (1000 U/mL) and IL-4 (500 U/mL) (R&D Systems) (Martinez et al., 2010). $DX5^+$ NK cells (2×10^5) were then cultured

with the bone-marrow-derived DCs (10^5) in a ratio of 2:1 in the presence or absence of VV at an MOI of 1 overnight at 37°C. IL-18 production was assayed by harvesting the supernatant for analysis by ELISA (eBioscience). IL-18R expression was determined by sort-purifying NK cells or DCs into TRI Reagent (Sigma) for RNA purification and semi-quantitative RT-PCR (Promega) PCR for IL-18R. Forward primer 5′-AGAGCTTCGTCTTGGTGAGAA-3′ and reverse primer 5′-TACCTGTTAGTGTCTCGTCTCTT-3′ were purchased from Integrated DNA Technologies to assess IL-18R expression. Recombinant mouse IL-18 protein was purchased from Life Technologies.

2.7 NK cell transfer experiment

$DX5^+CD3^-$ NK cells were first enriched from the naïve splenocytes of $CD45.1^+$mice by positive selection with PE-conjugated anti-DX5 and anti-PE microbeads (Miltenyi Biotec) and then purified via flow cytometry sorting on a FACS DiVA with a purity >95%. 5 to 10 x 10^5 NK cells were transferred by tail-vein intravenous injection into recipient $CD45.2^+$ C57BL/6 or $IL\text{-}18R^{-/-}$ mice. After two days, mice were infected by injection of 5 x 10^6 pfu VV i.p. or left naïve and sacrificed 24 hours post-infection for analysis.

2.8 DC Ablation

CD11c-DTR^+ mice were ablated of their $CD11c^+$ DCs by a single i.p. injection of 4 ng diphtheria toxin (DT, Sigma) per gram of body weight (~100 ng/mouse) in 100ul PBS as described (Jung et al., 2002). Control mice received PBS injections. One day after DT

administration, mice were infected by i.p. injection of 5 x 10^6 pfu VV or left naïve and sacrificed 24 hours post-infection for analysis. We observed ~90% reduction in $CD11c^+$ DCs upon DT administration and could not appreciate a concomitant effect on the relative or absolute number of NK cells.

2.9 Macrophage Culture

To attain macrophages for in vitro culture, peritoneal macrophages were harvested by peritoneal lavage with 5mL cold PBS using a 5mL syringe. Two to three days before harvesting, mice were injected intraperitoneally with 1.5mL 3% fluid thioglycollate medium (BD) to elicit a mild peritonitis and recruit macrophages into the peritoneum. All cultures of macrophages were performed in 96-well U-bottom plates as described. Chemical inhibitors of cell signaling molecules were incubated with macrophages for 1 h before the addition of agonist. The PI3K inhibitor Ly294002 (Calbiochem) was used at 10μM. The NF-κB inhibitor pyrrolidine dithiocarbamate, PDTC, (Sigma Aldrich) was used at 10μM. The GSK-3 inhibitor SB216763 (Sigma Aldrich) was used at 10μM. Recombinant mouse IL-18 protein (Life Technologies) was used at 5ng/mL. Lipopolysaccharide, LPS, (Sigma Aldrich) was used at 100ng/mL.

2.10 Statistical Analysis

All data are presented as means and standard errors. All indicated statistical tests, including unpaired student's t test, ANOVA, and ANCOVA, were performed using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA). Significance was assumed at $p<0.05$.

3. Role of IL-18 in NK cell activation to vaccinia virus infection

A modified version of this chapter is currently in revision with the *European Journal of Immunology.*

3.1 Introduction

NK cells play an important role in antiviral responses (Lanier, 2008; Lee et al., 2007). NK cells are particularly critical for the control of poxviruses. Previous studies have shown that NK cells are activated in response to poxviral infections and mobilized to the site of infection, leading to effective viral control (Bukowski et al., 1983; Martinez et al., 2008; Martinez et al., 2010; Natuk and Welsh, 1987). VV is the most studied member of the poxvirus family and is the live vaccine responsible for the successful elimination of smallpox (Fenner F, 1988). Efficient NK cell activation represents the initial step in the control of VV infection. We have recently shown that efficient activation of NK cells and subsequent control of VV infection in vivo requires both TLR-dependent and -independent pathways, as well as the NKG2D activating receptor that recognizes host stress-induced NKG2D ligands (Martinez et al., 2008; Martinez et al., 2010; Zhu et al., 2007a). However, while we recently showed how STAT1 could influence NKG2D ligand expression (Fortin et al., 2013), it remains largely unknown what controls the upregulation of NKG2D ligands in response to VV infection.

IL-18, originally called IFN-γ-inducing factor (Okamura et al., 1995), is a pro-inflammatory cytokine that plays an important role in innate and adaptive immune responses (Okamura et al., 1998). Studies have shown that IL-18 is critical for NK cell

activation and function both in vitro and in vivo (Dao et al., 1998; Takeda et al., 1998). This is accomplished by enhancing IFN-γ production by NK cells (Chaix et al., 2008; Pien et al., 2000; Tanaka-Kataoka et al., 1999), as well as by stimulating NK cell proliferation and cytotoxicity (Dao et al., 1998; French et al., 2006; Takeda et al., 1998). IL-18 is also required for the activation of NK cells in response to infections with VV and MCMV and plays a critical role in antiviral defense (Gherardi et al., 2003; Pien et al., 2000; Tanaka-Kataoka et al., 1999). But, how IL-18 contributes to the regulation of NK cell activation and function remains incompletely defined.

In a model of VV infection, we first showed that NK cell activation and function are severely compromised in IL-18 receptor-deficient (IL-18R$^{-/-}$) mice, leading to impaired viral clearance. We then demonstrated that IL-18 signaling on both NK cells and accessory cells such as DCs, is critical for efficient NK cell activation in vitro. We further showed in vivo that efficient NK cell activation to VV is dependent on DCs and IL-18 signaling in non-NK cells, suggesting an essential role for NK cell-extrinsic IL-18 signaling in NK cell activation. Furthermore, IL-18R$^{-/-}$ DCs failed to upregulate the expression of Rae-1, an NKG2D ligand, which is required for efficient NK cell activation upon VV infection via the NKG2D pathway. Collectively, the data presented here suggest a critical role for NK cell-extrinsic IL-18 signaling in NK cell activation through upregulation of NKG2D ligands.

3.2 Results

3.2.1 IL-18 is required for efficient NK cell activation and VV clearance

Previous studies have shown that recombinant IL-18 or IL-18-expressing VV promotes NK cell activation and VV clearance (Gherardi et al., 2003; Tanaka-Kataoka et al., 1999). However, it remains to be defined whether IL-18 signaling is necessary for NK cell activation and VV clearance. Here, we used IL-18R$^{-/-}$ to address this question. We first examined whether IL-18 signaling is critical for NK cell activation upon VV infection in vivo. WT or IL-18R$^{-/-}$ C57BL/6 mice were infected with VV intraperitoneally and splenic NK cells were analyzed 24 h later. WT NK cells produced significant amounts of GRB and IFN-γ (Fig. 2A, B), compared to the naïve control. However, the production of GRB and IFN-γ by IL-18R$^{-/-}$NK cells was significantly ($p < 0.01$) reduced compared to the WT controls (Fig. 2A, B). For the measurement of IFN-γ production NK cells intracellularly, we used PMA/ionomycin to re-stimulate the NK cells ex vivo in order to increase the sensitivity of intracellular IFN-γ detection. Without PMA/ionomycin, the production of IFN-γ is reduced (Fig. 3B, C) compared to that with PMA/ionomycin (Fig 2A, B). However, the addition of PMA/ionomycin did not stimulate naïve NK cells to produce IFN-γ, suggesting the specificity of the assay. We also found that VV-infected WT NK cells had enhanced lytic activity on YAC-1 target cells, compared to naïve control (Fig. 2C), whereas NK cells from VV-infected IL-18R$^{-/-}$ mice showed a significantly ($p < 0.05$) reduced lytic activity on YAC-1 cells (Fig. 2C). We further observed that the compromised NK cell activation in IL-18R$^{-/-}$ mice was

associated with a significantly ($p < 0.01$) higher viral load compared to WT mice (Fig. 2D). Viral loads were measured in the ovaries where the virus has been known to accumulate early in the infection (Martinez et al., 2010). These results indicate that IL-18 signaling is crucial for NK cell activation and the innate immune control of VV infection in vivo.

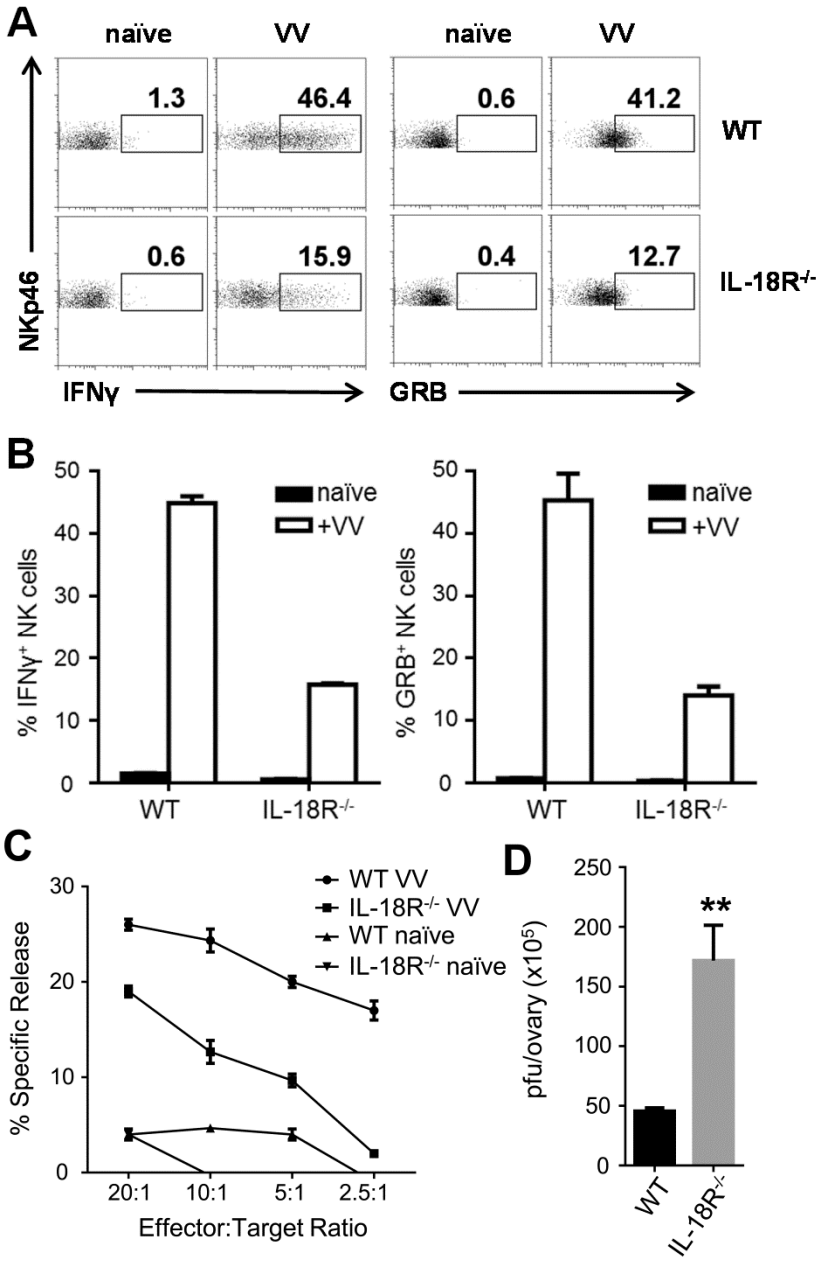

Figure 2: IL-18 is required for efficient NK cell activation and VV clearance. WT and IL-18R-/- C57BL/6 mice were infected by i.p. injection of 5 x 106 pfu VV or left uninfected (naïve). (A) 24 h after infection, splenocytes were assayed for IFN-γ and GRB production. Representative FACS plots showing the percentage of IFN-γ and GRB-positive NKp46^{+}CD3^{-} NK cells are shown. (B) The mean percentages ±s.e.m. of IFN-γ and GRB-positive NK cells (n=3 mice per group) are shown. Interaction term for two-way ANOVA is $p<0.01$ for IFN-γ and GRB. Data is representative of three independent experiments. (C) 48 h after infection, splenocytes were enriched for DX5+ cells and NK cell lytic activity was assayed on YAC-1 target cells by a standard 4-hour chromium release assay at different effector:target ratios. The mean

percentages ±s.e.m. of specific lysis are indicated (n=3 per group). ANCOVA comparing infected WT and IL-18R-/- mice shows $p<0.05$. Data is representative of two independent experiments. (D) 48 h after infection, ovaries of female mice were harvested for measurement of viral load by plaque assay using TK-143B cells. Data represents the mean viral titer ±s.e.m. as pfu per ovary (n=4 per group). Data is representative of two independent experiments. ** signifies a p-value <0.01 on an unpaired student's t test.

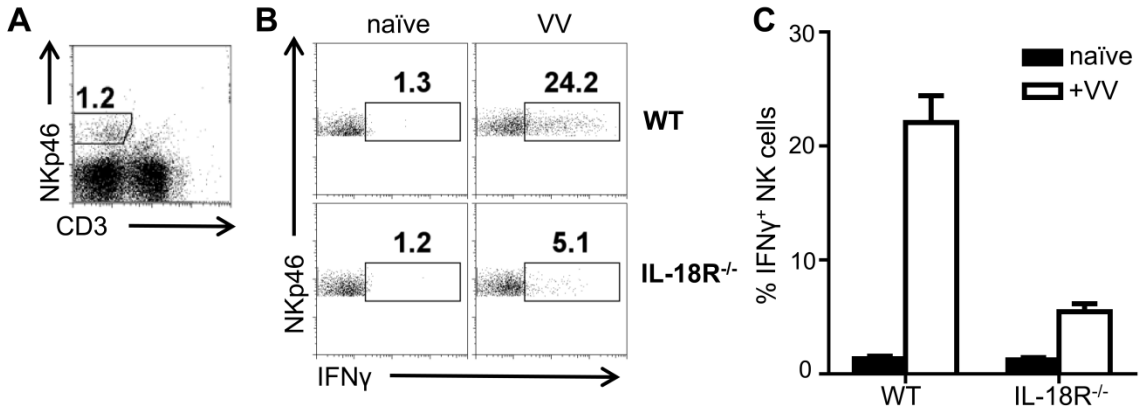

Figure 3: Validation of PMA/ionomycin restimulation to assess in vivo production of IFN-γ. WT and IL-18R$^{-/-}$ C57BL/6 mice were infected by i.p. injection of 5 x 10^6 pfu VV or left uninfected (naïve) as done in Figure 2. 24 h after infection, splenocytes were assayed for IFN-γ production without the use of ex vivo restimulation with PMA and ionomycin. (A) Representative FACS plot shows gating strategy to identify splenic NKp46$^+$CD3$^-$ NK cells. (B) Representative FACS plots showing the percentage of IFN-γ NKp46$^+$CD3$^-$ NK cells are shown. (B) The mean percentages ±s.e.m. of IFN-γ NK cells (n=3 mice per group) are shown. Interaction term for two-way ANOVA is $p<0.01$. Data is representative of three independent experiments.

3.2.2 IL-18 signaling on both NK cells and DCs is required for NK cell activation to VV in vitro

How does IL-18 signaling promote the activation of NK cells in response to VV infection? Previous studies in other models have suggested that IL-18 can act directly on NK cells for their activation (Chaix et al., 2008; Humann and Lenz, 2010; Ni et al., 2012). To address this question, we utilized an in vitro DC-NK cell co-culture system (Martinez et al., 2008). Purified WT or IL-18R$^{-/-}$ NK cells were co-cultured with WT or IL-18R$^{-/-}$ bone marrow-derived CD11c^{+} DCs, followed by infection with VV. Under these conditions, IL-18 was produced in the culture in response to the virus (Fig. 4A) and both DCs and NK cells express the IL-18R (Fig. 4B). NK cells were analyzed for the production of IFN-γ and GRB 18 h post-infection. Our results showed that NK cell activation was compromised ($p < 0.01$) when IL-18R$^{-/-}$ NK cells were used for stimulation (Fig. 5A, B), suggesting that direct IL-18 signaling on NK cells is important for their activation upon VV infection. This is consistent with previous reports in other settings (Chaix et al., 2008; Humann and Lenz, 2010; Ni et al., 2012). We further observed that when IL-18R$^{-/-}$ DCs were used for stimulation, the production of IFN-γ and GRB by WT NK cells in response to VV is also significantly ($p < 0.01$) reduced (5A, B), indicating IL-18 signaling on accessory DCs is also critical for NK cell activation. These results indicate that IL-18 signaling on both NK and accessory cells is required for the activation of NK cells by VV in vitro.

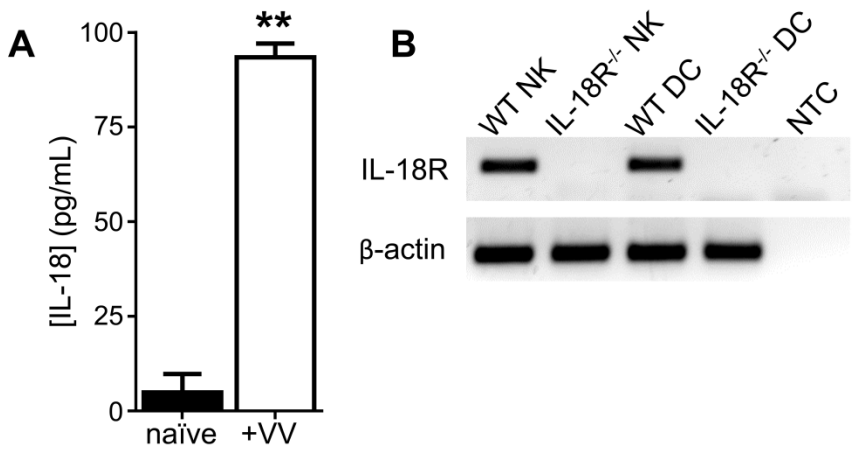

Figure 4: Validation of the NK-DC co-culture system. WT or IL-18R$^{-/-}$ NK cells were co-cultured with WT or IL-18R$^{-/-}$ bone-marrow derived CD11c^{+} DCs and stimulated with VV or left uninfected (naïve). (A) After 18h culture, supernatant of naïve and VV-infected wells was assessed for the presence of IL-18 by ELISA (n=3 wells). Data is representative of two independent experiments. Unpaired student's t-test produces $p<0.001$, represented by **. (B) RNA was extracted from sort-purified NK cells or DCs for semiquantitative RT-PCR to detect IL-18R (and control β-actin) expression. Data is representative of two independent experiments.

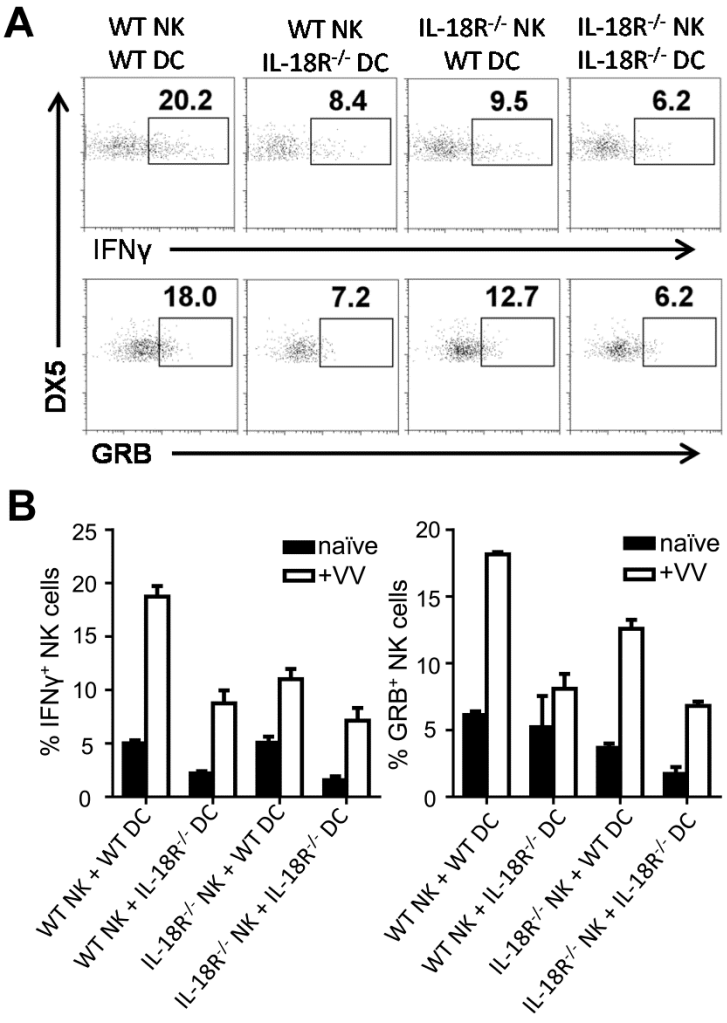

Figure 5: IL-18 signaling on both NK cells and DCs is required for NK cell activation to VV in vitro. WT or IL-18R$^{-/-}$ NK cells were co-cultured with WT or IL-18R$^{-/-}$ bone-marrow derived CD11c^{+} DCs and stimulated with VV or left uninfected (naïve) for 18 h. (A) The percentage of IFN-γ and GRB-positive DX5^{+}CD3^{-} NK cells are shown on representative FACS plots. (B) The mean percentages ±s.e.m. of IFN-γ and GRB-positive DX5^{+}CD3^{-} NK cells are shown (n=3 wells per condition). Data is representative of three independent experiments. Interaction term for two-way ANOVA is $p<0.01$ for both IFN-γ and GRB.

3.2.3 NK cell-extrinsic IL-18 signaling is critical for NK cell activation to VV infection in vivo

The observation that NK cell-extrinsic IL-18 signaling is also critical for NK cell activation in vitro led us to examine the physiological relevance of this finding in response to VV infection in vivo. NK cells were purified from $CD45.1^+$ C57BL/6 mice by FACS sorting and adoptively transferred into $CD45.2^+$ WT or $IL\text{-}18R^{-/-}$ recipient mice, followed by VV infection intraperitoneally 2 days later (Fig. 6A). The production of IFN-γ and GRB was assessed on the transferred $CD45.1^+$ WT NK cells 24 h after infection. WT NK cells transferred into WT recipients were able to mount an adequate response after VV infection (Fig. 6B, C). However, the production of IFN-γ and GRB was significantly ($p < 0.05$) reduced when WT NK cells were transferred into $IL\text{-}18R^{-/-}$ recipients (Fig. 6B, C), suggesting that IL-18 signaling on non-NK cells is required for NK cell activation. Thus, our results demonstrate that NK cell-extrinsic IL-18 signaling is required for NK cell responses during VV infection in vivo.

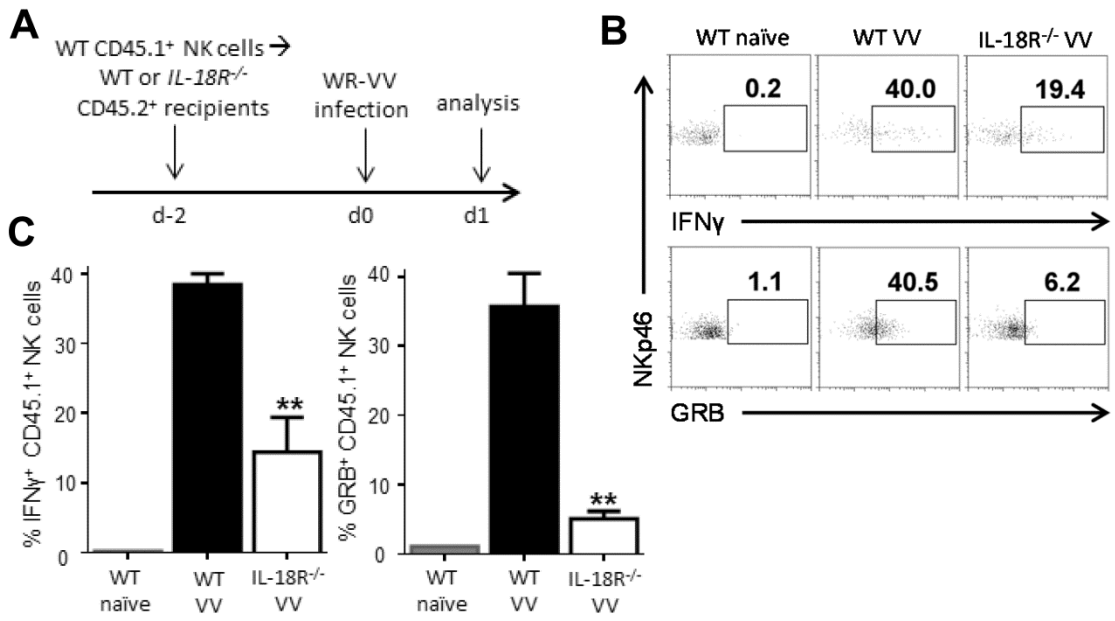

Figure 6: NK cell-extrinsic IL-18 signaling is critical for NK cell activation in vivo. (A) Five to 10 x 10^5 $DX5^+CD3^-$ NK cells from the spleens of $CD45.1^+$ WT C57BL/6 mice were purified by cell sorting and injected intravenously into WT or $IL\text{-}18R^{-/-}$ recipients, which are both $CD45.2^+$. Two days after cell transfer, recipient mice were infected with 5 x 10^6pfu VV i.p. or left uninfected (naïve). 24 h after infection, spleens were assayed for IFN-γ and GRB production by donor ($CD45.1^+$) NK cells. (B) The percentages of IFN-γ and GRB-positive $NKp46^+CD3^-$ NK cells from the WT $CD45.1^+$ donor NK cells are shown on representative FACS plots with recipient genotype and stimulation indicated above. (C) The mean percentages ±s.e.m. of IFN-γ and GRB-positive WT donor $NKp46^+CD3^-$ NK cells are shown (n=2 infected mice). Interaction term for one-way ANOVA is $p<0.01$ for IFN-γ and GRB production. Data is representative of four independent experiments. ** signifies a p-value <0.05 on post-hoc unpaired student's t test comparing WT donor NK cell activation between infected WT and $IL\text{-}18R^{-/-}$ recipients.

3.2.4 Dendritic cells are required for NK cell activation to VV in vivo

We next investigated which non-NK cell component is responsible for NK cell activation in response to VV infection in vivo. Conventional $CD11c^+$ DCs have been shown to play a critical role in NK cell activation (Andoniou et al., 2005; Lucas et al., 2007). Furthermore, our data in vitro also suggest that IL-18 signaling on DCs is critical for NK cell activation. Based on these findings, we hypothesized that DCs are required for the NK cell response to VV infection in vivo. To test this, we used mice carrying the CD11c-DTR transgene where the diphtheria toxin (DT) receptor is driven by the CD11c promoter so that it is expressed in $CD11c^+$ conventional DCs (Jung et al., 2002). This allows administration of DT to ablate DCs in vivo. While a few confounding cell populations do weakly express CD11c, upon administration of DT, we found DCs were efficiently depleted. The activation of NK cells assessed by the production of IFN-γ and GRB was significantly ($p < 0.01$) impaired in response to VV upon DC ablation (Fig. 7A, B). Relative and absolute numbers of NK cells were unaffected by DT treatment (data not shown). These results successfully replicate what others have shown (Lucas et al., 2007) and suggest, in the context of our work, that DCs do play an important role in NK cell activation in response to VV infection in vivo.

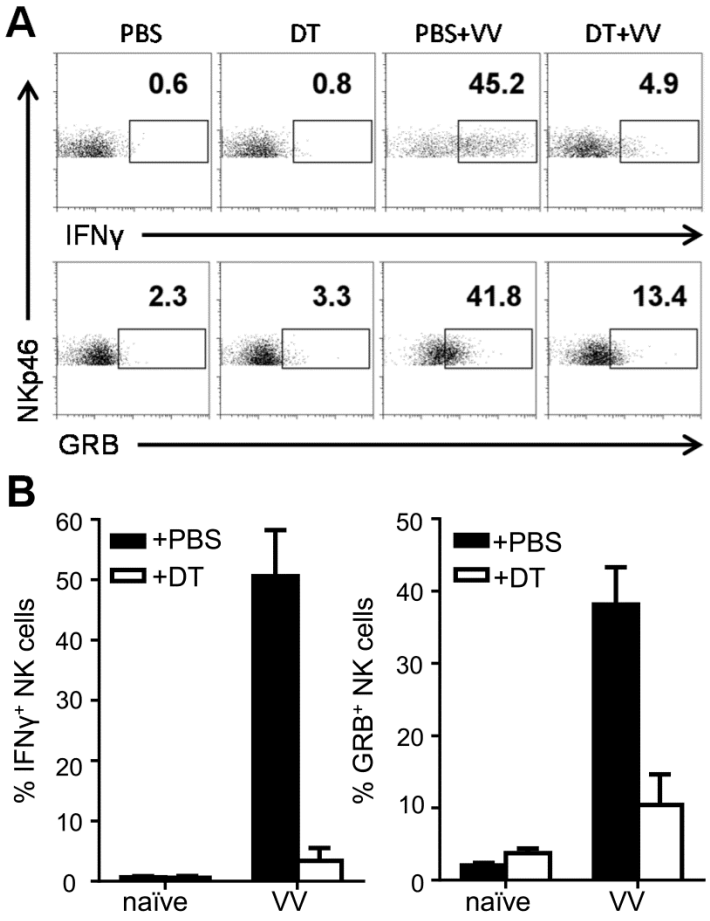

Figure 7: Dendritic cells are required for NK cell activation to VV in vivo. CD11c-DTR$^+$ transgenic mice were treated with diphtheria toxin (DT) and the next day were infected i.p. with 5 x 10^6pfu VV or left uninfected (naïve). Control mice were injected with PBS. 24 h after infection, splenocytes were assayed for IFN-γ and GRB production. In the naïve mice, CD11c$^+$ DCs, but not NK cells or total splenocytes, were depleted in mice carrying the transgene (data not shown). (A) Representative FACS plots with the percentages of IFN-γ and GRB-positive NKp46$^+$CD3$^-$ NK cells are shown. (B) The mean percentages ± s.e.m. of IFN-γ and GRB-positive NKp46$^+$CD3$^-$ NK cells (n=2 mice per group). Interaction term for two-way ANOVA is $p<0.01$ for IFN-γ and GRB. Data is representative of two independent experiments.

3.2.5 IL-18 signaling on DCs is required for Rae-1 upregulation to VV in vivo

The requirement of IL-18 signaling on DCs for NK cell activation prompted us to investigate the underlying mechanism. We have previously shown that the interaction between NKG2D ligand expressed on DCs and NKG2D on NK cells is critical for NK cell activation to VV infection (Martinez et al., 2010). NKG2D recognizes a diverse class of stress-induced host ligands that are all poorly expressed on healthy adult cells, but become upregulated during instances of cellular stress, such as viral infection (Andoniou et al., 2005; Guma et al., 2006). We hypothesized that upregulation of NKG2D ligands upon VV infection is dependent on IL-18 signaling on DCs. To test this, we examined the expression of the NKG2D ligand, Rae-1, on splenic $CD11c^{+}B220^{-}$ $CD11b^{+}$ conventional DCs in WT and $IL\text{-}18R^{-/-}$ mice 24 h after VV infection (Fig 8). We found a significant ($p < 0.01$) upregulation of Rae-1 expression in infected WT DCs compared to the uninfected naïve control (Fig. 9A, B). However, the upregulation of Rae-1 expression is significantly ($p < 0.01$) reduced in DCs from $IL\text{-}18R^{-/-}$ mice (Fig. 9A, B). To further demonstrate that IL-18 alone can directly act on DCs to upregulate Rae-1, we found that rIL-18 can be sufficient to upregulate Rae-1 expression on WT, but not $IL\text{-}18R^{-/-}$ DCs ($p < 0.0001$) in vitro (Fig. 9C). Thus, while IL-18 signaling on another cell-type may be contributing NKG2D ligand upregulation on the DC, DC-intrinsic IL-18 signaling can upregulate Rae-1 expression. These results suggest that IL-18 signaling on DCs is required for upregulation of NKG2D ligand expression, which in turn promotes NK cell activation via the NKG2D pathway.

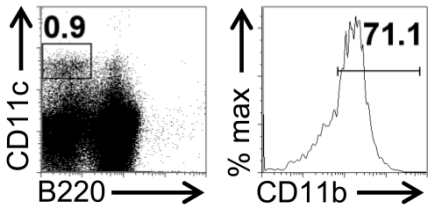

Figure 8: Gating strategy for splenic conventional DCs. Representative FACS plot show the gating strategy to identify $CD11b^{+}CD11c^{+}B220^{-}$ conventional DCs.

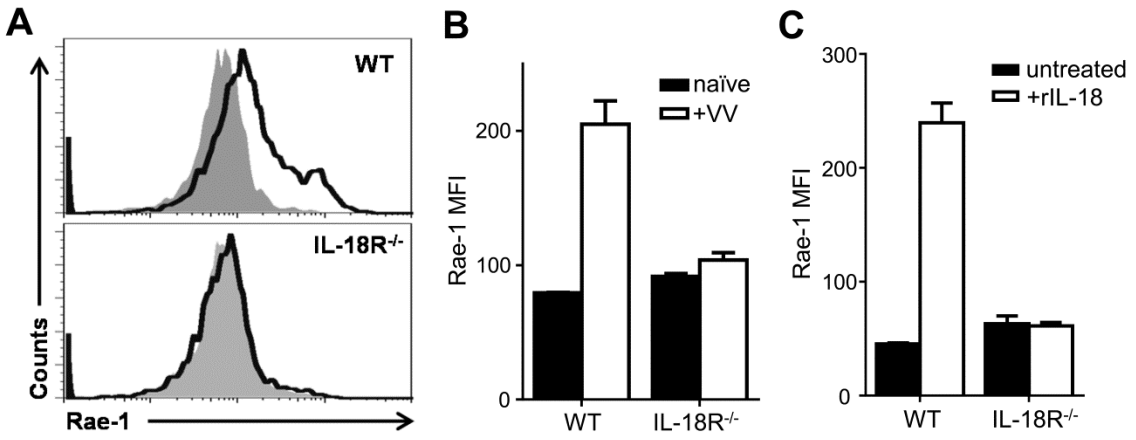

Figure 9: IL-18 signaling on DCs is required for Rae-1 upregulation to VV in vivo. WT or IL-18R$^{-/-}$ mice were infected with 5 x 10^{6}pfu VV i.p. or left uninfected (naïve). 24 h after infection, splenocytes were assayed for Rae-1 surface expression on CD11c$^+$CD11b$^+$B220$^-$ cDCs. (A) Histograms compare Rae-1 expression on cDCs from infected (thick line) and naïve (shaded histogram) WT (top panel) and IL-18R$^{-/-}$ (bottom panel) mice. (B) The mean fluorescent intensity ±s.e.m. of Rae-1 on cDCs is shown (n=3 infected mice). Data is representative of three independent experiments. Interaction term for two-way ANOVA is $p<0.01$. (C) The mean fluorescent intensity ±s.e.m. of Rae-1 on bone-marrow derived DCs cultured with or without 5ng/mL rIL-18 for four days is shown (n=3 wells). Data is representative of three independent experiments. Interaction term for two-way ANOVA is $p<0.0001$.

3.3 Discussion

In this report, we showed that IL-18 is critical for NK cell activation upon VV infection and the subsequent viral clearance in vivo. In addition to the requirement of NK cell-intrinsic IL-18 signaling for NK cell activation, we revealed a previously unknown mechanism by which IL-18 activates NK cells through DCs by promoting the expression of NKG2D ligands, which is required for NK cell activation via the NKG2D pathway. We showed that this NK cell-extrinsic IL-18 signaling is critical for NK cell response to VV infection.

Although IL-18 had been shown to play an important role in NK cell activation (Dao et al., 1998; Takeda et al., 1998) and antiviral defense (Gherardi et al., 2003; Pien et al., 2000; Tanaka-Kataoka et al., 1999), how IL-18 promotes NK cell activation and function during viral infections remained incompletely defined. This is the first report showing that IL-18 is critical for NK cell activation and the clearance of VV infection in vivo. Furthermore, we provided evidence that IL-18 signaling on both NK cells and DCs are important for NK cell activation. The NK cell-intrinsic role of IL-18 is consistent with previous reports in other models that IL-18 acts directly on NK cells for their activation (Chaix et al., 2008; Humann and Lenz, 2010; Ni et al., 2012). However, the NK cell-extrinsic role of IL-18 in NK activation upon viral infection represents a novel finding. This observation not only advances our understanding for how IL-18 works in promoting NK cell activation, but more importantly, may inform clinical applications of IL-18 in NK cell-based therapies. Since we have shown that IL-18 action on DCs is

required to fully activate NK cells, new treatments should exploit the same mechanism in order to achieve a maximal therapeutic effect.

NK cell activation in response to viral infection is regulated by engagement of NK activating receptors such as Ly49H, NKp46 and NKG2D (Brown et al., 2001; Guma et al., 2006; Mandelboim et al., 2001). We have previously shown that the interaction between NKG2D ligands on accessory cells such as DCs, and NKG2D on NK cells is critical for NK cell activation and function in the setting of VV infection (Martinez et al., 2010). However, it remained largely unclear how the upregulation of NKG2D ligands on DCs in response to VV infection is regulated. In this study, we provided evidence that IL-18 signaling on DCs is critical for upregulating NKG2D ligand expression upon VV infection. Recent studies have shown that TLR stimulation on macrophages can upregulate NKG2D ligand expression (Eissmann et al., 2010; Hamerman et al., 2004). Interestingly, both TLR and IL-18R signaling are mediated by the common adaptor, MyD88 (Adachi et al., 1998; Akira and Takeda, 2004). In addition, we have recently shown that STAT1 signaling in DCs is also critical for the upregulation of NKG2D ligands (Fortin et al., 2013), although this finding was in the 129 background. How IL-18R/TLR signaling promotes the upregulation of NKG2D ligands remains unknown. Thus, future studies are needed to define mechanisms responsible for IL-18R/TLR-mediated upregulation of NKG2D ligands. Similarly, a potentially synergistic role of STAT1 activation and IL-18/TLR signaling in NKG2D ligand upregulation requires further investigation.

It is important to note, however, that other accessory cells may also play roles in receiving IL-18 signals to activate NK cells via NKG2D ligand upregulation. Our data shows a clear role for DCs in receiving IL-18 signals to activate NK cells via NKG2D and, using the DTR ablation model, we have shown that NK cell activation depends on the presence of DCs in vivo. However, we have not entirely excluded a role for other accessory cells, such as macrophages, in contributing to NK cell activation by upregulating ligands to NKG2D in response to IL-18.

This work should also not exclude other mechanisms for cell-extrinsic IL-18 signaling in NK cell activation. We have demonstrated one route for how IL-18, acting on DCs, can boost NK cell activation by control of NKG2D ligand expression. However, this does not completely rule out other putative mechanisms, including IL-18-induced production of other pro-inflammatory cytokines also known to play roles in NK cell activation and proliferation such as IL-12 and IL-15. Furthermore, while IL-18 can upregulate Rae-1 expression on DCs in vitro, whether direct IL-18 signaling on DCs causes Rae-1 upregulation in vivo has not been conclusively demonstrated and IL-18 could conceivably act through another cell to induce the upregulation of NKG2D ligands on DCs.

In conclusion, we have shown that IL-18 is required for the innate immune control of VV in vivo. This is mediated by promoting the activation and effector function of NK cells. This is dependent on IL-18 signaling on both NK cells and DCs in response to VV in vitro. Furthermore, we showed in vivo that efficient NK cell activation to VV is dependent on DCs and IL-18 signaling in non-NK cells, suggesting an essential role for

NK cell-extrinsic IL-18 signaling in NK cell activation. In addition, IL-18 signaling on DCs promotes expression of Rae-1, an NKG2D ligand. Taken together, our results reveal a previously unknown role for NK cell-extrinsic IL-18 signaling in NK cell activation through upregulation of NKG2D ligands and may provide insights into the design of effective NK cell-based therapies for viral infections and cancer.

4. Mechanisms for IL-18-dependent NKG2D ligand upregulation on accessory cells

A version of this chapter is in preparation for submission.

4.1 Introduction

The NKG2D activation receptor plays a powerful role in antiviral immunity and tumor immune surveillance (Raulet, 2003). Loss of the receptor, found on all NK cells and some T cells, through genetic deletion or blocking antibody treatment can result in higher viral loads or larger tumors (Guerra et al., 2008; Martinez et al., 2010; Zhu et al., 2010). Artificially expressing ligands for the receptor on tumor cells, however, can make otherwise refractory tumors sensitive to NK cell-mediated clearance (Diefenbach et al., 2001).

Accessory cells – namely DCs and macrophages – express ligands to the NKG2D receptor for the purpose of boosting NK cell activation. TLR ligand stimulation can provoke the upregulation of the NKG2D ligands on the surface of human or murine macrophages (Eissmann et al., 2010; Hamerman et al., 2004). In mice, the NKG2D ligand Rae-1 is upregulated in response to TLR stimulation. We have previously shown that IL-18, whose receptor shares the MyD88 adaptor molecule with TLRs, can signal on DCs to upregulate Rae-1 in the context of VV infection.

However, the mechanism by which TLR/IL-18R signaling on the accessory cell contributes to Rae-1 upregulation remains incompletely defined. In one series of experiments, Eissmann and colleagues described how multiple mechanisms contributed to LPS-mediated upregulation of the NKG2D ligand MICA on human macrophages

(Eissmann et al., 2010). Specifically, they concluded that TLR4-MyD88 signaling controlled MICA expression via both transcriptional and post-transcriptional mechanisms. In this work, they also identified a novel pathway whereby LPS treatment resulted in the downregulation of microRNAs (miRNAs) that target MICA mRNA. But beyond this, little else is known about how TLR/IL-18R signaling results in the upregulation of NKG2Dligand expression.

In this paper, we first showed that – as we had previously shown in DCs – rIL-18 is sufficient to upregulate the surface expression of Rae-1 in macrophages. Since others had demonstrated that TLR signaling, which also uses the MyD88 adaptor, can upregulate Rae-1 expression in macrophages, we next compared the magnitude and kinetics of LPS and IL-18-mediated Rae-1 upregulation and confirmed that both do depend upon the MyD88 adaptor. Using chemical inhibitors to cell signaling molecules downstream of MyD88, we found that Rae-1 upregulation in response to LPS/IL-18 requires PI3K signaling. In particular, we show that LPS/IL-18 treatment correlates with the activating phosphorylation of Akt, a classic downstream signaling partner of PI3K. We next asked whether LPS and IL-18 signaling through PI3K could inhibit GSK-3, a possible negative regulator of Rae-1 expression. Consistent with this hypothesis, LPS and IL-18 treatment could be correlated with the inhibitory phosphorylation of GSK-3. Furthermore, chemical inhibition of GSK-3 proved sufficient to upregulate Rae-1 expression in both WT and MyD88-deficient macrophages. Finally, we found that many of our observations in macrophages also apply to IL-18-mediated Rae-1 upregulation in DCs. Collectively, our results reveal that TLR/IL-18R signaling through MyD88 can

control Rae-1 upregulation via PI3K-Akt-mediated suppression of GSK-3, a negative regulator of Rae-1.

4.2 Results

4.2.1 IL-18 increases macrophage expression of Rae-1

Previous studies have shown that TLRs, signaling through the adaptor MyD88, are capable of driving Rae-1 transcription and surface expression on murine macrophages. We have previously demonstrated that IL-18, whose receptor also signals through MyD88, can upregulate Rae-1 expression on DCs in the context of VV infection.

However, it remained to be demonstrated whether rIL-18 would be sufficient to upregulate surface expression of Rae-1 in macrophages. To test this, peritoneal macrophages from WT or IL-18R$^{-/-}$ mice were harvested by peritoneal lavage and cultured in the presence or absence of rIL-18. After 48 h of culture, the macrophages were assessed for their surface expression of Rae-1 (Fig 10A, B). We found Rae-1 expression increased roughly two-fold on WT macrophages upon IL-18 treatment (p<0.001). As expected, no upregulation was observed in IL-18R$^{-/-}$ macrophages, proving the specificity of this effect.

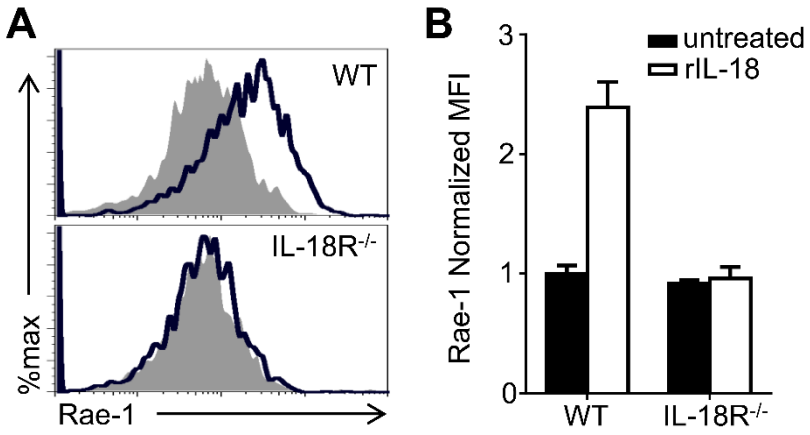

Figure 10: IL-18 increases macrophage expression of Rae-1. (A) Histograms of F4/80^{+}CD11b^{+} WT (top panel) and IL-18R-/- (bottom panel) peritoneal macrophages cultured for 48 h in the absence (shaded histogram) or presence (thick line) of 5ng/mL rIL-18 before analysis of Rae-1 expression. (B) The normalized mean fluorescent intensity ±s.e.m. of Rae-1 on macrophages is shown (n=3 wells). Data is normalized to untreated WT macrophages' expression of Rae-1. Data is representative of three independent experiments. Interaction term for two-way ANOVA is $p<0.001$.

4.2.2 The kinetics and magnitude of IL-18-mediated Rae-1 upregulation differ from LPS stimulation

IL-18R shares the adaptor molecule MyD88 with TLR signaling. However, IL-18 and TLR signaling may differ in their ability to control Rae-1 expression. Differences in the magnitude or kinetics of the response would enable macrophages to fine tune their response to different pro-inflammatory signals. Differences might also provide guidance for how IL-18 or TLR ligands could be used in a therapeutic setting to trigger NKG2D ligand upregulation.

We cultured macrophages with LPS, rIL-18, or left them untreated and harvested the cells at 12 h, 24 h, 36 h, and 48 h to assess their expression of Rae-1 (Fig 11A, B). Ultimately, LPS increased Rae-1 expression faster than IL-18, dramatically upregulating Rae-1 by 24 h of culture ($p<0.0001$). Furthermore, the magnitude of the increase in Rae-1 expression was slightly larger upon LPS treatment even at 48 h, the time of maximal effect for rIL-18. We then sought to confirm that – for both IL-18 and LPS signaling – the common adaptor MyD88 was indeed critical for Rae-1 upregulation (Fig 11C, D). We cultured WT and MyD88-deficient macrophages with LPS and rIL-18 for 48 h and found that while WT macrophages robustly upregulate Rae-1 expression in response to either LPS or IL-18 ($p<0.0001$), MyD88$^{-/-}$ macrophages fail to respond.

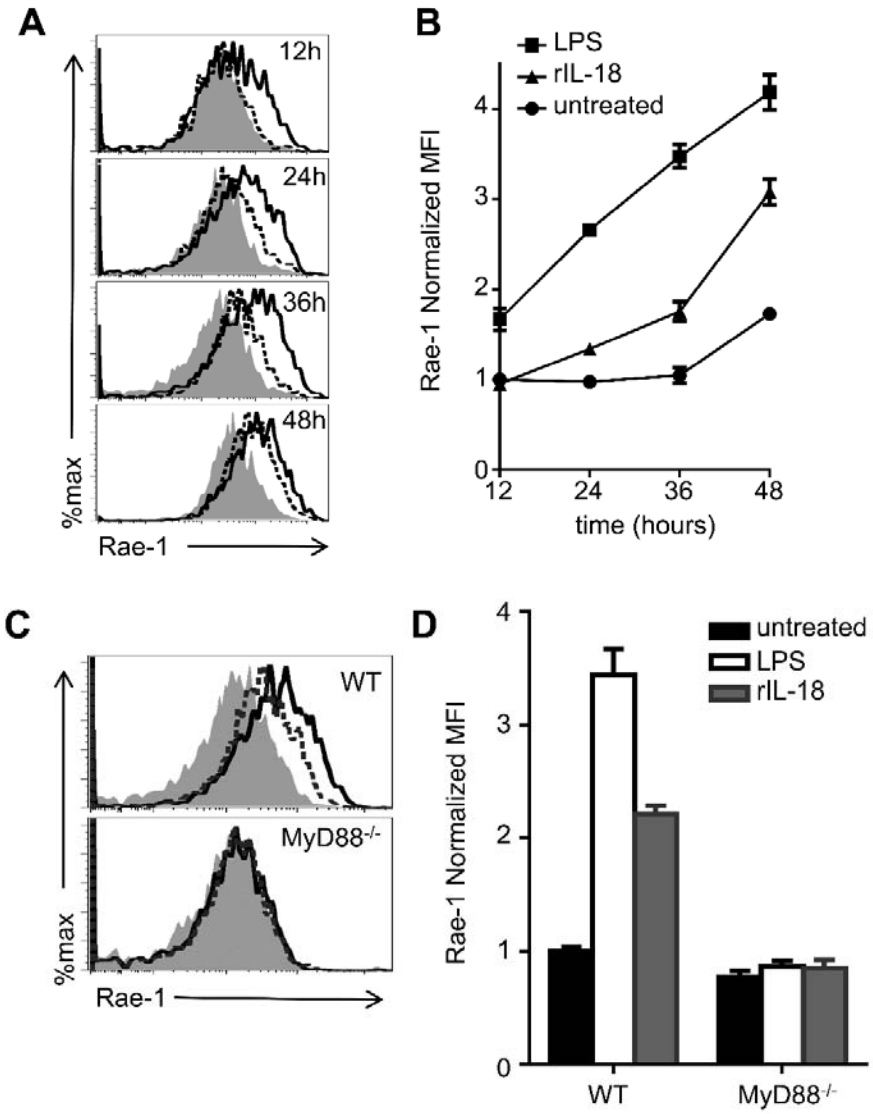

Figure 11: The kinetics and magnitude of IL-18-mediated Rae-1 upregulation differ from LPS stimulation. (A) Representative FACS plots of Rae-1 expression on F4/80^{+}CD11b^{+} peritoneal macrophages treated with 100ng/mL LPS (thick lines), 5ng/mL rIL-18 (dotted lines), or left untreated (shaded histograms) at 12 h, 24 h, 36 h, and 48 h of culture (B) Kinetics of Rae-1 expression shown by the normalized mean fluorescent intensity ±s.e.m. of Rae-1 on macrophages (n=3 wells). Data is normalized to untreated macrophages' expression of Rae-1 at 12 h. ANCOVA interaction term has $p<0.0001$. Data is representative of two independent experiments. (C) Representative FACS plots of Rae-1 expression on WT and MyD88$^{-/-}$ macrophages in response to LPS (thick lines), rIL-18 (dotted lines), or left untreated (shaded histograms). (D) The normalized mean fluorescent intensity ±s.e.m. of Rae-1 on macrophages is shown (n=3 wells). Data is normalized to untreated WT macrophages' expression of Rae-1. Interaction term for two-way ANOVA is $p<0.0001$.Data is representative of two independent experiments.

4.2.3 LPS and IL-18 signal through PI3K-Akt to upregulate Rae-1 expression

To describe the mechanism of LPS/IL-18-mediated Rae-1 upregulation, we began by examining known cell signaling molecules downstream of their common adaptor, MyD88. TLR-MyD88 signaling has been classically shown to follow a pathway beginning with homotypic recruitment of TIR domain-containing IRAK4 and culminating in the activation of NF-κB, p38, and JNK to promote the transcription of a panel of pro-inflammatory genes. However, MyD88 has also been shown to physically interact with PI3K through a SH2 binding motif within its TIR domain. This association can be used to signal through Akt and influence cytokine production and other effector functions in a variety of different leukocytes (Gelman et al., 2006; Guiducci et al., 2008; Martinez et al., 2010).

To test whether MyD88 might signal through PI3K to influence Rae-1 upregulation, we pre-treated macrophages with a chemical inhibitor of PI3K (Ly294002) for 1 h before culturing them with LPS or IL-18. After 48 h, we analyzed expression of Rae-1 on the macrophages (Fig 12A, B) and found that inhibition of PI3K abrogated LPS- or IL-18-mediated Rae-1 upregulation ($p<0.0001$). This suggests that MyD88 signaling through PI3K is necessary for Rae-1 upregulation in our model.

Since others have identified a role for PI3K in Rae-1 expression in the context of tumor cells and MCMV infection, we decided to further explore the TLR/IL-18R-MyD88-PI3K pathway's ability to influence Rae-1 upregulation (Tokuyama et al., 2011). We cultured macrophages with LPS or IL-18 and stained for the activating phosphorylation

of Akt (Serine 473), an immediate downstream effect of PI3K signaling. We found that both LPS and IL-18 are capable of leading to the phosphorylation of Akt ($p<0.01$), providing additional, though correlative, data for PI3K-Akt in LPS/IL-18-mediated Rae-1 upregulation (Fig 12C, D).

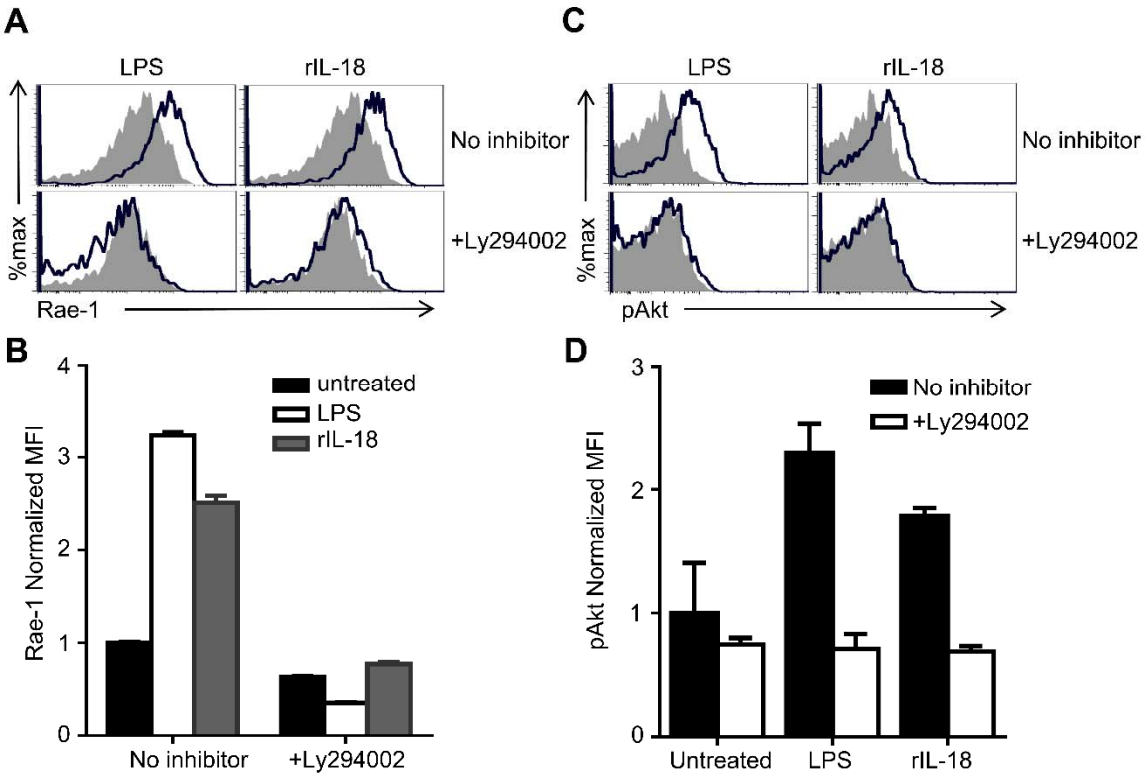

Figure 12: LPS and IL-18 signal through PI3K-Akt to upregulate Rae-1 expression. (A) Representative FACS plots of Rae-1 expression on macrophages cultured for 48 h with LPS (thick lines, left panels), rIL-18 (thick lines, right panels), or left untreated (shaded histograms) is shown either in the absence of inhibitors (top panels) or with 10μM Ly294002 (bottom panels). (B) The normalized mean fluorescent intensity ±s.e.m. of Rae-1 on macrophages cultured for 48 h is shown (n=3 wells). Data is normalized to untreated WT macrophages' expression of Rae-1. Interaction term for two-way ANOVA is $p<0.0001$. Data is representative of three independent experiments. (C) Representative histograms show staining with an antibody specific for the activating phosphorylation of Akt (S473) on macrophages treated with LPS (thick lines, left panels), rIL-18 (thick lines, right panels) or left untreated (shaded histograms) in the absence (top panels) or presence (bottom panels) of the PI3K inhibitor Ly294002 for 22 h. (D) Data is representative of two independent experiments. The normalized mean fluorescent intensity ±s.e.m. of pAkt on macrophages cultured for 22 h is shown (n=3 wells). Data is normalized to untreated WT macrophages' expression of pAkt. Interaction term for two-way ANOVA is $p<0.01$. Data is representative of two independent experiments.

4.2.4 GSK-3 inhibition increases Rae-1 expression

To identify the downstream signaling partners of the TLR/IL-18R-MyD88-PI3K-Akt pathway, we explored a rapidly expanding literature on the regulation of NKG2D ligand expression (Raulet et al., 2013). Two particular reports seemed relevant to our investigation. In one, the E2F transcription factors were found to be able to directly transcribe Rae-1 in proliferating fibroblasts (Jung et al., 2012). In another, the translation initiation complex was identified to play a crucial role in Rae-1 translation – given the mRNA's long 5' untranslated region (UTR) – during oncogenic Ras-mediated Rae-1 upregulation (Liu et al., 2012). Both E2F and the translation initiation complex are negatively regulated by GSK-3 – a pleiotropic kinase originally described in insulin signaling and glycogen metabolism (Diehl et al., 1998; Welsh et al., 1998; Woodgett, 2001). TLR-MyD88-PI3K signaling through Akt has already been shown to inhibit GSK-3 in macrophages to control cytokine production (Martin et al., 2005). We therefore hypothesized that MyD88-PI3K-Akt signaling could inhibit GSK-3 to release its putative breaks on Rae-1 transcription and translation. According to this hypothesis, we should be able to show that IL-18 and LPS can signal through PI3K to inhibit GSK-3 and that GSK-3 is a negative regulator of Rae-1 expression.

To test this hypothesis, we first therefore sought to correlate LPS and IL-18 treatment with the inhibitory Serine 9 phosphorylation of GSK-3 (Fig13A, B). We found that LPS or IL-18 can in fact drive the inhibitory phosphorylation of GSK-3 ($p<0.0001$). This increase in the inhibitory phosphorylation of GSK-3 can also be blocked upon

addition of the PI3K inhibitor Ly2940002 (Fig 13A, B). These data support our hypothesis that LPS or IL-18 can inhibit GSK-3 through PI3K.

Next, we wanted to test whether GSK-3 – which we have now shown to be inhibited by LPS and IL-18 treatment – is a negative regulator of Rae-1 expression. Our hypothesis would predict that inhibition of GSK-3 alone should be sufficient to upregulate Rae-1 expression. We therefore treated WT and MyD88$^{-/-}$ macrophages with a selective chemical inhibitor to GSK-3 (SB216763) and analyzed its effect on Rae-1 expression. When we analyzed Rae-1 expression 24 h after culture with the GSK-3 inhibitor, we observed an upregulation of Rae-1 expression in both WT and MyD88$^{-/-}$ macrophages ($p<0.01$, Fig 13C, D).

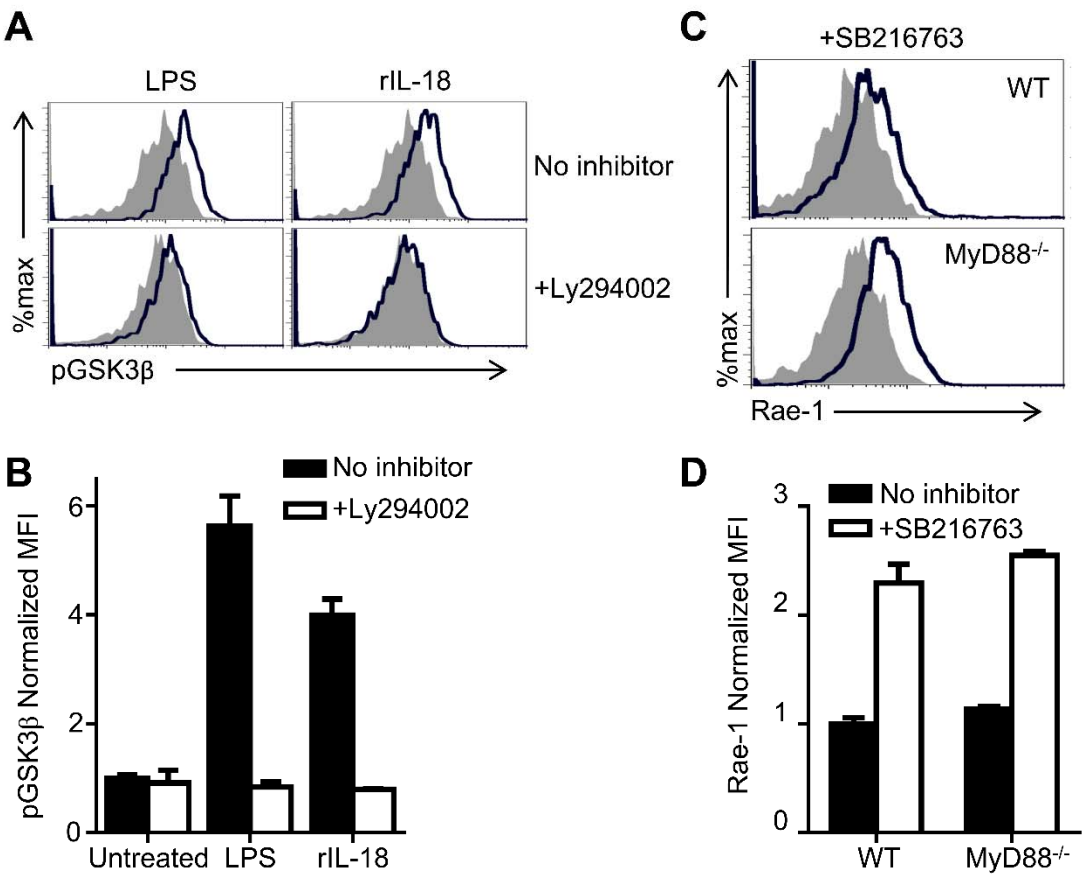

Figure 13: GSK-3 inhibition increases Rae-1 expression. (A) Representative FACS plots show staining with an antibody specific for the inhibitory phosphorylation of GSK-3β (S9) treated with LPS (thick lines, left panels), treated with rIL-18 (thick lines, right panels) or left untreated (shaded histograms) in the absence (top panels) or presence (bottom panels) of the PI3K inhibitor Ly294002 for 22 h. Data is representative of two independent experiments. (B) The normalized mean fluorescent intensity ±s.e.m. of pGSK-3 on macrophages cultured for 22 h is shown (n=3 wells). Data is normalized to untreated macrophages' expression of pGSK-3. Interaction term for two-way ANOVA is $p<0.0001$. Data is representative of two independent experiments. (C) Representative FACS plots show the expression of Rae-1 in the absence (shaded histograms) or presence of 10μM GSK-3 inhibitor SB216763 (thick lines) in WT (top panel) and MyD88$^{-/-}$ (bottom panel) F4/80^{+}CD11b^{+} peritoneal macrophages. (D) The normalized mean fluorescent intensity ±s.e.m. of Rae-1 on macrophages cultured for 24 h is shown (n=3 wells). Data is normalized to untreated WT macrophages' expression of Rae-1. Unpaired student's t-tests comparing no inhibitor to +SB216763 has $p<0.01$ for WT and MyD88$^{-/-}$ macrophages. Data is representative of three independent experiments.

4.2.5 IL-18 control of Rae-1 expression on DCs also signals through PI3K and GSK-3

While much of our work here – and previous work by others – has defined NKG2D ligand regulation in response to MyD88 stimulation in macrophages, DCs have a better defined in vivo role as accessory cells in NK cell activation. In particular, we have previously shown the importance of IL-18 signaling in the control of NKG2D ligands on DCs in NK cell activation to VV infection.

Therefore, we asked whether many of our key findings in macrophages apply to control of Rae-1 expression on DCs as well. We cultured bone marrow-derived DCs for 4 d in the absence or presence of rIL-18 and the PI3K inhibitor Ly294002. We found that the IL-18-mediated upregulation of Rae-1 expression on these DCs could be abrogated upon inhibition of PI3K signaling ($p<0.0001$, Fig 14A, B).

Furthermore, as we have shown in macrophages, chemical inhibition of GSK-3 signaling was sufficient to upregulate Rae-1 expression in DCs ($p<0.05$), suggesting that GSK-3 is a negative regulator of Rae-1 expression across different cell-types (Fig 14C, D).

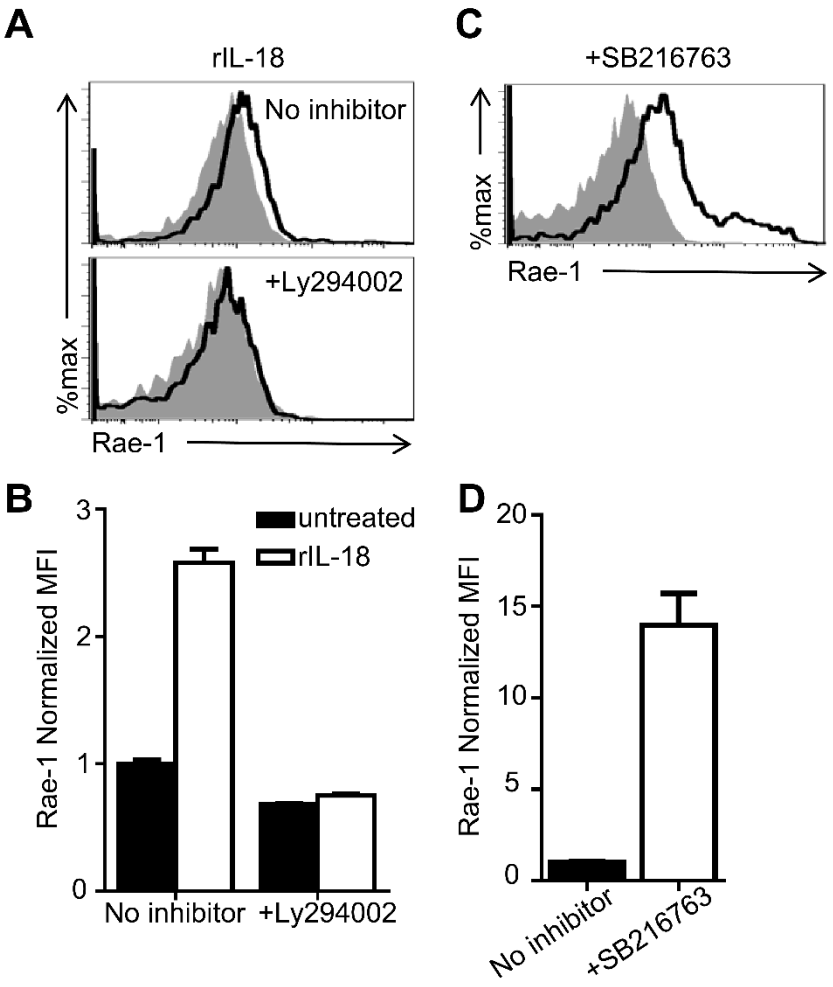

Figure 14: IL-18 control of Rae-1 expression in DCs also signals through PI3K and GSK-3. (A) Representative FACS plots show the expression of Rae-1 on $CD11c^{+}B220^{-}$ bone marrow-derived DCs in the absence (shaded histogram) or presence of rIL-18 (thick line) and the absence (top panel) or presence of the PI3K inhibitor Ly294002 (bottom panel) after 4 d of culture. (B) The normalized mean fluorescent intensity ±s.e.m. of Rae-1 on DCs cultured for 4 d is shown (n=3 wells). Data is normalized to untreated DCs' expression of Rae-1. Interaction term for two-way ANOVA is $p<0.0001$. Data is representative of two independent experiments. (C) Representative FACS plot shows the expression of Rae-1 on DCs in the absence (shaded histogram) or presence of 10uM GSK-3 inhibitor SB216763 (thick line) after 4 d of culture. (D) The normalized mean fluorescent intensity ±s.e.m. of Rae-1 on DCs cultured for 4 d is shown (n=3 wells). Data is normalized to untreated DCs' expression of Rae-1. Unpaired student's t-tests comparing no inhibitor to +SB216763 has $p<0.05$. Data is representative of two independent experiments.

4.3 Discussion

In this report, we revealed a previously unidentified pathway in TLR/IL-18R control of Rae-1 expression. Specifically, we first showed that IL-18 – like LPS – can upregulate the surface expression of Rae-1 on murine macrophages. The kinetics and magnitude of this upregulation were found to differ between LPS and IL-18, but both rely on their receptors' common adaptor MyD88. We next demonstrated that MyD88 signaling to PI3K is needed for LPS/IL-18-mediated Rae-1 upregulation and that LPS/IL-18 treatment correlates with the activating phosphorylation of Akt. We then showed that chemical inhibition of GSK-3 alone could upregulate Rae-1 expression on both WT and MyD88$^{-/-}$ macrophages and that LPS/IL-18 signals through PI3K to mediate inhibitory phosphorylation of GSK-3. Finally, we found that IL-18-mediated control of Rae-1 expression on DCs can also signal through PI3K and GSK-3. Taken together, we identified a novel pathway for how TLR/IL-18R can stimulate Rae-1 expression via MyD88-PI3K-Akt suppression of GSK-3.

We demonstrated how LPS and IL-18 signaling through their respective receptors and MyD88 can activate PI3K to inhibit GSK-3 to release its brakes on Rae-1 expression. While others had shown a role for PI3K signaling in NKG2D ligand expression in cancer cell lines and during viral infection, this is the first report to our knowledge of its role in TLR/IL-18-mediated Rae-1 control (Tokuyama et al., 2011). Furthermore, this is the first time GSK-3 has also been implicated in TLR/IL-18-mediated induction of Rae-1 expression. We hope that the identification of this novel cell signaling

pathway in Rae-1 regulation may broaden possible immunotherapies designed to harness NKG2D-mediated killing.

Despite the well-demonstrated importance of NKG2D activation signaling against viral infections and cancer, the literature has only recently begun to describe the mechanisms of control for its host-derived stress-mediated ligands (Raulet et al., 2013). The ability of TLR ligands and IL-18 to upregulate Rae-1 expression on accessory cells – which we have already shown to be important in NK cell activation during VV infection – may provide translatable opportunities to the clinic. But until now, only one group has investigated the mechanism by which TLR/IL-18 signaling results in the upregulation of NKG2D ligand expression (Eissmann et al., 2010). In that work, Eissmann and colleagues describe that MICA mRNA is increased in response to LPS treatment, though the increase was insufficient to account for the increase in the ligand's surface expression. Thus, in addition to controlling transcription, LPS appear to exert a post-transcriptional level of regulation, which they found to involve ATM/ATR molecules. They furthermore defined a mechanism of control via LPS-induced suppression of inhibiting miRNA. However, this description was far from complete and greater definition of this pathway can increase the number of putative strategies for pharmacologically increasing NKG2D ligand expression to boost NK cell activation therapeutically.

Since PI3K has been shown to both activate downstream of TLR signaling and play a role in NKG2D ligand expression in other contexts, our discovery of its role in IL-18/TLR-mediated Rae-1 control fits nicely with the literature (Gelman et al., 2006;

Tokuyama et al., 2011). Its previously defined role in NKG2D ligand regulation in MCMV infection and cancer cell lines, however, remained poorly understood. Exactly how PI3K signaling contributed to NKG2D ligand expression was not clear. Here, we propose one mechanism by which PI3K might influence Rae-1 expression by its inhibition of GSK-3. However, PI3K may control Rae-1 expression through multiple signaling pathways. The PI3K-Akt-GSK-3 pathway described here, though necessary, may not be the only one.

Furthermore, we make the novel observation that GSK-3 acts downstream of MyD88-PI3K-Akt in Rae-1 control. Again, this fits with the literature, which has established a TLR-MyD88-PI3K-Akt-GSK-3 pathway in other contexts and has shown GSK-3 capable in other settings of inhibiting transcription and translation factors others have associated with Rae-1 expression (Martin et al., 2005). However, while we have shown GSK-3 does negatively regulate Rae-1 expression, we have not explicitly addressed how GSK-3 mediates this control. Based on the good work in the literature, we speculate that GSK-3 inhibition of E2F transcription factors and the translation initiation complex may account for how GSK-3 blocks Rae-1 expression in resting cells (Diehl et al., 1998; Welsh et al., 1998; Woodgett, 2001). E2F transcription factors have already been shown to be critical for transcribing Rae-1 in proliferating fibroblasts and the translation initiation complex has been found necessary for the efficient translation of Rae-1 given its unwieldy 5′ UTRs (Jung et al., 2012; Liu et al., 2012). But there are alternative explanations for how GSK-3 may control Rae-1 expression. One group, which showed GSK-3 inhibition can upregulate MICA expression on human multiple myeloma

cell lines, correlated GSK-3 inhibition with a downregulation of STAT3, a negative regulator of MICA transcription, and found constitutively active STAT3 capable of preventing MICA upregulation in the face of GSK-3 inhibition (Fionda et al., 2013). This mechanism may prove true beyond human cancer cells, though it also may not apply here to the Rae-1 expression on murine macrophages. GSK-3 could also simply act upstream of NF-κB to influence Rae-1 expression (Martin et al., 2005).

In conclusion, we have shown that like TLR ligands, IL-18 signaling can upregulate Rae-1 on murine macrophages. Furthermore, we showed that while TLR and IL-18R control of Rae-1 both require the common adaptor MyD88, they differ in the kinetics and magnitude of their effect on Rae-1 expression. LPS/IL-18-mediated Rae-1 upregulation also depends upon PI3K signaling. Downstream, PI3K signals through Akt to inhibit GSK-3 – a negative regulator of Rae-1 expression. Many of these findings also appear to apply to Rae-1 expression on DCs – another important accessory cell in NK cell activation. Taken together, this unveils a novel pathway for TLR/IL-18 control of Rae-1 expression through a MyD88-PI3K-Akt-GSK3 signaling axis. We hope that our definition of this pathway broadens the opportunities to harness NKG2D in new immunotherapies.

5. Conclusions and Future Directions

NK cells exert powerful activities against viral infections and tumors. As a consequence, great interest has arisen in wielding these capabilities in new NK cell-based therapies. If NK cells are capable of controlling some viral infections and tumors, can they be pharmacologically stimulated and/or adoptively transferred to treat patients with viral infections or tumors that have escaped NK cell-mediated clearance?

To develop these tools, a greater understanding of the mechanisms of NK cell activation is required. NK cells have been shown to be particularly critical for the clearance of poxviral infections. We have therefore investigated the mechanisms of NK cell activation in the context of infection with VV, a well-studied member of the poxviral family and the vehicle of the vaccine responsible for the worldwide eradication of smallpox. NK cells are required to clear VV infection – the virus efficiently activates NK cells and recruits them to the site of the infection – making VV a suitable model to study the mechanisms of NK cell activation.

Before this work, we had already begun to define pathways in NK cell activation to VV infection and had identified both TLR-dependent and –independent mechanisms. Specifically, we had previously shown roles for direct TLR2-MyD88, type I IFN, and NKG2D signaling in NK cell activation to VV and viral clearance (Martinez et al., 2008; Martinez et al., 2010). What remained unclear in our model of NK cell activation to VV infection was how the NKG2D ligand is regulated during VV infection.

5.1 Inflammatory signaling from IL-18 can boost NK cell activation to VV by promoting Rae-1 expression on DCs

Does inflammatory signaling via IL-18 control NKG2D ligand expression on DCs boost NK cell activation and viral clearance?

IL-18 has previously been implicated in NK cell-mediated clearance of VV infection. Past work has shown that deletion of the IL-18bp from the genome of the virus can attenuate its infection in a fashion that depends upon NK cells (Reading and Smith, 2003). On the other hand, engineering the virus to express additional IL-18 promotes its clearance synergistically with IL-12 (Gherardi et al., 2003). However, neither of these reports actually showed that IL-18 is necessary for NK cell activation to VV or how IL-18 signaling might work. The available literature suggested that NK cells can respond directly to IL-18 signaling as they express the IL-18R and can be primed ex vivo with IL-18 to boost production of IFN-γ in response to IL-12 (Chaix et al., 2008). But is IL-18 actually necessary for NK cell activation in a physiologic setting?

To address this question, we compared NK cell activation in WT and IL-18R$^{-/-}$ mice infected with VV and found that NK cells in IL-18R$^{-/-}$ mice failed to mount the same response to the viral infection as WT. NK cells from IL-18R$^{-/-}$ mice produced lower amounts of IFN-γ and GRB and were less able to lyse YAC-1 target cells in a cytotoxicity assay. Altogether, since NK cell activity is necessary to control VV infection, weaker NK cell activation in IL-18R$^{-/-}$ mice translated into higher viral loads compared to WT. Thus, we concluded that IL-18 signaling is essential in NK cell activation to VV.

But these organismal knock-out studies still did not answer how IL-18 might be signaling to boost NK cell activation to the virus. To tease apart how IL-18 signaling resulted in NK cell activation, we cultured WT or IL-18R$^{-/-}$ NK cells with WT or IL-18R$^{-/-}$ DCs with the virus. We found that not only is NK cell activation impaired with IL-18R$^{-/-}$ NK cells, it is also impaired when the DC lacks the IL-18R. While the literature may have predicted a role for cell-intrinsic IL-18 signaling in NK cell activation, our discovery that IL-18 signaling on DCs is needed for NK cell activation in vitro is entirely novel. We followed this result with a cell-transfer experiment in which WT NK cells were transferred to either WT or IL-18R$^{-/-}$ mice. We found impaired activation in the donor WT NK cells upon transfer to the IL-18R$^{-/-}$ recipient, confirming our in vitro observation that cell-extrinsic IL-18 signaling is needed to efficiently activate NK cells to VV. We thus concluded that cell-extrinsic IL-18 signaling is required for NK cell activation to VV both in vitro and in vivo.

Since we showed in vitro that IL-18 signaling on DCs could boost NK cell activation, we asked whether DCs activate NK cells in vivo. Using a transgenic mouse model whereupon administration of diphtheria toxin specifically ablates CD11c^{+} cells, we showed that CD11c^{+} cDCs are necessary for NK cell activation in vivo.

But how would IL-18 signaling on DCs contribute to NK cell activation? Since we had already shown NGK2D to be critical to NK cell action against VV, we asked whether IL-18 could control the expression of NKG2D ligands on the surface of DCs. And we observed that while cDCs in WT mice upregulate the NKG2D ligand Rae-1 in response to viral infection, cDCs in IL-18R$^{-/-}$ mice fail to do so. We also noted that rIL-18

treatment could upregulate the expression of Rae-1 on DCs in culture. Taken together, we concluded that IL-18 signaling on DCs could boost NK cell activation to VV by controlling the expression of NKG2D ligands on the DC.

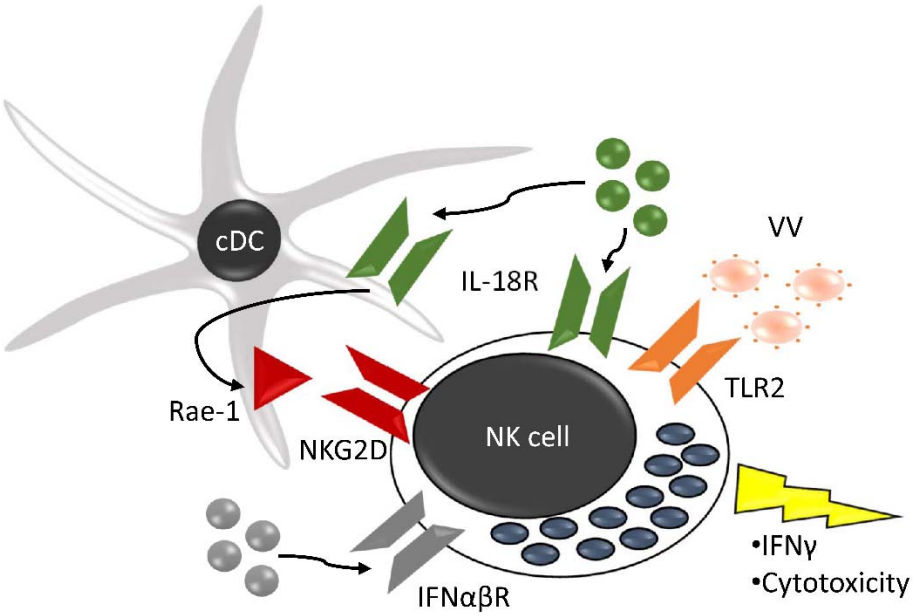

Figure 15: A cartoon summarizing known mechanisms of NK cell activation to VV infection.

Our conclusion that IL-18 signaling on DCs can control NKG2D ligand expression to boost NK cell activation to VV provides clarity to our question about how NKG2D ligands are regulated in the context of VV infection. This finding also complements the existing literature as TLR ligands, which signal through receptors that share the MyD88 adaptor with IL-18R, can also influence NKG2D ligand expression on accessory cells. We have provided definition about how NKG2D ligands can be induced in one powerful model of NK cell activation. We hope that lessons from this work – which showed IL-18 treatment of DCs capable of upregulating activating signals to NK cells – can be applied in the development of future NK cell-based therapies.

5.2 IL-18R-MyD88 signaling through PI3K-Akt inhibits GSK-3 to upregulate Rae-1 on accessory cells

Having established that IL-18 signaling on DCs can control NKG2D ligand expression to influence NK cell activation against VV infection, we next asked how does IL-18 signaling upregulate NKG2D ligand expression on an accessory cell. The answer to this question would not only clarify the mechanism by of IL-18-mediated NKG2D ligand control, but would hopefully inform strategies for the pharmacologic induction of NKG2D ligand expression against therapeutically desired targets.

Although others had shown how TLR ligands – which also signal through the common adaptor MyD88 – can control Rae-1 expression in macrophages, our understanding of the mechanism of IL-18/TLR control of Rae-1 remained limited (Hamerman et al., 2004). At the same time, a rapidly growing body of literature – cognizant of the importance of NKG2D signaling in viral and tumor control – has defined mechanisms of NKG2D ligand regulation in other settings.

To answer how IL-18 controls Rae-1 upregulation, we began by establishing a model where we could simultaneously explore the mechanism of TLR ligand and IL-18 induction of Rae-1. Since others have shown the ability of TLR ligands to control Rae-1 expression on macrophages – which provide important activation signals to NK cells as accessory cells - we sought to know whether IL-18 has a similar effect. Peritoneal macrophages were harvested and cultured in the presence and absence of rIL-18. We observed that – like LPS and other TLR ligands – IL-18 could upregulate expression of Rae-1 on these macrophages.

We next sought to compare LPS and IL-18-mediated Rae-1 upregulation side-by-side. We found that LPS could upregulate Rae-1 expression faster and to a greater degree than with IL-18. However we confirmed that both IL-18 and LPS signaling require the common adaptor MyD88 to influence Rae-1 expression. From these experiments, we concluded that both LPS and IL-18 signaling was MyD88-dependent, but differed in the magnitude and kinetics of their effects on Rae-1 expression.

MyD88 may be the common adaptor through which both IL-18 and LPS must signal to exert their effects, but it remained unclear how signaling downstream of it resulted in increased Rae-1 surface expression. To determine the downstream signaling pathway connecting MyD88 and Rae-1 upregulation, we pre-treated macrophages with a chemical inhibitor to PI3K to test the dependence of this signaling molecule on LPS and IL-18-mediated Rae-1 upregulation. We found that PI3K was necessary for LPS/IL-18-mediated Rae-1 upregulation. While others had shown a role for PI3K in NKG2D ligand expression in other settings, this is the first time it has been implicated in TLR/IL-18 control of Rae-1. If PI3K can now be concluded to be essential for NKG2D ligand expression across a variety of contexts, the use of PI3K inhibitors in cancer treatment may have the contradictory effect of hiding tumors from the immune system even while slowing PI3K-dependent tumor growth.

Left unanswered by others implicating PI3K signaling with NKG2D ligand expression is how PI3K exercises this control (Tokuyama et al., 2011). What's downstream of PI3K? Our literature search suggested that GSK-3 – which could inhibit transcription and translation factors associated with Rae-1 expression – might be

inhibited by PI3K-Akt signaling to release its brakes on Rae-1 expression. Ultimately, we found that chemical inhibition of GSK-3 was sufficient to upregulate Rae-1 expression on both WT and even MyD88-deficient macrophages, suggesting that GSK-3 negatively regulates Rae-1 expression. We further correlated the inhibitory phosphorylation of GSK-3 with LPS and IL-18 treatment and showed that it can be prevented by inhibiting PI3K signaling – allowing us to conclude that LPS and IL-18 can signal through PI3K to block GSK-3's suppression of Rae-1. This is particularly interesting as GSK-3 inhibitors have already been used in diabetes management and mood stabilization and could therefore be applied to other contexts for the purpose of boosting NK cell activation.

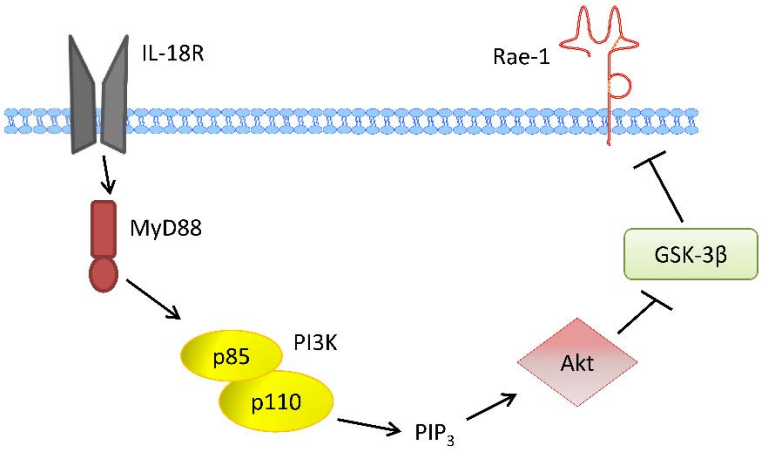

Figure 16: A cartoon representing a novel pathway of control for the NKG2D ligand Rae-1.

Overall, we can conclude that IL-18 and LPS can both signal through MyD88 to PI3K-Akt to inhibit GSK-3, a negative regulator of Rae-1 expression. The identification of these cell signaling pathways in NKG2D ligand expression augments the current literature and suggests new strategies that might harness NKG2D-mediated killing in future therapies.

5.3 Future Directions

Our work has established a novel mechanism for NK cell activation that will hopefully inform the design of future NK cell-based treatments. Specifically, we have uncovered a pathway whereby IL-18 can control NKG2D ligand expression on accessory cells via PI3K-dependent inhibition of GSK-3 to activate NK cells against VV infection. However, further work is necessary to complete this model – and to possibly define even more mechanisms of NK cell activation.

While our work has identified an essential role for DCs in NK cell activation – and demonstrated one mechanism by which DCs provide that necessary help – it remains unclear whether other cells act as accessory cells (including macrophages) in the context of VV infection. While others have shown important roles for macrophages in other settings, we have not addressed the role of macrophages – or other cell-types – in our model. Certainly, we have shown that macrophages – like DCs – can upregulate Rae-1 in response to IL-18 treatment. And, in unpublished work, we have noticed that macrophages (like DCs) do upregulate Rae-1 in response to VV infection in an IL-18-dependent fashion. But it remains difficult to determine whether macrophages are in fact necessary for NK cell activation to VV infection as we (and others) have shown for DCs. Our deficient knowledge of the role of macrophages has in part been due to the better tools available for DCs, namely a specific DTR ablation transgenic mouse model. The availability of a similar macrophage ablation mouse – an F4/80 promoter-driven DTR, for example – might facilitate analysis into how macrophages contribute to NK cell activation to VV. Comparing these results to the CD11c-DTR model data would allow a

more unbiased approach to understanding which cells are more important accessory cells in NK cell activation to VV. We could also try rescuing the impairment observed in our cell-transfer experiment (in which WT NK cells were impaired in IL-18R$^{-/-}$ recipients) by transferring WT DCs or macrophages to more conclusively determine which cell-type is responsible for the cell-extrinsic IL-18 signaling crucial to NK cell activation to VV. Furthermore, if we could obtain mice with a floxed IL-18R, we could cross them with mice carrying a NKp46, CD11c, or Lysozyme M promoter-driven Cre recombinase to conditionally delete IL-18R from NK cells, cDCs, or myeloid cells to more precisely determine the relative contribution of IL-18 signaling in these cell-types to NK cell activation.

Our work also proposes NKG2D ligand control of DC Rae-1 expression as a putative mechanism for cell-extrinsic IL-18 signaling though doesn't exclude other possible explanations. We could test the importance of NKG2D ligand control to NK cell activation from cell-extrinsic IL-18 signaling by determining whether lentivirally-driven expression of Rae-1 in IL-18R$^{-/-}$ DCs could rescue the deficiency in NK cell activation that we observed in our NK-DC co-culture. We could also look at whether IL-18R$^{-/-}$ DCs driven to express Rae-1 could be transferred back into IL-18R$^{-/-}$ mice to restore NK cell activation in donor WT NK cells.

Our experiments have been performed in a model where NK cells respond very efficiently to the inflammatory stimulus. It would be very interesting to test whether we can apply lessons from this model to a setting where NK cell fail to activate efficiently. For example, the B16 melanoma model is one where NK cells fail to control tumor

growth, which proceeds rapidly in this lethal tumor. However, others have demonstrated that B16 melanoma can be made controllable by NK cells if the tumor cells stably express NKG2D ligands. Having learned that IL-18 boosts NKG2D ligand expression against VV infection, we would be interested in observing whether transfer of rIL-18-treated DCs or intratumoral injection of rIL-18 might influence NK cell control of B16 melanoma. We could test whether this treatment might influence tumor infiltration, tumor growth, and mouse survival.

There also remains ambiguity in our model of NK cell responses to VV infection that invite further investigation. Specifically, it would be worthwhile to define the viral ligand for TLR2 in NK cell activation. Identification of this viral product would not only provide a possible new vaccine adjuvant, but might form part of a potentially new smallpox vaccine based on recombinant viral proteins rather than the live virus. Given the risks associated with current live viral vaccine – and the continuing need to guard against the threat of bioterrorist use of smallpox – the development of a recombinant vaccine with an improved safety profile would be an important pursuit (Fulginiti et al., 2003). Definition of the VV protein capable of engaging TLR2 and initiating an NK cell response would be a key accomplishment in the development of an alternative recombinant protein vaccine.

Additionally, there are several ways we could expand our study of the mechanism of IL-18 control of Rae-1 expression. For one, it would be interesting to clarify how MyD88-PI3K inhibition of GSK-3 acts to upregulate Rae-1 expression. How does GSK-3 tonically suppress Rae-1 in resting cells? Does this pathway affect Rae-1 at

the transcriptional or post-transcriptional level? Others have shown that E2F transcription factors can transcribe Rae-1 in proliferating fibroblasts. E2F transcription factors are downstream of cyclin D – a GSK-3 target for inhibition. Does GSK-3 inhibit E2F transcription factors to suppress Rae-1 expression?

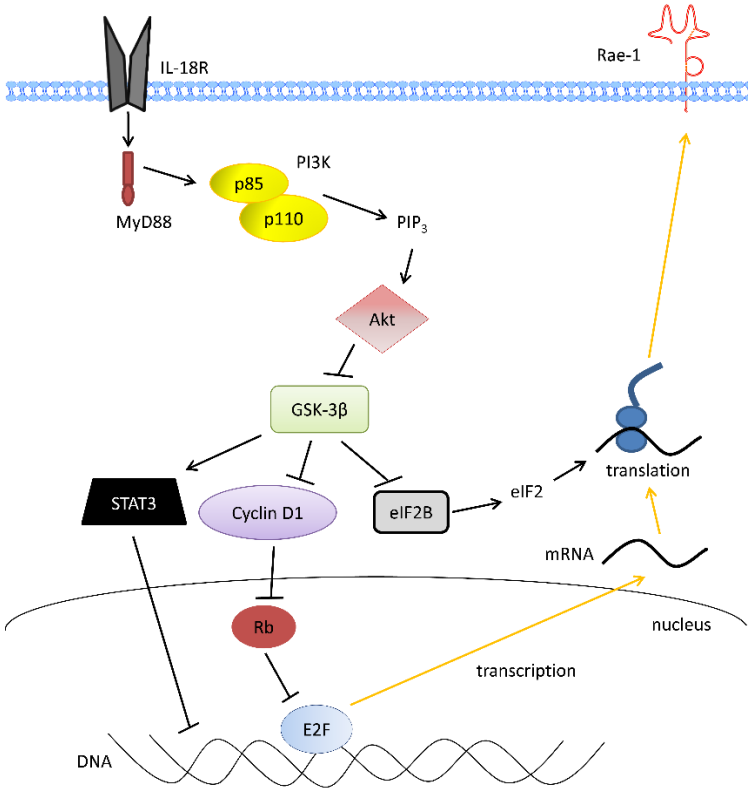

Figure 17: A cartoon depicting putative pathways by which GSK-3 may act as a negative regulator of Rae-1 expression.

To answer these questions about what's downstream of GSK-3, we would begin by determining whether GSK-3 inhibition with SB216763 can boost transcription of Rae-1. This would reveal at least part of the level of regulation by which GSK-3 tonically suppresses Rae-1. Specifically, we would assess how SB216763 treatment of peritoneal macrophages affects the level of Rae-1 mRNA by quantitative real-time PCR. We would also try treating macrophages in this experiment with the transcription inhibitor

actinomycin D to determine whether any increase in Rae-1 mRNA as a consequence of GSK-3 inhibition is due to improved mRNA stability or de novo transcription. As others have implicated E2F transcription factors in Rae-1 transcription in other contexts, we could then perform a chromatin immunoprecipitation to correlate LPS/IL-18 treatment and GSK-3 inhibition with E2F binding to the Rae-1 promoter.

However, even if we can link GSK-3 to transcription via control of E2F transcription factors, this would not exclude other putative mechanisms of action. Previous studies have shown in the case of Rae-1 upregulation due to oncogenic Ras that Rae-1 mRNA has an unwieldy 5′ UTR that depends upon the translation initiation complex for efficient translation. GSK-3 can inhibit a member of this complex, eIF2B, by phosphorylation. eIF2B, a guanine nucleotide exchange factor, is needed for eIF2 to return to its active, GTP-bound state when it can bind methionine t-RNA to the ribosome. Could GSK-3 inhibition cause Rae-1 upregulation by relieving a brake on Rae-1 translation? We would check whether LPS/IL-18 treatment and GSK-3 inhibition correlate with a decrease in the inhibitory phosphorylation of eIF2B by FACS analysis or Western blot. We could also use a commercially available inhibitor to eIF2-GTP-tRNA ternary complex and determine whether it can exercise any block on Rae-1 upregulation in response to GSK-3 inhibition or LPS/IL-18 treatment.

Another hypothesis for how GSK-3 mediates its suppression of Rae-1 was suggested by a group which observed GSK-3 inhibition capable of upregulating the NKG2D ligand MICA in human multiple myeloma cell lines. According to this model, GSK-3 activates STAT3 –a negative inhibitor of MICA transcription. This group found

that constitutively active STAT3 prevents GSK-3 inhibition from upregulating MICA. Does this apply to our model of Rae-1 expression in murine macrophages? First, we can check whether STAT3 inhibition is sufficient to upregulate Rae-1 in peritoneal macrophages. We can then test if LPS/IL-18 treatment and GSK-3 inhibition correlates with the activating phosphorylation of STAT3 previously observed in the multiple myeloma cell line. We may be limited in our ability to attempt other approaches (such as using constitutively active STAT3) due to the low efficiency of transfection of primary cells and the potential of lentiviral transduction to affect Rae-1 expression.

Finally, we have left unresolved whether our pathway controlling Rae-1 expression interacts with STAT1 – which our lab has also shown to be capable of controlling Rae-1 expression in DCs in response to VV infection. It is tempting to speculate that the two pro-inflammatory pathways might synergize to promote the expression of activating NKG2D ligands. However, our STAT1 work was done in different murine background (129) than what is discussed here. We can address whether these pathways interact by establishing first whether type I IFNs – which signal through STAT1 – can upregulate Rae-1 in our peritoneal macrophage model. We could then test whether there is an additive or synergistic effect on Rae-1 upregulation when type I IFNs and rIL-18 are added in combination to macrophages compared to when they are used alone. We could also look for overlap between their mechanisms by testing whether PI3K inhibition influences STAT1-mediated Rae-1 upregulation or whether type I IFN treatment correlates with the inhibitory phosphorylation of GSK-3.

Furthermore, we would like to investigate the functional consequences of our findings on the mechanism. We would be interested in testing the effects of in vivo treatment with the GSK-3 inhibitor SB216763 on NK cell activation. Most intriguingly, we would like to see if GSK-3 inhibition can rescue the activation of WT NK cells transferred into IL-18R$^{-/-}$ mice to VV and to test whether that rescue is NKG2D-dependent using a blocking antibody. More broadly, it would be worthwhile to investigate whether in vivo GSK-3 inhibition can boost NK cell activation to VV in WT and IL-18R$^{-/-}$ mice. We could also test whether DCs or macrophages – pre-treated with GSK-3 inhibitors and washed – can boost NK cell cytotoxicity to target cells in culture (and whether any enhanced cytotoxicity is NKG2D-dependent). It would also be worthwhile to explore the effects of GSK-3 inhibition in the setting of B16 melanoma – a tumor in which NK cells fail to properly activate to control its growth. Specifically, we could test whether GSK-3 inhibition – given systemically, intratumorally, or to pre-treated and transferred DCs or macrophages – would be capable of eliciting a response from NK cells that might effect tumor infiltration, tumor growth, and mouse survival. We could also go farther and cross floxed GSK-3 mice with mice carrying a CD11c or Lysozyme M promoter-driven Cre recombinase to conditionally delete GSK-3 from either the cDCs or myeloid cells to determine whether it can effect Rae-1 expression and NK cell responses. Overall, since GSK-3 inhibitors have been explored in other clinical contexts – mood stabilization and diabetes management – it would be especially exciting if we could use these to provoke NK cells against an otherwise refractory tumor.

Altogether, there are many exciting directions for us to build upon our current body of work. We particularly hope experiments attempting to translate our work in VV infection to a model of clinical interest where NK cells fail to properly respond are pursued. We sincerely believe insights gleaned into the mechanism of NK cell activation can be applied to inform the development of future therapeutic strategies that make use of the unique capabilities of NK cells.

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Biography

I was born on February 26, 1984 to Bonnie and Marty Brandstadter. I was raised in Port Washington, NY on Long Island with my younger sister, Rachel. While at Paul D. Schreiber High School, I had my first exposure to scientific research as an intern at a biotechnology company. I attended Wesleyan University in Middletown, CT where I completed a dual-major in molecular biology and economics, served as editor-in-chief of the campus newspaper, studied biochemistry for a full year at the University of Oxford (Corpus Christi College), and pursued my thesis research in a yeast genetics lab. At Oxford, I volunteered at a hospice, which sparked my interests in medicine and oncology. After graduating in 2006 from Wesleyan with a Bachelor of Arts with Honors, I returned to the University of Oxford (Green College) for a Master of Science in immunology. I then attended Duke University in Durham, NC as a member of the Medical Scientist Training Program. On May 25, 2013, I married my wife, Kaitlin. I am scheduled to receive a Doctor of Philosophy in Molecular Cancer Biology Program and Doctor of Medicine degrees from Duke in May 2015.

Publications

Brandstadter JD, Huang X, Yang Y. 2014. NK Cell-extrinsic IL-18 Signaling is Required for Efficient NK Cell Activation to Vaccinia Virus. *European Journal of Immunology. In Revision.*

Brandstadter JD, Huang X, Yang Y. 2014. LPS and IL-18 Signal through PI3K to Inhibit GSK3, a Negative Regulator of the NKG2D Ligand Rae-1 in Accessory Cells. *In Preparation.*

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Scientific Conferences

NK2013: 14th Meeting of the Society for Natural Immunity. September 18-22, 2013. Heidelberg, Germany. "IL-18 Signaling on Dendritic Cells is Required for Efficient NK Cell Activation to Vaccinia Virus." Poster Presentation.

NIAID Immune Mechanisms of Virus Control Program Kickoff Meeting. Conference Participant. November 30 – December 2, 2009. Washington, DC.

Presentations

Molecular Cancer Biology Program Student Seminar (MCB780). January 16, 2014. "NK Cell-extrinsic IL-18 Signaling is Required for Efficient NK Cell Activation to Vaccinia Virus."

Immunology Department Works in Progress. October 7, 2013. "IL-18 Signaling on Dendritic Cells is Required for Efficient NK Cell Activation to Vaccinia Virus."

Duke Center for Virology Works in Progress. February 25, 2013. "Mechanisms of Natural Killer Cell Activation to Viral Infection."

Molecular Cancer Biology Program Student Seminar (MCB780). November 11, 2012. "Mechanisms of Natural Killer Cell Activation to Viral Infection."

Awards

2007-2015 Duke Medical Scientist Training Program NIH Fellowship

2010-2011 Viral Oncology NIH Fellowship