The Innate Immune Response to Vaccinia 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 Immunology
in the Graduate School of Duke University

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ABSTRACT

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Abstract

Over the years, vaccinia virus (VV) has proven itself as a useful tool for the study of antiviral immunity, vaccine development, and potentially, cancer immunotherapy. VV is capable of eliciting a robust immune response; however, the mechanisms by which VV accomplishes this task remain unknown. The overall goal of this thesis project is to determine how VV activates the innate immune system, and how this activation contributes to viral clearance in vivo.

Plasmacytoid dendritic cells (pDCs) are critical mediators of antiviral immunity. The first aim of this thesis project was to determine the mechanism(s) by which VV activates pDCs for the production of type I IFNs and what role this activation plays in the clearance of VV in vivo. Our results demonstrate that murine pDC recognition of VV, a dsDNA virus, was MyD88-dependent, but TLR9-independent. Using the NFκB luciferase assay, we demonstrated that stimulation of TLR8-, but not TLR7-, transfected cells with either VV or VV-DNA resulted in substantial NFκB activation, and that siRNA-mediated knockdown of TLR8 expression in pDCs led to a complete ablation of VV-induced type I IFN production. We further identified that the VV genome was rich in poly A/T sequences, and synthetic poly A and poly T ODNs were capable of activating pDCs in a TLR8-dependent manner. In vivo, TLR8-MyD88 dependent pDC activation played a critical role in the innate immune control of VV infection. Our data demonstrates for the first time that TLR8 can serve as a DNA sensor in pDCs, and that murine TLR8 is functional in the context of a viral infection.

Type I IFNs play an important role in the activation of natural killer (NK) cells. However, the mechanisms underlying type I IFN-dependent NK cell activation remain largely unknown. Using a VV model of infection, the second aim of this project is to determine the mechanisms by which type I IFNs activate NK cells, as well as the role that type I IFN-activated NK cells play in VV clearance. We demonstrated that, rather than a cytoprotective role, type I IFN-dependent innate immune control of VV infection in vivo was mediated by activated NK cells. We further demonstrated that direct action of type I IFNs on NK cells, but not on DCs, is required for NK cell activation in response to VV infection, both in vitro and in vivo, leading to efficient VV clearance.

Given the importance of NK cells in VV clearance, the third aim of this thesis project is to determine how the TLR2-MyD88 pathway regulates NK cell activation and function in response to VV infection. We demonstrated that the TLR2-MyD88 pathway was critical for NK cell activation and the control of VV infection in vivo, independent of pro-inflammatory cytokine production. We further showed that intrinsic TLR2-MyD88 signaling on NK cells, but not on DCs, was necessary for NK cell activation and played a critical role in the control of VV infection in vivo. In addition, we showed that the NKG2D pathway was also important for efficient NK activation and function. Finally, we demonstrated that that VV could directly activate NK cells via TLR2 in vitro and TLR2-MyD88-dependent activation of NK cells by VV was mediated through the PI3K-ERK pathway. These results represent the first evidence that intrinsic TLR signaling is critical for efficient NK cell activation and function in the control of a viral infection in

vivo. Collectively, this thesis seeks to determine how VV, a potent stimulator of the innate immune system, initiates an efficient immune response and how this activation confers optimal viral control and clearance.

Dedication

I would like to dedicate this dissertation to my sister, Courtney Martinez Gillespie. I honestly could not have accomplished a single data point without her support, encouragement, and love. She has been and always will be my best friend and dearest confidant, and I feel extremely lucky to have such a wonderful person in my life.

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List of Abbreviations

Ad5	Adenovirus 5
APC	Antigen-presenting cell
BM	Bone marrow
CD	Cluster of differentiation
cDC	Conventional dendritic cell
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
ECTV	Ectromelia virus
ELISA	Enzyme-linked immunosorbant assay
EMCV	Encephalomyocardititis virus
ERK1/2	Extracellular signal-regulated kinase 1/2
FACS	Fluorescence activated cell sorting
Flt3L	FMS-like tyrosine kinase 3 ligand
GFP	Green fluorescent protein
GRB	Granzyme B
GM-CSF	Granulocyte-macrophage colony stimulating factor

HA	Hemagglutinin
HSV	Herpes simplex virus
IFN	Interferon
Ig	Immunoglobulin
IκB	Inhibitor of NFκB kinase
IKK	Inhibitor of NFκB kinase
IL	Interleukin
ip	Intraperitoneally
IRAK	Interleukin-1 receptor associated kinase
IRF	Interferon regulatory factor
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
iv	Intravenously
Kbp	Kilobase pair
LCMV	Lymphocytic choriomeningitis virus
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MAPK	Mitogen-activated protein kinase
MCMV	Murine cytomegalovirus
MCV	Molluscum contagiosum virus
MHC	Major histocompatibility complex

MMTV	Mouse mammary tumor virus
MOI	Multiplicity of infection
MVA	Modified vaccinia virus ankara
MyD88	Myeloid differentiation primary response gene 88
NDV	Newcastle disease virus
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells)
NK	Natural killer
NKG2D	NK group 2 member D
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerization domain
OAS	Oligo adenylate synthetase
ODN	Oligodeoxynucleotide
PAMP	Pathogen-associated molecular pattern
pDC	Plasmacytoid dendritic cell
PFN	Perforin
PGN	Peptidoglycan
PI3K	Phosphoinositide-3 kinase
PKR	RNA-dependent protein kinase
PMA	Phorbol myristate acetate
PRR	Pathogen recognition receptor
pAkt	Phospho-Akt

PCR	Polymerase chain reaction
PDCA-1	pDC Ag-1
pERK1/2	Phospho-ERK1/2
pfu	Particle forming units
RIG-I	Retinoic acid inducible gene I
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcription PCR
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Short interfering RNA
ssRNA	Single-stranded RNA
STAT	Signal Transducers and Activators of Transcription
TCR	T cell receptor
TGFβ	Transforming growth factor β
T_h	T helper
TIR	Toll/interleukin-1 receptor-like domain
TLR	Toll-like receptor
TRAF	TNF Receptor Associated Factor
T_{reg}	T regulatory

TRIF	TIR-domain-containing adapter-inducing IFNβ
UV-VV	UV-inactivated VV
VSV	Vesicular stomatitis virus
VV	Vaccinia virus
WNV	West Nile virus
WR	Western reserve
WT	Wild type

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Chapter 1: Introduction

1.1 The innate immune system

The immune system of vertebrates has evolved into two distinct, yet interconnected branches: the innate and adaptive immune systems. The adaptive immune system is characterized by its ability to generate, through genetic recombination, pools of antigen-specific B and T lymphocytes, as well as establish a long-lived pool of memory lymphocytes capable of a rapid, robust response upon repeated exposure [1]. Despite the unquestioned importance of adaptive immunity, the overwhelming majority of organisms lack this acquired immune response, and must solely rely on the ability of their innate immune system to eliminate invading pathogens [2]. A vital component of immunity, the innate immune response represents the initial line of defense against invading pathogens and is comprised of an army of multi-functional cell types, including phagocytic cells, such as macrophages, antigen presenting cells, such as conventional dendritic cells, and cytotoxic cells, such as natural killer cells [3]. This repertoire of innate immune cells eliminate pathogens via the engulfment and degradation of foreign particles, the processing and presentation of antigens to elicit an adaptive immune response, and the production of cytokines and interferons that activate, recruit, and destroy cells [1, 3-4]. Despite its lack of antigen-specific receptor recognition, the innate immune system represents a sophisticated network of multi-faceted cells, capable of distinguishing “self” from “non-self” by a collection of germline-encoded receptors, termed pattern recognition receptors (PRR), and mounting a robust immune response [3].

1.1.1 Pattern recognition receptors (PRR)

In terms of pathogen detection, the most important weapon in the innate immune system's artillery is the pattern recognition receptor (PRR). These non-clonal, germline-encoded receptors are capable of recognizing bacterial, fungal, parasitic, and viral components, known as pathogen-associated molecular patterns (PAMPs). These PAMPs constitute a "danger" signal to the innate immune cell and elicit an immune response, such as production of pro-inflammatory cytokines and type I interferons (IFNs). PRRs are expressed constitutively by both innate and adaptive immune cells and respond efficiently without the necessity for previous antigen exposure. The system of PAMP recognition is, in many ways, a flexible one. PRRs are capable of recognizing and responding to "non-self", molecular patterns present in a multitude of different pathogens, such as viral nucleic acids or bacterial cell wall components. For example, peptidoglycan (PGN), a component of both Gram-negative and Gram-positive bacteria, is a ligand for Toll-like receptor 2 (TLR2). Additionally, innate immune cells show unique PRR expression patterns, and activation of these signaling cascades results in multiple anti-pathogen responses. These PRRs and their basic signaling machinery can be found in a wide array of divergent species, including plants, fruit flies, fish, and mammals. This high degree of evolutionary conservation highlights its importance in pathogen resistance [3, 5]. In terms of viral recognition, there are three main families of PRRs: Toll-like receptors (TLR), retinoic acid-inducible gene I (RIG-I)-like receptors (RLR), and nucleotide oligermization domain (NOD)-like receptors (NLR).

1.1.1.1 Toll-like receptors (TLR)

Originally discovered in *Drosophila melanogaster* as a gene necessary for embryonic polarity, Toll (which is German for “awesome”) was later found to be a critical mediator of antifungal immunity in fruit flies [6]. At least 13 mammalian homologues, called Toll-like receptors (TLRs), have been identified, with recognition specificities spanning bacterial cell wall components, fungal components, parasites, viral nucleic acids, viral envelope proteins, and host stress factors. Table 1 summarizes the known microbial stimuli that activate TLRs and their cellular localization [3, 7].

Table 1: Summary of TLRs and their known ligands.

TLR	Localization	PAMP	Source of PAMP
TLR1/2	Plasma Membrane	Triacyl lipopeptides	Bacteria, mycobacteria
TLR2	Plasma Membrane	Peptidoglycan (PGN) Porins Lipoparabinomannan (LAM) Hemagglutinin Glycoproteins Phospholipomannan Glucuronoxylomannan Glycosylphosphophatidyl inositol mucin (tGPI-mucin)	Bacteria *Neisseria* Mycobacteria Viral Envelope (Influenza, Measles) Viral Envelope *Candida albicans* *Cryptococcus neoformans* *Trypanosoma*
TLR2/6	Plasma Membrane	LTA Diacyl lipopeptides Zymosan	*Streptococcus* Mycoplasma *Saccharomyces cerevisiae*
TLR3	Endosome	dsRNA	Viruses (WNV, RSV, MCMV)
TLR4	Plasma Membrane	Lipopolysaccharide Viral envelope proteins Heat-shock protein 60, 70 Fibrinogen	Gram-negative bacteria Viruses (RSV, MMTV) Host Host

Table 1: Summary of TLRs and their known ligands, continued.

TLR5	Plasma Membrane	Flagellin	Bacteria
TLR7	Endosome	ssRNA	Viruses (VSV, Influenza, RSV)
TLR8	Endosome	ssRNA	RNA viruses*
TLR9	Endosome	CpG DNA Viral dsDNA Hemozoin	Bacteria, mycobacteria Viruses (HSV, MCMV) *Plasmodium*
TLR10	Plasma Membrane	Unknown	Unknown
TLR11	Plasma Membrane	Uropathogenic bacteria Profillin-like molecule	Uropathogenic bacteria *Toxoplasma gondii*
TLR12	Plasma Membrane	Unknown	Unknown
TLR13	Plasma Membrane	Unknown	Unknown

*RNA viruses have been demonstrated to activate TLR8 in humans. A function for murine TLR8 has yet to be determined.

TLRs are type I integral glycoproteins, composed of an N terminal ectodomain of 16 to 28 leucine-rich repeats (LRR), a transmembrane domain, and a C terminal cytoplasmic domain termed the Toll/interleukin-1 (IL1) receptor (TIR) domain [4-5]. The LRR domain is responsible for ligand recognition and binding, with each LRR containing a β strand and an α helix, connected by loops. Whereas it is believed that ligand binding would occur in the concave groove formed by the LRR looping, recent crystallographic data for human TLR3 indicate that its ligand, dsRNA, binds the convex surface [3, 6]. The TIR domain shows significant homology to the IL1 receptor, and its recruitment and interaction with TIR domains in downstream adaptors is critical for the ensuing signaling cascade [4]. The TIR domain, of approximately 160 amino acids, is

composed of five β sheets surrounded by α helices, linked by flexible loops. There are two types of loops contained in each TIR domain: the "BB loop" and the "DD loop". Upon ligand binding, TLRs form homodimeric (TLR2-TLR2) or heterodimeric (TLR1-TLR2) structures; this dimerization is achieved via the binding of the BB loop of one TIR domain to the DD loop of the other TIR domain. For example, the BB loop of TLR1 interacts with the DD loop of TLR2, while the BB loop of TLR2 recruits and binds the TIR domain-containing adapter, myeloid differentiation primary-response gene 88 (MyD88). It is therefore presumed that successful signaling via TLRs requires functional TIR domains for dimerization and recruitment of downstream adaptors [10-11].

There are five known TIR-containing adaptors downstream of TLR signaling: MyD88, TIR-domain-containing adaptor protein-inducing IFNβ (TRIF), MyD88-adaptor-like (MAL), TRIF-related adaptor molecule (TRAM), and sterile α- and armadillo-motif-containing protein (SARM) [3, 7]. MyD88 is exclusively used by TLR1, 2, 5, 6, 7, 8, 9, and 11. TLR3 exclusively uses TRIF, while TLR4 utilizes both MyD88 and TRIF. The required adaptor molecules for TLR10, 12, and 13 remain unknown [3, 5, 7]. TLR2 and TLR4 require the bridge adaptor, MAL, for the successful recruitment and binding of MyD88 binding; while TRAM, a bridge adaptor associated with the plasma membrane, is required for proper recruitment and binding of TRIF to TLR4 [7]. TRAM, however, is not required for TLR3 signaling, despite being a TRIF-dependent pathway [3, 11]. The final TIR-containing adaptor, SARM, is unique in that it is a negative regulator of TRIF function [8].

The most common TLR signaling pathway occurs via a MyD88-dependent mechanism. Once MyD88 is recruited to and binds the TIR domain, it signals the recruitment of IL1R-associated kinase 4 (IRAK4), which in turn recruits IRAK1 and subsequently IRAK2. These kinases are quickly activated by IRAK4 phosphorylation, and are critical for TLR signaling [3, 5]. IRAK1 associates with and activates TNFR-associated factor 6 (TRAF6), an E3 ubiquitin protein ligase. It is at this point in the signaling cascade that TRAF6 mediates multiple pathways, leading to different transcriptional outcomes. During pro-inflammatory cytokine production, TRAF6, with an ubiquitination E2 enzyme complex, creates a K63-linked polyubiquitin chain on itself and IKK-γ/NFκB essential modulator (NEMO). Additionally, transforming growth factor-β (TGF-β)-activated kinase I (TAK1) and its binding proteins are recruited to TRAF6, and this TAK1 complex phosphorylates NEMO, mitogen-activated protein kinase (MAPK) kinase 6 (MKK6), and IKK-β, bound together in a complex referred to as the IKK complex. The activated IKK complex leads to phosphorylation, ubiquitination, and degradation of IκB, which consequently frees NFκB to translocate to the nucleus for the induction of pro-inflammatory gene transcription [3]. In addition to NFκB activation, the TAK1 complex also facilitates the activation of the MAPK, p38, and JNK, leading to translocation of AP-1 for the induction of pro-inflammatory gene transcription [3, 5].

In a manner similar to that of pro-inflammatory production, type I IFNs are induced by TLR-MyD88 signaling. Upon stimulation, MyD88, IRAK4, IRAK1, and TRAF6 form a complex at the TLR. However, this multimeric protein complex also

recruits members of the IRF family, which translocate to the nucleus and mediate type I IFN production. The use of IRF members is dependent on the type of cell being stimulated; IRF7, an inducer of both IFNα and IFNβ, is used predominantly by plasmacytoid dendritic cells, while IRF5 is used primarily by conventional dendritic cells. Furthermore, stimulation of the TLR3-TRIF pathway results in activation of yet another IRF member, IRF3, which only induces IFNβ [3-4]. The cell-specificity of transcription factors is just one explanation for the plasticity of the TLR response.

The two TLRs that use TRIF as an adaptor molecule, TLR3 and TLR4, are able to produce IFNβ in a MyD88-independent manner. Because TLR3 exclusively uses TRIF as its downstream adaptor, this TLR3-TRIF pathway is also capable of initiating production of pro-inflammatory cytokines [3, 9]. Upon stimulation with dsRNA, TLR3 recruits and binds TRIF via its TIR domains. Subsequently, TRIF associates with TRAF3, TRAF6, and the receptor-interacting protein 1 and 3 (RIP1 and RIP3). Together, TRAF6 and RIP1 are able to activate NFκB, resulting in its translocation to the nucleus and initiation of pro-inflammatory gene transcription. On the other hand, TRAF3 activates the TANK-binding kinase 1 (TBK1), which, in conjunction with inducible IκB kinase, IKK-ε, directly phosphorylates and activates IRF3 and IRF7. These IRFs homodimerize and translocate to the nucleus to for the induction of type I IFN gene transcription [3, 9].

TLR signaling on innate immune cells results in the production of pro-inflammatory cytokines and type I IFNs, which play pivotal roles in activating T cells, B

cell, and NK cells. Moreover, TLR stimulation results in the maturation of and co-stimulatory molecules upregulation on antigen-presenting cells (APCs), thus enhancing the APC-T cell interaction. Whereas the role of TLR signaling in innate immune cells is well-established, evidence is mounting in support of a direct role for the TLR pathway in the adaptive immune response. Stimulation of B lymphocytes by TLR ligands results in polyclonal activation and production of immunoglobulin M (IgM) antibodies, albeit of low affinity. Furthermore, human memory B cells, whose antigen specificity has already been determined, rapidly produce antibodies in response to CpG stimulation [10]. In the setting of vaccinia viral (VV) infection, it has been demonstrated that direct TLR2 signaling on $CD8^+$ T cells was necessary for their optimal clonal expansion and formation of a memory population. Upon VV stimulation, direct TLR2-MyD88 signaling on $CD8^+$ T cells promoted their proliferation and survival in a phosphatidylinositol 3-kinase (PI3K)-Akt-dependent manner in vitro. Indeed, $CD8^+$ T cell memory formation was dependent on the activation of Akt in response to VV infection in vivo [11]. These results concur with a previous study demonstrating that CpG DNA stimulation of $CD4^+$ T cells resulted in association of MyD88 with PI3K, leading to the downstream activation of Akt and glycogen synthetase kinase-3 (GSK-3). Moreover, a putative SH2 binding domain was identified in the TIR domain of MyD88, and ablation of this domain eliminated PI3K activation in response to CpG DNA stimulation [12]. The ability of TLR stimulation to access and activate such critical

mediators of effector function and survival adds yet another level of importance to TLR signaling [12-14].

The significance of TLR recognition and activation is accentuated by the inability of mice deficient in TLRs or their critical downstream molecules to control invading pathogens. As MyD88 is the most common adaptor of the TLR signaling pathway, mice deficient for MyD88 often display the most severe phenotype in terms of pathogen susceptibility. MyD88-/- mice exhibit decreased immune responses to infection by lymphocytoid choriomeningitis virus (LCMV), *Staphylococcus aureus*, *Toxoplasma gondii*, and herpes simplex virus (HSV), as evidenced by diminished IL12 and type I IFN production, defective NK cell activation, and increased viral load [7, 9, 15]. Mice deficient for TLR2, TLR3, TLR9, TRIF, or MyD88 all display a decreased resistance to murine cytomegalovirus (MCMV) infection [5, 7]. MCMV is just example of a pathogen activating innate immune pathways that converge and complement each other, thus synergistically establishing anti-pathogen state. Collectively, the TLR family provides a collaborative, crucial mechanism for recognizing and responding to a variety of "danger" signals, therefore setting the tone for pathogen clearance and aiding in the development of the ensuing adaptive immune response.

1.1.1.2 RIG-I like receptors (RLR) and NOD/NOD-like receptors (NLR)

Recent advances in innate immune recognition have uncovered the existence of two families of cytoplasmic PRRs, the RLR family and the NLR family. The NLR family includes NALPs, ICE-protease-activating factor (IPAF, also know as CARD12),

and nucleotide-binding oligomerization domain (NOD) proteins, and recognize bacterial components within the cytoplasm. NLRs are comprised of a trimeric domain structure. Like TLRs, NLRs possess a C-terminal LRR domain that is responsible for ligand recognition and binding. The N-terminal contains an effector domain, usually composed of a pyrin domain (PYD) or caspase-recruitment domain (CARD). There also exists a common NOD domain, which is believed to mediate oligermization. Stimulation of the NALP or IPAF pathway results in activation of caspase-1, which facilitates the processing of pro-IL1β to generate mature cytokines. Stimulation of the NOD pathway results in NFκB activation. In terms of biological significance, mutations in the human NOD2 gene have been associated with Crohn's disease, an inflammatory bowel disease [3, 5].

The RLR family is vital part of viral recognition in the innate immune system. To date, there are two members of the RLR family, retinoic-acid-inducible gene I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA5). These RLR members recognize viral dsRNA localized in the cytoplasm, and therefore inaccessible to the endosomal TLR3. RLRs are composed of an RNA-helicase domain, responsible for dsRNA detection, and a CARD domain, the effector domain. RLRs associate with a CARD-domain-containing adaptor called IPS-1, which is critical for an efficient RLR response. Activation of the RLR pathway results in potent type I IFN production, as well as NFκB activation. Pathologically, RLRs have been demonstrated to be important for

the recognition and immune control of vesicular stomatitis virus (VSV), Newcastle disease virus (NDV), and hepatitis virus C (HCV) [3, 9].

1.1.2 Cells of the innate immune system

1.1.2.1 Dendritic cells

Often referred to as the "sentinels" of the immune system, dendritic cells (DCs) are bone marrow-derived cells that are capable of shaping the adaptive immune response, exerting innate control of invading pathogens, and maintaining tolerance to self antigens. Based on their different hematopoietic origins, tissue localization, and receptor expression, DCs can be broadly divided into two subsets: conventional DC (cDCs) and plasmacytoid DC (pDCs).

1.1.2.1.1 Conventional dendritic cells (cDC)

During steady state conditions, resident cDCs can be further characterized into subsets based on their surface expression of receptors and tissue distribution. Mature, murine DCs express the classical DC marker and integrin α chain, CD11c, the co-stimulatory markers, CD80, CD86, and CD40, and the class II major histocompatibility complex (MHC class II) during steady state conditions. Upon infection, DCs upregulate these co-stimulatory markers and MHC class II, which vastly improve their ability to activate T cells and induce their proliferation [16]. Some subtypes of cDCs also express CD11b (the integrin αM chain of Mac-1) and CD205 (an endocytic receptor that mediates efficient processing and presentation of antigens in vivo) [16-17]. Interestingly, cDCs can also express the classical T cell markers, CD4 and CD8 (expressed as an αα

homodimer) [18]. The presence of these "lymphoid" surface markers has raised questions about the hematopoietic origin of cDCs, which were originally thought to be derived from common myeloid progenitors (CMPs). It was demonstrated that murine myeloid precursors could differentiate into DCs in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF); furthermore, bone marrow-derived CMPs can reconstitute irradiated mice of splenic and thymic DCs in vivo [19]. However, intravenously transferred bone marrow-derived common lymphoid progenitors (CLPs) were able to give rise to both $CD8^-$ and $CD8^+$ cDCs in the spleen and the thymus [23]. Therefore, cDCs can arise from both lymphoid and myeloid precursors, the origin of which has little effect on its mature phenotype.

Based on these markers, cDCs can be segregated into five subtypes. The most prevalent cDC subset is $CD11c^+CD8^+CD4^-CD205^+CD11b^-$ and is the predominant cDC subtype in the thymus, as well as being present in the spleen and lymph nodes. The spleen also contains two subpopulations of cDCs, characterized by their expression pattern of $CD11c^+CD8^-CD4^{+/-}CD205^-CD11b^+$. The dominant subsets of cDCs in the lymph nodes include those that are $CD11c^+CD8^-CD4^-CD205^-CD11b^+$ and $CD11c^+CD8^-CD4^-CD205^+CD11b^+$. More specifically, skin-draining lymph nodes contain "Langerhans" DCs, which are $CD11c^+CD4^-CD8^+CD205^+CD11b^+$ [19, 21]. The different location and specificity of these cDC subtypes allows for peripheral antigens to be sampled, processed, and presented to T cells in lymphoid tissues, thus connecting the site of pathogen entry to that of the immune response [20].

As members of the innate immune system, cDCs are armed with a repertoire of PRRs and are activated to perform a variety of important immunological roles. Upon sensing of "danger" signals, such as viral nucleic acids or bacterial components, cDCs produce a milieu of cytokines, including IL1, IL6, IL12, IL4, IL18, type I IFNs, and TGFβ [19, 24-25]. Subsequently, these cytokines can sculpt the T_H polarization environment, initiate the transcription needed to achieve an antiviral state, and assist in the activation of other cell types, such as NK cells, B cells, and T cells [19, 25]. In the case of MCMV infection, cDCs upregulate ligands for the activating receptor, NKG2D, resulting in effective activation of NK cells. Additionally, cDC-secreted IL12 and IL18 were required for optimal NK cell production of IFNγ and subsequent cytotoxicity and viral clearance [21]. Upon VV infection, cDCs produce the pro-inflammatory cytokines, IL6, IL1, and IL12, in a TLR2-MyD88-dependent manner; however, the mechanism by which they produce IFNβ is TLR-independent, suggesting that a non-TLR, cytosolic nucleic acid sensor could play a role in type I IFN production by cDCs in response to VV infection. Three days after VV infection, mice deficient for the TLR2/MyD88 pathway (and hence, pro-inflammatory cytokine production) or the type I IFN receptor (IFNα/βR) were unable to clear viral load as efficiently as their wild type counterparts In addition, upon VV infection, cDCs deficient in TLR2, MyD88, or IFNα/βR were unable to upregulate key co-stimulatory molecules, such as CD86, compared to wild type cDCs. This defect also resulted in diminished activation of antigen-specific $CD8^+$ T cells. Therefore, cDC-derived secretion of pro-inflammatory cytokines and type I interferons

play a pivotal role in mediating early antiviral immunity as well as shaping the ensuing adaptive immune response [22].

Antigen uptake, processing, and presentation are the classical, essential functions of cDCs, and it is for this reason that cDCs are referred to "professional" antigen presenting cells (APCs). Intracellular or endogenous antigens, such as viral particles, are processed into peptides of eight to ten amino acids and bound to the MHC class I molecule, which is ubiquitously expressed on most host cells. This peptide-MHC class I complex presents the antigen to and activates the effector function of $CD8^+$ T cells. On the other hand, $CD4^+$ cells are presented to and activated by peptides bound to the MHC class II molecule, whose expression is restricted to DCs, as well as some B cells and macrophages. Extracellular or exogenous antigens are phagocytosed by cDCs and processed by the lysosomal/endosomal pathway into peptides of thirteen to eighteen amino acids in size. They are then bound to the MHC class II molecule and transported to the surface, wherein they present antigen to $CD4^+$ T cells [16, 20]. Collectively, cDCs represent an important subset of the innate immune system, whose influence reaches both the early mediators of immune control as well as the antigen-specific cellular populations.

1.1.2.1.2 Plasmacytoid dendritic cells (pDC)

First identified in T cell zones of human lymphoid tissue as "plasma"-like cells, plasmacytoid dendritic cells (pDCs) are a subpopulation of dendritic cells, originally termed interferon-producing cells (IPCs), capable of producing large amounts of type I

interferons (IFNs) in response to viral stimulation [23-24]. In mice, pDCs are characterized by their expression of CD11c, B220, Ly6G/C, and mPDCA-1; their human counterparts, however, express CD4, BDCA2, and CD123, but not CD11c, B220, or Ly6G/C [23]. It is known that Flt3L is the chief cytokine responsible for pDC differentiation from hematopoietic stem cells in both humans and mice; however, the developmental mechanisms for pDC generation are not fully understood [23, 25]. There are numerous hypotheses concerning the origins of pDCs. Because pDCs express gene transcripts for pre-T cell receptor α (pTα) and Immunoglobulin H (IgH) diversity-joining rearrangements, one school of thought believes that pDCs are of lymphoid origin; these recombination associated genes are not expressed in cDCs, which are capable of being derived from both lymphoid and myeloid progenitors. Furthermore, inhibition of the E2A proteins, Id2 and Id3, resulted in a developmental block of T cells, B cells, and pDCs [26-27]. In contrast, studies have observed that $Flt3^+$ cells from either CMPs or CLPs can develop into both cDCs and pDCs. These observations, along with others, suggest that there exists a common DC precursor population [28-29]. The developmental pathway and the molecular regulation of pDC differentiation require further analysis.

pDCs are critical mediators of the antiviral immune response, as they are able to produce vigorous amounts of type I IFNs upon detection of the presence of viral nucleic acids. Innate immune cells possess a multitude of PRR families, including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs). However, pDCs only utilize one of these families, TLRs, to sense viral and microbial

nucleic acids. Moreover, pDCs have a more restricted repertoire of TLR expression and reactivity, as they preferentially express TLR7, TLR8, and TLR9, but not TLR2, TLR3, TLR4, or TLR5 [24, 30]. This limited TLR expression pattern indicates that pDCs are unable to respond to bacterial components, such as lipopolysaccharide (LPS), peptidoglycan (PGN), or flagellin. More intriguingly, pDCs are unable to respond to viral dsRNA, as sensed by TLR3. Taken together, this suggests that pDCs are a highly specialized cellular subset, evolved to detect certain "non-self" nucleic acids for the production of type I IFNs.

Production of type I IFNs by pDCs occurs strictly in a TLR-MyD88-dependent manner. More specifically, studies have demonstrated that production of type I IFNs by pDCs requires activation of the endosomal nucleic acid sensors, TLR7 and TLR9, which sense viral single-stranded RNA and unmethylated CpG-rich DNA (a mimic of bacterial DNA), respectively [28-29]. TLR9 has been demonstrated to be crucial in pDCs for type I IFN production in response to herpes simplex virus 1 (HSV-1), HSV-2, and MCMV [36-38]. Likewise, TLR7 is critical for recognition of the ssRNA viruses influenza, vesicular stomatitis virus (VSV), Sendai virus, and respiratory syncytial virus (RSV) [24]. Whereas human TLR7 and TLR8 have both been implicated in viral ssRNA recognition, a biological function for murine TLR8, which can respond to synthetic ligands such as R848, has yet to be identified [31]. Activation of the human TLR8 pathway has been implicated in the reversal of suppressive T_{reg} cell function [40]. Although TLR7 and TLR8 are closely related phylogenetically, murine TLR8 is not

stimulated by natural ligand ssRNA, as murine TLR7 is. However, a recent study demonstrated that oligodeoxynucleotides rich in poly T sequences were able to enhance murine TLR8 stimulation in vitro [32]. Biochemical analysis of human TLR8 established that ligand interaction with and activation of the TLR8 pathway requires a low pH environment, such as the late endosome [33]. This data differs from that of TLR9 signaling, where induction of type I IFN production was activated by TLR9 signaling in the early endosomes of pDCs; however, cDCs quickly translocate the activated TLR9-MyD88 complex to the late endosome/lysosome, resulting in cDC maturation, but not type I IFN production [34]. It is perhaps these differential ligand interaction and signaling requirements that distinguish endosomal TLR signaling in pDCs and cDCs. As pDCs produce type I IFNs in a TLR-MyD88-dependent manner, thus limiting their recognition catalog for viral nucleic acids to TLR7, TLR8, and TLR9, a physiological role for murine TLR8 seems forthcoming.

All of these endosomal TLRs recruit and signal through MyD88, and pDCs from MyD88-/- mice are unable to produce type I IFNs in response to TLR7, TLR8, or TLR9 ligands [23]. Upon engagement of the TLR and MyD88, a multi-protein signaling complex is assembled in the cytoplasm and includes IRAK1, IRAK4, TRAF6, and IRF7 [24, 30]. MyD88 recruits and interacts with IRAK4, which in turn phosphorylates IRAK1. IRF7 is phosphorylated by IRAK1, and it has been demonstrated that IRAK1-/- pDCs are unable to produce to type I IFNs in response to TLR7 or TLR9 ligands [35]. It has also been reported that the E3 ubiquitin ligase, TRAF6, is required to activate IRF7,

although its mechanisms remain unknown [30]. Upon activation, IRF7 translocates to the nucleus and initiates the transcription of type I IFN genes. IRF7, which has constitutively high expression in pDCs compared to cDCs, is required for type I IFN production by pDCs in response to viral nucleic acid stimulation [24, 30]. Furthermore, it has been reported that pDCs are capable of retaining nucleic acid ligands longer within their endosomes with the TLR-MyD88-IRF7 complex, thus resulting in a more robust type I IFN response [34-35].

In 2001, it was demonstrated that stimulation of pDCs with A-type CpG ODN, but not B-type CpG-ODN, resulted in a type I IFN production; stimulation with B-type CpG-ODN resulted in IL6 production and induced B cell proliferation and antibody production [36]. Furthermore, it was demonstrated that A-type CpG ODN specifically localizes with TLR9, MyD88, and IRF7 in the endosome, and this localization is necessary for type I IFN production [34]. This is not the case for B-type CpG ODN, which localizes to the lysosome, thereby bypassing the crucial TLR9-MyD88-IRF7 interaction in favor of the TLR9-MyD88-IRF5 pathway, which is predominant in cDCs [37]. However, when B-type CpG ODN is complexed to the cationic lipid, DOTAP, thus mimicking the endosomal trafficking of A-type CpG ODN, IRF7 activation and subsequent type I IFN production is achieved [34]. These differential stimulation profiles indicate that pDCs utilize unique mechanisms by which to generate type I IFN and pro-inflammatory responses. Indeed, the pDC has evolved specialized molecular recognition techniques, capable of eliciting a specific response based on its pathogen.

Upon activation, pDCs do adopt the traditional “dendritic” morphology, as well as upregulate co-stimulatory markers, CD80 and CD86, and MHC molecules. However, the expression level of these molecules is appreciably less than that of cDCs, and while they are capable, pDCs are not as efficient stimulators of T cell activation. One difference between the two DC subsets is their ability to acquire antigens. pDCs are fully functional in their presentation of endogenous antigens via MHC class I or MHC class II. However, pDCs have been described as poorly endocytic, and are, generally considered, poor presenters of exogenously-captured antigens, whether it be via MHC class II or cross-presentation on MHC class I. Therefore, while pDCs are capable of antigen presentation and T cell activation, the mechanisms that regulate this function seem to differ from that of cDCs, and these differences result in a comparatively less efficient capacity of antigen presentation and T cell activation [38]. Truly, pDCs are a unique subpopulation of DCs that have evolved to prevail over viral challenge.

1.1.2.2 Natural Killer Cells (NK cells)

Originally identified by their natural anti-tumor cytotoxicity, natural killer (NK) cells are large granular lymphocytes of the innate immune system capable of distinguishing normal “self” cells from abnormal cells. Our understanding of NK cell function has vastly improved, with their repertoire of reactivity expanding to include targets infected by viruses, bacterial, fungus, and parasites, in addition to tumors [48-49]. NK cells were once considered “non-specific” killers, able to lyse targets in a manner independent of antigen-specificity. Moreover, they constitutively express relatively

increased levels of the cytotoxicity mediators IFNγ, granzyme B, and perforin [39]. NK cells are localized throughout the lymphoid and nonlymphoid tissues, patrolling for the presence of transformed or infected cells and limiting the spread of infection and tissue damage. NK cells originate from the CLP lineage, and their development, survival, and homeostatic function is reliant upon IL15 [50-51].

In the mouse, NK cells express a variety of NK cell-associated molecules, such as CD49b (or DX5) and NK1.1; however, the expression of these molecules is often strain-specific. NK cells also express many non-NK cell-associated molecules, such as CD11b, CD11c, and TLRs [40-41]. Importantly, they do not express a T cell receptor (TCR), pan-T cell marker (CD3), or surface immunoglobulin (Ig) [42]. NK cells can also be identified by their expression of activating and inhibitory receptors, the engagement of which aids in the regulation of NK cell activity [43]. The discovery of this receptor system helped formulate the theory of "dynamic equilibrium", meaning that the balance of these signals determines NK cell effector function [43].

Inhibitory receptors, like killer cell immunoglobulin-like receptors (KIRs, in humans only), are Ig-like, type I glycoproteins, whereas members of the Ly49 family (such as Ly49A, Ly49C, Ly49F, Ly49Gg, and Ly49I, which are present in mice only) and CD94-NKG2A (present in both mouse and human) are type II glycoproteins containing a C-type lectin-like scaffold [44]. Inhibitory receptors were first identified by the observation that NK cells could lyse tumor target cells that had downregulated MHC class I expression, a common occurrence by tumor cells in an effort to avoid recognition

by cytotoxic $CD8^+$ T cells [45]. This observation culminated in the formation of the "missing self" hypothesis, in which NK cells survey tissue for normal expression of MHC class I molecules, which interact with inhibitory receptors. Successful interaction between an inhibitory receptor and its ligand results in an inactivated NK cell which views the MHC class I-expressing target cell as healthy and normal [44, 46]. Upon engagement of an inhibitory receptor with its ligand, the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) of the receptor are phosphorylated, presumably by Src kinases, and recruit the phosphatases, SHIP-1, SHP-1, or SHP-2, to the synapse of the NK cell and its target. These phosphatases dephosphorylate the protein substrates of tyrosine kinases associated with NK cell activating receptors, therefore inhibiting cytokine production, actin reorganization, degranulation, and proliferation [43]. When both inhibitory and activating receptors are engaged, NK cell activity is determined by which signal is most potent and can overcome its opponent [42].

The "missing self" hypothesis, however, cannot full explain the entirety of NK cell activation, as NK cells are capable of effectively recognizing and lysing tumor and virus-infected cells that display normal levels of MHC class I [39, 42]. Activating receptors, such as CD16, NKG2C, NKG2D, NKG2E, and NKp46 (expressed in both mouse and human), recognize the presence of ligands presented on target cells during times of "stress", such as transformation or viral infection [39, 43]. Upon ligand recognition, activating receptors recruit and interact with membrane-bound proteins via shared immunoreceptor tyrosine-based activation motifs (ITAMs), such as DAP12,

FcεRI-γ, and CD3-ζ, to form functional signaling complexes [39]. The signaling cascade downstream of these NK cell activating receptors is reminiscent of the pathways downstream of T and B lymphocytes; this is unsurprising, as all three cell types are of the lymphoid lineage and are effector cells. Upon NK cell activating receptor engagement, Src family kinases phosphorylate the tyrosines on the ITAM-containing proteins, which in turn bind and activate the tyrosine kinases Syk and ZAP-70. PI3K is subsequently activated; PI3K is a critical mediator of many different pathways, including survival, cytokine and chemokine gene transcription, actin reorganization, and degranulation of effector molecules such as granzyme B and perforin [39]. Upon degranulation, perforin attacks the target cell plasma membrane, forming a pore in which effector molecules, such as granzyme B, can enter. Upon entering the target cells, granzyme B, a serine protease, activates the apoptotic pathway to destroy the transformed or infected target cell [47]. Another effector molecule produced by NK cells is IFNγ, the sole member of the type II interferon family. IFNγ binds a heterodimeric receptor, IFNGR1/2, on the surface of target cells, and initiates multiple signaling pathways that result in the inhibition of viral replication, upregulation of PRRs, increased antigen processing and presentation, apoptosis, and immune cell trafficking [48]. Therefore, the NK cell is equipped with a multi-faceted system in which to eliminate abnormal cells and induce an immune response.

NKG2D, a type II transmembrane-anchored glycoprotein, is expressed ubiquitously on all murine NK cells and recognizes MHC class I-like, stress-induced self-

ligands, such as Rae1, H60, and MULT1 [39]. These ligands have been demonstrated to be upregulated on mousepox-infected cells and tumor cells, leading to enhanced NK cell cytotoxicity via the NKG2D pathway [49-50]. The long isoform of NKG2D, NKG2D-L, can also bind DAP10, a membrane-bound adaptor protein with a YINM-containing domain that shows significant sequence homology to DAP12, yet enough difference to confer specificity in receptor association [51]. The NKG2D-L-DAP10 pathway differs considerably from that of the DAP12 pathway, in that DAP10-mediated signaling does not utilize the Syk family kinases. DAP10 contains a binding site for both PI3K and the adaptor Grb2, and the ablation of either of these sites completely inhibits cytotoxic function. Calcium flux and subsequent actin polarization and degranulation requires both intact PI3K and Grb2 signaling [44].

Perhaps the most well characterized NK cell response is of that to MCMV infection. It has been clearly established that MCMV infection elicits TLR2, TLR3, and TLR9-dependent responses in DCs, resulting in type I IFN and pro-inflammatory cytokine production, both of which are key to NK cell effector function [39]. In addition, MCMV is capable of eliciting a more direct NK cell response. Target cells infected by MCMV express the CMV-encoded, MHC-like glycoprotein, m157, on their surface, and the NK cell activating receptor, Ly49H, recognizes and is activated upon m157 interaction. The importance of the *Ly49H* gene as a vital mediator of anti-MCMV immunity has been demonstrated through the use of Ly49H-deficient mouse strains, such as BALB/c. Compared to Ly49H$^+$ mice, such as C57BL/6, BALB/c mice are highly

susceptible to MCMV infection and display decreased NK cell cytotoxicity and increased viral load [39, 43, 52]. However, MCMV has devoted much evolutionary effort to circumventing NK cell recognition. MCMV-encoded glycoproteins, m145, m152, and m155, inhibit the expression of NKG2D ligands, such as Rae1, H60, and MULT1. Additionally, m157, the glycoprotein recognized by Ly49H, also acts as decoy ligand by interacting with the inhibitory receptor, Ly49I, thus antagonizing NK cell activation [39, 43]. Indeed, the equilibrium between inhibitory and activating receptors is a fundamental mechanism by which NK cells sense and respond to abnormal cells.

The role of PRRs in the indirect activation of NK cells has been well-documented. TLR-mediated induction of type I IFNs and pro-inflammatory cytokines by DCs and macrophages is a critical component to the NK cell response. However, evidence is mounting in support of direct TLR activation of NK cells. Freshly isolated NK cells from both humans and mice express TLR1-10 at varying degrees [41, 53]. It has been demonstrated that human NK cells could produce IFNγ in response to direct stimulation with a TLR2 ligand, the outer membrane protein A from *Klebsiella pneumoniae* (KpOmpA), and a TLR5 ligand, flagellin from *Escherichia coli*. Furthermore, activation of NK cells with KpOmpA occurred in a TLR2-dependent manner. Also, this induction of IFNγ by NK cells was enhanced by the accessory cell derived-cytokines, IL12, IL1β, IL2, and IFNα, suggesting that the cytokine environment created by other innate immune cells can regulate NK cell sensitivity to PAMPs [53]. A similar observation was made in murine NK cells. Hepatic NK cells, expanded in IL15 for 10 days, were able to produce

IFNγ in response to TLR2 (PGN), TLR3 (polyI:C), or TLR7 (loxoribine) stimulation in the presence of IL12. As in the human NK cell studies, the murine NK cell response to PGN was also TLR2-dependent [41]. The ability of TLRs to stimulate NK cells directly adds another level of complexity to the NK cell paradigm.

In the setting of poxviral infection, NK cells have been implicated as critical mediators of the immune response. Certain mouse strains, such as C57BL/6, are resistant to the poxvirus, ectromelia virus (ECTV), and this resistance has been linked to the NK gene complex [54]. Recently, the NK cell activating receptor, NKG2D, was shown to play a crucial role in NK cell-mediated resistance to ECTV, blockade of which resulted in increased viral titer, increased mortality, and decreased NK cell effector function in response to ECTV infection [59]. In the setting of VV infection, NK cells expand and become activated at the site of infection, and mice depleted of NK cells with anti-asialo GM1 antibody have increased susceptibility to VV infection [55]. However, poxviruses have evolved numerous strategies to overcome NK cell-mediated elimination. Both ECTV and molluscum contagiosum virus (MCV) encode IL18-binding proteins, p13 and MC53/54L, respectively. These proteins prevent IL18 from binding its cognate receptor and on NK cells, resulting in decreased NK cell cytotoxicity [42, 56-57]. In human cells, VV infection results in the downregulation of MHC class I surface expression, specifically HLA-C, whose interaction with KIRs constitute a major NK cell activation mechanism. Additionally, VV can infect NK cells at the site of infection, rendering them

less effective at target cell lysis. This observation suggests that VV can modify the NK cell response by direct infection, as well as target cell ligand downregulation [58].

Clinically, patients that are NK cell-deficient (*IL2RG* or *JAK3* deficiency) are susceptible to viral infection, especially herpesvirus infection. The redundancy of the immune system, however, oftentimes allows for eventual control of these infection [43]. Perhaps more relevant is the ability of NK cells to limit immunopathology. In the setting of Theiler's virus and coxsackie B3 virus infection, NK cells are responsible for controlling the onset of encephalitis and myocarditis, respectively [59-60]. Using a murine model, it was also demonstrated that the absence of functional NK cells resulted in activated macrophage accumulation and hemophagocytosis lymphohistiocytsosis (HLH)-like symptoms. In humans, HLH can be inherited (although rarely) or initiated by a common viral infection. NK cells may represent a mechanism by which hyperactivated cells are eliminated to prevent damaging immunopathology [43]. NK cells are now recognized as a multi-faceted innate cell type, governed by series of intricate mechanisms capable of activating and inhibiting their function.

1.1.3 Type I interferons and antiviral immunity

Type I interferons (IFNs) are a family of cytokines that constitute 13 and 17 IFNα subtypes in mice and humans, respectively, and one IFNβ in both species [61]. All type I IFNs signal through a cell-surface, transmembrane heterodimeric receptor composed of two subunits, IFNα/β receptor 1 (IFNα/βR1) and IFNα/βR2. Binding of type I IFNs to the receptor results in subunit dimerization and the subsequent activation of the kinases,

Janus-activated kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) at their cytoplasmic tails. The phosphorylation activity of these kinases results in the activation of signal transducers and activators of transcription 1 and 2 (STAT1 and STAT2), which in turn assemble with IRF9 into a complex known as the IFN-stimulated gene factor 3 (ISGF3). In addition, STAT1 can form a homodimer, termed the IFNγ-activated factor (GAF). STAT1 and STAT2 are the two best characterized signaling mediators of type I IFN activity, although other STAT proteins, such as STAT4, have been shown to play a role in the type I IFN response; it is believed that the redundancy and abundance of STAT molecules may allow for flexible usage of these downstream molecules [62]. These protein complexes, ISGF3 and GAF, translocate to the nucleus, where they bind DNA regulatory sequences containing IFN-stimulated response elements (ISRE) and IFNγ-activated sites (GAS), respectively. These interactions trigger a series of signaling cascades leading to the transcription of more than 100 IFN-stimulated genes (ISGs) [4, 72-73].

There are numerous ISGs downstream of the type I IFN signaling pathway, and among the best characterized are the myxovirus resistance gene (Mx), the serine/threonine protein kinase (PKR), and the 2'-5' oligoadenylate synthetases (OAS) [63]. Mx, a guanosine triphosphatase (GTPase), exerts its antiviral activity by localizing viral ribonucleoproteins to subcellular compartments for degradation. PKR, a serine-threonine kinase, activates substrates, such as the elongation initiation factor, EIF2α, that are capable of inhibiting protein translation. OAS synthesizes 2'-5' oligoadenylates,

which activates an enzyme, RNaseL, that destroys viral RNA [64]. Both OAS and PKR are activated by viral dsRNA sensed in the endosomal compartments (in response to TLR stimulation), but not in the cytoplasm (in response to RIG-I) [65]. The multiple pathways capable of inducing type I IFN production, coupled with the existence of multiple antiviral ISGs, indicates that the innate immune system has evolved surplus pathways by which to circumvent viral infection, therefore creating a situation in which the virus must evade more than one pathway to compete.

In addition to the direct antiviral effects, type I IFN signaling also mediates a variety of immunoregulatory effects. Perhaps the most well established, yet mechanistically unclear, effect of type I IFNs is its ability to promote NK cell cytotoxicity [63]. In murine pDCs, MCMV stimulation of the TLR9 pathway results in elevated NK cell cytolytic activity and increased IFNγ production, culminating in an early critical antiviral response [66]. It also has been demonstrated that pre-treatment of L929 cells with IFNα protects them from RNA virus infection, such as encephalomyocarditis virus (EMCV)-induced cell death, through inhibiting viral replication [76]. In addition, type I IFNs can promote IL15 production in a STAT1-dependent manner, resulting in increased NK cell proliferation [67].

Type I IFNs can also regulate the adaptive immune response. Type I IFNs, in conjunction with IL6, can enhance the isotype switching and antibody production of B cells [10, 23]. In the context of vaccinia viral infection, intrinsic STAT1 signaling was critical for the efficient expansion of Ag-specific $CD8^+$ T cells in vivo. It was shown that

the IFNα/β-STAT1 pathway promotes the survival of activated $CD8^+$ T cells; therefore, long-lived memory $CD8^+$ T cells could not develop in the absence of STAT1 signaling [68]. Furthermore, activation of STAT1 results in IL15 production, which promotes the short-term proliferation of memory $CD8^+$ T cells. Type I IFNs also have anti-proliferative properties, as they can restrict non-specific $CD8^+$ T cell expansion by a STAT1-dependent mechanism [79-80]. Type I IFNs can also enhance the T cell response, through the upregulation of CD69 and IFNγ production. Moreover, type I IFNs promote the maturation of and upregulation of co-stimulatory and antigen-presenting molecules on DCs, resulting in more efficient interactions with T cells [1, 35, 46]. The role of type I IFNs in T_H cell polarization is more controversial. Because type I IFNs can enhance $CD8^+$ T cell production of IFNγ, a T_H1 polarizing cytokine, it has been reported that type I IFNs could aid in T_H1 polarization. Indeed, mice deficient in MyD88 signaling fail to develop a T_H1 response [81-82]. However, it has been established that high amounts of type I IFN, such as that produced during viral infection, inhibits IL12 production, a cytokine critical for T_H1 polarization [37]. The role of type I IFNs in T cell polarization requires further study in order to fully understand its ability to mold the adaptive immune response.

1.2 Vaccinia Virus

Vaccinia virus (VV) is the most extensively characterized member of the poxvirus family and is the vaccine used in the successful 1977 eradication of smallpox worldwide. The ~190 kbp dsDNA genome encodes many of the enzymes and proteins required for

viral DNA replication and transcription. The central ~100 kbp portion of the VV genome encodes the genes required for virus replication, whereas the terminal portions of the genome play a role in virulence and regulation of the immune response [69-70]. The VV genome is capable of replicating exclusively in the cytoplasm of infected cells, occurring after approximately 2 hours after infection. The early viral genes encoded after cell entry are responsible for further uncoating of the virus and nucleotide biosynthesis enzymes. These early viral genes are necessary for the transcription of intermediate and late genes, which mediate the formation of new viral particles [71]. Morphogenesis of the VV virion progresses through three stages. The first form, the intracellular mature virus (IMV), is composed of VV DNA surrounded by the membrane cisterna derived from the intermediate compartment between the endoplasmic reticulum and the Golgi apparatus. These crescent-shaped structures condense to produce the IMV, which constitute the majority of infectious progeny [71-72]. A portion of the IMV particles, however, are further surrounded by endosomal or trans-Golgi network membranes to form the intracellular enveloped virion (IEV). This IEV translocates to the cell surface and fuses with the plasma membrane to become the double-encapsulated extracellular enveloped virus (EEV) [71]. While the EEV is critical for viral dissemination, the function of many encoded proteins of the EEV remains unknown. It has been demonstrated that antibodies directed against the EEV are able to inhibit viral release, rather than neutralize viral particles, as previously believed. Moreover, antibodies to EEV proteins protect animals from VV challenge more effectively than antibodies to IMV proteins [71, 73-74].

VV has been and continues to be a powerful tool for immunotherapy and vaccine development, but what remains unclear are the unique characteristics that contribute to its potency. In addition to its success as a vaccine against smallpox, the ability of recombinant viruses, engineered to express a specific antigen, to act as a vaccine vector was first demonstrated using VV. Recombinant VV encoding the influenza-derived hemagglutinin (HA) gene was shown to provide protection against subsequent influenza infection and elicit antibody titers equivalent to influenza vaccination [75]. As opposed to prophylactic vaccines against infectious pathogens, therapeutic cancer vaccines have not yet demonstrated the same level of efficacy. The most likely explanation for the difference between the two vaccines is the development of immune evasion techniques by cancer cells, including disruption of antigen presentation machinery and induction of T cell tolerance. However, using a peripheral tolerance model of mice expressing HA as a self-antigen, it was demonstrated that VV-HA can break $CD8^+$ T cell tolerance via persistent TLR signaling, leading to increased resistance to tumor challenge [76]. Additionally, VV activates the innate immune system through both TLR-dependent and TLR-independent pathways. The production of pro-inflammatory cytokines, such as IL6, IL1, and IL12, occur via a TLR2-MyD88-dependent mechanism, whereas type I interferons, such as IFNβ, are produced in a TLR-independent manner. Strikingly, both pathways, while independent of each other, were shown to be crucial for the clearance of vaccinia in vivo, as well as the development of the adaptive immune response; what

remains unknown are the mechanisms by which these pathways direct the innate immune response to vaccinia viral infection [22].

Like other viruses, VV has developed numerous strategies to evade immune detection and impede an immune response. Table 2 represents a summary of the proteins encoded by VV for immune evasion [69, 71, 77].

Table 2: Summary of proteins encoded by VV for immune evasion.

VV Factor Gene	Mechanism of Evasion
A46R	Sequesters MyD88, TRIF, and TRAM, prevents interactions with and signaling of TLR4
A52R	Binds and inhibits IRAK2 and TRAF6, prevents NFκB activation
A53R	TNFR mimic, binds TNF and prevents TNF binding to and signaling of the TNFR
B8R*	IFNγR mimic, binds IFNγ and prevents IFNγ binding to and signaling of the IFNγR
B13R	Inhibits ICE, inhibits caspase I, IL1β, and IL18
B14R	Prevents degradation of IκB, inhibits NFκB activation
B15R/B16R	IL1β receptor mimic, binds IL1β and prevents IL1β binding to and signaling of IL1βR
B18R/B19R**	IFNα/βR mimic, binds type I IFN and prevents IFNα/β binding to and signaling of the IFNα/βR
C7L	Inhibits eIF2α phosphorylation
C12L	Binds IL18 and prevents IL18-induced IFN-γ production and NK cell response
C23L	Binds to chemokines, such as CCL3 and CCL5, inhibits localization
CrmE	TNFR mimic
E3L	Binds dsRNA, inhibits PKR, ISG15, OAS, and ADAR signaling, inhibits IRF3 and IRF7 activation
H1L	Dephosphorylates STAT1
K1R	Prevents degradation of IκB, inhibits NFκB activation
K3L	eIF2α mimic, inhibits PKR signaling
K7R	Inhibits TBK1/IKK-ε-mediated IRF activation, prevents IFNβ induction
M2L	Prevents NFκB activation via the ERK2 pathway

Table 2: Summary of proteins encoded by VV for immune evasion, continued.

N1L	Binds to IKK complex, prevents NFκB activation Binds to TBK1, prevents IRF3

*B8R is not functional in mice. ** B18R/B19R has a lower affinity for type I IFN in mice.

The unparalleled success of smallpox eradication is now being threatened by bioterrorism, in which the deliberate reintroduction of smallpox, against which vaccination is no longer routine, would provide devastating effects on a population that is either immunologically naïve or possess diminished immunity [78-80]. However, the relatively high incidence of adverse events associated with the currently used live VV vaccine has encumbered the proposal for a return to smallpox vaccination [81-83]. Moreover, immunologically-compromised or cancer-afflicted patients are at more of a risk for adverse reactions [82]. It is of the utmost importance, therefore, to elucidate the host's defense mechanism(s) against vaccinia virus in vivo.

1.3 Thesis Prospectus

As the first line of defense against invading pathogens, the innate immune system is essential for the recognition and elimination of viral pathogens; it also plays an important role in sculpting the adaptive immune response, in terms of cellular polarization, activation, and formation of the memory cell population. Vaccinia virus is a potent stimulator of the innate immune response and has been demonstrated a powerful tool in vaccine development and immunotherapy. However, the mechanisms by which vaccinia virus activates the innate immune system remain undefined. In light of this

dearth of knowledge, the overall goal of this thesis was to determine how vaccinia virus stimulates the innate immune response and what effect this innate immune response had on viral clearance. In an effort to do so, we examined the ability of vaccinia virus to activate key, antiviral innate immune cells, as well as the molecular mechanisms required to achieve this activation. Finally, we analyzed the individual role that these cell types played in the clearance of vaccinia virus in vivo. We addressed this thesis project with the following three chapters:

Chapter 3

How does VV activate pDCs for the production of type I IFNs, and what role does pDC-produced type I IFNs play in VV clearance in vivo?

Chapter 4

How do type I IFNs activate NK cells in response to VV infection, and what role does type I IFN-dependent NK cell activation play in VV clearance in vivo?

Chapter 5

How does TLR2-MyD88 signaling regulate NK cell activation in response to VV infection and what role does TLR2-dependent NK cell activation play in VV clearance in vivo?

Chapter 2: Materials and Methods

2.1 *Mice*

CD45.1$^+$ and CD45.2$^+$, wild type (WT) C57BL/6 mice (H-2^b) were obtained from The Jackson Laboratory (Bar Harbor, ME). TLR2-/-, MyD88-/- and TLR9-/- mice, backcrossed onto C57BL/6 background for >9 generations, were kindly provided by Shizou Akira at Osaka University, Osaka, Japan. IL1R-/-, IL12-/-, and IL6-/- mice, on C57BL/6 background, were obtained from The Jackson Laboratory. Wild type 129/Sv mice (H-2^b) were purchased from Charles River Laboratories. IFNα/βR–/– mice, on 129/Sv background, were obtained from B&K Universal. Groups of 6- to 8-wk-old female mice were selected for this study. All experiments involving the use of mice were done in accordance with the A-052-09-02 protocol, and were approved by the Animal Care and Use Committee of Duke University.

2.2 *Viruses*

2.2.1 Vaccinia virus (VV)

The Western Reserve (WR) strain of vaccinia virus (VV) was purchased from American Type Culture Collection (ATCC). VV was grown in TK-143B cells (ATCC) and purified, and the titer was determined by plaque assay on TK-143B cells and stored at –80°C until use, as described [22].

2.2.2 UV-inactivated vaccinia virus (UV-VV)

UV-inactivation of VV was performed as described with some modifications [22]. Briefly, purified virus was resuspended in 1 μg/ml 8-methoxypsoralen (Sigma-Aldrich)

and then exposed to a 365 nm UV light source (UVP model UVGL-25) on ice for 3 min in a 24-well plate. For in vitro NK stimulations, both live VV and UV-inactivated VV were used at MOI of 1. The dose of UV-VV was based on pre-inactivation titer.

2.2.3 Vaccinia viral DNA Isolation (VV-DNA)

Isolation of vaccinia viral DNA was performed by incubating live WR vaccinia virus with 1% SDS and 200 μg/ml Proteinase-K for 4 hr at 37ºC. After incubation, phenol chloroform extraction followed by sodium acetate precipitation was performed to purify the vaccinia viral DNA. DNA concentration was assessed using a spectrophotometer.

2.2.4 Encephalomyocarditits virus (EMCV)

Encephalomyocarditits virus (EMCV) was purchased from American Type Culture Collection (ATCC). EMCV was grown in L929 cells (ATCC) and purified by centrifugation, and the titer was determined by plaque assay on L929 cells as described [22, 84].

2.3 Generation and culture of DC subsets using bone marrow (BM) culture

2.3.1 BM culture with Flt3L for pDC and cDC generation

DCs were generated as described [22]. Briefly, femurs and tibiae of mice were harvested and bone marrow cells were flushed out with DC medium (RPMI-1640 with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM HEPES, 50 μM β-mercaptoethanol, 100 IU/ml penicillin, and 100 IU/ml streptomycin). After lysis of red

blood cells, the bone marrow cells were cultured in 6-well plates at a density of 3×10^6/ml in 3 ml DC medium in the presence of recombinant murine Flt3L at a concentration of 100 ng/ml (R&D Systems, Minneapolis, MN) for 7 to 10 days.

2.3.2 BM culture with GM-CSF for cDC generation

DCs were generated as described [22]. Briefly, femurs and tibiae of mice were harvested and bone marrow cells were flushed out with DC medium. After lysis of red blood cells, the bone marrow cells were cultured in 6-well plates at a density of 3×10^6/ml in 3 ml DC medium in the presence of recombinant murine granulocyte macrophage–colony-stimulating factor (GM-CSF) at 1000 U/ml (R&D Systems, Minneapolis, MN). GM-CSF was replenished on days 2 and 4.

2.3.3 Harvest of DC subsets and stimulation

On day 7 to 10 of Flt3L BM culture, DCs were harvested, stained, and sorted into a pDC population ($CD11c^+B220^+PDCA1^+$) and a cDC population ($CD11c^+B220^-PDCA1^-$). Sorted pDCs and cDCs were plated in DC medium at a density of 1×10^6/ml in 96-well round bottom plates and stimulated for 24 hr with various reagents.

On day 5 of GM-CSF BM culture, DCs were harvested, stained, and sorted into a cDC population ($CD11c^+B220^-PDCA1^-$). Sorted cDCs were plated in DC medium at a density of 1×10^6/ml in 96-well round bottom plates and stimulated for 24 hr with various reagents.

2.4 Isolation of endogenous DCs

$CD11c^+B220^+$ $PDCA1^+$ pDCs were purified from splenocytes of naïve WT mice via flow cytometry sorting. Sorted pDCs were plated in DC medium at a density of 1×10^6/ml in 96-well round bottom plates and stimulated for 24 hours with various reagents.

2.5 Oligonucleotides

Phosphorothioate (*)-stabilized PolyA10 (5'-A*AAAA*A*A*A*A*A-3'), PolyC10 (5'-C*CCCC*C*C*C*C*C-3'), PolyG10 (5'-G*GGGG*G*G*G*G*G-3'), PolyT10 (5'-T*TTTT*T*T*T*T*T-3'), CpG-A ODN (5'-TCCATGACGTTCCTGATGCT-3'), and CpG ODN2088 (5'-TCCTGGCGGGGAAGT-3') were all generated by Integrated DNA Technologies (Coralville, IA). PolyA10, PolyC10, PolyG10, and PolyT10 were all used at a concentration of 5 μg/ml. CpG-A ODN was used at a concentration of 1 nM. CpG ODN2088 was used at a concentration of 1 μM.

2.6 Antibodies and flow cytometry

The following antibodies were purchased from BD Biosciences (San Diego, CA): FITC-conjugated anti-B220, PE-conjugated anti-CD80, PE-conjugated anti-CD86, streptavidin-conjugated PE-Cy5, biotin-conjugated anti-CD11c, FITC-conjugated anti-IFNγ, PE-conjugated anti-DX5 (CD49b), PE-Cy5-conjugated anti-CD3ε, biotin-conjugated CD45.2, FITC-conjugated anti-CD69, FITC-conjugated anti-CD62L, and streptavidin-conjugated APC were purchased from BD Biosciences (San Diego, CA).

FITC-conjugated anti-granzyme B, FITC-conjugated anti-perforin, and APC-conjugated anti-KLRG1 were purchased from eBioscience (San Diego, CA). PE-conjugated anti-PDCA-1 was obtained from Miltenyi Biotec (Germany). To assess production of IFNγ, granzyme B, and perforin intracellularly, splenocytes were incubated with 100 ng/ml of PMA, 250 ng/ml of ionomycin, and 5 μg/ml of Brefeldin A containing Golgi-plug (BD Biosciences) for 4 hr at 37°C. Intracellular staining was performed as previously described [85]. FACS Canto (BD Biosciences) was used for flow cytometry event collection, which was analyzed using FACS DiVA software (BD Biosciences).

2.7 Enzyme-linked immunosorbant assay (ELISA)

Production IFNα and IFNß by the DCs in response to various stimuli was detected in culture supernatants or sera by ELISA kits according to the manufacturers' standard protocols. Serum levels of IFNα produced in response to VV was detected 24 hr post i.v. infection by ELISA kits according to the manufacturers' standard protocols. IFNα and IFNß ELISA kits were obtained from PBL Biomedical Laboratories (Picataway, NJ).

2.8 NFκB luciferase assay

HEK293 cells (CRL-1573; American Type Culture Collection) were seeded in 6-well plates at 5 x 10^5 cell/well and co-transfected with 1 μg of murine TLR7 (pUNOmTLR7; Invivogen, San Diego, CA) or murine TLR8 (pUNO-mTLR8; Invivogen, San Diego, CA), along with 0.1 μg of NFκB-luciferase reporter (pNFκB-Luc; Clontech, Mountain View, CA) with Lipofectamine transfection reagent (Invitrogen) following the manufacturer's instructions. Forty-eight hours after transfection, cells

were then stimulated with live VV, VV-DNA, or a TLR7 agonist, CL087 (Invivogen). NFκB activation was determined by lysing the transfected HEK293 cells with reporter lysis buffer (Clontech), and the lysate was assayed for luciferase activity using an LMax Luminometer (Molecular Devices). NFκB activation is directly proportional to relative luciferase units.

2.9 RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from flow cytometry sorted cells using Trizol reagent according to manufacturer's instructions. One-step semi-quantitative RT-PCR was performed in the presence or absence of reverse transcriptase using template RNA and primers specific for the following murine sequences: TLR2 (forward 5'TTGCTCCTGCGAACTCCTAT-3', reverse 5'-CAATGGGAATCCTGCTCACT-3'), TLR3 (forward 5'-CCCCCTTTGAACTCCTCTTC-3', reverse 5'-TTTCGGCTTCTTTTGATGCT-3'), TLR4 (forward 5'-GCTTTCACCTCTGCCTTCAC-3', reverse 5'-CGAGGCTTTTCCATCCAATA-3'), TLR7 (forward 5'-GGTATGCCGCCAAATCTAAA-3', reverse 5'-TTGCAAAGAAAGCGATTGTG-3'), TLR8 (forward 5'-CAAACAACAGCACCCAAATG-3', reverse 5'-GGGGGCACATAGAAAAGGTT-3'), TLR9 (forward 5'-GCAAGCTCAACCTGTCCTTC-3', reverse 5'-TAGAAGCAGGGGTGCTCAGT-3') and β-actin (forward 5'AGCCATGTACGTAGCCATCC-3', reverse 5'-CTCTCAGCTGTGGTGGTGAA-3').

2.10 siRNA knockdown

2.10.1 Construction of lentiviral vectors encoding siRNA for murine TLR7 and TLR8

The lentiviral construct, pNL-SIN-GFP [86], was a generous gift from B. Cullen (Duke University, Durham, NC). Murine TLR8 siRNA constructs were generated in two PCR steps. The first PCR used genomic DNA from HEK293 cells to generate a fragment containing an Xba I restriction site, the RNA polymerase III-dependent H1 promoter, the TLR8-specific siRNA "sense" strand sequence, and the hinge region sequence. The primers used in this initial PCR are H1 forward primer (5'-TGGCAGGAAGATGGCTGTGA-3') and TLR8 siRNA1-Sense (5'-ACTGACAGGAAGACTACCCAGTTTACAAATCCGGGGATCTGTGGTCTCATACAGAACTTATAAGATTCCC-3') or TLR8 siRNA2-Sense (5'-CTGACAGGAAGAGCTTCGTTCTCTCAAGAAGGGGGATCTGTGGTCTCATACAGAACTTATAAGATTCCC-3'). The second PCR used the first PCR product as the template to add the TLR8 siRNA "anti-sense" strand sequence, terminal sequence, and a Cla I restriction site. The primers used in the second PCR are H1 forward primer and TLR8 siRNA1-Antisense (5'-GCGCATCGATAGCTGGCAAGGAAAAAGGATTTGTAAACTGGGTAGTTACTGACAGGAAGACTA-3') or TLR8 siRNA2-Antisense (5'-GCGCATCGATAGCTGGCAAGGAAAAACTTCTTGAGAGAACGAAGCTTACTGACAGGAAGAGCT-3'). Similarly, primers for generating TLR7 siRNA are: Sense (5'-

ACTGACAGGAAGACAAAAGTACCTGAGAGATTTTGGGGATCTGTGGTCTCAT ACAGAACTTATAAGATTCCC-3') and Anti-sense (5'-GCATCGATAGCTGGCAAGGAAAAAAATCTCTCAGGTACTTTTGTTGACAGGA AGACAA-3'). The TLR8 and TLR7 siRNA expression cassettes were then digested with Xba I and Cla I and subcloned into pNL-SIN-GFP to generate pNL-SIN-GFP-TLR8 siRNA1 (or 2) and pNL-SIN-GFP-TLR7 siRNA. Insertion and sequence were confirmed by restriction enzyme digestion and DNA sequencing.

2.10.2 Lentivirus-mediated siRNA knockdown

Lentiviruses were produced by co-transfecting plasmids pNL-SIN-GFP-TLR8 or TLR7 siRNA (16 μg), pcRev (800 ng), pcTat (800 ng), and pHIT/G (400 ng) into human 293T cells. The viral supernatants were then used to infect day 2 Flt3L BM cultures in the presence of 8 μg/ml polybrene. After 24 hr, the lentivirus-containing media was replaced with DC medium and Flt3L. This procedure was repeated on day 4 of Fl3L BM culture. GFP-expressing $CD11c^+$ $B220^+$ pDCs were isolated on day 8 by flow cytometry sorting. Empty vector controls were generated by performing the above procedure with empty plasmid, pNL-SIN-GFP.

2.11 Viral genome analysis

Vaccinia virus (Accession No. NC_006998) and herpes simplex virus-2 (Accession No. NC_001798) were analyzed by the DNA analysis software pDRAW32 (AcaClone). Whole genomes were analyzed for G/C and A/T content and expressed as a percentage. In order to determine the frequency of G/C- or A/T-rich sequences, the VV

genome was analyzed for instances where ten consecutive base pairs included 5 or more G/C or A/T (defined henceforth as an “island”). The percentage of the VV genome that exists in G/C-rich or A/T-rich islands is calculated by dividing the total number of base pairs that exist in such islands by the total number of base pairs in the VV genome.

2.12 Reconstitution of cells in vivo

2.12.1 Reconstitution of pDC

WT and MyD88-/- pDCs were generated from BM cultures in the presence of Flt3L and purified by flow cytometry sorting as described above. One x 10^6 sorted pDCs were adoptively transferred into MyD88-/- hosts intravenously, followed by infection with 1 x 10^7 pfu of VV intravenously. Serum, spleen, and liver were harvested at 48 hr post-infection for analysis.

For the reconstitution of pDCs after pDC depletion, mice received 150 µg of anti-Ly6G/C on days -3 and -1 intravenously. On day 0, 5 x 10^5 sorted TLR7-knockdown, TLR8-knockdown, or TLR9-/- pDCs were adoptively transferred into pDC-depleted hosts intravenously, followed by infection with 1 x 10^7 pfu live VV intravenously. Serum, spleen, and liver were harvested at 48 hr post-infection for analysis.

2.12.2 Reconstitution of NK cells

$DX5^+CD3^-$ NK cells were purified from splenocytes of naive mice via flow cytometry sorting. Two x 10^5 $DX5^+CD3^-$ NK cells were administered i.v. to recipients, which were subsequently injected i.p. with 1 x 10^7 pfu VV. For the reconstitution of NK cells after NK cell depletion, mice received only a single dose of anti-NK1.1 (200 µg) or

anti-asialo GM1 (250 µg) on day -2, and were reconstituted with purified NK cells (5 x 10^5) on day 0 as described [87].

2.13 VV-DNA quantitative Real-Time PCR

Total genomic DNA was isolated from the spleen and the liver tissues as described [88]. Real-time quantitative PCR was used to measure the amount of VV genomic DNA in the spleen and liver using primers located in the VV A33R gene. Amounts of VV-DNA were normalized to β-actin gene within each sample. The sequences of the forward and reverse primers for A33R, were 5' -TATTACTGACGCCGCTGTTG – 3' and 5' -GTGTTGATGATTCCGCAGTG – 3', respectively. The sequences of the forward and reverse primers for β-actin gene were 5'-AGCCATGTACGTAGCCATCC-3' and 5'-CTCTCAGCTGTGGTGGTGAA-3', respectively. Normalized value for VV-DNA in each sample was calculated as the relative quantity of vaccinia viral DNA divided by the relative quantity of β-actin gene.

2.14 VV Titer assay

Viral loads in the ovaries, spleen, and liver were measured by plaque-forming assay as described [22]. Briefly, mice were sacrificed 1 to 2 days after infection, and organs were harvested and stored at -80ºC. Organs from individual mice were homogenized and freeze-thawed 3 times. Serial dilutions were performed on confluent TK-143B cells, and viral titers were then determined 2 days later by crystal violet staining.

2.15 IFNα protection assay

L929 cells plated at 1 x 10^5 cell/ml were pre-treated with 1 x 10^3 U/ml recombinant murine IFNα (R&D Systems) for 24 hr. After removal of the IFNα-containing supernatant, the cells were infected for 24 hr with either VV or EMCV with a multiplicity of infection (MOI) of 62.5, 12.5, 2.5, 0.5, or 0.1. Survival was determined by FACS analysis 24 hr later.

2.16 In vivo depletion of cells

2.16.1 pDC depletion

Mice were depleted of pDCs with 150 μg of anti-Ly6G/C (clone RB6-8C5, a generous gift of D. Cain and G. Kelsoe, originally from R. Coffman) administered intravenously to WT mice on days -3 and -1 [22, 89]. Depletion was confirmed by FACS.

2.16.2 NK cell depletion

For depletion of NK cells in vivo, mice received 250 μg of anti-asialo GM1 antiserum (Wako Chemicals) or 200 μg of functional grade purified anti-mouse NK1.1 (eBioscience) injected intravenously on day -3 and day 0 of infection with VV. Before infections, peripheral blood and splenic cells were analyzed by FACS to confirm elimination of $DX5^+CD3^-$ NK cells.

2.17 NK cell cytotoxicity assay

NK cell cytotoxicity assay was performed as previously described [85]. In brief, splenocytes were enriched for $DX5^+$ NK cells by positive selection with PE-conjugated

anti-DX5 and anti-PE microbeads (Miltenyi Biotec). $DX5^+$ splenocytes were then incubated with ^{51}Cr-labeled NK sensitive targets, YAC-1 cells (ATCC), at different effector:target ratios for 4 hr at 37°C. In some experiments, L929 cells (ATCC) infected with VV at an MOI of 20 for 2 hr, were used for targets as described [90]. $DX5^+$ splenocytes were incubated with ^{51}Cr-labeled VV-infected L929 targets, at different effector:target ratios for 4 hr at 37°C, in the presence or absence of anti-NKG2D antibody (10 μg/ml, eBioscience) or anti-NKp46 Fc (30 μg/ml, R&D Systems). The specific ^{51}Cr-release was calculated as (experimental cpm – spontaneous cpm) / (maximum cpm – spontaneous cpm) x 100.

2.18 In vitro NK:DC co-culture system

cDCs were generated from the bone marrow cells in the presence of GM-CSF, as described above. In brief, bone marrow cells were harvested from femurs and tibiae of mice and cultured in the presence of murine GM-CSF at a concentration of 1000 U/ml (R&D Systems) for 5 days. On day 5, $CD11c^+$ cDCs were harvested for NK cell stimulation. NK:DC co-culture was performed as described with some modifications [21, 85] . In brief, $DX5^+$ NK cells were enriched from splenocytes of naïve mice by positive selection with PE-conjugated anti-DX5 and anti-PE microbeads (Miltenyi Biotec). $DX5^+CD3^-$ NK cells were purified from the enriched $DX5^+$ cells via flow cytometry sorting on a FACS DiVA. NK cells (5×10^5) were co-cultured with $CD11c^+$ cDCs (2.5×10^5) at an NK:DC ratio of 2:1 in CTL medium (RPMI-1640 with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM sodium pyruvate, 50 μM β-

mercaptoethanol, 100 IU/ml penicillin, and 100 IU/ml streptomycin). The co-culture was subsequently infected with VV (MOI of 1) or LPS (100 ng/ml), for 48 hr at 37°C. In some experiments, NK:DC co-cultures were inhibited with 10 μg/ml of functional grade purified anti-mouse NKG2D (eBioscience) or 30 μg/ml of recombinant mouse NKp46/NCR1/Fc Chimera (R & D Systems) as described [49, 91].

2.19 Accessory cell-free NK cell culture

NK cell alone culture was performed as described with some modifications [21]. Briefly, $DX5^{+}CD3^{-}$ NK cells were purified from splenocytes of naïve mice via flow cytometry sorting on a FACS DiVA. A purity of >98% was confirmed by FACS. NK cells (1×10^{6}) were cultured in CTL medium in the presence of recombinant murine IL2 at 50 U/ml and IFNα at 2000 U/ml (both from R&D Systems). NK cells were infected with VV with MOI of 2 for 48 hr at 37ºC. In some experiments, the PI3-K inhibitor, LY294002 (10 μM, Calbiochem), the ERK1/2 inhibitor, PD98059 (50 μM, Calbiochem) or functional grade anti-mouse TLR2 antibody (50 μg/ml, eBioscience) were used to block their respective functions in vitro.

2.20 Generation of mixed bone marrow (BM) chimeras

$CD45.1^{+}$ C57BL/6 recipient mice were irradiated with 1200 cGy of gamma irradiation and reconstituted with 1×10^{6} of CD45.1+ WT and 1×10^{6} of $CD45.2^{+}$ MyD88-/- or TLR2-/- bone marrow cells. Mice were allowed to reconstitute their hematopoietic cell population for 6 to 8 weeks. Chimerism was confirmed prior to experimental use. Mixed chimeric mice were injected with 1×10^{7} pfu VV

intraperitoneally, and their spleens were harvested and assayed for NK cell function 48 hr later. Analysis of intracellular IFNγ and granzyme B was performed as described above. NK cell cytotoxicity assay was performed (with ^{51}Cr-labeled YAC-1 targets as described above) after FACS sorting for WT (CD45.1^{+}DX5^{+}CD3^{-}) and MyD88-/- or TLR2-/- (CD45.2^{+}DX5^{+}CD3^{-}) NK cells.

2.21 In vivo inhibition of NK cell activity with anti-NKG2D

For inhibition of NKG2D activity in vivo, mice received 100 μg of functional grade purified anti-mouse NKG2D (eBioscience) intravenously 24 hr and 6 hr prior to infection with VV.

2.22 Western blot analysis

NK cells (1 x 10^{6}) were cultured in CTL medium and infected with VV with MOI of 1 for the indicated time periods at 37ºC. Western blot analysis was conducted as previously described [11]. Briefly, samples were transferred to a nitrocellulose membrane following separation on SDS-PAGE gels. Membranes were blocked for 1 hr at 22 ºC with 1% fish gelatin. Membranes were then blotted overnight at 4ºC with anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (E10) Mouse mAb or Phospho-Akt (Thr308) Rabbit pAb (Cell Signaling Technologies), washed three times, probed with an Alexa-Fluor 680-conjugated anti-mouse Ig secondary antibody (for pERK1/2) or an Alexa-Fluor 680-conjugated anti-rabbit Ig secondary antibody (for pAkt) (Molecular Probes), followed by visualizing the Odyssey infrared imaging system (LI-COR).

Membranes were then stripped and probed with an anti-total Erk1/2 or anti-total Akt antibody (Cell Signaling Technologies) to serve as a loading control.

2.23 Statistical Analysis

A two-sided, two sample student t-test with 95% confidence bounds was used for statistical analysis. Data are presented as mean ± sd. All statistical analyses were performed using the SAS/STAT software (SAS Institute, Cary, NC) as we previously described [76].

Chapter 3: Toll-like receptor 8-mediated activation of murine plasmacytoid dendritic cells by vaccinia viral DNA

The following text was slightly modified from its original manuscript, "Toll-like receptor 8-mediated activation of murine plasmacytoid dendritic cells by vaccinia viral DNA," currently under review at Proceedings of the National Academy of Sciences (PNAS).

3.1 Introduction

Plasmacytoid dendritic cells (pDCs), originally called interferon-producing cells, are a specialized type of dendritic cells that play a critical role in antiviral immunity, in that they are capable of rapidly producing large amounts of type I interferons (IFNs) upon viral infection [23]. Unlike other innate immune cells, pDCs produce type I IFNs via a TLR-MyD88-dependent mechanism, and therefore do not utilize other PRRs, such as the RLR or NLR pathways to sense viral infection [24]. pDCs preferentially express TLR7, TLR8, and TLR9, which sense viral nucleic acids in the endosomal compartments in a MyD88-dependent manner [24, 30]. TLR7 recognizes specific sequences in guanosine- and uridine-rich ssRNA, whereas TLR9 senses an unmethylated CpG motif in DNA, commonly found in bacteria and mimicked by viruses[24, 92-95]. It has been demonstrated TLR8 also plays a role in sensing viral ssRNA, such as HIV, in humans [93, 96]. Interestingly, a biological function for murine TLR8, which can respond to the synthetic ligands, R848, but not the natural ligand, ssRNA, has yet to be identified [31]. However, a recent study demonstrated that polyT-rich oligodeoxynucleotides were able

to enhance murine TLR8 stimulation in vitro. This observation is especially novel, as ssRNA does not contain thymine [32]. Furthermore, biochemical analysis of human TLR8 established that ligand interaction with and activation of the TLR8 pathway requires an acidic environment, such as the late endosome [33]. This data differs from that of TLR9 signaling, where induction of type I IFN production was activated by TLR9 signaling in the early endosomes of pDCs, which contain a slightly more basic environment than the late endosome [34]. It is perhaps these differential ligand interaction and signaling requirements that distinguish the endosomal TLR signaling in pDCs. As pDCs produce type I IFNs in a TLR-MyD88-dependent manner, thus limiting their recognition catalog for viral nucleic acids to TLR7, TLR8, and TLR9, a physiological role for murine TLR8 seems forthcoming.

Vaccinia virus (VV) is a member of the poxvirus family that includes smallpox (variola) virus, monkeypox virus, cowpox virus, and mousepox (ectromelia) virus (ECTV) [70]. It is an enveloped, dsDNA virus with a genome of approximately 200 kbp. VV is the most studied member of the poxvirus family and is responsible for the successful elimination of smallpox worldwide in the late 1970s [70-71]. Despite this extensive research, mechanisms underlying the control of VV infection remain largely undiscovered. Using a murine model of VV infection, we have recently shown that VV activates the TLR2-MyD88 pathway on conventional DCs (cDCs), leading to production of the pro-inflammatory cytokines IL6, IL1, and IL12. In addition, VV also stimulates cDCs to produce IFNβ in a TLR-independent manner [22]. However, cDCs are not the

primary producers of type I IFNs in the setting of viral infection, and it has been established that pDCs do not preferentially express TLR2 [24, 30]. Given the importance of pDCs in antiviral immunity, it remains to be defined whether and how VV activates pDCs.

In these studies, it is demonstrated that both VV and VV-DNA were capable of activating pDCs, leading to MyD88-dependent secretion of type I IFNs. Unexpectedly, it was found that pDC recognition of VV-DNA was TLR9-independent, despite VV being a DNA virus. Using HEK293 cells transfected with a plasmid expressing murine TLR7 or TLR8 and an NFκB luciferase reporter construct, it was demonstrated that stimulation of TLR8-transfected cells with VV or VV-DNA resulted in substantial NFκB activation, whereas stimulation of TLR7-transfected cells did not. Moreover, siRNA knockdown of TLR8 expression in murine pDCs resulted in a severe decrease in type I IFN production. Additionally, functional blockade of TLR8 on pDCs with a TLR8 antagonist, ODN2088, led to a complete ablation of type I IFN production by pDCs upon stimulation with VV or VV-DNA. Further analysis of the VV genome revealed that it was rich in poly A/T sequences, and synthetic polyA and polyT ODNs (but not polyC or polyG ODN) were capable of activating pDCs in a TLR8-dependent fashion. In vivo, TLR8-MyD88-dependent activation of pDCs played a critical role in innate immune control of VV infection. Taken together, these data demonstrate for the first time that TLR8 in pDCs can function as a DNA sensor likely through recognition of polyA and polyT motifs, and that murine TLR8 is functional in the context of a viral infection.

3.2 Results

3.2.1 Activation of pDCs by VV occurs in a MyD88-dependent, but TLR9-independent manner

In order to first establish a role of pDCs in the immune response to VV, we examined whether VV was capable of activating pDCs, as they play a critical role in antiviral immunity [24]. pDCs, identified as $CD11c^{+}B220^{+}mPDCA\text{-}1^{+}$, were generated from bone marrow cells in the presence of Flt3 ligand (Flt3L) and purified by FACS sorting. These pDCs were then stimulated with live VV, and the culture supernatants were assayed for the secretion of IFNα and IFNβ by ELISA. Indeed, pDCs stimulated with VV produced high levels of IFNα (Figure 1A) and IFNβ (Figure 1B), and upregulated the expression of CD86 (Figure 1C), indicating that they were activated upon VV infection. We next investigated whether TLRs were involved in the induction of type I IFNs by pDCs upon VV infection. Since all TLR signaling in pDCs is mediated by MyD88 [24], pDCs generated from the bone marrow of MyD88-/- mice were tested for their ability to produce type I IFNs upon VV infection. The production of both IFNα (Figure 1A) and IFNβ (Figure 1B) by MyD88-/- pDCs was abolished, demonstrating that the production of type I IFNs by pDCs in response to VV infection is TLR-mediated and dependent on MyD88. We further demonstrated that endogenous splenic pDCs also secreted type I IFNs in a MyD88-dependent fashion upon VV infection and to an equivalent degree as bone marrow-derived pDCs (Figure 1D). However, in line with our previous observations, cDCs ($CD11c^{+}B220^{-}mPDCA\text{-}1^{-}$) activated with VV produced

lower levels of IFNβ in a MyD88-independent manner and failed to produce any significant IFNα (Figure 1A, B).

We next sought to determine which TLR was responsible for the production of type I IFNs by pDCs in response to VV infection. Among all TLRs characterized to date, only TLR7, TLR8, and TLR9 are known to mediate MyD88-dependent production of type I IFNs by pDCs [3]. Since the known ligands for TLR7 and TLR8 are ssRNA, and VV is dsDNA virus, we first examined if VV or DNA isolated from VV, VV-DNA, activated TLR9 in pDCs to produce type I IFNs. pDCs generated from TLR9-/- mice secreted similar levels of IFNα (Figure 1E) and IFNβ (Figure 1F) upon stimulation with VV or VV-DNA compared to the WT controls, indicating that TLR9 is not involved in induction of type I IFNs by VV-DNA. Taken together, the above observations indicate that VV-induced production of type I IFNs by pDCs is MyD88-dependent, yet TLR9-independent.

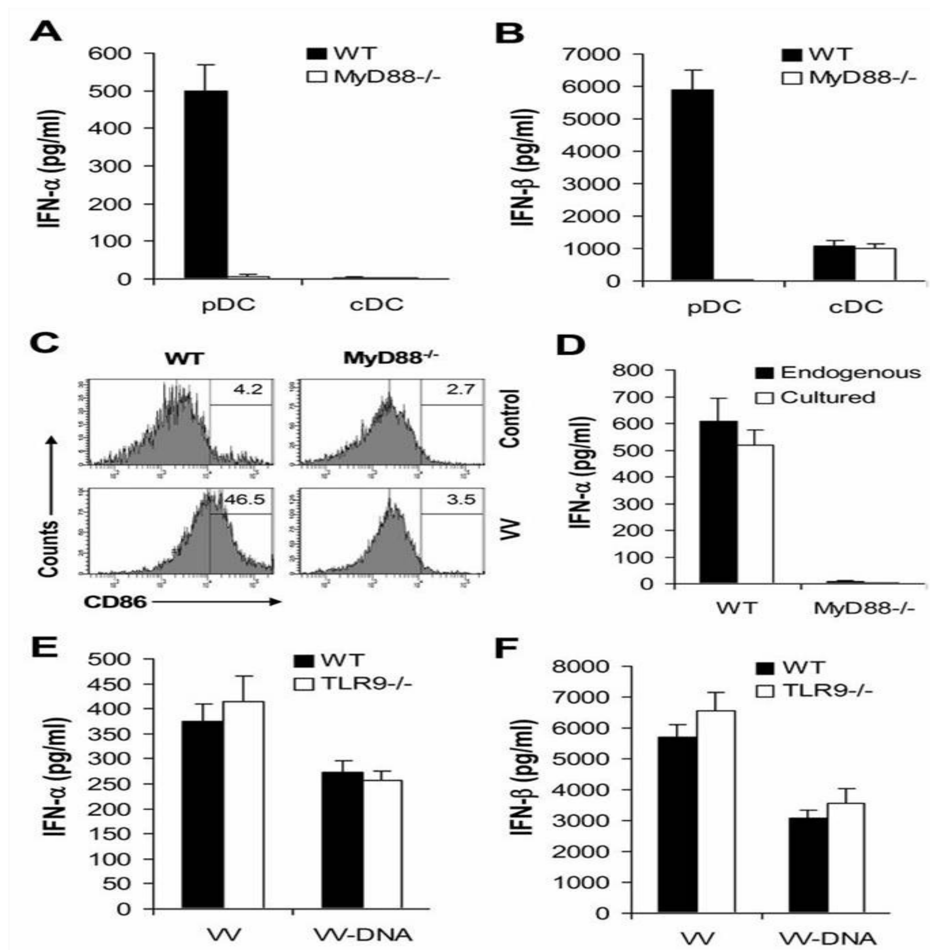

Figure 1: MyD88-dependent, but TLR9-independent activation of pDCs by VV. (A-C) DCs were generated from bone marrow cells of WT or MyD88-/- C57BL/6 mice in the presence of Flt3L for 9 days. Cells were stained with anti-CD11c and anti-B220, sorted into pDC and cDC populations by flow cytometry. Purified cells were then stimulated with live VV at an MOI of 1 for 24 hr. Culture supernatants were analyzed by ELISA for secretion of IFNα (A) and IFNβ (B). In addition, pDCs were stained with anti-CD86 antibodies, and analyzed for CD86 expression. The percentages of pDCs expressing CD86 are indicated (C). (D) Endogenous splenic pDC were isolated from C57BL/6 (WT) and MyD88-/- mice by flow cytometry sorting and stimulated with live VV at an MOI of 1 for 24 hr. Culture supernatants were analyzed by ELISA for secretion of IFNα in comparison with Flt3L cultured pDCs. (E-F) WT or TLR9-/- pDCs were stimulated with live VV (MOI 1) or VV-DNA (25 μg/ml) for 24 hr. Culture supernatants were analyzed by ELISA for secretion of IFNα (E) and IFNβ (F). Results are expressed as mean ±SD. Data shown is representative of three independent experiments (n=4 per data point).

3.2.2 VV or VV-DNA activates pDC via TLR8

The MyD88-dependent, TLR9-independent sensing of VV by pDCs suggested that the TLR responsible for pDC activation would likely be TLR7 or TLR8, as both of them are expressed in murine pDCs [30]. To address this question, we transfected HEK293 cells with a plasmid encoding either murine TLR7 or TLR8, in combination with an NFκB luciferase reporter construct. The transfected cells were subsequently stimulated with VV or VV-DNA, and cell lysates were assayed for luciferase activity. Stimulation of the TLR7-transfected cells with a synthetic TLR7 ligand, CL087, yielded high levels of luciferase activity compared to the NFκB luciferase reporter construct only control (Figure 2A). However, no significant levels of luciferase activity were produced by TLR7-transfected cells stimulated with VV or VV-DNA (Figure 2A). In contrast, stimulation of TLR8-transduced cells with VV or VV-DNA resulted in high levels of luciferase activity (Figure 2A). These results suggest that TLR8, but not TLR7, can be activated by VV or VV- DNA in this system.

Whereas this system was important for initially examining TLR8 reactivity to VV stimulation, it was also important to examine the ability of VV to activate the TLR8 pathway in pDCs. Therefore, we then tested whether murine TLR8 was also responsible for sensing of VV or VV-DNA by pDCs, using two experimental approaches to address this question. We first evaluated the effect of small interfering RNA (siRNA)-mediated knockdown of TLR8 in pDCs on the induction of type I IFNs by VV or VV-DNA. pDCs were transduced by a GFP-containing lentiviral construct encoding siRNA specific for

murine TLR8 or a control siRNA, and the transduced GFP^{+} pDCs were sorted and assayed for the expression of TLR8 by RT-PCR. No expression of TLR8 was detected in pDCs transduced with two different siRNA constructs specific to TLR8, compared to the control siRNA-transduced or untransduced pDCs (Figure 2B), thus confirming the knockdown of TLR8 in pDCs. The pDCs were then stimulated with VV or VV-DNA and assayed for IFNα secretion. The production of IFNα was essentially abolished in pDCs transduced with TLR8-specific siRNAs, compared with the control siRNA (Figure 2C). However, the TLR8-knockdown pDCs retained the ability to secret IFNα in response to stimulation with the TLR7 ligand, CL087, or the TLR9 ligand, CpG DNA (Figure 3), suggesting the specificity of TLR8 knock-down by siRNA.

These results suggest that the sensing of VV-DNA in pDCs is mediated by TLR8. To further support this, we examined whether TLR8 blockade with the inhibitor, ODN2088, suppressed the ability of pDCs to secrete type I IFNs upon stimulation with VV or VV-DNA. The production of IFNα was significantly reduced in the presence of ODN2088 (Figure 2D). Since ODN2088 disrupts the colocalization of CpG ODNs with TLRs, including TLR9, in endosomal vesicles without affecting cellular binding and uptake, we repeated the experiment using TLR9-/- pDCs. Similar results were obtained with TLR9-/- pDCs compared to the WT controls (Figure 2D), confirming that sensing of VV-DNA by pDCs is dependent on TLR8, but not TLR9.

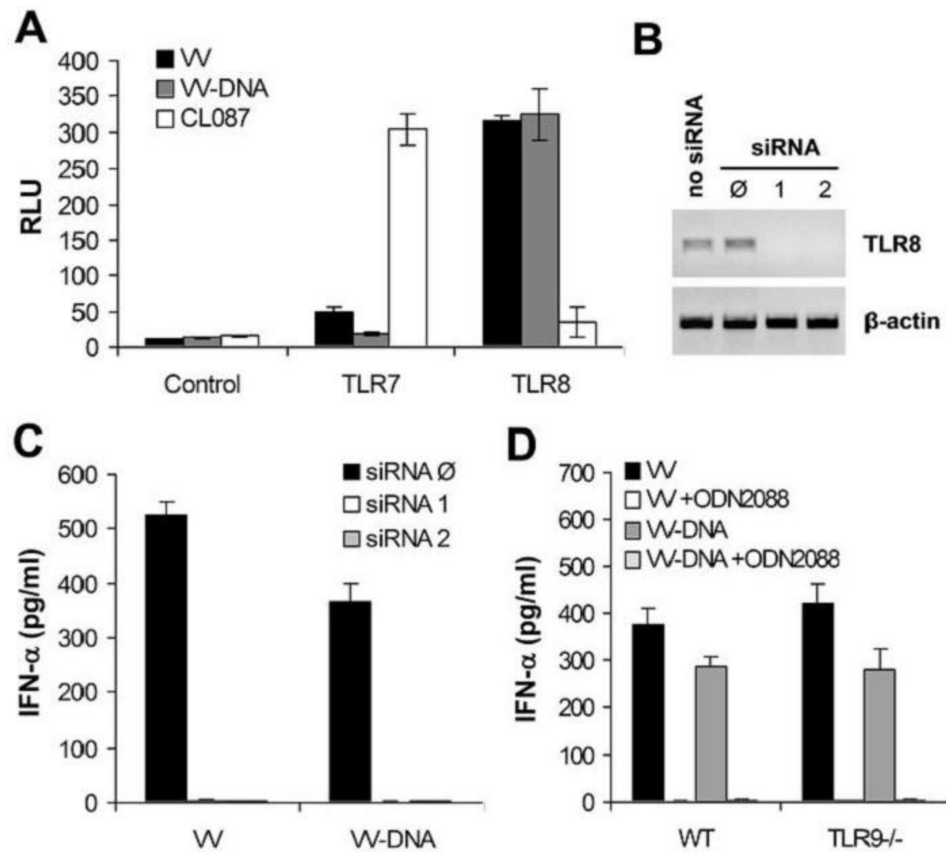

Figure 2: VV or VV DNA activates pDCs via TLR8. (A) HEK293 cells were transfected with a plasmid encoding either murine TLR7 or TLR8 and an NFκB-Luciferase reporter construct. Forty-eight hours after transfection, cells were stimulated with live VV (MOI 1), VV-DNA (25 μg/ml), or a TLR7 ligand, CL087 (0.1 μg/ml) for 12 hr. Cells were then lysed and NFκB activation was analyzed by a luminometer. Data is presented as mean relative luciferase units (RLU) ±SD. (B-C) pDCs generated from bone marrow cells were transduced with two different lentiviral constructs encoding siRNA specific for murine TLR8, pNL-SIN-GFP-siRNA1 (siRNA1) or pNL-SIN- EGFP-siRNA2 (siRNA2), or a control virus pNL-SIN-GFP without siRNA (siRNAØ), or left untransduced (no siRNA). RNA was purified from the transduced EGFP+ pDCs and subjected to TLR8 expression by RT-PCR (B). Sorted EGFP+ pDCs were stimulated with live VV (MOI 1) or VV-DNA (25 μg/ml) for 24 hr and the culture supernatants were analyzed by ELISA for secretion of IFNα (C). (D) pDCs generated from bone marrow cells of WT or TLR9-/- mice were stimulated with live VV (MOI 1) or VV-DNA (25 μg/ml) in the presence or absence of ODN2088 (1 μM) for 24 hr. Culture supernatants were analyzed by ELISA for secretion of IFNα. Results are expressed as mean ±SD. Data shown is representative of three independent experiments (n=3 per data point).

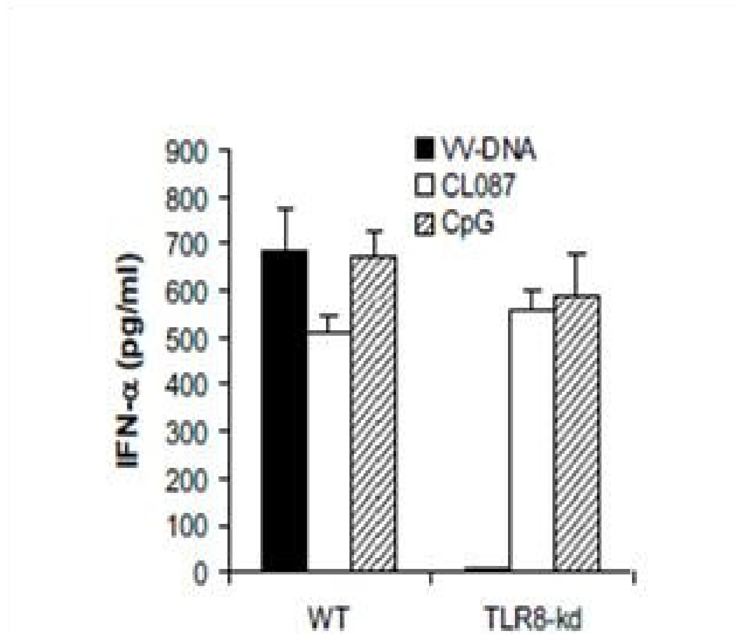

Figure 3: TLR8-knockdown pDCs are responsive upon stimulation with TLR7 and TLR9 ligands. WT or TLR8-knockdown (TLR8-kd) pDCs were stimulated with VV-DNA (25 μg/ml), TLR7 ligand CL087 (0.1 μg/ml), or TLR9 ligand CpG (1 nM) for 24 hr, and the culture supernatants were assayed for IFNα secretion by ELISA. Data shown is representative of three independent experiments (n=2 per data point).

3.2.3 The polyA and polyT motifs in the VV genome are responsible for activation of TLR8 on pDCs

What are the potential sequence motifs in VV-DNA required for activation of TLR8? Since polyT ODN has been shown to enhance human TLR8 responsiveness to small molecule ligands [32], we first examined the VV genome for the A/T and G/C compositions. Indeed, approximately 67% of the VV genome is composed of A/T sequences (Figure 4A), indicating the VV genome is rich in A/T sequences. This is in contrast to only 44% of the human adenovirus type 5 (Ad5, Figure 4A) and 30% of the herpes simplex virus type 2 (HSV-2) genomes (data not shown). It has been demonstrated that both Ad5 and HSV-2 activate TLR9 in pDCs via their CpG motif [97-98]. Indeed, we showed here that activation of pDCs by Ad5-DNA was dependent on TLR9, but not TLR8 (Figure 4B). We further found that about 45% of the VV genome is composed of A/T rich islands, defined as 10 base pair stretches that contain 5 or more A/T (either consecutively or non-consecutively), compared to only about 1% of G/C rich islands (Figure 4C). These results suggest that polyA or polyT-DNA might contribute to the activation of TLR8. To test this, polyA10, polyT10, polyC10, and polyG10 ODN were generated and used to stimulate WT or TLR8-knockdown pDCs, and the culture supernatants were analyzed for secretion of IFNα. Stimulation of WT, but not TLR8-knockdown, pDCs with poly A10 or poly T10 ODN led to high levels of IFNα production, comparable to those stimulated with VV-DNA (Figure 4D). However, no significant levels of IFNα were secreted when WT pDCs were stimulated with polyC10

or polyG10 (Figure 4D). These results suggest that polyA and polyT-DNA are responsible for the activation of murine pDCs via TLR8.

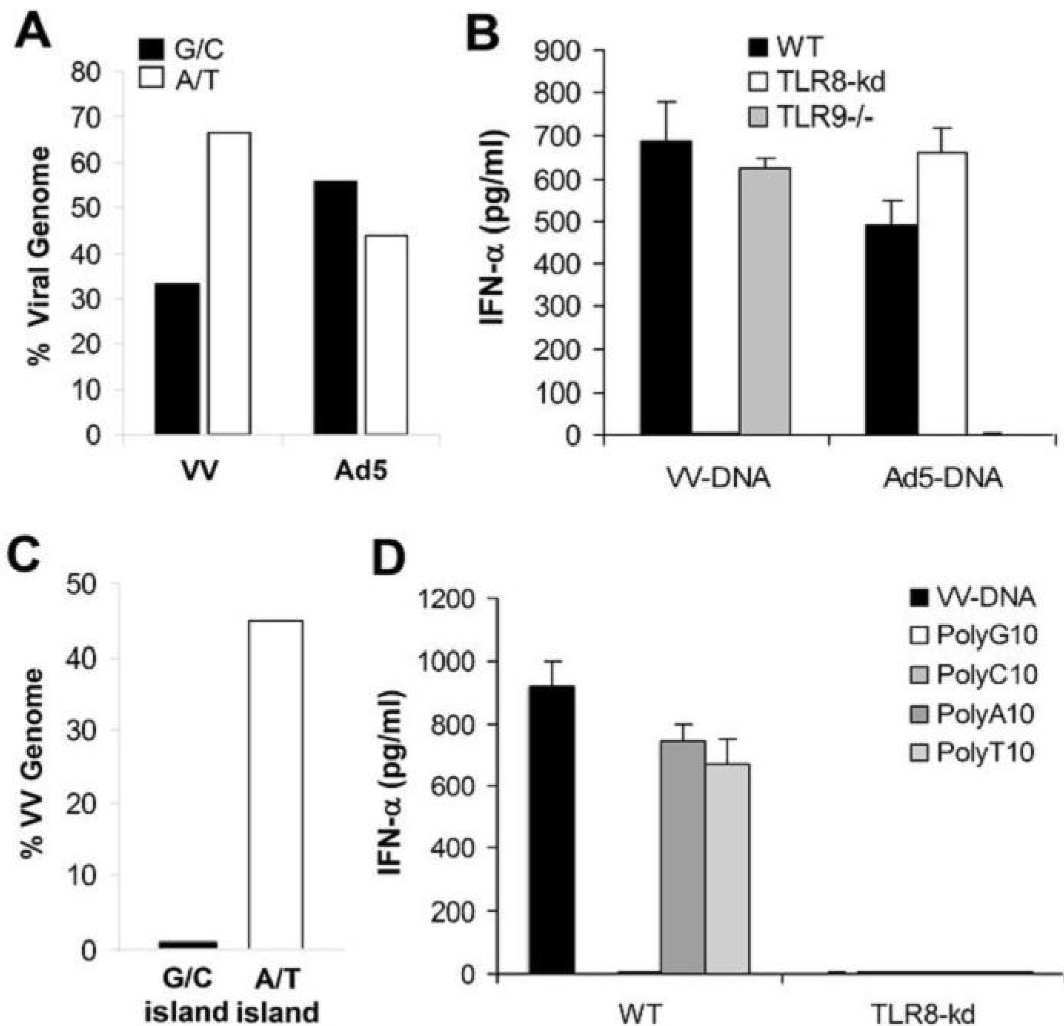

Figure 4: The polyA and polyT motifs in the VV genome are possibly responsible for activation of TLR8 on pDCs. (A) The complete genomes of VV and human adenovirus type 5 (Ad5) were analyzed for A/T and G/C contents and expressed as a percentage using pDRAW32 software. (B) WT, TLR8-knockdown (TLR8-kd), or TLR9-/- pDCs were stimulated with VV-DNA (25 µg/ml) or Ad5-DNA (5 µg/ml) for 24 hr, and culture supernatants were assayed for IFNα secretion. (C) The VV genome was analyzed for A/T- or G/C-rich islands, defined as 10 base pair stretches with at least 5 A/T or G/C. (D) WT or TLR8-knockdown (TLR8-kd) pDCs were stimulated with VV-DNA (25 µg/ml), PolyG10, PolyC10, PolyA10, or PolyT10 (all at 5 µg/ml) for 24 hr. Culture supernatants were analyzed by ELISA for secretion of IFNα. Data shown is representative of three independent experiments (n=2 per data point).

3.2.4 WT, but not TLR8-knockdown, pDCs restore VV clearance in MyD88-/- mice

We next investigated the functional significance of TLR8-dependent activation of pDCs by VV in vivo. WT or MyD88-/- mice were injected with 1 x 10^7 pfu of VV intravenously. Forty-eight hr later, serum was assayed for IFNα secretion, and the spleen was harvested for determination of the viral titers by the plaque assay. Sera from VV-infected WT mice were found to contain significant levels of IFNα (Figure 5A). However, IFNα levels were greatly diminished in VV-infected, MyD88-/- mice (Figure 5A). This was accompanied by a significant (p <0.001, Figure 5B) increase in viral titers in the spleen of MyD88-/- mice. Adoptive transfer of WT pDCs into MyD88-/- mice led to restoration of IFNα secretion (Figure 5A) and reduction of viral titers in the spleen (Figure 5B). However, transfer of TLR8-knockdown pDCs into MyD88-/- mice did not induce IFNα secretion or reduction of viral load (Figure 5A, B). These results suggest that TLR8-MyD88-dependent pDCs activation is critical for innate immune control of VV infection in vivo.

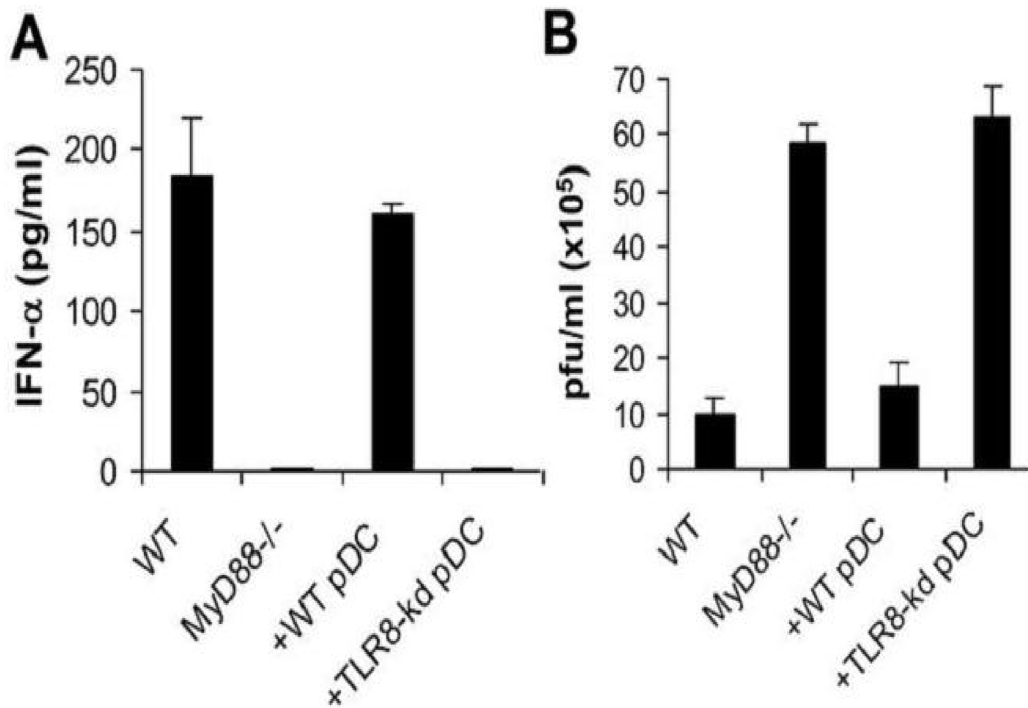

Figure 5: WT, but not TLR8-knockdown, pDCs restore VV clearance in MyD88-/- mice. MyD88-/- mice were reconstituted with WT or TLR8-knockdown (TLR8-kd) pDCs, and infected with $1x10^7$ pfu of live VV, intravenously. WT and MyD88-/- mice were also infected with VV and used as controls. Forty-eight hours after infection, serum was analyzed by ELISA for secretion of IFNα (A), and the spleen was evaluated for viral titers by the plaque forming assay (B). Results are expressed as mean ±SD (n=5). Data shown is representative of two independent experiments.

3.2.5 TLR8-knowdown pDCs fail to restore VV clearance in pDC-depleted mice

To further confirm the significance of TLR8-dependent pDCs activation in innate immune control of VV in vivo, we first depleted WT mice of pDCs using anti-Ly6G/C Ab as described [99-100], and then reconstituted the pDC-depleted mice with TLR7-knockdown, TLR8-knockdown, or TLR9-/- pDCs and examined for their ability to restore innate immune control of VV infection in vivo. Indeed, we found that injecting mice twice with Ly6G/C Ab successfully depleted >92% of pDCs in vivo (Figure 6). pDC depletion abrogated IFNα secretion in response to VV infection (Figure 7A) and resulted in a significant increase in viral titers and viral DNA in the spleen (Figure 7B, C). Previous studies have shown that the liver is also a target organ for VV upon intravenous infection [101]. Thus, we also examined viral titer and DNA in the liver and obtained similar results (Figure 7D, E). These results further illustrate the importance of pDCs in VV control in vivo. Reconstitution of pDC-depleted mice with TLR7-knockdown or TLR9-/- pDCs restored IFNα secretion (Figure 7A) and resulted in a significant reduction of viral titers and viral DNA in the target organs (Figure 7B-E). However, transfer of TLR8-knockdown pDCs into pDC-depleted mice failed to elicit the production of IFNα (Figure 7A) or a reduction of viral DNA or viral titers in target organs (Figure 7B-E). Taken together, these data further support the conclusion that TLR8-dependent pDC activation is critical for innate immune control of VV infection in vivo.

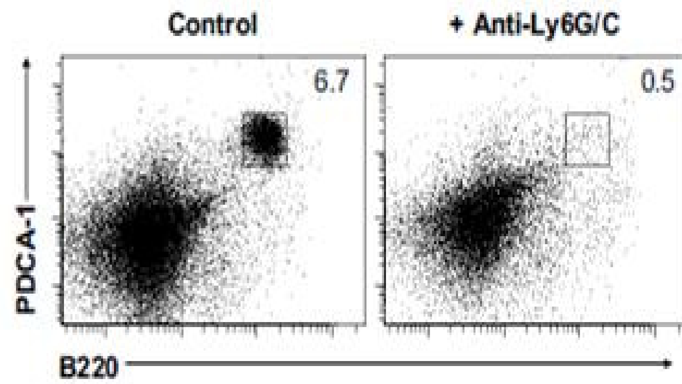

Figure 6: Depletion of pDCs in vivo. Mice were injected with 150 μg of anti-Ly6G/C or a control antibody intravenously on days -3 and -1. On day 0, splenocytes were harvested and stained with anti-CD11c, anti-B220 and anti-PDCA-1 and subjected to FACS analysis. The FACS plots are gated on $CD11c^+$ cells. The percentage of $B220^+PDCA\text{-}1^+$ pDCs is shown. Data is representative of three independent experiments.

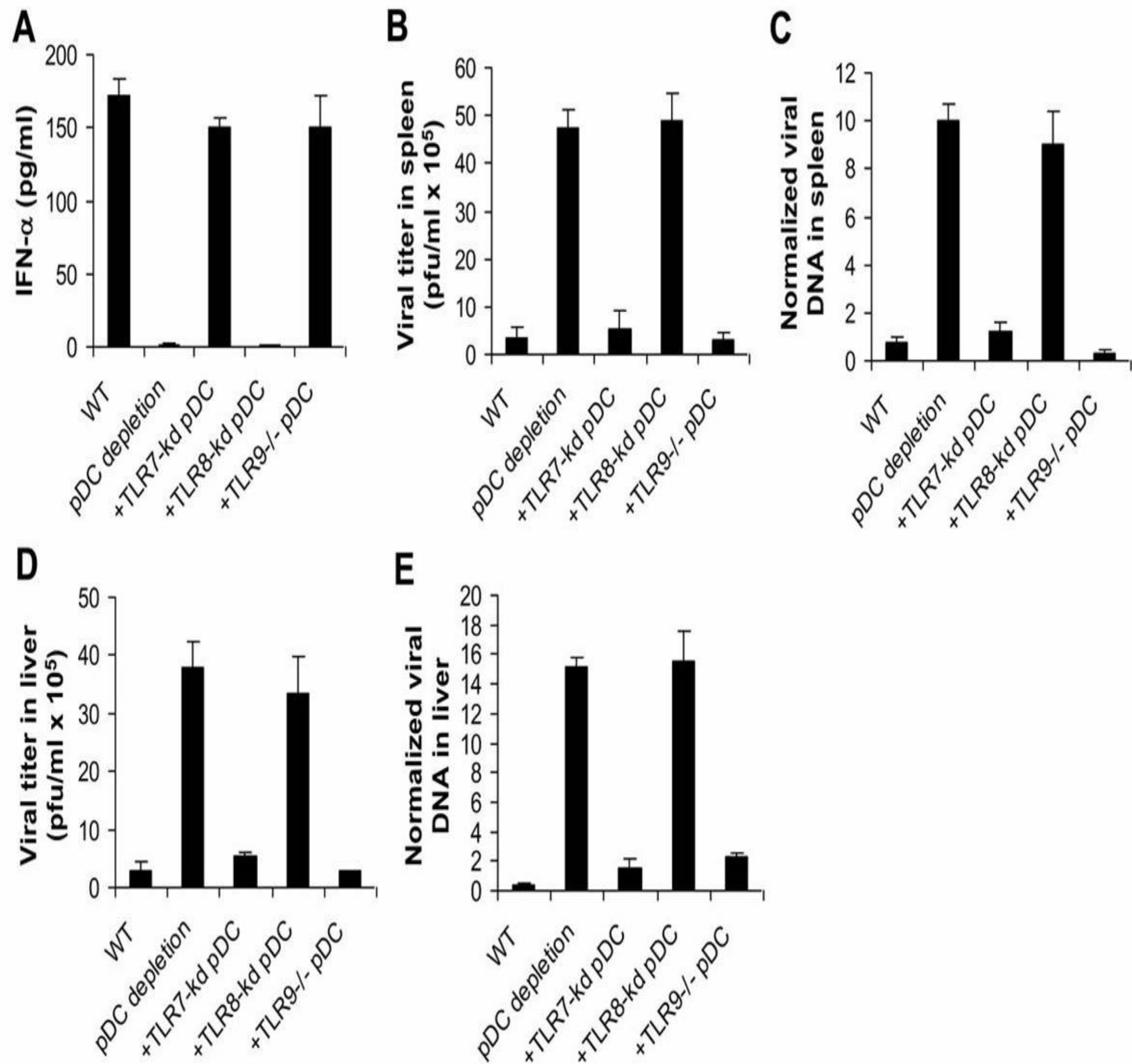

Figure 7: TLR8-knockdown pDCs fail to restore VV clearance in pDC-depleted mice. WT mice were depleted of pDCs with anti-Ly6G/C on days -3 and -1, and reconstituted on day 0 with TLR7-knockdown (TLR7-kd), TLR8-knockdown (TLR8-kd), or TLR9-/- pDCs, or left unreconstituted (pDC depletion). These mice were subsequently infected with $1x10^7$ pfu of live VV, intravenously. WT mice were also infected with VV and used as a positive control. Forty-eight hours after infection, serum was analyzed by ELISA for secretion of IFNα (A), and the spleen (B, C) and the liver (D, E) were evaluated for viral titers by the plaque forming assay (B, D) and viral DNA by real-time quantitative PCR (C, E). Results are expressed as mean ±SD (n=5). Data shown is representative of two independent experiments.

3.3 Discussion

In this study, we found that recognition of VV-DNA by pDCs was MyD88-dependent, yet TLR9-independent. We identified that pDC sensing of VV-DNA was mediated by TLR8, possibly through recognition of polyA/T-rich motifs. We further demonstrated that TLR8-MyD88 dependent pDC activation was crucial to the control of VV infection in vivo. Previous studies have shown that type I IFN production by pDCs in response to a viral infection is linked to endosomal TLR7, which senses guanosine- and uridine-rich ssRNA [92-93], and TLR9, which recognizes unmethylated CpG DNA [94-95]. Although TLR8, another endosomal TLR, has been shown to sense viral ssRNA in humans, it was thought that TLR8 was non-functional in mice [93, 96]. A recent study has indicated, however, that murine TLR8 can be activated by a combination of imidazoquinoline TLR8 ligands and polyT ODN [32]. Our findings that the polyA/T-rich VV-DNA alone is sufficient to activate murine pDCs via TLR8 not only further extend this observation, but more importantly, demonstrate for the first time that TLR8 can serve as a DNA sensor in recognition of polyA and polyT motifs in pDCs and that murine TLR8 is functional in the context of a viral infection. Using bulk, unsorted, Flt3L cultured DCs, a recent study indicated that the production of type I IFNs by these DCs upon infection with modified VV Ankara (MVA) was TLR9-independent, but partially dependent on MyD88 [102]. Since bulk Flt3L cultured DCs contain both pDCs and cDCs populations [98], our finding that pDC recognition of VV-DNA is dependent on the TLR8-MyD88 pathway, leading to production of both IFNα and IFNβ, whereas cDC

sensing of VV-DNA is TLR-independent, resulting in secretion of only IFNβ, may provide explanation for their observation [22]. In fact, our data may also help explain the observed TLR7- and TLR9-independent, but MyD88-dependent pDC activation in response to some other viral infections [24]. On the other hand, a recent report showed that pDC recognition of ECTV (mousepox), but not MVA, was dependent on TLR9 [103]. It is not clear why pDC recognition of VV/MVA vs. ECTV is mediated through different TLRs. It may reflect differences between VV/MVA and ECTV virus in terms of their genetic compositions (i.e. A/T islands, CpG contents) and endosomal trafficking pathways, as type I IFN production by TLR8 and TLR9 requires signaling via in the late and early endosomes, respectively [33-34]. Consistent with our previous observations [22], cDCs can also secret IFNβ, albeit at lower levels, independent of TLR upon VV infection. Thus, the requirement of TLR8-dependent pDC activation in the control of VV infection in vivo suggests that the low levels of IFNβ produced by non-pDCs may not be sufficient for the control of VV in vivo. Alternatively, pDCs could exert type I IFN-independent anti-viral mechanisms. Future studies will be needed to delineate the underlying mechanisms.

The observation that TLR8-dependent pDC activation is critical for innate immune control of VV infection in vivo suggests that different pathogens have evolved to adopt unique mechanisms to effectively activate the innate immune system. Although our results establish a role for TLR8 in pDC recognition of VV in mice, human pDCs only express TLR7 and TLR9. However, human TLR8 is widely expressed in non-pDCs,

such as cDCs and monocytes [10]. At present, it is not clear whether TLR8-mediated recognition of VV by non-pDCs is of importance in VV control in humans or whether human pDCs use alternative TLRs in recognition of VV-DNA. Thus, it will be important to address these questions in future studies. In conclusion, our study reveals a previously unappreciated role for murine TLR8 in pDC activation, possibly through recognition of polyA/T-rich motifs in response to VV infection and suggests a novel strategy of polyA and polyT ODN-mediated pDC activation for the efficient viral control in vivo.

Chapter 4: Direct Action of Type I IFN on NK Cells is Required for Their Activation in Response to Vaccinia Viral Infection in vivo

The following text was slightly modified from its original publication, "Direct Action of Type I IFN on NK Cells is Required for Their Activation in Response to Vaccinia Viral Infection in vivo," published in Volume 180 of The Journal of Immunology (2008) [85].

4.1 Introduction

Vaccinia virus (VV) represents one of the medically important viruses. It is a member of the poxvirus family, which includes smallpox (variola) virus, monkeypox virus, cowpox virus, and ectromelia virus (ECTV), and has a large, complex dsDNA genome that replicates exclusively in the cytoplasm [104]. VV is the most studied member of the poxvirus family and is the vaccine responsible for successful eradication of smallpox worldwide in the late 1970s [105]. This unparalleled success is now being threatened by bioterrorists deliberately reintroducing smallpox, against which vaccination is no longer routine [78-80]. The revival of smallpox vaccination has been countermanded by the relatively high incidence of adverse events associated with the currently used live VV vaccine [81-83]. Thus, to effectively control poxviral infections, it is necessary to elucidate the host's defense mechanism(s) against poxviruses in vivo.

Recovery from viral infections depends on the host's ability to mount effective innate antiviral responses that can eliminate, or at least control, the invading pathogen.

NK cells represent an important component of the innate immune system. It has been shown that NK cells play a critical role in innate immune defense against various viral infections in vivo [106]. NK cells have also been implicated in the response to poxviruses. Upon poxviral infection, NK cells are activated, expand and accumulate at the site of infection, and the activated NK cells are important for recovery of the infection [101, 107-109]. However, it remains poorly understood how NK cell activation is regulated upon poxviral infection.

Type I interferons (IFNs), produced by host cells early after viral infection, represent a key player in anti-viral defense [63]. They are a family of cytokines that constitute 13 and 17 IFNα subtypes in mice and humans, respectively, and one IFNβ in both species [61]. All type I IFNs signal through a heterodimeric receptor composed of two subunits, IFNα/β receptor 1 (IFNα/βR1) and IFNα/βR2. Stimulation of IFNα/βR with type I IFNs triggers a series of signaling cascades leading to the transcription of more than 100 IFN-stimulated genes (ISGs) [63]. The serine/threonine protein kinase (PKR) and the 2'-5' oligoadenylate synthetases (OAS), both of which are activated by viral dsRNA, are among the best characterized ISG with antiviral activity through suppression of viral replication in infected cells by inhibiting RNA and protein synthesis [110-111].

In addition to the direct antiviral effects, type I IFN signaling also mediates a variety of immunoregulatory effects [63], including regulation of NK cell activation [112]. How type I IFNs regulate NK cell activation is yet to be fully elucidated. A recent

study suggested that action of type I IFNs on accessory dendritic cells (DCs), but not on NK cells, was required for NK cell activation in response to synthetic TLR ligands [113]. In a murine model of VV infection, we have recently shown that type I IFNs play a critical role in the innate immune control of VV infection [22]. In this study, we showed that type I IFNs did not directly protect cells from VV infection in vitro and that type I IFN-dependent innate immune control of VV infection in vivo was mediated through the activation of NK cells. We further demonstrated that type I IFN signaling directly on NK cells was necessary for their activation and effector function both in vitro and in vivo.

4.2 Results

4.2.1 Type I IFNs do not protect cells from VV infection in vitro

To understand how type I IFNs confer innate immune defense against VV infection in vivo, we first examined if IFNα interfered with the replication of VV in a permissive cell line, L929 cells. It has been shown that IFNα pre-treatment protects L929 from RNA viruses such as Encephalomyocarditis virus (EMCV)-induced cell death through inhibiting viral replication [114-115]. We found that infection of L929 monolayer with EMCV at MOI as low as of 0.1 led to cell death for the majority of cells 2 days later, suggesting that cell death was dependent on viral replication and cell-to-cell spread in the monolayer (Figure 8A). Indeed, pretreatment with IFNα resulted in a significant ($p < 0.01$) reduction in cell death even at MOI of 62.5, confirming that IFNα can directly protect cells from EMCV infection. However, under similar conditions, IFNα pretreatment did not alter VV-induced cell death, suggesting that IFNα did not

prevent VV replication in L929 cells (Figure 8B). Thus, IFNα does not directly protect cells against VV infection in vitro.

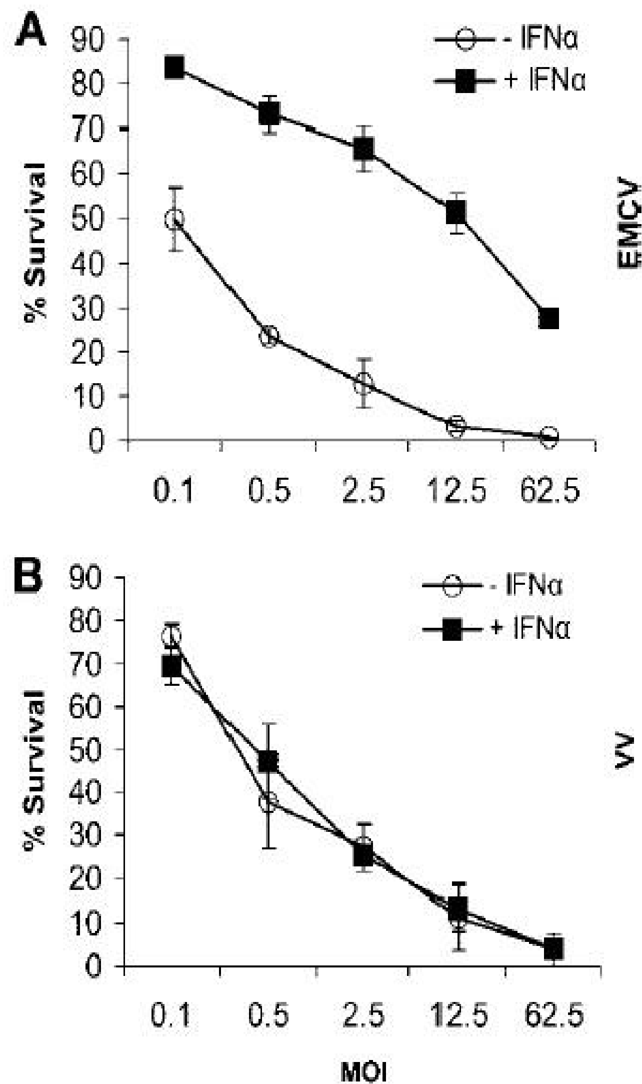

Figure 8: IFNα pre-treatment protects L929 cells from EMCV, but not VV, infection. L929 cells were cultured in the presence (+IFNα) or absence (-IFNα) of IFNα for 24 hr. The supernatant was then removed, and the cells were infected with either EMCV (*A*) or VV (*B*) at different multiplicities of infection (MOI) for another 48 hr. The mean percentage of survival ±SD is indicated (n=4). Data shown is representative of three independent experiments.

4.2.2 Type I IFN-dependent innate immune control of VV infection in vivo is mediated through regulating NK cell activation

Our observation that IFNα did not directly protect cells against VV infection in vitro suggests that the type I IFN-dependent innate immune control of VV infection in vivo likely operates through a different mechanism. As type I IFNs have been shown to mediate immunoregulatory effects on NK cells in other models of infection [101, 112] and NK cells have been implicated in innate immunity against poxviruses [101, 107-109], we hypothesized that type I IFN-dependent innate immunity against VV infection in vivo was mediated by activated NK cells. We first tested whether VV infection in WT mice led to NK cell activation. Forty-eight hours after infection with 1 $x10^7$ pfu VV i.p., splenic NK cells expanded more than twice (Figure 9, A and B), produced significantly ($p < 0.001$) higher amounts of effector molecules (Figure 9C), and demonstrated lytic function on NK-sensitive YAC-1 cells (Figure 9D) compared with the naive controls, confirming that indeed NK cells are activated upon VV infection. These activated NK cells were critical for VV clearance as WT mice depleted of NK cells (Figure 10A) showed a defect in NK lytic activity (Figure 10B) and had a significantly ($p < 0.001$) higher viral titer than the control mice (Figure 10C). We next determined whether activation of NK cells upon VV infection was regulated by type I IFN signaling. IFNα/βR-/- mice were infected with $1x10^7$ pfu VV i.p. and splenic NK cells were analyzed 48 hr later. No significant expansion of NK cells was observed in IFNα/βR-/- mice upon VV infection (Figure 9B). In addition, IFNα/βR-/- NK cells failed to produce

any significant amounts of effector molecules (Figure 9C) or lytic activity (Figure 9D) over the background levels in the naive mice, indicating that type I IFNs was critical for NK cell activation in response to VV infection. We further observed that IFNα/βR-/- mice and WT mice depleted of NK cells displayed similar levels of viral titer that was significantly ($p < 0.001$) elevated compared with the WT mice (Figure 10C). Collectively, our data support the conclusion that type I IFN-dependent innate immune control of VV infection in vivo is mediated through regulating NK cell function.

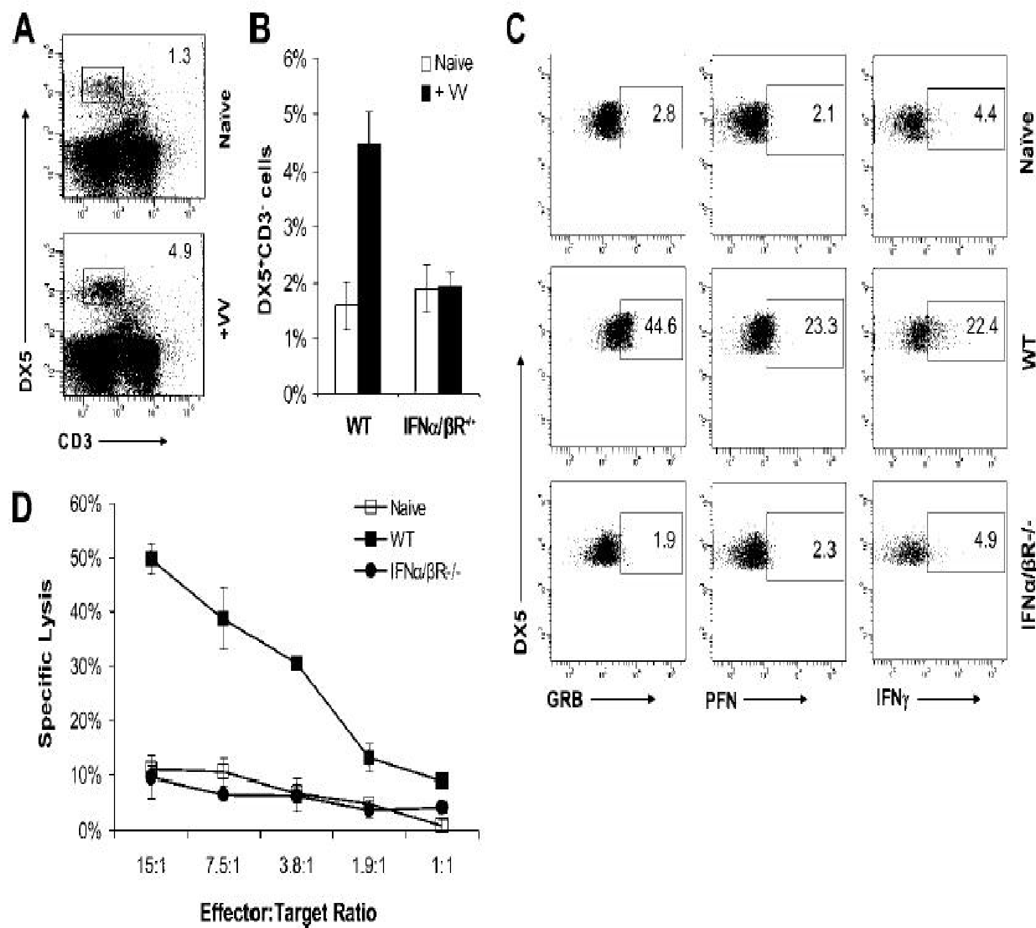

Figure 9: Type I IFN signaling is required for NK cell activation in response to VV infection in vivo. Mice were infected with VV (+VV) or left uninfected (Naive). Splenocytes were harvested 48 hr later and stained for NK cells with anti-DX5 and anti-CD3 Abs. (A) The percentage of NK ($DX5^+CD3^-$) cells among total lymphocytes from WT mice is indicated. (B) The mean percentage ± SD of NK ($DX5^+CD3^-$) cells among total lymphocytes from WT and IFNα/βR-/- mice is indicated (n=4). (C) Splenocytes from WT and IFNα/βR-/- mice were assayed for intracellular granzyme B (GRB), perforin (PFN), and IFNγ production by NK cells. The percentage of GRB-, PFN-, or IFNγ-positive cells among $DX5^+CD3^-$ cells is indicated. (D) Splenocytes were assayed for NK lytic activity on YAC-1 cells for 4 hr at different effector:target ratios. Naive splenocytes (Naive) were used as a control. The mean percentage ±SD of specific lysis is indicated (n=5). Data shown are representative of three independent experiments.

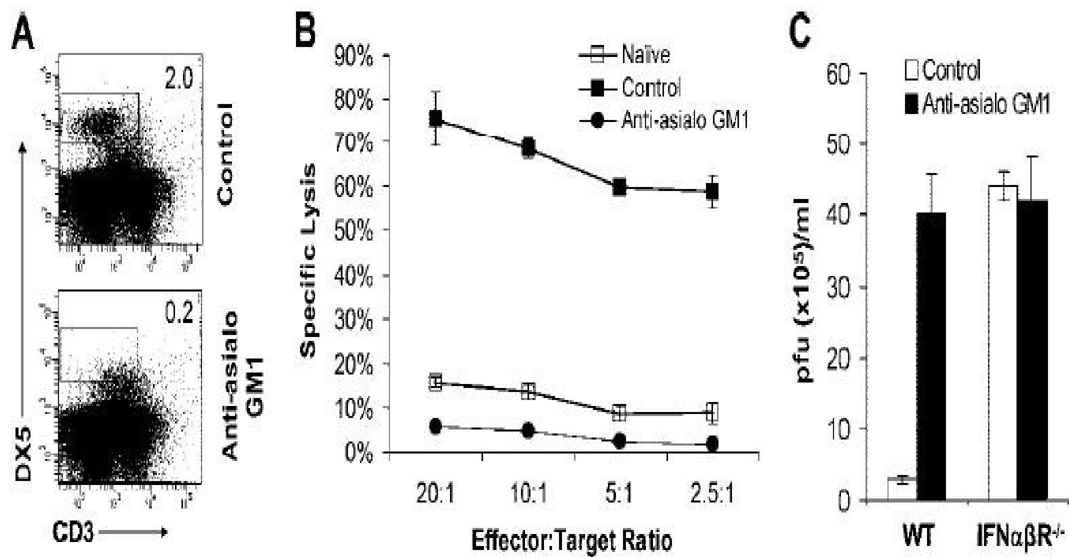

Figure 10: NK cells are required for efficient clearance of VV in vivo. (A) Mice were treated with 250 µg of anti-asialo GM1 Ab on days 0 and 3 (anti-asialo GM1) or left untreated (Control). On day 5, splenocytes were stained with anti-DX5 and anti-CD3 Abs. The percentage of $DX5^{+}CD3^{-}$ cells among total lymphocytes is indicated. (B) Mice were depleted of NK cells on days 0 and 3 (anti-asialo GM1) or left untreated (Control), and infected with VV. Forty-eight hours later, splenocytes were assayed for NK lytic activity on YAC-1 cells for 4 hr at different effector:target ratios. Naive splenocytes (Naive) were used for comparison. The mean percentage ±SD of specific lysis is indicated (n=3). (C) Female WT or IFNα/βR-/- mice were depleted of NK cells on days 0 and 3 (anti-asialo GM1) or left untreated (Control), followed by VV infection. After 48 hr, their ovaries were harvested for measurement of viral load. Data represents viral titer ±SD as pfu per milliliter of cell lysate (n=3). Data shown is representative of three independent experiments.

4.2.3 Activation of NK cells upon VV infection in vitro is dependent on type I IFN signaling through NK cells

We next investigated how type I IFNs regulated the activation of NK cells upon VV infection. Conventional $CD11c^+$ DCs have been shown to play a critical role in NK cell activation [21, 113]. A recent report has suggested that type I IFN signaling through DCs may be important in the activation of NK cells in response to stimulation with various TLRs [113]. To address whether the same is true in NK cell response to VV infection, we used an in vitro NK:DC co-culture system. Purified $DX5^+CD3^-$ NK cells were co-cultured in vitro with conventional $CD11c^+$ DCs generated from bone marrow cells in the presence of GM-CSF, followed by infection with VV. Forty-eight hours after infection, NK cells produced much higher amounts of granzyme B, perforin, and IFNγ compared with the uninfected control (Figure 11). Because infection of DCs with VV in vitro also elicits type I IFN production [22], we next examined whether type I IFNs acted on DCs or NK cells for NK cell activation in response to VV infection. To address this question, purified WT or IFNα/βR-/- NK cells were co-cultured with WT or IFNα/βR-/- DCs, followed by infection with VV. The activation of NK cells was analyzed 48 hr after infection. Our data showed that IFNα/βR-/- DCs elicited similar amounts of granzyme B (Figure 12, A and B), perforin (Figure 12C), and IFNγ (Figure 12D) production by NK cells compared with the WT counterparts, suggesting NK cell activation is independent of type I IFN signaling on DCs in response to VV infection. In contrast, NK cell activation was severely compromised when IFNα/βR-/- NK cells were used for

stimulation (Figure 12), indicating that direct action of type I IFNs on NK cells is required for their activation upon VV infection.

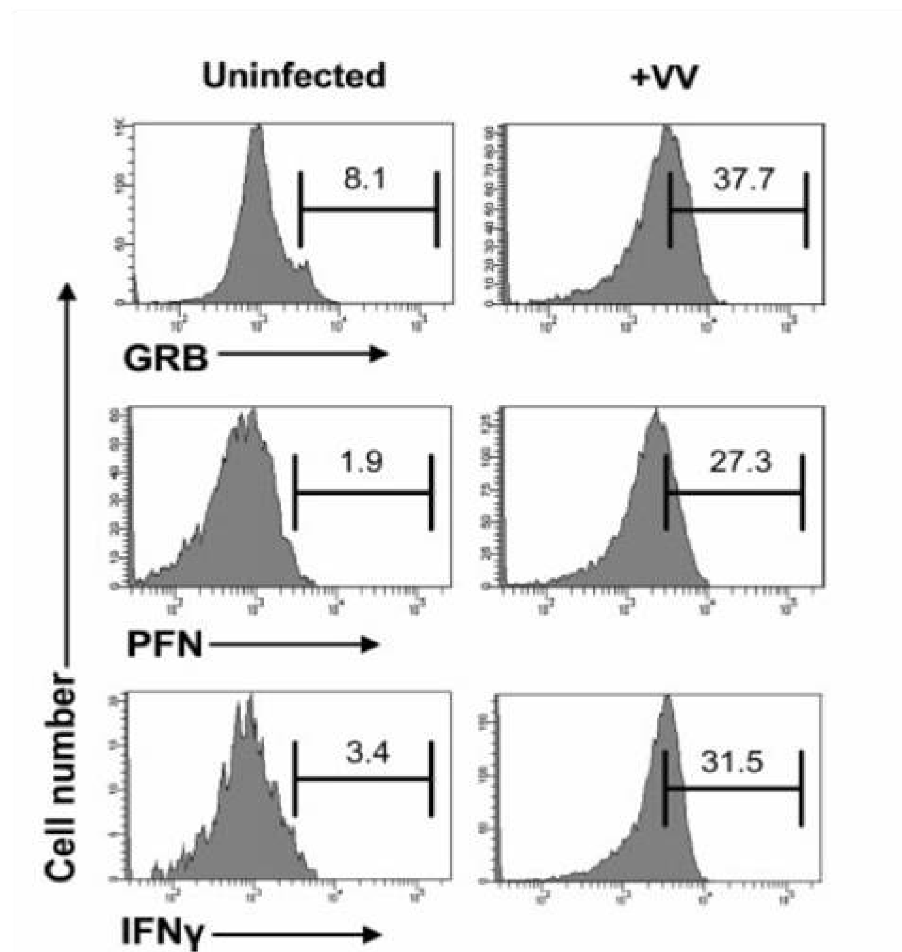

Figure 11: Activation of NK cells by DCs upon VV infection in vitro. Purified $DX5^+CD3^-$ NK cells were co-cultured with $CD11c^+$ DCs and infected with VV (+VV) or left uninfected (Uninfected). NK cells were assayed for intracellular granzyme B (GRB), perforin (PFN), and IFNγ 48 hr later. The percentage of GRB-, PFN-, or IFNγ- positive cells among $DX5^+CD3^-$ cells is indicated. Data shown are representative of three independent experiments (n=3).

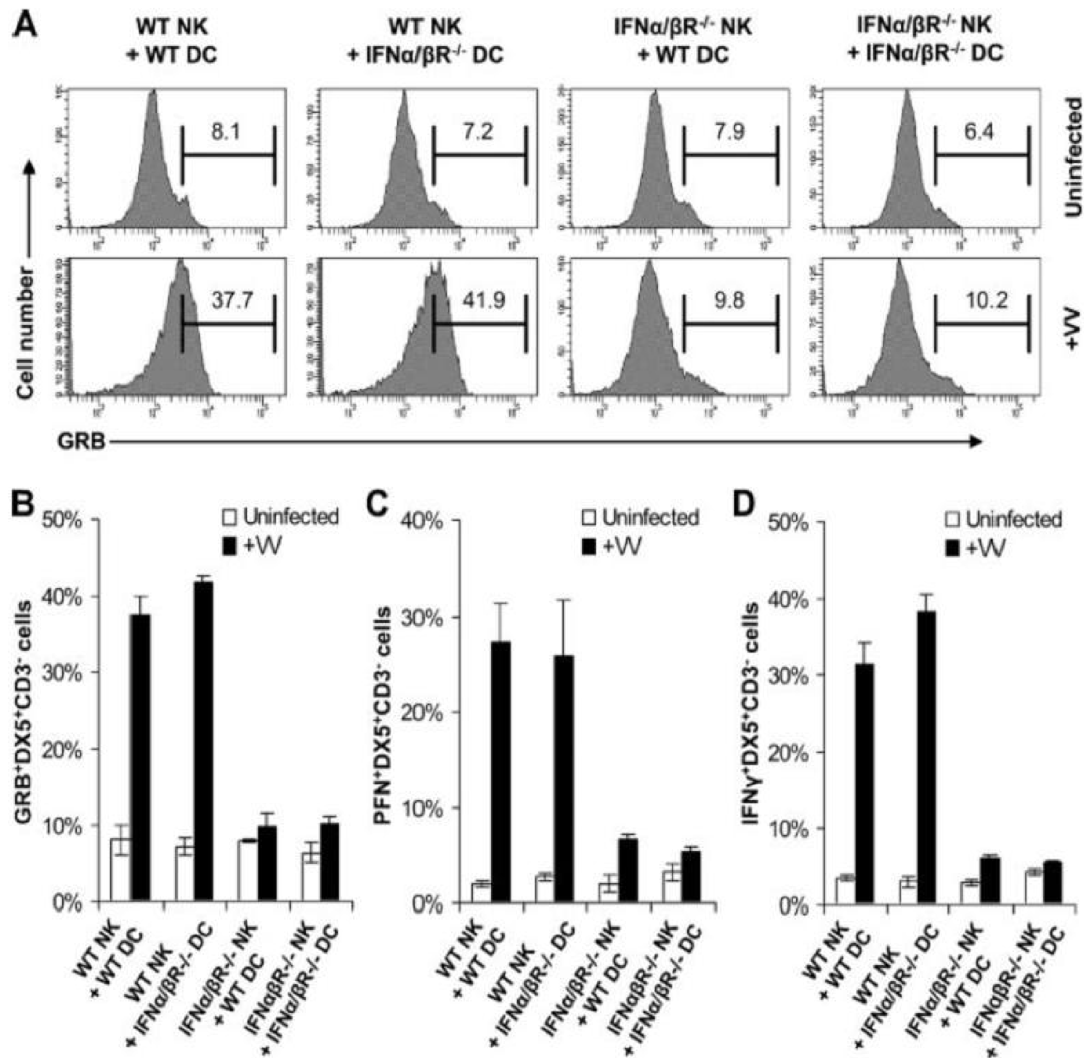

Figure 12: NK cell activation upon VV is dependent on type I IFN signaling on NK cells in vitro. WT or IFNα/βR-/- $DX5^+CD3^-$ NK cells were co-cultured with WT or IFNα/βR-/- $CD11c^+$ DCs and infected with VV (+VV) or left uninfected (Uninfected). Forty-eight hours after infection, NK cells were assayed for intracellular granzyme B (GRB), perforin (PFN), and IFNγ. (A) The FACS plots of GRB is shown with the percentage of GRB positive cells among $DX5^+CD3^-$ cells indicated. (B-D) The percentage ±SD of GRB- (B), PFN- (C), or IFNγ- (D) positive cells among $DX5^+$ $CD3^-$ cells is shown (n=4). Data shown are representative of two independent experiments.

4.2.4 Direct action of type I IFN on NK cells is required for their activation upon VV infection in vivo

We next sought to examine whether direct type I IFN signaling on NK cells was also required for NK cell activation upon VV infection in vivo. We have previously shown that IFNα/βR-/- mice produce large amounts of type I IFN upon VV infection [22]. Thus, if type I IFNs act directly on NK cells for their activation in vivo, adoptive transfer of WT NK cells into IFNα/βR-/- mice should restore NK cell activation and result in a significant reduction of viral load. To address this question, $DX5^{+}CD3^{-}$ NK cells were purified from the spleens of WT or IFNα/βR-/- 129s/v mice by FACS sorting. Two x 10^5 WT or IFNα/βR-/- NK cells were transferred into IFNα/βR-/- mice i.v., which were subsequently infected i.p. with 1 x 10^7 pfu VV. After 48 hr, the spleens and ovaries from these recipient mice were analyzed for NK cell activation and viral titer. In IFNα/βR-/- mice reconstituted with WT NK cells, the production of granzyme B, perforin, and IFNγ by splenic NK cells neared that in WT mice (data not shown). Furthermore, splenic NK cells harvested from IFNα/βR-/- mice reconstituted with WT, but not IFNα/βR-/-, NK cells were capable of lysing YAC-1 targets to a level equivalent to that of WT mice (Figure 13A). These data indicated that WT NK cells were functionally activated in otherwise IFNα/βR-/- mice. When VV titer in the ovaries was assessed, IFNα/βR-/- mice reconstituted with WT NK cells were able to clear VV in vivo similarly to WT mice, whereas IFNα/βR-/- mice or IFNα/βR-/- mice reconstituted with IFNα/βR-/- NK cells failed to clear the virus (Figure 13B). Taken together, these data

support the conclusion that direct action of type I IFN on NK cells is required for activation of NK cells in response to VV infection in vivo.

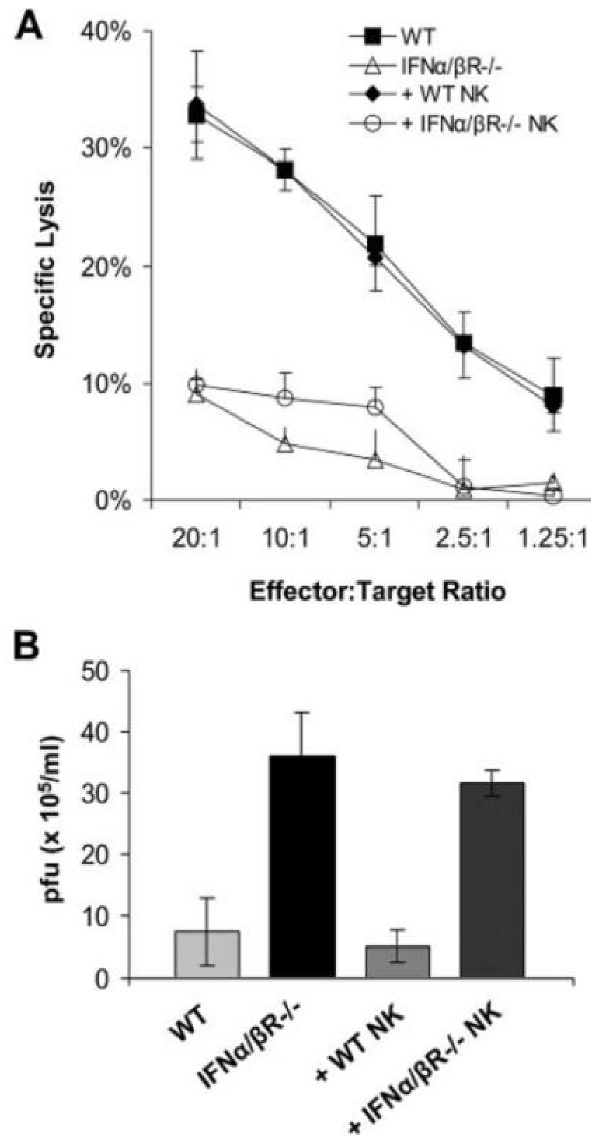

Figure 13: Type I IFNs act directly on NK cells for their activation in response to VV infection in vivo. IFNα/βR-/- mice were reconstituted with WT NK cells (+ WT NK) or IFNα/βR-/- NK cells (+IFNα/βR-/- NK) followed by infection with VV. WT and IFNα/βR-/- mice infected with VV were used as controls. (A) Forty-eight hours later, splenocytes were assayed for NK lytic activity on YAC-1 cells at different effector:target ratios. The mean percentage ±SD of specific lysis is indicated (n=2). (B) The ovaries of female mice were harvested for measurement of viral load. Data represents viral titer ±SD as pfu per milliliter of cell lysate (n=2). Data are representative of two independent experiments.

4.3 Discussion

In this study, we have presented evidence that type I IFNs do not protect L929 cells directly from VV infection in vitro. In vivo, type I IFN-dependent innate immune clearance of VV infection is mediated by regulating NK cell activation. We have further shown that type I IFNs act directly on NK cells for their activation and effector function in response to VV infection both in vitro and in vivo. The observation that type I IFNs did not directly protect L929 cells against VV infection in vitro may not rule out completely the possibility of a direct role for type I IFNs in anti-viral defense in vivo. However, our data that mice defective for type I IFN signaling and those depleted of NK cells showed similar levels of elevated viral titer, and that WT NK cells were sufficient to clear VV infection in IFNα/βR-/- mice supports our conclusion that type I IFN-dependent NK cell activation is mainly responsible for innate immune defense against VV infection in vivo. This is in contrast to a previous report that IFN-mediated prophylaxis against VV or murine cytomegalovirus (MCMV) infections is NK cell independent [106, 116]. What contributes to the differences is not entirely clear, but might be related to the timing (before vs. during the infection), the dose, and/or the source (exogenously administered vs. endogenously induced upon VV infection) of type I IFNs.

Previous studies have demonstrated a critical role of NK cells in innate immune defense against viral infection [106]. It has been shown that NK cell activation upon MCMV infection is mediated by NK cell activation receptor, Ly49H, which specifically recognizes the m157 gene product of MCMV [106, 117]. Furthermore, a recent report

has suggested a role of NKG2D-activating receptor in NK cell activation in response to MCMV infection [21]. Consistent with previous observations [101, 107-109], we showed in this study that NK cells are activated upon VV infection, which is critical for innate immune defense against VV infection in vivo. How VV activates NK cells remains to be defined. Thus, it will be important to identify what component of VV is responsible for NK cell activation and the corresponding NK cell activation receptor. Identification of these will help in the design of effective NK cell-based strategies to control poxviral infections in vivo.

In addition to direct stimulation through NK cell receptors, the activation of NK cells is also regulated by cytokines, particularly type I IFNs [112]. It has been shown that type I IFNs directly enhance NK cell cytotoxicity and induces IL-15 to promote NK cell proliferation during MCMV infection [67]. We showed in this study that NK cell activation in response to VV infection is also critically dependent on type I IFNs. We further demonstrated that this is achieved by direct action of type I IFNs on NK cells, but not on accessory DCs. Our observation is in contrast to a recent report that type I IFN signaling on DCs may be important in the activation of NK cells in response to stimulation with various synthetic TLR ligands [113]. The reasons for the discrepancy are not clear, but could be related to the agents (live pathogen vs. synthetic TLR ligands) used for in vitro and in vivo NK cell activation. Indeed, NK activation upon viral infections is a more complex process, which involves pathogen-derived gene products, a NKG2D-activating receptor in addition to cytokines, such as type I IFNs [106]. In

addition, the secretion of other cytokines upon stimulation with a TLR ligand vs. VV would be quite different, which could influence the dependency of DCs vs. NK cells on type I IFN signals for NK cell activation.

In summary, we have demonstrated that type I IFN-dependent innate immune control of VV infection in vivo is mediated through activation of NK cells. We have further shown that direct action of type I IFNs on NK cells is required for their activation and function upon VV infection. These results may suggest potential strategies for the control of poxviral infections in vivo.

Chapter 5: Direct TLR2 Signaling Is Critical for NK Cell Activation and Function in Response to Vaccinia Viral Infection

The following text was slightly modified from its original publication, "Direct TLR2 Signaling is Critical for NK Cell Activation and Function in Response to Vaccinia Viral Infection," accepted for publication on January 14, 2010 at Public Library of Science, Pathogens (PLoS Pathogens).

5.1 Introduction

Vaccinia virus (VV) is a member of the *Orthopoxvirus* genus of the Poxviridae family, including smallpox (variola) virus, monkeypox virus, cowpox virus, and mousepox (ectromelia) virus. It has a large and complex, double-stranded DNA genome, measuring about 200 Kb, that encodes most of the genes required for cytoplasmic replication of the virus [118]. VV is the most studied member of the poxvirus family and is the live vaccine responsible for successful elimination of smallpox in the late 1970s [105]. This triumph is now being threatened by bioterrorists deliberately reintroducing smallpox, against which vaccination is no longer routine [78-79, 119]. Thus, widespread public vaccination is being considered to counter this potential threat. However, the currently used live VV vaccine is associated with a relatively high incidence of severe adverse events, particularly in individuals with eczema and immunodeficiency [81-83, 120]. Therefore, there is an imminent need to explore new

and safe approaches to control, not only the actual smallpox infection, but also the potential complications from smallpox vaccination with the live VV.

Critical for the development of novel strategies is a better understanding of the host's defense mechanism(s) against poxviruses in vivo. Recent advances have shown that recovery from viral infections depends on the host's ability to mount effective innate immune responses. NK cells represent an important component of the innate immune system and play a critical role in innate immune defense against various viral infections in vivo [106, 121]. Clinically, individuals who are NK cell-deficient suffer from severe, recurrent viral infections [122]. NK cells are also crucial in the control of poxviruses. Upon poxviral infection, NK cells are activated, expand, and accumulate at the site of infection, and these activated NK cells are important for the clearance of the infection [85, 101, 109, 123]. Activation of NK cells is tightly controlled by both inhibitory and activating receptors [44]. Previous studies have shown that upon murine CMV (MCMV) infection, NK cell activation is mediated by the NK cell activating receptor, Ly49H, which specifically recognizes the m157 gene product of MCMV expressed on the surface of infected cells [117, 124]. Similarly, recognition of influenza virus hemagglutinin on virus-infected cells by another activating receptor, NKp46, activates lysis by human NK cells [125], and the murine NKp46 equivalent, NCR1, is required for protection against lethal influenza infection [126]. In addition, the NKG2D activating receptor has been shown to recognize host stress proteins induced upon viral infections, including human CMV and MCMV infections [21, 127].

How NK cells are activated upon poxviral infection remains poorly understood. It is known that Ly49H is not involved in the control of VV in mice [107, 128]. Studies in vitro have shown that recognition of VV-infected cells by human NK cells is, in part, mediated by NKp30, NKp44, and NKp46, but not NKG2D [108]. On the other hand, recent studies have suggested that NKG2D is partially involved in NK cell-mediated control of mousepox virus [49]. Thus, mechanisms underlying NK cell responses upon poxviral infection remain largely undefined. In a murine model of VV infection, we have previously shown that VV activates the innate immune system through both the Toll-like receptor (TLR)-dependent and -independent pathways [129]. The TLR pathway is mediated by TLR2 and dependent on MyD88, leading to production of pro-inflammatory cytokines, IL6, IL1, and IL12, whereas activation of the TLR-independent pathway results in the secretion of type I IFNs. More importantly, pro-inflammatory cytokines and type I IFNs are required for innate immune control of VV infection [22]. We have shown that a critical role in innate immune control of VV is mediated by type I IFNs, which acts directly on NK cells to regulate their activation and function [85]. However, how the TLR2-MyD88 pathway contributes to innate immune control of VV remains unclear.

In this report, we provided evidence that the TLR2-MyD88 pathway is also critical for NK cell activation and function in response to VV infection in vivo. This was independent of TLR2-induced production of pro-inflammatory cytokines. We showed that direct TLR2 signaling on NK cells was necessary for efficient NK cell activation

upon stimulation with VV and played a critical role in VV control in vivo. In addition, we showed that the NKG2D pathway was also required for efficient NK activation and function, as well as recognition of VV-infected targets. Furthermore, we demonstrated that that VV could directly activate NK cells via TLR2 in the presence of cytokines in vitro and TLR2-dependent activation of NK cells by VV was mediated through the MyD88-PI3K-ERK pathway. Collectively, these results suggest that efficient NK cell activation depends on multiple pathways in response to VV infection and that direct TLR2 activation on NK cells is critical for their function and could lead to the development of novel strategies to combat poxviral infection.

5.2 Results

5.2.1 NK cell activation and function upon VV infection depends on the TLR2-MyD88 pathway

Using depleting antibodies to asialo GM1, but not NK1.1, previous studies have shown that NK cells are critical for the control of VV infection in vivo [85, 101, 109, 123]. Anti-asialo GM1 depletes NK cells and some T cells, but not NKT cells, whereas anti-NK1.1 depletes NK and NKT cells, but not T cells. Thus, to further confirm the role of NK cells in VV control in vivo, we depleted mice of NK cells with anti-NK1.1 antibodies (PK136), followed by infection with VV intraperitoneally. Here we showed that mice depleted of NK cells with anti-NK1.1 also had a defect in NK cell lytic activity (Figure 14A) and a significantly ($p < 0.001$) higher viral titer than the control mice (Figure 14B).

We next investigated how the TLR2-MyD88 pathway contributed to innate immune control of VV. We hypothesized that TLR2-dependent control of VV infection was also mediated through the regulation of NK cell activation. To test this hypothesis, WT, TLR2-deficient (TLR2-/-), or MyD88-/- mice were infected with VV intraperitoneally, and splenic NK cells were analyzed for their activation and function. We first showed that splenic NK cell numbers (Figure 15A) and phenotypic markers (Figure 16) from naïve TLR2-/- or MyD88-/- mice were similar to those from WT mice. Forty-eight hours after infection, at which time splenic NK cell activation peaked upon VV infection as shown previously [85], splenic NK cells from WT mice produced significantly ($p < 0.001$) higher amounts of effector molecules such as IFNγ and granzyme B, compared to the uninfected naïve control (Figure 15B, C), indicating that these NK cells are activated upon VV infection in vivo. In addition, these NK cells were functionally active, as they demonstrated lytic activities with NK-sensitive YAC-1 cells or VV-infected L929 cells (Figure 15D). In contrast, the production of IFNγ and granzyme B by splenic NK cells from TLR2-/- or MyD88-/- mice was significantly ($p < 0.001$) reduced, compared to the WT controls (Figure 15B, C). Furthermore, splenic NK cells from VV-infected TLR2-/- or MyD88-/- mice displayed drastically diminished lytic activities (Figure 15D), leading to a significant ($p < 0.001$) increase in viral load in the ovary (Figure 15E). Collectively, these results indicate that NK cell activation and their effector function in response to VV infection is critically dependent on the TLR2-

MyD88 pathway in vivo. However, the expansion of NK cells upon VV infection appeared unchanged in the absence of TLR2 signaling (Figure 15A).

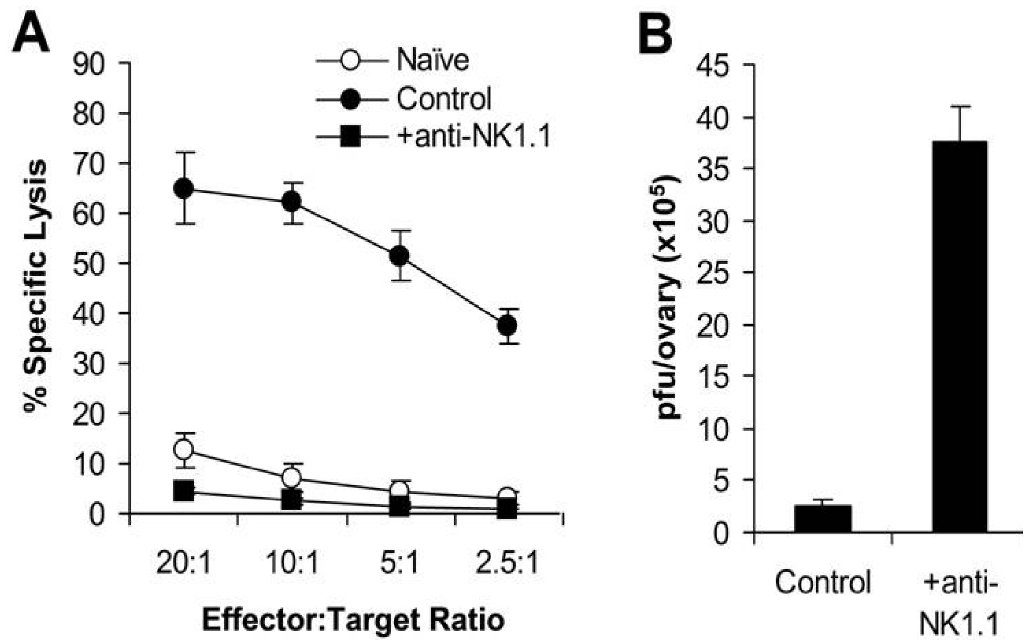

Figure 14: NK cells are required for the control of VV infection in vivo. Female C57BL/6 mice were depleted of NK cells with anti-NK1.1 antibodies on days -3 and 0 (+anti-NK1.1) or left untreated (Control), followed by infection with VV on day 0. (A) Forty-eight hours after infection, splenocytes were assayed for NK lytic activity on YAC-1 cells for 4 hr at different effector:target ratios. Naïve splenocytes (Naïve) were used for comparison. The percentage of specific lysis is shown (n=3). (B) The ovaries were assayed for viral load. Data represents viral titer ± SD as pfu per ovary (n=3).

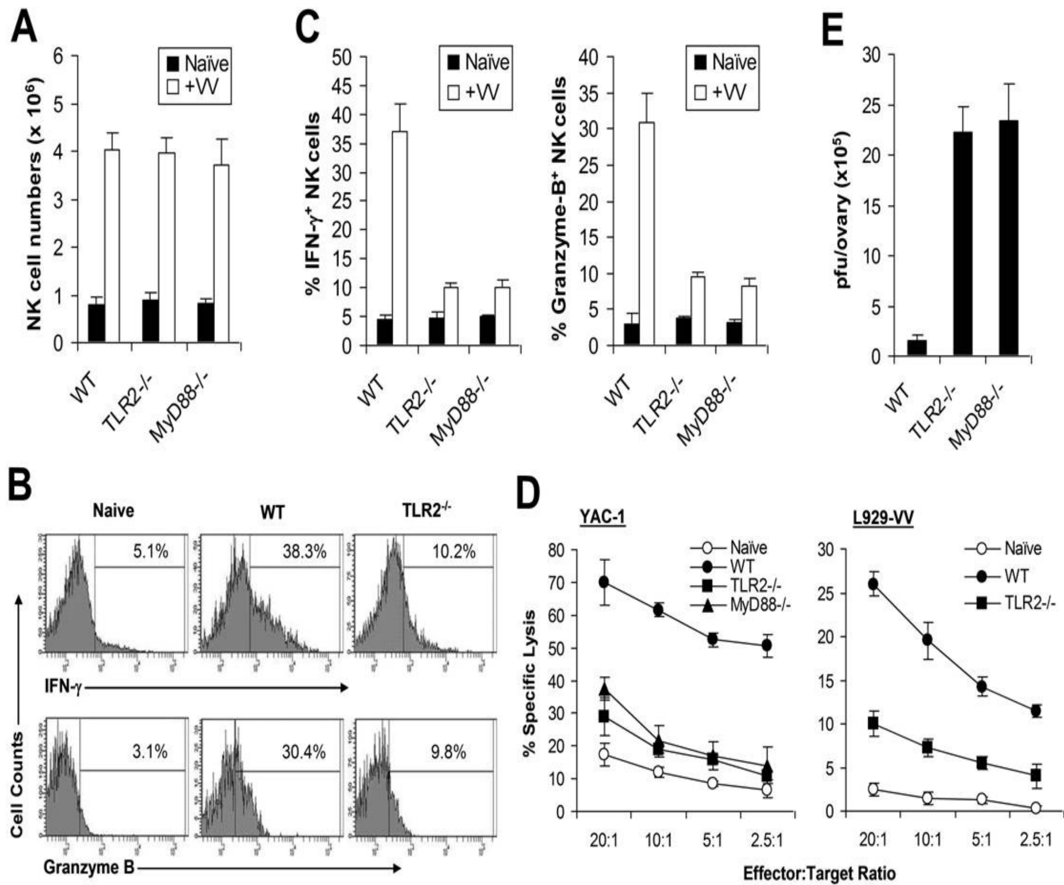

Figure 15: NK cell activation and function in response to VV in vivo requires an intact TLR2-MyD88 pathway. (A-C) Wild-type (WT), TLR2-/-, or MyD88-/- mice were infected with VV (+VV) or left uninfected (Naïve). Forty-eight hours later, splenocytes were assayed for total NK cell numbers, intracellular IFNγ and granzyme B production by NK cells, as well as NK cell lytic assay. (A) The mean numbers ± SD of total $DX5^{+}CD3^{-}$ NK cells per spleen are indicated (n = 6 per group). (B) FACS plots of intracellular IFNγ and granzyme B production by NK cells with the percentage of IFNγ- or granzyme B-positive cells among $DX5^{+}CD3^{-}$ NK cells indicated. (C) The mean percentage ±SD of IFNγ- or granzyme B-positive cells among $DX5^{+}CD3^{-}$ cells is indicated (n = 6 per group). (D) Forty-eight hours after infection, splenocytes were harvested and NK cell lytic activity was assayed on YAC-1 cells or VV-infected L929 cells (L929-VV) for 4 hr at different effector:target ratios. Naïve splenocytes (Naïve) were used as a control. The mean percentage ±SD of specific lysis is indicated (n = 6 per group). (E) The ovaries of female mice were harvested for measurement of viral load. Data represents the mean viral titer ±SD as plaque-forming units (pfu) per ovary (n = 6 per group). Data shown is representative of three independent experiments.

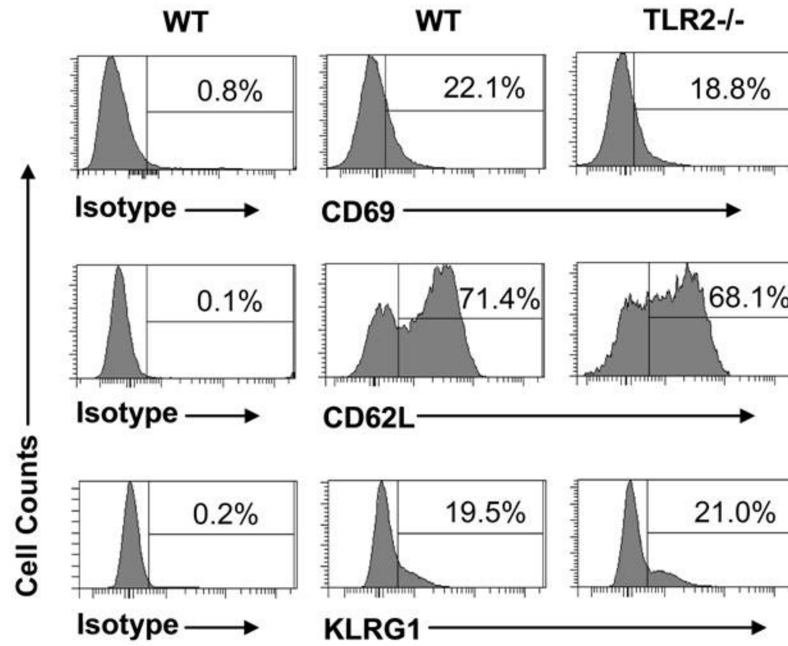

Figure 16: Phenotypic analysis of WT and TLR2-/- NK cells. $DX5^+CD3^-$ NK cells from WT or TLR2-/- mice were stained with anti-CD69, anti-CD62L, or anti-KLRG1, as well as their corresponding isotype controls, and subjected to FACS analysis. The percentages of CD69-, CD62L- and KLRG1-postive NK cells among $DX5^+CD3^-$ NK cells are indicated (n=3).

5.2.2 NK cell activation is independent of TLR2-induced pro-inflammatory cytokines

What then is responsible for TLR2-dependent NK cell activation and function in response to VV infection in vivo? We have previously shown that activation of TLR2 on DCs and macrophages by VV leads to the production of pro-inflammatory cytokines, including IL6, IL1, and IL12 [22]. Since IL12 and IL1 have been implicated in regulating NK cell activation and function in other models of viral infection [106, 121], we investigated whether the TLR2-dependent NK cell response to VV infection was due to TLR2-induced secretion of pro-inflammatory cytokines. WT, IL1 receptor-deficient (IL1R-/-), IL12-/-, or IL6-/- mice were infected with VV intraperitoneally, and NK cells were analyzed 48 hr later. No significant differences ($p > 0.05$) were observed in the production of IFNγ or granzyme B by splenic NK cells from IL1R-/-, IL12-/-, or IL6-/- mice, compared to the WT controls (Figure 17A). In addition, splenic NK cells from IL1R-/-, IL12-/-, or IL6-/- mice displayed similar levels of lytic activities on YAC-1 targets compared to WT mice (Figure 17B), and no differences in viral load were observed among all mice (data not shown). These results suggest that TLR2-induced IL12, IL1, and IL6 are not critical for NK cell activation and effector function in response to VV infection in vivo.

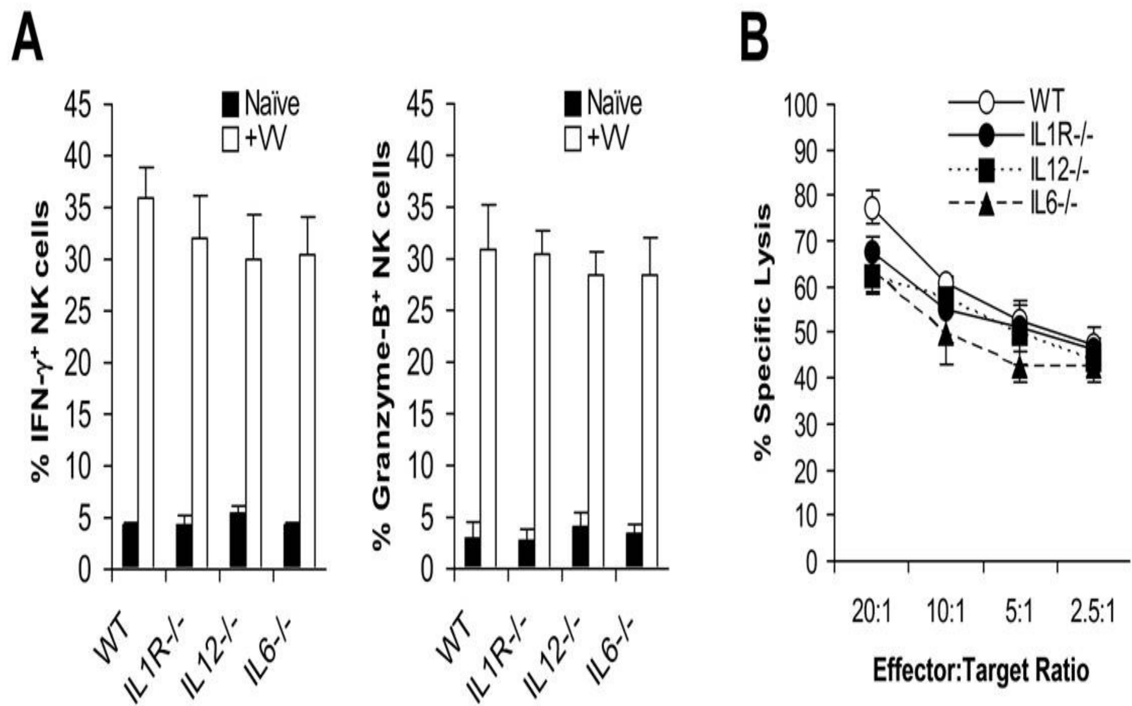

Figure 17: NK cell activation upon VV infection is independent of TLR2-induced production of pro-inflammatory cytokines. (A) WT, IL1R-/-, IL12-/-, and IL6-/- mice were infected with VV (+VV) or left uninfected (Naïve), and splenocytes were assayed for intracellular IFNγ and granzyme B production by NK cells 48 hr later. The percentage ±SD of IFNγ- or granzyme B-positive cells among $DX5^{+}CD3^{-}$ cells is indicated (n = 6). (B) Splenocytes were harvested 48 hr after infection with VV, and NK lytic activity was assayed on YAC-1 cells for 4 hr at different effector:target ratios. The mean percentage ±SD of specific lysis is indicated (n = 6). Data shown is representative of three independent experiments.

5.2.3 TLR2 signaling on NK cells, but not on DC, is required for NK cell activation

The observation that NK cell activation and function upon VV infection in vivo is independent of TLR2-induced IL1, IL6, and IL12 production may not completely rule out the role of TLR2 signaling on accessory cells, such as DCs, in NK cell activation, as VV could stimulate TLR2 on DCs to secrete other cytokines or activate other pathways that may be critical for NK cell activation. To address this question, we utilized an in vitro NK:DC co-culture system as described [85]. $DX5^{+}CD3^{-}$ splenic NK cells were isolated by FACS sorting with a purity of >98% (Figure 18). Purified WT or TLR2-/- NK cells were co-cultured in vitro with WT or TLR2-/- $CD11c^{+}$ DCs, followed by infection with VV. Forty-eight hours later, NK cells were analyzed for the production of IFNγ and granzyme B. Our data showed that similar amounts of IFNγ and granzyme B were produced by WT NK cells when cultured with TLR2-/- DCs to those with WT DCs (Figure 19A, B). In addition, WT NK cells co-cultured with TLR2-/- DCs displayed similar levels of lytic activity on YAC-1 targets to those with WT DCs (data not shown). These results suggest NK cell activation is independent of TLR2 signaling on DCs in response to VV infection. In contrast, NK cell activation was severely compromised when TLR2-/- NK cells were used for stimulation (Figure 19A, B), indicating that direct TLR2 signaling on NK cells is required for their activation upon VV infection. The lack of IFNγ and granzyme B production by TLR2-/- NK cells was not due to their inherent inability to be activated as TLR2-/- NK cells stimulated with TLR4 ligand, LPS,

produced similar levels of IFNγ and granzyme B compared to the WT NK cells (Figure 19B). Taken together, these results suggest that TLR2 signaling on NK cells, but not accessory DCs, is required for the activation of NK cells by VV.

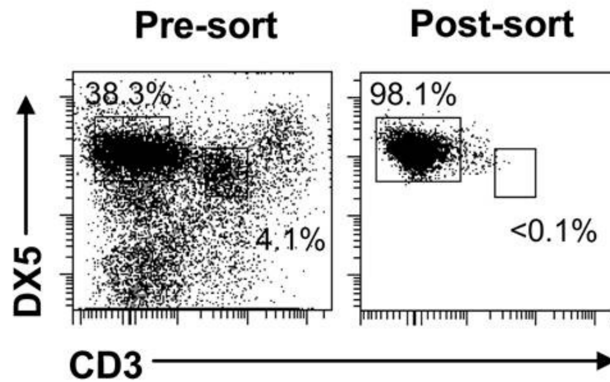

Figure 18: Purity of sorted NK cells. Splenic NK cells were first enriched using anti-DX5-PE and PE-microbeads. The $DX5^+$ cells were then stained with anti-CD3-FITC and subjected to FACS sorting gated on the $DX5^+CD3^-$ population. The percentages of $DX5^+CD3^-$ vs. $DX5^+CD3^+$ populations before (Pre-sort) and after (Post-sort) sorting are indicated (n=3).

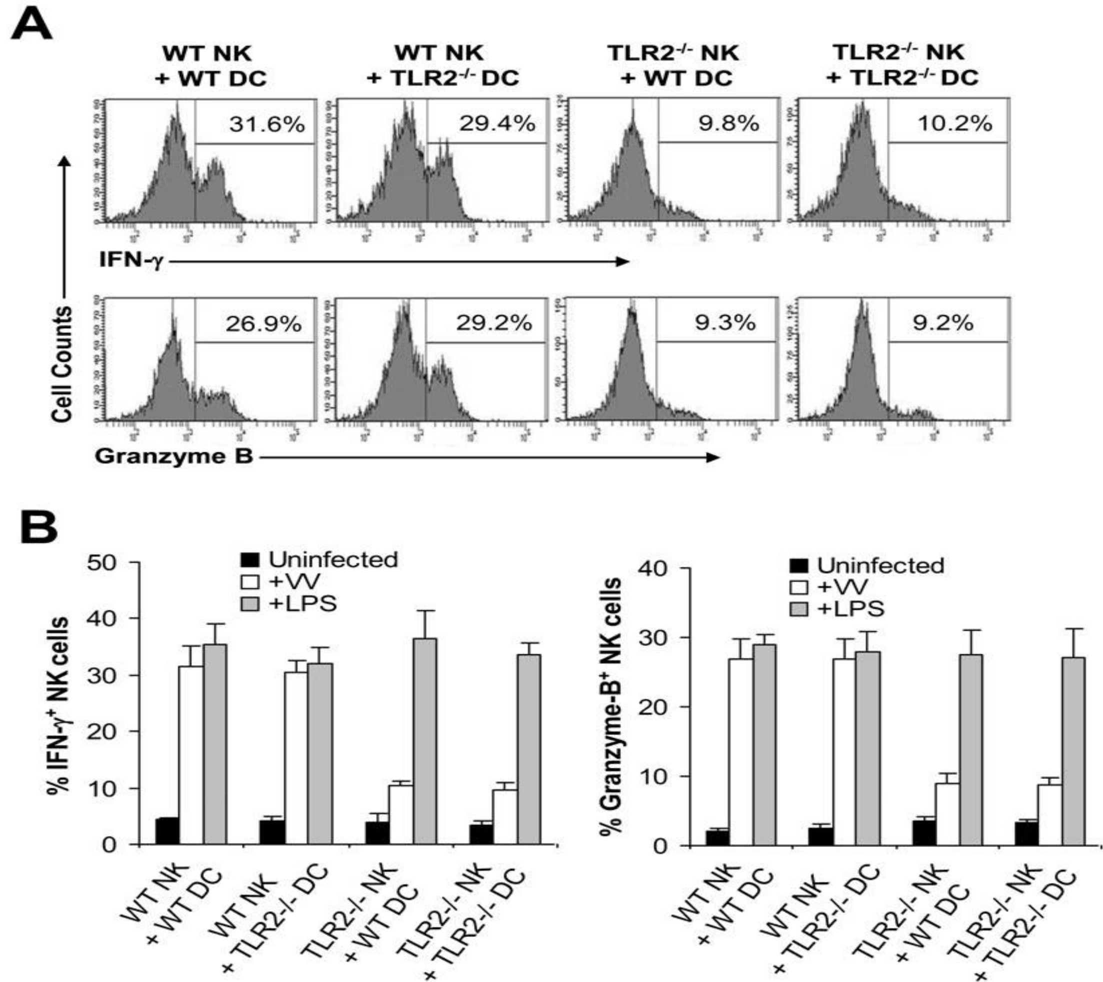

Figure 19: TLR2 signaling on NK cells, but not on DCs, is required for NK cell activation to VV in vitro. WT or TLR2-/- $DX5^+CD3^-$ NK cells were co-cultured with WT or TLR2-/- $CD11c^+$ DCs and stimulated with VV (+VV), LPS (+LPS), or left uninfected (Uninfected). Forty-eight hours after infection, NK cells were assayed for intracellular IFNγ and Granzyme B. (A) The percentage of IFNγ- or granzyme B-positive cells among $DX5^+CD3^-$ cells is indicated. (B) The mean percentage ±SD of IFNγ- or granzyme B-positive cells among $DX5^+CD3^-$ cells is shown (n=4). Data shown is representative of two independent experiments.

5.2.4 Direct TLR2-MyD88 signaling is required for NK cell activation and function in response to VV infection in vivo

We next investigated the in vivo relevance of direct TLR2 signaling on NK cell activation and the control of VV infection in vivo. We first evaluated if TLR2-MyD88 signaling on NK cells was critical for NK cell activation in mixed TLR2-/- or MyD88-/-, and WT bone marrow chimeric mice. $CD45.1^+$ recipient mice were irradiated with 1200 cGy and reconstituted with equal numbers of bone marrow cells harvested from $CD45.1^+$ WT and $CD45.2^+$ TLR2-/- (or MyD88-/-) mice. Mice were allowed to reconstitute their hematopoietic cell populations for 6 to 8 weeks. Mixed chimeric mice were then infected with VV intraperitoneally, or left uninfected as controls. Forty-eight hours later, splenocytes were analyzed for activation of WT ($CD45.1^+$), and TLR2-/- or MyD88-/- ($CD45.2^+$) $DX5^+CD3^-$ NK cells. In addition, WT and TLR2-/- (or MyD88-/-) NK cells were purified by FACS and assayed for cytotoxicity on NK-sensitive YAC-1 cells. WT NK cells from VV-infected recipients produced significantly (p <0.001) higher amounts of IFNγ and granzyme B, compared to the uninfected control (Figure 20A, B). However, the production of IFNγ and granzyme B by TLR2-/- (Figure 20A) or MyD88-/- (Figure 20B) NK cells was significantly (p <0.001) reduced compared to the respective WT controls. In addition, TLR2-/- (Figure 20C) or MyD88-/- (Figure 20D) NK cells showed drastically diminished lytic activity compared to the WT NK cells. These results suggest that direct TLR2-MyD88 signaling on NK cells is critical for NK cell activation and their function in vivo.

To further support the role of direct TLR2 signaling on NK cell activation and function, we examined if transfer of WT NK cells into TLR2-/- mice restored NK cell function and resulted in a significant reduction of viral load. $DX5^{+}CD3^{-}$ NK cells were purified from the spleens of WT or TLR2-/- mice by FACS sorting. Purified WT or TLR2-/- NK cells were then transferred into TLR2-/- mice intravenously, which were subsequently infected with VV intraperitoneally. After 48 hr, the spleens and ovaries from these recipient mice were analyzed for NK cell activation and viral titer. In TLR2-/- mice reconstituted with WT NK cells, the production of IFNγ and granzyme B by splenic NK cells neared that in WT mice (data not shown). Furthermore, splenic NK cells harvested from $TLR2^{-/-}$ mice reconstituted with WT, but not TLR2-/-, NK cells were capable of lysing YAC-1 targets to a level equivalent to that of WT mice (Figure 20E). When VV titer in the ovaries was assessed, TLR2-/- mice reconstituted with WT NK cells were able to clear VV in vivo similarly to WT mice, whereas TLR2-/- mice or TLR2-/- mice reconstituted with TLR2-/- NK cells failed to clear the virus (Figure 20F). Taken together, these data support the conclusion that intrinsic TLR2-MyD88 signaling on NK cells is required for NK cell activation and function in response to VV infection in vivo.

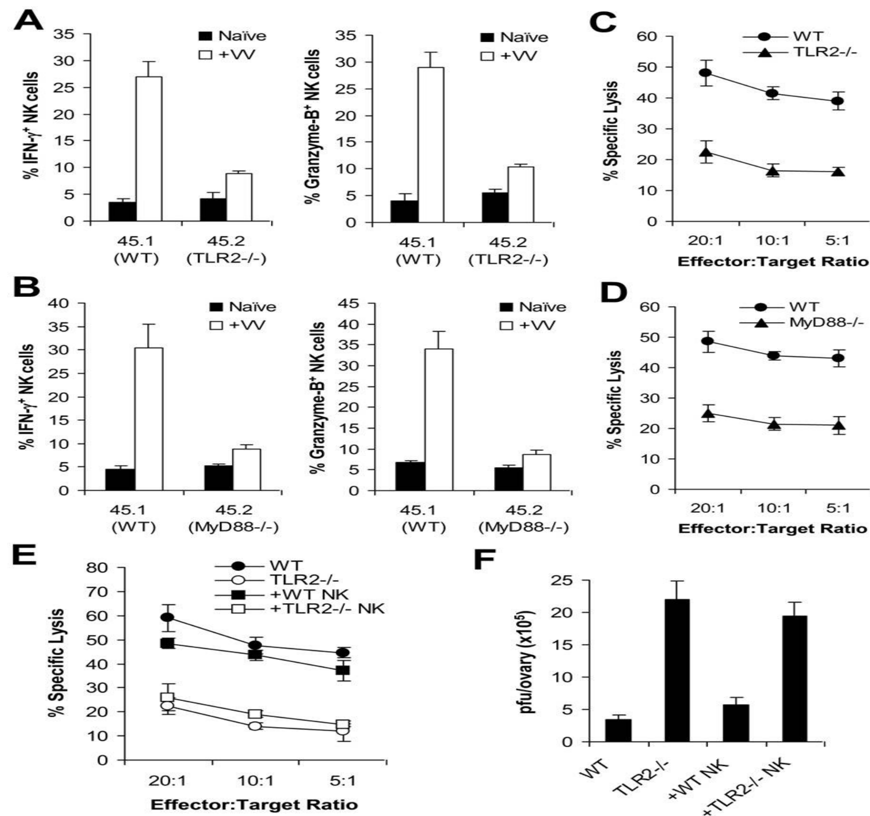

Figure 20: Direct TLR2-MyD88 signaling is required for NK cell priming in response to VV in vivo. (A-D) Bone marrow chimeric mice were generated by reconstituting irradiated CD45.1$^+$ WT mice with bone marrow cells from CD45.1$^+$ WT and CD45.2$^+$ TLR2-/- (A, C) or MyD88-/- (B, D) mice at a 1:1 ratio. Splenocytes were assayed for intracellular IFNγ and granzyme B production by NK cells. The mean percentage ±SD of IFNγ- or granzyme B-positive cells among CD45.1$^+$ or CD45.2$^+$ DX5$^+$CD3$^-$ cells is indicated (n = 4 per group) (A, B). Splenocytes were sorted into CD45.1$^+$ or CD45.2$^+$ DX5$^+$CD3$^-$ populations 48 hr after VV infection, and NK lytic activity was assayed on YAC-1 cells for 4 hr at different effector:target ratios. The mean percentages of specific lysis ±SD are indicated (n = 4 per group) (C, D). (E-F) TLR2-/- mice were reconstituted with WT NK cells (+ WT NK) or TLR2-/- NK cells (+ TLR2-/- NK) followed by infection with VV. WT and TLR2-/- mice infected with VV were used as controls. Forty-eight hours later, splenocytes were assayed for NK lytic activity on YAC-1 cells at different effector:target ratios. The mean percentage of specific lysis ±SD is indicated (n = 4) (E). The ovaries of female mice were harvested for measurement of viral load. Data represents the mean viral titer ±SD as pfu per ovary (n = 4) (F). Data is representative of two independent experiments.

5.2.5 Efficient NK cell activation and function upon VV infection is also dependent on the NKG2D pathway

Despite a critical role for the direct TLR2 signaling in NK cell activation, the production of IFNγ and granzyme B by TLR2-/- NK cells in response to VV infection was 2-3 fold above the background (Figures 15, 19), suggesting NK cell activation is not completely abolished in the absence of TLR2 signaling. Indeed, depletion of NK cells with anti-NK1.1 (Figure 21A) or anti-asialo GM1 (data not shown) in TLR2-/- mice led to an increased viral titer over the control TLR2-/- mice (Figure 21A). Furthermore, transfer of TLR2-/- NK cells into NK cell-depleted WT mice also resulted in a small reduction in viral load (Figure 21B). Collectively, these data suggest the existence of a TLR2-independent pathway for efficient NK cell activation.

Previous studies have shown that NKG2D is partially involved in NK cell-mediated control of mousepox virus in vivo [49], and that recognition of VV-infected cells by human NK cells is, in part, mediated by natural cytotoxicity receptors, NKp30, NKp44, and NKp46 [108]. Among natural cytotoxicity receptors, only NKp46 is expressed in mice. Thus, we investigated whether NKG2D or NKp46 contributed to TLR2-independent NK cell activation in response to VV infection. We first tested this in vitro with the NK-DC co-culture system. Purified NK cells from WT or TLR2-/- mice were co-cultured in vitro with WT CD11c$^+$ DCs in the presence of a blocking anti-NKG2D antibody or a blocking NKp46-Fc fusion protein, followed by infection with VV. The activation of NK cells was analyzed 24 hr later. The production of IFNγ and

granzyme B by WT NK cells was significantly (p <0.01) decreased in the presence of anti-NKG2D compared to the control without anti-NKG2D (Figure 22A). In addition, the production of IFNγ and granzyme B by TLR2-/- NK cells was completely abolished with the NKG2D blocking antibody (Figure 22A). However, blocking with NKp46-Fc had no effect on activation of WT or TLR2-/- NK cells (Figure 20A). These results indicating that NKG2D, but not NKp46, is also involved in NK cell activation upon VV infection.

We next examined the role of NKG2D in NK cell activation and function in vivo. WT or TLR2-/- mice were injected with the blocking anti-NKG2D antibody intravenously 24 hr and 6 hr prior to infection with VV intraperitoneally, and splenic NK cells were analyzed for their activation and function 48 hr after infection. In WT mice, NK cells produced significantly (p <0.01) less IFNγ and granzyme B in the presence of the NKG2D blocking antibody, compared to the control without NKG2D blocking (Figure 22B), leading to a significant (p <0.001) increase in viral load in the ovaries (Figure 22C). Furthermore, in TLR2-/- mice, NK cell activation was completely abolished with NKG2D blocking (Figure 22B), leading to a further increase in viral load in the ovaries (Figure 22C). These data indicate that the NKG2D pathway is also important in the activation of NK cells and the control of VV infection in vivo.

To address whether NKG2D or NKp46 is involved in the NK cell recognition of VV-infected targets, NK cells harvested from VV-infected WT mice were assayed for their lytic activities on VV-infected L929 targets in vitro in the presence of NKG2D or

NKp46 blocking antibodies. A significant reduction in cytolytic activities was observed with the NKG2D, but not NKp46, blocking antibodies (Figure 22D), suggesting NKG2D is also critical for the recognition of VV-infected cells.

Collectively, these results suggest that the TLR2-independent NKG2D pathway is also required for efficient NK cell activation, recognition of VV-infected targets, and VV control in vivo.

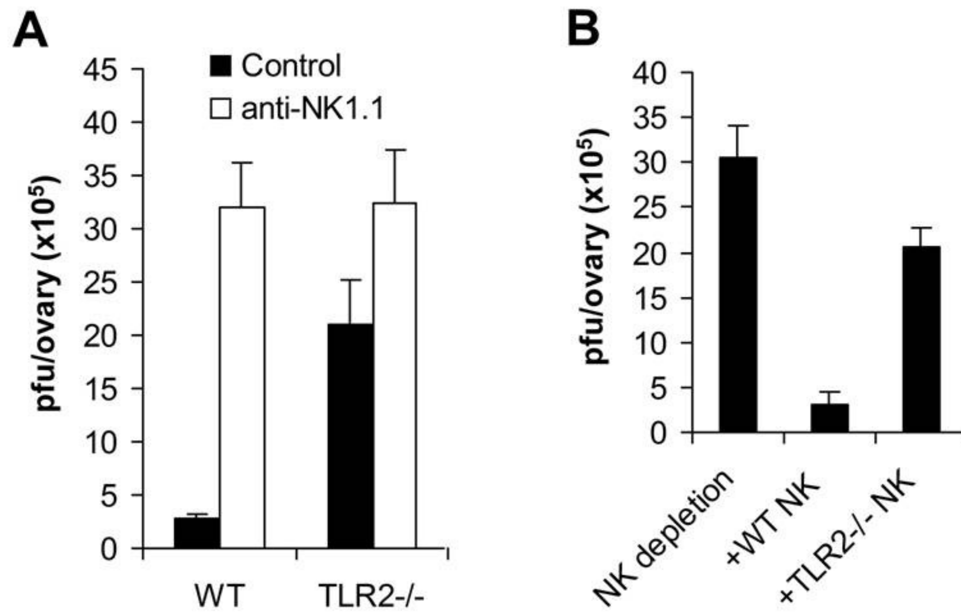

Figure 21: The role of TLR2-independent NK cell activation in VV control. (A) Female WT or TLR2-/- mice were depleted of NK cells with anti-NK1.1 antibodies on days -3 and 0 (+anti-NK1.1) or left untreated (Control), followed by infection with VV. Forty-eight hours after infection, the ovaries were assayed for viral load. Data represents viral titer ±SD as pfu per ovary (n=2). (B) Female WT mice were depleted of NK cells on days -2 with anti-NK1.1 antibodies. On day 0, NK cell-depleted mice were reconstituted with highly purified NK cells, followed by infection with VV. Forty-eight hours after infection, the ovaries were assayed for viral load. Data represents viral titer ±SD as pfu per ovary (n=2).

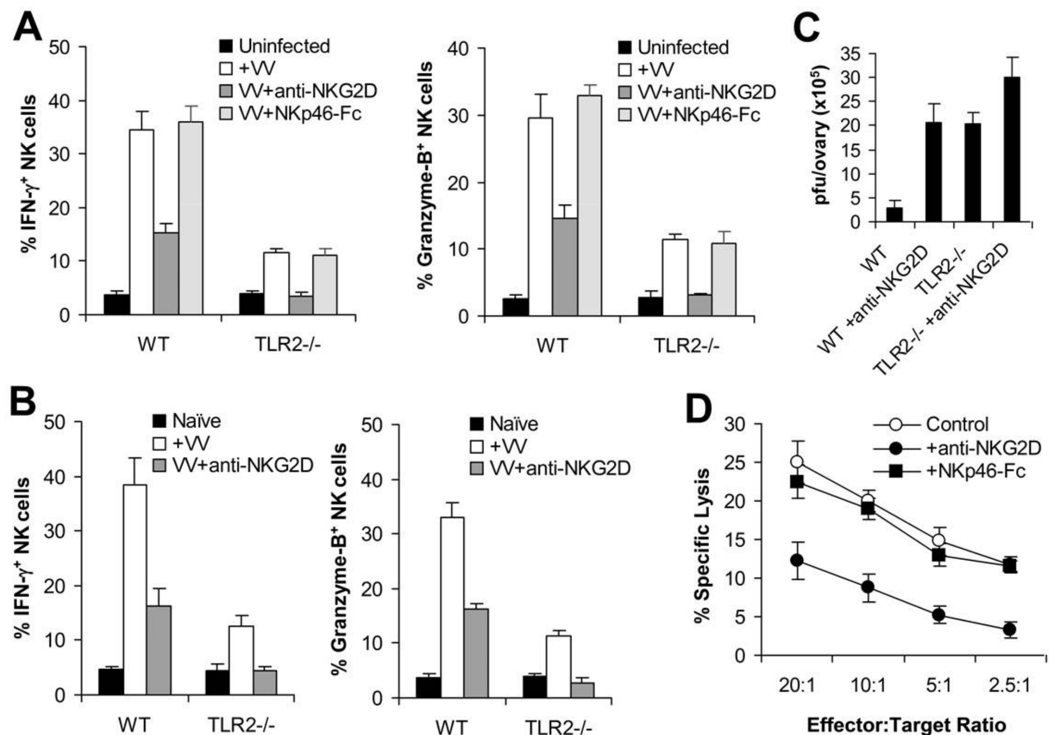

Figure 22: The NKG2D pathway is required for efficient NK activation and function in response to VV infection. (A) WT or TLR2-/- DX5^{+}CD3^{-} NK cells were co-cultured with WT CD11c^{+} DCs and stimulated with VV alone (+VV), VV in the presence of anti-NKG2D (VV+anti-NKG2D), or NKp46-Fc chimera (VV+NKp46-Fc), or left uninfected (Uninfected). Twenty-four hours later, NK cells were assayed for intracellular IFNγ and granzyme B. The mean percentage ±SD of IFNγ- or granzyme B-positive cells among DX5^{+}CD3^{-} cells is shown (n=2). (B-C) WT or TLR2$^{-/-}$ mice were infected with VV (+VV) or left uninfected (Naïve). Some mice were pre-treated with anti-NKG2D antibodies 24 and 6 hr prior to infection with VV (VV+anti-NKG2D). Forty-eight hours after infection, splenic NK cells were analyzed for IFNγ and granzyme B production. The mean percentage ±SD of IFNγ- or granzyme B-positive cells among DX5^{+}CD3^{-} cells is indicated (n = 4 per group) (B). The ovaries of female mice were harvested for measurement of viral load. Data represents the mean viral titer ±SD as pfu per ovary (n = 4 per group) (C). (D) Forty-eight hours after infection, splenocytes from WT mice were assayed for NK cell lytic activity on VV-infected L929 cells in the presence of anti-NKG2D antibodies (+anti-NKG2D) or NKp46-Fc chimera (+NKp46-Fc), for 4 hr at different effector:target ratios. The mean percentage ±SD of specific lysis is indicated (n = 4 per group). Data shown is representative of two independent experiments.

5.2.6 VV can directly activate NK cells via TLR2 in the presence of cytokines in vitro

The observation that direct TLR2 signaling on NK cells is required for NK cell activation and function in response to VV infection suggests that VV may directly activate NK cells via TLR2. To address this question, an accessory cell-free NK culture system is required. However, an accessory cell-free system would lack NKG2D stimulation, as NKG2D ligands are usually expressed by accessory cells upon viral infection. To compensate for lack of NKG2D stimulation, we established an accessory cell-free NK culture system in the presence of low dose IL2 and IFNα, as they have been used for the activation of NK cells in vitro. FACS-purified splenic $DX5^{+}CD3^{-}$ NK cells were stimulated with VV and assayed for NK cell activation 48 hr later. No significant IFNγ or granzyme B production was detected in the presence of IL2 and IFNα compared to the medium control (Figure 23A, B), suggesting that IL2 and IFNα alone do not activate NK cells. However, addition of VV stimulated NK cells to produce significantly ($p < 0.001$) higher levels of IFNγ and granzyme B (Figure 23A, B). These results indicate that VV can directly activate NK cells in the presence of cytokines. Similar results were obtained when UV-inactivated VV was used for stimulation (Figure 23B), suggesting that NK cell stimulation by VV is independent of newly synthesized viral products after infection.

We next investigated whether VV activated NK cells via TLR2. It has been shown NK cells express multiple TLRs [130-132]. Indeed, we showed here that TLR2,

TLR4, TLR8, and TLR9 were expressed at the RNA level in NK cells (Figure 24A). Furthermore, we found that TLR2 protein is expressed on the surface of freshly isolated NK cells by FACS and that the levels of TLR2 expression remained constant after incubation with cytokines or stimulation with VV (Figure 24B). When purified NK cell from TLR2-/- or TLR9-/- mice were stimulated with VV, the production of IFNγ and granzyme B by TLR2-/- (Figure 23C), but not TLR9-/- (data not shown), NK cells was significantly (p <0.001) reduced, compared to WT controls. To more directly confirm that VV activate NK cells via TLR2, WT NK cells were pre-treated with a blocking TLR2 antibody and stimulated with VV. Our result showed that TLR2 blocking led to a significant reduction in IFNγ and granzyme B production by WT NK cells (Figure 23C). Taken together, these data indicate that VV directly activates NK cells via TLR2.

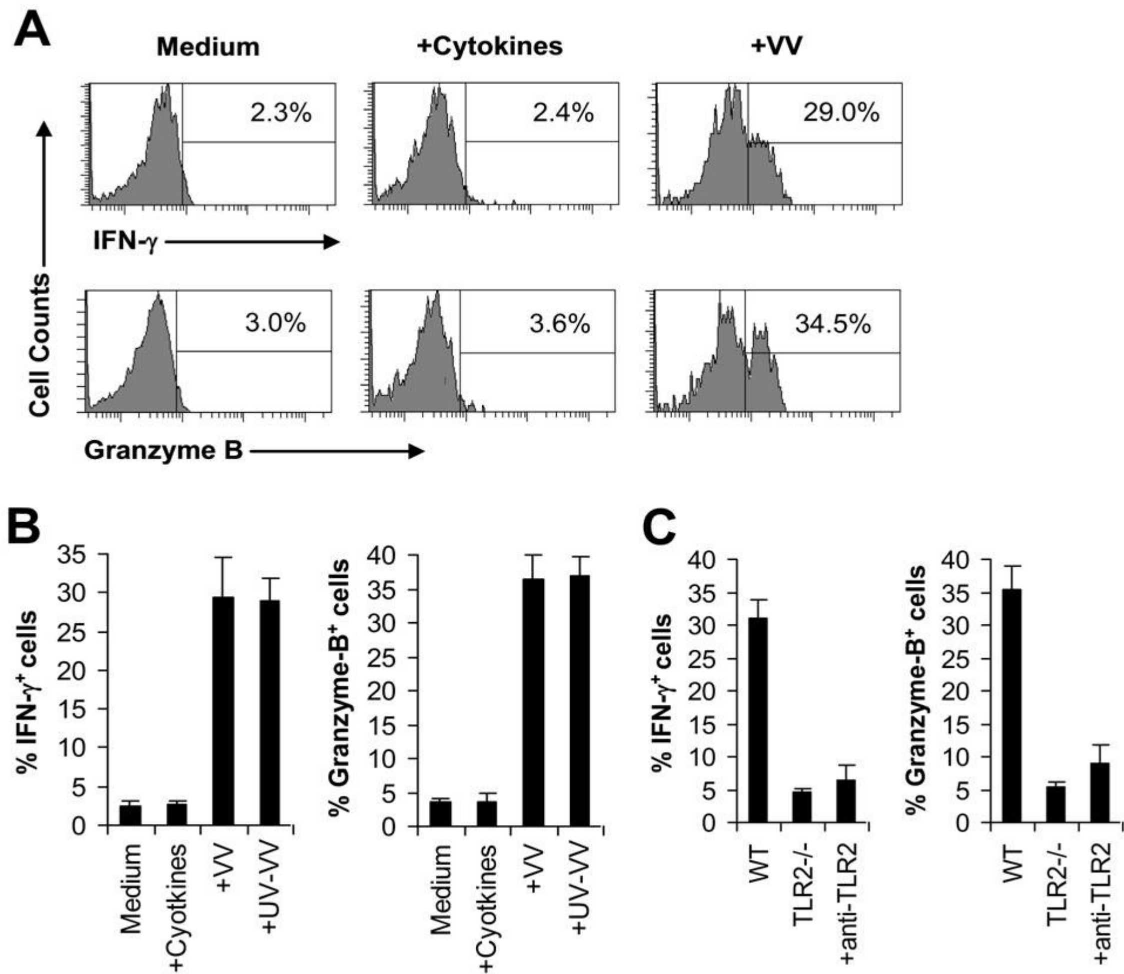

Figure 23: VV activates NK cells directly via TLR2 in vitro. (A-B) $DX5^{+}CD3^{-}$ NK cells were cultured in the medium alone (Medium) or medium supplemented with IL2 and IFNα (+Cytokines), or infected with live VV (+VV) or UV-inactivated VV (+UV-VV) in the presence of IL-2 and IFNα. Forty-eight hours later, NK cells were assayed for intracellular IFNγ and granzyme B. The percentage of IFNγ- or granzyme B-positive cells among $DX5^{+}CD3^{-}$ cells is indicated (n=4) (A). The mean percentage ±SD of IFNγ- or granzyme B-positive cells among $DX5^{+}CD3^{-}$ cells is shown (n=4) (B). (C) $DX5^{+}CD3^{-}$ NK cells from WT or TLR2-/- mice were stimulated with VV for 48 hr and assayed for intracellular IFNγ and granzyme B. Some WT NK cells were stimulated with VV in the presence of a blocking TLR2 antibody (+anti-TLR2). The percentage ±SD of IFNγ- or granzyme B-positive cells among $DX5^{+}CD3^{-}$ cells is shown (n=4). Data shown is representative of three independent experiments.

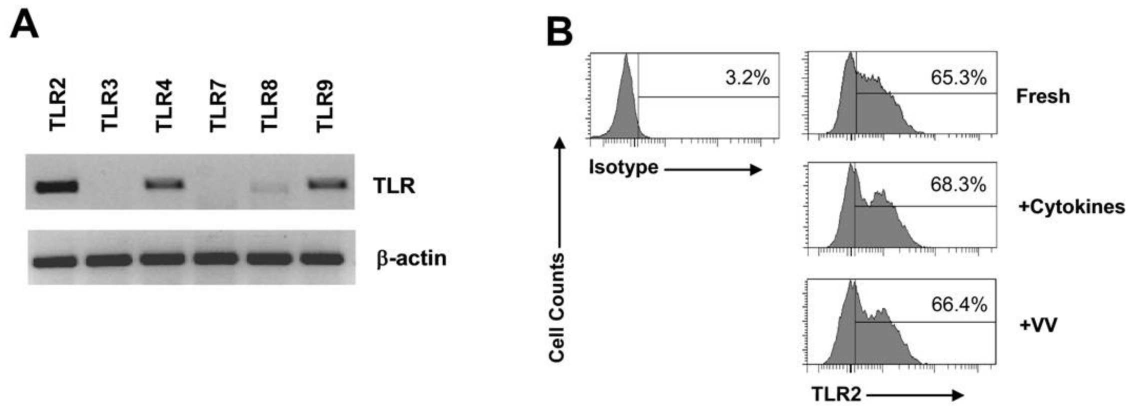

Figure 24: TLR2 expression on NK cells. (A) RNA was isolated from purified $DX5^{+}CD3^{-}$ NK cells and subjected to RT-PCR for expression of TLR2, 3, 4, 7, 8, and 9. (B) Purified NK cell24: were infected with VV (+VV) or left uninfected in the presence of IL2 and IFNα. Forty-eight hours later, cells were stained with anti-TLR2 and subjected FACS. Untreated freshly isolated NK cells (Fresh) were stained with anti-TLR2 or an isotype antibody as controls. The percentages of TLR2-expressing cells are indicated (n=3).

5.2.7 TLR2-MyD88-dependent NK cell activation by VV is mediated by the PI3K-ERK pathway

How does stimulation of TLR2-MyD88 pathway on NK cells by VV lead to NK cell activation? Recent studies have shown that in T cells, MyD88 can interact with phosphatidylinositol 3-kinase (PI3K) and activate the PI3K pathway, leading to enhanced T cell activation and survival upon TLR stimulation [12], and PI3K is the common signaling mediator downstream of activating NK receptors, such as ITAM-bearing NK receptors and NKG2D [44]. Furthermore, the PI3K-ERK pathway has been shown to play an important role in NK cell activation and cytotoxicity [13, 133]. Thus, we hypothesized that TLR2-MyD88 signaling on NK cells activated the downstream PI3K-ERK pathway, leading to activation of NK cells upon VV infection. To test this hypothesis, we first examined if activation of NK cells by VV was mediated by PI3K and ERK. FACS-purified NK cells were stimulated with VV in the presence of the PI3K inhibitor, LY294002, or the ERK1/2 inhibitor, PD98059, and analyzed for their activation 48 hr later. Indeed, the production of IFNγ and granzyme B by NK cells was significantly ($p < 0.001$) reduced in the presence of LY294002 or PD98059, compared to the control without inhibitors (Figure 25A), suggesting that both PI3K and ERK are involved in NK cell activation by VV.

We next tested whether stimulation of NK cells with VV led to the activation of both PI3K and ERK in a TLR2-MyD88-dependent manner. Stimulation of NK cells with VV resulted in the activation of PI3K, evidenced by phosphorylation of Akt, an

immediate downstream target of PI3K (indicative of PI3K activation), peaked at 4 hr after stimulation (Figure 25B). Similarly, ERK was also activated upon stimulation with VV, peaked at 18 hr after the infection (Figure 25C). The phosphorylation of both PI3K and ERK was critically dependent on an intact TLR2-MyD88 pathway, as the activation of Akt (Figure 25D) and ERK (Figure 25E) was greatly diminished in TLR2-/- or MyD88-/- NK cells stimulated with VV, compared to that in the WT controls. Furthermore, ERK phosphorylation was severely compromised in the presence of LY294002 (Figure 25E), confirming that ERK is downstream of PI3K. Taken together, these data indicate that TLR2-MyD88 dependent NK cell activation by VV is mediated by the PI3K-ERK pathway.

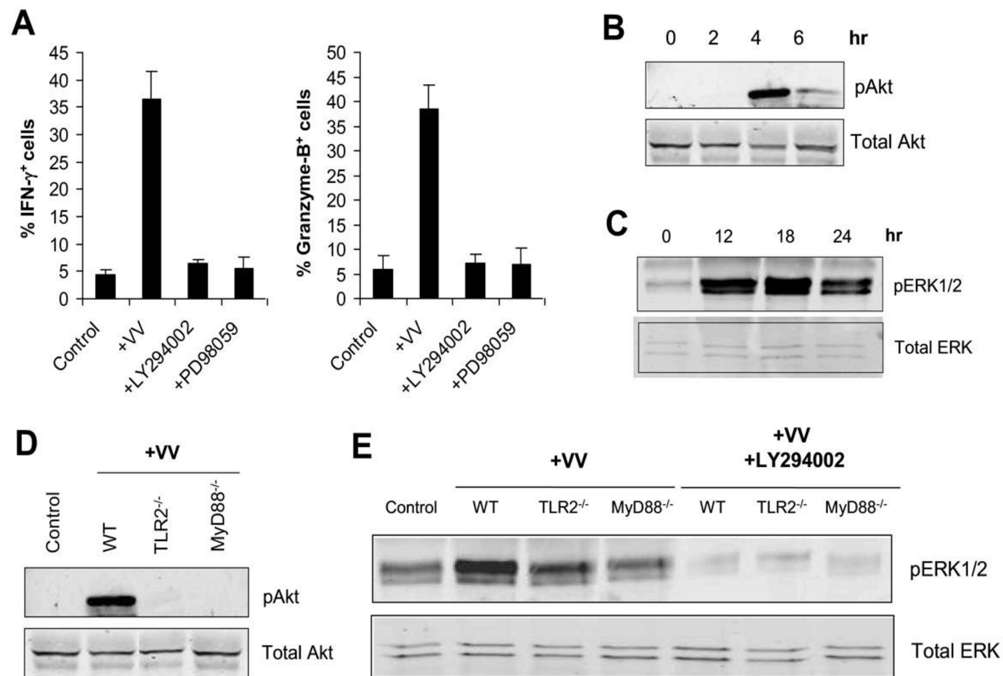

Figure 25: TLR2-dependent activation of NK cells by VV is mediated by PI3K and ERK. (A) $DX5^+CD3^-$ NK cells were infected with VV (+VV), VV in the presence of the PI3K inhibitor, LY294002 (+LY294002), or the ERK inhibitor, PD98059 (+PD98059) in vitro. Forty-eight hours after infection, NK cells were assayed for intracellular IFNγ and granzyme B. The mean percentage ±SD of IFNγ- or granzyme B-positive cells among $DX5^+CD3^-$ cells is shown (n=6). Data shown is representative of three independent experiments. (B-C) $DX5^+CD3^-$ NK cells were stimulated in vitro with VV for the indicated time periods. After stimulation for 2, 4, and 6 hr, NK cells were removed from culture, and total cell lysates were collected for Western blot analysis of phosphorylated Akt (pAkt), as well as total Akt (Total Akt), which served as a loading control (B). After stimulation for 12, 18, and 24 hr, NK cells were removed from culture, and total cell lysates were collected for Western blot analysis of phosphorylated ERK1/2 (pERK1/2), as well as total ERK (Total ERK) (C). (D) WT, TLR2-/-, and MyD88-/- $DX5^+CD3^-$ NK cells were stimulated in vitro with VV (+VV), or left unstimulated (Control) for 4 hr. NK cells were removed from culture, and total cell lysates were collected for Western blot analysis of phosphorylated Akt (pAkt), as well as total Akt (Total Akt). (E) WT, TLR2-/-, and MyD88-/- $DX5^+CD3^-$ NK cells were stimulated in vitro with VV (+VV), VV and the PI3K inhibitor LY294002 (+LY294002), or left unstimulated (Control) for 18 hr. NK cells were removed from culture, and total cell lysates were collected for Western blot analysis of phosphorylated ERK1/2 (pERK1/2), as well as total ERK (Total ERK). Data shown is a representative blot of five independent experiments.

5.3 Discussion

NK cells play a critical role in the control of poxviral infection in vivo [85, 101, 109, 123]. We have previously shown that type I IFNs, induced upon VV infection, acts directly on NK cells to regulate their activation and function [85]. However, the mechanism(s) by which NK cells are activated and function in response to poxviral infection remains poorly understood. In this study, we provided evidence that activation of the TLR2-MyD88 innate immune pathway is critical for NK cell activation and function in response to VV infection in vivo. Since VV activates TLR2 on accessory cells, such as DCs and macrophages, to secrete pro-inflammatory cytokines IL6, IL1, and IL12 [129], this dependence on TLR2 pathway could be mediated indirectly through IL1 and IL12, as these cytokines have been shown to play a role in NK cell activation [106, 121]. However, our data demonstrates that NK cell activation and function is independent of TLR2-induced IL1 and IL12 in vivo and that TLR2 signaling on NK cells, but not on DCs, is necessary for NK cell activation upon stimulation with VV in vitro. This data suggest a role for direct TLR2 stimulation on NK cells by VV in NK cell activation and function.

Indeed, the observations that TLR2 is expressed on NK cells, that VV can directly activate NK cells via TLR2 in the absence of accessory cells, and that intrinsic TLR2-MyD88 signaling is required for NK cell activation and function in the control of VV in vivo, support a critical role for direct TLR2 stimulation on NK cells for their activation and function. Although it has been shown that TLR3, TLR7, TLR8, and TLR9 are also

expressed on human NK cells and that ligands for these TLRs can activate human NK cells in vitro [130-132], our observations reveal for the first time that direct TLR stimulation is critical for NK cell activation and function in the control of viral infection in vivo. Thus, in the context of sensing VV, TLR2 may represent a novel class of NK cell activating receptor that is distinct from NKG2D, which detects stress-induced ligands [106, 121], as well as Ly49H and NKp46, both of which recognize pathogen-derived products expressed on infected cells [117, 124-126].

Although NK cell activation in response to MCMV is mediated by NK cell activation receptor, Ly49H, which specifically recognizes the m157 gene product of MCMV [117, 124], Ly49H is not involved in the control of VV in mice [107, 128]. The role of other NK activating receptors, such as NKp46 [125-126] and NKG2D [21, 127], in NK cell activation and recognition of poxviruses remain controversial [49, 108], and their precise role in vivo remains to be defined. Here we showed that NK cell activation, recognition of VV-infected target cells by NK cells and NK cell-mediated control of VV infection in vivo are also dependent on NKG2D, but not NKp46. Thus, multiple pathways are required for efficient activation of NK cells as well as their function in the control of VV infection in vivo. Future work is required to delineate how TLR2-dependent and independent pathways cooperate to achieve efficient NK cell activation in the control of VV infection in vivo.

We have further shown that direct TLR2-MyD88 signaling on NK cells activates the downstream PI3K-ERK pathway, leading to activation of NK cells upon VV

infection. This is in line with previous observations that PI3K is the common signaling mediator downstream of activating NK receptors, such as ITAM-bearing NK receptors and NKG2D [44]. Furthermore, the PI3K-ERK pathway has been shown to play an important role in NK cell activation and cytotoxicity [13, 133]. How does stimulation of TLR2 on NK cells lead to activation of the PI3K-ERK pathway? Recent studies have shown that in CD4+ T cells, MyD88 can directly interact with PI3K and activate the PI3K pathway, leading to enhanced T cell activation and survival upon TLR stimulation [12]. Similarly, we have recently demonstrated that VV can directly stimulate TLR2 on CD8+ T cells, which activates the PI3K-Akt pathway, leading to enhanced proliferation and survival of activated CD8+ T cells [11]. Whether MyD88 interacts directly or indirectly with PI3K in NK cells requires further investigation.

What component(s) of VV then is responsible for the activation of TLR2 on NK cells? In addition to live VV, UV-VV can also activate NK cells via TLR2, suggesting that the stimulation of TLR2 on NK cells by VV is independent of newly synthesized viral gene products after infection. Although NK cells also express TLR9, the observation that NK cell activation by VV is independent of TLR9 (data not shown), suggesting that VV-DNA is unlikely the ligand to activate NK cells. These results, coupled with our previous observation that both live VV and UV-VV can induce TLR2-dependent production of pro-inflammatory cytokines, peaking at 6 hr after infection in vivo [22], suggest that the envelope and/or membrane structural components of VV might be responsible for activating TLR2 during virus-cell contact. Indeed, previous

studies on other viruses have shown that viral envelope glycoproteins can trigger TLR2 or TLR4 responses [134-136]. Specifically, the fusion protein of respiratory syncytial virus (RSV) [134], the envelope protein of mouse mammary tumor virus (MMTV) [136], and hemagglutinin protein of measles virus [135] have been identified as ligands for activating the TLR responsiveness in their respective systems. Thus, it will be important to identify what component(s) of VV triggers NK cell activation in the future. Identification of such a ligand will help us design effective NK cell-based strategies to control poxviral infections in vivo.

In conclusion, we have shown that VV can directly activate NK cells via TLR2 and this intrinsic TLR2-MyD88 signaling pathway was critical for NK cell activation and function in the control of VV infection in vivo. This TLR2-MyD88-dependent NK cell activation by VV was mediated by the PI3K-ERK pathway. Furthermore, there is a TLR2-independent NK cell activation pathway mediated by NKG2D, which is also important for efficient NK cell activation and function in response to VV infection. These results identify for the first time that direct stimulation of TLR on NK cells are critical for their activation and function following a viral infection in vivo and may shed light on the design of effective strategies to combat poxviral infections.

Chapter 6: Overall Conclusions and Future Directions

As the most thoroughly studied member of the Poxviridae family and the vaccine used to achieve the only successful eradication of a human disease, vaccinia virus (VV) is a powerful tool for immunotherapy and vaccine development. The efficacy of recombinant viruses, engineered to express a specific antigen, to act as a vaccine vector was first demonstrated using VV. Protection against influenza infection was provided by vaccination with recombinant VV encoding the influenza-derived hemagglutinin gene; moreover, HA-expressing recombinant VV was capable of eliciting antibody titers equivalent to that of traditional influenza vaccination [75]. This clearly elucidates the powerful nature of VV as immunotherapeutic. However, there are numerous adverse side effects associated with VV administration; therefore, further understanding of the immunogenic properties of VV are required to establish a safer tool. Whereas prophylactic vaccines against infectious pathogens have been, by and large, successful, therapeutic cancer vaccines have not yet achieved such victory. The most likely explanation for the difference between the two vaccines is the development of immune evasion techniques by cancer cells. One manner in which tumor cells escape detection and, therefore the immune response, is the induction of tumor antigen-specific T cell tolerance. It was demonstrated that VV can break $CD8^+$ T cell tolerance via persistent TLR signaling, leading to increased resistance to tumor challenge [76]. Although VV first made its name by successfully eradicating smallpox in the 1970's, it has proven

itself as a useful tool for the study of antiviral immunity, vaccine development, and potentially cancer immunotherapy.

By what mechanisms does VV elicit such a robust immune response? It has been demonstrated by several studies that VV is a potent stimulator of the TLR pathway, in both the adaptive and innate immune systems, the activation of both being required for the efficient control of VV in vivo. Upon VV infection in vivo, direct TLR2-MyD88 signaling in $CD8^+$ T cells was required for their efficient clonal expansion, survival, and differentiation into a memory population. Furthermore, antigen-specific $CD8^+$ T cell proliferation, survival, and memory cell formation occurred in a manner dependent on the PI3K-Akt pathway [11]. These observations represent a unique mechanism by which the memory population is generated, as TLRs are most commonly associated with the innate immune response. Indeed, VV activation of the innate immune system is necessary for an optimal T cell response. VV activates cDCs via both TLR-dependent and TLR-independent pathways. The production of pro-inflammatory cytokines, such as IL6, IL1, and IL12, occur via a TLR2-MyD88-dependent mechanism, whereas type I IFN, such as IFNβ, are produced in a TLR-independent manner. Strikingly, both pro-inflammatory cytokines and type I IFNs, despite using pathways independent of each other, are crucial to the clearance of vaccinia in vivo. However, it remains to be determined how these pathways direct the innate immune response to vaccinia viral infection [22].

While production of IFNβ by cDCs occurs in a TLR-independent manner in response to VV, cDCs are not the primary producers of type I IFNs. Plasmacytoid DCs

are a subset of DCs, originally identified by their ability to produce large amounts of type I IFNs in response to viral infection [24]. Unlike other innate immune cells, pDCs produce type I IFNs through a strict TLR-MyD88-dependent mechanism. Moreover, pDCs preferentially express the endosomal TLR7, TLR8, and TLR9. These three TLRs utilize the downstream adaptor, MyD88 and recognize viral nucleic acids. TLR9 recognizes the CpG motif (mimic of bacterial DNA) in viral dsDNA, such as HSV and MCMV. TLR7 and TLR8 have both been implicated in ssRNA recognition, although a biological role for murine TLR8 has yet to be determined [4]. Considering the importance of type I IFNs in the antiviral immunity and the established role of pDCs in type I IFN production, the first aim of this thesis project was to examine the role of pDCs in innate immune control of VV infection, as well as identify the mechanisms by which VV activates pDCs.

In response to VV stimulation, pDCs produce robust amounts of both IFNα and IFNβ, in quantities significantly greater than those produced by cDCs, which produces solely IFNβ. Furthermore, pDCs produced type I IFNs in a MyD88-dependent manner, whereas production of IFNβ by cDCs occurred in a TLR-independent manner. In addition, pDCs upregulated the co-stimulatory marker, CD86, in a MyD88-dependent manner. These observations established that pDCs were indeed activated by VV infection to produce vigorous amounts of type I IFNs, far greater than that of cDCs, and aid in the establishment of the antiviral state. Given the fact that VV is a dsDNA virus, we first investigated the role of TLR9 in VV-mediated activation of pDCs, as TLR9

senses viral DNA [63]. Surprisingly, TLR9-deficient pDCs were able to produce equivalent amounts of type I IFNs compared to WT pDCs. Thus, VV activates pDCs to produce type I IFNs in a MyD88-dependent, but TLR9-independent manner.

Having established the requirement for MyD88 but not TLR9 for pDC activation, we next examined the possibility of TLR7 or TLR8 involvement in type I IFN production in response to VV. Both of these endosomal TLRs signal via MyD88, yet have only been characterized as ssRNA sensors [24]. Likewise, murine TLR8 has been described as biologically nonfunctional, although recent studies have demonstrated that murine TLR8 can be activated by certain synthetic imidazoquinolines [24, 32]. In order to first determine the reactivity of murine TLR7 or TLR8 to VV, we utilized the NFκB luciferase assay system. In response to VV or VV-DNA stimulation, HEK293 cells transfected with TLR8, but not TLR7, activated NFκB significantly over empty controls. This observation suggested that VV was capable of activating the TLR8 pathway, a pathway previously thought of as nonfunctional. In order to establish a role for TLR8 in pDC activation to VV, we utilized lentiviral transduction to knockdown TLR7 or TLR8 expression via siRNA in pDCs. Type I IFN production was completely abolished in pDCs in which TLR8, but not TLR7, was knocked down by siRNA. Yet, these pDCs were still capable of type I IFN production in response to non-TLR8 ligands, such as CpG-A ODN, indicating that the siRNA was specific for TLR8 and the pDCs were still functional. These results reveal a role for TLR8 in the recognition of VV, more specifically VV-DNA, and the activation of pDCs. This data was further bolstered by the

ability of the synthetic inhibitor, ODN2088, to ablate type I IFN production by pDCs in response to VV or VV-DNA stimulation, in a TLR9-independent manner. Collectively, these results demonstrate that VV, using its DNA as a ligand, is capable of activating pDCs via a TLR8-mediated mechanism. This data represent, for the first time, that murine TLR8 is functional in the context of viral infection and that murine TLR8 is capable of sensing viral DNA. While evolutionarily similar, perhaps TLR7 and TLR8 have evolved separately to sense different pathogenic ligands, and the evolutionary similarity between these 2 TLRs has masked their differences.

What then are the possible motifs present in VV-DNA capable of stimulating TLR8? A recent report demonstrated that oligodeoxynucleotides rich in thymidine (polyT ODN) enhanced TLR8-mediated activation of NFκB [32]. Therefore, we next analyzed the composition of VV-DNA. Approximately 67% of VV-DNA is composed of A/T's, while only 33% is composed of G/T's. This differs greatly from known TLR9 activating viruses, such as Ad-5 (44% A/T) and HSV-2 (30% A/T). Additionally, 45% of VV-DNA consisted as A/T "islands", defined here as tracts of 10 base pairs containing 5 or more A/T (consecutively and non-consecutively). In order to correlate the polyA- and polyT-rich sequences to TLR8 responsiveness, polyA, polyT, polyG, and polyC ODN were generated and used to stimulate both WT and TLR8-knockdown pDCs. Both polyA and polyT ODN were able to stimulate pDCs production of IFNα in a TLR8-dependent manner, while polyG and polyC ODN were not able to stimulate pDCs at all. Therefore,

the A/T rich composition of VV-DNA could represent a potential motif capable of activating the TLR8 pathway.

It has been well-established that TLR9 is a sensor of bacterial and viral DNA, specifically CpG motifs. It is possible that likewise, TLR8 has evolved as a sensor for A/T motifs within pathogenic DNA. Therefore, there exist within the endosomal TLR system DNA sensors for both C/G-rich motifs and A/T-rich motifs. Clearly, the viruses that have been demonstrated as TLR9-dependent are C/G-rich, which is unsurprising as TLR9 is known to detect CpG-rich DNA. The A/T-rich nature for VV-DNA, far greater than the genomic composition of other viruses we examined, could be a unique characteristic, as no other virus has been associated with murine TLR8 activation. Moreover, VV-DNA contains "islands" of A/T-rich areas, defined in this thesis as 10 base pair stretches containing 5 or more A/Ts (either consecutively or non-consecutively). It is possible that these "islands" are the areas in VV-DNA that serve as activating motifs for TLR8 sensing, although further characterization of the VV genome is required. It would also be of interest to synthesize fragments of VV-DNA with mutations in the A/T-rich areas and determine the ability of these mutated VV-DNA ODNs to stimulate type I IFN production in a TLR8-dependent manner.

How does TLR8-mediated activation of pDCs control VV infection in vivo? Type I IFNs are critical mediators of the antiviral innate immunity, as well development of the adaptive immune response [63]. Firstly, we demonstrated that pDCs were crucial to the clearance of VV in vivo, as mice depleted of pDCs with anti-Ly6G/C Ab exhibited

higher viral titer, higher viral replication, and lower serum IFNα levels upon VV infection. These defects were corrected by adoptive transfer of WT, TLR9-/-, or TLR7-knockdown pDCs, resulting in viral titer, viral replication, and serum IFNα levels similar to WT mice. In addition, mice deficient for MyD88 display significantly decreased levels of IFNα in the serum, as well as increased viral titers in response to VV infection; adoptive transfer of WT pDCs, but not TLR8-knockdown pDCs, rescued IFNα production and decreased viral titers to WT levels. Hence, TLR8 activation of pDCs by VV is crucial for the clearance of VV in vivo. This observation suggests that different pathogens have evolved to adopt unique mechanisms to effectively activate the innate immune system. It remains to be determined whether human, non-pDCs utilize the TLR8 pathway for recognition of VV or whether human pDC use alternative PRRs in response to VV. These studies represent, for the first time, a biological role for murine TLR8 in the recognition of VV, a dsDNA virus, potentially through recognition of polyA/T-rich motifs. These results suggest a novel strategy of polyA and polyT ODN-mediated pDC activation for the efficient viral control in vivo.

Why does VV use different TLRs to activate different cell types? One explanation is that VV is not a single entity, but a complex pathogen comprised on envelope glycoproteins, membrane components, and nucleic acids. Each one of these parts is capable of activating the appropriate PRRs to elicit a specific response. Additionally, the ability of the VV-DNA to reach the endosomal TLR8 in pDCs may provide a unique opportunity to elicit the type I IFN response. More research into the

trafficking of VV-DNA to the endosomes and subsequent TLR8 signaling is required to fully illuminate this pathway.

Extensive research has demonstrated that type I IFNs are important molecules for establishing the antiviral state. Binding of type I IFNs to the shared heterodimeric receptor, IFNα/βR, triggers a series of signaling cascades leading to the transcription of more than 100 IFN-stimulated genes (ISGs), which promote antiviral immunity by degrading viral ribonucleoproteins, destroying viral RNA, and inhibiting translation [63, 137-138]. Additionally, type I IFN signaling also mediates a variety of immunoregulatory effects. In the setting of VV infection, the IFNα/β-STAT1 pathway was critical for efficient expansion, survival, and memory formation of Ag-specific CD8+ T cells *in vivo* [68]. Moreover, type I IFNs promote the maturation of and upregulation of co-stimulatory and antigen-presenting molecules on DCs, resulting in more efficient interactions with T cells. As early as 1 day after infection, type I IFNs are required for clearance of VV in vivo [1, 22, 37]. How type I IFNs control the innate immune response upon VV infection remains undefined, however.

Perhaps the most well established, yet mechanistically unclear, effect of type I IFNs is its ability to promote NK cell cytotoxicity [63]. NK cells have long been associated with the control of viral infection in vivo, and it has been demonstrated that poxviral infection requires NK cell activity [42]. Taking these factors into account, the second aim of this thesis project was to examine the role of type I IFNs in the activation of NK cells in the context of VV infection. It has been shown that IFNα pre-treatment

protects L929 from RNA viruses, such as EMCV-induced cell death, through inhibiting viral replication [114-115]. However, we demonstrated that IFNα pre-treatment does not protect against cell death in the context of VV infection in vitro, suggesting that IFNα does not prevent VV replication in cells. Thus, IFNα does not directly protect cells against VV infection in vitro [85].

This observation implies that the type I IFN-dependent innate immune control of VV infection in vivo operates through a different mechanism. As NK cells have been implicated in the control of poxviruses in vivo, we next examined the ability of VV infection to activate NK cells [107]. Indeed, NK cells expanded and produced effector molecules, such as IFNγ, granzyme B, and perforin, upon VV infection in vivo. This activation functionally translated to significant cytotoxicity against NK-specific target cells, YAC-1, compared to naïve controls. We further examined whether or not NK cells were required for VV clearance in vivo. Depletion of NK cells from WT mice resulted in significantly increased viral titer over NK-sufficient mice, indicating that NK cells were a necessary component of innate immune control of VV in vivo. However, NK cells from IFNα/βR-/- mice did not expand or produce IFNγ, granzyme B, or perforin upon VV infection. Hence, NK cells from IFNα/βR-/- mice did not demonstrate cytolytic function in response to VV infection. Depletion of NK cells from IFNα/βR-/- mice had not effect on viral titer in vivo, suggesting that type I IFNs exert their effect directly on NK cells. Taken together, this data supports the conclusion that type I IFN-dependent innate

immune control of VV infection in vivo is mediated through regulating NK cell function [85].

How do type I IFNs regulate the activation of NK cells upon VV infection? It had been reported that cDCs play a critical role in NK cell activation, and type I IFN signaling on cDCs may aid in the activation of NK cells in response to TLR stimuli [21, 113]. In order to examine how type I IFNs activate in NK cells in the context of VV infection, we used an in vitro NK-DC co-culture system. Using this system with WT and IFNα/βR-/- NK cells and DCs, we were able to determine whether type I IFNs acted on DCs or NK cells for NK cell activation in response to VV infection. Our data showed that NK cell activation was significantly reduced when IFNα/βR-/- NK cells were used for stimulation, even with WT DCs. However, IFNα/βR-/- DCs elicited similar amounts of IFNγ, granzyme B, and perforin production by NK cells compared with the WT counterparts, suggesting NK cell activation is independent of type I IFN signaling on DCs in response to VV infection. This data indicates that direct action of type I IFNs on NK cells is required for their activation upon VV infection [85].

In order to establish an in vivo role for type I IFN-dependent activation of NK cells, we examined the ability of WT NK cells to rescue the defect in VV clearance in IFNα/βR-/- mice. Adoptive transfer of WT NK cells into IFNα/βR-/- mice resulted in increased NK cell cytotoxicity, indicating that WT NK cells were able to be activated and function even in an IFNα/βR-deficient host. Furthermore, IFNα/βR-/- reconstituted with WT NK cells were able to clear VV to levels similar to WT mice. Therefore, direct type

I IFN signaling on NK cells is required for the control of VV in vivo. These results may contribute to future, potential strategies for the control of poxviral infections in vivo [85].

This report represents the first evidence that direct action of type I IFNs on NK cells is required for the optimal activation in response to VV in vivo. Further studies are required to determine what ISGs are activated in NK cells by type I IFN signaling, and how these signaling pathways contribute to the production of effector molecules and ultimately cytotoxicity. Having previously identified pDCs as the key producers of type I IFNs in response to VV (or more specifically, VV-DNA), it is possible, that while cDC can act as NK cell activating accessory cells, pDC may be more important in terms of NK cell activation. Therefore, a possible strategy to enhance NK cell activation in response to VV infection is to use VV-DNA as a vaccine, thus activating pDCs in a TLR8-dependent manner for the robust production of type I IFNs and hence activating NK cells. This approach also bypasses many of the adverse effects of VV vaccination, as many of the immunogenic components of VV will not be present.

Previous reports from our laboratory demonstrated that VV was capable of activating the TLR2-MyD88 pathway in cDCs, resulting in the production of pro-inflammatory cytokines, and this pathway is critical for the efficient clearance of VV in vivo [22]. The mechanisms by which the TLR2-MyD88 pathway control VV clearance in vivo, however, remains unknown. Pro-inflammatory cytokines, such as IL1 and IL12, have been implicated in the enhancing NK cell activation in response to viral infection [53]. However, stimulation of cDCs with VV results in only a modest production of

IL12, and VV encodes an IL1β-receptor mimic (B15R) that inhibits endogenous IL1β signaling [22, 71]. Importantly, recent reports have demonstrated that direct TLR stimulation can activate or enhance the activation of NK cells; the majority of these studies, however, have been performed in human NK cells [41, 53]. Therefore, the third aim of this thesis project is to examine the role of TLR2-MyD88 signaling in NK cells activation and its effect on clearance of VV in vivo.

Having previously established that clearance of VV in vivo requires NK cell activation and function, we next examined the role of TLR2-MyD88 signaling on the NK cell function in response to VV. As earlier described, NK cells from WT mice expand, produce effector molecules, and display cytolytic function upon VV infection. However, NK cells from TLR2-/- or MyD88-/- mice fail to become fully activated, producing significantly less IFNγ and granzyme B and displaying reduced cytotoxicity. This reduction in effector function translated to an increased viral titer in TLR2-/- and MyD88-/- mice compared to WT counterparts. Because the TLR2-MyD88 pathway is critical for pro-inflammatory cytokine production by cDCs [22], we next examined whether or not NK cells from IL1R-/-, IL6-/-, and IL12-/- mice acquired effector function and viral clearance upon VV infection. NK cells from IL1R-/-, IL6-/-, and IL12-/- mice displayed similar levels of lytic activities and viral titer compared to WT mice. These results suggest that TLR2-induced pro-inflammatory cytokine production is not critical for NK cell activation and effector function in response to VV infection in vivo. However, these results do not exclude the possibility that 2 or more of these pro-

inflammatory cytokines having a combined effect on NK cell activation. Only mice deficient in multiple pro-inflammatory cytokines (such as an IL12-/- IL1R-/- mouse) could answer this question.

Is TLR2-MyD88 signaling on accessory cells, such as DCs, or directly on NK cells critical for NK cell activation in response to VV? In order to determine where TLR2 signaling was necessary, we again utilized the NK:DC co-culture system, using WT and TLR2-/- cells. NK cells isolated from the spleens of WT mice were able to produce normal levels of IFNγ and granzyme when cultured with either WT or TLR2-/- cDCs. This observation suggests that TLR2-MyD88 signaling on cDCs is not required for NK cell activation in response to VV infection. These results are in line with our previous in vivo observation that NK cells from IL1R-/-, IL12-/-, and IL6-/- mice display levels of cytotoxicity equivalent to that of WT controls. However, TLR2-/- NK produced decreased amounts of IFNγ and granzyme B when cultured with either WT or TLR2-/- cDCs, indicating that direct TLR2 signaling on NK cells is required for their efficient activation in response to VV infection. In order to "mimic" this in vitro co-culture system in an in vivo setting, mixed bone marrow chimeras were generated, with approximately 50% of the hematopoietic system derived from WT cells and approximately 50% of the hematopoietic system derived from TLR2-/- or MyD88-/- cells. Upon VV infection, WT cells from the mixed chimera were able to produce normal amounts of IFNγ and granzyme B, as well as lyse targets efficiently in a cytotoxicity assay. However, TLR2-/- or MyD88-/- NK cells from the same mixed

chimera produced significantly lower amounts of IFNγ and granzyme B and displayed reduced cytolytic function, even in the presence of WT accessory cells within the chimera. Therefore, efficient activation and function of NK cells in response to VV infection requires intrinsic TLR2-MyD88 signaling. These results are quite important, as they demonstrate that TLR2-/- NK cells, within a normal environment, fail to become fully activated in response to VV infection; while WT NK cells, from within the same mouse, do.

In order to establish the in vivo relevance of TLR2-MyD88 signaling on NK cells for the efficient clearance of VV, TLR2-/- mice were adoptively transferred with splenic WT or TLR2-/- NK cells, and lytic activity and viral titer were assayed 48 hr post-infection. Splenic NK cells harvested from TLR2-/- mice reconstituted with WT, but not TLR2-/-, NK cells were capable of lysing target cells in a cytotoxicity assay, in a manner similar to WT counterparts. Moreover, reconstitution of TLR2-/- mice with WT NK cells resulted in a significantly decreased viral titer, whereas reconstitution with TLR2-/- NK cells did not. Therefore, intrinsic TLR2-MyD88 signaling on NK cells is required for the efficient clearance of VV in vivo.

Despite its obvious defects, TLR2-/- NK cells still retain a modest capacity for activation and function, as its effector molecule production is 2 – 3 fold higher than naïve controls. Depletion of NK cells with anti-NK1.1 in TLR2-/- mice led to an increased viral titer over the NK-sufficient TLR2-/- mice. Moreover, transfer of TLR2-/- NK cells into NK cell-depleted WT mice also resulted in a small reduction in viral load.

Collectively, these data suggest the existence of a TLR2-independent pathway for efficient NK cell activation. Recently, the NK cell activating receptor, NKG2D, has been implicated in the control of mousepox virus in vivo [49]. In humans, NK cell recognition of VV is in part regulated by members of the NCR1 complex, NKp30, NKp44, and NKp46, although only NKp46 is expressed in mice [108]. Using the NK:DC co-culture system, we then investigated the possible roles of either NKG2D or NKp46 in the activation of NK cells in response to VV infection. Inhibition of the NKG2D pathway with the anti-NKG2D blocking antibody resulted in a decrease in effector molecule production by WT NK cells and a complete ablation of effector molecule production by TLR2-/- NK cells in response to VV infection. However, inhibition of the NKp46 pathway with blocking NKp46-Fc fusion protein had no effect on effector molecule production by either WT or TLR2-/- NK cells. This data then suggests that NK cells also utilize the NKG2D pathway, but not the NKp46 pathway, in their recognition and activation in response to VV infection. We further examined the role of NKG2D in the in vivo NK cell response to VV. WT mice, treated with the anti-NKG2D blocking antibody, produced less IFNγ and granzyme B in response to VV infection, and subsequently displayed a decreased ability to lyse target cells in a cytotoxicity assay. This reduced activation and function translated to an increase in viral titer. Furthermore, TLR2-/- mice, treated with the anti-NKG2D blocking antibody, produce no significant amounts of effector molecules, and displayed lytic function on par with that of naïve controls. In addition, these mice demonstrated an even higher viral titer, compared to

WT mice with anti-NKG2D treatment and untreated TLR2-/- mice. Collectively, these results indicate that the NKG2D pathway is also important in the activation of NK cells and the control of VV infection in vivo. As “natural killers”, NK cells have many ways in which they can be activated. Therefore, the existence of multiple pathways that contribute to NK cell activation in response to VV infection is no surprising, as NK cells are vital to the initial control of VV in vivo. What is of utmost importance is to delineate how each pathway contributes to NK cell cytotoxicity, and to determine if (and how) these 2 pathways converge and cooperate.

We then examined the ability of VV to directly activate NK cells, in the absence of accessory cells. We devised an accessory cell-free NK cell culture system, using flow cytometry-sorted NK cells and the supplemental cytokines, IL2 and IFNα, which we demonstrated were incapable of activating NK cells alone. WT NK cells stimulated with VV in vitro produced high amounts of IFNγ and granzyme B, whereas TLR2-/- NK cells produced significantly reduced amounts of IFNγ and granzyme B, indicating that VV can directly activate TLR2 on NK cells. To more directly demonstrate that VV activates NK cells via TLR2, WT NK cells were pre-treated with a blocking TLR2 antibody and stimulated with VV. TLR2 blocking led to a significant reduction in IFNγ and granzyme B production by WT NK cells. Collectively, these data indicate that VV directly activates NK cells via TLR2.

How then does direct TLR2 signaling on NK cells confer effector function? Recent studies have shown that in T cells, MyD88 can directly interact with and activate

PI3K, leading to enhanced T cell activation and survival upon TLR stimulation [12]. Additionally, the PI3K-ERK pathway has been shown to play an important role in NK cell activation and cytotoxicity [13, 133]. Hence, we hypothesized that TLR2-MyD88 signaling on NK cells activated the downstream PI3K-ERK pathway, leading to activation of NK cells upon VV infection. Indeed, pharmacological inhibition of PI3K or ERK1/2 (with L7249002 or PD98059, respectively) significantly decreased NK cell production of IFNγ and granzyme B in response to VV stimulation in vitro. Moreover, we demonstrated that VV stimulation resulted in activation of PI3K (as evidenced by activation of its downstream target, Akt) and ERK1/2. However, the activation of PI3K and ERK1/2 was significantly reduced in TLR2-/- and MyD88-/- NK cells upon VV stimulation, indicating that TLR2-MyD88-dependent NK cell activation by VV is mediated by the PI3K-ERK pathway. The PI3K-ERK pathway undoubtedly contains other signaling components that contribute to NK cell cytotoxicity. It will important to determine how PI3K activates ERK1/2, and how activated ERK1/2 influences NK cell cytotoxicity. ERK1/2 has been demonstrated to play a role in actin depolarization and cytoskeletal rearrangements, mechanisms critical for the movement and degranulation of effector molecules, such as granzyme B and perforin [133]. Therefore, it is possible that key exocytosis molecules, such as Rac1, PAK1, and Rho, could play a role in TLR2-dependent NK cell activation.

Collectively, we were able to demonstrate that VV was capable of directly activating NK cells via the TLR2-MyD88 pathway, leading to NK cell activation and

effector function mediated by the PI3K-ERK pathway. We were also able to demonstrate that while TLR2 signaling on NK cell was necessary for efficient clearance of VV in vivo, there existed a TLR2-independent pathway for NK cell activation, mediated by NKG2D. Together, these two pathways are required for optimal NK cell activation and functionality, leading to successful clearance of VV in vivo.

Why does VV activate multiple pathways, such as numerous TLRs, to elicit an immune response? One evolutionary reason could be to keep its host alive. Although this reasoning seems counterintuitive to a virus, a virus can only spread and propagate via living hosts. Therefore, it is in the virus's best interest to infect, yet maintain the life, of its host, especially during the critical early phases of the infection (hence the viral replication time perios). VV, being a complex pathogen with multiple possible immunogens, has the opportunity to elicit an innate immune response using multiple different components activating numerous pathways.

Further understanding of the mechanisms by which VV activates the innate immune system is critical to developing novel immunotherapeutics and safer vaccines. Collectively, this thesis sought to examine how VV, a potent stimulator of the innate immune system, initiates an immune response and how this activation conferred efficient viral control. In the first aim of this thesis project, we determined that VV activated pDCs in a TLR8-MyD88-dependent manner to produced type I IFNs, possible via its polyA- and polyT-rich genomic motif. We also determined that TLR8-dependent type I IFNs production by pDCs was crucial for VV clearance in vivo. The second aim of this

thesis project determined that this production of type I IFNs was also required for efficient NK cell activation and function. NK cells were necessary for VV clearance in vivo, and direct action of type I IFNs on NK cells was required for NK cell function and subsequent viral clearance. Given the importance of NK cells in viral clearance and the established role of the TLR2-MyD88 pathway in VV immunity, the third aim of this these project sought to determine the role of the TLR2-MyD88 pathway in NK cell activation in response to VV infection. We determined that VV was capable of directly activating the TLR2-MyD88 pathway on NK cells, in a manner independent of accessory cell production of pro-inflammatory cytokines, IL1, IL12, and IL6. Direct TLR2 signaling on NK cells resulted in activation of the PI3K-ERK1/2 pathway, leading to NK cell activation and cytotoxicity. While this direct activation of NK cells was required for optimal VV clearance, we also discovered a TLR2-independent mechanism for NK cell activation in response to VV; we determined that the NKG2D pathway was also required for optimal NK cell activation and viral clearance in vivo. Together, these two pathways cooperate to achieve efficient NK cell activation, and therefore, viral clearance in vivo. Figure 26, summarizing these mechanisms for VV activation of the innate immune system, is illustrated below.

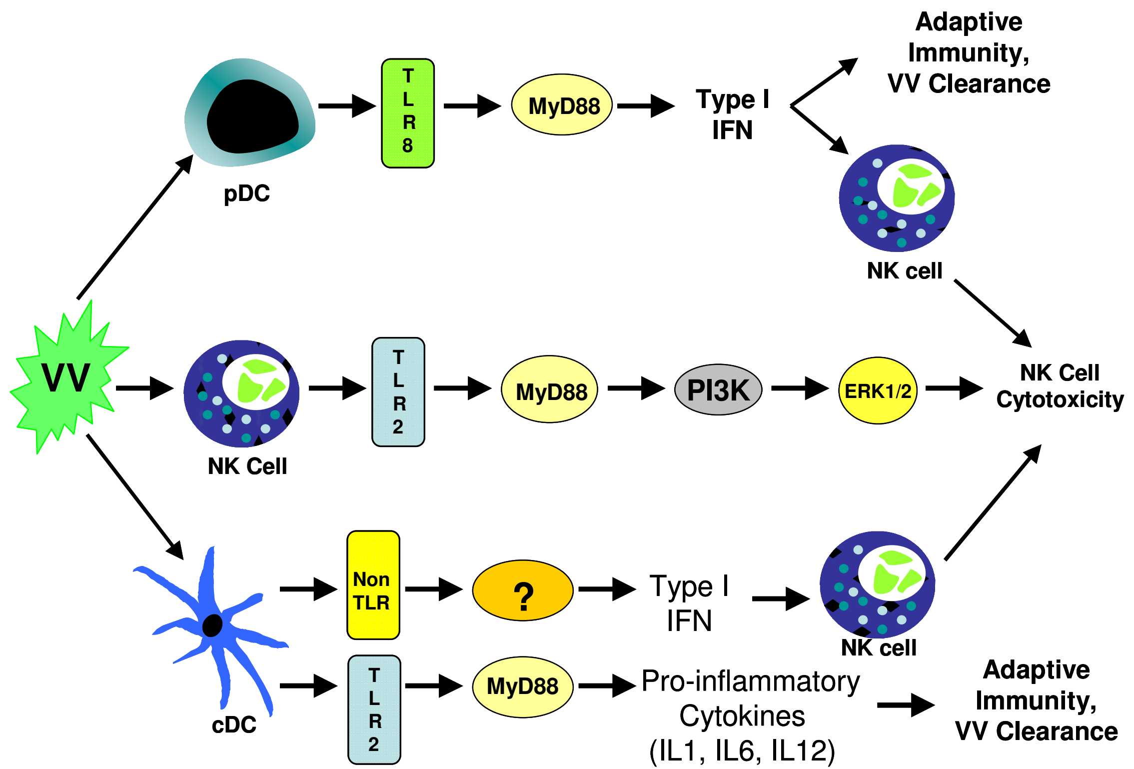

Figure 26: A summary of the mechanisms by which VV activates the innate immune system.

In order to fully understand how VV initiates the innate immune response, further studies will be needed. Whereas TLR8 has been described as an ssRNA sensor, data presented in this thesis project has illustrated that VV-DNA can activate the TLR8 pathway in pDCs, leading to the production of type I IFNs. We have identified polyA- and polyT-rich motifs in the VV genome as the possible ligands for TLR8 activation, although further genomic analysis is required to fully confirm this phenomenon. Genetically mutated ODNs from VV, such as those lacking these A/T-rich sequences, should be generated, and its ability to activate the TLR8 pathway should be analyzed. Moreover, the ability of other viruses, such as HSV-2 and Ad5, to acquire TLR8 reactivity through the addition of A/T-rich sequences should be examined. Moreover, the use of VV-DNA as a vaccine could prove useful, in that it is capable of eliciting a robust type I IFN response, likely without many of the negative effects seen in whole virus immunization.

It has been demonstrated that viral envelope glycoproteins can activate the TLR2 and TLR4 pathways [3-4], and a proteomic analysis of VV has identified numerous glycoprotein candidates [139]. The identification of endogenous VV TLR2 ligands could prove to be a useful tool for vaccine development and immunotherapy, as well as shed light onto the activation of T cells, cDCs, and NK cells. As the TLR2 response is critical to VV clearance in vivo, these glycoproteins could also serve as vaccines, as antibodies to VV glycoproteins have proven to provide a substantial protective effect. Moreover, these TLR2-dependent glycoproteins will likely lack many of the factors of the whole virus

that result in adverse effects. In addition, further analysis into the NKG2D-dependent activation of NK cells in response to VV needs to be performed. It will be critical to identify the NKG2D ligand, such as Rae1 or MULT1, responsible for activation of the NKG2D pathway, as well as the factor that lead to NKG2D ligand upregulation.

From the first successful eradication of a human disease to its potential role in a cancer immunotherapy, VV has demonstrated itself a potent inducer of immunity. However, the mechanisms behind its tremendous ability have yet to be fully realized. This thesis project has demonstrated just a fraction of this virus's power, but has uncovered numerous techniques by which it activates the immune system. Optimal utilization of VV as a future immunotherapeutic will require extensive research into its detailed mechanisms, allowing us to create more efficient and safer treatments.

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Biography

I was born Jennifer Ashley Martinez on February 4, 1979 in Hattiesburg, MS, the first child of Bill and Jan Martinez. I had their undivided attention until 1981, when my sister, Courtney, was born, who turned out to be a far greater gift than the bicycle I had originally wanted. I graduated from Oak Grove High School in 1997 and moved to my family's hometown, New Orleans, LA to attend Tulane University. Needless to say, I very much enjoyed my time there.

In 2001, I received my Bachelor's of Science in Cellular and Molecular Biology and moved to Seattle, WA. I had always enjoyed my laboratory work in multiple developmental biology labs at Tulane University, so I began work at a small biotech company called CellExSys. It was my connection at CellExSys that allowed me to get my next position at the University of Washington in the Gene and Cell Therapy Core Laboratory. Both positions allowed me the opportunity to work in the field of immunology, which inspired me to pursue an advanced degree in the field.

After applying to and interviewing with numerous graduate programs, I was honored and ecstatic to be offered a place in the Department of Immunology at Duke University. In 2005, I moved to Durham, NC to pursue my Ph.D. at Duke University. After 4 ½ years in the laboratory of Dr. Yiping Yang, MD Ph.D., I received my doctorate in immunology. I will be moving to Memphis, TN to begin a post-doctoral fellowship at St. Jude Children's Research Hospital in the laboratory of Dr. Douglas Green, Ph.D., working in the field of autophagy.

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EDUCATION

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RESEARCH EXPERIENCE

Duke University — **2005 to 2010**
Department of Immunology — Laboratory of Dr. Yiping Yang, M.D., Ph.D.

Determine the mechanisms by which the innate immune response recognize and control vaccinia viral (VV) infection

- Demonstrate the requirements for optimal Natural killer (NK) cell activation in response to VV infection via FACS analysis, cytotoxicity assays, NK:DC co-cultures, bone marrow chimera reconstitution, Western Blot analysis, adoptive transfer, and depletion experiments
- Clarify the mechanism by which plasmacytoid dendritic cells produce type I interferons in response to VV infection via FACS analysis, ELISA, siRNA design and implementation, synthetic inhibition in vitro, and Luciferase Assay analysis.

Determine which envelope glycoproteins on the vaccinia viron elicits the production of pro-inflammatory cytokines via the Toll-like receptor-2 pathway

- Cloning and production of recombinant glycoproteins from VV DNA
- GST-purification of recombinant glycoproteins for in vitro stimulation
- In vitro stimulation of conventional dendritic cells with titered concentrations of recombinant glycoproteins

Determine the role of Akt2 in the maturation of endosomes, as well the signaling of endosomal receptors using FACS analysis, ELISA, immunofluorescence, and confocal microscopy

Design and generation of an NK-specific Cre-recombinase mouse to utilize with various floxed mouse strains

University of Washington **2003 to 2005**
Gene and Cell Therapy Core Laboratory Research Scientist

Develop and perform a variety of protocols within the ISO class 7 facility for re-infusion into the subject during Phase I/II clinical trials, such as:

- Rapid expansion of HER-2/neu specific T Cells for the therapeutic treatment of breast cancer
- AAV-mediated delivery of a micro-dystrophin gene into muscles of patients with Duchenne muscular dystrophy
- HBVcore-specific Cytotoxic T-Lymphocytes for cell therapeutic treatment of chronic Hepatitis B
- Autologous PBMC culture with OKT3 and IL-2, transfected with DNA plasmid vector expressing chimeric anti-CD-20 receptor

Organized and developed clinical documents, including standard operating procedures, batch production records, and quality control documents

Assisted in the preparation and submission of three FDA-approved IND (Introduction of New Drug)

Assisted in the creation of a Drug Master File for the Gene and Cell Therapy Core Laboratory

Participated in development of a sterile, high-speed cell sorter to produce pure population of human cells for re-infusion

CellExSys, Inc. **2001 to 2003**
Process Development Research Associate I

Initiate and maintain Donor-Specific REM (Rapid Expansion Method) Cycles in order to produce and analyze HBVcore-specific Cytotoxic T-Lymphocytes for cell therapeutic treatment of chronic Hepatitis B patients via FACS analysis, Chromium51 Release Cytotoxicity Assays, cell culture, and maturation of PBMCs into APCs via viral transformation.

PUBLICATIONS

Martinez J, Huang X, and Yang Y. Direct TLR2 Signaling Is Critical for NK Cell Activation and Function in Response to Vaccinia Viral Infection. *PLoS Pathogens*. (Accepted for publication on January 14, 2010).

Martinez J, Huang X, and Yang Y. Toll-like receptor 8-mediated activation of murine plasmacytoid dendritic cells by vaccinia viral DNA. *PNAS*. (Preparation of reviewers' comments in progress).

Quigley M, Martinez J, Huang X, and Yang Y. A critical role for direct TLR2-MyD88 signaling in CD8 T cell clonal expansion and memory formation following vaccinia viral infection. *Blood.* 2008 23 Oct. (in press).

Martinez J, Huang X, and Yang Y. Direct action of type I IFN on NK cells is required for their activation in response to vaccinia viral infection in vivo. *J Immunol.* 180(3):1592-7, 2008.

Zhu J, Martinez J, Huang X, and Yang Y. Innate immunity against vaccinia virus is mediated by TLR2 and requires TLR-independent production of IFN-beta. *Blood* 109(2):619-25, 2007.

Hardwick A, McMillen D, Martinez J, Austin A, Posey A, Ave-Teel C, Maples P, and Schneider S. Clinical-Scale Production of Antigen-Specific T Cells Directed Against Hepatitis B Virus. *BioProcessing Journal* 2: 27 – 31, 2003.

SCIENTIFIC CONFERENCES

Martinez J, Zhu J, and Yang Y. Immune Mechanisms of Virus Control Program Kickoff Meeting. Conference Participants. NIH, Washington, DC, USA. (12/2009)

Martinez J, Huang X, and Yang Y. Direct action of type I IFN on NK cells is required for their activation in response to vaccinia viral infection in vivo. Poster Presentation. Keystone Symposia: NK and NKT Cell Biology. Keystone, CO, USA. (02/2008)

AWARDS/HONORS

G. Bernard Amos Graduate Student Poster Winner	05/2009
Duke University Graduate Student Symposium Chosen Speaker	11/2008
Keystone Symposia Education Fund Scholarship	02/2008

ADDITIONAL EXPERIENCE

Beyond the Classroom Scientific Lecture	03/2008 to 02/2010
G. Bernard Amos Poster Participant	03/2008 to 05/2009
Mentor, Duke University undergraduate/graduate research	02/2008 to 02/2010
Mentor, Women in Mathematics Mentoring	01/2008 to 02/2010
Guest AP Biology Lecturer, Collierville High School	12/2007 to present
Department of Immunology Faculty Recruit Student Liaison	03/2007 to 02/2010
Teaching Assistant, Principles of Immunology, Duke University	08/2007 to 12/2007
Committee Chair, Dept. of Immunology Retreat Planning Committee	01/2007 to 10/2007
Gordon G. Hammes Teaching Award Committee	08/2005 to 08/2007

COMMUNITY SERVICE/OTHER ACTIVITIES

- Participated in Doughman Competition for Durham SEEDS Project (May 2009)
- Participated in and completed the Joints in Motion Marathon for the Arthritis Foundation in Dublin, Ireland (October 2004)
- Performed volunteer work for the local Seattle National Public Radio station (2001 to 2004)
- Involved in the Cystic Fibrosis Stair Climb fundraising effort (2001 to 2004)