TYPE I IFN REGULATES INNATE IMMUNITY DURING HSV-2 INFECTION

UNDERSTANDING THE ROLE OF TYPE I INTERFERON IN REGULATING THE INNATE IMMUNE RESPONSE DURING HERPES SIMPLEX VIRUS TYPE 2 INFECTION

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LAY ABSTRACT:

Type I interferons (IFN) are a group of proteins that are rapidly produced early during infection and is important for combatting virus infections. We show that type I IFN is not just an antiviral molecule, but can modulate the initial immune response to virus infection. As part of the initial immune response, Natural killer (NK) cells are immune cells that respond rapidly to infection and are a key element in controlling the early stages of infection. We found that type I IFN is critical for activating NK cell function by signaling through an intermediary cell, but can also suppress that same function by directly acting on NK cells. We also found that type I IFN is critical for suppressing a dysregulated immune response that causes severe virus-induced vaginal pathology. Overall, our data suggests that type I IFN is a key antiviral molecule that shapes the immune response to virus infection.

ABSTRACT:

Type I interferons (IFN) are a potent antiviral cytokine group that are key regulators of the immune response against virus infection. Not only does this group activate antiviral states within target cells, it can modulate the innate immune response. In the studies presented, we investigate the effects of type I IFN on the innate immune system during a mucosal vaginal virus infection, herpes simplex virus type 2 (HSV-2), a prominent sexually transmitted infection that causes genital herpes and increases risk of human immunodeficiency virus acquisition. It is well known that type I IFN is critical for natural killer (NK) cell activation. These cells contribute to the antiviral response by suppressing virus replication and aiding in the initiation of the adaptive immune response, particularly through the release of IFN-γ. In the work presented, we demonstrate that type I IFN does not act on NK cells directly for their activation, but instead activates NK cell IFN-γ production by inducing inflammatory monocytes to release IL-18, which in turn, signals NK cells to release IFN-γ during a mucosal HSV-2 infection. Rather, direct action of type I IFN on NK cells serves to negatively regulate their IFN-γ response. We also found that type I IFN was critical for suppressing virus-induced innate immunopathology during HSV-2 infection. Overall, our studies further our understanding of type I IFN and the many roles it plays during virus infection, which has become more relevant as specific therapies altering type I IFN are being used in the clinic. Further, we provide a fundamental understanding of type I IFN and its ability to shape the innate immune response to virus infection by suppressing dysregulated and immunopathological functions while promoting beneficial innate immune responses that can help fight the infection.

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LIST OF ABBREVIATIONS AND SYMBOLS

ACK	Ammonium-chloride-potassium
AHR	Airway hyperresponsiveness
ANOVA	Analysis of variance
B6	C57BL/6
CAF	Central animal facility
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
cDC	conventional DC
CFSE	5,6-carboxyfluorescein diacetate succinimidyl ester
cGAS	Cyclic GMP-AMP synthase
CIHR	Canadian Institutes of Health and Research
CLEC4C-DTR	C-type lectin domain family 4, member C-diphtheria toxin receptor
CMV	Cytomegalovirus
CNS	Central nervous system
CXCL	Chemokine (C-X-C) ligand
CXCR	C-X-C chemokine receptor
DAI	DNA-dependent activator of IFN regulatory factor
DC	Dendritic cell
DMPA	Medroxyprogesterone acetate
DR5	Death receptor 5
ds	Double-stranded
EC	Epithelial cell
ELISA	Enzyme-linked immunosorbent assay
ESD	Extreme studentized deviate
FBS	Fetal bovine serum
FcyRIII	Fc gamma receptor III
g	Grams
gB	Glycoprotein B
H&E	Hematoxylin and eosin
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
hr	Hour
HSE	Herpes simplex encephalitis
HSK	HSV-induced stromal keratitis
HSV-2	Herpes simplex virus type 2

i.p.	Intraperitoneally
i.v.	Intravenously
IFI16	Interferon gamma inducible protein 16
IFN	Interferon
IFNAR	type I IFN receptor
Ig	Immunoglobulin
ILC2	Innate lymphoid cell group 2
IRF-3	Interferon regulatory factor 3
ISG	Interferon stimulated gene
ISGF3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated response element
ITAM	Immunoreceptor-tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
ivag	Intravaginally
JAK	Janus kinase
KIR	Killer immunoglobulin receptor
LCMV	Lymphocytic choriomeningitis
LN	Lymph node
MALT	Mucosal associated lymphoid tissue
MAPK	p38 mitogen-activated protein kinase
MAVS	Mitochondrial antiviral signaling protein
MCMV	Murine cytomegalovirus
MCP-1	Monocyte chemoattractant protein-1
MDA5	Melanoma differentiation-associated gene 5
MDSC	Myeloid derived suppressor cell
MERS	Middle Eastern respiratory syndrome
MHC	Major histocompatibility complex
MIC	MHC class I chain related
mL	Milliliter
MMP	Matrix metalloproteinase
MOI	Multiplicity of infection
MyD88	Myeloid differentiation primary response gene 88
n.s.	Not significant
NET	Neutrophil extracellular traps
NFκB	Nuclear factor kappa B
ng	Nanograms
NK	Natural killer
NO	Nitric oxide
NRG	*Nod-Rag1$^{-/-}\gamma c^{-/-}$*

p.i.	Post-infection
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PDCA-1	plasmacytoid DC antigen 1
PDL1	Programmed death ligand 1
PFA	Paraformaldehyde
Pfu	Plaque forming units
pg	Picograms
PI3-kinase	Phosphatidylinositol 3'-kinase
PKR	Protein kinase RNA-regulated
PMA	Phorbol myristate acetate
poly I:C	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptor
RIG-I	Retinoic acid-inducible gene I
RIPA	radioimmunoprecipitation assay
RNA pol III	RNA polymerase III
RNaseL	Ribonuclease L
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcription polymerase chain reaction
s.c.	Subcutaneously
SARS	Severe acute respiratory syndrome
SEM	Standard error of the mean
SIV	Simian immunodeficiency virus
SLE	Systemic lupus erythematosus
SM	Submucosa
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
STI	Sexually transmitted infection
STING	Stimulator of interferon genes
TAM	Tyro3, Axl, Mer
TBK1	TANK-binding kinase 1
TGF-b	Transforming growth factor beta
TIP DCs	TNF and NO producing DCs
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha

TRAIL	TNF-related apoptosis-inducing ligand
TYK	Tyrosine kinase gene
U	International Units
ug	Microgram
uNK	Uterine NK
UTR	Untranslated region
VL	Vaginal lumen
WT	Wild-type
α	Alpha
α-MEM	Minimum essential medium eagle – alpha modification
β	Beta
γ	Gamma
δ	Delta
ε	Epsilon
ζ	Zeta
κ	Kappa
τ	Tau
ω	Omega

DECLARATION OF ACADEMIC ACHIEVEMENT:

This thesis is prepared in the "sandwich" format as outlined in the "Guide for the Preparation of a Master's and Doctoral Theses". Within this thesis, I have written an over-arching introduction to my area of research, deemed "Chapter 1". Following the introduction, I have included 3 independent manuscripts that I have written and conducted the majority of the experiments included in these studies. The experiments presented were designed by both A. Ashkar and myself. Where others have contributed, I will discuss in the preamble to each manuscript. The first manuscript is a published work in "Chapter 2", while the other two manuscripts, Chapters 3 and 4, are ready for submission. In "Chapter 5", I conclude my thesis by discussing how each of the manuscripts are linked and contribute to an overall picture and understanding about the effects of type I IFN during virus infection. This chapter also discusses the limitations and future directions of the research presented as well as the overall implications.

CHAPTER 1: INTRODUCTION

1.1 Herpes Simplex Virus Type 2

HSV-2 is a sexually transmitted infection, which along with HSV-1, is the predominant cause of genital ulcer disease.[1] HSV-2 transmission is difficult to prevent as it is a lifelong infection with no cure and those with asymptomatic infections unknowingly infect other individuals.[1 2] Symptomatic infections can manifest as painful bilateral lesions which can eventually lead to genital ulcer disease.[1] Moreover, infection in newborns and those that are immunocompromised can result in severe and potentially fatal infections.[3, 4] Individuals with HSV-2 have an increased likelihood of contracting human immunodeficiency virus (HIV), a virus that selectively depletes CD4+ T-cells leading to an increased risk of contracting fatal opportunistic infections.[5] Further, HSV-2 ulcers have been found to release higher quantities of HIV in co-infected individuals, thereby leading to increased transmission of HIV.[6, 7] Despite many years of research, there is no vaccine or cure currently available to combat HSV-2.[8] In order to develop an effective vaccine strategy and effective therapeutics, we need a thorough understanding of the interaction between the virus and host immune response.

1.1.1 Epidemiology

In 2012, there was an estimated 417 million individuals infected with HSV-2, with 19.2 million new infections annually.[9] There is an estimated worldwide seroprevalence of 11.3%, though certain geographical regions have a much higher seroprevalence.[9] The HSV-2 seroprevalence in women (14.8%) is double that of men (8%), which is particularly important, as women can transmit HSV-2 to their neonates causing neurological damage

and/or death if not treated early.[3, 9] Approximately 80% of individuals infected with HSV-2 are unaware they have contracted the virus, as it can be shed asymptomatically.[10] It is suggested that HSV-2 is frequently transmitted asymptomatically to other individuals.[1, 10]

1.1.2 Diagnosis

Individuals with a primary symptomatic infection with HSV-2 can present with clustered vesicles with localized adenopathy located in and around the genitalia. Systemically, this infection can lead to malaise and/or fever-like symptoms.[11] Though many individuals have asymptomatic infections, serological screening for HSV-2 is not recommended, as the test does not have optimal sensitivity, and it was determined that the benefits of screening did not outweigh the harms of false positives.[12] Serological testing is only recommended in certain cases, where individuals are at a higher risk of contracting HSV-2 (i.e. discordant couples), or as part of a sexually transmitted infection (STI) evaluation panel.[12] Diagnosis is generally confirmed by PCR in those presenting with lesions, in order to appropriately treat the infection.[12, 13]

1.1.3 Standard Treatments

The standard antiviral treatment for HSV-2 involves different nucleoside analogues, including acyclovir and valacyclovir, which specifically target the HSV-2 DNA polymerase in infected cells.[1, 14] These treatments can be taken as an ongoing suppressive therapy or during symptomatic episodes.[15] Both treatment regimens have been shown to significantly reduce virus shedding, risk of transmission, genital herpes symptoms, and rates of recurrences.[15] These treatments, however, do not completely eliminate the latent HSV-2 reservoirs.[1] Further, subclinical virus shedding still occurs while receiving antiviral

treatments, even in those who are compliant and given the maximum dose.[16, 17] There have also been reports of infections that are resistant to acyclovir.[18] As many individuals undergoing treatment continue to have breakthrough reactivations, there is still a need for the generation of more effective vaccines and/or treatments.

1.1.4 Site of Infection – The Vaginal Mucosa

As women have a 2-fold increased prevalence of HSV-2, the female genital tract is an area of intense research interest.[9] This higher prevalence in women can be attributed to a number of factors, such as hormonal and anatomical differences.[19] The immune system and subsequent response in the female genital tract is influenced by female hormone cycles.[19] Further, with more than 50 million women using progesterone-based forms of contraception, such as depot medroxyprogesterone acetate (DMPA), there are studies suggesting that these women may have an increased risk of contracting HSV-2.[20] Studies in mice have found that DMPA administration led to increased HSV-2 virus dissemination due to decreased mucosal thickness and decreased cell-to-cell adhesion factors.[20] In humans, Quispe Calla *et al*., compared female genital mucosae prior to and after DMPA administration and found that DMPA decreased cell-to-cell adhesion factors and increased transcription of proinflammatory factors.[20]

Structurally, the female genital tract is divided into upper and lower compartments, each with distinct structures, epithelial architecture, and underlying immune cell populations.[19, 21] The upper female genital tract includes the endocervix, the uterus, and fallopian tubes, while the lower female genital tract consists of the ectocervix and the vagina.[19, 21] The upper tract is composed of type I mucosa, which is an impermeable layer

of columnar epithelium joined by tight junctions.[19, 21] Moreover, this columnar epithelial layer is covered in secretory immunoglobulin (Ig) A and has underlying networks of mucosal associated lymphoid tissue (MALT; clusters of B-cells, CD8+ T-cells, and macrophages).[19, 21] Along with MALT, dendritic cells (DCs), macrophages, and memory lymphocytes underlie the type I mucosal surface in the upper female genital tract.[19, 21, 22] On the other hand, the lower female genital tract primarily consists of type II mucosa.[19, 21] Rather than a single layer of columnar epithelium, stratified squamous epithelial cells are arranged in multiple layers and dominated by IgG antibody secretion.[19, 21] Under the type II mucosa of the lower tract, there are fewer DCs, macrophages, and lymphocytes as well as an absence of MALT.[19, 21] Natural killer (NK) cells are found throughout the female genital tract.[19, 22] A layer of mucous, consisting of high molecular weight glycoproteins called mucins, covers the epithelium of both the upper and lower female genital tracts to trap microbes, though they are produced by goblet cells in the upper tract and epithelial cells in the lower tract.[19, 23] This layer also contains antimicrobial peptides, immunoglobulins, lysozyme, and lactoferrins to combat pathogens.[19, 24] Along with mucous, commensal microbiota colonize the female genital tract, predominantly the lower anatomical region.[19] Lactobacilli are the dominant species in the vaginal mucosa and are responsible for reducing the pH of the environment (~pH of 4-5) through the production of lactic acid.[25, 26] This acidic environment hinders the ability of pathogenic bacteria to colonize the vaginal tract.[26]

1.1.5 HSV-2 Lifecycle

HSV-2 is a double-stranded (ds) DNA virus, approximately 150Kb in size, which primarily infects mucosal epithelial cells of the genital mucosa and establishes latency in the neuronal cell bodies that innervate the mucosa.[27, 28] The structure of the HSV-2 virion is composed of viral DNA enclosed in a capsid that is surrounded by tegument (contains viral proteins for host modulation and gene expression) and encased in an outer envelope.[29] On the outer envelope, glycoprotein B (gB) and gC bind to specific areas on heparan sulfates, while gD binds to herpes virus entry mediators (HVEM) and nectin-1 and -2.[30] Upon tethering and fusion with the cell membrane, the tegument, capsid, and viral DNA are released into the cell.[27, 30] The capsid and viral DNA are subsequently shuttled to the nucleus and the DNA is released into the nucleus.[27] Within the nucleus, HSV-2 utilizes host machinery for replication and transcription of the viral genome.[27] Three waves of viral gene expression occur and are classified as immediate-early (α), early (β), and late genes (γ).[27] First, α genes are transcribed and lead to activation of early gene expression, in which the majority of the enzymes and factors associated with viral DNA transcription are transcribed along with viral DNA replication (second wave).[27] β gene expression is followed by γ gene transcription, which includes most of the structural proteins.[27] It is estimated that infection, entry, and release of new progeny occurs within 24 hours.[27]

Once the virion components and DNA are assembled as viral progeny, they are released from the infected cell either by exocytosis or lysis of the cell.[31] Free viral progeny infects other epithelial cells and can infect neuronal cells innervating the local environment.[27] Within the neuronal cell bodies, HSV-2 can enter a latent state, wherein the virus exists as circular DNA in the nucleus.[27] Replication is held to a minimum in which

only latent-associated transcripts and microRNAs are expressed.[27] In this form, the virus is able to evade immune detection and elimination, thus allowing for its long-term survival within the host.[27] HSV-2 reactivation and infection of the epithelium was originally thought to occur infrequently, as symptomatic recurrences appear, on average, less than ten times per year.[32] However, recent data suggests that HSV-2 reactivates more frequently, approximately every ten days, resulting in subclinical virus shedding.[33] The mathematical model that best fits the clinical shedding data suggests that HSV-2 reactivates from latently infected neurons at different times that contribute to a "slow viral drip".[34] The majority of these subclinical reactivations are cleared by the immune response within 2-12 hours.[33]

1.1.6 Immune Recognition of HSV-2

Recognition of HSV-2 as a foreign and dangerous pathogen is paramount in being able to generate an immune response to combat the infection. To this end, the body employs the use of pattern recognition receptors (PRRs) capable of recognizing specific patterns or structures that are commonly found in pathogenic bacteria and viruses, but not in molecular components of the human body.[35] Upon recognition of HSV-2, type I interferon (IFN) and pro-inflammatory cytokines and chemokines are produced in order to activate an immune response.[36] As HSV-2 is a virus that can be found in both extracellular and intracellular locations, there is an array of PRRs that target molecules/genetic material involved in specific steps of virus entry and replication.[35] Additionally, immune cells surveying the environment can express specific PRRs for pathogen recognition.[35]

Toll-like receptors (TLRs) are membrane-bound PRRs that can be located on the surface of a cell or within intracellular endosomes.[35] Starting with entry of HSV-2 into

epithelial or neuronal cells, TLR2 expressed on the plasma membrane recognizes microbial lipopetide structures and has been shown to recognize HSV-2 envelope receptors gB, gH, and gL.[37] Through the nuclear factor kappa B (NFκB) signalling transduction pathway, TLR2 ligand recognition leads to inflammatory cytokine production.[37]

Once inside the cell, there are a variety of sensors that can recognize HSV-2 DNA and RNA. When HSV-2 DNA is released into the nucleus for transcription, interferon gamma inducible protein 16 (IFI16 or the mouse IFI16 p204 analog) can detect the viral DNA and induce a type I IFN response.[38] Absence of IFI16 p204 in mice leads to increased virus titers after vaginal infection with HSV-2.[38] Viral DNA can also be detected in the cytoplasm by the cytosolic sensor cyclic GMP-AMP synthase (cGAS), again leading to type I IFN production.[39] Both IFI16 and cGAS both signal through the adaptors, stimulator of interferon genes (STING) and TANK-binding kinase 1 (TBK1), which results in interferon regulatory factor (IRF)-3 phosphorylation, dimerization, and induction of type I IFN.[38, 39, 40] As HSV-2 is a DNA virus and replicates in the nucleus, it is not currently known how viral DNA is able to access the cytoplasm during productive infection. In myeloid cells, however, Horan *et al* found that HSV-1 capsid-containing DNA underwent proteosomal degradation in the cytoplasm, allowing interaction between the viral DNA and DNA sensors.[41] Though a multitude of DNA sensors have recently been discovered, such as DNA-dependent activator of IFN regulatory factor (DAI), RNA polymerase III, and the DExD/H-Box RNA Helicases, IFI16 and cGAS have been shown to be the predominant nuclear and cytosolic DNA sensors critical for protection against HSV-1 and HSV-2 viral infection.[38, 39] From nuclear transcription, viral RNA is produced and can be detected in

the cytoplasm by the RNA sensors, retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), which recognize RNA with a 5` triphosphate group and higher order structure RNA, respectively.[40, 42, 43] Upon recognition of viral RNA, these sensors signal through the adaptor, mitochondrial antiviral signaling protein (MAVS), which subsequently leads to IRF3 and NFκB activation to induce production of type I IFN and pro-inflammatory cytokines.[40]

A recent publication documented an antiviral mechanism that occurs independently of TLRs, traditional DNA and RNA sensors, and type I IFN.[44] Iversen *et al.* found that O-linked glycans on the surface of HSV-2 were rapidly detected by epithelial cells after infection and induced CXCL9 and CXCL10 prior to type I IFN induction.[44] This led to the early recruitment of neutrophils, which played a protective role against infection.[44]

Along with epithelial cells, innate immune cells express PRRs that can recognize viral antigens to become key modulators of the immune response. Endosomal TLR9 expression by plasmacytoid dendritic cells (pDCs) has been implicated in HSV-2 recognition and results in IFN-α production.[45] TLR9 recognizes viral hypomethylated CpG, which is found predominantly in microbial DNA.[45] TLR9 recognition of viral DNA results in IFN-α production in pDCs that is dependent upon myeloid differentiation response gene 88 (MyD88) and IRF7 signaling.[45, 46] TLR3 expression by immune cells results in recognition of dsRNA, which leads to type I IFN and pro-inflammatory cytokine production.[47]

These PRRs implicated in recognition of HSV-2 are expressed by both non-hematopoietic and hematopoietic compartments, but their signaling can have vastly

different outcomes. For example, recognition of HSV by peripheral blood mononuclear cells (PBMCs) leads to type I and III IFN induction, but not in fibroblasts.[48] In both humans and mice, TLR3 deficiency can lead to increased susceptibility to herpes simplex encephalitis (HSE) or increased central nervous system (CNS) infection, respectively, despite minimal difference in peripheral control of HSV infection.[48, 49] This would suggest that TLR3 has a unique role in protecting the CNS from HSV infection. Indeed, it was found that astrocyte TLR3 detection of HSV-2 and subsequent type I IFN production was critical for controlling infection in the CNS of mice.[49] Further research is required to understand why similar TLR expression can have different outcomes depending on cell type.

1.1.7 Immune Response to HSV-2

The primary immune response to HSV-2 infection involves both innate and adaptive components, in which the former controls early virus infection and replication while the latter clears the infection. Upon immune recognition of the virus through a diverse array of PRRs, inflammatory cytokines and type I IFNs are released to recruit and modulate the immune cells at the site of infection, as well as initiate the adaptive immune response.[36] Of the various cytokines released, type I IFN is integral for protection and regulation of the immune response, as it places surrounding cells into a protective antiviral state and modulates the antiviral immune response.[50] Production of IFN-β occurs early during primary infection (between 6-12 hours post-infection), which can stimulate the release and amplification of further type I IFN production, including IFN-α.[51, 52] pDCs are recruited to the vaginal mucosa during HSV-2 infection and are the primary producers of

IFN-α, though their requirement for protection is controversial.[53, 54] Previous work by Lund *et al* found that depletion of pDCs using a monoclonal antibody targeting plasmacytoid DC antigen 1 (PDCA-1) resulted in increased pathology and decreased survival, as well as decreased IFN-α production in the vaginal mucosa.[53] Using C-type lectin domain family 4C-diphtheria toxin receptor (CLEC4C-DTR) transgenic mice, in which pDCs are depleted after diphtheria toxin administration, Swiecki *et al* demonstrated that the loss of pDC had no impact on vaginal virus replication, survival, and local IFN-α production.[54] IFN-ε, which is hormonally regulated and constitutively expressed in both mouse and human vaginal epithelium, has been recently demonstrated to be protective against HSV-2 infection.[55]

During a vaginal HSV-1 infection, type I IFN induces resident macrophages and DCs to produce CCL2 and recruit inflammatory monocytes.[56] These inflammatory monocytes then produce a larger amount of CCL2 to induce further infiltration of inflammatory monocytes. Type I IFN also induces production of CCL3, CCL4, and CCL5 from the vaginal epithelium to recruit NK cells in a CCR5-dependent manner.[56] NK cells are an essential component of the innate response to HSV-2.[57, 58] Depletion of these cells using either anti-NK1.1 antibody or through the use of *Il15*$^{-/-}$ mice resulted in increased susceptibility to genital HSV-2 infection.[57, 58] These cells are rapidly recruited to the vaginal mucosa via CCR2 and CCR5 receptors, and upon activation, these cells are capable of inducing cytotoxicity and can release IFN-γ, perforin, and granzyme.[56, 59, 60] However, it is the release of IFN-γ that has been observed to be largely important in controlling early HSV-2 infection.[58] This release of IFN-γ can then induce the production of nitric oxide

(NO), an inhibitor of HSV-2 replication, from cells in the environment, and help initiate antiviral adaptive immune responses.[61, 62, 63] In humans, NK cells are found to accumulate at the site of HSV-2 infection.[64]

The adaptive immune response involves both B- and T-cells, which are predominantly responsible for clearing the viral infection and generating immunological memory against HSV-2. There are numerous papers demonstrating the importance of CD4+ and CD8+ T-cells in clearing HSV-2 viral infection.[64, 65] Uninfected CD11b+ DCs have been shown to present antigen to T-cells in the lymph node in order to activate them.[66] Activated CD4+ T-cells subsequently release IFN-γ in the vaginal mucosa to induce secretion of chemokines CXCL9 and CXCL10 to recruit CD8+ T-cells.[67] These CD8+ T-cells are then able to clear the HSV-2 infection by releasing IFN-γ themselves, as well as inducing apoptosis through Fas-mediated mechanisms.[68]

T-regulatory cells are often considered suppressors of inflammation, though their exact role in HSV-2 infection remains controversial.[69, 70] There is evidence suggesting that they can suppress CD4+ and CD8+ T-cell functions; however, in the absence of these cells, mice become more susceptible to HSV-2 infection due to a delay in innate immune cell migration to the site of infection.[69, 70] Like T-regulatory cells, the exact function of B-cells has yet to be clarified. Though B-cells produce IgG and IgA antibodies capable of neutralizing HSV-2, their role in clearing HSV-2 infection is controversial.[71] Neutralizing antibodies against HSV can bind to the virus in the gap between neurones and the epithelial layer and maternal neutralizing antibodies can reduce neonatal transmission of HSV-2.[72, 73] However, vaccines that have been built upon generating neutralizing antibodies against

HSV-2 have been ineffective in protecting against infection.[8] In an alternate role, B-cells have been observed to activate CD4+ T-cells to release IFN-γ, suggesting a function outside of antibody production.[74]

After primary infection, HSV-2 can remain latent within neurons and reactivate to re-infect the vaginal epithelium. Memory CD4+ and CD8+ T-cells have been shown to remain resident in the vaginal mucosa after primary virus infection.[75, 76, 77] In a mouse model of vaginal HSV-2 immunization, resident memory CD4+ T-cells were found to reside in clusters with macrophages and responded quicker to re-infection and as a result provide better protection.[75] Mouse vaginal infections with either vaccinia virus or lymphocytic choriomeningitis (LCMV) resulted in the accumulation of resident memory CD8+ T-cells, which were responsible for increasing lymphocyte recruitment and activation of effector innate immune cells through cytokine production.[76] In the context of HSV-2 infection, Chen *et al* found that splenic memory CD4+ T-cells post-HSV-2 immunization were able to reactivate NK cell IFN-γ production via a mechanism dependent on IL-2.[78]

In humans, both CD4+ and CD8+ effector memory T-cells have been found to accumulate around symptomatic areas of infection and express markers of resident memory T-cells (Trm), including CD69 and CD103.[79] Of the T-cells in the vaginal tract, women seropositive for HSV-2 had HSV-2-specific CD3+ T-cells in their vaginal mucosa, of which an average of 91.3% were CD4+ and 3.9% were CD8+.[80] CD8+ T-cells have been observed to linger around sites of reactivation, generally peripheral nerve endings, for up to 8 weeks post-infection.[81] A transcriptional analysis of these CD8+ T-cells during a non-

lesion reprieve revealed that they have an upregulation of antiviral and functional markers (e.g. perforin and granzyme).[82] Even those that are asymptomatic have resident memory CD4+ T-cells in their vaginal mucosa.[83] It is likely that HSV-2 lifelong infection is characterized by frequent viral reactivation episodes that are for the most part rapidly cleared by the resident memory T-cell populations that reside in the vaginal mucosa.[33, 75, 84]

1.2 Type I Interferon

Type I IFNs are composed of a group of structurally similar cytokines that all signal through the same receptor.[50] This group consists of IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-δ, IFN-ζ, IFN-τ, and 13-14 subtypes of IFN-α (IFNα1-IFNα13/14).[50] Of the type I IFNs, IFN-α and IFN-β are the most well-researched in terms of their production and functional effects and are the main focus of my thesis. As they signal through a common receptor, all IFN-α subtypes and IFN-β induce a similar array of antiviral interferon stimulated genes (ISGs). However, a growing amount of evidence suggests that each type I IFN subtype can also have differential immunomodulatory effects, known as "tunable" interferon responses, despite signaling through the same receptor.[85] The next few sections will discuss the production and signaling mechanisms of type I IFN as well as its role as a key modulator of the antiviral immune response.

1.2.1 Production

In the context of virus infection, type I IFNs are largely produced in response to PRR virus ligand recognition.[38, 39, 45] There are large differences in production between

IFN-α and IFN-β in terms of the transcription factors required for their production, timing of their production, and the cell types that produce them. Downstream of the various PRRs, TBK1 has been implicated in phosphorylation of the key transcription factors responsible for induction of IFN-α and IFN-β.[86] It is generally thought that IRF3 phosphorylation and NFκB activation is required for an initial wave of IFN-β and IFN-α4 production, which leads to IRF7 phosphorylation that is largely responsible for the positive feedback loop that results in increasing amounts of type I IFN production, including IFN-α.[87, 88, 89] Though most cell types are capable of producing IFN-β (e.g. epithelial cells, macrophages, and DCs), leukocytes are the main producers of IFN-α.[90, 91] pDCs in particular are capable of producing large amounts of IFN-α during HSV-2 infection.[53] During vaginal HSV-2 infection, IFN-β is rapidly expressed between 6-12 hrs post-infection and is upregulated again at 48 hrs post-infection, along with IFN-α.[51, 92] Both subtypes are downregulated at the 72 hr mark of infection.[51, 92] IFN-ε is an interesting type I IFN subtype that has been implicated in protection against HSV-2.[55] Unlike IFN-α and -β which require either PRR or cytokine stimulation for expression, IFN-ε is constitutively expressed by the female reproductive epithelium and is hormonally regulated via estrogen.[55]

1.2.2 Signaling

All the type I IFN subtypes signal through a common type I IFN receptor (IFNAR), composed of the IFNAR1 and IFNAR2 subunits.[93] Type I IFN ligation with IFNAR induces endocytosis of the receptor complex and activation of tyrosine kinase gene (TYK)2 and janus kinase (JAK)2 (associated with IFNAR1 and IFNAR2, respectively).[94] The canonical type I IFN signaling pathway involves TYK2 and JAK2 phosphorylation and

dimerization of IFNAR2-associated STAT1 and STAT2.[41] The heterodimer of STAT1 and STAT2 then forms the IFN-stimulated gene factor 3 (ISGF3) complex with IRF9.[93] The ISGF3 complex then translocates to the nucleus to induce the transcription of canonical interferon stimulated genes.[93] Along with this well-known signaling cascade, many other transcription factors can be activated in response to type I IFN ligation.[93] IFNAR signaling can lead to activation, homo- and heterodimerization of other STAT proteins, including STAT3, 4, 5, and 6.[93] Further, type I IFN can signal through CRK proteins, which can modulate downstream cell anti-proliferation effects.[95] Type I IFN can also signal through the p38 mitogen-activated protein kinase (MAPK) pathway to activate interferon stimulated genes.[96] There is documented evidence demonstrating that type I IFN can signal through a myriad of other factors, including Vav, RAC1, and phosphatidylinositol 3' (PI3)-kinase.[97, 98, 99]

The ability of type I IFNs to have both similar antiviral effects, yet different "tunable" functions, depends on a number of factors, including cell type, timing of production, amount produced and length of type I IFN signaling, interaction with other signaling pathways, binding affinity to IFNAR, and receptor expression. In particular, the differential binding affinities between the different type I IFN subtypes to IFNAR has been shown to have a large impact on their downstream effects.[85] Though all the subtypes bind to IFNAR in a structurally similar way, there is a 1000-fold difference in binding affinities, with IFN-β having the strongest affinity to IFNAR2 and IFN-α1 with the weakest.[85, 100, 101] All type I IFN subtypes can induce an antiviral response, or a "robust" response, as this effect requires a low threshold of IFNAR signaling, in which even low affinity subtypes

can induce an antiviral response.[85, 102] The differential "tuning" effects between type I IFN subtypes requires a much higher threshold of activation, where subtypes with a higher affinity and higher concentrations of type I IFN are more likely to induce these effects.[85, 102] Additionally, a low level of IFNAR receptor expression has been shown to be sufficient for an antiviral response, but an antiproliferative "tunable" response requires a larger level of receptor expression.[103] Downstream of the receptor, promotors of genes leading to these immunomodulatory effects did not contain binding sites for IFN-stimulated response element (ISRE), a canonical transcription factor downstream of ISGF3.[104] Further, of the IFN subtypes and mutants tested, the subtle changes in STAT phosphorylation were not sufficient to explain the large difference in 'tunable' antiproliferative function.[102] Of notable interest, it was recently found that IFN-β can signal through IFNAR1, without IFNAR2, and activate a pathway independent of JAK-STAT.[105]

1.2.3 Direct Antiviral Functions

Type I IFN is well known for its antiviral effects. Indeed, its discovery over 50 years ago was for its ability to "interfere" with influenza infection.[50] During virus infection, type I IFNs are known to induce antiviral responses by responding to type I IFN production in an autocrine and paracrine fashion. Type I IFN signalling activates a number of ISGs that have direct antiviral effects by targeting host functions that viruses require for reproduction. Induction of protein kinase RNA-regulated (PKR) downstream of type I IFN signaling inhibits cellular translation.[106] In mice treated with an IFN-α1 transgene, absence of PKR during vaginal HSV-2 infection led to increased viral titers in the vaginal tissue and brainstem and decreased survival compared to controls, suggesting IFN-α1 induction

of PKR can limit HSV-2 replication *in vivo*.[107] Another "classical" ISG, the 2',5'-oligoadenylate synthetase family, activates ribonuclease L (RNase L), which when activated also inhibits cellular translation by cleaving single stranded RNA.[108, 109] Their role during vaginal HSV-2 infection, however, promotes virus-induced immunopathology, as mice lacking RNase L show decreased pathology and increased survival.[110] Use of a triple knock-out of the classical ISGs (PKR, RNase L, and Mx1) have demonstrated that other ISGs have antiviral activity.[111] Since then, over 300 ISGs have been identified and has demonstrated antiviral and immunomodulatory effects.[112, 113]

1.2.4 Indirect Antiviral Functions – Regulation of the Immune Response

Along with its direct antiviral effects, type I IFN is a key modulator of the immune response, though its effects can range from activating or enhancing a specific function to suppression. These broad and paradoxical effects of type I IFN can be explained by the subtype of type I IFN produced and the context in which type I IFN signals. Recent evidence has shown that the effects of type I IFN differ between subtypes, the length of viral persistence (acute vs. chronic), and the cell type responding to type I IFN.

As mentioned previously, the subtypes of type I IFN have been shown to have different "tunable" functions. In reference to virus infection, James *et al* treated mice with different subtypes of type I IFN (α1, α2, α4, α5, α6, α9, and β) and subsequently challenged these mice with influenza.[114] Though the same amount of each subtype was administered, their effect on virus replication greatly differed. IFN- α5 and IFN- α6 pre-treatment resulted in the greatest virus reduction, while IFN- α1 had very little effect.[114] Further, treatment of

DCs with IFN-α1, -α2, -α8, -α21, or –β, resulted in varying profiles of DC surface receptor expression and cytokine production.[115]

The effects of type I IFN differ between an acute infection and chronic infection and even between a low IFN signature and high IFN signature. Chronic infections, such as simian immunodeficiency virus (SIV), hepatitis C virus (HCV), and LCMV (Clone 13), can have high IFN signatures, where there is an observed persistent type I IFN signature.[116, 117, 118] During an acute primary infection, the type I IFN antiviral response is essential for limiting virus replication.[119] During a chronic infection, however, a high and persistent type I IFN signature during LCMV infection led to lymphoid structure deterioration, inhibition of T-cell responses through increased PD-L1 expression on DCs and increased IL-10 levels.[118] In fact, inhibition of the type I IFN receptor or IFN-β rescued lymphoid structure and led to an increased number of antiviral T-cells ultimately resulting in an earlier clearance of infection.[118, 120] A similar phenomena has also been observed during SIV infection in rhesus macaques, where type I IFN function early during infection resulted in suppression of systemic infection but chronic type I IFN activation led to an increased SIV viral load and increased CD4+ T-cell depletion.[116, 121] Though HSV-2 is a lifelong infection, biopsies of genital lesions have a low type I IFN signature.[122] Outside of virus infection, there is a growing amount of evidence demonstrating that select autoimmune diseases, such as systemic lupus erythematosus (SLE), have an elevated type I IFN signature that contributes to the disease.[123] Moreover, a recently developed antibody targeting human IFNAR has had a significant therapeutic effect in individuals with SLE, particularly those with a high type I IFN signature.[124]

Adding further complexity to the type I IFN fabric, the effects of type I IFN are different for each cell type and within that cell type, a pattern has emerged where type I IFN suppresses proliferation of naïve adaptive immune cells, but is involved in their activation. In the case of CD8+ T-cells, type I IFN suppresses cytokine secretion of naïve CD8+ T-cells and can even induce apoptosis of these cells.[125, 126] Further, presence of type I IFN during APC polarization of naïve CD4+ T-cells, resulted in suppression of Th1 cytokine production.[127, 128] During the T-cell activation and polarization process, type I IFN is involved in DC maturation and upregulation of major histocompatibility complex (MHC) expression, thus it is critical for generating optimal antiviral T-cell responses.[125, 129, 130] Further, type I IFN signaling within antiviral CD8+ T-cells protects these cells from NK cell lysis.[131] Similar to naïve cells, type I IFN can inhibit proliferation and can lead to apoptosis in memory CD8+ T-cells, but has also been shown to be required for proliferation of effector CD8+ T-cells.[125, 132] While exposure of immature B-cells to type I IFN suppresses B-cell survival and maturation, during virus infection, type I IFN enhances B-cell activation.[133, 134]

Within the innate immune cell compartment, type I IFN is critical for NK cell function, but there is evidence suggesting that type I IFN can also negatively regulate these cells – this will be addressed in further detail in the NK cell chapter of the introduction.[135, 136] During infection, type I IFN induces expression of CCR2 ligands, which lead to the recruitment of inflammatory monocytes.[60] Once recruited to the inflamed tissue, these inflammatory monocytes differentiate into CD11c+ MHC II+ APCs.[60] During acute infection and TLR stimuli (e.g. polyinosinic:polycytidylic acid (poly I:C)), type I IFN

mediates DC maturation and upregulates costimulatory molecules as well as MHC II-antigen complex expression.[129, 130, 137] While type I IFN plays a role in promoting antiviral DC functions, it has a largely suppressive effect on macrophages. Type I IFN dampens the effects of IFN-γ on macrophages, thus suppressing the ability of IFN-γ to upregulate MHC II expression, as well as secretion of proinflammatory cytokines IL-12, IL-1β, and TNF-α. This was largely established through the downregulation of IFN-γ receptor expression.[138, 139] During mycobacterial leprae and mycobacterial tuberculosis infections, type I IFN induces IL-10 production, which suppresses the ability of IFN-γ to activate macrophage antimicrobial functions.[140]

1.2.5 Type I IFN Deficiency

Type I interferon is critical for protection against HSV-2, as mice lacking the type I IFN receptor (*Ifnar*$^{-/-}$) have increased vaginal virus replication and decreased survival.[141] In humans, individuals with STAT1 deficiency have a deficient response to IFN-α, as STAT1 is a key component of the ISGF3 signaling complex.[142] These individuals are susceptible to both viral and bacterial infections, with one individual developing herpes simplex encephalitis (HSE).[142, 143] Individuals with a deficiency in UNC-93B, involved in endocytosis and signaling of TLR3, 7, 8, and 9, have significantly decreased levels of type I and III interferon production when their fibroblasts or PBMCs are stimulated with TLR agonists and developed HSE.[144] Fibroblasts from individuals with a TLR3 deficiency have a defective type I and III IFN response.[48] Similar to a UNC-93B deficiency, individuals with a TLR3 deficiency have been observed to develop HSE.[48]

1.3 Natural Killer Cells

NK cells are a key component of the innate immune response and are known to mediate the primary immune response to eliminate both virally infected and tumor cells.[145] Since their discovery over 45 years ago, an immense body of literature has been dedicated to understanding the biology, function, and regulation of these cells.[145, 146] They differ from the T-cell lineage in that they do not express antigen-specific receptors generated from rearranged genes nor do they require prior antigen-exposure for their activation or elicitation of their functions.[147] Instead, their functions are regulated through the integration of signals from both activation and inhibitory stimuli.[148] As they play a critical role during virus infection, it is important to understand the mechanisms by which these cells are activated to help clear the infection and negatively regulated to prevent them from becoming immunopathogenic.

1.3.1 Development

NK cells are largely differentiated from hematopoietic stem cells (HSC) in the bone marrow, though recent evidence has detected NK cell progenitors in circulation, suggesting that NK cells can differentiate at other sites, such as the lymph node and thymus.[149, 150] The timely expression of various transcription factors directs the differentiation of NK cells.[151] Beginning as a multipotent progenitor with the capacity to differentiate into numerous cells types, NK cells go through a series of intermediate stages that are defined by specific patterns of cell-surface expression markers, to finally become mature, functional NK cells.[151] In the mouse model, the process of NK cell differentiation begins with HSC lineage tapering into the common lymphoid progenitor.[151] From there, early NK cell development

continues through pre-pro NK cell and NK progenitor stages to the immature NK cell, where NK cells gain expression of NK1.1 and eventually DX5.[151, 152] Throughout this process, NK cell responsiveness to IL-15 is a cytokine critical for NK cell survival and proliferation.[153, 154]

From an immature NK cell state, NK cells undergo a maturation process, defined by the expression of CD27 and CD11b, to gain functional competence.[155] Immature subsets are classified as a double-negative population.[155] As NK cells mature, they express CD27, and subsequently CD11b.[155] The double positive subset is considered to be the highest functional effector state, characterized by having the lowest threshold of activation.[155] Expression of CD11b alone, when CD27 expression is lost, defines the most mature terminally differentiated subset of NK cells.[155] Though some groups have found that expression of CD27 is found on the most cytotoxic and cytokine-secreting cells, others have determined that expression of CD11b characterizes NK cells with the highest effector potential.[155, 156] Along with the expression of CD11b, NK cells gain expression DX5 and KLRG1 as they progress to the later stages of maturation.[151, 152, 157] The expression of transcription factors T-bet and Eomes are critical for maintaining a mature NK cell phenotype.[158]

Less is known about human NK cell development as intermediate precursors have been difficult to detect.[159] Human NK cells can be subdivided based on their expression of CD56 and CD16. $CD56^{bright}CD16^{-}$ NK cells are known for their cytokine-secreting capabilities and are less cytotoxic than their $CD56^{dim}CD16^{+}$ counterparts.[150] Some studies suggest that $CD56^{bright}$ NK cells are the precursors to a more mature $CD56^{dim}$ subset, as

$CD56^{bright}$ NK cells have been shown to contain longer telomeres and emerge prior to $CD56^{dim}$ NK cells after a HSC transplant.[159, 160, 161] However, a lineage study on rhesus macaques suggested that $CD56^{bright}$ and $CD56^{dim}$ arise from separate precursors, as the $CD56^{dim}$ circulating population had a distinct and separate clonal pattern.[159, 162]

NK cells have an array of variegated receptors that can be expressed in different combinations to create a diverse number of expression patterns and therefore a pool of NK cells that can have varying responses.[163] From birth, NK cell diversity increases with age; however, this increase in diversity has not been found to enhance antiviral responses.[163] In fact, a study of a female cohort in Kenya found that increased NK cell repertoire diversity was associated with a heightened risk of HIV infection.[163]

1.3.2 Kinetics and Compartmental Distribution

NK cells are found throughout the body in lymphoid tissues (eg. bone marrow, spleen and secondary lymphoid tissue), mucosal tissue (eg. female genital tract), and organs (eg. liver and lung).[159, 164] In mice, the highest proportion of mature NK cells are found in the liver and lung; however, the highest total number of NK cells was found in the spleen.[164] In humans, NK cells constitute a range between 5-20% of lymphocytes in the blood.[165] 90% of these NK cells are the more cytotoxic $CD56^{dim}$CD16+ phenotype, while the remaining 10% are the cytokine-producing $CD56^{bright}$CD16- cells.[150, 165] Similar to mice, human NK cells constitute a large proportion of the immune cells that can be found in the liver with distinct phenotype and function.[166]

Steady state trafficking of mouse NK cells involves egress of NK cells from the BM (where they largely are differentiated from) into circulation.[167] In the bone marrow, a

large proportion of immature CD27-CD11b- NK cells reside, where they express CXCR4 to maintain their retention within the bone marrow environment.[167] Mayol *et al* found that as NK cells differentiate, they lose expression of CXCR4 and gain expression of sphingosine-1 phosphate receptor 5 (S1P5), which allows for their egress from the BM.[167] S1P5 was also found to be required for NK cell recruitment to the blood, spleen, and lung as well as inflamed non-lymphoid tissue.[168] In the periphery, circulating NK cells express CD62L and CCR7, which allows for their trafficking to lymphoid tissues through high endothelial venules.[169, 170] During inflammation, a number of chemokine receptors are involved in the recruitment of NK cells to the inflamed tissue, including CCR2 and CCR5.[57, 60]

Resident NK cells can be found within tissues, in particular the uterus, liver, and secondary lymphoid tissues.[159] In mice, we and others have shown that NK cells, classified as CD3-NK1.1+ cells, are detectable in the mouse female genital tract.[60] In humans, these resident NK cells usually express high levels of CD56 and minimal levels of CD16, and are considered to be ILC1-like. Further, they express receptors that maintain their residence within these tissues, such as CD69, CXCR6, and CCR5.[159] Within the female genital tract, there lies a specific phenotype of NK cells, known as uterine NK cells (uNK).[159, 171] These uNK cells are typically found in the endometrium of non-pregnant women or the decidua of pregnant women.[159, 171] These uNK cells constitute approximately 30% of lymphocytes in the endometrium and constitute a large proportion of lymphocytes in the decidua of pregnant females.[159, 171] Like other tissue-resident NK cells, they belong to the $CD56^{bright}$ phenotype of NK cells and express CD151 along with CD9, part of the transmembrane 4

super family.[172] In contrast to circulating NK cells, they have a limited cytolytic capability and do not produce IFN-γ to the same level as their blood counterparts, respectively.[173] Importantly, these uNK cells are important for pregnancy, as they mediate trophoblast invasion and spinal artery remodeling via IFN-γ expression and other factors.[174]

Beyond the endometrium, NK cells can be found throughout the female genital tract and have been found to express different surface receptors depending on whether they reside in the upper or lower tracts.[175] In the upper tract of women, NK cells expressed CD94 and CD69 but low levels of CD16, whereas NK cells in the lower tract expressed CD16 but lacked CD94 expression.[175] Both endocervix and ectocervix NK cells were capable of producing IFN-γ upon stimulation.[175] In rhesus macaques, the majority of NK cells within the female genital mucosa were CD56+ and either had CD16 expression or lacked CD16 expression.[176] NK cells with high expression of CD56 had a greater tendency to produce IFN-γ when stimulated.[176]

1.3.3 Activation of NK cells

In order to respond to stimuli, such as virally infected cells, NK cells are required to recognize an inhibitory self-ligand stimuli, via Ly49 receptors in mice and killer immunoglobulin receptors (KIRs) in humans (both of which recognize MHC I), that enables them to become functionally competent – a process known as "NK cell education or licensing".[177] Absence of these inhibitory signals creates hyporesponsive, yet self-tolerant, NK cells, thereby preventing autoimmunity.[177, 178] NK cell education is an ongoing process that is dictated by signals in the environment.[177] The transfer of mature and licensed

mouse NK cells to an MHC I-deficient mouse reverts the transferred NK cells into a hyporesponsive state.[177, 179]

NK cells are a plastic cell type not only in terms of their education, but in their activation process as well.[180] Their activation and functional state is very much a reflection of their environment.[180] As mentioned previously, NK cells can express both activating and inhibitory receptors, and the integration of these signals dictates their activation status; inhibitory signals (e.g. MHC I-specific receptors), which exert a dominant influence, will suppress their cytotoxic function against healthy cells, while an overwhelming activation signal will trigger their function against virally infected cells or cells under duress (e.g. cells expressing stress ligands along with downregulation of MHC-I).[145, 148, 180] Indeed, cells that have limited or loss of MHC class I expression become targets for NK cell lysis.[180] The well-known inhibitory receptors in humans consist of KIRs (with long cytoplasmic tails) as well as NKG2A, whereas in mice they are predominantly constituted by the Ly49 family and NKG2A.[180, 181] These inhibitory receptors recognize self human leukocyte antigen (HLA) or MHC I as ligands and signal through immunoreceptor tyrosine-based inhibition motifs (ITIMs) in their cytoplasmic tails.[180, 181] Activation receptors predominantly recognize ligands that are upregulated on cells under stress, such as MHC class I chain-related (MIC)-A or MICB, ligands for NKG2D.[180, 181] These receptors include the short cytoplasmic tail KIR family in humans, as well as the natural cytotoxicity receptors (NKp44, NKp46, and NKp30), NKG2D, DNAM-1, CD16, and NKC2C among many others.[180, 181] Upon ligation, signaling mainly occurs through a cytoplasmic immunoreceptor-tyrosine-based activation motif (ITAM).[180]

Activating cytokines, such as IL-12, IL-15, IL-18, and type I IFN, are also capable of inducing NK cell effector functions.[145] Moreover, specific microRNAs have been implicated in NK cell activation.[182] NK cells can also become activated in response to specific viral components, such as m157 on murine cytomegalovirus (MCMV)-infected cells.[183] Lastly, NK cells can respond to cells opsonized with antibody through their FcγRIIIA (i.e. CD16), which triggers antibody-dependent cellular cytotoxicity (ADCC).[183]

A variety of immune cell types have been observed to activate NK cells during virus infection. A wide range of evidence suggests that DCs, monocytes, and neutrophils are all capable of activating NK cells, and eliciting IFN-γ production, in a number of conditions involving different types of viral infections.

DCs are the core subset of immune cells that bridge the innate and adaptive immune response.[184] Armed with a number of pattern recognition receptors, they are capable of detecting pathogens in peripheral tissues, which triggers them to migrate to the lymph node and activate the adaptive immune response.[185] Along with initiating the adaptive immune response, DCs have been observed to play an important role in activating NK cells as well.[184, 185] Plasmacytoid DC production of type I IFN has been observed to induce NK cell activation, particularly cytotoxicity.[184, 186] Moreover, conventional dendritic cells (cDCs) can be activated to produce NK cell activating cytokines, such as IL-12, IL-15, and IL-18, all of which can induce NK cell IFN-γ production as well as cytotoxicity.[184, 185] In particular, the trans-presentation of IL-15 by CD11c+ DCs is a well-known and well-referenced mechanism by which NK cells are activated during virus infection and TLR ligand stimulation.[187, 188] DCs have also been shown to activate NK cells through NKG2D

ligand expression.[189] In a mouse model of vaccinia virus infection, both IL-18 and NKG2D ligand-receptor interaction were required for the activation of NK cells.[189]

Infiltrating monocytes have also been implicated in activating NK cells during bacterial and viral infections.[60, 190] These cells, originating from the bone marrow, are known to circulate within the peripheral blood.[191] They replenish macrophages and DCs within tissues during the steady state and are heavily recruited to sites of inflammation.[191] Geissmann *et al* were able to functionally and phenotypically categorize monocytes into two populations: an 'inflammatory' monocyte population and a 'resident' monocyte population.[192] Both monocyte populations stably express CD11b and CD115 and have moderate expression of F4/80.[192] Additionally, the inflammatory monocyte population highly expresses Ly6C, CD62L, and CCR2 with low expression of CX3CR1, while the resident monocyte population has high expression of CX3CR1 with low expression of CCR2, CD62L, and Ly6C.[192] The inflammatory population, akin to its name, is quickly recruited to sites of inflammation, where they can begin to upregulate DC markers, such as MHC II and CD11c, and produce inflammatory cytokines.[192] On the other hand, resident monocytes replenish and differentiate into macrophages and DCs during the steady state.[192] In terms of NK cell activation, the differentiation of monocytes into DCs can be a mechanism by which monocytes indirectly activate NK cells, as DC-NK cell interactions are well documented.[187, 188] Soudja *et al* found that a depletion of the inflammatory monocyte population via CCR2 led to a decrease in NK cell IFN-γ and granzyme B production during *Listeria monocytogenes* immunization.[190] Likewise, *ccr2*$^{-/-}$ mice, which have a reduction in circulating monocytes, had a significant decrease in NK cell IFN-γ

production during vaginal HSV-2 infection.[60] However, the study also observed a decrease in NK cell recruitment, which the researchers attributed to a need for CCR2 in NK cell homing to the vaginal tract.[60] Two groups have also observed that depletion of monocytes from PBMCs in an *in vitro* model of HCV infection reduced NK cell IFN-γ production.[193, 194] Serti *et al* observed that monocyte production of IL-18 was responsible for activating the NK cells.[193] On the other hand, Zhang *et al* suggested that type I IFN was required to sensitize NK cells to monocyte IL-15 stimulation.[194]

Macrophages have also been implicated in activating NK cells during infection.[195, 196] Co-culturing of macrophages and NK cells under various stimulatory or infection conditions have revealed that macrophages can increase NK cell activating receptor expression as well as activation of NK cell effector functions.[195, 196] Macrophage ligation of the NK cell NKG2D receptor, along with many other activating receptors, can activate NK cell cytotoxicity, as well as the release of pro-inflammatory cytokines, particularly IFN-γ.[196, 197, 198] In particular, researchers have found that macrophages infected with intracellular bacteria can upregulate NK cell receptor ligands to trigger NK cell-mediated cell death of the infected macrophages.[199] The secretion of IL-18 has been detected from macrophages stimulated with TLR ligands, which is involved in triggering the release of NK cell IFN-γ production.[200]

Though less well documented and known, neutrophils have been observed to activate natural killer cells during infection.[201] Like monocytes, neutrophils are rapidly recruited to sites of inflammation where they have a host of different functions capable of combatting infection.[202] Functionally, they are capable of phagocytosing pathogens,

deploying granules containing antimicrobial and antibacterial factors, releasing neutrophil extracellular traps (NETs) to capture microbes in antibacterial-laced chromatin nets, and releasing cytokines to help modulate the inflammatory environment.[202] Neutrophil depletion has led to decreased NK cell IFN-γ production in a number of infections.[203, 204] In the case of *L. pneumophila*, the decrease in IFN-γ production was due to diminished neutrophil IL-18 production, while in the case of the *L. monocytogenes* infection, the decrease was due to a lack of neutrophil IL-12 production.[203, 204] Recently, Jaeger *et al* observed that neutrophil depletion prevented NK cells from fully maturing as well as inhibited their ability to produce IFN-γ in response to activating receptor ligation.[205] Importantly, they were able to corroborate their data in humans with severe congenital neutropenia.[205] Lastly, there is also evidence that neutrophils can indirectly stimulate NK cell IFN-γ production by activating DCs to produce IL-12.[206]

1.3.4 Antiviral Functions

Upon activation, NK cells utilize a number of effector functions to both eliminate virally infected cells and contribute to the pro-inflammatory environment to help activate adaptive immune mechanisms that will ultimately clear the infection.[145] NK cells can induce cell death through the release of perforin and granzyme molecules and expression of death receptor ligands.[145, 151] In the former, NK cells form an immunological synapse with their target cell. This induces the release of perforin, which creates pores in the target cell membrane, allowing the entry of granzymes into the cell which are responsible for inducing cellular apoptosis.[151] In the latter, NK cells can express the ligands for either Fas or TNF-related apoptosis-inducing ligand (TRAIL) receptors, which when ligated will

induce the activation of caspases and cellular apoptosis in the receptor-expressing cells.[151] Type I IFN-induced NK cell cytotoxicity is heavily dependent on the transcription factor STAT1, as NK cells from *Stat1*$^{-/-}$ mice have impaired cytotoxicity against target cells.[207] Further, an interesting study by Choi *et al* found that the majority of target cell death resulted from bursts of NK cell killing of target cells, where NK cells would become activated and become "serial killers" and induce apoptosis of subsequent target cells that were not only in close proximity, but also in quick succession.[208]

NK cells can release pro-inflammatory cytokines, such as IFN-γ and TNF-α among many other cytokines, which have antiviral and inflammatory effects on other cells in the microenvironment.[145] During virus infection, the release of early IFN-γ is one of the hallmarks of NK cell function.[135] This IFN-γ release has been shown to promote the priming of Th1 cells by inducing maturation and the antigen-presenting function of DCs as well as promote Th1 priming in the lymph node (LN).[62, 63] Further, NK cell IFN-γ production is essential for controlling initial virus replication, as absence during the early phases of infection results in increased virus.[135] This release of IFN-γ can also induce the production of NO, an inhibitor of virus replication, from cells in the environment.[61] IFN-γ production from NK cells is dependent upon the transcription factor STAT4, where an absence of STAT4 reduces the level of IFN-γ detected from NK cells.[209]

While promoting virus clearance and shaping the adaptive immune response, NK cells use these effector functions to eliminate infected macrophages and DCs, along with immature DCs during infection.[199, 210, 211, 212] Further, they have also been found to induce apoptosis of both CD4+ and CD8+ T-cells to potentially limit the amount of

immunopathology caused by the adaptive immune response.[131, 210, 213] Thus, NK cells have a hand in regulating both the innate and adaptive immune responses as well as reducing viral burden during the early stages of infection.

1.3.5 Negative Regulation of NK cells

As mentioned previously, the predominant method by which NK cell cytolytic function is suppressed is through the ligation of NK cell inhibitory receptors.[148] However, there are numerous other mechanisms by which other immune cells or factors can suppress NK cell function. Immune cells in the local microenvironment are capable of secreting immunoregulatory cytokines, such as transforming growth factor (TGF)-β, IL-10, and type I IFN, all of which have been shown to suppress NK cell antiviral functions.[136, 214, 215] Moreover, a plethora of microRNAs have been implicated in dampening NK cell effector function.[216]

Specific microRNAs (miR 132, 212, and 200) were observed to reduce STAT4, pSTAT4, and IFN-γ expression within NK cells when upregulated after IL-12 activation.[216] Alternatively, other microRNAs (29, 223, 27) have been found to inhibit IFN-γ, perforin, or granzyme B production by targeting and repressing their specific 3' untranslated regions (UTRs).[217] Additionally, T-regulatory cells have been shown to regulate NK cell proliferation and activation through the secretion of TGF-β.[214, 218] Furthermore, T-regulatory cells express the IL-2 receptor to a high degree, which consumes and competes for IL-2 available in the environment. In numerous studies, depletion of T-regulatory cells results in increased activation and proliferation of NK cells, while co-culture with T-regulatory cells decreases NK cell cytotoxicity.[214, 218, 219, 220]

Within the last 15 years, there has been a growing body of evidence suggesting that myeloid derived suppressor cells (MDSCs) are capable of inhibiting NK cell activation and function, particularly within the tumor microenvironment.[221] MDSCs are made up of a heterogeneous population of immature or progenitor myeloid cells, best known for their ability to negatively regulate T-cell function.[221] In the steady state, immature myeloid cells produced in the bone marrow differentiate into macrophages, dendritic cells, or granulocytes; however, in areas of inflammation or pathological conditions, such as cancer, MDSCs expand due to an obstruction in the differentiation process from immature to mature myeloid cells.[221] There is evidence to suggest that MDSCs are capable of suppressing NK cell function, in both cancerous and infectious conditions.[222, 223] In a mouse model of adenocarcinoma, Mundy-Bosse *et al* observed that NO production by MDSCs decreased the responsiveness of lymphocytes, including NK cells, to respond to type I IFN.[222] Additionally, Yiping Yang's group observed that MDSCs directly repressed NK cell activity, particularly IFN-γ production, through the production of reactive oxgen species (ROS) during vaccinia virus infection and in response to adenovirus vectors.[223, 224]

1.3.6 Antiviral Role of NK cells during HSV-2 infection

NK cells are an important component of the innate immune response during herpes virus infections, including HSV-2. Pediatric patients have been observed to have an increased susceptibility to herpes virus infections if they have NK cell defects.[225] In a humanized mouse model, human NK cells do indeed home to the vaginal tract during infection.[226] Further, human NK cells in LN were able to produce IFN-γ during an *in vitro* HSV-2 infection.[226] In a mouse model of HSV-2 infection, depletion of NK cells through

an anti-NK1.1 antibody rendered mice more susceptible to HSV-2 infection, as they had increased viral titers.[57] In addition, *I115*$^{-/-}$ and *Rag2*$^{-/-}\gamma c^{-/-}$ mice, both of which lack NK cells, had a significantly higher mortality rate in response to a non-lethal infection with HSV-2.[58] Gill *et al* determined that NK cells respond to HSV-2 infection with IFN-γ production, particularly during day 2 post-infection, as depletion of NK cells abrogates this early IFN-γ response.[135] IFN-γ has been observed to be important during the antiviral response, as *Ifnγ*$^{-/-}$ mice have increased susceptibility to HSV-2 infection.[58] Interestingly, it was observed that NK cells can respond directly to HSV-2 antigens through TLR2.[227] In responding directly to HSV-2 antigens, these NK cells were then observed to present antigen to CD4+ T-cells and augment DC function.[227] During a recall response, Chen *et al* found that the NK cell IFN-γ response during a secondary challenge was mediated by antigen-specific CD4+ T-cell IL-2 production.[78]

1.3.6 Type I IFN and NK cell activation

Type I IFN has a wide range of effects on NK cells, including maturation, proliferation, activation, and inhibition of function. NK cells found within mice deficient in IFNAR have a reduced proportion of mature (i.e. CD11b+) NK cells in the spleen, however, whether a similar phenomena is observed in other peripheral tissues has yet to be determined.[228] During virus infection, type I IFN has an essential role in the activation of NK cells.[135] Not only do *Ifnar*$^{-/-}$ mice have increased susceptibility to HSV-2 infection, but Gill *et al* also observed that the NK cells of *Ifnar*$^{-/-}$ mice were unable to produce IFN-γ in response to HSV-2 infection.[135] This has also been observed in the cases of murine cytomegalovirus (MCMV), vaccinia virus, and adenovirus infections, among many others

infections.[188, 229, 230] Further, NK cells derived from *Ifnar*$^{-/-}$ mice have reduced cytolytic ability against target cells *in vitro*.[231] Recently, Madera *et al* found that type I IFN receptor was essential for protecting NK cells against fracticide during MCMV infection.[232]

In the literature, the mechanism by which type I IFN activates NK cell IFN-γ production during viral infection is widely discussed and researched, yet a consensus has not been reached. There are currently three hypothesized scenarios by which type I IFN can activate NK cells: type I IFN can directly bind to IFNAR on NK cells, type I IFN indirectly activates NK cells by activating an intermediary cell to induce NK cell activation, or NK cells require both direct and indirect type I IFN signalling in order to become optimally activated.

In some of the first papers to address the topic, Martinez *et al* and Zhu *et al* both observed that direct type I IFN ligation on NK cells was essential for their activation of both cytotoxicity and IFN-γ production in response to vaccinia virus and adenovirus vectors.[229, 230] More recently, this observation was corroborated for the secretion of NK cell IFN-γ in LCMV infection.[233] Moreover, Gibbert *et al* observed that a specific subtype of IFN-α11 directly activated NK cell cytotoxic killing of cells infected with Friend virus.[234] Thus, various subtypes of type I IFN may yield varying effects. On the other hand, Lucas *et al* determined that NK cell activation of IFN-γ, granzyme release and specific lysis of target cells required type I IFN activation of DCs and trans-presentation of IL-15 in response to TLR ligand stimulations.[187] Baranek *et al* recently corroborated this observation during MCMV infection, wherein infected mice had upregulated expression of type I IFN stimulated genes in cDCs, while NK cells had a higher expression of IL-15-

related genes.[188] As a potential resolution, Beuneu *et al* determined that type I IFN ligation was required on both accessory and NK cells to induce optimal NK cell activation.[235] A similar scenario was observed in an *in vitro* HCV model of infection, wherein the authors suggested that type I IFN induced NK cell IFN-γ production through a mechanism that required type I IFN to sensitize NK cells in order to respond to monocyte IL-15 production.[194]

1.3.7 Type I IFN and NK cell Negative Regulation

Along with activating NK cells, type I IFN can paradoxically negatively regulate NK cell function as well. Recently, there has been a surge in the number of studies that have examined the negative regulatory effects of type I IFN. As both an activator and negative regulator of NK cell function, it is likely that the subtype and timing of type I IFN produced, the magnitude produced, and the cells that it acts on plays a role in whether type I IFN functions as an activator or negative regulator of NK cell antiviral function.

During virus infection, Teijaro *et al* showed that blocking IFNAR during LCMV infection rescued NK cell IFN-γ production, suggesting that type I IFN has immunosuppressive properties.[118] During bacterial infection, Teles *et al* observed that type I IFN production was harmful during *Mycobacterium leprae* infection, as it led to an enhanced production of IL-10 and subsequent inhibition of beneficial IFN-γ and the Th1 response.[140] It has also been demonstrated that type I IFN can upregulate expression of ligands for PD1 receptors, which can be found on NK cells and can negatively regulate NK cell function.[236]

The negative regulatory effects of type I IFN may depend on the magnitude of type I IFN produced. Marshall *et al* observed that NK cells stimulated with both supernatants from CpG-stimulated pDCs, along with additional IFN-α, inhibited NK cell IFN-γ production.[237] Further, the mechanism of type I IFN negative regulation may also depend on the kinetics of type I IFN production. This was observed during *Listeria monocytogenes* infection, wherein a late wave of IFN-β production at 24 hours post-infection impaired the NK cell response.[238] Exogenous IFN-β administered earlier during infection, however, activated NK cells to clear the bacteria and improve overall survival.[238]

Miyagi *et al* (2007) proposed that the ability of type I IFN to regulate NK cell activation was due to a shifting balance in transcription factors STAT1 and STAT4.[136] They found that there was an abundance and preferential association of STAT4 with IFNAR in NK cells.[136] Type I IFN ligation to IFNAR on NK cells induced STAT4 phosphorylation, subsequently leading to IFN-γ production.[136] Upon phosphorylation of STAT4, STAT1 replaced its association to IFNAR, which inhibited IFN-γ production from NK cells.[136] As increasing amounts of type I IFN are produced during viral infection, the association with IFNAR shifts from STAT4 to STAT1, leading to a gradual inhibition of IFN-γ release from NK cells.[136]

In humans, individuals with HCV can be treated with pegylated IFN-α2a therapy.[239] Alhenstiel *et al* examined NK cell IFN-γ production and cytotoxicity prior to and after initiation of type I IFN therapy to examine the impact of type I IFN on human NK cells in an *ex vivo* environment.[239] They found that shortly after pegylated IFN therapy (6-24 hours) there was a boost in NK cell activating receptor expression, Trail expression, and CD107a

expression (a protein found in the lysosomal membrane and a marker of granule release).[239] However, they also observed a significant decrease in the proportion of IFN-γ+ NK cells found in the peripheral blood of these patients.[239]

1.4 Virus-induced immunopathology

The antiviral mechanisms that are adept and proficient at protecting the body from virus infections can also cause significant and debilitating immunopathology at the sites of infection, if not appropriately regulated or overwhelmed by a chronic virus infection. The best documented cases of virus-induced immunopathology are in the lung, where immunopathology can range from necrosis and sloughing off of epithelial cells and alveolar edema to extensive hemorrhaging.[240]

1.4.1 Virus-induced Immunopathology

While immune responses to acute respiratory viruses infecting the lung microenvironment, such as influenza and coronaviruses, are sufficient for clearing virus infections in healthy individuals, they can also cause significant tissue damage leading to severe lung immunopathology in others.[241] In the case of specific pandemics, such as the 2009 H1N1 and the 2005 H5N1 pandemics, researchers believe that the pathology and fatalities associated with these strains of influenza stemmed from severe lung immunopathology caused by uncontrolled inflammation – or a cytokine storm.[241, 242, 243] In each of these cases, individuals affected most severely by the infection had increased levels of pro-inflammatory cytokines, such as IL-6, compared to individuals who suffered from moderate illness and recovered.[241, 242, 243] However, these increases in pro-inflammatory

cytokine production were accompanied by an increase in viral load, which could be driving the increased cytokine levels.[242, 243]

Coronaviruses, such as the Severe Acute Respiratory Syndrome (SARS) or the Middle East Respiratory Syndrome (MERS), can cause severe pneumonia, acute respiratory distress syndrome, significant lung damage, and can lead to mortality in some individuals, particularly those that were elderly or had co-morbidities.[244] The severe respiratory condition associated with coronaviruses is unlikely due to virus replication, as replication actually decreases prior to individuals progressing to acute respiratory distress syndrome (ARDS).[244, 245] Individuals who contracted SARS had increased levels of pro-inflammatory cytokines present in their serum, including IL-6, along with significant neutrophil and macrophage cell infiltrates in their lungs in comparison to either control individuals or individuals with community acquired pneumonia.[240, 244, 246, 247, 248] In a mouse model of SARS, infection with this coronavirus led to delayed type I IFN production, which was accompanied by increased pathogenic inflammatory monocyte recruitment, IL-1, IL-6, and IFN-γ production.[249] This dysregulated type I IFN response is thought to have a significant impact on the resultant lung pathology.[249] Indeed, treatment of MERS-infected rhesus macaques with type I IFN and ribavirin 8 hours after infection was able to reduce viral load and pro-inflammatory cytokine production in serum and the lung.[250]

Ebola and Dengue viruses cause hemorrhagic fever and have high mortality rates in those they infect.[251] In the case of Ebola virus, the pathology is due to both the cytopathic effects of the virus and an uncontrolled immune response.[252, 253, 254] Dengue virus, on the other hand, does not cause severe disease with the initial primary infection.[251] Instead, a

second infection with a different serotype of the virus can lead to immunopathology and severe disease.[251] The amount of virus replication is correlated with disease severity, where it is thought to partially dictate the magnitude of an uncontrolled immune response and cytokine storm, as there is increased production of pro-inflammatory cytokines (e.g. soluble TNF receptors and IFN-γ).[255, 256] As a primary infection predisposes individuals to a severe secondary infection, it is likely that the adaptive immune response and the immunity generated against the first infection plays a large role in the immunopathology observed during the second infection.[251]

Chronic viral infections, such as those caused by hepatitis B (HBV) and hepatitis C viruses, can lead to tissue immunopathology. In these cases, CD8+ T-cell killing of infected hepatocytes and Th17 production of IL-22 can cause liver damage.[257, 258, 259] HSV, though a life-long infection, does not cause any significantly severe immunopathology at the oral and genital mucosal surfaces of infection. In the eye, however, HSV-1 infection can cause HSV-induced stromal keratitis (HSK), a condition that leads to vision impairment. CD4+ Th1 and Th17 release of their prototypic cytokines, IFN-γ and IL-17, respectively, have been implicated in the pathology from this infection.[260, 261]

Several mechanisms exist to negatively regulate antiviral immune responses in order to limit immunopathology. As an example, cytomegalovirus (CMV) in humans or MCMV infection in mice is a lifelong asymptomatic infection with very little immunopathology.[262] MCMV infection leads to the induction of Treg cells that are responsible for modulating the antiviral response during this chronic infection.[262] Depletion of these regulatory cells leads to significant immunopathology and tissue damage in the

liver of mice infected with MCMV.[262] IL-10 and TGF-β have regulatory properties in a myriad of inflammatory conditions, including virus infection.[140, 263] Along with Treg cells, IL-10 was found to dampen and prevent further excessive immune responses in HSK.[264] Lastly, increased PDL1 expression can dampen CD8+ T-cell activity through CD8+ T-cell expression of PD-1 receptor.[265]

1.4.2 Role of Immune cells

Numerous cell types have been implicated in the development and progression of virus-induced immunopathology. As mentioned previously, the antiviral effector functions of adaptive immune responses, including Th1, Th17, and CD8+ T-cells, can lead to significant tissue damage if not regulated appropriately. Macrophages, TIP DCs (produce TNF-a and iNOS), and inflammatory monocytes can produce vast amounts of pro-inflammatory cytokines, contributing to the inflammation and destructive tissue damage.[266] During influenza infection, macrophages have been found to contribute to tissue destruction in the lung through the induction of TRAIL.[267] This TRAIL ligates to epithelium expressed death receptor 5 (DR5), inducing apoptosis and ultimately creating a permeable lung environment.[267] During respiratory infections, neutrophils are rapidly recruited to the lung and can contribute to lung immunopathology through the release of NETs.[268] However, this release of chromatin material can also act as a danger signal and promote further inflammation.[268] Depletion of neutrophils has been shown to alleviate virus-induced immunopathology during influenza infection.[268] During respiratory syncytial virus (RSV) and influenza infection, NK cells have been shown to contribute to

influenza-induced lung pathology, as depletion of NK cells significantly alleviates immunopathology.[269, 270]

1.4.3 Type 2 Immune Responses and Virus-induced Immunopathology

Type 2 immune responses are not typically associated with virus infections nor virus-induced immunopathology. Type 2 immune responses are typically associated with defence against parasitic infections and are pathogenic in diseases involving allergic responses.[271]

Type 2 responses are generally associated with innate lymphoid cell group 2 (ILC2) and Th2 cells, with the former modulating type 2 innate responses and the latter involved in type 2 adaptive immune responses.[271] These responses are characterized by the production of type 2 cytokines, including IL-4, IL-5, IL-9, and IL-13, which result in significant airway inflammation and the influx of eosinophils and neutrophils.[271] Th2 cells ordinarily produce IL-4, IL-5, IL-9, and IL-13, whereas ILC2 cells have been shown to produce IL-5, IL-6, IL-9, and IL-13.[271] Both ILC2 and Th2 cells have been implicated in the development of allergic inflammation in a variety of conditions, including asthma.[271]

In neonates, RSV infection can lead to hospitalization of infants and is associated with a higher likelihood of developing asthma later in life.[272, 273] In this specific age group, RSV infections generate a type 2 biased immune response, with the production of IL-13, rather than an antiviral type 1 immune response.[274, 275] A study by Stier *et al* found that infection of neonatal mice with RSV induced expansion and IL-13 production from ILC2s compared to adult mice.[275] In both humans and mice, rhinovirus infection induced the production of type 2 cytokines, in particular IL-4, IL-5, and IL-13. It was found that IL-33

derived from infected bronchial epithelial cells was sufficient to induce type 2 cytokine production from both Th2 and ILC2 cells.[276] Thus, specific respiratory virus infections induce type 2 immune responses which contribute to the lung pathology observed in these infections.

1.4.4 Type I IFN and Virus-induced Immunopathology

The timing and magnitude of the type I IFN response during virus infection is critical as it can influence whether type I IFN contributes to or suppresses virus-induced immunopathology. Influenza virus infection with an immense type I IFN response, for example, is associated with pDC and monocyte upregulation of TRAIL, inducing epithelial cell apoptosis and lung immunopathology.[267, 277, 278] In severe coronavirus infections, such as SARS, there is a delay in the type I IFN response, as these viruses have viral proteins that can suppress the type I IFN response. [244] As previously discussed, this dysregulated type I IFN response can lead to severe immunopathology in a mouse model of SARS infection through the excessive recruitment and pro-inflammatory cytokine-producing inflammatory monocytes.[249] Lastly, an absence or significant decrease in type I IFN signaling can result in significant lung immunopathology during neonate RSV infection and influenza infection.[279]

Cells from neonates have significantly dampened type I IFN responses, and when infected with RSV, a type 2 response is induced and this leads to both immunopathology and a predisposition for developing asthma later in life.[274, 280, 281] Through this, it is thought that a traditional type I IFN response is required to activate an antiviral Th1 response that can clear the RSV infection.[282] Further, absence of type I IFN receptor or its signaling

component STAT1 led to increased lung immunopathology after RSV infection.[283, 284] In a mouse model of influenza A infection, Duerr *et al* found that in the absence of type I IFN signaling, ILC2 production of type 2 cytokines dominates the response and leads to severe lung immunopathology, characterized by increased influx of both neutrophils and eosinophils.[279] Type I IFN was required to directly suppress ILC2 cytokine production.[279] Stifter *et al* found that in the absence of type I IFN receptor, along with an influx of neutrophils, the $Ly6C^{lo}$ monocyte population had a significant upregulation of NOS2 and other pro-inflammatory receptors.[285] Thus, type I IFN is also required not only to polarize antiviral responses towards a Th1 response, but to dampen excessive inflammation. Type I IFN can lead to production of immunomodulatory cytokines, such as IL-10, and IL-27, both of which can suppress inflammatory responses.[140] IL-27 in particular has been shown to repress Th17 polarization and ILC2 cytokine expression.[286, 287] It can also upregulate programmed death ligand 1 (PDL1) expression on epithelial cells, which can dampen CD8+ T-cell effector functions and upregulate the expression of Tyro 3, Axl, and Mer (TAM) receptors on immune cells.[265] TAM receptor expression can hijack the type I IFN receptor and its signaling components to induce transcription of suppressor of cytokine signaling (SOCS) protein 1 and SOCS3, which can then suppress other cytokine and TLR signaling pathways.

Overall, type I IFN has been shown to have a dual role during virus-induced immunopathology, where it has been demonstrated to both dampen and contribute to immunopathology in different infectious scenarios. In the context of chronic LCMV infection, a chronic type I IFN response leads to destruction of lymphoid structure and

dampening of an antiviral immune response.[118] However, in the complete absence of type I IFN receptor, mice have significantly decreased survival in response to many virus infections, including HSV-2.[288] Further to that, absence of type I IFN receptor in a mouse model of influenza infection led to ILC2-mediated lung immunopathology.[279] Thus, type I IFN production during virus infection is beneficial when it is appropriately produced at an early time point; however, can be detrimental if produced in a prolonged fashion.

1.5 Rationale, Hypothesis, and Specific Thesis Objectives

Rationale:

HSV-2 is a sexually transmitted lifelong infection with no cure and no readily available preventative vaccine.[8] With an estimated 400 million individuals worldwide currently infected, where over half of those infected are women, it is imperative to understand the immune response to HSV-2 in order to develop efficacious treatments and vaccines for the future.[9]

Numerous studies have shown that the innate immune response has a key role in directing the antiviral response to virus infections. In particular, type I IFN, a pleiotropic cytokine produced early during virus infection, has been shown to be critical for not only limiting virus replication, but influencing innate and adaptive immune cell functions during infection.[130, 141] Type I IFN is critical for defence against virus infections, including HSV-2, where absence of the type I IFN receptor leads to decreased survival and increased virus titers.[135, 141] Previously, we and other have shown that type I IFN is necessary for the activation of NK cell function during virus infection, particularly HSV-2 infection.[135, 230] The NK cell antiviral response is a critical component of the innate immune response

against HSV-2. In particular, their ability to produce IFN-γ has not only been shown to decrease virus replication, but also help to initiate a Th1 adaptive immune response, which is ultimately responsible for clearing the infection.[62, 63, 135] However, despite years of research, it is still not clear how type I IFN is able to regulate NK cell function, as well as the antiviral innate immune response. Furthermore, type I IFN involvement in antiviral NK cell activation has yet to be explored in the context of the vaginal mucosal environment. We hypothesize that type I IFN is able to activate NK cell IFN-γ production through an indirect mechanism involving an accessory cell type and cytokines that are typically linked to inducing NK cell IFN-γ production (e.g. IL-12, IL-15, or IL-18). We further hypothesize that direct action of type I IFN on NK cells is able to negatively regulate their IFN-γ release as well as innate immunopathology induced by HSV-2 infection.

In this project, we examined the influence and underlying mechanisms by which type I IFN impact NK cell antiviral function and the innate antiviral immune response. In understanding how type I IFN modulates the innate antiviral immune response, we not only improve our fundamental understanding of the host antiviral immune response, but we can also use this knowledge to develop future therapeutics and vaccines against HSV-2.

Hypothesis:

Type I IFN activates NK cells through an indirect mechanism, but is also required to negatively regulate NK cell function and innate immunopathology.

Specific Thesis Objectives:

1. Understand the underlying mechanism by which type I IFN induces IFN-γ production by NK cells during a mucosal viral infection
2. Investigate the role of type I IFN in suppressing virus-induced immunopathology
3. Understand the mechanism by which NK cells are negatively regulated during a mucosal virus infection

CHAPTER 2

Inflammatory monocytes require type I interferon receptor signalling to activate NK cells via IL-18 during a mucosal viral infection

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Abbreviations: NK, natural killer; DC, dendritic cell; IFN, interferon; HSV-2, herpes simplex virus type 2; MCMV, mouse cytomegalovirus; LCMV, lymphocytic choriomeningitis virus; TLR, toll-like receptor; HCV, hepatitis C virus; WT, wild-type; Rα, receptor alpha; PBMC, peripheral blood mononuclear cells

Short ~40-word summary:

Though type I interferon is critical for NK cell activation, the underlying mechanism is under debate and unknown during a mucosal infection. Lee *et al* has determined that type I interferon induces inflammatory monocytes to produce IL-18 to directly activate NK cells to combat viral infections.

Author Contributions:

AJ Lee designed and performed experiments, analyzed the data, and wrote the manuscript. B Chen designed and performed experiments for Figure 1B and C. MV Chew, NG Barra, MM Shenouda, and T Nham provided technical help and expertise for experiments. N van Rooijen provided the clodronate and PBS liposomes. M Jordana edited the manuscript and provided the *Gata1*$^{-/-}$ mice. KL Mossman edited the manuscript and provided *Irf9*$^{-/-}$ mice. RD Schreiber provided the *Ifnar*$^{f/f}$ Itgax cre and *Ifnar*$^{f/f}$ mice. M Mack provided the α-CCR2 antibody. AA Ashkar conceived the study, designed and performed experiments, edited the manuscript, and is the corresponding author.

Abstract:

The requirement of type I IFN for NK cell activation in response to viral infection is known, but the underlying mechanism remains unclear. Here, we demonstrate that type I IFN signalling in inflammatory monocytes, but not in dendritic cells (DCs) or NK cells, is essential for NK cell function in response to a mucosal HSV-2 infection. Mice deficient in type I IFN signalling, *Ifnar*$^{-/-}$ and *Irf9*$^{-/-}$ mice, had significantly lower levels of inflammatory monocytes, were deficient in IL-18 production, and lacked NK cell-derived IFN-γ. Depletion of inflammatory monocytes, but not DCs or other myeloid cells, resulted in lower levels of IL-18 and complete abrogation of NK cell function to HSV-2 infection. Moreover, this resulted in higher susceptibility to HSV-2 infection. While *IL18*$^{-/-}$ and *Il18r1*$^{-/-}$ mice had normal levels of inflammatory monocytes, their NK cells were unresponsive to HSV-2 challenge. This study highlights the importance of type I IFN signaling in inflammatory monocytes and the induction of the early innate antiviral response.

Introduction

Natural Killer (NK) cells are an important component of the innate immune response as they are rapidly activated upon viral infection and can directly recognize infected cells and eliminate them (Vivier et al., 2008). Additionally, they can release pro-inflammatory cytokines, which activate other immune cells and facilitate the initiation of the adaptive immune response (Vivier et al., 2008). In particular, their ability to produce interferon (IFN)-γ during the early stages of an infection has been shown to be critical for the defence against viral infections. (Gill et al., 2011; Orange et al., 1995; Thapa et al., 2007). Indeed, the absence of NK cells, in *Il15*$^{-/-}$ mice or through NK cell depletion, results in significantly increased susceptibility to HSV-2 infection (Ashkar and Rosenthal, 2003; Thapa et al., 2007). Depletion of NK cells in mice led to increased HSV-2 viral titers found in the vaginal tract, spinal cord, and brain stem (Thapa et al., 2007). Further, *Ifng*$^{-/-}$ mice have increased mortality rates when infected with HSV-2 (Ashkar and Rosenthal, 2003). As a critical component of the innate immune response, it is important to understand how NK cells are activated, particularly to produce IFN-γ early in the response.

The functional state of NK cells is greatly influenced by their microenvironment. An overwhelming increase in activation signals over inhibitory signals will cause activation of their anti-viral functions (Pegram et al., 2011). On the other hand, a plethora of inhibitory signals will prevent NK cell activation (Pegram et al., 2011). Cytokines, including type I IFN, IL-15, IL-12, IL-18, and ISG15, have all been shown to activate NK cell function, particularly IFN-γ production (Pegram et al., 2011). Alternatively, inhibitory

receptor recognition of MHC class I on target cells inhibits NK cell activation (Pegram et al., 2011).

Type I IFNs are central to the activation of NK cells during viral infections, including MCMV, adenovirus, vaccinia virus, and HSV infections (Baranek et al., 2012; Gill et al., 2011; Lucas et al., 2007; Martinez et al., 2008; Zhu et al., 2008). Type I IFNs comprise a family of cytokines that includes IFN-β and numerous subtypes of IFN-α (Platanias, 2005). These cytokines signal through their specific IFNAR1 and IFNAR2 receptors, which together form the type I IFN receptor (Platanias, 2005). Type I IFNs are rapidly produced upon viral infection and play an essential role in the anti-viral innate immune response (Platanias, 2005). Though type I IFNs are required for NK cell activation, the underlying mechanism is still controversial. Evidence in the literature suggests that type I IFNs directly activate NK cells during vaccinia virus, adenovirus, and LCMV infection (Mack et al., 2011; Martinez et al., 2008; Zhu et al., 2008). However, it has also been reported that type I IFNs act on dendritic cells (DCs) to produce and trans-present IL-15, which leads to NK cell activation in response to TLR ligand stimulation and MCMV infection (Baranek et al., 2012; Lucas et al., 2007). This suggests that type I IFN signalling is required for IL-15 induction following viral infection. However, the majority of studies examining NK cell activation have used intravenous, intraperitoneal, or subcutaneous routes of viral infection. The mechanism underlying NK cell activation during a mucosal infection has yet to be explored.

Inflammatory monocytes (defined by the phenotype $CCR2^{+}$, $Ly6C^{hi}$) are rapidly recruited to sites of inflammation and produce a plethora of inflammatory cytokines in

order to combat infection. Once in the inflammatory environment, these inflammatory monocytes can differentiate into DCs, which can aid in the development of adaptive immunity against infection. However, their role in stimulating innate antiviral immunity, particularly NK cell anti-viral responses, has been relatively unexplored. Depletion of inflammatory monocytes from human PBMCs during an *in vitro* HCV infection suppressed NK cell responses, suggesting that these cells are capable of activating NK cells (Serti et al., 2014; Zhang et al., 2013).

Here, we describe a thorough mechanism by which NK cells are activated during a mucosal viral infection *in vivo*. We clearly show that type I IFN does not directly act on NK cells or DCs to activate NK cells. Instead, during vaginal HSV-2 infection, type I IFNs signal through inflammatory monocytes to produce IL-18, which then activates NK cells to produce IFN-γ and augment host defense.

Results

IFNAR and IRF9 are essential for NK cell IFN-γ production during HSV-2 infection

Production of IFN-γ by NK cells is the hallmark of the host innate immune response to genital HSV-2 infection (Ashkar and Rosenthal, 2003; Gill et al., 2011), Indeed, NK cells have been shown to be the main producers of IFN-γ during viral infections as in many cases the absence or depletion of these cells diminishes IFN-γ production (Krug et al., 2004; Mack et al., 2011; Martinez et al., 2008; Zhu et al., 2008). We first confirmed that IFN-γ is produced directly by NK cells in response to HSV-2 infection (Fig. 1A) (Gill et al., 2011). Depletion of NK cells using anti-NK1.1 or anti-IL-15 antibodies significantly

diminished IFN-γ levels in the vaginal lavages of HSV-2 infected mice (Fig. 1B and C). We have previously shown that IFNAR is required for NK cell IFN-γ production during HSV-2 infection. (Gill et al., 2011). Production of IFN-γ was completely abrogated in *Ifnar*$^{-/-}$ mice during vaginal infection with HSV-2 (Fig. 1D). This deficiency in vaginal IFN-γ level was not a result of decreased NK cell recruitment to the vaginal tract (Gill et al., 2011). Additionally, blocking type I IFN receptor in C57BL/6 (B6) mice prior to infection resulted in a complete abrogation of IFN-γ production, similar to that observed in genetically deficient type I IFN receptor mice (Fig. 1E). We determined whether the type I IFNs' downstream canonical signalling cascade, which involves IRF9, is also required for NK cell function during infection. There was a complete abrogation of NK cell IFN-γ production in the vaginal lavages of HSV-2 infected *Irf9*$^{-/-}$ mice, similar to that observed in *Ifnar*$^{-/-}$ mice (Fig. 1F). Additionally, the total number of NK cells within the vaginal mucosa between wildtype (WT) and *Irf9*$^{-/-}$ B6 mice was not significantly different (Fig. 1G). This suggests that the deficiency in IFN-γ production was not due to a reduction in the vaginal NK cell numbers. We also determined that both *Ifnar*$^{-/-}$ and *Irf9*$^{-/-}$ mice have decreased survival after infection compared to WT mice (Fig. 1H and I). It has been reported that levels of vaginal HSV-2 viral replication are proportional to the amount of IFN-γ released from NK cells (Gillgrass et al., 2003). At odds with this finding, we detected similar, if not higher, levels of HSV-2 viral replication within the vaginal mucosa in *Ifnar*$^{-/-}$ and *Irf9*$^{-/-}$ mice compared to WT mice, despite a lack of NK cell IFN-γ production in *Ifnar*$^{-/-}$ and *Irf9*$^{-/-}$ mice (Fig. 1J). Overall, our data suggests that type I IFN signalling is essential for NK cell IFN-γ production. It has been shown that IFN-β is rapidly produced

in the vaginal tract in response to HSV-2 infection or TLR challenge (Gill et al., 2011; Gill et al., 2006). IFN-β production occurs at 6 hrs p.i., and is then downregulated at 12 and 24hrs p.i., which would suggest the involvement of IFN-β in activating NK cell IFN-γ production. However, when *Ifnb*$^{-/-}$ mice were infected with HSV-2, they produced similar amounts of IFN-γ compared to WT mice (Fig. 1K) (Gill et al., 2011; Gill et al., 2006). This would suggest that IFN-β is not essential for activating NK cells during HSV-2 infection.

Expression of IFNAR on NK cells is not required for their activation during HSV-2 infection

After establishing the requirement of type I IFN signalling for NK cell function, we inquired whether type I IFN activates NK cell IFN-γ production through a direct or indirect mechanism. To address this question, we adoptively transferred either WT or *Ifnar*$^{-/-}$ NK cells into *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice, which lack all lymphocytes including NK cells. In transferring *Ifnar*$^{-/-}$ NK cells into these mice, we retain type I IFN signalling in the environment, but the adoptively transferred NK cells are unable to respond to type I IFN. Upon infection, *Ifnar*$^{-/-}$ NK cells produced IFN-γ in response to HSV-2 infection when transferred to an environment with functional type I IFN signalling (Fig. 2A). In fact, *Ifnar*$^{-/-}$ NK cells produced a significantly greater amount of IFN-γ compared to their WT controls, suggesting that IFNAR on NK cells is not required for NK cell activation and IFN-γ production (Fig 2A). Additionally, we were able to detect these adoptively transferred cells at day 3 p.i. (Fig. 2B). As further evidence, we adoptively transferred WT NK cells (able to respond to type I IFNs) into *Ifnar*$^{-/-}$ mice (unable to respond to type I

IFNs) and observed that WT NK cells in an *Ifnar*$^{-/-}$ environment are unable to produce IFN-γ in response to HSV-2 infection (Fig. 2C). The presence of these adoptively transferred cells persisted in the *Ifnar*$^{-/-}$ mice to day 2 p.i. both in the spleen and vaginal mucosa (Fig. 2D and E).

NK cell activation does not require type I IFN activation of DCs nor IL-15 trans-presentation

Previously, Lucas *et al.* (2007) and Baranek *et al.* (2012) reported that during TLR stimulation and MCMV infection, type I IFN stimulates DCs in order to trans-present IL-15 to NK cells and induce their production of IFN-γ (Baranek et al., 2012; Lucas et al., 2007). To determine whether IFNAR is required on DCs for the subsequent activation of NK cells during a mucosal vaginal infection, we used a mouse model wherein only DCs are deficient in IFNAR. As detailed in Diamond *et al.* (2011), we crossed *Ifnar*$^{f/f}$ mice with CD11c-Cre^{+} transgenic mice to generate offspring that are selectively IFNAR deficient in DCs (Diamond et al., 2011). No IFNAR expressing CD11c+ dendritic cells could be identified by flow cytometry in these mice (data not shown). Infection of these mice yielded no significant difference in vaginal NK cell IFN-γ production in comparison to controls, suggesting that DCs are not required to induce NK cell IFN-γ production (Fig. 3A). To determine whether IL-15 trans-presentation is involved in the activation of NK cells during HSV-2 infection, we provided *Ifnar*$^{-/-}$ and WT mice with a trans-presentation mimic, IL-15 in complex with its specific receptor alpha (Rα), in an attempt to 'rescue' NK cell IFN-γ production in *Ifnar*$^{-/-}$ mice. IL-15/IL-15Rα complexes (IL-15/Rα) have been shown to

induce rapid proliferation as well as induction of IFN-γ production in NK cells (Elpek et al., 2010). However, we were unable to rescue IFN-γ production in *Ifnar*$^{-/-}$ mice, despite finding NK cell proliferation in both the spleen and vaginal tract (Fig. 3B-F). Significant increases in proportion of NK cells were observed in the spleen. Thus, IL-15/Ra complexes were sufficient to stimulate NK cell proliferation, but not IFN-ɣ production.

IL-18 is required for NK cell IFN-γ production during HSV-2 infection

Several factors, including IL-15, IL-12, ISG15, and IL-18, have been shown to induce NK cell IFN-γ production, particularly during viral infection (Pegram et al., 2011). We have determined that IL-15 is not sufficient to activate NK cells during genital HSV-2 infection (Fig. 3B). Further, we previously reported that *Ifnar*$^{-/-}$ mice can produce IL-15 in the vaginal mucosa following genital HSV-2 infection (Gill et al., 2011). IL-12 is a well-known activator of IFN-γ production, but we have previously shown that HSV-2 infection of *Il12*$^{-/-}$ mice had no impact on NK cell IFN-γ production by NK cells (Bogunovic et al., 2012; Gill et al., 2011). Here, we examined the role of ISG15 in the activation of NK cells, as human ISG15 deficiency has been observed to lead to decreased NK cell IFN-γ production during mycobacterial infection (Bogunovic et al., 2012). HSV-2 infection of *Isg15*$^{-/-}$ mice yielded no difference in NK cell IFN-γ production in comparison to WT mice (data not shown). We investigated whether IL-18 is required for NK cell activation in response to HSV-2 infection. We first compared IL-18 content in the vaginal lavages of *Ifnar*$^{-/-}$ and WT B6 mice and detected significantly lower levels of IL-18 in *Ifnar*$^{-/-}$ vaginal lavages, particularly at day 2 p.i. (Fig. 4A). To further investigate the role of IL-18 in the induction

of IFN-γ production by NK cells, we infected mice that lacked IL-18 or IL-18R. There was a significant abrogation in the production of IFN-γ by NK cells in both *Il18*$^{-/-}$ and *Il18r1*$^{-/-}$ mice infected with HSV-2, compared to WT mice (Fig. 4B). This decrease was not due to a lack of NK cell recruitment to the vaginal mucosa in *Il18*$^{-/-}$ or *Il18ra*$^{-/-}$ mice (Fig. 4C and D). These data demonstrate that IL-18 is required for the activation of NK cell IFN-γ production during HSV-2 infection.

NK cells respond to IL-18 directly in order to produce IFN-γ

To investigate whether IL-18 directly activates NK cells, we adoptively transferred either *Il18ra*$^{-/-}$ or WT B6 NK cells into *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice prior to infection with HSV-2. Following infection, *IL18r1*$^{-/-}$ NK cells adoptively transferred to *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice had significantly decreased IFN-γ production compared to WT NK cells (Fig. 4E). However, *Il18r1*$^{-/-}$ NK cells produced copious amounts of IFN-γ when stimulated with PMA and ionomycin (Fig 4F). These findings suggest that *IL18r1*$^{-/-}$ NK cells are not inherently incapable of producing IFN-γ, but lack the required signalling via IL-18R to do so during HSV-2 infection.

Hematopoietic cells respond to type I IFN and produce IL-18 in order to activate NK cells

Because our data clearly indicated that type I IFNs are required but do not act directly on NK cells, we investigated the cell types that produce IL-18 in response to type I IFN. We first used bone marrow chimeric mice to determine whether hematopoietic or non-hematopoietic cells were required. We created chimeric mice by reconstituting WT and

Ifnar$^{-/-}$ mice with either WT or *Ifnar*$^{-/-}$ bone marrow and examined reconstitution in the peripheral blood (Fig. 5A). After HSV-2 infection, WT recipients reconstituted with *Ifnar*$^{-/-}$ bone marrow were unable to produce IFN-γ at day 2 and 3 p.i., whereas *Ifnar*$^{-/-}$ recipients reconstituted with WT bone marrow did produce IFN-γ (Fig. 5B). To determine whether the hematopoietic cell compartment is also required to produce IL-18, we created bone marrow chimeric mice between WT and *IL18*$^{-/-}$ mice and examined reconstitution in the peripheral blood (Fig. 5C). Upon infection with HSV-2, NK cells from *Il18*$^{-/-}$ mice that received WT bone marrow produced IFN-γ, whereas WT mice reconstituted with *Il18*$^{-/-}$ bone marrow did not (Fig. 5D). These data suggested that the cells responding to type I IFN, as well as producing IL-18, belong to the hematopoietic cell compartment. Furthermore, this intermediary cell type is likely of myeloid lineage as *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice are alymphoid, yet the transfer of WT or *Ifnar*$^{-/-}$ NK cells into these mice yielded IFN-γ production (Fig. 2A).

Decreased vaginal recruitment of inflammatory monocytes in *Ifnar*$^{-/-}$ mice

It has been documented that macrophages can activate NK cells during infection (Hamerman et al., 2004; Siren et al., 2004). However, we found that depletion of macrophages by clodronate administration had no impact on NK cell IFN-γ production during HSV-2 infection (data not shown). Eosinophils are a cell type prominent within the reproductive tract and may play a role against infection (Robertson et al., 2000). However, similar to macrophages, we found no difference in vaginal NK cell production of IFN-γ between *Gata1*$^{-/-}$ mice, which lack eosinophils, and WT controls (data not shown). When

we compared the composition of myeloid immune cells within the vaginal mucosa between WT and *Ifnar*$^{-/-}$ mice during infection, we observed a significantly decreased recruitment of inflammatory monocytes in *Ifnar*$^{-/-}$ mice at both days 2 and 3 p.i. (Fig. 6A and B). In agreement with this, we also found that *Ifnar*$^{-/-}$ mice had a significantly decreased vaginal content of MCP-1, the chemokine responsible for recruiting inflammatory monocytes, during infection (Fig. 6C). In contrast, there was no significant difference in MCP-1 levels and inflammatory monocyte recruitment in *Il18*$^{-/-}$ mice compared to WT mice at days 2 and 3 p.i. (Fig. 6A, D and E).

Inflammatory monocytes, but not neutrophils, are essential for NK cell IFN-γ production

Because the recruitment of inflammatory monocytes is significantly reduced in *Ifnar*$^{-/-}$ mice, we examined whether these cells were involved in activating NK cells during infection. Initially, we depleted both inflammatory monocytes and neutrophils using the anti-GR-1 depletion antibody RB6-8C5 in WT mice prior to infection with HSV-2 (Fig. 7 A-D) (Daley et al., 2008). This led to a drastic reduction in NK cell IFN-γ production, with no impact on NK cell recruitment to the vaginal tissue or proportion of vaginal CD11c+ cells during infection (Fig. 7 E-G). To assess the role of inflammatory monocytes, we used a recently developed anti-CCR2 antibody that has been shown to successfully deplete inflammatory monocytes while leaving the neutrophil cell population intact (Mack et al., 2001; Schumak et al., 2015). This antibody depleted the inflammatory monocyte population in the vaginal mucosa without impacting the neutrophil population during infection (Fig 8 A-D). In the absence of inflammatory monocytes, there was an almost

complete abrogation of IFN-γ production by NK cells within the vaginal mucosa, while the percentage of NK cells and CD11c+ cells in the vaginal mucosa during infection were similar (Fig. 8 E-G). Furthermore, when we examined vaginal HSV-2 viral titers in the absence of inflammatory monocytes, we found that there was a significant increase in HSV-2 viral titer level compared to mice given an isotype-matched control Ig (Fig. 8 H). This suggests that inflammatory monocytes are essential in activating NK cell IFN-γ production, which is necessary for controlling HSV-2 infection in the innate immune response.

Depletion of inflammatory monocytes abrogates IL-18 production during HSV-2 infection

To determine whether inflammatory monocytes are responsible for producing IL-18 during vaginal HSV-2 infection, we examined IL-18 levels in the vaginal lavages of mice depleted of inflammatory monocytes. Compared to mice given an isotype-matched control Ig, mice given the anti-CCR2 antibody had significantly decreased IL-18 content in their vaginal lavages, particularly at day 2 and 3 p.i. (Fig. 8 I). We have previously published that Ifnar$^{-/-}$ mice have significantly lower levels of vaginal IL-12 at d2 and d3 p.i. compared to WT mice (Gill et al., 2011). We examined whether depletion of inflammatory monocytes impacted induction of IL-12 during HSV-2 infection. Upon depletion of inflammatory monocytes, however, we observed no significant difference in levels of IL-12 (Fig 8J). Overall, these data suggest that inflammatory monocytes are responsible for IL-18, but not IL-12, production in the vaginal mucosa during HSV-2 infection.

Discussion

Early IFN-γ production from NK cells is the hallmark of the innate immune response and required for protection during vaginal HSV-2 infection (Ashkar and Rosenthal, 2003; Gill et al., 2011). We and others have found that the presence of IFNAR is absolutely required for the activation of IFN-γ production by NK cells (Baranek et al., 2012; Gill et al., 2011; Lucas et al., 2007; Martinez et al., 2008; Zhu et al., 2008). Here, we demonstrate a mechanism by which type I IFN activates IFN-γ production by NK cells during a mucosal viral infection. Although IFNAR is not required on NK cells for their activation, we have also determined that type I IFN does not go through the canonical pathway of DC activation and IL-15 trans-presentation in our model (Baranek et al., 2012; Lucas et al., 2007). Instead, type I IFN is necessary to recruit inflammatory monocytes, as well as signal through these cells, to release IL-18, which then acts directly on NK cells through their IL-18R to induce their production of IFN-γ.

Previously, several studies have suggested that type I IFN signals directly on NK cells to induce their activation (Mack et al., 2011; Martinez et al., 2008; Zhu et al., 2008). Through our adoptive transfer system, we found that NK cells do not require IFNAR expression in order to become activated to produce IFN-γ during HSV-2 infection. This is similar to previously published results by Guan *et al*, where they demonstrated that IFNAR was not required on NK cells for their activation in the context of MCMV infection (Guan et al., 2014). Surprisingly, our results showed that *Ifnar*$^{-/-}$ NK cells have increased expression of IFN-γ when adoptively transferred to *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice compared to WT NK cells. Type I IFN has been shown to negatively regulate IFN-γ production from NK

cells, explaining the increase in IFN-γ expression from *Ifnar*$^{-/-}$ NK cells (Nguyen et al., 2000; Teles et al., 2013). On the other hand, Lucas *et al.* (2007) and Baranek *et al.* (2012) reported that type I IFN stimulates DCs to trans-present IL-15, which in turn activates NK cells (Baranek et al., 2012; Lucas et al., 2007). However, we found the mucosal route involves inflammatory monocytes and IL-18, rather than DCs and IL-15 trans-presentation. This is supported by previous evidence showing that IL-15 production in the vaginal mucosa of *Ifnar*$^{-/-}$ mice is similar, if not higher, to that of WT mice during HSV-2 infection (Gill et al., 2011). Furthermore, we found similar amounts of NK cell IFN-γ production between mice that had a specific absence of IFNAR on CD11c+ cells versus wildtype controls, indicating that DCs were not responsible for responding to type I IFN. Additionally, administration of IL-15- IL-15 receptor complexes, although leading to significant NK cell proliferation, did not rescue NK cell IFN-γ production in *Ifnar*$^{-/-}$ mice. The difference in findings may lie in the route of viral infection, which involves infecting through a mucosal surface, the vaginal mucosa. The majority of articles examining the relationship between type I IFN and NK cell activation administer viral infection or TLR stimulants through *in vitro* or a systemic, i.p., or subcutaneous routes of infection and found contradicting outcomes (Baranek et al., 2012; Lucas et al., 2007; Martinez et al., 2008; Zhu et al., 2008). Though we observed a decrease in IFN-γ production in WT mice after administration of IL-15/IL-15Rα, it is known that IL-15 is able to reduce HSV-2 virus replication both *in vivo* and *in vitro* independent of its effects on NK cells. As NK cell IFN-γ production is correlated with HSV-2 replication, IL-15's ability to reduce HSV-2 viral titers subsequently reduces IFN-γ expression from NK cells (Gill et al., 2005).

IFN-β and numerous subtypes of IFN-α have both been shown to signal through the type I IFN receptor and are produced upon virus infection (Perry et al., 2005). Indeed, Gill *et al* has previously shown that vaginal HSV-2 infection rapidly induces IFN-β production at 6 hrs p.i., but is rapidly controlled at 12 hrs and 24 hrs p.i. (Gill et al., 2011). As IFN-β is produced early during HSV-2 infection, we were interested in determining the role of IFN-β in activating NK cells. However, we found that IFNβ$^{-/-}$ mice had comparable levels of IFN-β production to WT mice. It is likely that IFN-α is able to compensate for the absence of IFN-β.

During vaginal HSV-2 infection, we observed that DCs were not required to respond to IFNAR, but instead, inflammatory monocytes were necessary for the activation of NK cell IFN-γ production. In agreement with Iijima *et al.* (2011), we found that *Ifnar*$^{-/-}$ mice had decreased MCP-1 production and a reduction in inflammatory monocyte recruitment during HSV-2 infection (Iijima et al., 2011). They also showed that type I IFN induced CCR2 ligand production (MCP-1, CCL7, CCL8, and CCL12), which was ultimately responsible for inflammatory monocyte recruitment (Iijima et al., 2011). When we depleted inflammatory monocytes using an anti-CCR2 antibody, we observed a complete abrogation of NK cell IFN-γ production. However, Iijima *et al.* (2011) also observed that *Ccr2*$^{-/-}$ mice, which have significant deficiencies in inflammatory monocyte recruitment, also had decreased NK cell IFN-γ production (Iijima et al., 2011). Although they found that CCR2 was required for NK cell recruitment to the vaginal mucosa, our depletion of inflammatory monocytes did not impact NK cell recruitment to the vaginal mucosa (Iijima et al., 2011). Furthermore, we did not observe any difference in proportion

of CD11c+ cells in the vaginal mucosa when depleting inflammatory monocytes. We also found that depletion of inflammatory monocytes increased vaginal HSV-2 viral titer levels at day 2 p.i., suggesting that inflammatory monocyte activation of NK cell IFN-γ production is critical for limiting HSV-2 virus replication during the early stages of infection. In humans, depletion of monocytes from PBMCs in an *in vitro* model of HCV infection reduced NK cell IFN-γ production (Serti et al., 2014; Zhang et al., 2013). In particular, Serti *et al.* (2014) observed that monocyte production of IL-18 was responsible for activating the NK cells (Serti et al., 2014).

IL-18 is a well-known cytokine activator of NK cell IFN-γ production (Okamura et al., 1995). Interestingly, like IL-1β, IL-18 is first produced intracellularly as an immature protein without a signal peptide and in this form is constitutively expressed (Dinarello et al., 2013). In order to be secreted, the IL-18 precursor needs to be cleaved into a mature and bioactive protein, usually by caspase-1 (Dinarello et al., 2013). Once secreted from the cell, IL-18 can bind to its two specific receptors, IL-18α and IL-18β, and cause signal transduction through MyD88 within the receiving cell (Dinarello et al., 2013). During a mucosal HSV-2 infection, *Il18*$^{-/-}$ mice have significantly increased shedding of HSV-2 in their vaginal washes and decreased survival when compared to control mice (Harandi et al., 2001). Accordingly, we found that type I IFN-induced IL-18 production was a critical factor for NK cell activation. Similar to our findings, others have shown that a deficiency in IL-18 production leads to decreased NK cell IFN-γ production *in vivo* during vaccinia virus, *Francisella tularensis*, and *Chlamydia muridarum* infections (Brandstadter et al., 2014; Nagarajan et al., 2011; Pierini et al., 2013). Even when using a natural mouse

pathogen, orthopoxvirus ECTV, *Il18*$^{-/-}$ mice produced significantly less NK cell IFN-γ (Wang et al., 2009). Using an intravenous HSV-1 infection model, Barr *et al.* observed that IL-18 production by CD11c+ cells was responsible for IFN-γ production by NK cells in an *ex vivo* splenocyte culture (Barr et al., 2007).

Much of the data regarding the relationship between type I IFN and IL-18 suggests that type I IFN activates cleaving of pro-IL-18 into active IL-18 (Fang et al., 2014; Fernandes-Alnemri et al., 2010). Recently, Fang *et al.* (2014) observed that type I IFN produced during *Streptococcus pneumoniae* infection led to IL-18 production through the activation of caspase-1 (Fang et al., 2014). During *F. tularensis* infection, Fernandes-Alnemri *et al.* (2010) observed that *Ifnar*$^{-/-}$ mice were defective in their ability to induce caspase-1 activation, which in turn inhibited their ability to cleave pro-IL-18 into active IL-18 (Fernandes-Alnemri et al., 2010). They attributed this to the ability of type I IFN to activate the absent in melanoma 2 inflammasome complex, which is responsible for activating caspase-1 (Fernandes-Alnemri et al., 2010). Further studies will be required to determine whether a similar mechanism occurs during viral infection and NK cell activation.

Our findings provide a complete mechanism detailing the steps that type I IFN takes to activate NK cells. Type I IFN produced during a viral infection stimulates MCP-1 production, which is responsible for inflammatory monocyte migration to the site of inflammation. Once recruited, type I IFN stimulates inflammatory monocytes to produce IL-18, which then signals through the IL-18R expressed by NK cells to induce their production of IFN-γ. In understanding the mechanism of NK cell activation during mucosal

vaginal HSV-2 infection, this information can be applied to other infections, cancers, and ailments that involve IL-18 and the activation of NK cells. Moreover, this information furthers our understanding of the innate immune response to mucosal virus infections.

Experimental Procedures

Mice Six to eight week-old C57BL/6 (B6) mice were purchased from Charles River Laboratory. *Il18*$^{-/-}$ and *Il18r1*$^{-/-}$ mice on a B6 background were purchased from Jackson Laboratories. Breeding pairs of *Ifnar*$^{-/-}$ mice on a B6 background were kindly provided by Dr. Laurel Lenz (University of Colorado, USA) and then were bred at McMaster's Central Animal Facility (CAF). Breeding pairs of *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice on a BALB/c background were provided by Dr. Ito (Central Institute for Experimental Animals, Kawasaki, Japan) and then bred in our CAF. Breeding pairs of *Irf9*$^{-/-}$ mice were provided by Dr. Peter Liu to Dr. Mossman and a colony was established at McMaster's CAF. Breeding pairs of *Gata1*$^{-/-}$ mice on a B6 background were established in Dr. Jordana's lab at McMaster's CAF. *Ifnb*$^{-/-}$ mice on a B6 background were generously provided by Dr. Eleanor Fish (University of Toronto, ON, Canada). *Isg15*$^{-/-}$ mice were generously provided by Dr. Philippe Gros (McGill University, QC, Canada). *Ifnar*$^{f/f}$ *Itgax*-cre+ and *Ifnar*$^{f/f}$ control mice were provided by Dr. Schreiber and bred in our CAF. All mice were housed in specific pathogen-free conditions with a 12 hr day and 12 hr night cycle. All experiments were performed in accordance with Canadian Council on Animal Care guidelines and approved by the Animal Research Ethics Board at McMaster University.

Genital HSV-2 Infection Mice were injected subcutaneously with 2 mg of Depo provera (medroxyprogesterone acetate) 5 days prior to HSV-2 infection. Mice were then infected with HSV-2, 333 strain, and assessed for genital pathology and survival. Genital pathology was scored on a scale of 5 according to severity of redness, swelling, lesion development, hair loss, ulceration, and lower limb paralysis. Ulceration of a lesion and/or lower limb paralysis was considered endpoint.

Vaginal Viral titration Vero cells were grown in a monolayer to confluency in 12 well plates in α-MEM supplemented with 1% (v/v) each of L-glutamine, penicillin and streptomycin, and hepes. Vaginal lavages were serially diluted and incubated with the monolayer for two hours. The vero cells were then overlayed with α-MEM and subsequently incubated for 48hrs. After incubation, the cells were fixed and stained with crystal violet and plaques were quantified using an inverted microscope.

Vaginal lavages During days 0-3 p.i., vaginal lavages were performed by flushing the vaginal cavity with 30 µL of PBS. Two 30 µL washes were taken per mouse per day. Samples were subsequently centrifuged at 800g for 5 min and supernatants were removed for IFN-γ or IL-18 measurement by ELISA.

***In vivo* treatments** 0.5 µg of recombinant mouse IL-15 was incubated with 1 µg of recombinant mouse IL-15Rα, respectively, in PBS at 37º C for 30 minutes. Each mouse was administered 0.5 µg IL-15 with 1 µg IL-15 Rα complex i.p. on day 0. To deplete

neutrophils and inflammatory monocytes, anti-GR-1 antibody or isotype-matched control antibodies were administered i.p. days -2, -1, and +1 post-infection. To deplete inflammatory monocytes, 10-20 µg of anti-CCR2 antibody or isotype control antibodies were administered i.p. on days -1, 0, +1, and +2 p.i.. To block IFNAR *in vivo*, B6 mice were administered 500 µg anti-IFNAR antibody or isotype-matched control antibody d-1, d 0, d +1, and 250 µg of each antibody d +3 and d +5. To deplete NK cells, 200 µg of anti-NK1.1 antibody was given i.p. d -2 and d -1 prior to infection. The anti-IL-15 was generated by Eric Butz and provided by Amgen. Forty µg of the anti-IL-15 antibody was then given i.p. to mice d -1 prior to infection.

Cell isolation from tissues Blood and splenocytes were obtained and processed to a single-cell suspension. Red blood cells were removed using ACK lysis buffer. Vaginal tissue was isolated, minced, and incubated in a digestion mixture of RPMI-1640 with 10% FBS, 1% penicillin and streptomycin, 1% L-glutamine, 1% HEPES, and 0.030 mg/mL of collagenase A for two separate 1 hour incubations. Cells were collected after each incubation and passed through a 40 µm filter.

NK cell adoptive transfer mouse splenocytes were obtained and processed into a single-cell suspension in 2% BSA in PBS before isolation of NK cells. NK cells were isolated using Stem Cell EasySep pan-NK cell isolation or EasySep PE selection (using PE-conjugated anti-NK1.1) kits with magnetic separation. 2-3x10^6 B6, *Ifnar*$^{-/-}$ or *Il18r1*$^{-/-}$ NK

cells were adoptively transferred intravenously (i.v.) into *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice or *Ifnar*$^{-/-}$ mice).

Ex-vivo **stimulation** WT, Ifnar$^{-/-}$, and Il-18r$^{-/-}$ NK cells were isolated as previously described. NK cells were stimulated with 50 ng/mL PMA and 500 ng/mL ionomycin for 24hrs ex-vivo. Supernatants were collected and assayed for IFN-γ levels.

Cytokine detection IFN-γ in either supernatants or vaginal lavages was detected by R&D Duoset ELISA kits. IL-18 in vaginal lavages was detected by the IL-18 ELISA from MBL. CCL2 in vaginal lavages was detected by the CCL2 and MCP-1 R&D Duoset ELISA kit.

Flow cytometry staining Briefly, cells were plated at 1x10^6 cells/well in 0.2% BSA in PBS. Non-specific antibody binding was blocked with anti-CD16+CD32 antibody 2.4G2. Extracellular cell staining was conducted by using combinations of Alexa 700 or PE-Texas Red-conjugated anti-CD45, Alexa-Fluor-700 or PE Texas Red-conjugated anti-CD3, PE- or Brilliant Violet 421-conjugated anti-NK1.1, APC-conjugated DX5 mAb, PerCp-conjugated anti-NKp46, PE-Cy-7 or PerCp-Cy5.5-conjugated anti-CD11b, FITC, Alexa 700, or APC-conjugated anti-F4/80 , Alexa 700 or APC, PE or Pe-Cy-7-conjugated anti-CD11c, Brilliant Violet 421 or Alexa Fluor 700-conjugated anti-GR1, FITC or BV510-conjugated anti-Ly6G, Pacific Blue-conjugated anti-Ly6C, APC-conjugated anti-CCR2, and PE-conjugated anti-IFNAR. For intracellular staining, cells were permeabilized using BD cytofix/cytoperm and then stained with APC-conjugated anti- IFN-γ. Cells were fixed

in 1% PFA in PBS and run on the BD Biosciences FACSCanto or BD LSRII. Data were analyzed using FlowJo software.

Generation of bone marrow chimeras Bone marrow was collected from WT, *Ifnar*$^{-/-}$, and *IL18*$^{-/-}$ B6 mice and T-cell depleted using anti-CD4 (GK1.5, anti-CD8 (2.43), and anti-Thy1.2 (BD Biosciences) and low-tox guinea pig complement (Cedarlane). Recipient mice were irradiated with 2 doses of 550 rads with a three hour interval between irradiations and reconstituted with $1x10^7$ T-cell depleted bone marrow cells after the second dose of irradiation.

Statistical analyses Differences were assessed using either student's t-test (parametric data), Mann-Whitney test (non-parametric data), one-way ANOVA (if more than two groups were being analyzed), or two-way ANOVA (if more than two groups were being analyzed with more than one independent variables). If post-statistical analysis was required, a Bonferroni post-test was applied. Outliers were determined using the extreme studentized deviate (ESD) method and removed if deemed a significant outlier (0.05). All statistical analyses were completed using GraphPad Prism 4.0. Statistical significance is indicated as ****$p<0.0001$, ***$p<0.001$, **$p<0.01$, *$p<0.05$, or n.s. (not significant).

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Figures

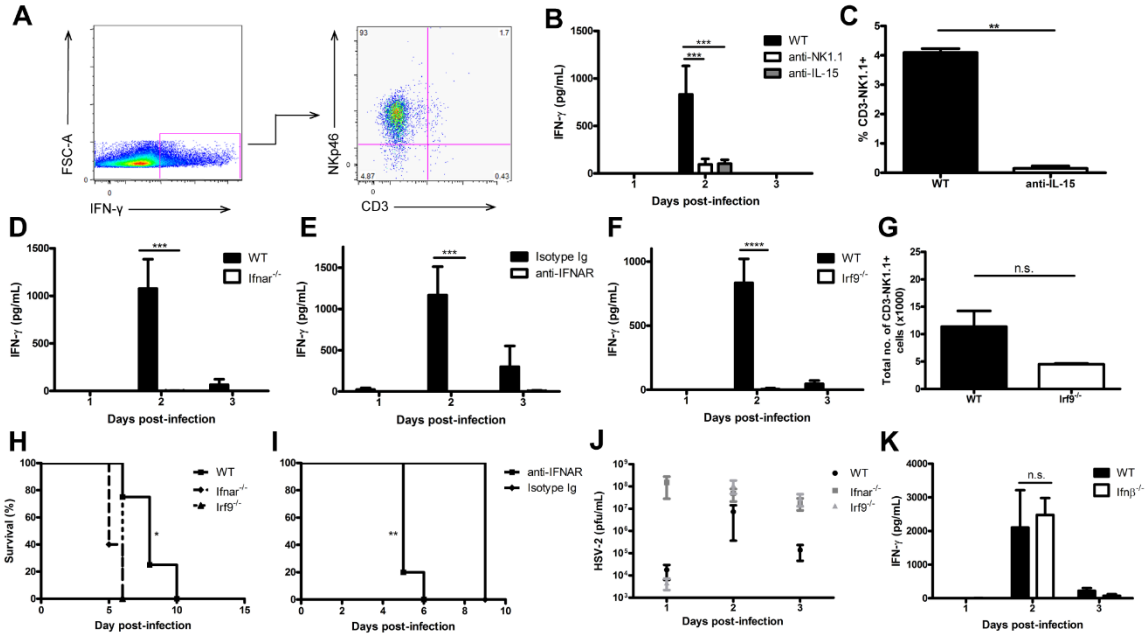

Figure 1. Type I IFN receptor and its respective signalling through IRF9 is required for NK cell IFN-γ production during HSV-2 infection. WT B6 mice were infected with $1x10^4$ pfu HSV-2 intravaginally (ivag). On day 2 p.i., vaginal tracts were processed and examined for NKp46, CD3, and IFN-γ expression (A). WT B6 mice were depleted of NK cells using an anti-NK1.1 Ab or IL-15 using an anti-IL-15 Ab and then infected with $1x10^4$ pfu HSV-2 ivag. Days 0-3 post-infection vaginal lavages were examined for IFN-γ levels (B; n=5). Peripheral blood was examined for NK cells in mice given anti-IL-15 Ab (C; n=5). WT and *Ifnar*$^{-/-}$ mice were infected with $1x10^4$ pfu HSV-2 ivag and examined for IFN-γ production on days 0-3 post-infection vaginal lavages (D; n=3; repeated twice with similar results). WT B6 mice were administered anti-IFNAR antibody or the respective isotype-matched control Ig on days -1 through 2 i.p., and then infected with $1x10^4$ pfu HSV-2 ivag. Their vaginal lavages were examined for IFN-γ content (E; n=4; repeated once with similar

results). WT and *Irf9*$^{-/-}$ mice were infected with 1x10^4 pfu HSV-2 ivag and examined for IFN-γ levels on days 0-3 p.i. vaginal lavages (F; n=3; repeated once with similar results). WT and *Irf9*$^{-/-}$ mice were infected with 1x10^4 pfu HSV-2 ivag. At day 3 post-infection, the vaginal mucosa was examined for CD3-NK1.1+ NK cells (G; n=3; repeated once with similar results). WT, *Ifnar*$^{-/-}$, and *Irf9*$^{-/-}$ mice were infected with 1x10^4 pfu HSV-2 ivag and followed for survival (H; n=5; repeated once with similar results). WT mice were administered IFNAR antibody or the respective isotype control Ig days -1 through 2, and infected with 1x10^4 pfu HSV-2 ivag. Mice were followed for survival (I; n=4). After infection with HSV-2, vaginal lavages were collected from WT, *Ifnar*$^{-/-}$, and *Irf9*$^{-/-}$ mice and assessed for HSV-2 level via plaque assay (J; n=5). WT and *Ifnb*$^{-/-}$ mice were infected with 1x10^4 pfu HSV-2 ivag. Days 0-3 vaginal washes were collected and examined for IFN-γ amount (K; n=4; repeated once with similar results). Data in (B), (D-F), and (K) are displayed as mean +/- SEM and analyzed using a two-way ANOVA; n.s. not significant, *p<0.05, ***p<0.001, ****p<0.0001. Data in (C) and (G) are displayed as mean +/- SEM and analyzed using an unpaired t-test and a Mann-Whitney test (for non-parametric data), respectively; n.s. not significant, **p<0.01. Data in (J) are displayed as mean +/- SEM and were analyzed using a one-way ANOVA. Data in (H) and (I) were analyzed using a log-rank test; *p<0.05, **p<0.01.

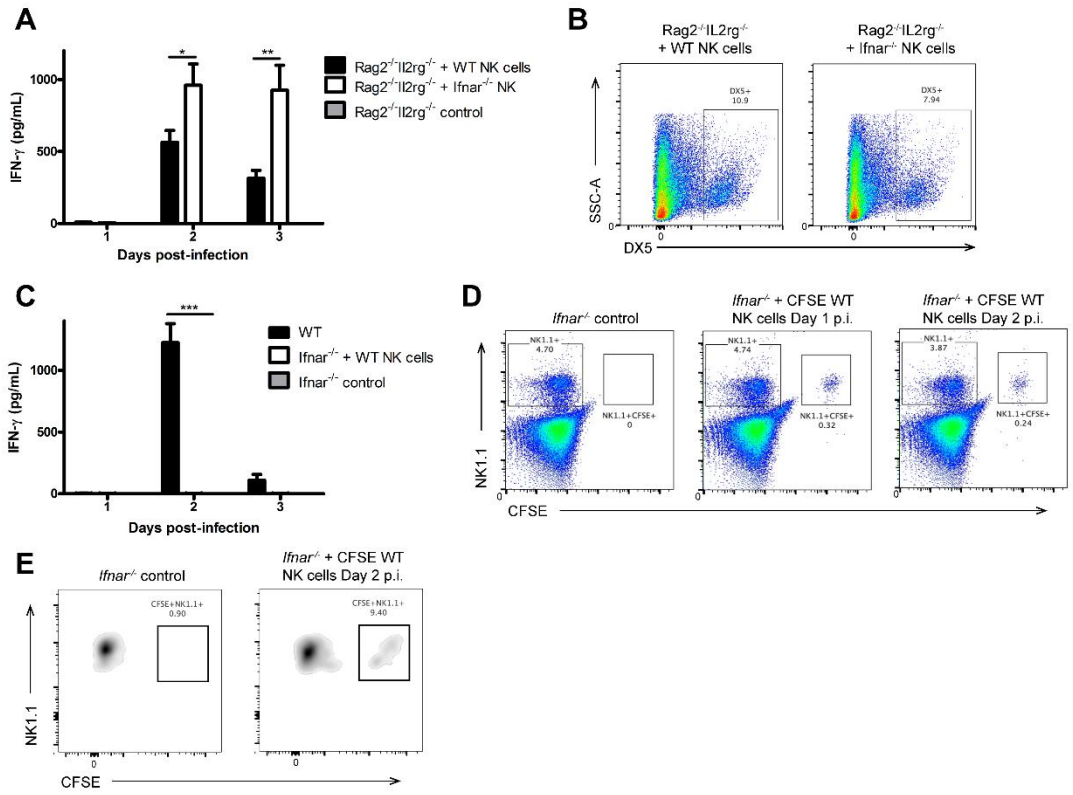

Figure 2. IFNAR is not required directly on NK cells in order to activate their IFN-γ production. NK cells were isolated from WT or *Ifnar*$^{-/-}$ spleens and adoptively transferred into *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice i.v. 24 hours post-transfer, mice were infected with 1x10^4 pfu HSV-2 ivag and on days 0-3 vaginal lavages were examined for IFN-γ levels (A; n=3; repeated twice with similar results). Spleens were collected d 3 p.i. and analyzed for DX5 expression (B). NK cells were isolated from WT spleens, CFSE-labeled, and then adoptively transferred into *Ifnar*$^{-/-}$ mice. Twenty-four hours post-transfer, *Ifnar*$^{-/-}$ mice given WT NK cells and WT controls were infected with 1x10^4 pfu HSV-2 ivag. d 0-3 p.i. vaginal lavages were examined for IFN-γ content (C; n=3; repeated once with similar results). Spleens were examined for CFSE+NK1.1+ adoptively transferred cells days 1 and 2 post-infection (D; representative of two independent experiments). Vaginal tissue from *Ifnar*$^{-/-}$ mice with or

without adoptive transfer of CFSE-labeled NK cells was examined on d2 p.i. for CFSE+NK1.1+ cells (E; representative of two independent experiments). Vaginal cells were first gated on the CD45+CD3-NK1.1+ population, and then examined for CFSE expression. Data in (A) and (C) are displayed as mean +/- SEM and analyzed using a two-way ANOVA; *p<0.05, **p<0.01, ****p<0.0001.

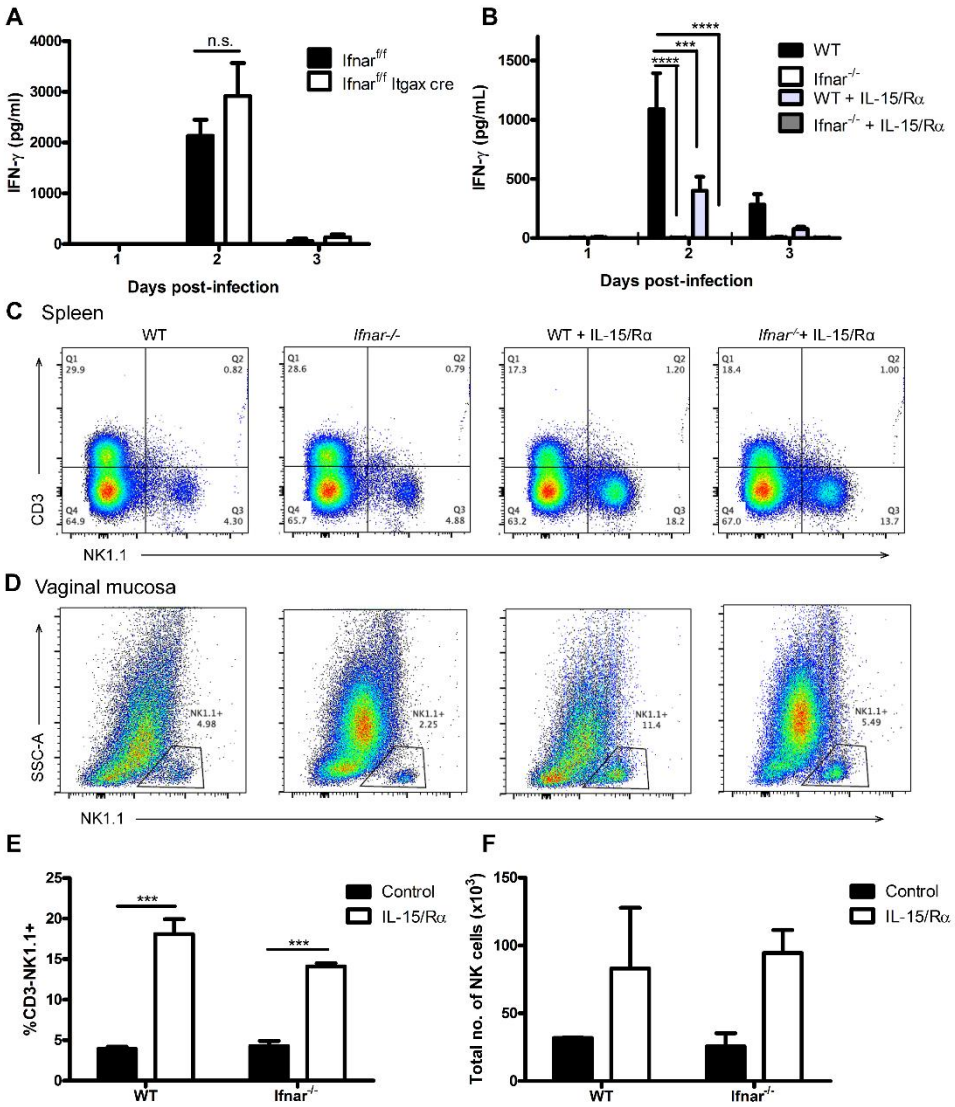

Figure 3. CD11c+ cells are not required to respond to type I IFN nor trans-present IL-15 during the activation of NK cell IFN-γ production during infection. *Ifnar*$^{f/f}$ and *Ifnar*$^{f/f}$ *Itgax*-cre mice were infected with 1x10^4 pfu HSV-2 ivag and examined for IFN-γ in the vaginal lavages collected d 0-3 p.i. (A; n=5; repeated once with similar results). WT and *Ifnar*$^{-/-}$ mice were given 0.5 µg IL-15 in complex with 1 µg IL-15Rα i.p. and subsequently infected with 5x10^4 pfu HSV-2 ivag on the same day. Vaginal washes were collected d 0-3 post-infection and examined for IFN-γ levels (B; n=4; repeated once with similar results). Spleen (C; n=3) and vaginal tissue (D; n=2) were collected on d 3 p.i. and examined for CD45+CD3-NK1.1+ NK cells as shown in the representative flow plots. Flow data are quantitatively shown for spleen and vaginal mucosa (E and F, respectively). Data in (A), (B), (E), and (F) are displayed as mean +/- SEM and analyzed using a two-way ANOVA; n.s. not significant, ***p<0.001, ****p<0.0001.

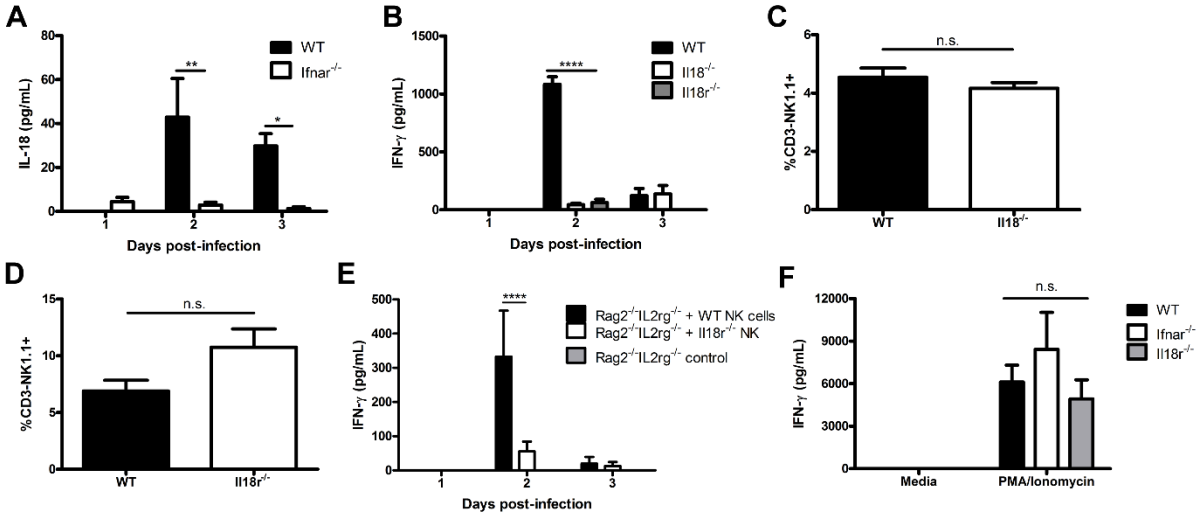

Figure 4. IL-18 and IL-18R are both required for NK cell IFN-γ production during HSV-2 infection. WT and *Ifnar*$^{-/-}$ mice were infected with 1x10^4 pfu HSV-2 ivag and d 0-3 vaginal lavages were examined for IL-18. Data was normalized to *Il18*$^{-/-}$ data (A; n=5; repeated once with similar results). WT, *Il18*$^{-/-}$, and *Il18r1*$^{-/-}$ B6 mice were infected with 1x10^4 pfu HSV-2 ivag and on d 0-3 p.i. vaginal lavages were examined for IFN-γ content (B; n=5; repeated once with similar results). Vaginal tissue was isolated on d 3 p.i. and examined for CD45+CD3-NK1.1+ cells in *Il18*$^{-/-}$ (C; n=3) and *Il18r1*$^{-/-}$ (D; n=3) mice. WT and *IL18r1*$^{-/-}$ NK cells were isolated from the spleen and adoptively transferred into *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice i.v. 24 h p.i., the mice were infected with 1x10^4 pfu HSV-2 ivag. On d 0-5 p.i., vaginal lavages were collected and examined for IFN-γ (E; n=6). NK cells were isolated from WT, *Ifnar*$^{-/-}$ and *Il-18r*$^{-/-}$ spleens and stimulated with 50 ng/mL PMA and 500 ng/mL ionomycin for 24hrs *ex vivo*. Supernatants were collected and assayed for IFN-γ (F, n=3). Data in (A), (B), and (E) are displayed as mean +/- SEM and analyzed using a two-way ANOVA; *p<0.05, **p<0.01, ****p<0.0001. Data in (C) and (D) are displayed as mean +/- SEM and analyzed using an unpaired t-test; n.s. not significant. Data in (F) is displayed as mean +/- SEM and analyzed using a one-way ANOVA; n.s. not significant.

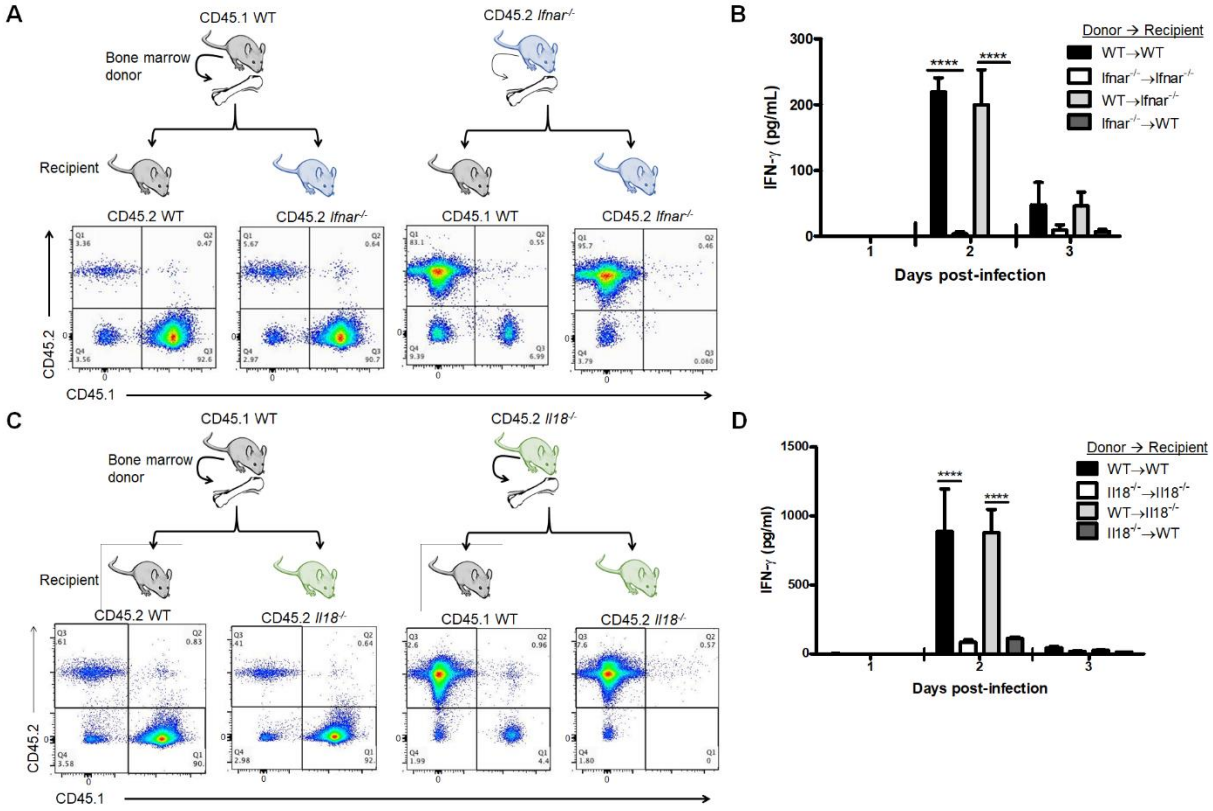

Figure 5. The hematopoietic cell compartment is required to respond to type I IFN and produce IL-18 in order to activate IFN-γ production during HSV-2 infection. WT and *Ifnar*$^{-/-}$ mice were lethally irradiated and reconstituted with either WT or *Ifnar*$^{-/-}$ bone marrow. Mice were allowed to reconstitute for 6-8 weeks and peripheral blood was assessed for reconstitution by examining the frequency of cells expressing CD45.1 or CD45.1 (A). Mice were then infected with 5x10^4 pfu HSV-2 ivag. On d 1-3 vaginal lavages were examined for IFN-γ content in NK cells (B; n=3; repeated once with similar results). WT and Il18$^{-/-}$ mice were lethally irradiated and reconstituted with either WT or *Il18*$^{-/-}$ bone marrow. Six to eight weeks post-reconstitution, mice were examined for CD45.1 and CD45.2 expression (C). Mice were then infected with 1x10^4 pfu HSV-2 ivag. On d 1-3 p.i., vaginal

lavages were examined for IFN-γ in NK cells (D; n=7). Data in (B) and (D) are displayed as mean +/- SEM and analyzed using a two-way ANOVA; ****p<0.0001.

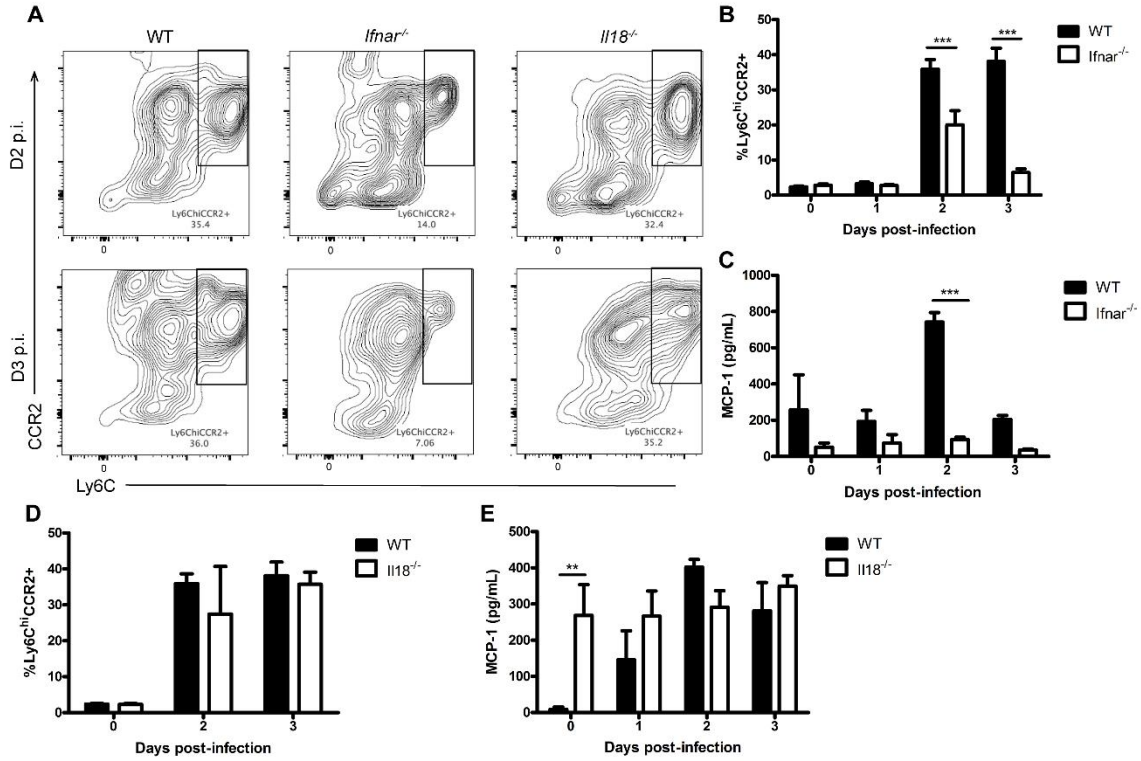

Figure 6. Decreased vaginal inflammatory monocyte infiltration in *Ifnar*$^{-/-}$ mice during HSV-2 infection. WT and *Ifnar*$^{-/-}$ mice were infected with 1x10^4 pfu HSV-2 and vaginal tissue was collected at baseline and on d 1-3 p.i. and examined for inflammatory monocytes, defined as CD45+CD11c-CD11b+Ly6G-Ly6C^{hi}CCR2+ cells as shown in representative flow plots (A). Flow cytometry data were quantified and graphically represented in (B; n=3; repeated once with similar results). Vaginal lavages were collected on d 0-3 p.i. and examined for MCP-1 (C; n=3; repeated once with similar results). WT and *Il18*$^{-/-}$ mice were infected with 1x10^4 pfu HSV-2 ivag and on d 0-3 p.i. vaginal lavages

were examined for MCP-1 (D; n=4). WT and *Il18*$^{-/-}$ mice were infected with HSV-2 ivag and vaginal tissue was collected at baseline, d 2, and d 3 p.i. and examined for inflammatory monocytes (E; n=3). Data in (B) - (E) are displayed as mean +/- SEM and analyzed using a two-way ANOVA; **p<0.01, ***p<0.001.

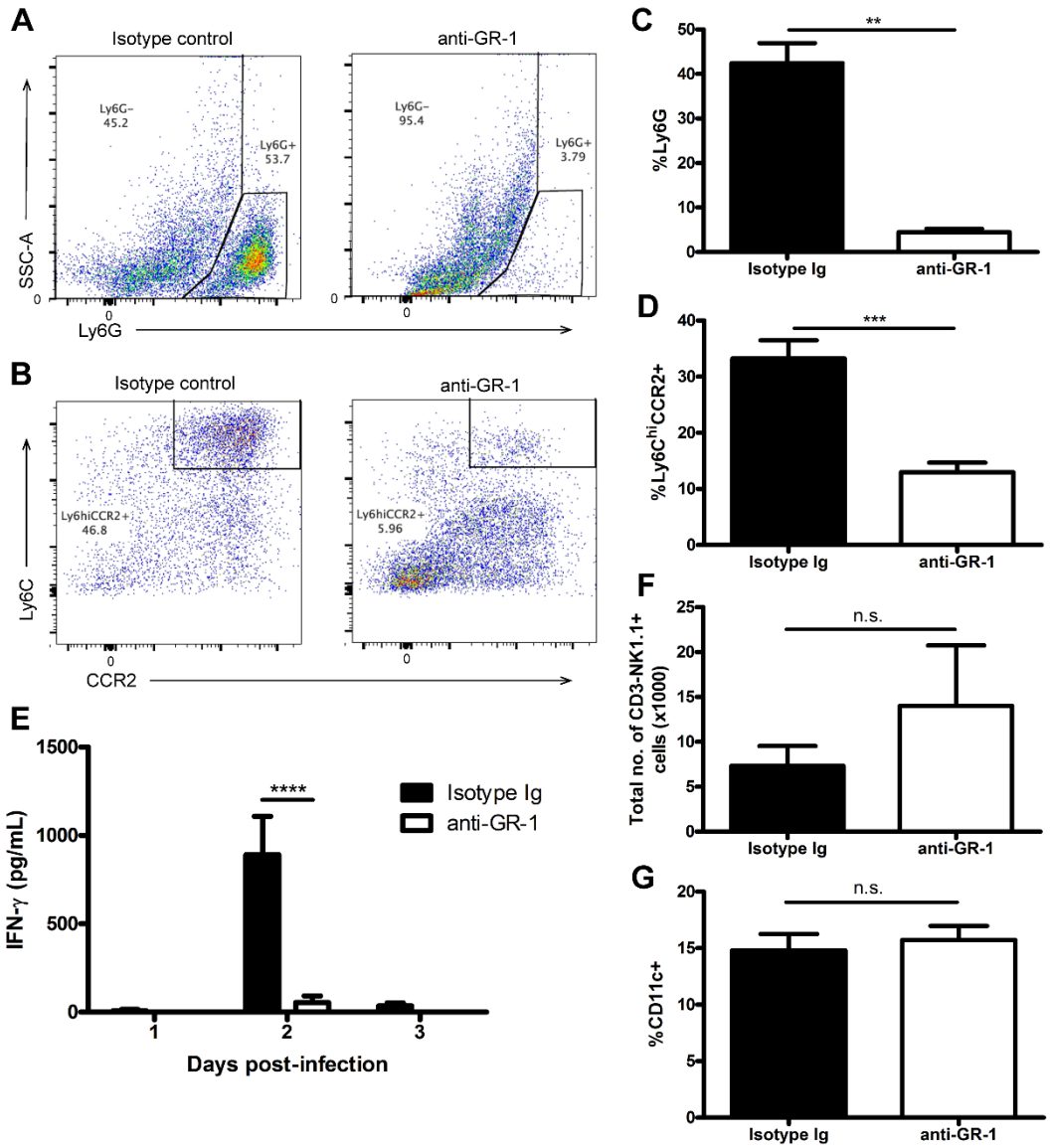

Figure 7. Inflammatory monocytes are required for activating NK cell IFN-γ production during HSV-2 infection. WT B6 mice were given an anti-GR-1 antibody or its respective isotype-matched control Ig and infected with $1x10^4$ pfu HSV-2 ivag. Vaginal tissue was examined for neutrophil (CD45+CD11c-CD11b+Ly6G+) and inflammatory monocyte (CD45+CD11c-CD11b+Ly6G-Ly6ChiCCR2+) depletion on d 3 p.i. Representative flow plots of vaginal neutrophil and inflammatory monocyte populations are shown, respectively, in (A) and (B). Vaginal neutrophil and inflammatory monocyte populations are displayed graphically in (C) and (D), respectively (n=5; repeated once with similar results). On d 0-3 p.i., vaginal washes were examined for IFN-γ (E; n=5; repeated twice with similar results). Vaginal tissue collected on d 3 p.i. was also examined for total vaginal NK cell number (F; CD45+CD3-NK1.1+; n=5) and CD11c+ cells (G; CD45+CD11c+; n=5; repeated once with similar results). Data in (C), (D), (F), and (G) are displayed as mean +/- SEM and analyzed using an unpaired t-test in (D) and (G) and a Mann-Whitney test (for non-parametric data) in (C) and (F); n.s. not significant, **p<0.01, ***p<0.001. Data in (E) are displayed as mean +/- SEM and analyzed using a two-way ANOVA; ****p<0.0001**.**

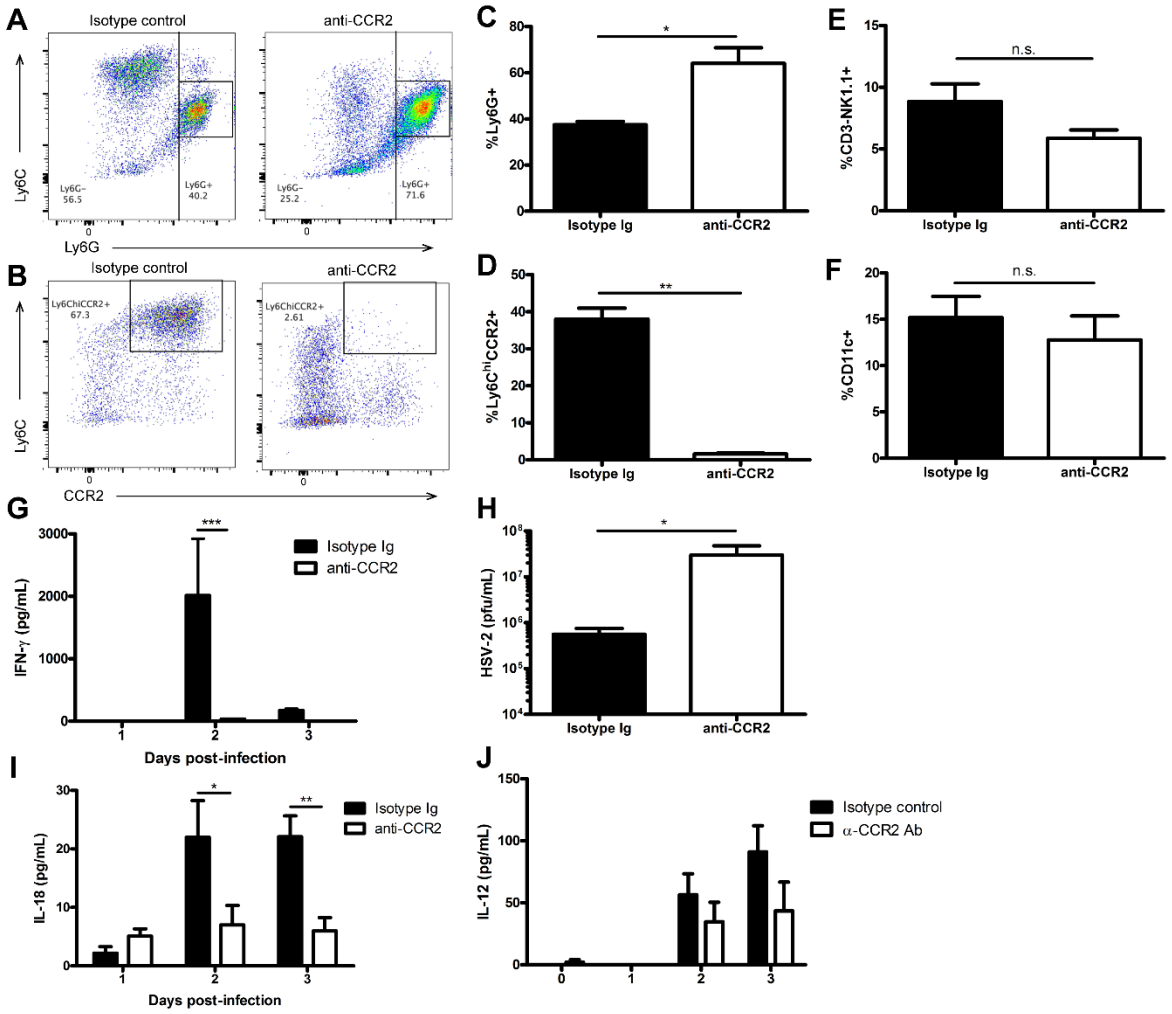

Figure 8. Inflammatory monocytes are required for activation of NK cell IFN-γ production during infection. WT B6 mice were given an anti-CCR2 antibody or the respective isotype-matched control Ig to deplete inflammatory monocytes and then infected with $1x10^4$ pfu HSV-2 ivag. Vaginal tissue was collected on d 3 p.i. and examined for neutrophil and inflammatory monocyte populations. Representative flow plots are respectively shown in (A) and (B), and graphically in (C) and (D), respectively (n=5). On d 3 p.i. vaginal cells were also examined for NK cells (E; n=5) and CD11c+ cells (F; n=5). On d 0-3 p.i. vaginal lavages were examined for IFN-γ levels (G; n=4; repeated once with similar results). Day

2 p.i. vaginal washes were examined for HSV-2 viral titers using a plaque assay method (H; n=4; repeated once with similar results). Day 0-3 p.i. vaginal lavages were also examined for IL-18 levels (I; n=4; repeated once with similar results) and IL-12 levels (J; n=5). Data in (C)-(F) and (H) are displayed as mean +/- SEM and analyzed using an unpaired t-test in (F) and a Mann-Whitney test (for non-parametric data) in (C) - (E) and (H); n.s. not significant, *p<0.05, **p<0.01. Data in (G), (I), and (J) are displayed as mean +/- SEM and analyzed using a two-way ANOVA; *p<0.05, **p<0.01, ***p<0.001.

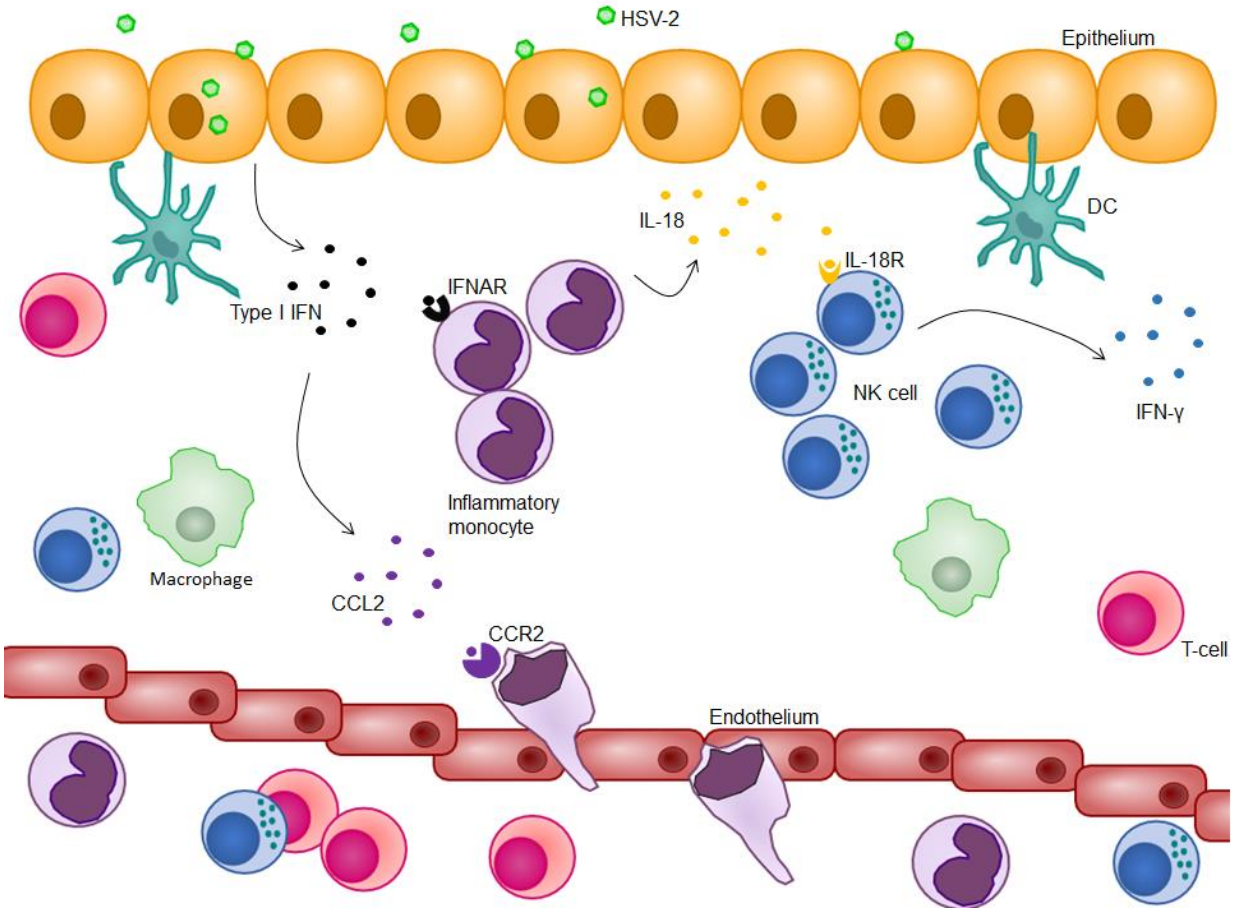

Figure 9. Type I IFN activates NK cell IFN-γ production through the stimulation of IL-18 production in inflammatory monocytes during vaginal HSV-2 infection. Rapidly after HSV-2 infection, type I IFN is produced in the vaginal mucosa. Type I IFN induces CCL2 production from a cell type or types and recruits inflammatory monocytes to the site of infection. Type I IFN binds IFNAR on inflammatory monocytes and signals through IRF9 to induce their release of IL-18. IL-18 then ligates to IL-18R on NK cells to induce their production of IFN-γ.

References:

Ashkar, A.A., and K.L. Rosenthal. 2003. Interleukin-15 and natural killer and NKT cells play a critical role in innate protection against genital herpes simplex virus type 2 infection. *J Virol* 77:10168-10171.

Baranek, T., T.P. Manh, Y. Alexandre, M.A. Maqbool, J.Z. Cabeza, E. Tomasello, K. Crozat, G. Bessou, N. Zucchini, S.H. Robbins, E. Vivier, U. Kalinke, P. Ferrier, and M. Dalod. 2012. Differential responses of immune cells to type I interferon contribute to host resistance to viral infection. *Cell Host Microbe* 12:571-584.

Barr, D.P., G.T. Belz, P.C. Reading, M. Wojtasiak, P.G. Whitney, W.R. Heath, F.R. Carbone, and A.G. Brooks. 2007. A role for plasmacytoid dendritic cells in the rapid IL-18-dependent activation of NK cells following HSV-1 infection. *Eur J Immunol* 37:1334-1342.

Bogunovic, D., M. Byun, L.A. Durfee, A. Abhyankar, O. Sanal, D. Mansouri, S. Salem, I. Radovanovic, A.V. Grant, P. Adimi, N. Mansouri, S. Okada, V.L. Bryant, X.F. Kong, A. Kreins, M.M. Velez, B. Boisson, S. Khalilzadeh, U. Ozcelik, I.A. Darazam, J.W. Schoggins, C.M. Rice, S. Al-Muhsen, M. Behr, G. Vogt, A. Puel, J. Bustamante, P. Gros, J.M. Huibregtse, L. Abel, S. Boisson-Dupuis, and J.L. Casanova. 2012. Mycobacterial disease and impaired IFN-gamma immunity in humans with inherited ISG15 deficiency. *Science* 337:1684-1688.

Brandstadter, J.D., X. Huang, and Y. Yang. 2014. NK cell-extrinsic IL-18 signaling is required for efficient NK-cell activation by vaccinia virus. *Eur J Immunol* 44:2659-2666.

Daley, J.M., A.A. Thomay, M.D. Connolly, J.S. Reichner, and J.E. Albina. 2008. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. *J Leukoc Biol* 83:64-70.

Diamond, M.S., M. Kinder, H. Matsushita, M. Mashayekhi, G.P. Dunn, J.M. Archambault, H. Lee, C.D. Arthur, J.M. White, U. Kalinke, K.M. Murphy, and

R.D. Schreiber. 2011. Type I interferon is selectively required by dendritic cells for immune rejection of tumors. *J Exp Med* 208:1989-2003.

Dinarello, C.A., D. Novick, S. Kim, and G. Kaplanski. 2013. Interleukin-18 and IL-18 binding protein. *Front Immunol* 4:289.

Elpek, K.G., M.P. Rubinstein, A. Bellemare-Pelletier, A.W. Goldrath, and S.J. Turley. 2010. Mature natural killer cells with phenotypic and functional alterations accumulate upon sustained stimulation with IL-15/IL-15Ralpha complexes. *Proc Natl Acad Sci U S A* 107:21647-21652.

Fang, R., H. Hara, S. Sakai, E. Hernandez-Cuellar, M. Mitsuyama, I. Kawamura, and K. Tsuchiya. 2014. Type I interferon signaling regulates activation of the absent in melanoma 2 inflammasome during Streptococcus pneumoniae infection. *Infect Immun* 82:2310-2317.

Fernandes-Alnemri, T., J.W. Yu, C. Juliana, L. Solorzano, S. Kang, J. Wu, P. Datta, M. McCormick, L. Huang, E. McDermott, L. Eisenlohr, C.P. Landel, and E.S. Alnemri. 2010. The AIM2 inflammasome is critical for innate immunity to Francisella tularensis. *Nat Immunol* 11:385-393.

Gill, N., M.J. Chenoweth, E.F. Verdu, and A.A. Ashkar. 2011. NK cells require type I IFN receptor for antiviral responses during genital HSV-2 infection. *Cell Immunol* 269:29-37.

Gill, N., P.M. Deacon, B. Lichty, K.L. Mossman, and A.A. Ashkar. 2006. Induction of innate immunity against herpes simplex virus type 2 infection via local delivery of Toll-like receptor ligands correlates with beta interferon production. *J Virol* 80:9943-9950.

Gill, N., K.L. Rosenthal, and A.A. Ashkar. 2005. NK and NKT cell-independent contribution of interleukin-15 to innate protection against mucosal viral infection. *J Virol* 79:4470-4478.

Gillgrass, A.E., A.A. Ashkar, K.L. Rosenthal, and C. Kaushic. 2003. Prolonged exposure to progesterone prevents induction of protective mucosal responses following intravaginal immunization with attenuated herpes simplex virus type 2. *J Virol* 77:9845-9851.

Guan, J., S.M. Miah, Z.S. Wilson, T.K. Erick, C. Banh, and L. Brossay. 2014. Role of type I interferon receptor signaling on NK cell development and functions. *PLoS One* 9:e111302.

Hamerman, J.A., K. Ogasawara, and L.L. Lanier. 2004. Cutting edge: Toll-like receptor signaling in macrophages induces ligands for the NKG2D receptor. *J Immunol* 172:2001-2005.

Harandi, A.M., B. Svennerholm, J. Holmgren, and K. Eriksson. 2001. Interleukin-12 (IL-12) and IL-18 are important in innate defense against genital herpes simplex virus type 2 infection in mice but are not required for the development of acquired gamma interferon-mediated protective immunity. *J Virol* 75:6705-6709.

Iijima, N., L.M. Mattei, and A. Iwasaki. 2011. Recruited inflammatory monocytes stimulate antiviral Th1 immunity in infected tissue. *Proc Natl Acad Sci U S A* 108:284-289.

Krug, A., A.R. French, W. Barchet, J.A. Fischer, A. Dzionek, J.T. Pingel, M.M. Orihuela, S. Akira, W.M. Yokoyama, and M. Colonna. 2004. TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. *Immunity* 21:107-119.

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

Mack, E.A., L.E. Kallal, D.A. Demers, and C.A. Biron. 2011. Type 1 interferon induction of natural killer cell gamma interferon production for defense during lymphocytic choriomeningitis virus infection. *MBio* 2:

Mack, M., J. Cihak, C. Simonis, B. Luckow, A.E. Proudfoot, J. Plachy, H. Bruhl, M. Frink, H.J. Anders, V. Vielhauer, J. Pfirstinger, M. Stangassinger, and D. Schlondorff. 2001. Expression and characterization of the chemokine receptors CCR2 and CCR5 in mice. *J Immunol* 166:4697-4704.

Martinez, J., X. Huang, and Y. Yang. 2008. Direct action of type I IFN on NK cells is required for their activation in response to vaccinia viral infection in vivo. *J Immunol* 180:1592-1597.

Nagarajan, U.M., J. Sikes, D. Prantner, C.W. Andrews, Jr., L. Frazer, A. Goodwin, J.N. Snowden, and T. Darville. 2011. MyD88 deficiency leads to decreased NK cell gamma interferon production and T cell recruitment during Chlamydia muridarum genital tract infection, but a predominant Th1 response and enhanced monocytic inflammation are associated with infection resolution. *Infect Immun* 79:486-498.

Nguyen, K.B., L.P. Cousens, L.A. Doughty, G.C. Pien, J.E. Durbin, and C.A. Biron. 2000. Interferon alpha/beta-mediated inhibition and promotion of interferon gamma: STAT1 resolves a paradox. *Nat Immunol* 1:70-76.

Okamura, H., K. Nagata, T. Komatsu, T. Tanimoto, Y. Nukata, F. Tanabe, K. Akita, K. Torigoe, T. Okura, S. Fukuda, and et al. 1995. A novel costimulatory factor for gamma interferon induction found in the livers of mice causes endotoxic shock. *Infect Immun* 63:3966-3972.

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

Pegram, H.J., D.M. Andrews, M.J. Smyth, P.K. Darcy, and M.H. Kershaw. 2011. Activating and inhibitory receptors of natural killer cells. *Immunol Cell Biol* 89:216-224.

Perry, A.K., G. Chen, D. Zheng, H. Tang, and G. Cheng. 2005. The host type I interferon response to viral and bacterial infections. *Cell Res* 15:407-422.

Pierini, R., M. Perret, S. Djebali, C. Juruj, M.C. Michallet, I. Forster, J. Marvel, T. Walzer, and T. Henry. 2013. ASC controls IFN-gamma levels in an IL-18-dependent manner in caspase-1-deficient mice infected with Francisella novicida. *J Immunol* 191:3847-3857.

Platanias, L.C. 2005. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol* 5:375-386.

Robertson, S.A., V.J. Mau, I.G. Young, and K.I. Matthaei. 2000. Uterine eosinophils and reproductive performance in interleukin 5-deficient mice. *J Reprod Fertil* 120:423-432.

Schumak, B., K. Klocke, J.M. Kuepper, A. Biswas, A. Djie-Maletz, A. Limmer, N. van Rooijen, M. Mack, A. Hoerauf, and I.R. Dunay. 2015. Specific depletion of Ly6C(hi) inflammatory monocytes prevents immunopathology in experimental cerebral malaria. *PLoS One* 10:e0124080.

Serti, E., J.M. Werner, M. Chattergoon, A.L. Cox, V. Lohmann, and B. Rehermann. 2014. Monocytes activate natural killer cells via inflammasome-induced interleukin 18 in response to hepatitis C virus replication. *Gastroenterology* 147:209-220 e203.

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

Teles, R.M., T.G. Graeber, S.R. Krutzik, D. Montoya, M. Schenk, D.J. Lee, E. Komisopoulou, K. Kelly-Scumpia, R. Chun, S.S. Iyer, E.N. Sarno, T.H. Rea, M. Hewison, J.S. Adams, S.J. Popper, D.A. Relman, S. Stenger, B.R. Bloom, G. Cheng, and R.L. Modlin. 2013. Type I interferon suppresses type II interferon-triggered human anti-mycobacterial responses. *Science* 339:1448-1453.

Thapa, M., W.A. Kuziel, and D.J. Carr. 2007. Susceptibility of CCR5-deficient mice to genital herpes simplex virus type 2 is linked to NK cell mobilization. *J Virol* 81:3704-3713.

Vivier, E., E. Tomasello, M. Baratin, T. Walzer, and S. Ugolini. 2008. Functions of natural killer cells. *Nat Immunol* 9:503-510.

Wang, Y., G. Chaudhri, R.J. Jackson, and G. Karupiah. 2009. IL-12p40 and IL-18 play pivotal roles in orchestrating the cell-mediated immune response to a poxvirus infection. *J Immunol* 183:3324-3331.

Zhang, S., B. Saha, K. Kodys, and G. Szabo. 2013. IFN-gamma production by human natural killer cells in response to HCV-infected hepatoma cells is dependent on accessory cells. *J Hepatol* 59:442-449.

Zhu, J., X. Huang, and Y. Yang. 2008. A critical role for type I IFN-dependent NK cell activation in innate immune elimination of adenoviral vectors in vivo. *Mol Ther* 16:1300-1307.

CHAPTER 3

Type I Interferon regulates a Type 2-Independent Mechanism of Virus-induced Innate Immunopathology

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AJ Lee designed and performed all experiments, analyzed the data, and wrote the manuscript. MV Chew, SM Poznanski, and E Ayaub provided technical help and expertise. A Dubey performed western blotting experiments in Supplementary Figure 3. R Jimenez-Saiz provided technical expertise. M Jordana and CD Richards provided experimental design and guidance for the overall theme of the manuscript. AA Ashkar designed experiments, edited the manuscript, and is the corresponding author.

Abstract

As part of the antiviral response, type I IFN is critical for protection and activation of immune cells; however, recent evidence has demonstrated that type I IFN is a potent negative regulator of virus-induced immunopathology in the lung, typically caused by exacerbated type 2 immune responses. In the vaginal mucosa, we found that absence of the type I IFN receptor (IFNAR) led to the development of significant vaginal immunopathology during HSV-2 infection. Though we observed significantly increased levels of IL-5 and IL-13 cytokines in *Ifnar*$^{-/-}$ mice, these levels were very (~1-15 pg/mL). However, we found high levels of IL-6 (~4500 pg/mL). Additionally, this immunopathology correlated with a higher influx of neutrophils, but not eosinophils, in *Ifnar*$^{-/-}$ mice. We found that this atypical immune-mediated pathology was independent of CD4+ T-cells and ILC2 cells, as blocking IFNAR in NRG (*Nod/Rag*$^{-/-}$/$\gamma c^{-/-}$) mice, which lack all lymphoid cells, still led to a significant level of vaginal immunopathology. IL-6 contributes to the immunopathological process, as *Il6*$^{-/-}$ mice have a decreased level of vaginal immunopathology in the absence of IFNAR. In addition to the absence of type I IFN receptor, *Ifnar*$^{-/-}$ mice completely lack NK cell IFN-γ produced during the early stages of infection. We found that rescuing NK cell IFN-γ production in *Ifnar*$^{-/-}$ mice was able to reduce the level of virus-induced immunopathology. In this paper, we demonstrate that type I IFN promotes an antiviral Th1 response by actively suppressing ILC2-independent innate immunopathology.

Introduction

Type I IFN has traditionally been known as a potent antiviral cytokine capable of inducing a plethora of antiviral responses, including but not limited to the induction of the 2-5A system and IFN-γ production from natural killer (NK) cells.[1, 2] Type I IFN is composed of a family of cytokines that include 13-14 subtypes of IFN-α and a single homologue of IFN-β.[3] These cytokines all signal through the two chain type I IFN receptor (IFNAR1 and IFNAR2), which when ligated activate a series of downstream signalling proteins that eventually lead to the formation of the IFN-stimulated gene factor 3 (ISGF3) complex to activate transcription of IFN-stimulated genes.[3] However, type I IFN has recently been shown to be a versatile cytokine, with accounts of type I IFN as a driver of negative regulation. Indeed, there have been numerous independent reports demonstrating that type I IFN receptor (*Ifnar*) is essential for dampening a type 2 immune response, as mice lacking this receptor (*Ifnar*$^{-/-}$) show immense lung pathology when challenged with a RNA virus.[4, 5, 6]

Type 2 immunopathology has been well documented in the lung mucosa in the case of airway hyperresponsiveness (AHR) and airway obstruction in response to viral, bacterial, or even innocuous stimuli.[7] It is characterized by the production of type 2 cytokines, including IL-5 and IL-13, which result in significant airway inflammation and the influx of eosinophils and neutrophils.[7] Though CD4+ Th2 cells have previously been thought of as the main producers of type 2 cytokines, the discovery of ILC2s has revealed another source of type 2 cytokines. Indeed, ILC2s have been shown to direct inflammation

in allergic inflammatory responses in the lung through the release of IL-5 and IL-13, along with IL-6 production.[5, 6]

Recently, Duerr *et al* and Moro *et al* have both independently shown that type I IFN is a potent negative regulator of ILC2s in the lung mucosa when challenging with an influenza A virus or stimulation with IL-33 *in vivo*.[5, 6] They also show that type I IFN ligates IFNAR directly on ILC2s to restrain their production of type 2 cytokines, as well as their proliferation.[5, 6] STAT1 and IRF9 downstream of the type I IFN receptor are critical for ILC2 negative regulation.[5, 6] In addition to type I IFN, IL-27 and IFN-γ have also been shown to negatively regulate ILC2 cytokine production after stimulation with IL-33.[5, 6]

The vaginal tract is a unique mucosal environment regulated by reproductive hormones and dominated by lactobacillus species of microflora.[8] It is the site of numerous sexually transmitted infections, including HSV-2.[8] HSV-2 is a sexually transmitted DNA virus that is a difficult disease to prevent, as it is a lifelong infection with no cure and those with asymptomatic infections unknowingly infect other individuals.[9, 10, 11] Individuals with symptomatic infections can suffer from painful lesions, which can eventually lead to genital ulcer disease.[12] Furthermore, HSV-2 infection has been implicated in abetting HIV infection and transmission.[13, 14]

In the absence of type I IFN, mouse models of HSV-2 have increased viral titers and decreased survival compared to wild-type mice.[2, 15] As well, NK cells in the absence of type I IFN receptor are unable to produce IFN-γ in response to a viral infection, including HSV-2.[16, 17, 18, 19] Here, we demonstrate another critical function of type I IFN during vaginal virus infection. In this paper, we show that a DNA virus, HSV-2, induces

significant immune-mediated pathology in the vaginal mucosa of *Ifnar*$^{-/-}$ mice. However, this inflammation and pathology is independent of both ILC2 and Th2 cells, and the type 2 cytokines that they produce, as well as all other adaptive immune cells. We did detect a significant upregulation of IL-6, which we found to be a contributor to the vaginal immunopathology in the absence of type I IFN. Further, we found that restoration of NK cell function during vaginal HSV-2 infection was able to partially rescue the immunopathology observed in *Ifnar*$^{-/-}$ mice. Overall, our data suggests that not only is type I IFN a potent activator of the antiviral immune response, it also serves to suppress exuberant innate immune responses that can severely damage mucosal tissue.

Results

Absence of IFNAR leads to significant vaginal immunopathology during HSV-2 infection

Though others have shown that the absence of the type I IFN receptor leads to a severe type 2 immunopathological response in the lung mucosa after a RNA virus infection, we wanted to determine whether a similar response would occur using a DNA virus and a geographically distant mucosal surface, the vaginal mucosa.[4, 5] Infection of HSV-2 led to observable swelling and inflamed vaginal tracts from *Ifnar*$^{-/-}$ mice isolated at day 3 p.i. (Fig 1A). Upon closer examination of vaginal tract cross-sections stained with H&E, we found immense inflammation in the *Ifnar*$^{-/-}$ vaginal mucosa at d3 p.i., characterized by a large influx of immune cells, destruction of the epithelial lining, hemorrhaging, and an overall breakdown in vaginal tract structure in comparison to WT mice (Fig 1B). At baseline, however, we found very little inflammation in both the WT and *Ifnar*$^{-/-}$ vaginal mucosa

cross-sections (Fig 1B). We further examined collagen arrangement in both WT and *Ifnar*$^{-/-}$ mice at baseline and d3 p.i. and found decreased collagen staining in *Ifnar*$^{-/-}$ mice at d3 p.i. (Fig 1C). This suggests that in the absence of type I IFN receptor, HSV-2 induced immunopathology results in increased collagen degradation within the vaginal submucosa. To quantitatively assess inflammation, we examined the influx of CD45+ cells to the vaginal mucosa at d3 p.i. We detected a significantly increased number of total CD45+ cells in the vaginal mucosa of *Ifnar*$^{-/-}$ mice compared to WT mice at d3 p.i. (Fig 1D). This increase in total CD45+ cells in *Ifnar*$^{-/-}$ mice coincides with the intense immunopathology we observed in these mice at d3 p.i. As we found that type I IFN is essential to dampen virus-induced vaginal pathology, we examined the production of type I IFN during HSV-2 infection. Previously, we had published that IFN-β is produced at 6-12 hrs post-infection.[2] Further, Oh *et al* found that IFN-β is upregulated at 48 hrs as well.[22] Here we found that in WT mice infected with HSV-2, however, there was a significant release of IFN-α in the vaginal washes at 48hrs post-infection (Fig 1E).

Increased vaginal immunopathology in *Ifnar*$^{-/-}$ mice is not linked to increased virus shedding

We next wanted to determine whether an increased level of virus replication was responsible for the tissue immunopathology observed in *Ifnar*$^{-/-}$ mice. To examine this, we compared vaginal histological cross-sections between *Ifnar*$^{-/-}$ and *Il15*$^{-/-}$ mice, both of which have been shown to have similar levels of viral titers within their vaginal washes at d1 p.i.[2] We examined vaginal viral titers between WT, *Ifnar*$^{-/-}$, and *Il15*$^{-/-}$ mice and observed

no significant difference between *Ifnar*$^{-/-}$ and *Il15*$^{-/-}$ mice at d2 p.i (Fig 2A). However, when we compared histological cross-sections of d3 p.i. vaginal tracts between WT, *Ifnar*$^{-/-}$, and *Il15*$^{-/-}$ mice, there was little vaginal tissue destruction found in *Il15*$^{-/-}$ cross sections and similar levels of inflammation compared to what is observed in WT mice (Figure 2B). On the other hand, *Ifnar*$^{-/-}$ vaginal cross-sections continued to display immense inflammation and vaginal tissue destruction (Figure 2B). Therefore, despite having similar vaginal viral titers, *Ifnar*$^{-/-}$ mice display a larger scale of vaginal immunopathology compared to *Il15*$^{-/-}$ mice, suggesting that increased virus replication is not correlated with the immunopathology observed in *Ifnar*$^{-/-}$ mice.

Increased influx of neutrophils in *Ifnar*$^{-/-}$ mice does not mediate vaginal immunopathology

To further characterize the inflammation observed in *Ifnar*$^{-/-}$ mice, we examined the influx of neutrophils and eosinophils at different days post-infection in the vaginal mucosa of WT and *Ifnar*$^{-/-}$ mice. Similar to what Duerr *et al* observed in the lung, we detected a significantly increased proportion and total number of neutrophils (Ly6G+ cells) in the vaginal mucosa of *Ifnar*$^{-/-}$ mice at d2 p.i. in comparison to WT mice (Fig 3A-C).[5] Neutrophils have an arsenal of immune effector mechanisms, including phagocytosis, netosis, and the production of antimicrobials, proteases, and oxidants.[23] While beneficial for combatting bacterial infections, these mechanisms can also cause tissue damage, particularly the release of matrix metalloproteases.[24] To determine whether the increased proportion of neutrophils in *Ifnar*$^{-/-}$ mice play a role in the vast tissue damage observed at d3 p.i., we depleted neutrophils using an α-Ly6G antibody, which specifically targets

neutrophils.[25] Using this antibody *in vivo*, we observed significant depletion of neutrophils at d3 p.i. in the blood, spleen, and vaginal tissue of WT mice (Supplementary Fig1). However, in the absence of neutrophils, we still found a high degree of vaginal tissue destruction and pathology in *Ifnar*$^{-/-}$ mice comparable to *Ifnar*$^{-/-}$ mice that were given an isotype control antibody (Fig 3D). Thus, while neutrophil levels are significantly increased, they are not necessary for generating vaginal tissue pathology. Using CD11b and Siglec-F, we examined the eosinophil population in the vaginal mucosa at different days post-infection between WT and *Ifnar*$^{-/-}$ mice. We found no difference in proportion nor total cell number of eosinophils between the two groups (Fig 4A-C).

IL-6 signaling contributes to the vaginal pathology in *Ifnar*$^{-/-}$ mice

In the lung microenvironment, infection with influenza virus induced a significantly increased level of type 2 cytokine secretion (IL-5 and IL-13), as well as IL-6, in *Ifnar*$^{-/-}$ mice.[5] When infecting *Ifnar*$^{-/-}$ mice intravaginally with HSV-2, though we observed an upregulation of these cytokines in the vaginal washes collected (IL-5 and IL-13), the levels of these stereotypical type 2 cytokines were very low (Fig 5A and B). There was no difference in levels of IL-4 and a significant increase in IL-9 strictly at baseline in *Ifnar*$^{-/-}$ mice compared to control mice (Fig 5C and D). On the other hand, *Ifnar*$^{-/-}$ mice had a significantly higher level of IL-6 compared to WT mice (Fig 5E). Despite the high levels of IL-6 observed in *Ifnar*$^{-/-}$ mice, we did not detect any difference in levels of IL-1β or TNF-α between WT and *Ifnar*$^{-/-}$ mice, as these cytokines are typically produced together to induce an acute phase response (Supplementary Fig 2). Similar to what we have found in

previous papers, *Ifnar*$^{-/-}$ mice had a significant abrogation of IFN-γ production compared to WT mice (Fig 5F).[2, 26] As the timely increase in IL-6 coincides with the immunopathology found in *Ifnar*$^{-/-}$ mice, we examined the role of IL-6 in contributing to the vaginal immunopathology. When we blocked the type I IFN receptor in WT mice, we observed a similar degree of vaginal immunopathology in comparison to genetically *Ifnar* knockout mice. Upon blocking the type I IFN receptor in *Il6*$^{-/-}$ mice, however, we found a decrease in vaginal immunopathology with a lower degree of tissue destruction, particularly at the epithelial lining (Fig 6). Overall, our data suggests that IL-6 is highly induced in *Ifnar*$^{-/-}$ mice during HSV-2 infection and IL-6 plays a role in the development of vaginal immunopathology.

ILC2 cells and CD4+ T-cells do not mediate the vaginal immunopathology in *Ifnar*$^{-/-}$ mice

Recently, Duerr *et al* and Moro *et al* both determined that the absence of *Ifnar* led to significantly higher levels of ILC2 cells in the lung upon virus infection.[5, 6] Further, they found that these ILC2s were responsible for the increased production of type 2 cytokines.[5, 6] Though we did not observe a high level of the prototypical type 2 cytokines (i.e. IL-5 and IL-13), there was still a significant increase in these type 2 cytokines in *Ifnar*$^{-/-}$ mice when infected with HSV-2. To this end, we examined the levels of ILC2 cells in the vaginal mucosa between WT and *Ifnar*$^{-/-}$ mice at days 0, 2, and 3 p.i (Fig 7A and B). Though we detected a significant increase in the proportion of ILC2 cells between WT and *Ifnar*$^{-/-}$ mice, upon examination of the total number of ILC2 cells, there was no difference between the two groups (Fig 7C and D). Further, we detected no differences in the levels of vaginal

IL-33, a potent stimulator of ILC2 cells, between WT and *Ifnar*$^{-/-}$ mice at d1 p.i. (Supplementary Fig 3). Th2 CD4+ T-cells are well known producers of type 2 cytokines, particularly during allergic and parasitic immune responses. However, WT and *Ifnar*$^{-/-}$ mice had comparable levels of CD3+ T-cells, both in proportion and total CD3+ T-cell number, in their vaginal mucosa at both d2 and d3 p.i (Figure 8A and B). We attempted to examine the levels of vaginal CD4+ T-cells, however, we were unable to detect any CD4+ staining on our CD3+ cell population (Supplementary Fig 4). Further, despite successfully depleting CD4+ T-cells in *Ifnar*$^{-/-}$ mice, we observed no difference in the degree of vaginal immunopathology between mice receiving the α-CD4 depletion antibody and mice who received and isotype control (Fig 8C-E). To definitively determine the involvement of any lymphoid cells in the development of vaginal immunopathology in the absence of type I IFN receptor signaling, we used NRG (*Nod/Rag*$^{-/-}$*/γc*$^{-/-}$) mice, which lack all lymphoid lineage cells including both ILC2s and CD4+ T-cells. We administered the α-IFNAR antibody to the mice and found that the blockade of type I IFN receptor signaling was still sufficient in inducing significant vaginal immunopathology, despite the absence of lymphoid lineage cells (Fig 9A). Blocking type I IFN receptor signaling in NRG mice yielded decreased collagen staining in comparison to mice receiving an isotype control. This indicates in the absence of both IFNAR and lymphoid cells, HSV-2-induced immunopathology resulted in collagen degradation (Fig 9B) Overall, our data suggests that not only are ILC2 and Th2 cells not involved in governing the immunopathological process in the absence of type I IFN receptor signaling, but other adaptive and innate lymphoid cells are also not involved.

Ifnar$^{-/-}$ vaginal tissue has increased numbers of F4/80+ cells and CD206 staining at d3 p.i.

As our previous data has shown that adaptive immune cells and cells of the lymphoid lineage are not involved in mediating the immunopathology in *Ifnar*$^{-/-}$ mice, the immunopathology is likely regulated by myeloid innate immune cells. Further, we have shown that neutrophils and eosinophils are unlikely contributors to this process. In instances of virus-induced lung immunopathology, macrophages have been implicated in the immunopathological process through the release of pro-inflammatory cytokines and through the upregulation of TRAIL.[27, 28] To determine if there was a difference in vaginal macrophage populations, we examined the level of F4/80+ cells in the vaginal mucosa at d0 and d3 post-infection in both *Ifnar*$^{-/-}$ and WT mice. Though we did not find a difference in the proportion of macrophages between WT and *Ifnar*$^{-/-}$ mice, we found a significantly increased total number of F4/80+ cells in *Ifnar*$^{-/-}$ vaginal tissue at d3 post-infection (Fig. 10A and B). With this higher number of F4/80+ macrophages in *Ifnar*$^{-/-}$ mice, we examined whether these macrophages had a higher expression of M2-like phenotypic markers, such as CD206 and Arginase.[29] We found higher levels of CD206 staining in cross-sections of *Ifnar*$^{-/-}$ vaginal tissue at d3 p.i. compared to WT mice and baseline controls (Fig. 10C). CD206, or the macrophage mannose receptor, is a pattern recognition receptor expressed on a number of innate immune cells, including macrophages and DCs.[29, 30] Upon activation of the receptor, CD206 triggers phagocytosis or endocytosis of the bound ligand.[30] With respect to arginase expression, we found no difference between WT and *Ifnar*$^{-/-}$ mice both at baseline and at d3 p.i. (Supplementary Fig 5). Overall, our data suggests that *Ifnar*$^{-/-}$ mice

have a significantly increased level of F4/80+ macrophages at d3 post-infection, along with an increased expression of CD206. This may indicate a potential role for macrophages in the pathogenesis of HSV-2-induced immunopathology.

IL-12 and IL-18 activation of NK cells partially suppresses HSV-2-induced immunopathology

Previous studies looking at virus-induced immunopathology in the lung of *Ifnar*$^{-/-}$ mice found that administration of recombinant IFN-γ was able to suppress the ILC2-mediated secretion of pathology-inducing type 2 cytokines.[5, 31] Furthermore, not only is there an absence of type I IFN receptor signaling in *Ifnar*$^{-/-}$ mice, but we have also shown that there is a complete absence of early NK cell IFN-γ production during vaginal HSV-2 infection.[2, 26] Thus, we wanted to examine the effect of restoring NK cell IFN-γ function on virus-induced immunopathology if *Ifnar*$^{-/-}$ mice. To rescue NK cell IFN-γ production, we administered a potent IFN-γ-inducing combination of IL-12 and IL-18 intraperitoneally. The combined stimulation of IL-12 and IL-18 was sufficient to recover early IFN-γ production both at d2 and d3 p.i. in *Ifnar*-/- mice (Fig 11A). Moreover, this combined stimulation significantly reduced vaginal HSV-2 viral titers at d2 p.i. in comparison to *Ifnar*$^{-/-}$ controls (Fig 11B). Upon examination of the vaginal tissue pathology at d3 p.i., we found that the combined administration of IL-12 and IL-18 was able to partially rescue the HSV-2 vaginal immunopathology in *Ifnar*$^{-/-}$ mice, characterized by decreased inflammation and partial restoration of the vaginal epithelial lining (Fig 11C). Further, there was an increase in collagen staining between *Ifnar*$^{-/-}$ mice treated with IL-12 and IL-

18 in comparison to control *Ifnar*$^{-/-}$ mice (Fig 11D). This partial rescue phenomena was partially dependent on NK cells, as depletion of NK cells in *Ifnar*$^{-/-}$ mice receiving IL-12 and IL-18 augmented their immunopathology (Fig 12). Thus, along with type I IFN, type II IFN may be capable of suppressing virus-induced immunopathology in the vaginal tract.

Discussion

Overall, our research shows that type I IFN is necessary to suppress an immunopathological innate immune response, as well as promote an antiviral response. Further, we found that activation of NK cells with IL-12 and IL-18 is able to partially suppress the immunopathology, indicating a role for NK cells, likely through IFN-γ production, in promoting polarization of an antiviral immune response, but suppressing detrimental innate responses.

Similar to an influenza infection in the lung, vaginal HSV-2 infection in the absence of type I IFN receptor leads to severe vaginal immunopathology characterized by a complete loss of the epithelial lining, hemorrhage, inflammation, collagen degradation, and recruitment of neutrophils.[5, 6] In WT mice, we found that IFN-α is significantly upregulated at 48hrs post-infection and could be involved in suppressing immunopathogenic immune responses. The immunopathology in *Ifnar*$^{-/-}$ mice was not correlated with increased virus shedding, as *Il15*$^{-/-}$ mice, which have similar levels of virus shedding, did not show any signs of immunopathology. Unlike the lung environment, however, we found that this virus-induced pathology was not mediated by any lymphoid cells, including ILC2 or Th2 cells. Depletion of CD4+ T-cells and infection of alymphoid mice yielded no difference in

vaginal immunopathology in mice deficient in type I IFN receptor signaling. Additionally, we found little difference in the total number of ILC2 cells present in the vaginal tract at both d2 and d3 p.i. This is a large departure from the findings of Duerr *et al* and Moro *et al*, as they found that type I IFN impedes ILC2 production of type 2 cytokines during influenza infection in the lung.[5, 6] Further, the nature of the immunopathology significantly differed from what was found in the lung. Rather than a prototypical type 2 immune response, we detected very low levels of IL-4. Despite detecting elevated levels of IL-5, IL-9, and IL-13, in *Ifnar*$^{-/-}$ mice, these cytokine levels were abundantly low. Instead, we found significantly high levels of IL-6 in the vaginal washes of *Ifnar*$^{-/-}$ mice. We also determined that this IL-6 was partially responsible for mediating the pathological response. In line with a low level of IL-6 production, we found very little difference in eosinophil recruitment between WT and *Ifnar*$^{-/-}$ mice, again differing from the respiratory influenza infection, where researchers found a significant recruitment of eosinophils to the site of infection. Lastly, activation of *Ifnar*$^{-/-}$ NK cells through the combined administration of IL-12 and IL-18 was sufficient to partially suppress the vaginal immunopathology, likely through the production of IFN-γ.

The difference in profile of immunopathology in *Ifnar*$^{-/-}$ mice between our study and those done in the respiratory tract could be due to a difference in mucosal environment. The lung environment is a highly oxygenated and vascularized organ in comparison to the vaginal tract, allowing for rapid recruitment and circulation of immune cells.[32] Further, there are specialized subsets of cells in the lung, including alveolar macrophages and type II alveolar cells that are not present in the vaginal mucosa.[33] The vaginal tract, on the other

hand, is a unique mucosal environment as it is heavily influenced by reproductive hormones and predominantly colonized with commensal lactobacillus species of bacteria.[8] These factors can have a significant impact on the type of immune response generated in response to microbial infections. Further, our studies examine the impact of a DNA virus infection, whereas influenza is an RNA virus. Thus, different virus classifications may yield different profiles of immunopathology.

Type I IFN is rapidly produced upon detection of a viral pathogen and is best known for its antiviral effects.[3] Along with inducing an antiviral response within target cells, it polarizes the response towards type 1 immunity by activating NK cell IFN-γ production, inducing maturation and upregulation of MHC II and costimulatory molecules.[26, 34] Recently, however, ongoing research has found that type I interferon is also capable of negatively regulating immune responses, through the production of IL-10 and increased expression of PD-L1.[35, 36] It is unlikely that type I IFN suppresses HSV-2-induced innate immunopathology through IL-10, as there is neither an upregulation of IL-10 in the vaginal washes of WT mice during HSV-2 infection nor is there a significant difference in levels of IL-10 in comparison to *Ifnar*$^{-/-}$ mice (unpublished data). Further research would be required to examine the role of PD-L1 in suppressing innate immunopathology.

During HSV-2 infection, IFN-β is produced at 6-12 hrs post-infection, while IFN-α and another wave of IFN-β is produced at a later time point, 48 hrs post-infection.[22, 37] The second wave of type I IFN (IFN-α) corresponds to the severe immunopathology observed at d3 post-infection. This would suggest that either IFN-α or –β could be involved in suppressing dysregulated innate immune cell function that eventually leads to vaginal

pathology during HSV-2 infection. In the future, it would be interesting to investigate the roles of IFN-α and IFN-β in suppressing virus-induced innate immunopathology.

We found that type I IFN also suppresses IL-6 production during infection, which has a role in mediating the immunopathology seen in the absence of type I IFN receptor signaling. IL-6 is part of the gp130 cytokine family and is known for its pathogenic role in inflammatory arthritis.[38] It can signal through a classical model, in which IL-6 binds to its specific membrane-bound IL-6Rα, which then joins and dimerizes gp130 to induce signal transduction and activation of its effects.[38] Alternatively, a soluble form of IL-6Rα can bind to IL-6 and signal through the membrane bound gp130, allowing IL-6 to trans-signal through a multitude of cell types.[38] IL-6 has been shown to be involved in both the recruitment and apoptosis of neutrophils.[38] The increased vaginal levels of IL-6 likely play a role in inducing apoptosis of the recruited neutrophils in *Ifnar*$^{-/-}$ mice; however, IL-6 may not play a role in neutrophil recruitment in this model, as neutrophils appear as early as day 2 p.i., whereas IL-6 is not detected until d3 p.i. Overall, our data suggests that IL-6 has a detrimental effect during virus-induced immunopathology both in the vaginal mucosal environment during HSV-2 infection.

We found that the uncontrolled immunopathology in *Ifnar*$^{-/-}$ mice was not mediated by a type 2 immune response, differing from the findings of Duerr *et al* in the lung.[5] Additionally, depletion of neutrophils had no impact on the vaginal immunopathology outcome. It has previously been shown that type I IFN is able to suppress CXCL1 and CXCL2 chemokine production, both of which are involved in neutrophil recruitment, which may explain the increased neutrophil population at d2 post-infection in *Ifnar*$^{-/-}$

mice.[39] We did, however, detect a significant increase in total number of F4/80+ macrophages at d3 p.i. and an increase in vaginal CD206 (mannose receptor) expression in *Ifnar*$^{-/-}$ mice, which would indicate a potential role for macrophages in mediating the virus-induced immunopathology in the vaginal tract. Further, this would indicate that type I IFN plays a key role in preventing this exaggerated macrophage response. However, further macrophage or cell-expressing CD206 depletion studies would be required to confirm their involvement.

In a seminal research article by Herold *et al*, they found that macrophages contributed to influenza-induced lung pathology through the release of TRAIL, which induced significant lung permeability through the increased apoptosis of epithelial cells.[27] In terms of mediating immunopathology and collagen degradation, Madsen *et al* found that macrophages can rapidly internalize and degrade collagen through their expression of CD206.[40] CD206 is not only a pattern recognition receptor for microbial carbohydrate moieties, it can also bind to collagen through its fibronectin type II domains.[41] Moreover, IL-6 has been found to promote the differentiation of macrophages, as well as their upregulation of CD206.[42] In individuals with hepatitis B or C virus-induced chronic liver inflammation, it was observed that their hepatic CD206+ cells had increased expression of pro-inflammatory genes and spontaneously produced pro-inflammatory cytokines, including IL-6, compared to healthy controls. Thus, macrophages and CD206 expression may play a role in either mediating destruction of the collagen structure during immunopathogenesis or aiding in cleaning up the already degraded collagen. Alternatively, a model of LPS-induced endotoxemia found that CD206+ cells played a protective anti-

inflammatory role.[43] Further work is required to delineate the exact role of macrophages and CD206 and how type I IFN is able to suppress their function.

The massive epithelial and collagen destruction found in the vaginal tissue points to the potential involvement of metalloproteinases and their ability to degrade collagen. Matrix metalloproteinase (MMP)-2 and MMP9 both contain fibronectin type II binding domains and can degrade collagen and extracellular matrix.[41] Kothari *et al* found that IL-6 was able to upregulate expression of MMP9 in peritoneal macrophages in a dose-dependent fashion.[44] Further, both type I and II IFNs can inhibit MMP9 expression in a sarcoma cell line and B lymphocytic leukemia cells.[45, 46] On the other hand, IFN-β treatment of lung epithelial cells induced expression of a variety of MMPs.[47] Further work will need to examine the role of proteases, including MMPs, in degradation of the vaginal tissue matrix.

We and others had published that NK cell IFN-γ production was significantly decreased in the absence of type I IFN receptor signaling.[2, 26] IL-12 and IL-18 activation of NK cells was able to dampen the vaginal immunopathology in *Ifnar*$^{-/-}$ mice as well as rescue vaginal IFN-γ production at d2 p.i. Previous studies have found that IFN-γ is a potent inhibitor of ILC2- and Th2-mediated type 2 immunopathology.[5, 31] Further, IFN-γ is a contributor to M1 macrophage polarization and induction of antiviral Th1 immune responses.[48, 49] The mechanism of NK cell-mediated suppression of pathology is likely through IFN-γ production, as IL-12 and IL-18 induce a large amount of this cytokine; however, IL-12 and IL-18 stimulation induces a wide range of factors from NK cells, including high levels of IL-8. Further work will be required to delineate the exact mechanism of NK cell-mediated suppression.

Overall, we have found a novel and critical immunomodulatory function of type I IFN in suppressing innate immunopathology during a mucosal virus infection. As we delve into modulating the type I IFN response in cancer, autoimmunity, and infectious disease, it is essential to thoroughly understand the full impacts of type I IFN as it may impact therapeutic efficacy.

Experimental Procedures

Mice: Six to twelve week old C57BL/6 (WT) mice were purchased from Charles River Laboratory. *Ifnar*$^{-/-}$ mice were bred onto a B6 background and breeding pairs were provided by Dr. Laurel Lenz (University of Colorado). *Il15*$^{-/-}$ mice were purchased from Taconic. *Nod-Rag1*$^{-/-}$*γc*$^{-/-}$ (NRG) mice were purchased from Jackson Laboratory. Colonies of *Ifnar*$^{-/-}$ *Il15*$^{-/-}$, and NRG mice were established at the McMaster Central Animal Facility (McMaster University). Mice were housed in specific pathogen-free conditions on a 12 hour light/12 hour dark cycle. All experiments were performed in accordance with Canadian Council on Animal Care guidelines and approved by the Animal Research Ethics Board at McMaster University.

Genital HSV-2 infection: Mice were injected with 2mg Depo Provera subcutaneously (s.c.) 5 days prior to infection. Mice were then infected with $1x10^4$pfu HSV-2 333 intravaginally (ivag) in a 10uL volume. Genital pathology was assessed as previously described.[20]

Vaginal tissue collection and histology: Vaginal canals were isolated from mice at day 0 or day 3 post-infection (p.i.) and fixed in a 2% paraformaldehyde solution for 48hrs. Vaginal tissue was then embedded in paraffin, cross-sectioned, and stained with H&E or Picrosirius red. Cross-sections were also assayed for CD206 or Arginase-1 expression. Sections were imaged at a 10x or 20x magnification using either a Leica microscope or scanned using a VS210 Olympus Slide Scanner.

Vaginal cell isolation: Vaginal tissue was isolated and processed into smaller pieces, which were then subjected to enzymatic digestion using a solution of RPMI supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) L-glutamine, penicillin and streptomycin, and hepes, and 0.03mg/mL Collagenase A. Tissues were mechanically stirred with the digestion mixture for two separate one hour incubation periods. Resulting cells were then passed through a 40um filter.

Flow cytometry staining: Vaginal cells were resuspended in PBS with 0.2% bovine serum albumin. Cells were then blocked with anti-CD16/CD32 to prevent non-specific binding of antibodies. Cells were then extracellularly stained with combinations of the following antibodies: PE-Texas Red or Alexa Fluor 700-conjugated anti-mouse CD45, Alexa Fluor 700-conjugated anti-mouse CD3, PE-Cy7-conjucated anti-mouse CD11c, Percp-cy5.5-conjugated anti-mouse CD11b, FITC-conjugated anti-mouse Ly6G, FITC-conjugated anti-mouse F4/80, FITC-conjugated anti-mouse lineage markers, Brilliant Violet 605-conjugated anti-mouse Sca-1, Percp-cy5.5-conjugated anti-mouse ST2, APC anti-mouse

CD127, APC-Cy7-conjugated anti-mouse CD90.2, APC-Cy7-conjugated anti-mouse CD4, PE-conjugated anti-mouse CD25, PE-conjugated anti-mouse Siglec-F, and BV-510-conjugated anti-mouse CD11b.

Viral titering from vaginal lavages: 30uL of PBS was pipetted in and out of the mouse vaginal canal twice. Serial dilutions of the vaginal lavage were incubated on a confluent monolayer of Vero cells with α-MEM supplemented with a 1% L-glutamine, penicillin and streptomycin, and hepes. After a two hour incubation, vero cells were then overlayed with α-MEM supplemented with the above additives as well as 5% (v/v) fetal bovine serum and 0.05% human immune serum. 48 hours later, the cells were fixed and stained with crystal violet and plaques were quantified using an inverted microscope.

In-vivo treatments: To deplete neutrophils, 250 µg of α-Ly6G antibody or isotype control antibodies were administered i.p. days -2, -1, and +1 post-infection. To deplete NK cells, 200 µg of α-NK1.1 antibody or isotype control anitbodies were administered i.p. days -2, -1, and +1 post-infection. To block type I IFN receptor, 500 µg of α-IFNAR Ab or an isotype control antibody were administered i.p d-1, d0, d1, d2 post-infection. To deplete CD4+ T-cells, 200 µg of α-CD4 Ab or an isotype control antibody was administered i.p. d-2, d-1, and d1 post-infection. To stimulate NK cells *in vivo*, 1 mg of IL-18 and 200 µg of IL-12 were given i.p. at d1 and d2 p.i.

Cytokine detection: Vaginal lavages were subjected to a 32-plex luminex (Eve Technologies) including IL-4, IL-5, IL-6, IL-9, IL-13, IFN-γ, TNF-α, and IL-1β. IFN-γ and IL-33 were assessed using R&D Systems ELISA kits.

Immunoblot analysis of protein: Vaginal tissues were removed at days 0 and 1 post-infection, snap frozen in liquid nitrogen, and then homogenized. Homogenized tissue was then lysed in 1mL of cold radioimmunoprecipitation assay (RIPA) buffer with protease inhibitors. Protein concentration was then determined using a Bradford Assay. The Immunoblot procedure was then conducted as previously described by Richards *et al.*[21] Blots were probed for anti-mouse IL-33 using a 1:1000 dilution at 4 C overnight. Primary antibodies were detected using an anti-goat IRDye infrared secondary antibody at a 1:10 000 dilution and then imaged with the LI-COR Odyssey infrared scanner. Band densiometry was assessed using software from Image Studio Lite (LI-COR).

Statistics: Statistically significant differences were determined using a student's t-test for differences between two groups, a Mann-Whitney test for differences between two groups of non-parametric data, a one-way ANOVA for differences between more than two groups, or a two-way ANOVA for differences between two groups with more than one independent variable. All data was analyzed using GraphPad Prism 4.0. Statistical significance is indicated as n.s. not significant, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

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References

1. Kerr, I.M. & Brown, R.E. pppA2'p5'A2'p5'A: an inhibitor of protein synthesis synthesized with an enzyme fraction from interferon-treated cells. *Proc Natl Acad Sci U S A* **75**, 256-260 (1978).

2. Gill, N., Chenoweth, M.J., Verdu, E.F. & Ashkar, A.A. NK cells require type I IFN receptor for antiviral responses during genital HSV-2 infection. *Cell Immunol* **269**, 29-37 (2011).

3. Platanias, L.C. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol* **5**, 375-386 (2005).

4. Arimori, Y. *et al.* Type I interferon limits influenza virus-induced acute lung injury by regulation of excessive inflammation in mice. *Antiviral Res* **99**, 230-237 (2013).

5. Duerr, C.U. *et al.* Type I interferon restricts type 2 immunopathology through the regulation of group 2 innate lymphoid cells. *Nat Immunol* **17**, 65-75 (2016).

6. Moro, K. *et al.* Interferon and IL-27 antagonize the function of group 2 innate lymphoid cells and type 2 innate immune responses. *Nat Immunol* **17**, 76-86 (2016).

7. Wynn, T.A. Type 2 cytokines: mechanisms and therapeutic strategies. *Nat Rev Immunol* **15**, 271-282 (2015).

8. Iwasaki, A. Antiviral immune responses in the genital tract: clues for vaccines. *Nat Rev Immunol* **10**, 699-711 (2010).

9. Wald, A. *et al.* Reactivation of genital herpes simplex virus type 2 infection in asymptomatic seropositive persons. *N Engl J Med* **342**, 844-850 (2000).

10. Johnston, C. *et al.* Standard-dose and high-dose daily antiviral therapy for short episodes of genital HSV-2 reactivation: three randomised, open-label, cross-over trials. *Lancet* **379**, 641-647 (2012).

11. Belshe, R.B. *et al.* Efficacy results of a trial of a herpes simplex vaccine. *N Engl J Med* **366**, 34-43 (2012).

12. Knipe, D.M., Howley, P. M. (ed). *Fields Virology*. Lippincott Williams & Wilkins: Philadelphia, 2007.

13. Gray, R.H. *et al.* Determinants of HIV-1 load in subjects with early and later HIV infections, in a general-population cohort of Rakai, Uganda. *J Infect Dis* **189**, 1209-1215 (2004).

14. Nagot, N. *et al.* Reduction of HIV-1 RNA levels with therapy to suppress herpes simplex virus. *N Engl J Med* **356**, 790-799 (2007).

15. Conrady, C.D., Halford, W.P. & Carr, D.J. Loss of the type I interferon pathway increases vulnerability of mice to genital herpes simplex virus 2 infection. *J Virol* **85**, 1625-1633 (2011).

16. Zhu, J., Huang, X. & Yang, Y. A critical role for type I IFN-dependent NK cell activation in innate immune elimination of adenoviral vectors in vivo. *Mol Ther* **16**, 1300-1307 (2008).

17. Martinez, J., Huang, X. & 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**, 1592-1597 (2008).

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

19. Baranek, T. *et al.* Differential responses of immune cells to type I interferon contribute to host resistance to viral infection. *Cell Host Microbe* **12**, 571-584 (2012).

20. Abdul-Careem, M.F. *et al.* Genital HSV-2 infection induces short-term NK cell memory. *PLoS One* **7**, e32821 (2012).

21. Richards, C.D. *et al.* Regulation of IL-33 by Oncostatin M in Mouse Lung Epithelial Cells. *Mediators Inflamm* **2016**, 9858374 (2016).

22. Oh, J.E. *et al.* Dysbiosis-induced IL-33 contributes to impaired antiviral immunity in the genital mucosa. *Proc Natl Acad Sci U S A* **113**, E762-771 (2016).

23. Kruger, P. *et al.* Neutrophils: Between host defence, immune modulation, and tissue injury. *PLoS Pathog* **11**, e1004651 (2015).

24. Korematsu, S. *et al.* Cell distribution differences of matrix metalloproteinase-9 and tissue inhibitor of matrix metalloproteinase-1 in patients with Kawasaki disease. *Pediatr Infect Dis J* **31**, 973-974 (2012).

25. Daley, J.M., Thomay, A.A., Connolly, M.D., Reichner, J.S. & Albina, J.E. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. *J Leukoc Biol* **83**, 64-70 (2008).

26. Lee, A.J. *et al.* Inflammatory monocytes require type I interferon receptor signaling to activate NK cells via IL-18 during a mucosal viral infection. *J Exp Med* **214**, 1153-1167 (2017).

27. Herold, S. *et al.* Lung epithelial apoptosis in influenza virus pneumonia: the role of macrophage-expressed TNF-related apoptosis-inducing ligand. *J Exp Med* **205**, 3065-3077 (2008).

28. Gong, J.H. *et al.* Influenza A virus infection of macrophages. Enhanced tumor necrosis factor-alpha (TNF-alpha) gene expression and lipopolysaccharide-triggered TNF-alpha release. *J Immunol* **147**, 3507-3513 (1991).

29. Biswas, S.K. & Mantovani, A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol* **11**, 889-896 (2010).

30. Tan, M.C. *et al.* Mannose receptor-mediated uptake of antigens strongly enhances HLA class II-restricted antigen presentation by cultured dendritic cells. *Eur J Immunol* **27**, 2426-2435 (1997).

31. Molofsky, A.B. *et al.* Interleukin-33 and Interferon-gamma Counter-Regulate Group 2 Innate Lymphoid Cell Activation during Immune Perturbation. *Immunity* **43**, 161-174 (2015).

32. Button, B.M. & Button, B. Structure and function of the mucus clearance system of the lung. *Cold Spring Harb Perspect Med* **3** (2013).

33. Weitnauer, M., Mijosek, V. & Dalpke, A.H. Control of local immunity by airway epithelial cells. *Mucosal Immunol* **9**, 287-298 (2016).

34. Simmons, D.P. *et al.* Type I IFN drives a distinctive dendritic cell maturation phenotype that allows continued class II MHC synthesis and antigen processing. *J Immunol* **188**, 3116-3126 (2012).

35. Staples, K.J. *et al.* Viral infection of human lung macrophages increases PDL1 expression via IFNbeta. *PLoS One* **10**, e0121527 (2015).

36. McNab, F.W. *et al.* Type I IFN induces IL-10 production in an IL-27-independent manner and blocks responsiveness to IFN-gamma for production of IL-12 and bacterial killing in Mycobacterium tuberculosis-infected macrophages. *J Immunol* **193**, 3600-3612 (2014).

37. Gill, N., Deacon, P.M., Lichty, B., Mossman, K.L. & Ashkar, A.A. Induction of innate immunity against herpes simplex virus type 2 infection via local delivery of Toll-like receptor ligands correlates with beta interferon production. *J Virol* **80**, 9943-9950 (2006).

38. Hunter, C.A. & Jones, S.A. IL-6 as a keystone cytokine in health and disease. *Nat Immunol* **16**, 448-457 (2015).

39. Shahangian, A. *et al.* Type I IFNs mediate development of postinfluenza bacterial pneumonia in mice. *J Clin Invest* **119**, 1910-1920 (2009).

40. Madsen, D.H. *et al.* M2-like macrophages are responsible for collagen degradation through a mannose receptor-mediated pathway. *J Cell Biol* **202**, 951-966 (2013).

41. Napper, C.E., Drickamer, K. & Taylor, M.E. Collagen binding by the mannose receptor mediated through the fibronectin type II domain. *Biochem J* **395**, 579-586 (2006).

42. Deng, W. *et al.* Mesenchymal stem cells promote CD206 expression and phagocytic activity of macrophages through IL-6 in systemic lupus erythematosus. *Clin Immunol* **161**, 209-216 (2015).

43. Tan-Garcia, A. *et al.* Intrahepatic CD206+ macrophages contribute to inflammation in advanced viral-related liver disease. *J Hepatol* (2017).

44. Kothari, P. *et al.* IL-6-mediated induction of matrix metalloproteinase-9 is modulated by JAK-dependent IL-10 expression in macrophages. *J Immunol* **192**, 349-357 (2014).

45. Sanceau, J., Boyd, D.D., Seiki, M. & Bauvois, B. Interferons inhibit tumor necrosis factor-alpha-mediated matrix metalloproteinase-9 activation via

interferon regulatory factor-1 binding competition with NF-kappa B. *J Biol Chem* **277**, 35766-35775 (2002).

46. Bauvois, B., Dumont, J., Mathiot, C. & Kolb, J.P. Production of matrix metalloproteinase-9 in early stage B-CLL: suppression by interferons. *Leukemia* **16**, 791-798 (2002).

47. Foronjy, R.F. *et al.* Type-I interferons induce lung protease responses following respiratory syncytial virus infection via RIG-I-like receptors. *Mucosal Immunol* **8**, 161-175 (2015).

48. Mills, C.D., Kincaid, K., Alt, J.M., Heilman, M.J. & Hill, A.M. M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol* **164**, 6166-6173 (2000).

49. Martin-Fontecha, A. *et al.* Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. *Nat Immunol* **5**, 1260-1265 (2004).

Figures

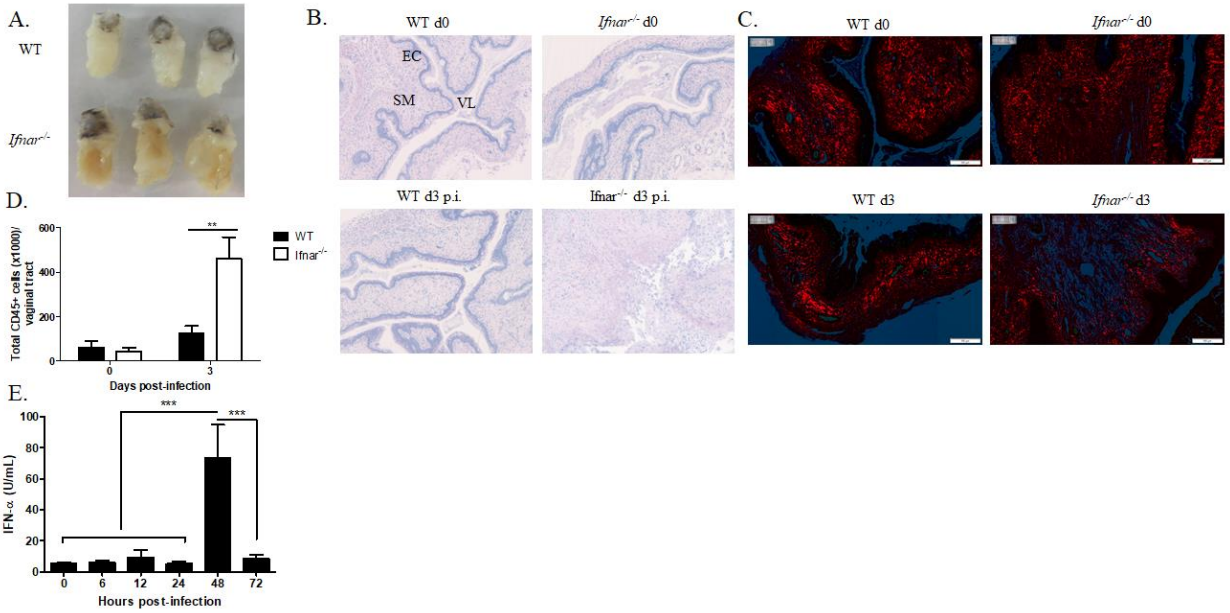


Figure 1. *Ifnar*$^{-/-}$ mice infected with HSV-2 show greater vaginal tissue immunopathology in comparison to WT mice during HSV-2 infection. WT and *Ifnar*$^{-/-}$ mice were infected with HSV-2 ivag and vaginal tracts were isolated at d3 p.i. and fixed in PFA for 48hrs. Isolated vaginal tracts are displayed in (A; n=3). Baseline and d3 p.i. vaginal tracts were sectioned and stained with H&E and imaged at 10x magnification (B; repeated once with similar results). Cross-sections were examined for collagen structure by staining for picrosirius red. Sections were then imaged at a 10x magnification (C). At d0 and d3 p.i., vaginal tracts were isolated, processed, and stained with anti-mouse CD45. Total number of CD45+ cells were quantified per vaginal tract at d3 (D) p.i. (n=3). WT mice were infected with HSV-2 ivag and vaginal lavages were collected at the time points indicated and examined for IFN-α (E; n=5). EC epithelial cell, SM submucosa, VL vaginal lumen; **p<0.01, ***p<0.001

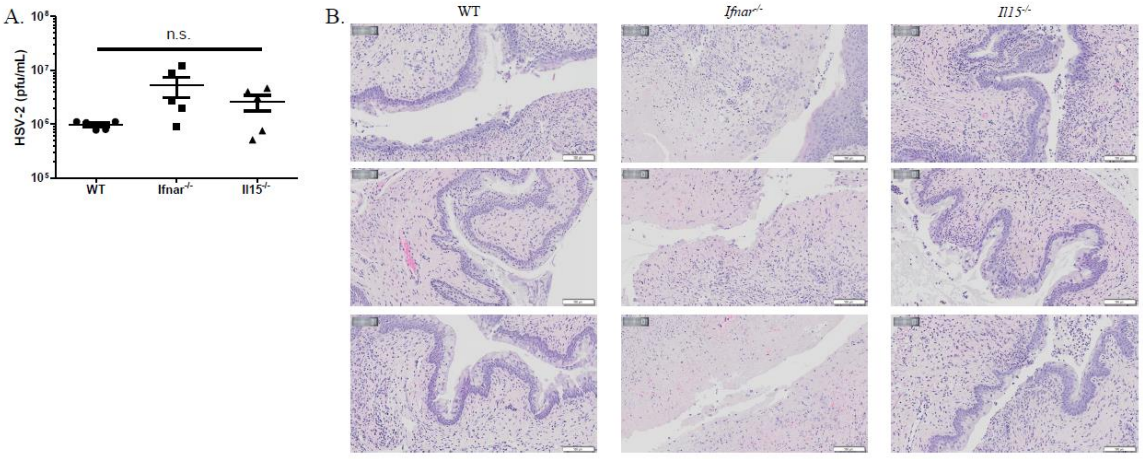


Figure 2. Significant pathology in *Ifnar*$^{-/-}$ vaginal mucosa is not linked to increased HSV-2 virus shedding. WT, *Ifnar*$^{-/-}$, and *Il15*$^{-/-}$ mice were infected with HSV-2 ivag. On days 2 p.i., vaginal lavages were collected and titered for HSV-2 (A; n=4). On day 3 p.i., vaginal tissue was isolated, fixed, and then sectioned. Sections were stained with H&E and images were taken at a 10x magnification (B; n=4). All images shown are from individual mice. n.s. not significant.

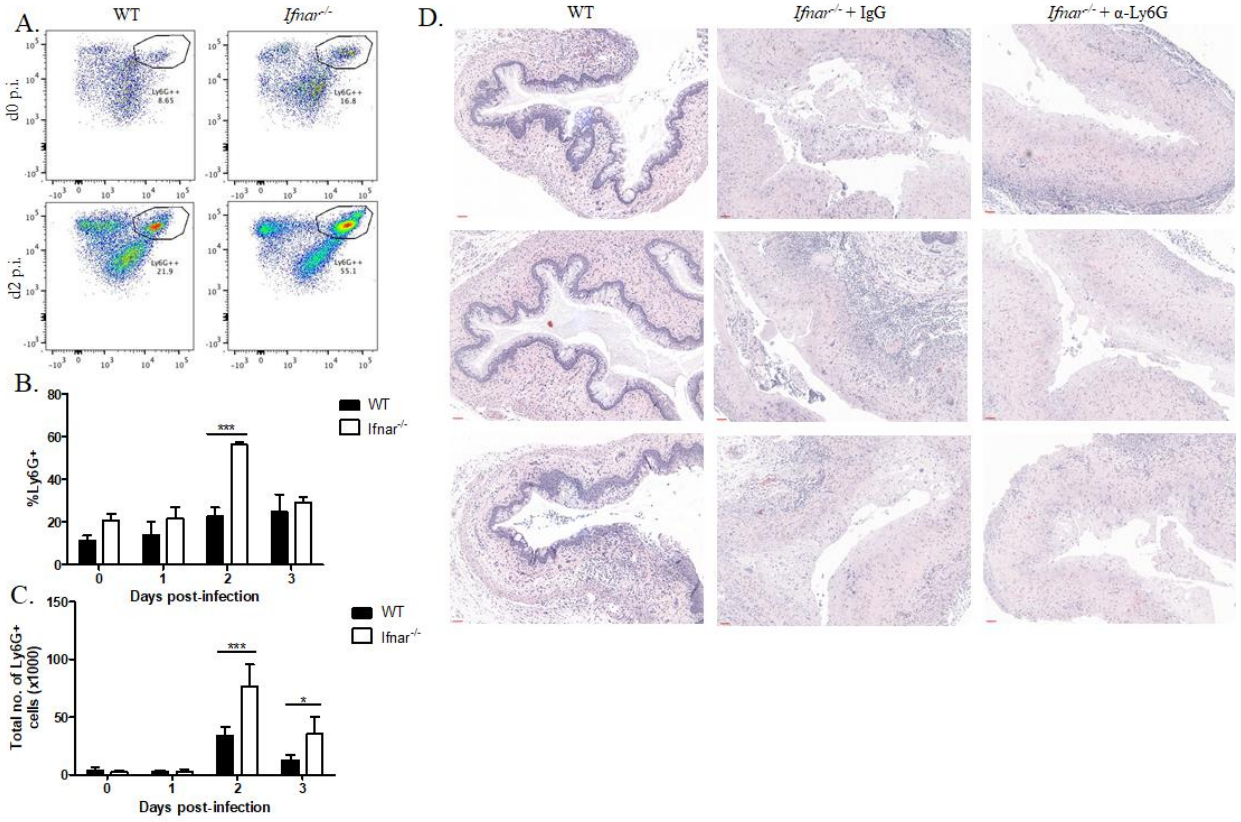


Figure 3. Increased proportion of neutrophils in *Ifnar-/-* vaginal tissue do not mediate the vaginal immunopathology observed in *Ifnar*$^{-/-}$ mice. WT and *Ifnar*$^{-/-}$ mice were infected ivag with HSV-2. At baseline, d1, d2, and d3 p.i., vaginal tracts were isolated, processed and stained with anti-mouse CD45, CD11c, CD11b, Ly6G. Neutrophils were gated as CD45+, CD11c-, CD11b+, Ly6G+. Representative flow plots of baseline and d2 p.i. are shown in (A). Proportion of Ly6G+ neutrophils are graphically shown in (B; n=3; repeated once with similar results). Total number of neutrophils are shown in (C; n=3). *Ifnar*$^{-/-}$ mice were given α-Ly6G antibody or its respective isotype control d-1 and d+1 p.i. and infected with HSV-2 ivag, alongside WT controls. On d3 p.i., vaginal tracts were isolated,

sectioned, and stained with H&E. Sections were then imaged at a 10x magnification (D; n=3) All images shown are from individual mice. *p<0.05; ***p<0.001

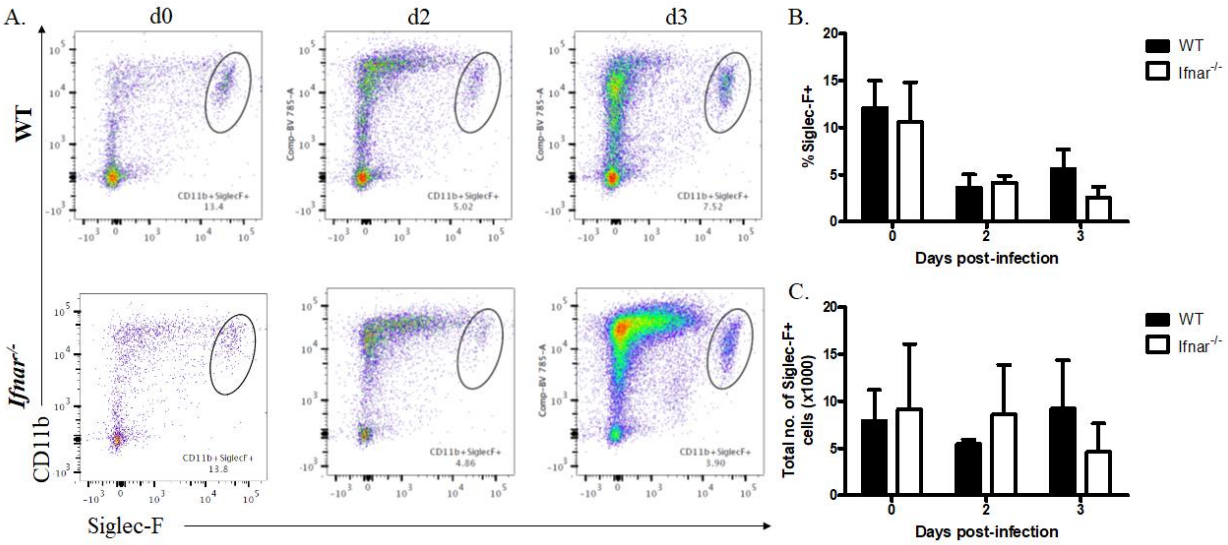


Figure 4. No significant difference in total number or proportion of eosinophils in the vaginal mucosa between *Ifnar*$^{-/-}$ mice and controls. WT and *Ifnar*$^{-/-}$ mice were infected with HSV-2 ivag. Day 0, 2, and 3 p.i. vaginal tissues were isolated and examined for eosinophils (CD45+CD11b+Siglec-F+). Representative flow plots are shown in (A). Proportions of eosinophils are graphically represented in (B; n=3) and total number of eosinophils are shown in (C; n=3).

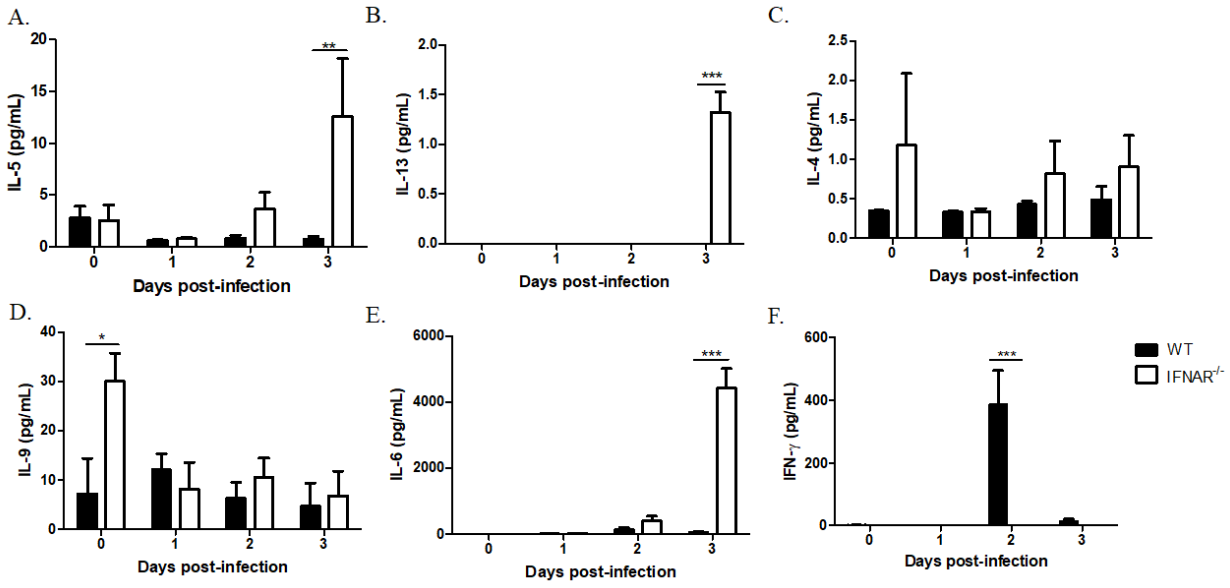


Figure 5. Significant upregulation of IL-6 in *Ifnar*$^{-/-}$ vaginal washes during HSV-2 infection. WT and *Ifnar*$^{-/-}$ mice were infected with HSV-2 ivag and d0-d3 p.i. vaginal washes were collected. Levels of IL-5 are shown in (B), IL-4 shown in (B), IL-6 shown in (C), IL-13 shown in (D), IL-9 shown in (E), and IFN-γ shown in (F). n=3. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

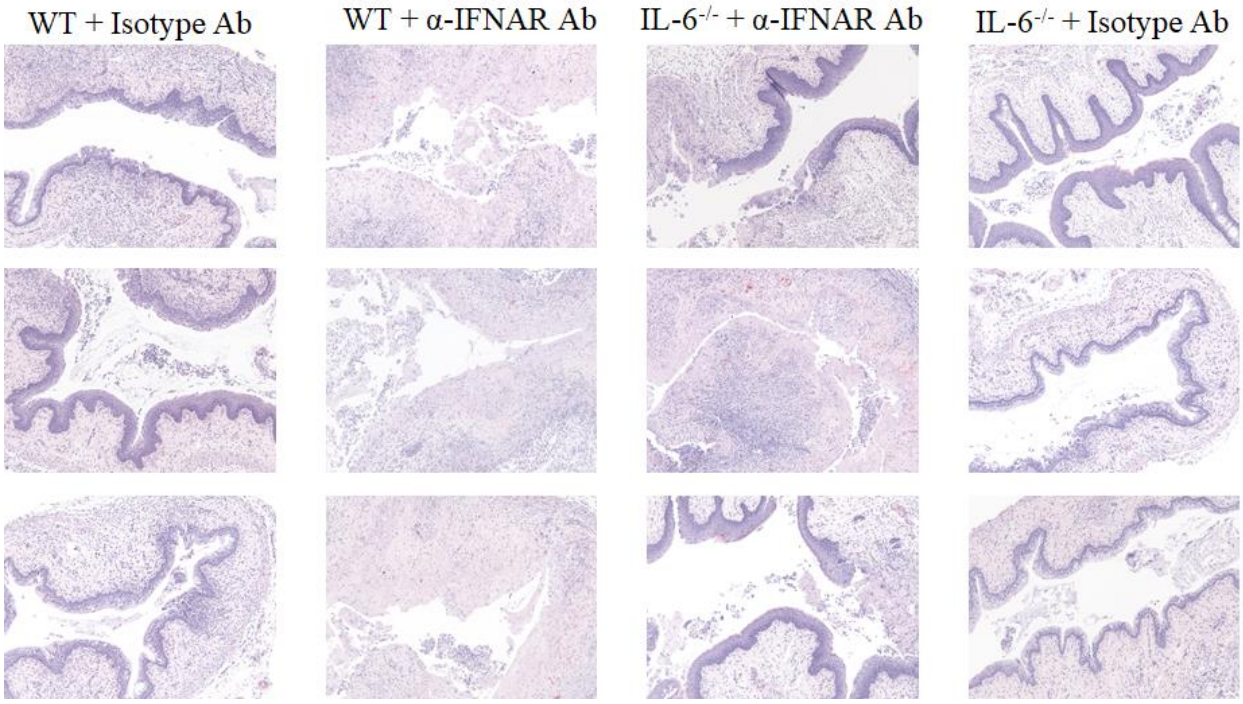


Figure 6. Absence of IL-6 in mice given α-IFNAR Ab partially rescues the immunopathology observed in *Ifnar*$^{-/-}$ mice. WT and Il6-/- mice were given 500ug of either α-IFNAR or an isotype control antibody d-1 pre-infection through to d3 post-infection. Mice were infected with HSV-2 ivag. Vaginal tissue was collected at d3 p.i., fixed in 2% PFA. Tissue was cross-sectioned and stained with H&E. Sections were imaged at a 10x magnification. All images shown are from individual mice.

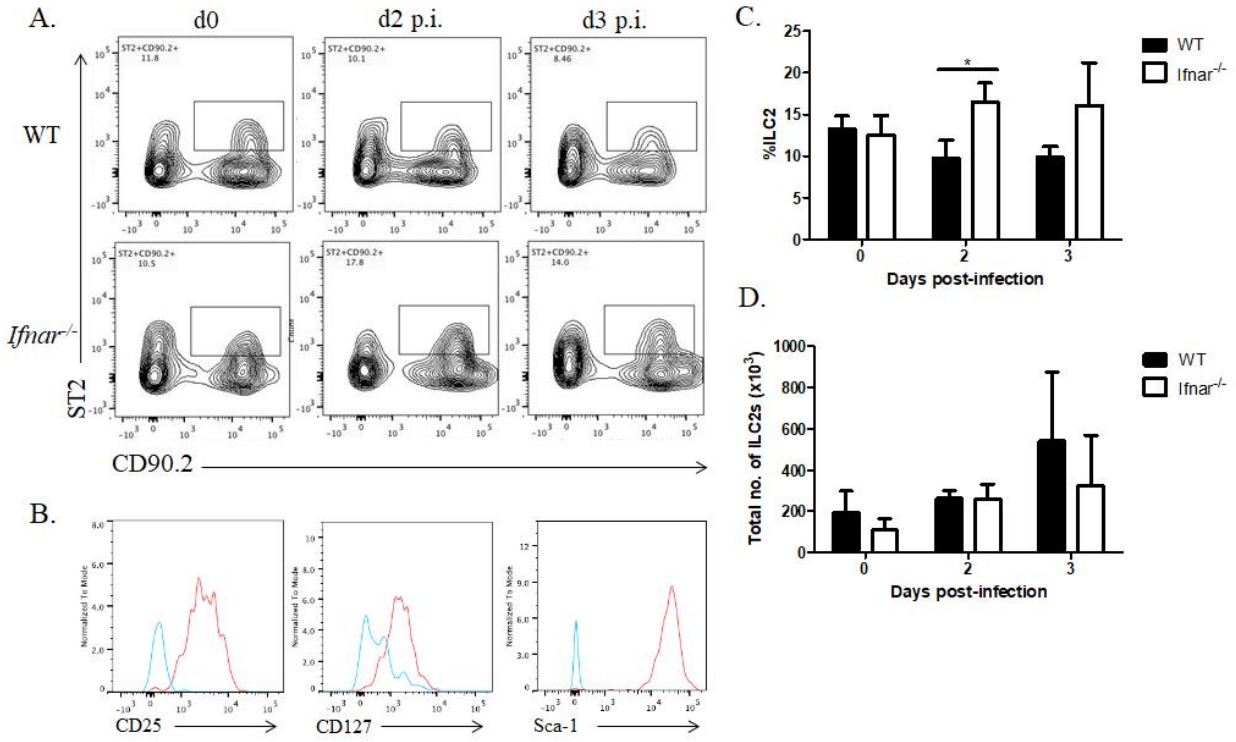


Figure 7. *Ifnar*$^{-/-}$ mice have similar levels of total vaginal ILC2s compared to WT mice during HSV-2 infection. WT and *Ifnar*$^{-/-}$ mice were infected with HSV-2 ivag. Baseline, d2, and d3 p.i. vaginal tracts were isolated, processed, and stained for anti-mouse CD45, lineage markers, ST2, CD90.2, CD25, CD127, and Sca-1. ILC2 cells were gated as CD45+, Lineage- (anti-mouse CD3, GR-1, CD11b, B220, mTer-119), ST2+, CD90.2+. Representative flow plots are shown in (A). Gated ILC2 cells were further examined for CD25, CD127, and Sca-1 expression (blue = isotype control Ab; red = anti-mouse CD25, CD127, or Sca-1). Representative flow plots are shown in (B). Proportion of ILC2s are shown graphically in (C; n=3). Total number of ILC2 cells are graphically shown in (D).*$p<0.05$

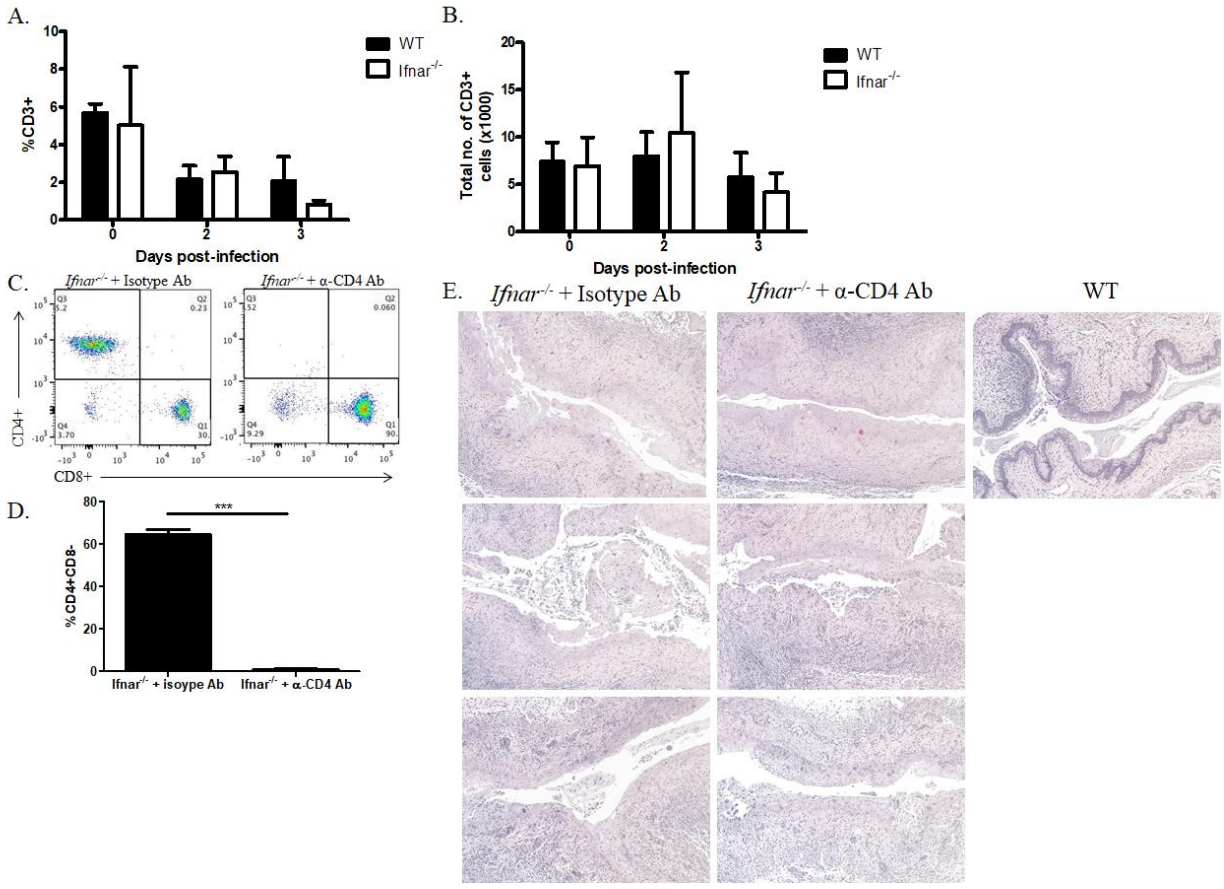


Figure 8. CD4+ T-cells are not required for the immunopathology observed in *Ifnar*$^{-/-}$ mice. WT and *Ifnar*$^{-/-}$ mice were infected with HSV-2 ivag. At d0, d2, and d3 p.i., vaginal tissue was collected, processed, and stained with anti-mouse CD45 and CD3. CD3+ cells were gated as CD45+CD3+ cells. Proportion of CD3+ T-cells are shown in (A; n=3) and total CD3+ cell number is graphically shown in (B; n=3). *Ifnar*$^{-/-}$ mice were administered either an α-CD4 depletion antibody or an isotype control antibody d-2, d-1 and d+1 post-infection. Mice were infected with HSV-2 ivag. On d3 p.i. blood was collected and stained with CD45, CD3, CD4, and CD8. Blood CD4+ T-cells were examined as CD45+CD3+CD4+CD8-. Representative flow plots are shown in (C) and graphically

shown in (D; n=3). Vaginal tissue was collected on d3 p.i., fixed in 2% PFA, sectioned, and stained for H&E. Images were taken at a 10x magnification (E). All images shown are from individual mice. ***p<0.001

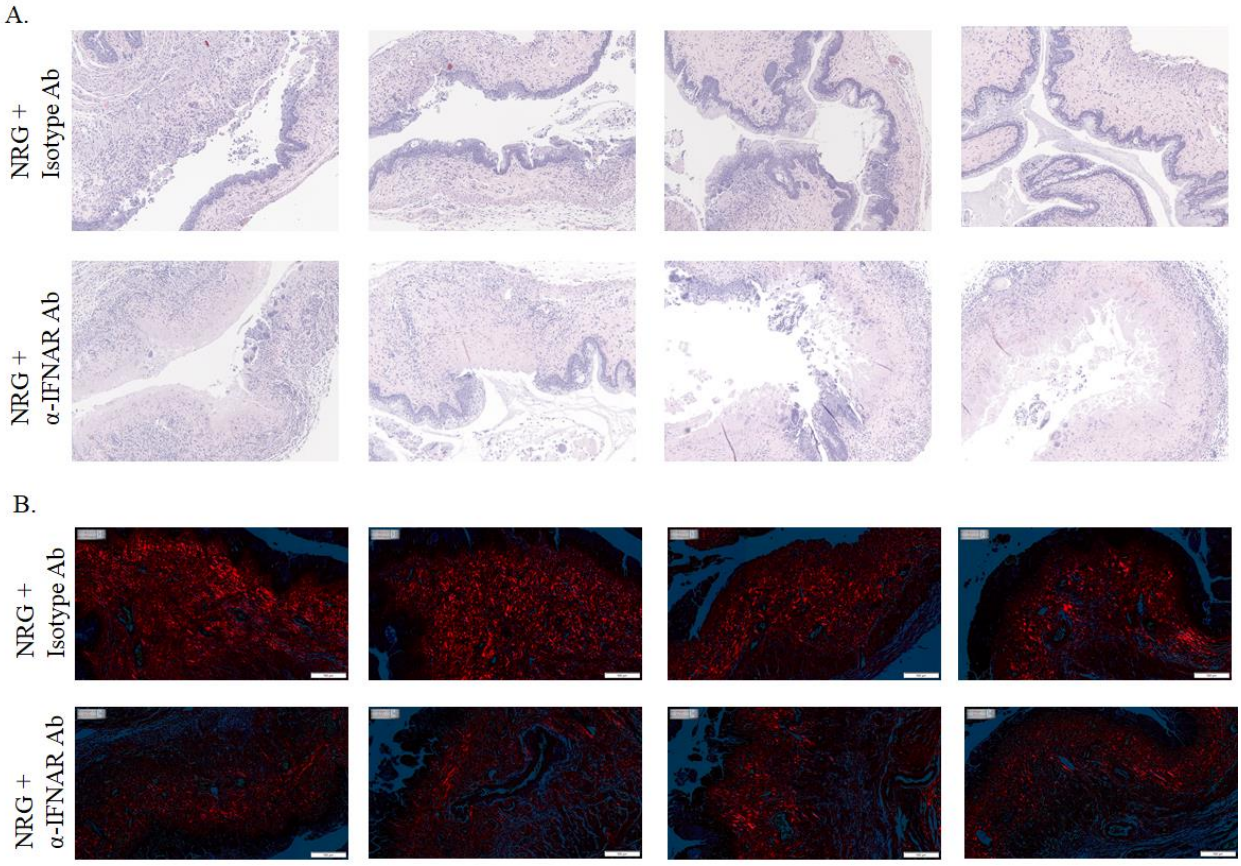


Figure 9. Blocking IFNAR in NRG mice leads to significant vaginal tissue immunopathology during HSV-2 infection. NRG mice were given either α-IFNAR Ab or an Isotype control Ab d-1 through to d2 p.i. NRG mice were infected with HSV-2 ivag. Vaginal tissue was collected on d3 p.i., fixed in 2% PFA, sectioned, and stained with H&E (A). Sections were stained with picrosirius red for collagen (B). Images were taken at a 10x magnification. All images shown are from individual mice.

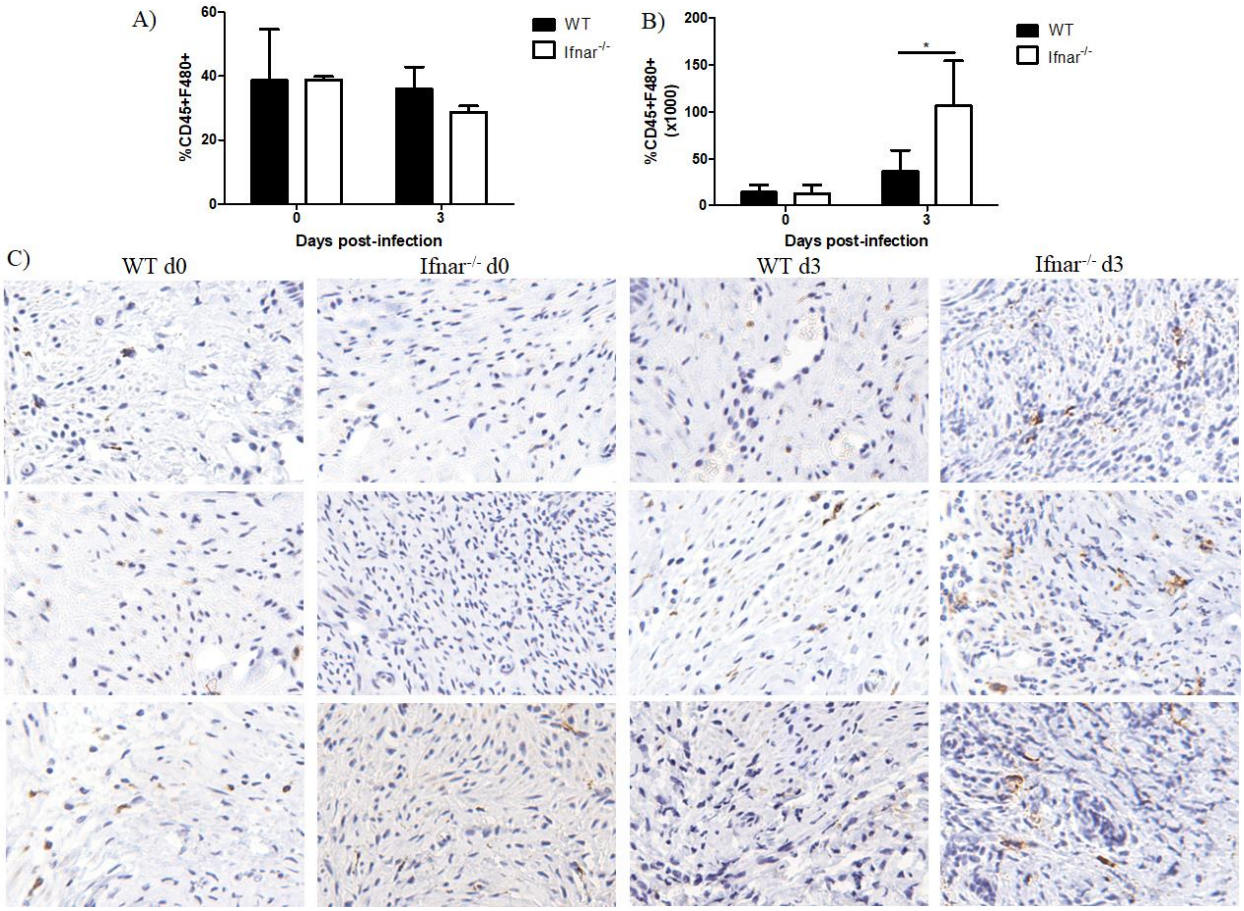


Figure 10. Increased number of F4/80+ cells and expression of CD206 in *Ifnar*$^{-/-}$ vaginal tissue at d3 p.i. WT and *Ifnar*$^{-/-}$ mice were infected with HSV-2 ivag. Vaginal tissue was collected at d0 and d3, processed, and stained for CD45 and F480. F480+ cells were gated on CD45 and F480. Proportion of F480+ cells is shown in (A; n=3). Total number of F480+cells is shown in (B; n=3). WT and *Ifnar*$^{-/-}$ mice were infected with HSV-2 ivag. Vaginal tissue was collected from d0 and d3 p.i. WT and *Ifnar*$^{-/-}$ mice, fixed in 2% PFA, sectioned, and stained for CD206. Images of sections were taken at a 20x magnification (C). All images shown are from individual mice. *$p<0.05$

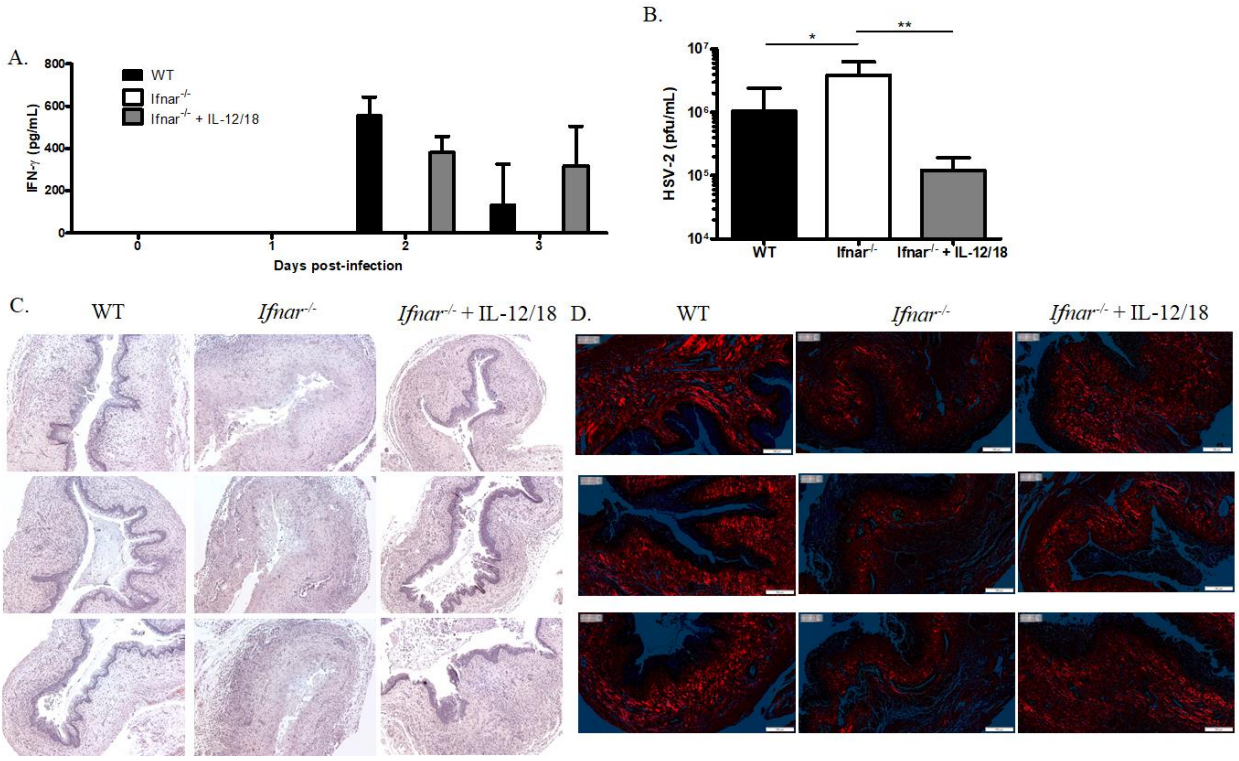


Figure 11. Administration of IL-12 and IL-18 to *Ifnar*$^{-/-}$ mice partially rescues the HSV-2-induced immunopathology in *Ifnar*$^{-/-}$ mice. WT and *Ifnar*$^{-/-}$ mice were infected with HSV-2 ivag. A group of *Ifnar*$^{-/-}$ mice were given 1mg of IL-18 with 200ug of IL-12 i.p. d1 and d2 p.i. Vaginal washes were collected d0-d3 post-infection and examined for IFN-γ production (A; n=5). HSV-2 viral titers were examined from vaginal washes collected at d2 p.i (B; n=5). Vaginal tissue was collected from WT, *Ifnar*$^{-/-}$, and *Ifnar*$^{-/-}$ given IL-12 and IL-18 i.p. at d3 p.i. Vaginal tissues were fixed in 2% PFA, sectioned, and stained for H&E. Images were captured at a 10x magnification, representative images are shown in (C). Sections were assayed for collagen arrangement through picrosirius red staining (D). All images shown are from individual mice. *p<0.05; **p<0.01

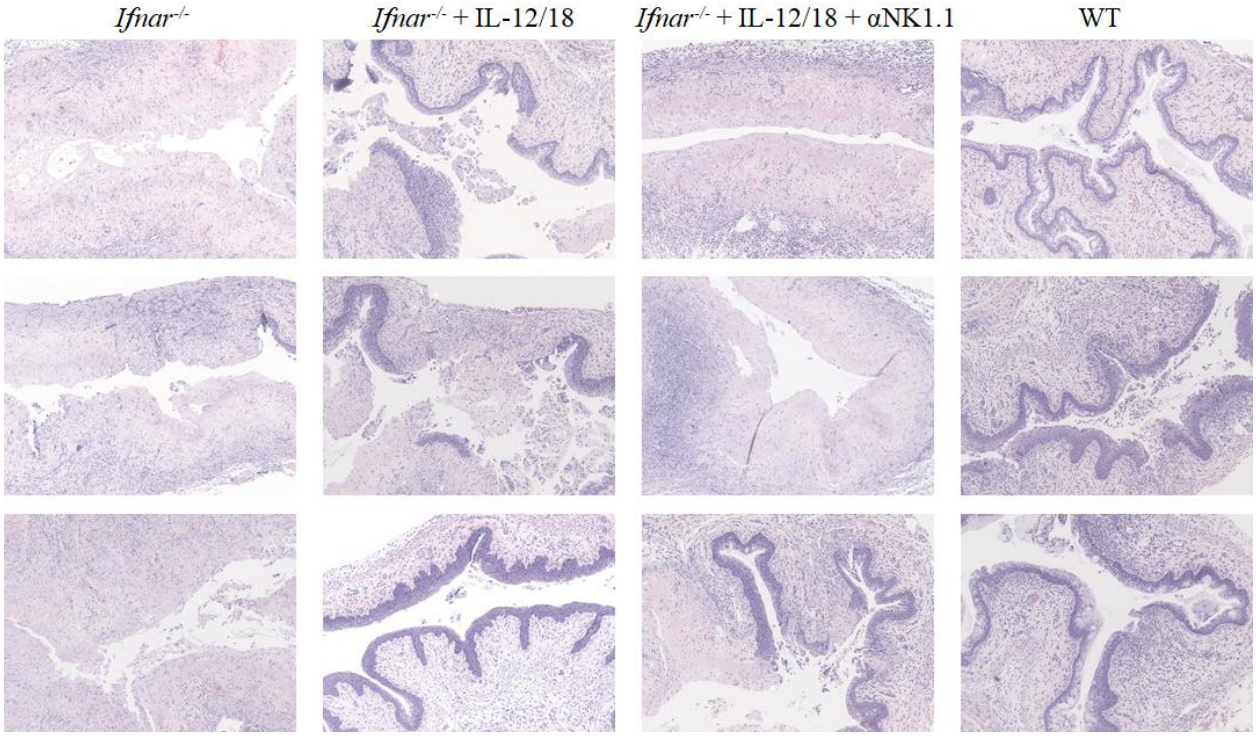
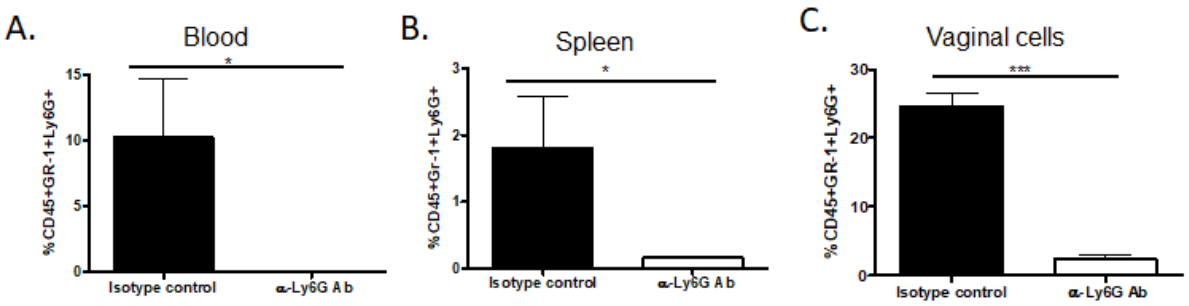
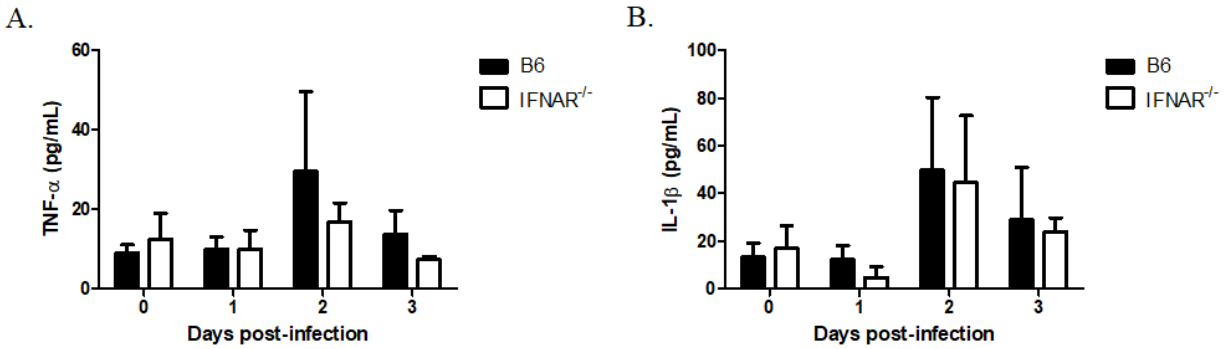


Figure 12. Suppression of immunopathology in IL-12 and IL-18-treated *Ifnar*$^{-/-}$ mice is dependent on NK cells. *Ifnar*$^{-/-}$ mice and WT mice were infected with HSV-2 ivag. *Ifnar*$^{-/-}$ mice given 1mg IL-18 with 200ug of IL-12 i.p. d1 and d2 p.i. were also given either α-NK1.1 Ab or an isotype control Ab i.p. d-2, d-1 and d+1 post-infection. Vaginal tissue was collected at d3 p.i., fixed in 2% PFA, sectioned, and stained for H&E. Images were captured at a 10x magnification. All images shown are from individual mice.

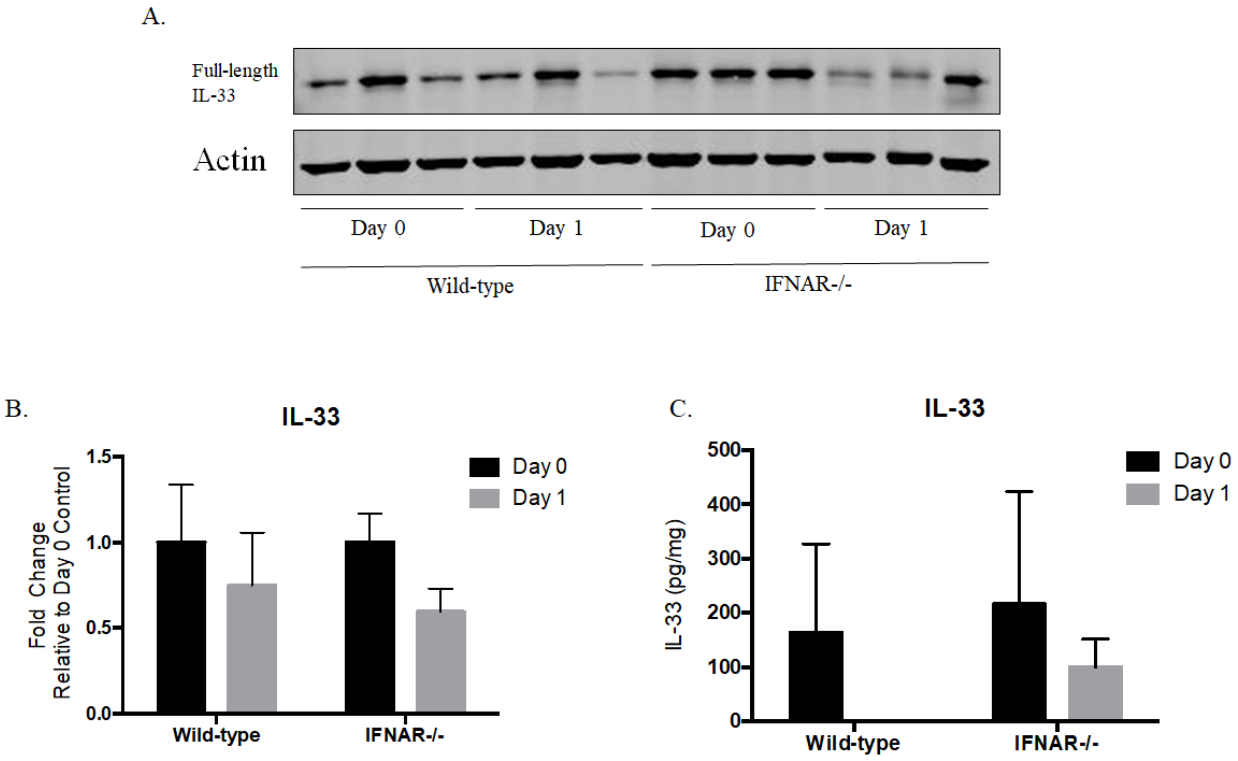
Supplementary Figures



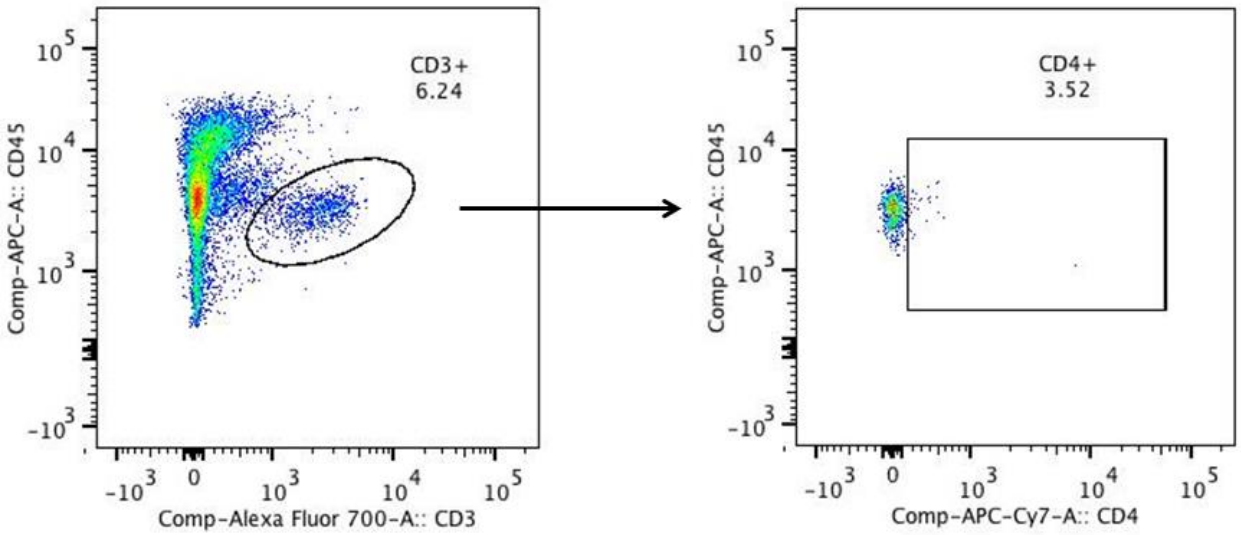
Supplementary Figure 1. Depletion of neutrophils in the blood, spleen, and vaginal tissue of WT mice. WT mice were given an α-Ly6G antibody or isotype control antibody to deplete neutrophils. Mice were infected with HSV-2 ivag and examined for neutrophil depletion in blood (A; n=4), spleen (B; n=4), and vaginal cells (C; n=3) at d3 p.i. *$p<0.05$; ***$p<0.001$



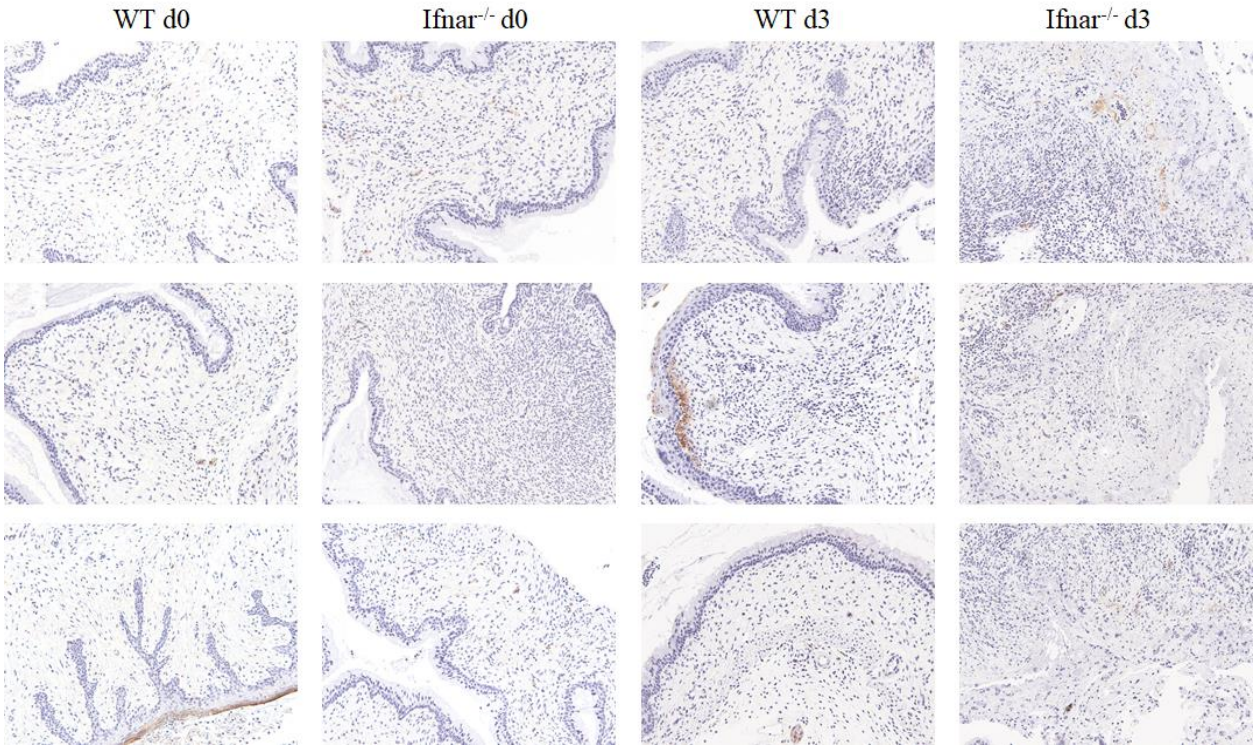
Supplementary Figure 2. No difference in levels of vaginal TNF-α and IL-1β between WT and *Ifnar*$^{-/-}$ mice. WT and *Ifnar*$^{-/-}$ mice were infected with HSV-2 ivag. Days 0-3 p.i. vaginal washes were collected and examined for TNF-α (A; n=3) and IL-1β (B; n=3).



Supplementary Figure 3. No difference in levels of vaginal IL-33 between WT and *Ifnar*$^{-/-}$ mice during HSV-2 infection. WT and *Ifnar*$^{-/-}$ mice were infected with HSV-2 ivag. Baseline and d1 p.i. vaginal tissue was collected, homogenized, lysed, and examined for IL-33 protein levels via immunoblot. Gel image is shown in (A) and band densiometry is shown in (B; n=3). Whole vaginal tissue protein was also analyzed for IL-33 via ELISA (C; n=3).



Supplementary Figure 4. Little levels of CD4+ T-cells found in vaginal tissue of infected mice at d3 p.i. WT mice were infected with HSV-2 ivag and examined for CD4+ T-cells in the vaginal tissue at d0 and d3 p.i. Vaginal tissue was collected, processed, and stained with CD45, CD3, and CD4. A representative flow plot is shown.



Supplementary Figure 5. No difference in vaginal arginase expression between WT and *Ifnar*$^{-/-}$ mice at d3 p.i.. WT and *Ifnar*$^{-/-}$ mice were infected with HSV-2 ivag. Baseline and d3 p.i. vaginal tissue was collected, fixed, cross-sectioned, and stained for Arginase-1. All images shown are from individual mice.

CHAPTER 4

Type I Interferon receptor on NK cells negatively regulates interferon-γ production

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AJ Lee designed and performed all experiments except for Figures 5A, 5C, and Figure 7. AJ Lee analyzed all the data and wrote the manuscript. F Mian designed and performed experiments for Figure 7. SM Poznanski, and MV Chew provided technical help and expertise. T Chan designed and performed experiments for Figure 5A. M Stackaruk designed and performed experiments for Figure 5 C. AA Ashkar designed experiments, edited the manuscript, and is the corresponding author.

Abstract

NK cells are a key antiviral component of the innate immune response to HSV-2, particularly through their production of IFN-γ. It is still commonly thought that type I IFN activates NK cell function. However, rather than requiring the type I IFN receptor themselves, we have previously found that type I IFN activates NK cells through an indirect mechanism involving inflammatory monocytes and IL-18. Here, we show that direct action of type I IFN on NK cells negatively regulates IFN-γ production during HSV-2 infection and cytokine stimulation. During infection, IFN-γ is rapidly induced from NK cells at day 2 post-infection and then immediately downregulated at day 3 post-infection. We found that this downregulation of NK cell IFN-γ release is not due to a loss of NK cells at day 3 post-infection, but due to negative regulation through IFN signaling on NK cells. Co-culture of *Ifnar*$^{-/-}$ NK cells with *Rag2*$^{-/-}\gamma c^{-/-}$ immune cells led to a significantly increased level of IFN-γ compared to WT NK cells after HSV-2 infection. Further, priming of NK cells with type I IFN was able to suppress IL-15-induced IFN-γ production from both human and mouse NK cells. This was not mediated by the production of IL-10; however, type I IFN did induce a significant increase in Axl expression on NK cells. Overall, our data suggests that type I IFN negatively regulates NK cell IFN-γ production *in vitro* and during HSV-2 infection.

Introduction

Natural killer (NK) cells are innate lymphocytes that are capable of killing virally infected and malignant cells.[1] NK cells are an important component of the innate antiviral response, as they can directly eliminate infected cells and release pro-inflammatory cytokines that can alter the microenvironment and activate other immune cells.[1] During virus infection, NK cell release of IFN-γ during the innate response can induce nitric oxide from macrophages to inhibit virus replication, as well as promote maturation of dendritic cells (DCs) and polarize Th1 cell adaptive immune responses.[2, 3, 4] IFN-γ is a critical cytokine as part of the immune response against Herpes Simplex Virus type 2 (HSV-2) infection, as *Ifnγ*$^{-/-}$ mice have significantly decreased survival after HSV-2 infection, a lifelong virus infection that causes genital ulcer disease and increases the risk of HIV acquisition.[5, 6, 7, 8]

NK cell function can be mediated through the ligation and signaling of activation and inhibitory receptors, wherein an overwhelming activation signal will activate their effector functions.[9] Cytokines within the local environment can also regulate NK cell antiviral function, where cytokines such as type I interferon (IFN), IL-12, IL-15, and IL-18 are known to activate NK cell function, particularly IFN-γ production.[9] Type I IFN has been shown to be critical for NK cell activation during virus infection, though the mechanism by which type I IFN activates NK cells is still under debate. We have previously shown that type I IFN does not activate NK cells directly during HSV-2 infection.[10] Instead, type I IFN induces inflammatory monocytes to release IL-18, which then activates IFN-γ production from NK cells.[10] Further, we also found that type I IFN

receptor on NK cells was required to negatively regulate their IFN-γ release, as transfer of *Ifnar*$^{-/-}$ NK cells into alymphoid mice led to significantly increased and sustained levels of IFN-γ production in comparison to WT NK cells during HSV-2 infection.[10] This suggested to us that type I IFN signaling on NK cells, rather than activating them, negatively regulates their ability to produce IFN-γ. Thus, type I IFN can potentially differentially modulate NK cell function through direct or indirect mechanisms.

Type I IFN consists of a family of structurally similar cytokines, including a single IFN-β and 13-14 subtypes of IFN-α, that all signal through the same type I IFN receptor (IFNAR).[11] Type I IFN, though considered to be a potent antiviral cytokine, has also been found to negatively regulate immune cells. In particular, Teijaro *et al* found that blocking the type I IFN receptor during chronic LCMV infection was able to restore IFN-γ production, suggesting that type I IFN can suppress antiviral effector functions.[12] Others have found that type I IFN can negatively regulate NK cell function, where exogenous type I IFN was able to suppress NK cell IFN-γ release.[13, 14] Further, a late wave of IFN-β during a *Listeria monocytogenes* infection suppressed NK cell function.[15] In an intracellular bacterial infection, type I IFN led to IL-10 production, which contributed to suppression of an IFN-γ response.[16] Type I IFN has also been shown to upregulate TAM (Tyro 3, Axl, and Mer) receptor expression and can co-opt the type I IFN receptor signaling cascade to induce expression of suppressor of cytokine signaling (SOCS) proteins.[17] In humans, administration of pegylated IFN-α therapy to hepatitis C patients leads to a significant reduction in IFN-γ positive NK cells in the peripheral blood of these patients.[18, 19]

In this study, we found that rather than activating NK cells, NK cells required the type I IFN receptor to negatively regulate their IFN-γ production during HSV-2 infection and during IL-15 and IL-18 stimulation. During infection, NK cell IFN-γ levels peak at day 2 post-infection and are subsequently negatively regulated at day 3 post-infection. We found that culturing *Ifnar*$^{-/-}$ NK cells with *Rag2*$^{-/-}\gamma c^{-/-}$ cells during HSV-2 infection led to a significant upregulation of IFN-γ in comparison to WT NK cells. Further, type I IFN pre-treatment prior to cytokine stimulation led to a significant reduction in NK cell IFN-γ production in both isolated NK cells and splenocytes, suggesting that type I IFN can both directly suppress NK cell function alone and in the presence of other immune cells. Further, we found that type I IFN does not mediate its negative regulatory function through IL-10 induction, but did lead to a significant increase in Axl expression. Thus, type I IFN negatively regulates NK cell IFN-γ release during HSV-2 infection both *in vivo* and *in vitro*, potentially through the upregulation of Axl expression. In summary, our papers show a new fundamental understanding of how type I IFN directly and indirectly modulates NK cell function.

Results

NK cell IFN-γ levels are down-regulated at day 3 post-infection

During a vaginal HSV-2 infection, we have previously shown that NK cells are activated to release an early wave of IFN-γ at day 2 post-infection.[10, 20] Infection of WT mice with HSV-2 intravaginally yielded a rapid upregulation of IFN-γ at d2 post-infection, as previously described. We also found a similarly rapid downregulation of IFN-γ at d3 post-

infection (Fig 1). Indeed, we observed an almost complete abrogation of IFN-γ levels at d3 post-infection. This would suggest that the activated NK cells are disappearing, becoming exhausted, or negatively regulated during infection.

Reduction in IFN-γ is not due to a loss in NK cell number at day 3 post-infection

A loss of NK cells at day 3 post-infection could account for the rapid reduction in IFN-γ. Therefore, we examined the number of NK cells within the vaginal tract between the peak of IFN-γ production (day 2 post-infection) and the time point at which IFN-γ is abrogated (day 3 post-infection). We found very low levels of NK cells at baseline and one day after infection (Fig 2A and B). At day 2 post-infection, we observed a significant accumulation of NK cells in the vaginal mucosa that was maintained at day 3 post-infection (Fig 2A and B). This suggests that the abrogation in IFN-γ levels at d3 post-infection is not due to a reduction of NK cells within the vaginal tract. As NK cells mature, their progression can be determined by examining their expression of CD27 and CD11b.[21, 22] Immature NK cells start out as a double-negative population lacking expression of both CD27 and CD11b (CD27-CD11b-).[22] As they mature, they gain expression of CD27 (CD27+CD11b-) and progress to a double-positive population with expression of both CD27 and CD11b (CD27+CD11b+).[22] The most mature subset of NK cells has lost expression of CD27, but maintains expression of CD11b (CD27-CD11b+).[22] Groups have suggested that NK cells expressing CD27 are good cytokine producers; conversely, others have suggested that CD11b+ NK cells have the highest cytotoxic effector potential.[21, 22] While the abrogation of NK cell IFN-γ production was not due to a reduction in NK cell number, we examined

whether a difference in NK cell maturation status could contribute to the difference in IFN-γ levels at d2 and d3 post-infection. We found that NK cells at baseline and d1 post-infection have a higher proportion of immature double-negative NK cells compared to NK cells at the peak of IFN-γ production (d2 p.i.; Fig 2A and B). There was no significant difference levels of CD27+CD11b- or the double-positive NK cell populations at d2 and d3 post-infection (Fig 2A, C, and D). We did find, however, a significant increase in the most mature NK cell population (CD27-CD11b+) at d2 post-infection in comparison to d3 post-infection (Fig 3E). Thus, despite finding similar numbers of NK cells at days 2 and 3 post-infection, there was a decrease in the proportion of mature CD11b+CD27- NK cells at d3 post-infection in comparison to d2 post-infection. The decrease in the most mature phenotype related to high NK cell effector function could contribute to the downregulation of IFN-γ production at d3 post-infection.

Type I IFN receptor on NK cells negatively regulates their release of IFN-γ

We had previously found that during HSV-2 infection, though the type I IFN receptor was required to activate NK cell IFN-γ production, its action was not required directly on NK cells.[10] Instead, type I IFN activated inflammatory monocytes to release IL-18, which was responsible for NK cell activation. However, we did observe that transfer of NK cells lacking IFNAR into alymphoid *Rag2*$^{-/-}$*γc*$^{-/-}$ mice yielded significantly increased levels of IFN-γ at both d2 and d3 post-infection in comparison to WT NK cells.[10] This suggested to us that in an *in vivo* setting, the type I IFN receptor was required on NK cells in order to negatively regulate their IFN-γ response to virus infections. We found a similar

phenomenon occurring in *vitro*, where HSV-2 infection of co-cultured WT or *Ifnar*$^{-/-}$ NK cells with *Rag2*$^{-/-}\gamma c^{-/-}$ splenocytes led to significantly increased levels of IFN-γ from co-cultures with *Ifnar*$^{-/-}$ NK cells in comparison to WT NK cells (Fig 4). Overall, our data suggests that NK cells require type I IFN receptor in order to negatively regulate their release of IFN-γ during virus infection.

Type I IFN pre-treatment dampens cytokine-induced NK cell IFN-γ production

Turning to an *in vitro* model, we further explored the ability of type I IFN to suppress NK cell IFN-γ production. We wanted to determine if the negative regulatory ability of type I IFN was restricted to infection or could be observed with other stimuli. We pre-treated isolated NK cells with IFN-β for 12 hours prior to stimulating with an activating cytokine, such as IL-15 or IL-18. Treatment of NK cells with IFN-β prior to cytokine stimulation completely abrogated their ability to produce IFN-γ in response to varying doses of IL-15, and significantly reduced the level of IFN-γ after IL-18 treatment (Fig 5A and B). Further, we found that in the presence of other immune cells, pre-treating splenocytes with IFN-β was able to significantly reduce IL-15-induced IFN-γ production, even with low concentration of IFN-β (1U; Fig 5C). We were concerned that IFN-β treatment was reducing the survival of NK cells, as type I IFN can be a pro-apoptotic factor. We examined NK cell survival after 12 hrs of IFN-β treatment and did not observe a large difference in survival between media treatment (63% viable) and IFN-β treatment (59% viable; data not shown). We examined the impact of type I IFN on NK cell IFN-γ production when given at the same time as an activating cytokine stimulus. Treatment of both isolated NK cells

and splenocytes with type I IFN and IL-15 at the same time yielded no difference in IFN-γ levels (Fig 6A and B, respectively). Overall, our data suggest that type I IFN pre-treatment is able to negatively regulate IFN-γ from NK cells in both a direct manner and in the presence of other immune cells. Further, the negative regulatory capacity of type I IFN spans across different stimulatory conditions.

Type I IFN pre-treatment suppresses human NK cell IFN-γ production

Though we and others have found that type I IFN and is able negatively regulate NK cell function, most of the data involves mouse modeling. Thus, we wanted to determine if type I IFN was able to negatively regulate human NK cells as well. PBMC-isolated NK cells were treated with IFN-β for 12 hrs prior to IL-15 stimulation. IFN-β pre-treatment significantly reduced NK cell IFN-γ production in comparison to IL-15 stimulation alone (Fig 7A). Moreover, IFN-β pre-treatment of PBMCs with IFN-β yielded significantly less IFN-γ in comparison to IL-15 stimulation (Fig 7B). To rule out the pro-apoptotic effects of type I IFN, we examined viability after 18hrs of IFN-β treatment and observed no difference in viability between media and IFN-β treatment groups (Supplementary Fig 1). Our data suggests that type I IFN is able to negatively regulate human NK cell IFN-γ release. Further, we detected a greater IFN-β-induced reduction in IFN-γ levels in the presence of other immune cells.

IL-10 is not induced after IFN-β treatment nor during HSV-2 infection

Type I interferon has been shown to induce the immunosuppressive cytokine, IL-10, during LCMV infection and mycobacterial infections.[12, 16] During the mycobacterial infections, type I IFN has been shown to upregulate IL-10 and suppress IFN-γ production.[16] Further, IL-10 has been shown to suppress NK cell IFN-γ production by acting on NK cells or suppressing maturation and/or activating cytokines (e.g. IL-12 and IL-18) from myeloid cells.[23, 24, 25, 26] From this, we wanted to determine whether type I IFN could mediate its immunosuppressive effects through the induction of IL-10. IFN-β treatment of isolated NK cells did not induce significant IL-10 production (Fig 8A). Further, there was no significant difference in IL-10 levels between splenocytes that were cultured in media or cells treated with IFN-β (Fig 8B). In the context of HSV-2 infection, we did not see an induction of IL-10 in WT vaginal lavages, nor did we see a significant difference in levels of IL-10 between WT and *Ifnar*$^{-/-}$ mice (Fig 8C). Overall, our data suggest that IL-10 is not involved in the mechanism by which type I IFN is able to suppress NK cell IFN-γ production both *in vitro* and during an *in vivo* HSV-2 infection.

Type I IFN stimulation upregulates Axl expression on NK cells

Type I IFN has been shown to upregulate TAM receptors (Tyro3, Axl, and Mer) on immune cells.[17] The expression of TAM can redirect type I IFN signaling to induce the expression of SOCS proteins, which are known to suppress pro-inflammatory signaling pathways downstream of TLR and cytokine activation.[17] We examined Tyro3, Axl, and Mer expression on NK cells after stimulating NK cells alone or PBMCs with IFN-β. We found no significant difference in Tyro3 expression on NK cells following type I IFN

stimulation in either the NK cells alone or PBMCs (Fig 9A). We did detect a significant upregulation of Axl expression on NK cells after stimulation of NK cells or PBMCs with IFN-β (Fig 9B). Similar to Tyro3, we found no difference in NK cell Mer expression after IFN-β stimulation in either the NK cell or PBMC group (Fig 9C). Overall, our data suggests that type I IFN stimulation can upregulate Axl expression on human NK cells.

Discussion

We had previously found that type I IFN was critical for the activation of IFN-γ release from NK cells during HSV-2 infection.[20] We determined that type I IFN activates inflammatory monocytes to release IL-18, which was necessary for activating NK cells.[10] However, we also found that expression of type I IFN receptor on NK cells was required for the negative regulation of IFN-γ production during infection.[10] We demonstrated that *Ifnar*$^{-/-}$ NK cells had significantly increased and sustained levels of IFN-γ production during HSV-2 infection. In WT mice, we normally observe a peak of NK cell IFN-γ levels at d2 post-infection and an abrogation of IFN-γ at day 3 post-infection. Our data suggests that this down-regulation of IFN-γ is somewhat mediated by type I IFN rather than a loss of NK cells at d3 post-infection, as there was no difference in vaginal NK cell number between d2 and d3 post-infection. However, we did find a significantly increased proportion of mature (CD27-CD11b+) NK cells at d2 post-infection in comparison to d3 post-infection. Further, co-culturing of *Ifnar*$^{-/-}$ NK cells with *Rag2*$^{-/-}$*γc*$^{-/-}$ splenocytes yielded significantly more IFN-γ compared to WT NK cells during HSV-2 infection. Additionally, pre-treatment of both mouse and human isolated NK cells and splenocytes

(or PBMCs) with type I IFN suppressed cytokine-induced IFN-γ production. In trying to resolve the mechanism by which type I IFN negatively regulates NK cells, we found that there was no difference in IL-10 production between untreated cells and cells treated with type I IFN. Further, we found no induction or difference in IL-10 production between WT and *Ifnar*$^{-/-}$ mice during HSV-2 infection. We did, however, detect a significant increase in NK cell Axl expression after type I IFN treatment of both NK cells and PBMCs. Overall, our data provides a novel mechanism by which type I IFN can both activate and negatively regulate NK cell function

NK cell development can be classified by expression of CD27 and CD11b. Reports have found that expression of CD11b not only identifies mature NK cells, but is also found on NK cells with the highest cytotoxic effector potential.[22] On the other hand, Hakayawa *et al* found that NK cells with high expression of CD27 have increased cytokine secretion, among other effector functions, in comparison to NK cells with low CD27 expression.[21] Further work will be needed to determine whether the CD11b+CD27- NK cell subset is responsible for producing IFN-γ during HSV-2 infection and determining whether that subset is lost at d3 post-infection.

Other groups have found that along with their antiviral function, type I IFN can negatively regulate immune cells. It has been reported that type I IFN can suppress NK cell function through a number of different mechanisms. During chronic virus infection, such as LCMV, type I IFN suppresses IFN-γ production and blockade of the type I IFN receptor restores the antiviral IFN-γ response.[12] Moreover, type I IFN has also been shown to induce IL-10 production, which can dampen IFN-γ levels during *Mycobacterium leprae*

infections.[16] In our study, we found no difference in IL-10 production when cells were stimulated with type I IFN, nor during HSV-2 infection between WT and *Ifnar*$^{-/-}$ mice. It was also found that type I IFN is able to upregulate TAM receptor expression on immune cells, which can suppress their function, as TAM receptors hijack the type I IFN signaling pathway to induce activation of SOCS proteins to repress antiviral function.[17] We detected a significant increase in Axl expression on NK cells after stimulating both isolated human NK cells and PBMCs with type I IFN. To determine if TAM expression is involved in negatively regulating NK cell IFN-γ production during HSV-2 infection, NK cell Axl expression and TAM receptor ligands, Gas6 and Protein S, should be examined at d2 and d3 post-infection. In a study by Miyagi *et al,* they found that STAT4 was preferentially associated with the type I IFN receptor, and upon stimulation with type I IFN, there was a shift to STAT1 association with the type I IFN receptor, which was responsible for inhibiting NK cell IFN-γ production.[27] Further, Nguyen *et al* found that type I IFN inhibition of NK cell function was dependent upon STAT1.[14] Further work should examine the relative expression of STAT1 and STAT4 expression within NK cells during HSV-2 infection to determine their involvement.

In our studies, we also found that type I IFN stimulation of NK cells, in the presence of other immune cells, was still able to suppress cytokine-induced IFN-γ production. Cousens *et al* found that both IFN-α and IFN-β suppressed IL-12 production, which is a potent stimulator of NK cell IFN-γ production.[28] Further, others have found that type I IFN can suppress IL-12 production from DCs and IL-12p40 production from PBMCs.[29, 30] Type I IFN has also been found to upregulate PD ligand 1 (PDL1), which can suppress NK cell

function by ligating the PD1 receptor on NK cells.[12] Thus, type I IFN can not only inhibit NK cell function through NK cell type I IFN receptor expression, but type I IFN can also induce other immune cells to suppress NK cell function by increasing IL-10 or PDL1 expression or by suppressing IL-12 levels.

Recent evidence suggests that the subtypes of type I IFN can have very different modulatory effects on immune cells. While all subtypes can potently induce an antiviral response, Garcin *et al* found that treating DCs with different IFN subtypes led to significantly different profiles of receptor expression and cytokine production.[31] Further, the timing of type I IFN production, as well as the array of immune cells present during type I IFN production, can impact the effect of type I IFN on a specific immune cell.[15] During HSV-2 infection, a first wave of IFN-β occurs between 6-12 hrs post-infection, followed by a wave of IFN-α production at 48 hrs post-infection.[32] In addition, NK cell number is significantly increased in the vaginal tract at d2 post-infection. The wave of IFN-α production at 48 hrs post-infection could be responsible for negatively regulating NK cell IFN-γ production during HSV-2 infection, but further work will be required to delineate the effects of IFN-β and IFN-α on NK cell function.

Overall, our data suggests that type I IFN can suppress NK cell IFN-γ production during HSV-2 infection and cytokine stimulation. We found that type I IFN does not induce IL-10 production; however, it does increase expression of Axl receptor on NK cells, which has previously been shown to suppress immune cell function. It is important to understand how NK cell function is negatively regulated in order to prevent dysregulated function and the potential development of immunopathology. However, understanding how immune

cells are negatively regulated, can also serve to identify strategies to unleash their function to inhibit virus replication or eliminate tumor cells. Importantly, we demonstrate that type I IFN does not directly activate NK cells, rather, it negatively regulates their IFN-γ production.

Experimental Procedures

Mice: Six to eight week old C57BL/6 (WT) mice were purchased from Charles River Laboratory. *Ifnar*$^{-/-}$ mice were generously provided by Dr. Laurel Lenz (University of Colorado, Boulder, CO), backcrossed onto a C57BL/6 background, and a breeding colony was established at McMaster University's Central Animal Facility (CAF). *Rag2*$^{-/-}\gamma c^{-/-}$ mice on a Balb/c background were generously provided by Dr. M. Ito (Central Institute for Experimental Animals, Kawasaki, Japan) and a breeding colony was established at McMaster University's CAF. Mice were housed in specific pathogen free conditions on a 12 hour day and 12 hour night cycle. All experiments were performed in accordance with Canadian Council on Animal Care guidelines and with approval from McMaster University's Animal Research Ethics Board.

HSV-2 *in vivo* infection: Mice were given 2mg of Depo-Provera (medroxyprogesterone acetate) subcutaneously 5 days prior to infection. Mice were then infected with HSV-2 333 intravaginally. Vaginal lavages were collected at baseline through to day 3 post-infection once daily. For vaginal lavage collection, 30 µL of PBS were flushed in and out of the

vaginal tracts twice. Samples were centrifuged at 800 g for 5 min and supernatants were collected and assayed for protein via ELISA.

Tissue processing: Spleens were mechanically crushed into a single cell suspension and red blood cells were removed using an ACK lysis buffer. Vaginal tissue was isolated and processed into smaller pieces and then digested using a mixture of RPMI-1640 with 10% fetal bovine serum, 1% penicillin, 1% streptomycin, 1% L-glutamine, 1% hepes and collagenase A. Tissue was digested at 37 C for 1 hour twice.

Flow cytometry: Mouse cells were blocked with anti-CD16/CD32 antibody to prevent non-specific binding. Extracellular surface markers were then stained with APC-conjugated anti-mouse CD45, Alexa Fluor 700-conjugated anti-mouse CD3, PE-conjugated anti-mouse NK1.1, PE-cy7-conjugated anti-mouse CD11b, Percp-conjugated anti-mouse CD27. Human cells were stained with PE-CF594-conjugated anti-human CD56, APC-H7-conjugated anti-mouse CD3, PE-conjugated anti-human Tyro3, APC-conjugated anti-human Mer, and PE-conjugated anti-human Axl. Viability was assessed using a fixable viability dye (efluor 520). Cells were fixed in 1% paraformaldehyde prior to running the samples on a BD Biosciences FACSCanto, BD LSR II, or BD LSR Fortessa. Samples were analyzed using FlowJo software.

PBMC isolation: Peripheral blood was collected with written-informed consent and with approval by the Hamilton Integrated Research Ethics Board at McMaster University.

Peripheral blood was collected in an ACD solution A vacutainer (BD Biosciences) to prevent clotting. Blood was then diluted with 2% FBS in PBS and then separated using a Lymphoprep density gradient centrifugation method (Stem Cell Technologies). Isolated PBMCs were then used for in vitro assays or sent through an NK cell isolation kit.

NK cell isolation: Mouse NK cells were isolated using a PE-magnetic selection kit (Stem Cell Technologies) using an PE-conjugated anti-NK1.1 antibody. Human NK cells were isolated using a CD56+ magnetic selection kit (Stem Cell Technologies).

HSV-2 *in vitro* infection: *Rag2*$^{-/-}\gamma c^{-/-}$ splenocytes were isolated and infected with HSV-2 333 at an MOI of 3 in RPMI-1640 with 1% penicillin, 1% streptomycin 1% L-glutamine, and 1% hepes. Cells were infected for 2 hrs. NK cells isolated from WT or *Ifnar*$^{-/-}$ splenocytes were co-cultured with the infected *Rag2*$^{-/-}\gamma c^{-/-}$ splenocytes at a 1:4 ratio, respectively, in RPMI-1640 with 10% fetal bovine serum, 1% penicillin, 1% streptomycin, 1% L-glutamine, and 1% hepes. Cells were cultured for 48 hrs. Supernatants were collected and examined for IFN-γ levels.

Cytokine stimulation: Splenocytes, PBMCs, or isolated NK cells (both human and mouse) were cultured in RPMI-1640 with 10% fetal bovine serum, 1% penicillin, 1% streptomycin, 1% L-glutamine, and 1% hepes. Cells were pre-treated with 100 U (or the indicated concentrations) of IFN-β for 0, 4, 6, or 12 hrs and then stimulated with the

indicated concentrations of IL-15 or IL-18 for 24 hrs. Cells were also stimulated with 100 U of IFN-β for 18 hrs or 24 hrs.

ELISA: Mouse and human IFN-γ levels were measured using an R&D Systems ELISA kit. Mouse IL-10 levels were assayed using an R&D Systems ELISA kit.

Statistical Analysis: Differences were assessed using either a student's t-test, a one-way ANOVA if multiple groups were being analyzed, or a two-way ANOVA if more than two groups were being analyzed with two or more independent variables. If post-statistical analysis was required, a bonferroni post-test was applied. All statistical analyses were completed using GraphPad Prism 4.0. Statistical significance is indicated as ***$p<0.001$, **$p<0.01$, *$p<0.05$, or n.s. (not significant).

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References

1. Vivier, E., Tomasello, E., Baratin, M., Walzer, T. & Ugolini, S. Functions of natural killer cells. *Nat Immunol* **9**, 503-510 (2008).

2. Croen, K.D. Evidence for antiviral effect of nitric oxide. Inhibition of herpes simplex virus type 1 replication. *J Clin Invest* **91**, 2446-2452 (1993).

3. Goldszmid, R.S. *et al.* NK cell-derived interferon-gamma orchestrates cellular dynamics and the differentiation of monocytes into dendritic cells at the site of infection. *Immunity* **36**, 1047-1059 (2012).

4. Martin-Fontecha, A. *et al.* Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. *Nat Immunol* **5**, 1260-1265 (2004).

5. Ashkar, A.A. & Rosenthal, K.L. Interleukin-15 and natural killer and NKT cells play a critical role in innate protection against genital herpes simplex virus type 2 infection. *J Virol* **77**, 10168-10171 (2003).

6. Wald, A. *et al.* Reactivation of genital herpes simplex virus type 2 infection in asymptomatic seropositive persons. *N Engl J Med* **342**, 844-850 (2000).

7. Gray, R.H. *et al.* Determinants of HIV-1 load in subjects with early and later HIV infections, in a general-population cohort of Rakai, Uganda. *J Infect Dis* **189**, 1209-1215 (2004).

8. Nagot, N. *et al.* Reduction of HIV-1 RNA levels with therapy to suppress herpes simplex virus. *N Engl J Med* **356**, 790-799 (2007).

9. Pegram, H.J., Andrews, D.M., Smyth, M.J., Darcy, P.K. & Kershaw, M.H. Activating and inhibitory receptors of natural killer cells. *Immunol Cell Biol* **89**, 216-224 (2011).

10. Lee, A.J. *et al.* Inflammatory monocytes require type I interferon receptor signaling to activate NK cells via IL-18 during a mucosal viral infection. *J Exp Med* **214**, 1153-1167 (2017).

11. Platanias, L.C. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol* **5**, 375-386 (2005).

12. Teijaro, J.R. *et al.* Persistent LCMV infection is controlled by blockade of type I interferon signaling. *Science* **340**, 207-211 (2013).

13. Marshall, J.D., Heeke, D.S., Abbate, C., Yee, P. & Van Nest, G. Induction of interferon-gamma from natural killer cells by immunostimulatory CpG DNA is mediated through plasmacytoid-dendritic-cell-produced interferon-alpha and tumour necrosis factor-alpha. *Immunology* **117**, 38-46 (2006).

14. Nguyen, K.B. *et al.* Interferon alpha/beta-mediated inhibition and promotion of interferon gamma: STAT1 resolves a paradox. *Nat Immunol* **1**, 70-76 (2000).

15. Pontiroli, F. *et al.* The timing of IFNbeta production affects early innate responses to Listeria monocytogenes and determines the overall outcome of lethal infection. *PLoS One* **7**, e43455 (2012).

16. Teles, R.M. *et al.* Type I interferon suppresses type II interferon-triggered human anti-mycobacterial responses. *Science* **339**, 1448-1453 (2013).

17. Rothlin, C.V., Ghosh, S., Zuniga, E.I., Oldstone, M.B. & Lemke, G. TAM receptors are pleiotropic inhibitors of the innate immune response. *Cell* **131**, 1124-1136 (2007).

18. Ahlenstiel, G. *et al.* Early changes in natural killer cell function indicate virologic response to interferon therapy for hepatitis C. *Gastroenterology* **141**, 1231-1239, 1239 e1231-1232 (2011).

19. Edlich, B. *et al.* Early changes in interferon signaling define natural killer cell response and refractoriness to interferon-based therapy of hepatitis C patients. *Hepatology* **55**, 39-48 (2012).

20. Gill, N., Chenoweth, M.J., Verdu, E.F. & Ashkar, A.A. NK cells require type I IFN receptor for antiviral responses during genital HSV-2 infection. *Cell Immunol* **269**, 29-37 (2011).

21. Hayakawa, Y. & Smyth, M.J. CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity. *J Immunol* **176**, 1517-1524 (2006).

22. Chiossone, L. *et al.* Maturation of mouse NK cells is a 4-stage developmental program. *Blood* **113**, 5488-5496 (2009).

23. Peppa, D. *et al.* Blockade of immunosuppressive cytokines restores NK cell antiviral function in chronic hepatitis B virus infection. *PLoS Pathog* **6**, e1001227 (2010).

24. Spallanzani, R.G. *et al.* Regulatory Dendritic Cells Restrain NK Cell IFN-gamma Production through Mechanisms Involving NKp46, IL-10, and MHC Class I-Specific Inhibitory Receptors. *J Immunol* **195**, 2141-2148 (2015).

25. Tripp, C.S., Wolf, S.F. & Unanue, E.R. Interleukin 12 and tumor necrosis factor alpha are costimulators of interferon gamma production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist. *Proc Natl Acad Sci U S A* **90**, 3725-3729 (1993).

26. Chiu, B.C., Stolberg, V.R. & Chensue, S.W. Mononuclear phagocyte-derived IL-10 suppresses the innate IL-12/IFN-gamma axis in lung-challenged aged mice. *J Immunol* **181**, 3156-3166 (2008).

27. Miyagi, T. *et al.* High basal STAT4 balanced by STAT1 induction to control type 1 interferon effects in natural killer cells. *J Exp Med* **204**, 2383-2396 (2007).

28. Cousens, L.P., Orange, J.S., Su, H.C. & Biron, C.A. Interferon-alpha/beta inhibition of interleukin 12 and interferon-gamma production in vitro and endogenously during viral infection. *Proc Natl Acad Sci U S A* **94**, 634-639 (1997).

29. McRae, B.L., Semnani, R.T., Hayes, M.P. & van Seventer, G.A. Type I IFNs inhibit human dendritic cell IL-12 production and Th1 cell development. *J Immunol* **160**, 4298-4304 (1998).

30. Wang, X., Chen, M., Wandinger, K.P., Williams, G. & Dhib-Jalbut, S. IFN-beta-1b inhibits IL-12 production in peripheral blood mononuclear cells in an IL-10-

dependent mechanism: relevance to IFN-beta-1b therapeutic effects in multiple sclerosis. *J Immunol* **165**, 548-557 (2000).

31. Garcin, G. *et al.* Differential activity of type I interferon subtypes for dendritic cell differentiation. *PLoS One* **8**, e58465 (2013).

32. Gill, N., Deacon, P.M., Lichty, B., Mossman, K.L. & Ashkar, A.A. Induction of innate immunity against herpes simplex virus type 2 infection via local delivery of Toll-like receptor ligands correlates with beta interferon production. *J Virol* **80**, 9943-9950 (2006).

Figures

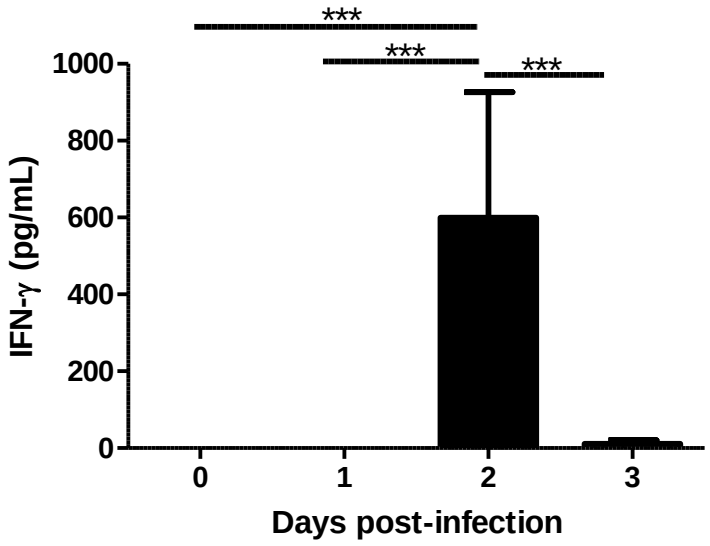


Figure 1. HSV-2 induced IFN-γ production is upregulated at day 2 post-infection and abrogated at day 3 post-infection. WT mice were infected with HSV-2 ivag. Days 0-3 p.i. vaginal lavages were collected and assayed for IFN-γ (n=5). ***p<0.001

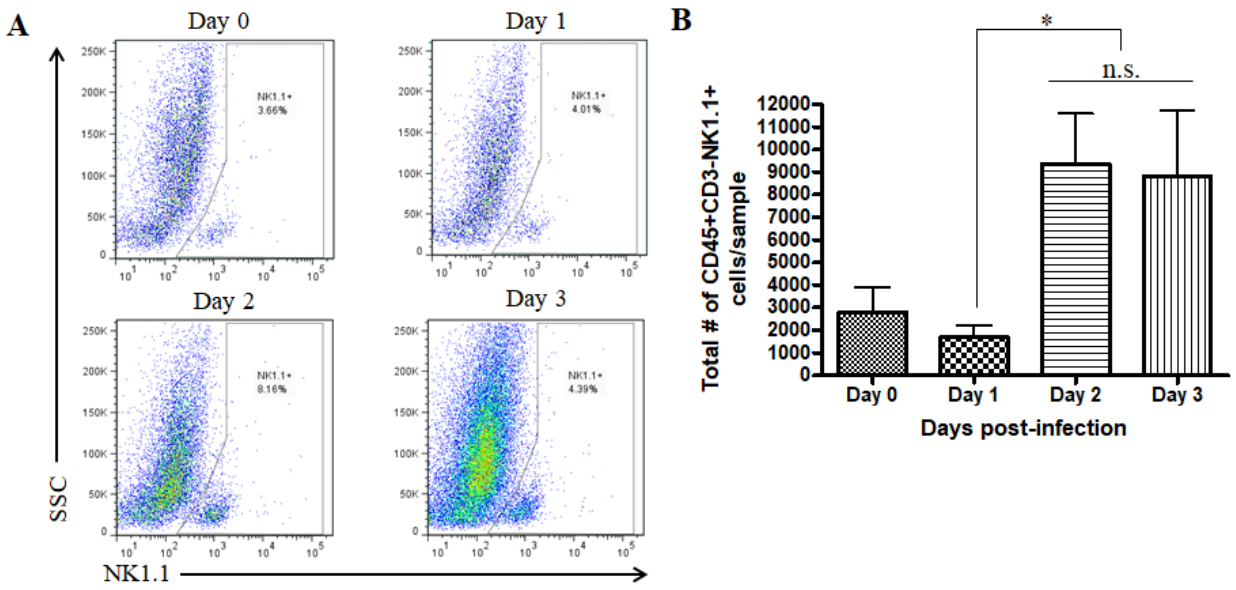


Figure 2. No significant difference in total number of vaginal NK cells between day 2 and day 3 post-infection. WT mice were infected with HSV-2 ivag. Vaginal tissue was isolated and processed at baseline through to d3 p.i. and stained for CD45, CD3, and NK1.1. NK cells were gated as CD45+, CD3- and NK1.1+. Representative flow plots are shown in (A) and total number of NK cells is shown in (B; n=4). n.s. not significant; *$p<0.05$.

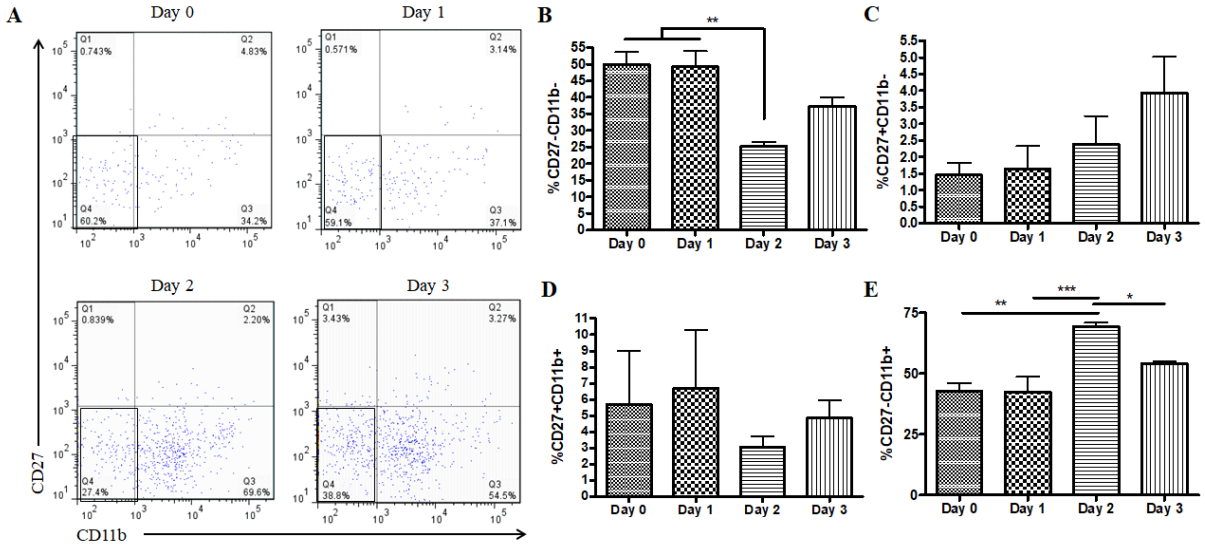


Figure 3. Increased proportion of CD27-CD11b+ NK cells at day 2 post-infection. WT mice were infected with HSV-2 ivag and vaginal cells were isolated at baseline through to day 3 post-infection. Vaginal cells were stained for CD45, CD3, NK1.1 CD27, and CD11b. Cells were first gated on CD45+, CD3, and NK1.1 to determine the NK cell population. NK cells were then examined for CD27 and CD11b expression. Representative flow plots are shown in (A). Proportion of double-negative NK cells (CD27-CD11b-) is shown in (B; n=4). Proportion of CD27+ (CD27+CD11b-) NK cells is shown in (C; n=4). Proportion of double-positive (CD27+CD11b+) NK cells is shown in (D; n=3). Proportion of CD11b+ (CD27-CD11b+) NK cells is shown in (E; n=4). *p<0.05; **p<0.01; ***p<0.001.

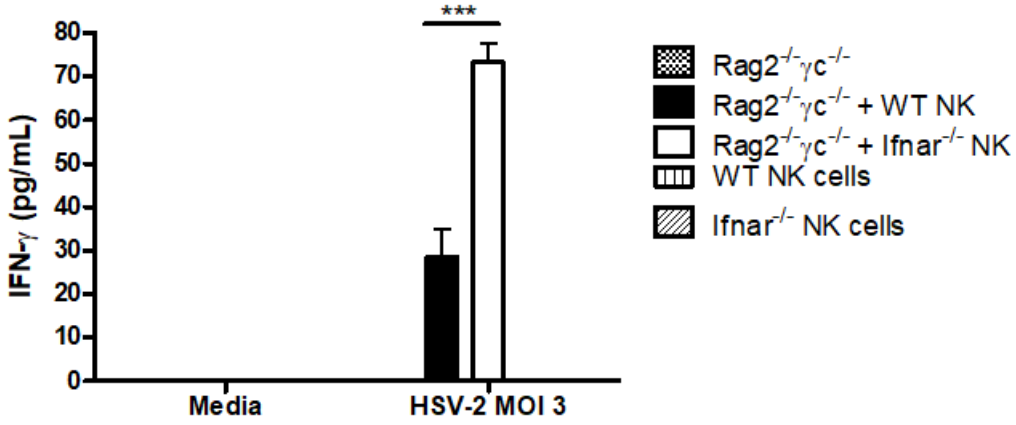


Figure 4. Absence of type I IFN receptor on NK cells allows for a significant increase in IFN-γ production from NK cells. *Rag2*$^{-/-}$*γc*$^{-/-}$ splenocytes were isolated and infected with HSV-2 MOI 3. Infected *Rag2*$^{-/-}$*γc*$^{-/-}$ splenocytes were then co-cultured with isolated WT or *Ifnar*$^{-/-}$ NK cells for 48hrs. As controls, Rag2$^{-/-}$γc$^{-/-}$ splenocytes, WT, and *Ifnar*$^{-/-}$ NK cells were each infected in separate wells for 48hs. After 48hrs of incubation, supernatants were collected and assay for IFN-γ production (A; n=3 repeated once with similar results). WT mice were infected with HSV-2 ivag and vaginal lavages were collected at different hours post-infection as indicated. Vaginal lavages were assayed for IFN-α levels (n=5). ***p<0.001

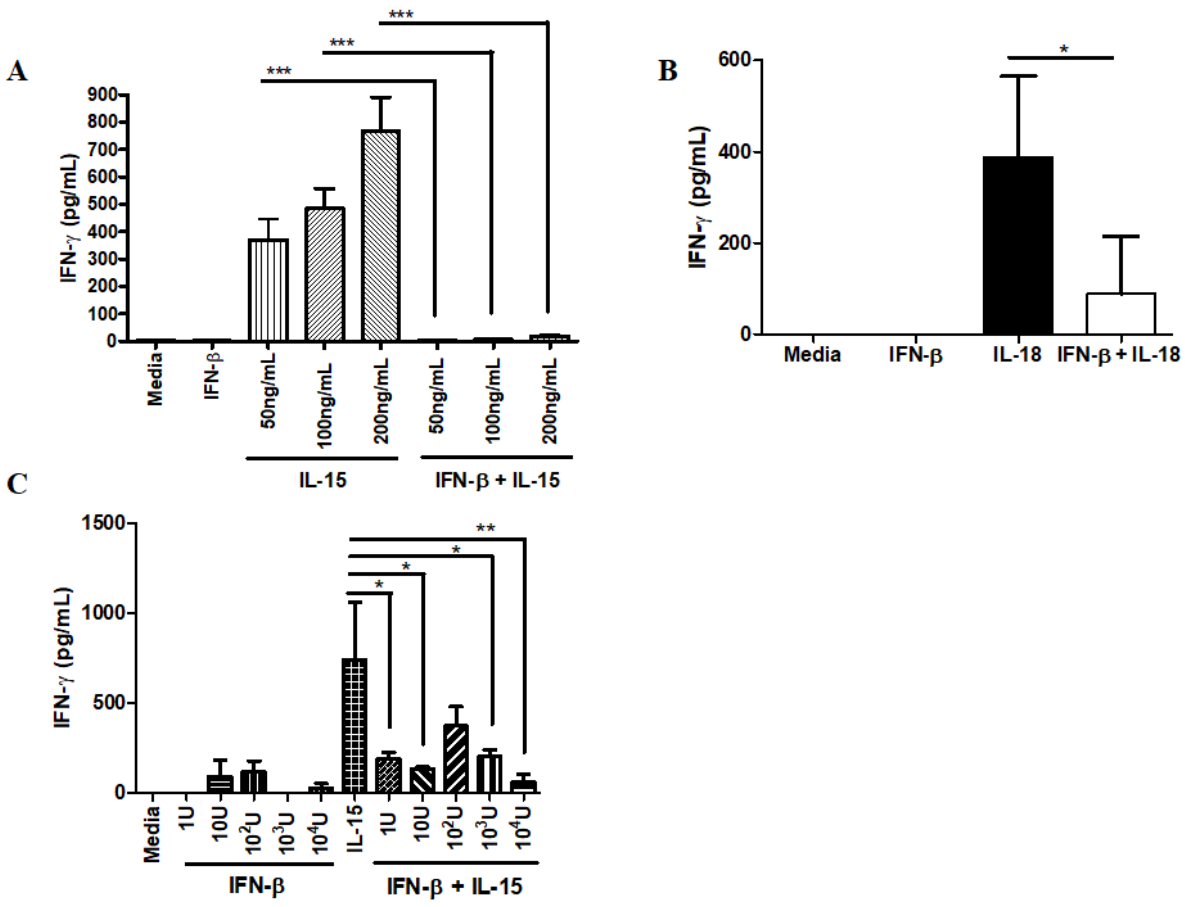


Figure 5. Type I IFN pre-treatment suppresses IL-15- and IL-18-induced IFN-γ production from NK cells. NK cells were isolated from WT spleens and pre-treated with 100U of IFN-β for 12 hrs. After the 12-hr pre-treatment, NK cells were stimulated with the indicated doses of IL-15 (A; n=3, repeated once with similar results) or 25 ng/mL IL-18 (B; n=3, repeated once with similar results) for 24 hrs. Supernatants were collected and assayed for IFN-γ production. WT splenocytes were pre-treated with 100 U IFN-β for 4 hrs. After 4 hrs of pre-treatment, splenocytes were stimulated with 250 ng/mL of IL-15 for 24 hrs. Supernatants were collected and assayed for IFN-γ production (C; n=3, repeated once with similar results). *$p<0.05$

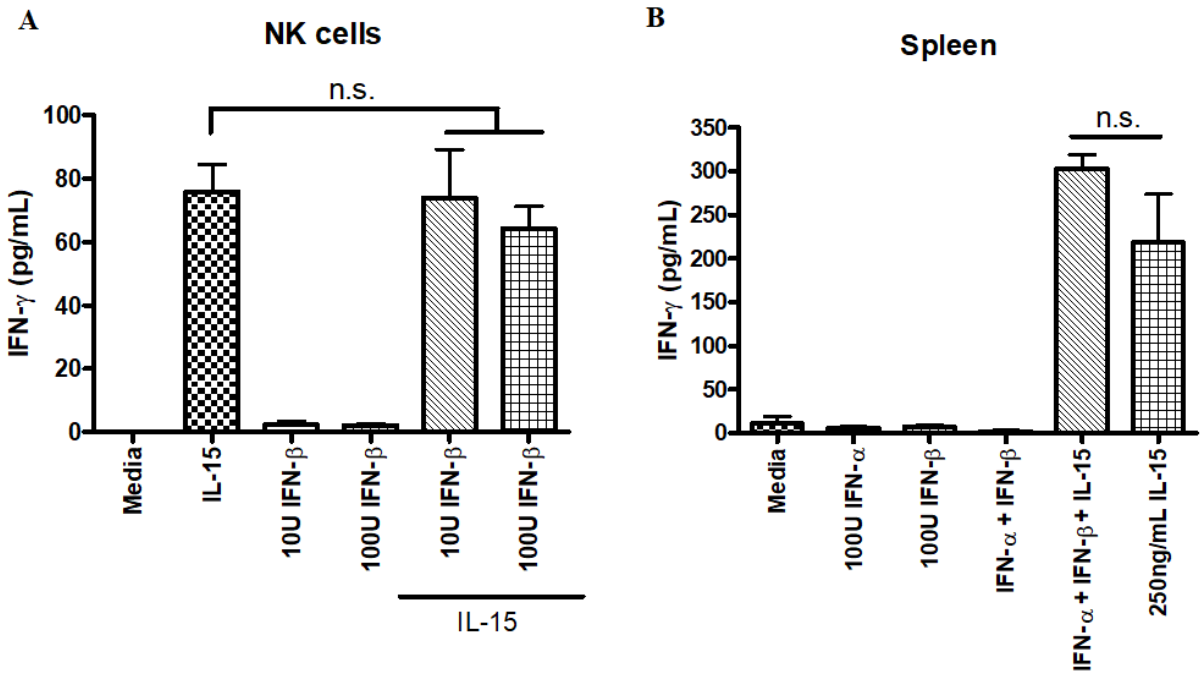


Figure 6. No difference in NK cell IFN-γ production when stimulating NK cells with type I IFN and IL-15 at the same time. NK cells were isolated from WT spleen and stimulated with either 200 ng/mL IL-15 alone, different doses of IFN-β or IL-15 and IFN-β at the same time for 24hrs. Supernatants were assayed for IFN-γ (A; n=3). WT splenocytes were isolated and stimulated with either 100 U IFN-α, 100 U IFN-β, 250ng/mL IL-15, or a combination of IFN-α, IFN-β, and IL-15 at the same time. After 24 hrs of stimulation, supernatants were collected and assayed for IFN-γ levels (B; n=3). n.s. not significant.

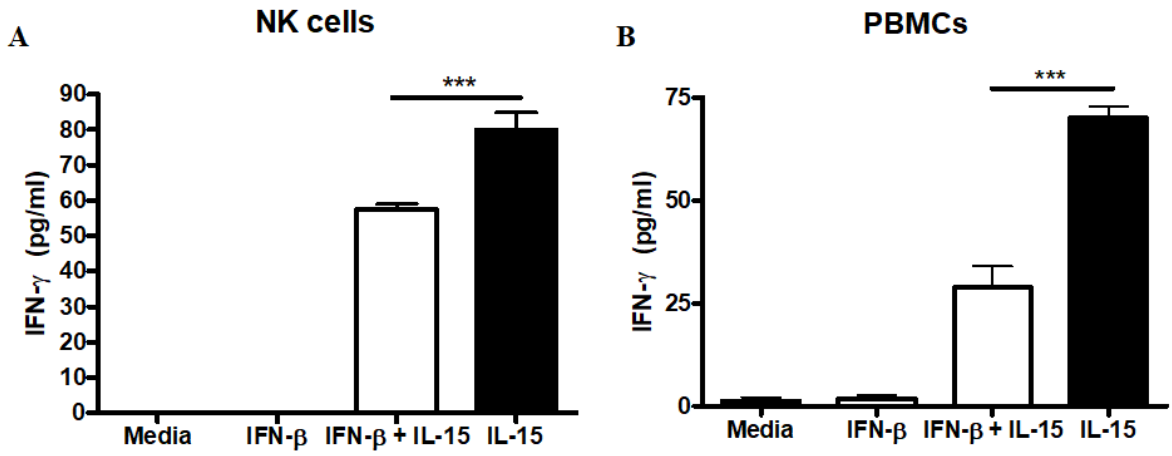


Figure 7. Type I IFN pre-treatment suppresses IL-15-induced human NK cell IFN-γ production. CD56+ cells were isolated from PBMCs and pre-treated with 100 U of IFN-β for 12 hrs. After 12 hrs, NK cells were then stimulated with 250 ng/mL IL-15 for 24hrs. Supernatants were collected and assayed for human IFN-γ levels (A; n=4). PBMCs were isolated and pre-treated with 100 U IFN-β for 12 hrs. After 12 hrs of treatment, PBMCs were stimulated with 250 ng/mL IL-15 for 24 hrs. Supernatants were collected and assayed for IFN-y levels (B; n=3). ***$p<0.001$

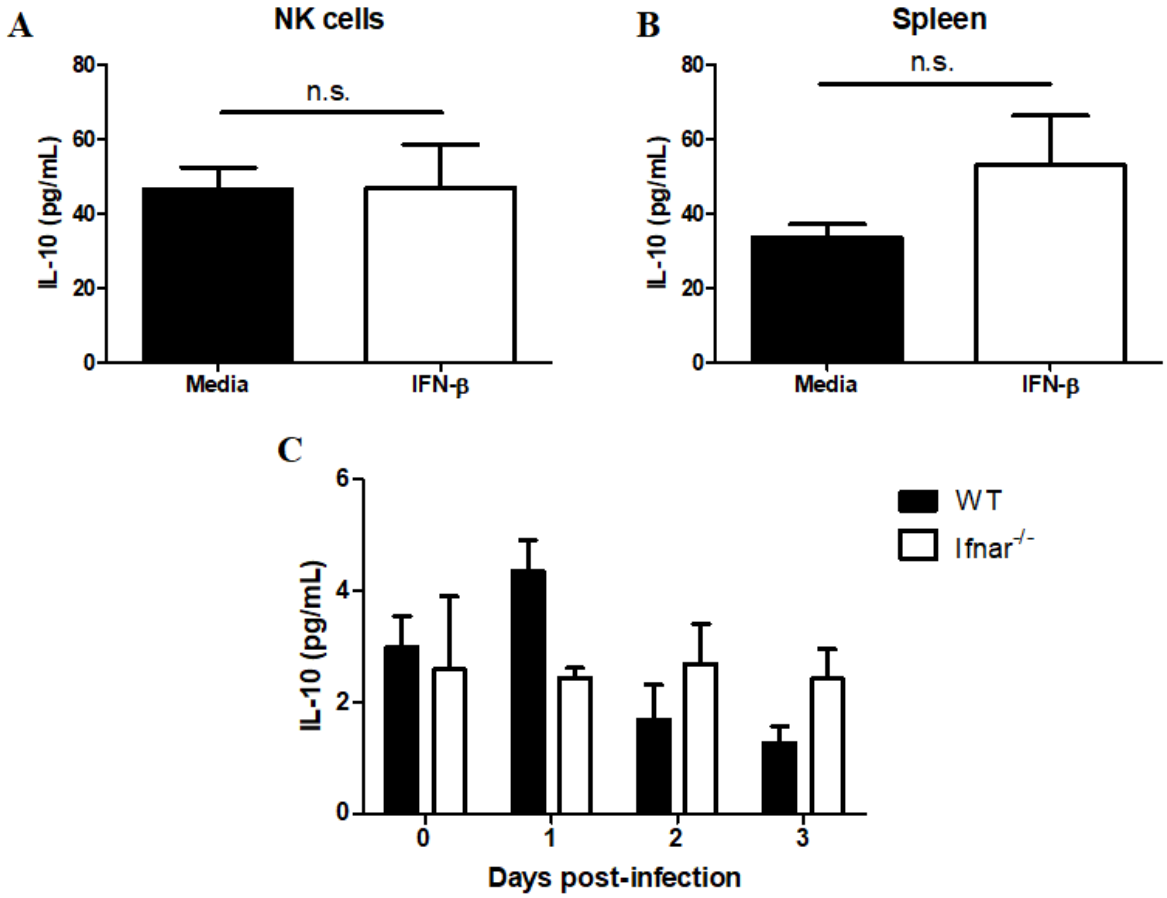


Figure 8. Type I IFN does not induce significant levels of IL-10. Isolated WT splenocyte NK cells (A; n=3, repeated once with similar results) and splenocytes (B) were stimulated with media or IFN-β for 24hrs. Supernatants were assayed for IL-10 production (B; n=3). WT and *Ifnar*$^{-/-}$ mice were infected with HSV-2 ivag. Vaginal lavages were collected at baseline through to d3 p.i. and examined for IL-10 levels (C; n-3). n.s. not significant.

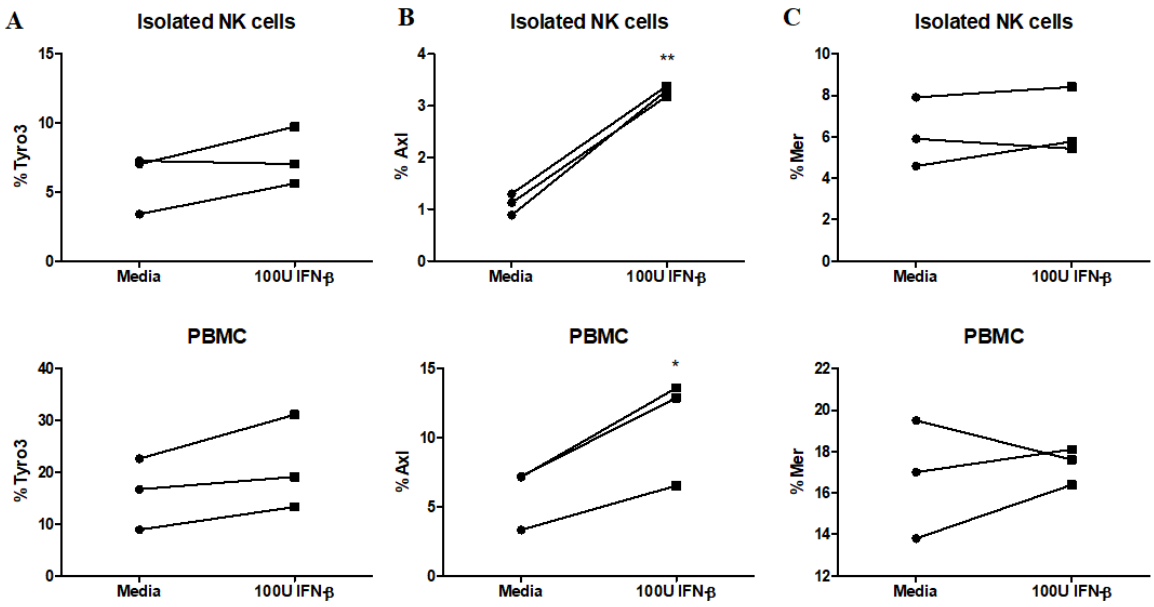
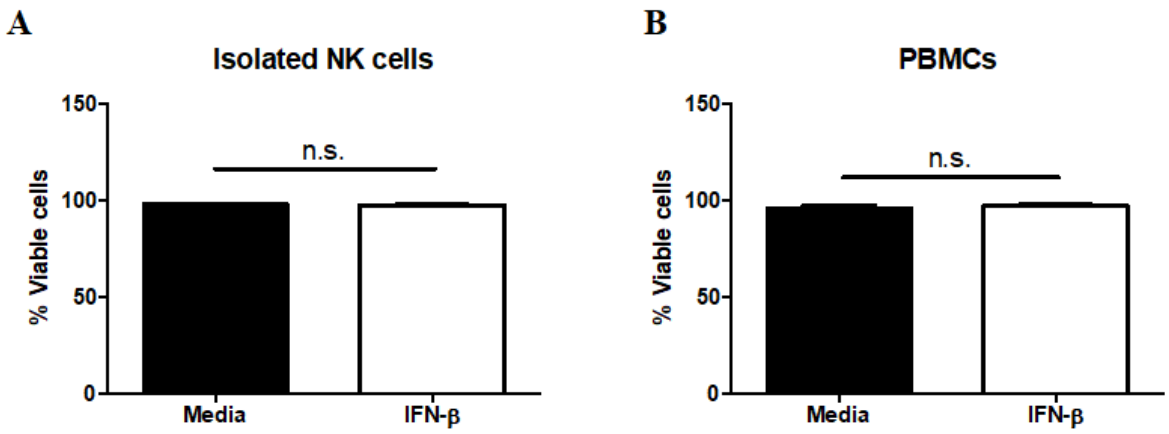


Figure 9. Significantly increased expression of Axl on NK cells after type I IFN stimulation. PBMC-isolated NK cells and PBMCs were stimulated with 100U of IFN-β for 18 hours. After stimulation, cells were stained with CD56, CD3, Tyro3, Axl, and Mer. Cells were first gated as CD56+CD3- NK cells and then examined for expression of Tyro3 (A; n=3), Axl (B; n=3), or Mer (C; n=3).

Supplementary Figures



Supplementary Figure 1. IFN-β stimulation does not affect NK cell and PBMC viability. NK cells were isolated from PBMCs and stimulated with 100U IFN-β for 18 hrs. After 18 hrs of stimulation, cells were stained with a fixable viability dye. The proportion of viable cells is graphically shown in (A; n=3). PBMCs were isolated from peripheral blood and stimulated with 100U of IFN-β for 18 hrs. After, they were stained with a fixable viability dye. The proportion f viable cells is graphically shown in (B; n=3). n.s. not significant.

CHAPTER 5: DISCUSSION

At the outset of my PhD project, we sought to understand the mucosal innate immune response to a vaginal HSV-2 infection, as women have a higher prevalence of HSV-2 infection and the vaginal tract is the primary site of infection.[9] As NK cells play an important role in the innate immune response to virus infection, both in limiting initial virus replication and also in helping initiate an adaptive immune response, we wanted to understand how the antiviral function of these cells are modulated.[57, 62, 63, 135] It is well known that type I IFN is critical for activating the antiviral function of NK cells; until this point, however, their effector mechanisms during virus infection were still under debate and their function during HSV-2 infection had yet to be explored.[135] Through the studies presented in this thesis, we have developed a fundamental understanding about the role of type I IFN in not only regulating NK cell function, but in shaping the immune response against a mucosal HSV-2 infection.

5.1 Type I IFN is a Key Regulator of Innate Immunity

In the three manuscripts presented in this thesis, we show three different, yet complementary roles of type I interferon in modulating and shaping the antiviral immune response. In directing a potent antiviral response, type I IFN is able to induce antiviral responses in infected and surrounding cells, which target cellular functions that are necessary for virus reproduction. As part of the innate immune response, type I IFN regulates the innate antiviral response. Type I IFN can induce cells to express CCR2 ligands to recruit inflammatory monocytes, as well as induce DC maturation and antigen-

presentation, which impacts activation of the adaptive immune response.[60, 62] Beyond that, we and others have found that type I IFN is required for NK cell antiviral functions, in particular IFN-γ production.[187, 230] We found that, unlike previous literature, type I IFN activates NK cells through an indirect mechanism that is not dependent upon DCs or IL-15. Instead, type I IFN activates inflammatory monocytes to release IL-18, which then activates NK cells through their IL-18R (Figure 1).

Along with involvement in recruiting innate immune cells and activating the antiviral response, type I IFN has immunomodulatory functions that dampen detrimental immune responses and promote antiviral responses. We found that during virus infection, type I IFN suppresses virus-induced immunopathology. In another infection model, Duerr *et al* and Moro *et al* found that *Ifnar*$^{-/-}$ mice challenged with a respiratory virus had intense lung immunopathology mediated by a type 2 immune response and ILC2s.[279, 289] They found that type I IFN directly suppressed ILC2 function and immunopathology in the lung.[279] In the context of HSV-2 infection in the vaginal mucosa, we found that type I IFN negatively regulated pathology caused by the innate immune response; however, this immunopathological response was independent of ILC2 cells. Further, as type I IFN was critical for inducing IFN-γ release from NK cells during HSV-2 infection, activating NK cells through IL-12 and IL-18 administration was able to not only rescue NK cell IFN-γ production, but also suppress virus-induced vaginal pathology. Thus, while promoting a beneficial antiviral response, type I IFN also suppresses exaggerated immune functions that can cause tissue pathology, likely through both direct and indirect mechanisms (Figure 1).

Finally, we demonstrated that not only is type I IFN a critical factor for NK cell activation, it is also responsible for negatively regulating IFN-γ release from NK cells during HSV-2 infection. While type I IFN mediated NK cell activation through an indirect mechanism involving inflammatory monocytes, we found that direct action of type I IFN on NK cells could suppress IFN-γ production during both virus infection and in response to exogenous cytokine stimulus. We also provided further evidence that type I IFN does not activate NK cells directly, as stimulation of isolated NK cells with type I IFN did not elicit an IFN-γ response (Figure 1).

Overall, our three papers provide further evidence that type I IFN is a key regulator of the innate antiviral immune response. It serves to not only activate antiviral responses within infected and surrounding cells and limit virus replication, but to polarize the immune system towards a Th1 response to combat virus infection while dampening detrimental responses that can harm the host.

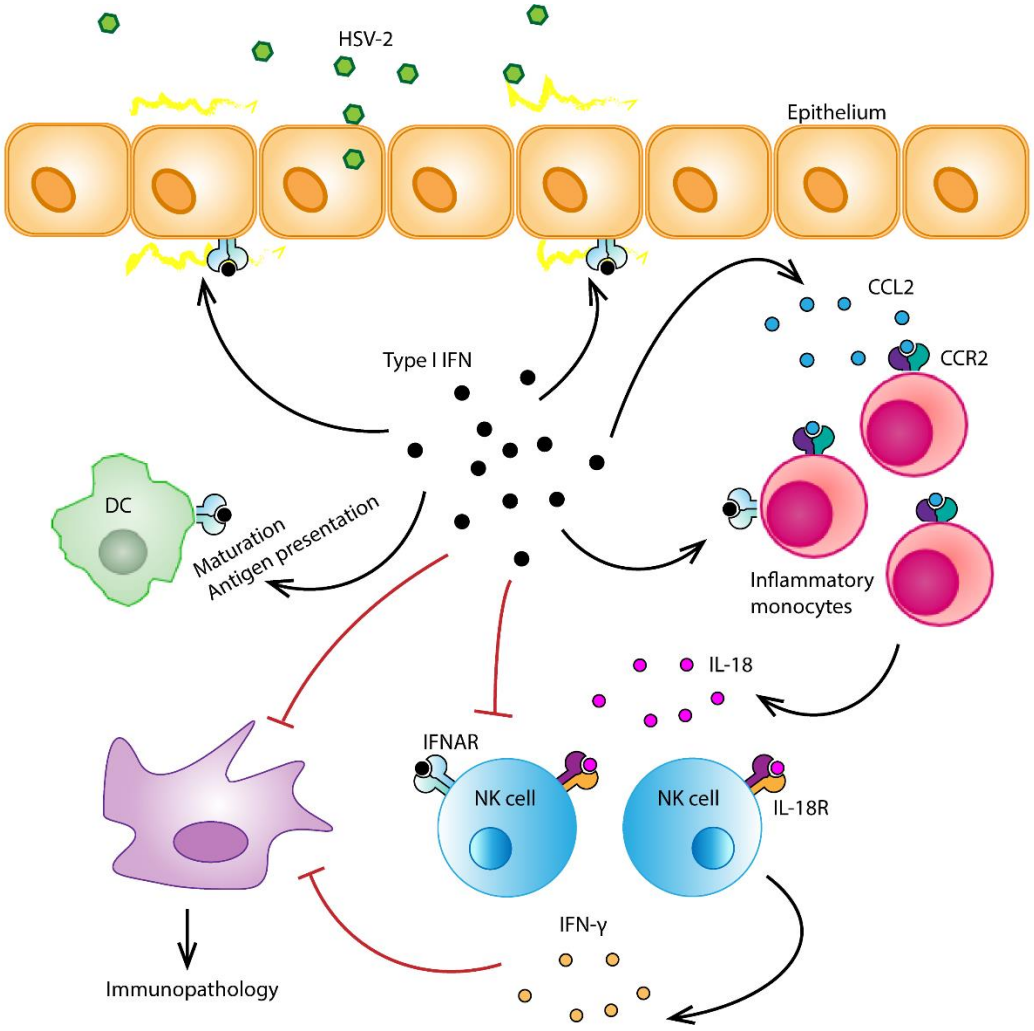


Figure 1. Type I IFN is a key modulator of the innate immune response during virus infection. After HSV-2 infection, type I IFN is rapidly produced and has a multitude of effects on the innate immune system. It places surrounding target cells into an antiviral state and induces maturation and increased antigen presentation functions of DCs. Type I IFN also prompts production of CCL2 to recruit inflammatory monocytes. These inflammatory monocytes respond to type I IFN to release IL-18 and induce NK cell IFN-γ production. Direct type I IFN action on NK cells, however, suppresses the release of IFN-γ. Further, type I IFN and IFN-γ suppress vaginal virus-induced immunopathology.

5.2 Teasing apart the dual role of type I IFN as both an activator and negative regulator of immune cell function

Through our studies and the research of others, we came to question how a single cytokine group, signaling through the same receptor, can result in such a diverse range of outcomes for the immune system. In the case of NK cells, type I IFN can serve to both activate and inhibit the same function, IFN-γ production, during the same virus infection, HSV-2. Recent literature has found that the route by which type I IFN signals, the subtype of type I IFN induced, the cells present at the time of type I IFN production, and the amount and length of type I IFN signaling can have a large impact on their resultant downstream effects and can dictate whether type I IFN will act as an activator or a negative regulator.

In our studies, we found that type I IFN can have differing effects on NK cells depending on the mechanism through which it signals. In the case of induction of IFN-γ from NK cells, type I IFN receptor is required to signal through an immune cell intermediary, inflammatory monocytes, which then release IL-18 to activate NK cells.[288] The same type I IFN receptor is also required to dampen NK cell IFN-γ production, however, through a direct mechanism involving NK cell IFNAR expression. Thus, the route of type I IFN signaling can determine the impact it has on NK cell function.

Recent research studies have found that type I IFN subtypes can have varying immune modulatory effects on immune cells. Though all subtypes were found to induce an antiviral response, not all subtypes were capable of inducing the same effect on specific immune cells. Indeed, treatment of DCs with different type I IFN subtypes resulted in

different levels and profiles of receptor and cytokine expression.[115] Further, through the use of specific blocking antibodies against IFN-α and IFN-β, IFN-α was determined to be the key type I IFN subtype responsible for regulating the antiviral response against West Nile Virus infection.[290] Differences in subtype immune modulation can stem from differences in affinity binding to the type I interferon receptor.[102] There is a large range in affinity between the interferon subtypes, with IFN-β having the highest affinity to IFNAR.[102] Thomas *et al* demonstrated that mutating IFN-α2 and IFN-ω, and altering affinity binding to the type I IFN receptor, resulted in large variations in anti-proliferative effects, which require much higher binding affinity.[102, 291] In contrast, all subtypes of type I IFN are capable of inducing an antiviral response, despite the large variation in binding affinities between them.[102, 291] Moreover, there is data to suggest that IFN-β can signal through IFNAR1 alone, which results in activation of a signaling pathway independent of JAK-STAT.[105]

In the context of type I IFN and its ability to modulate NK cell function during HSV-2 infection, we and others have found that IFN-α and IFN-β are produced at different time points during infection.[51, 92] Gill *et al* found that an early wave of IFN-β is produced at the 6-12 hour post-infection time point, while IFN-α is significantly increased at 48 hours post-infection.[51] Oh *et al* also showed that IFN-β is upregulated at the 48 hour time point.[92] It would be of interest to determine whether different subtypes are responsible for NK cell activation and negative regulation.

The immune cells present in the microenvironment when type I IFN is produced can have a large impact on the type of effect type I IFN will have. During HSV-2 infection,

IFN-β is produced rather early during infection (6-12 hrs post-infection), a time point at which NK cells are not yet recruited to the site of infection.[51] Instead, this early wave of type I IFN may be sufficient to activate inflammatory monocytes, which produce IL-18 at 48 hours post-infection, which corresponds to the significant increase in NK cell number in the vaginal tract during infection.[51, 288] However, NK cells in the vaginal microenvironment are likely exposed to IFN-α or the second wave of IFN-β produced at 48 hours post-infection, which could account for the negative regulatory effect type I has on NK cell IFN-γ production.[92] This induction of type I IFN at 48 hours post-infection may also be key for dampening the immunopathology found in the vaginal tract which we found occurs when type I IFN receptor is absent (Figure 2). Further investigation into the expression of type I IFN subtypes and their role in NK cell antiviral function and suppression of immunopathology would contribute greatly to our understanding of type I IFN during virus infection.

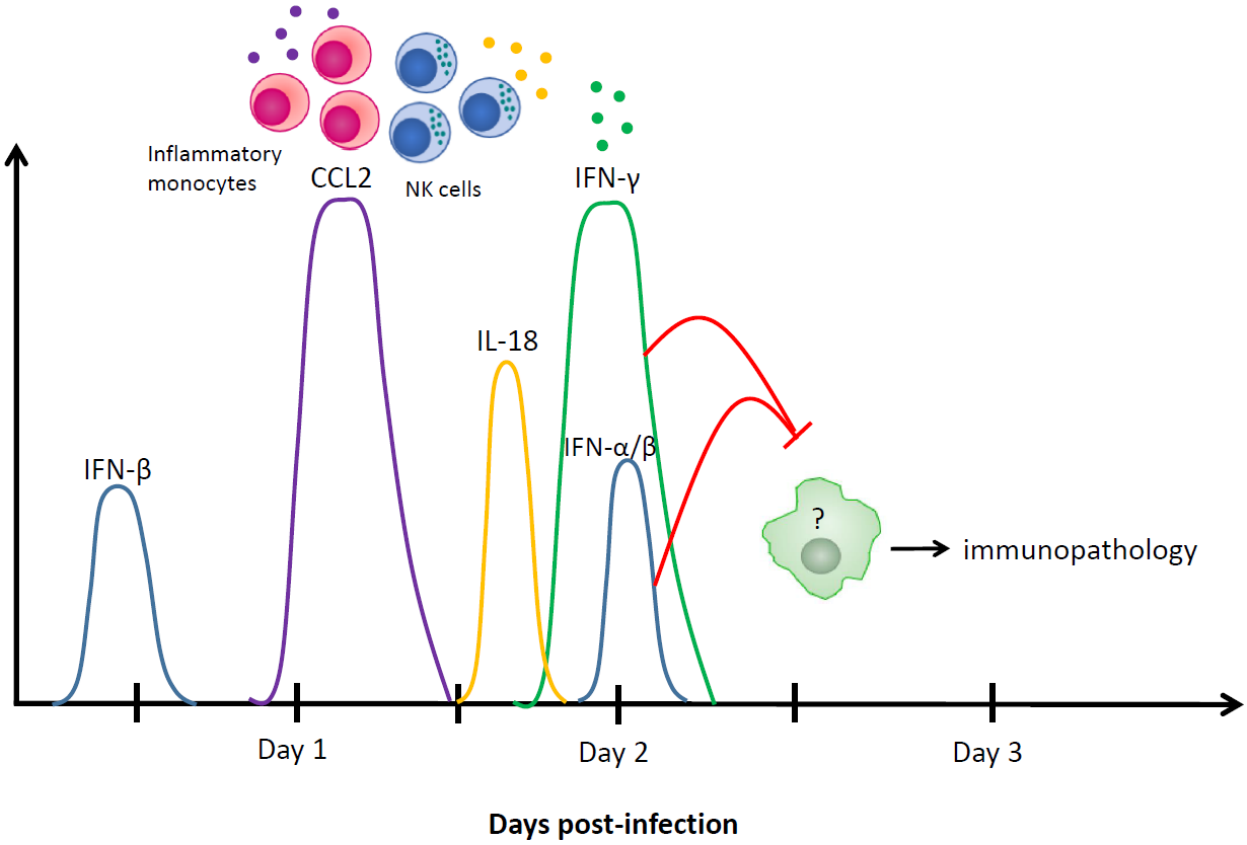


Figure 2. Timing of cytokine production and immune cell recruitment during HSV-2 infection.

After intravaginal HSV-2 infection, IFN-β is rapidly produced at 6-12 hrs post-infection. This is followed by production of CCL2 between day 1 and day 2 post-infection and recruitment of inflammatory monocytes and NK cells. Then inflammatory monocytes produce IL-18 between 24 and 48hrs post-infection, which activates NK cell IFN-γ production at 48hrs post-infection. A second wave of type I IFN, IFN-α, is produced at day 2 post-infection, and may play a role in negatively regulating NK cell IFN-γ release as well as virus-induced immunopathology.

The amount of type I IFN produced and the length of type I IFN signaling can result in vastly different outcomes that can tip the scale from being a beneficial antiviral response to a detrimental effect on the host. During chronic infections, a high type I IFN signature can lead to a detrimental response, where type I IFN can induce IL-10 production, increase PD-L1 expression, and suppress antiviral T-cell responses.[118] Further, it has been found that specific autoimmune diseases have an elevated type I IFN signature which contributes to the development and severity of the disease.[123] During SIV infection in rhesus macaques, administration of type I IFN early during infection was beneficial in combatting the infection, while chronic type I IFN administration resulted in an increased SIV load.[116, 121] Thus, in the context of another genital infection, the amount and length of type I IFN signaling has been descried to result in vastly different outcomes for the immune response.

5.3 Limitations and Future Directions

In the first manuscript presented, we examined the mechanism by which type I IFN induces NK cell IFN-γ production during HSV-2 infection. We presented data to suggest that type I IFN activates inflammatory monocytes to release IL-18, which then induces NK cell IFN-γ production.[288] Though selective depletion of inflammatory monocytes resulted in significantly decreased levels of IL-18 and IFN-γ production, this data would be nicely complemented by data showing that inflammatory monocytes are indeed responsible for producing IL-18 by examining intracellular IL-18 levels in inflammatory monocytes either during infection or in response to type I IFN stimulation. This could further be shown by the adoptive transfer of WT inflammatory monocytes into *Il18*$^{-/-}$ mice during HSV-2

infection or the selective depletion of IL-18 in inflammatory monocytes. However, a mouse model with the selective elimination of IL-18 expression in inflammatory monocytes currently does not exist and our attempted transfers of either *Rag2*$^{-/-}$-*γc*$^{-/-}$ cells or cultured monocytes into *Il18*$^{-/-}$ mice did not yield a sufficient recruitment of transferred inflammatory monocytes to the vaginal tract during infection.

NK cells have recently been reclassified as part of the ILC1 subset, where both NK cells and ILC1 cells have been shown to both produce IFN-γ when either stimulated or in the context of infection. In my first manuscript, I attributed IFN-γ production to NK cells, which express NK1.1/NKp46 and lack of CD3 expression. However, both ILC1 cells and NK cells have been shown to express these markers, and therefore either are potentially responsible for early IFN-γ production during HSV-2 infection. CD127 and Eomes have been suggested to differentiate between the two, where ILC1 cells express CD127 and lack Eomes expression, while NK cells lack CD127 expression and express Eomes.

In finding that type I IFN is able to suppress innate virus-induced immunopathology, we determined that in the vaginal tract, ILC2 cells and Th2 cells were not contributors to the immunopathology that occurred in the absence of type I IFN receptor, despite the upregulation of IL-6. Further, we found that neutrophils and eosinophils were similarly not involved. We also found that the increased amount of IL-6 is partially involved in mediating the immunopathology in *Ifnar*$^{-/-}$ mice. It would be of interest to examine how IL-6 is involved in mediating the immunopathological process, as well as the involvement of other mediators. We also found an increase in F4/80+ macrophages and CD206 expression in *Ifnar*$^{-/-}$ mice in comparison to WT mice. It would

be of interest to examine the role of macrophages in mediating the immunopathology in *Ifnar*$^{-/-}$ mice by depleting macrophages via clodronate administration. Once the mechanism of immunopathology is determined, next steps should examine how type I IFN is able to inhibit this mechanism.

We also found that activating NK cells in *Ifnar*$^{-/-}$ mice via IL-12 and IL-18 was able to dampen the immunopathology in *Ifnar*$^{-/-}$ mice. Though we found increased levels of IFN-γ after administration of IL-12 and IL-18, the involvement of IFN-γ in suppressing vaginal pathology could be further characterized through the use of either *Ifnγ*$^{-/-}$ mice or a blocking anti-IFN-γ antibody. To further this, it would be useful to examine other cytokines that are released by NK cells after IL-12 and IL-18 stimulation and determine their involvement in suppressing the pathological process in the absence of type I IFN receptor.

One important limitation to this study is the unexplored contribution of virus lysis to the vaginal immunopathology seen in *Ifnar*$^{-/-}$ mice. As type I IFN is critical for inducing an antiviral response in target cells, as well as activating an antiviral response, the absence of type I IFN receptor signaling allows viruses to replicate uncontrollably, creating the formation of syncytia and cellular lysis as new virus progeny exit the cell.[27, 51] This in itself can create vaginal pathology during infection. Though we showed that there is no difference in virus shedding between WT and *Ifnar*$^{-/-}$ mice during HSV-2 infection, it would be of interest to quantify the level of intracellular virus replication. In order to tease apart the role of virus-induced pathology and virus-induced innate immunopathology, innate immunopathology in the absence of virus replication can be examined by infecting

with UV-inactivated HSV-2 or stimulating the vaginal tract with TLR ligands, such as poly I:C or CpG.

In the third manuscript of this thesis, we again provide further support that type I IFN does not activate NK cells through a direct mechanism. Instead we found that direct action of type I IFN on NK cells actually suppresses the release of IFN-γ during HSV-2 infection and in response to cytokine stimuli. We also found that type I IFN upregulates Axl expression on NK cells, though it is not known whether Axl expression and signaling is responsible for suppressing IFN-γ production from NK cells. It would be interesting to examine NK cell TAM receptor expression after type I IFN and cytokine (e.g. IL-15 or IL-18) treatment and during HSV-2 infection. Further, it would be of interest to examine the levels of STAT1 and STAT4 expression as well as the signaling cascade downstream of TAM receptors, SOCS1 and SOCS3.

One limitation that encompasses all three studies is the fact that we strictly examine IFN-γ production as a marker of NK cell activation. NK cells can release other pro-inflammatory cytokines, such as TNF-α and GM-CSF, for example, and possess other antiviral cytotoxic functions, such as the expression of death receptor ligands or release of perforin and granzyme. Other studies have found that type I IFN activates NK cell cytolytic activity against target cells.[231] It would be interesting to examine the impact of type I IFN on NK cell cytotoxicity, production of other pro-inflammatory cytokines, and upregulation of death receptor ligands during HSV-2 infection.

Another over-arching future direction will be to examine the role of specific type I IFN subtypes during antiviral innate immune responses. The data presented here deals

specifically with a complete absence of type I IFN signaling with no differentiation between subtypes of type I IFN. Though we have previously found that IFN-β is produced at 6-12 hours post-infection and IFN-α is produced at 48 hours post-infection, it would be of interest to examine the expression of specific type I IFN subtypes via ELISA, western blot, or RT-PCR during infection.[51] From there, we could also examine the role of IFN-α and IFN-β, through the use of specific blocking antibodies, in modulating the innate immune response during HSV-2 infection.[290] The more we understand about the effects of type I IFN, the clearer it becomes that there is much to be explored regarding the effects of specific type I IFN subtypes, and it is important to do so in the contexts of infection, cancer, autoimmunity, and other diseases.

5.4 Clinical Relevance

As a potent modulator of the innate immune response, it is important to understand how type I IFN modulates both innate and adaptive immunity in order to aid in the development of future therapeutics and vaccines against virus infection. Further, understanding how NK cells are modulated both by type I IFN and during infection will not only further our knowledge of these cells, but how we can appropriately harness these cells in the future. As an example, we now know that type I IFN will not directly activate IFN-γ production from these cells, but will require the appropriate accessory components in order to become activated. Further, we now understand that type I IFN can directly negatively regulate NK cell IFN-γ production, as well as suppress innate immunopathology during infection

As new type I IFN based therapeutics are being produced, it becomes more crucial to understand their effects on the immune system, particularly during infection. Pegylated IFN-α is used as a therapeutic to treat HCV infection and IFN-β is used as a therapeutic for multiple sclerosis.[239, 292] Understanding the effects of type I IFN on the innate immune system is critical for extrapolating these effects in humans. Further, an antibody blocking type I IFN receptor has recently been developed for individuals with systemic lupus erythematosus and has been shown to have clinical benefit.[124] As type I IFN is crucial for NK cell activation and suppression of innate immunopathology during HSV-2 infection, this is important to keep in mind as this blocking antibody heads to the clinic and is administered to more patients.

5.5 Concluding Remarks

The current consensus in the literature regarding type I IFN and NK cells is that type I IFN is an activator of NK cell function, including IFN-γ production. Our data challenges this concept, as we clearly show that type I IFN does not directly activate IFN-γ release from NK cells during infection or NK cell stimulation. We present a mechanism by which type I IFN instead activates inflammatory monocytes to produce IL-18 and this IL-18 is necessary for NK cell IFN-γ production. We also show that direct action of type I IFN on NK cells actually suppresses NK cell IFN-γ production, rather than leading to activation. Further, we show a novel function of type I IFN in suppressing ILC2- and Th2-independent innate immune response-mediated pathology during infection. Overall, this

data significantly contributes to our understanding of the biological interplay between type I IFN, NK cells, and virus infection.

CHAPTER 6: REFERENCES

1. Johnston, C. & Corey, L. Current Concepts for Genital Herpes Simplex Virus Infection: Diagnostics and Pathogenesis of Genital Tract Shedding. *Clin Microbiol Rev* **29**, 149-161 (2016).

2. Wald, A. *et al.* Reactivation of genital herpes simplex virus type 2 infection in asymptomatic seropositive persons. *N Engl J Med* **342**, 844-850 (2000).

3. Caviness, A.C., Demmler, G.J. & Selwyn, B.J. Clinical and laboratory features of neonatal herpes simplex virus infection: a case-control study. *Pediatr Infect Dis J* **27**, 425-430 (2008).

4. Zuckerman, R., Wald, A. & Practice, A.S.T.I.D.C.o. Herpes simplex virus infections in solid organ transplant recipients. *Am J Transplant* **9 Suppl 4**, S104-107 (2009).

5. Kapiga, S.H. *et al.* The role of herpes simplex virus type 2 and other genital infections in the acquisition of HIV-1 among high-risk women in northern Tanzania. *J Infect Dis* **195**, 1260-1269 (2007).

6. Schacker, T. *et al.* Frequent recovery of HIV-1 from genital herpes simplex virus lesions in HIV-1-infected men. *JAMA* **280**, 61-66 (1998).

7. Nagot, N. *et al.* Roles of clinical and subclinical reactivated herpes simplex virus type 2 infection and human immunodeficiency virus type 1 (HIV-1)-induced immunosuppression on genital and plasma HIV-1 levels. *J Infect Dis* **198**, 241-249 (2008).

8. Belshe, R.B. *et al.* Efficacy results of a trial of a herpes simplex vaccine. *N Engl J Med* **366**, 34-43 (2012).

9. Looker, K.J. *et al.* Global estimates of prevalent and incident herpes simplex virus type 2 infections in 2012. *PLoS One* **10**, e114989 (2015).

10. Xu, F. *et al.* Trends in herpes simplex virus type 1 and type 2 seroprevalence in the United States. *JAMA* **296**, 964-973 (2006).

11. Corey, L., Adams, H.G., Brown, Z.A. & Holmes, K.K. Genital herpes simplex virus infections: clinical manifestations, course, and complications. *Ann Intern Med* **98**, 958-972 (1983).

12. Groves, M.J. Genital Herpes: A Review. *Am Fam Physician* **93**, 928-934 (2016).

13. Wald, A., Huang, M.L., Carrell, D., Selke, S. & Corey, L. Polymerase chain reaction for detection of herpes simplex virus (HSV) DNA on mucosal surfaces: comparison with HSV isolation in cell culture. *J Infect Dis* **188**, 1345-1351 (2003).

14. Elion, G.B. *et al.* Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl) guanine. *Proc Natl Acad Sci U S A* **74**, 5716-5720 (1977).

15. Sexually Transmitted Diseases: Summary of 2015 CDC Treatment Guidelines. *J Miss State Med Assoc* **56**, 372-375 (2015).

16. Celum, C. *et al.* Acyclovir and transmission of HIV-1 from persons infected with HIV-1 and HSV-2. *N Engl J Med* **362**, 427-439 (2010).

17. Johnston, C. *et al.* Standard-dose and high-dose daily antiviral therapy for short episodes of genital HSV-2 reactivation: three randomised, open-label, cross-over trials. *Lancet* **379**, 641-647 (2012).

18. Reyes, M. *et al.* Acyclovir-resistant genital herpes among persons attending sexually transmitted disease and human immunodeficiency virus clinics. *Arch Intern Med* **163**, 76-80 (2003).

19. Iwasaki, A. Antiviral immune responses in the genital tract: clues for vaccines. *Nat Rev Immunol* **10**, 699-711 (2010).

20. Quispe Calla, N.E. *et al.* Medroxyprogesterone acetate and levonorgestrel increase genital mucosal permeability and enhance susceptibility to genital herpes simplex virus type 2 infection. *Mucosal Immunol* **9**, 1571-1583 (2016).

21. Deruaz, M. & Luster, A.D. Chemokine-mediated immune responses in the female genital tract mucosa. *Immunol Cell Biol* **93**, 347-354 (2015).

22. Givan, A.L. *et al.* Flow cytometric analysis of leukocytes in the human female reproductive tract: comparison of fallopian tube, uterus, cervix, and vagina. *Am J Reprod Immunol* **38**, 350-359 (1997).

23. Gipson, I.K. *et al.* Mucin genes expressed by human female reproductive tract epithelia. *Biol Reprod* **56**, 999-1011 (1997).

24. Ming, L. *et al.* Purification of antimicrobial factors from human cervical mucus. *Hum Reprod* **22**, 1810-1815 (2007).

25. Brotman, R.M., Ravel, J., Bavoil, P.M., Gravitt, P.E. & Ghanem, K.G. Microbiome, sex hormones, and immune responses in the reproductive tract:

challenges for vaccine development against sexually transmitted infections. *Vaccine* **32**, 1543-1552 (2014).

26. O'Hanlon, D.E., Moench, T.R. & Cone, R.A. In vaginal fluid, bacteria associated with bacterial vaginosis can be suppressed with lactic acid but not hydrogen peroxide. *BMC Infect Dis* **11**, 200 (2011).

27. Knipe, D.M., Howley, P. M. (ed). *Fields Virology*. Lippincott Williams & Wilkins: Philadelphia, 2007.

28. Johnston, C., Koelle, D.M. & Wald, A. HSV-2: in pursuit of a vaccine. *J Clin Invest* **121**, 4600-4609 (2011).

29. Whitley, R.J. & Roizman, B. Herpes simplex virus infections. *Lancet* **357**, 1513-1518 (2001).

30. Connolly, S.A., Jackson, J.O., Jardetzky, T.S. & Longnecker, R. Fusing structure and function: a structural view of the herpesvirus entry machinery. *Nat Rev Microbiol* **9**, 369-381 (2011).

31. Paludan, S.R., Bowie, A.G., Horan, K.A. & Fitzgerald, K.A. Recognition of herpesviruses by the innate immune system. *Nat Rev Immunol* **11**, 143-154 (2011).

32. Schiffer, J.T. & Corey, L. Rapid host immune response and viral dynamics in herpes simplex virus-2 infection. *Nat Med* **19**, 280-290 (2013).

33. Mark, K.E. *et al.* Rapidly cleared episodes of herpes simplex virus reactivation in immunocompetent adults. *J Infect Dis* **198**, 1141-1149 (2008).

34. Schiffer, J.T. *et al.* Frequent release of low amounts of herpes simplex virus from neurons: results of a mathematical model. *Sci Transl Med* **1**, 7ra16 (2009).

35. Murphy, K. & Weaver, C. *Janeway's immunobiology*, 2017.

36. Koelle, D.M. & Corey, L. Herpes simplex: insights on pathogenesis and possible vaccines. *Annu Rev Med* **59**, 381-395 (2008).

37. Leoni, V., Gianni, T., Salvioli, S. & Campadelli-Fiume, G. Herpes simplex virus glycoproteins gH/gL and gB bind Toll-like receptor 2, and soluble gH/gL is sufficient to activate NF-kappaB. *J Virol* **86**, 6555-6562 (2012).

38. Conrady, C.D., Zheng, M., Fitzgerald, K.A., Liu, C. & Carr, D.J. Resistance to HSV-1 infection in the epithelium resides with the novel innate sensor, IFI-16. *Mucosal Immunol* **5**, 173-183 (2012).

39. Sun, L., Wu, J., Du, F., Chen, X. & Chen, Z.J. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science* **339**, 786-791 (2013).

40. Ma, Y. & He, B. Recognition of herpes simplex viruses: toll-like receptors and beyond. *J Mol Biol* **426**, 1133-1147 (2014).

41. Horan, K.A. *et al.* Proteasomal degradation of herpes simplex virus capsids in macrophages releases DNA to the cytosol for recognition by DNA sensors. *J Immunol* **190**, 2311-2319 (2013).

42. Rasmussen, S.B. *et al.* Herpes simplex virus infection is sensed by both Toll-like receptors and retinoic acid-inducible gene- like receptors, which synergize to induce type I interferon production. *J Gen Virol* **90**, 74-78 (2009).

43. Melchjorsen, J. *et al.* Early innate recognition of herpes simplex virus in human primary macrophages is mediated via the MDA5/MAVS-dependent and MDA5/MAVS/RNA polymerase III-independent pathways. *J Virol* **84**, 11350-11358 (2010).

44. Iversen, M.B. *et al.* An innate antiviral pathway acting before interferons at epithelial surfaces. *Nat Immunol* **17**, 150-158 (2016).

45. Lund, J., Sato, A., Akira, S., Medzhitov, R. & Iwasaki, A. Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. *J Exp Med* **198**, 513-520 (2003).

46. Honda, K. *et al.* IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature* **434**, 772-777 (2005).

47. Yamamoto, M. *et al.* Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* **301**, 640-643 (2003).

48. Zhang, S.Y. *et al.* TLR3 deficiency in patients with herpes simplex encephalitis. *Science* **317**, 1522-1527 (2007).

49. Reinert, L.S. *et al.* TLR3 deficiency renders astrocytes permissive to herpes simplex virus infection and facilitates establishment of CNS infection in mice. *J Clin Invest* **122**, 1368-1376 (2012).

50. Teijaro, J.R. Type I interferons in viral control and immune regulation. *Curr Opin Virol* **16**, 31-40 (2016).

51. Gill, N., Deacon, P.M., Lichty, B., Mossman, K.L. & Ashkar, A.A. Induction of innate immunity against herpes simplex virus type 2 infection via local delivery of Toll-like receptor ligands correlates with beta interferon production. *J Virol* **80**, 9943-9950 (2006).

52. Sato, M. *et al.* Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. *Immunity* **13**, 539-548 (2000).

53. Lund, J.M., Linehan, M.M., Iijima, N. & Iwasaki, A. Cutting Edge: Plasmacytoid dendritic cells provide innate immune protection against mucosal viral infection in situ. *J Immunol* **177**, 7510-7514 (2006).

54. Swiecki, M., Wang, Y., Gilfillan, S. & Colonna, M. Plasmacytoid dendritic cells contribute to systemic but not local antiviral responses to HSV infections. *PLoS Pathog* **9**, e1003728 (2013).

55. Fung, K.Y. *et al.* Interferon-epsilon protects the female reproductive tract from viral and bacterial infection. *Science* **339**, 1088-1092 (2013).

56. Uyangaa, E. *et al.* Distinct Upstream Role of Type I IFN Signaling in Hematopoietic Stem Cell-Derived and Epithelial Resident Cells for Concerted Recruitment of Ly-6Chi Monocytes and NK Cells via CCL2-CCL3 Cascade. *PLoS Pathog* **11**, e1005256 (2015).

57. Thapa, M., Kuziel, W.A. & Carr, D.J. Susceptibility of CCR5-deficient mice to genital herpes simplex virus type 2 is linked to NK cell mobilization. *J Virol* **81**, 3704-3713 (2007).

58. Ashkar, A.A. & Rosenthal, K.L. Interleukin-15 and natural killer and NKT cells play a critical role in innate protection against genital herpes simplex virus type 2 infection. *J Virol* **77**, 10168-10171 (2003).

59. Hayakawa, Y., Huntington, N.D., Nutt, S.L. & Smyth, M.J. Functional subsets of mouse natural killer cells. *Immunol Rev* **214**, 47-55 (2006).

60. Iijima, N., Mattei, L.M. & Iwasaki, A. Recruited inflammatory monocytes stimulate antiviral Th1 immunity in infected tissue. *Proc Natl Acad Sci U S A* **108**, 284-289 (2011).

61. Croen, K.D. Evidence for antiviral effect of nitric oxide. Inhibition of herpes simplex virus type 1 replication. *J Clin Invest* **91**, 2446-2452 (1993).

62. Goldszmid, R.S. *et al.* NK cell-derived interferon-gamma orchestrates cellular dynamics and the differentiation of monocytes into dendritic cells at the site of infection. *Immunity* **36**, 1047-1059 (2012).

63. Martin-Fontecha, A. *et al.* Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. *Nat Immunol* **5**, 1260-1265 (2004).

64. Koelle, D.M. *et al.* Clearance of HSV-2 from recurrent genital lesions correlates with infiltration of HSV-specific cytotoxic T lymphocytes. *J Clin Invest* **101**, 1500-1508 (1998).

65. Parr, M.B. & Parr, E.L. Mucosal immunity to herpes simplex virus type 2 infection in the mouse vagina is impaired by in vivo depletion of T lymphocytes. *J Virol* **72**, 2677-2685 (1998).

66. Zhao, X. *et al.* Vaginal submucosal dendritic cells, but not Langerhans cells, induce protective Th1 responses to herpes simplex virus-2. *J Exp Med* **197**, 153-162 (2003).

67. Nakanishi, Y., Lu, B., Gerard, C. & Iwasaki, A. CD8(+) T lymphocyte mobilization to virus-infected tissue requires CD4(+) T-cell help. *Nature* **462**, 510-513 (2009).

68. Dobbs, M.E., Strasser, J.E., Chu, C.F., Chalk, C. & Milligan, G.N. Clearance of herpes simplex virus type 2 by CD8+ T cells requires gamma interferon and either perforin- or Fas-mediated cytolytic mechanisms. *J Virol* **79**, 14546-14554 (2005).

69. Lund, J.M., Hsing, L., Pham, T.T. & Rudensky, A.Y. Coordination of early protective immunity to viral infection by regulatory T cells. *Science* **320**, 1220-1224 (2008).

70. Fernandez, M.A. *et al.* T regulatory cells contribute to the attenuated primary CD8+ and CD4+ T cell responses to herpes simplex virus type 2 in neonatal mice. *J Immunol* **180**, 1556-1564 (2008).

71. Morrison, L.A., Zhu, L. & Thebeau, L.G. Vaccine-induced serum immunoglobin contributes to protection from herpes simplex virus type 2 genital infection in the presence of immune T cells. *J Virol* **75**, 1195-1204 (2001).

72. Mikloska, Z., Sanna, P.P. & Cunningham, A.L. Neutralizing antibodies inhibit axonal spread of herpes simplex virus type 1 to epidermal cells in vitro. *J Virol* **73**, 5934-5944 (1999).

73. Brown, Z.A. *et al.* Neonatal herpes simplex virus infection in relation to asymptomatic maternal infection at the time of labor. *N Engl J Med* **324**, 1247-1252 (1991).

74. Iijima, N. *et al.* Dendritic cells and B cells maximize mucosal Th1 memory response to herpes simplex virus. *J Exp Med* **205**, 3041-3052 (2008).

75. Iijima, N. & Iwasaki, A. T cell memory. A local macrophage chemokine network sustains protective tissue-resident memory CD4 T cells. *Science* **346**, 93-98 (2014).

76. Schenkel, J.M. *et al.* T cell memory. Resident memory CD8 T cells trigger protective innate and adaptive immune responses. *Science* **346**, 98-101 (2014).

77. Shin, H., Kumamoto, Y., Gopinath, S. & Iwasaki, A. CD301b+ dendritic cells stimulate tissue-resident memory CD8+ T cells to protect against genital HSV-2. *Nat Commun* **7**, 13346 (2016).

78. Chen, B., Lee, A.J., Chew, M.V. & Ashkar, A.A. NK cells require antigen-specific memory CD4+ T cells to mediate superior effector functions during HSV-2 recall responses in vitro. *J Leukoc Biol* **101**, 1045-1052 (2017).

79. Posavad, C.M. *et al.* Enrichment of herpes simplex virus type 2 (HSV-2) reactive mucosal T cells in the human female genital tract. *Mucosal Immunol* (2017).

80. Posavad, C.M. *et al.* Persistence of mucosal T-cell responses to herpes simplex virus type 2 in the female genital tract. *Mucosal Immunol* **8**, 115-126 (2015).

81. Zhu, J. *et al.* Virus-specific CD8+ T cells accumulate near sensory nerve endings in genital skin during subclinical HSV-2 reactivation. *J Exp Med* **204**, 595-603 (2007).

82. Peng, T. *et al.* An effector phenotype of CD8+ T cells at the junction epithelium during clinical quiescence of herpes simplex virus 2 infection. *J Virol* **86**, 10587-10596 (2012).

83. Shannon, B. *et al.* Impact of asymptomatic herpes simplex virus type 2 infection on mucosal homing and immune cell subsets in the blood and female genital tract. *J Immunol* **192**, 5074-5082 (2014).

84. Zhu, J. *et al.* Immune surveillance by CD8alphaalpha+ skin-resident T cells in human herpes virus infection. *Nature* **497**, 494-497 (2013).

85. Schreiber, G. & Piehler, J. The molecular basis for functional plasticity in type I interferon signaling. *Trends Immunol* **36**, 139-149 (2015).

86. Fitzgerald, K.A. *et al.* IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol* **4**, 491-496 (2003).

87. Honda, K., Takaoka, A. & Taniguchi, T. Type I interferon [corrected] gene induction by the interferon regulatory factor family of transcription factors. *Immunity* **25**, 349-360 (2006).

88. Tamura, T., Yanai, H., Savitsky, D. & Taniguchi, T. The IRF family transcription factors in immunity and oncogenesis. *Annu Rev Immunol* **26**, 535-584 (2008).

89. McNab, F., Mayer-Barber, K., Sher, A., Wack, A. & O'Garra, A. Type I interferons in infectious disease. *Nat Rev Immunol* **15**, 87-103 (2015).

90. Siegal, F.P. *et al.* The nature of the principal type 1 interferon-producing cells in human blood. *Science* **284**, 1835-1837 (1999).

91. Hoffmann, H.H., Schneider, W.M. & Rice, C.M. Interferons and viruses: an evolutionary arms race of molecular interactions. *Trends Immunol* **36**, 124-138 (2015).

92. Oh, J.E. *et al.* Dysbiosis-induced IL-33 contributes to impaired antiviral immunity in the genital mucosa. *Proc Natl Acad Sci U S A* **113**, E762-771 (2016).

93. Platanias, L.C. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol* **5**, 375-386 (2005).

94. Marchetti, M. *et al.* Stat-mediated signaling induced by type I and type II interferons (IFNs) is differentially controlled through lipid microdomain association and clathrin-dependent endocytosis of IFN receptors. *Mol Biol Cell* **17**, 2896-2909 (2006).

95. Ahmad, S., Alsayed, Y.M., Druker, B.J. & Platanias, L.C. The type I interferon receptor mediates tyrosine phosphorylation of the CrkL adaptor protein. *J Biol Chem* **272**, 29991-29994 (1997).

96. Uddin, S. *et al.* Activation of the p38 mitogen-activated protein kinase by type I interferons. *J Biol Chem* **274**, 30127-30131 (1999).

97. Uddin, S. *et al.* Interferon-alpha engages the insulin receptor substrate-1 to associate with the phosphatidylinositol 3'-kinase. *J Biol Chem* **270**, 15938-15941 (1995).

98. Platanias, L.C. & Sweet, M.E. Interferon alpha induces rapid tyrosine phosphorylation of the vav proto-oncogene product in hematopoietic cells. *J Biol Chem* **269**, 3143-3146 (1994).

99. Uddin, S. *et al.* The Rac1/p38 mitogen-activated protein kinase pathway is required for interferon alpha-dependent transcriptional activation but not serine phosphorylation of Stat proteins. *J Biol Chem* **275**, 27634-27640 (2000).

100. Piehler, J. & Schreiber, G. Mutational and structural analysis of the binding interface between type I interferons and their receptor Ifnar2. *J Mol Biol* **294**, 223-237 (1999).

101. Lavoie, T.B. *et al.* Binding and activity of all human alpha interferon subtypes. *Cytokine* **56**, 282-289 (2011).

102. Thomas, C. *et al.* Structural linkage between ligand discrimination and receptor activation by type I interferons. *Cell* **146**, 621-632 (2011).

103. Levin, D., Harari, D. & Schreiber, G. Stochastic receptor expression determines cell fate upon interferon treatment. *Mol Cell Biol* **31**, 3252-3266 (2011).

104. Levin, D. *et al.* Multifaceted activities of type I interferon are revealed by a receptor antagonist. *Sci Signal* **7**, ra50 (2014).

105. de Weerd, N.A. *et al.* Structural basis of a unique interferon-beta signaling axis mediated via the receptor IFNAR1. *Nat Immunol* **14**, 901-907 (2013).

106. Meurs, E. *et al.* Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. *Cell* **62**, 379-390 (1990).

107. Carr, D.J., Tomanek, L., Silverman, R.H., Campbell, I.L. & Williams, B.R. RNA-dependent protein kinase is required for alpha-1 interferon transgene-induced resistance to genital herpes simplex virus type 2. *J Virol* **79**, 9341-9345 (2005).

108. Mangan, N.E. & Fung, K.Y. Type I interferons in regulation of mucosal immunity. *Immunol Cell Biol* **90**, 510-519 (2012).

109. Carroll, S.S. *et al.* Cleavage of oligoribonucleotides by the 2',5'-oligoadenylate-dependent ribonuclease L. *J Biol Chem* **271**, 4988-4992 (1996).

110. Duerst, R.J. & Morrison, L.A. Herpes simplex virus type 2-mediated disease is reduced in mice lacking RNase L. *Virology* **360**, 322-328 (2007).

111. Zhou, A., Paranjape, J.M., Der, S.D., Williams, B.R. & Silverman, R.H. Interferon action in triply deficient mice reveals the existence of alternative antiviral pathways. *Virology* **258**, 435-440 (1999).

112. de Veer, M.J. *et al.* Functional classification of interferon-stimulated genes identified using microarrays. *J Leukoc Biol* **69**, 912-920 (2001).

113. Schoggins, J.W. *et al.* A diverse range of gene products are effectors of the type I interferon antiviral response. *Nature* **472**, 481-485 (2011).

114. James, C.M. *et al.* Differential activities of alpha/beta IFN subtypes against influenza virus in vivo and enhancement of specific immune responses in DNA vaccinated mice expressing haemagglutinin and nucleoprotein. *Vaccine* **25**, 1856-1867 (2007).

115. Garcin, G. *et al.* Differential activity of type I interferon subtypes for dendritic cell differentiation. *PLoS One* **8**, e58465 (2013).

116. Jacquelin, B. *et al.* Nonpathogenic SIV infection of African green monkeys induces a strong but rapidly controlled type I IFN response. *J Clin Invest* **119**, 3544-3555 (2009).

117. Chen, L. *et al.* Hepatic gene expression discriminates responders and nonresponders in treatment of chronic hepatitis C viral infection. *Gastroenterology* **128**, 1437-1444 (2005).

118. Teijaro, J.R. *et al.* Persistent LCMV infection is controlled by blockade of type I interferon signaling. *Science* **340**, 207-211 (2013).

119. Arimori, Y. *et al.* Type I interferon limits influenza virus-induced acute lung injury by regulation of excessive inflammation in mice. *Antiviral Res* **99**, 230-237 (2013).

120. Ng, C.T. *et al.* Blockade of interferon Beta, but not interferon alpha, signaling controls persistent viral infection. *Cell Host Microbe* **17**, 653-661 (2015).

121. Sandler, N.G. *et al.* Type I interferon responses in rhesus macaques prevent SIV infection and slow disease progression. *Nature* **511**, 601-605 (2014).

122. Peng, T. *et al.* Evasion of the mucosal innate immune system by herpes simplex virus type 2. *J Virol* **83**, 12559-12568 (2009).

123. Lee-Kirsch, M.A. The Type I Interferonopathies. *Annu Rev Med* **68**, 297-315 (2017).

124. Furie, R. *et al.* Anifrolumab, an Anti-Interferon-alpha Receptor Monoclonal Antibody, in Moderate-to-Severe Systemic Lupus Erythematosus. *Arthritis Rheumatol* **69**, 376-386 (2017).

125. Welsh, R.M., Bahl, K., Marshall, H.D. & Urban, S.L. Type 1 interferons and antiviral CD8 T-cell responses. *PLoS Pathog* **8**, e1002352 (2012).

126. Marshall, H.D., Urban, S.L. & Welsh, R.M. Virus-induced transient immune suppression and the inhibition of T cell proliferation by type I interferon. *J Virol* **85**, 5929-5939 (2011).

127. McRae, B.L., Semnani, R.T., Hayes, M.P. & van Seventer, G.A. Type I IFNs inhibit human dendritic cell IL-12 production and Th1 cell development. *J Immunol* **160**, 4298-4304 (1998).

128. Nagai, T. *et al.* Timing of IFN-beta exposure during human dendritic cell maturation and naive Th cell stimulation has contrasting effects on Th1 subset generation: a role for IFN-beta-mediated regulation of IL-12 family cytokines and IL-18 in naive Th cell differentiation. *J Immunol* **171**, 5233-5243 (2003).

129. Kurche, J.S., Haluszczak, C., McWilliams, J.A., Sanchez, P.J. & Kedl, R.M. Type I IFN-dependent T cell activation is mediated by IFN-dependent dendritic cell OX40 ligand expression and is independent of T cell IFNR expression. *J Immunol* **188**, 585-593 (2012).

130. Simmons, D.P. *et al.* Type I IFN drives a distinctive dendritic cell maturation phenotype that allows continued class II MHC synthesis and antigen processing. *J Immunol* **188**, 3116-3126 (2012).

131. Xu, H.C. *et al.* Type I interferon protects antiviral CD8+ T cells from NK cell cytotoxicity. *Immunity* **40**, 949-960 (2014).

132. Kolumam, G.A., Thomas, S., Thompson, L.J., Sprent, J. & Murali-Krishna, K. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. *J Exp Med* **202**, 637-650 (2005).

133. Lin, Q., Dong, C. & Cooper, M.D. Impairment of T and B cell development by treatment with a type I interferon. *J Exp Med* **187**, 79-87 (1998).

134. Purtha, W.E., Chachu, K.A., Virgin, H.W.t. & Diamond, M.S. Early B-cell activation after West Nile virus infection requires alpha/beta interferon but not antigen receptor signaling. *J Virol* **82**, 10964-10974 (2008).

135. Gill, N., Chenoweth, M.J., Verdu, E.F. & Ashkar, A.A. NK cells require type I IFN receptor for antiviral responses during genital HSV-2 infection. *Cell Immunol* **269**, 29-37 (2011).

136. Miyagi, T. *et al.* High basal STAT4 balanced by STAT1 induction to control type 1 interferon effects in natural killer cells. *J Exp Med* **204**, 2383-2396 (2007).

137. Longhi, M.P. *et al.* Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. *J Exp Med* **206**, 1589-1602 (2009).

138. Rayamajhi, M., Humann, J., Penheiter, K., Andreasen, K. & Lenz, L.L. Induction of IFN-alphabeta enables Listeria monocytogenes to suppress macrophage activation by IFN-gamma. *J Exp Med* **207**, 327-337 (2010).

139. McNab, F.W. *et al.* Type I IFN induces IL-10 production in an IL-27-independent manner and blocks responsiveness to IFN-gamma for production of IL-12 and bacterial killing in Mycobacterium tuberculosis-infected macrophages. *J Immunol* **193**, 3600-3612 (2014).

140. Teles, R.M. *et al.* Type I interferon suppresses type II interferon-triggered human anti-mycobacterial responses. *Science* **339**, 1448-1453 (2013).

141. Svensson, A., Bellner, L., Magnusson, M. & Eriksson, K. Role of IFN-alpha/beta signaling in the prevention of genital herpes virus type 2 infection. *J Reprod Immunol* **74**, 114-123 (2007).

142. Chapgier, A. *et al.* A partial form of recessive STAT1 deficiency in humans. *J Clin Invest* **119**, 1502-1514 (2009).

143. Dupuis, S. *et al.* Impaired response to interferon-alpha/beta and lethal viral disease in human STAT1 deficiency. *Nat Genet* **33**, 388-391 (2003).

144. Casrouge, A. *et al.* Herpes simplex virus encephalitis in human UNC-93B deficiency. *Science* **314**, 308-312 (2006).

145. Vivier, E., Tomasello, E., Baratin, M., Walzer, T. & Ugolini, S. Functions of natural killer cells. *Nat Immunol* **9**, 503-510 (2008).

146. Kiessling, R., Klein, E., Pross, H. & Wigzell, H. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur J Immunol* **5**, 117-121 (1975).

147. Spits, H., Bernink, J.H. & Lanier, L. NK cells and type 1 innate lymphoid cells: partners in host defense. *Nat Immunol* **17**, 758-764 (2016).

148. Pegram, H.J., Andrews, D.M., Smyth, M.J., Darcy, P.K. & Kershaw, M.H. Activating and inhibitory receptors of natural killer cells. *Immunol Cell Biol* **89**, 216-224 (2011).

149. Freud, A.G. *et al.* A human CD34(+) subset resides in lymph nodes and differentiates into CD56bright natural killer cells. *Immunity* **22**, 295-304 (2005).

150. Freud, A.G., Yu, J. & Caligiuri, M.A. Human natural killer cell development in secondary lymphoid tissues. *Semin Immunol* **26**, 132-137 (2014).

151. Goh, W. & Huntington, N.D. Regulation of Murine Natural Killer Cell Development. *Front Immunol* **8**, 130 (2017).

152. Rosmaraki, E.E. *et al.* Identification of committed NK cell progenitors in adult murine bone marrow. *Eur J Immunol* **31**, 1900-1909 (2001).

153. Lodolce, J.P. *et al.* IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity* **9**, 669-676 (1998).

154. DiSanto, J.P., Muller, W., Guy-Grand, D., Fischer, A. & Rajewsky, K. Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor gamma chain. *Proc Natl Acad Sci U S A* **92**, 377-381 (1995).

155. Chiossone, L. *et al.* Maturation of mouse NK cells is a 4-stage developmental program. *Blood* **113**, 5488-5496 (2009).

156. Hayakawa, Y. & Smyth, M.J. CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity. *J Immunol* **176**, 1517-1524 (2006).

157. Huntington, N.D. *et al.* NK cell maturation and peripheral homeostasis is associated with KLRG1 up-regulation. *J Immunol* **178**, 4764-4770 (2007).

158. Gordon, S.M. *et al.* The transcription factors T-bet and Eomes control key checkpoints of natural killer cell maturation. *Immunity* **36**, 55-67 (2012).

159. Melsen, J.E., Lugthart, G., Lankester, A.C. & Schilham, M.W. Human Circulating and Tissue-Resident CD56(bright) Natural Killer Cell Populations. *Front Immunol* **7**, 262 (2016).

160. Romagnani, C. *et al.* CD56brightCD16- killer Ig-like receptor- NK cells display longer telomeres and acquire features of CD56dim NK cells upon activation. *J Immunol* **178**, 4947-4955 (2007).

161. Nguyen, S. *et al.* NK-cell reconstitution after haploidentical hematopoietic stem-cell transplantations: immaturity of NK cells and inhibitory effect of NKG2A override GvL effect. *Blood* **105**, 4135-4142 (2005).

162. Wu, C. *et al.* Clonal tracking of rhesus macaque hematopoiesis highlights a distinct lineage origin for natural killer cells. *Cell Stem Cell* **14**, 486-499 (2014).

163. Strauss-Albee, D.M. *et al.* Human NK cell repertoire diversity reflects immune experience and correlates with viral susceptibility. *Sci Transl Med* **7**, 297ra115 (2015).

164. Gregoire, C. *et al.* The trafficking of natural killer cells. *Immunol Rev* **220**, 169-182 (2007).

165. Lugthart, G. *et al.* Human Lymphoid Tissues Harbor a Distinct CD69+CXCR6+ NK Cell Population. *J Immunol* **197**, 78-84 (2016).

166. Tanimine, N. *et al.* Functional Behavior of NKp46-Positive Intrahepatic Natural Killer Cells Against Hepatitis C Virus Reinfection After Liver Transplantation. *Transplantation* **100**, 355-364 (2016).

167. Mayol, K., Biajoux, V., Marvel, J., Balabanian, K. & Walzer, T. Sequential desensitization of CXCR4 and S1P5 controls natural killer cell trafficking. *Blood* **118**, 4863-4871 (2011).

168. Walzer, T. *et al.* Natural killer cell trafficking in vivo requires a dedicated sphingosine 1-phosphate receptor. *Nat Immunol* **8**, 1337-1344 (2007).

169. Chen, S., Kawashima, H., Lowe, J.B., Lanier, L.L. & Fukuda, M. Suppression of tumor formation in lymph nodes by L-selectin-mediated natural killer cell recruitment. *J Exp Med* **202**, 1679-1689 (2005).

170. Campbell, J.J. *et al.* Unique subpopulations of CD56+ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. *J Immunol* **166**, 6477-6482 (2001).

171. Manaster, I. *et al.* Endometrial NK cells are special immature cells that await pregnancy. *J Immunol* **181**, 1869-1876 (2008).

172. Koopman, L.A. *et al.* Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential. *J Exp Med* **198**, 1201-1212 (2003).

173. Vacca, P. *et al.* Analysis of natural killer cells isolated from human decidua: Evidence that 2B4 (CD244) functions as an inhibitory receptor and blocks NK-cell function. *Blood* **108**, 4078-4085 (2006).

174. Ashkar, A.A., Di Santo, J.P. & Croy, B.A. Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. *J Exp Med* **192**, 259-270 (2000).

175. Mselle, T.F. *et al.* Unique characteristics of NK cells throughout the human female reproductive tract. *Clin Immunol* **124**, 69-76 (2007).

176. Reeves, R.K. *et al.* CD16- natural killer cells: enrichment in mucosal and secondary lymphoid tissues and altered function during chronic SIV infection. *Blood* **115**, 4439-4446 (2010).

177. Tu, M.M., Mahmoud, A.B. & Makrigiannis, A.P. Licensed and Unlicensed NK Cells: Differential Roles in Cancer and Viral Control. *Front Immunol* **7**, 166 (2016).

178. Fernandez, N.C. *et al.* A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. *Blood* **105**, 4416-4423 (2005).

179. Elliott, J.M., Wahle, J.A. & Yokoyama, W.M. MHC class I-deficient natural killer cells acquire a licensed phenotype after transfer into an MHC class I-sufficient environment. *J Exp Med* **207**, 2073-2079 (2010).

180. Long, E.O., Kim, H.S., Liu, D., Peterson, M.E. & Rajagopalan, S. Controlling natural killer cell responses: integration of signals for activation and inhibition. *Annu Rev Immunol* **31**, 227-258 (2013).

181. Guillerey, C., Huntington, N.D. & Smyth, M.J. Targeting natural killer cells in cancer immunotherapy. *Nat Immunol* **17**, 1025-1036 (2016).

182. He, S. *et al.* MicroRNAs activate natural killer cells through Toll-like receptor signaling. *Blood* **121**, 4663-4671 (2013).

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

184. Ferlazzo, G. & Munz, C. Dendritic cell interactions with NK cells from different tissues. *J Clin Immunol* **29**, 265-273 (2009).

185. Altfeld, M., Fadda, L., Frleta, D. & Bhardwaj, N. DCs and NK cells: critical effectors in the immune response to HIV-1. *Nat Rev Immunol* **11**, 176-186 (2011).

186. Rasmussen, S.B. *et al.* Type I interferon production during herpes simplex virus infection is controlled by cell-type-specific viral recognition through Toll-like receptor 9, the mitochondrial antiviral signaling protein pathway, and novel recognition systems. *J Virol* **81**, 13315-13324 (2007).

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

188. Baranek, T. *et al.* Differential responses of immune cells to type I interferon contribute to host resistance to viral infection. *Cell Host Microbe* **12**, 571-584 (2012).

189. Brandstadter, J.D., Huang, X. & Yang, Y. NK cell-extrinsic IL-18 signaling is required for efficient NK-cell activation by vaccinia virus. *Eur J Immunol* **44**, 2659-2666 (2014).

190. Soudja, S.M., Ruiz, A.L., Marie, J.C. & Lauvau, G. Inflammatory monocytes activate memory CD8(+) T and innate NK lymphocytes independent of cognate antigen during microbial pathogen invasion. *Immunity* **37**, 549-562 (2012).

191. Gordon, S. & Taylor, P.R. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* **5**, 953-964 (2005).

192. Geissmann, F., Jung, S. & Littman, D.R. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* **19**, 71-82 (2003).

193. Serti, E. *et al.* Monocytes activate natural killer cells via inflammasome-induced interleukin 18 in response to hepatitis C virus replication. *Gastroenterology* **147**, 209-220 e203 (2014).

194. Zhang, S., Saha, B., Kodys, K. & Szabo, G. IFN-gamma production by human natural killer cells in response to HCV-infected hepatoma cells is dependent on accessory cells. *J Hepatol* **59**, 442-449 (2013).

195. Coombes, J.L., Han, S.J., van Rooijen, N., Raulet, D.H. & Robey, E.A. Infection-induced regulation of natural killer cells by macrophages and collagen at the lymph node subcapsular sinus. *Cell Rep* **2**, 124-135 (2012).

196. Michel, T., Hentges, F. & Zimmer, J. Consequences of the crosstalk between monocytes/macrophages and natural killer cells. *Front Immunol* **3**, 403 (2012).

197. Hou, X., Zhou, R., Wei, H., Sun, R. & Tian, Z. NKG2D-retinoic acid early inducible-1 recognition between natural killer cells and Kupffer cells in a novel murine natural killer cell-dependent fulminant hepatitis. *Hepatology* **49**, 940-949 (2009).

198. Zhou, Z., Zhang, C., Zhang, J. & Tian, Z. Macrophages help NK cells to attack tumor cells by stimulatory NKG2D ligand but protect themselves from NK killing by inhibitory ligand Qa-1. *PLoS One* **7**, e36928 (2012).

199. Vankayalapati, R. *et al.* Role of NK cell-activating receptors and their ligands in the lysis of mononuclear phagocytes infected with an intracellular bacterium. *J Immunol* **175**, 4611-4617 (2005).

200. Tu, Z. *et al.* TLR-dependent cross talk between human Kupffer cells and NK cells. *J Exp Med* **205**, 233-244 (2008).

201. Costantini, C. & Cassatella, M.A. The defensive alliance between neutrophils and NK cells as a novel arm of innate immunity. *J Leukoc Biol* **89**, 221-233 (2011).

202. Amulic, B., Cazalet, C., Hayes, G.L., Metzler, K.D. & Zychlinsky, A. Neutrophil function: from mechanisms to disease. *Annu Rev Immunol* **30**, 459-489 (2012).

203. Sporri, R., Joller, N., Hilbi, H. & Oxenius, A. A Novel Role for Neutrophils As Critical Activators of NK Cells. *The Journal of Immunology* **181**, 7121-7130 (2008).

204. Emoto, M. *et al.* Highly biased type 1 immune responses in mice deficient in LFA-1 in Listeria monocytogenes infection are caused by elevated IL-12 production by granulocytes. *J Immunol* **171**, 3970-3976 (2003).

205. Jaeger, B.N. *et al.* Neutrophil depletion impairs natural killer cell maturation, function, and homeostasis. *J Exp Med* **209**, 565-580 (2012).

206. Bennouna, S., Bliss, S.K., Curiel, T.J. & Denkers, E.Y. Cross-talk in the innate immune system: neutrophils instruct recruitment and activation of dendritic cells during microbial infection. *J Immunol* **171**, 6052-6058 (2003).

207. Liang, S., Wei, H., Sun, R. & Tian, Z. IFNalpha regulates NK cell cytotoxicity through STAT1 pathway. *Cytokine* **23**, 190-199 (2003).

208. Choi, P.J. & Mitchison, T.J. Imaging burst kinetics and spatial coordination during serial killing by single natural killer cells. *Proc Natl Acad Sci U S A* **110**, 6488-6493 (2013).

209. Thierfelder, W.E. *et al.* Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* **382**, 171-174 (1996).

210. Pallmer, K. & Oxenius, A. Recognition and Regulation of T Cells by NK Cells. *Front Immunol* **7**, 251 (2016).

211. Piccioli, D., Sbrana, S., Melandri, E. & Valiante, N.M. Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells. *J Exp Med* **195**, 335-341 (2002).

212. Andrews, D.M. *et al.* Innate immunity defines the capacity of antiviral T cells to limit persistent infection. *J Exp Med* **207**, 1333-1343 (2010).

213. Rabinovich, B.A. *et al.* Activated, but not resting, T cells can be recognized and killed by syngeneic NK cells. *J Immunol* **170**, 3572-3576 (2003).

214. Ghiringhelli, F. *et al.* CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. *J Exp Med* **202**, 1075-1085 (2005).

215. Mandaric, S. *et al.* IL-10 suppression of NK/DC crosstalk leads to poor priming of MCMV-specific CD4 T cells and prolonged MCMV persistence. *PLoS Pathog* **8**, e1002846 (2012).

216. Huang, Y. *et al.* MicroRNA regulation of STAT4 protein expression: rapid and sensitive modulation of IL-12 signaling in human natural killer cells. *Blood* **118**, 6793-6802 (2011).

217. Beaulieu, A.M. *et al.* MicroRNA function in NK-cell biology. *Immunol Rev* **253**, 40-52 (2013).

218. Kerdiles, Y., Ugolini, S. & Vivier, E. T cell regulation of natural killer cells. *J Exp Med* **210**, 1065-1068 (2013).

219. Smyth, M.J. *et al.* CD4+CD25+ T regulatory cells suppress NK cell-mediated immunotherapy of cancer. *J Immunol* **176**, 1582-1587 (2006).

220. Sitrin, J., Ring, A., Garcia, K.C., Benoist, C. & Mathis, D. Regulatory T cells control NK cells in an insulitic lesion by depriving them of IL-2. *J Exp Med* **210**, 1153-1165 (2013).

221. Ostrand-Rosenberg, S. & Sinha, P. Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol* **182**, 4499-4506 (2009).

222. Mundy-Bosse, B.L. *et al.* Myeloid-derived suppressor cell inhibition of the IFN response in tumor-bearing mice. *Cancer Res* **71**, 5101-5110 (2011).

223. Fortin, C., Huang, X. & Yang, Y. NK cell response to vaccinia virus is regulated by myeloid-derived suppressor cells. *J Immunol* **189**, 1843-1849 (2012).

224. Zhu, J., Huang, X. & Yang, Y. Myeloid-derived suppressor cells regulate natural killer cell response to adenovirus-mediated gene transfer. *J Virol* **86**, 13689-13696 (2012).

225. Ornstein, B.W., Hill, E.B., Geurs, T.L. & French, A.R. Natural killer cell functional defects in pediatric patients with severe and recurrent herpesvirus infections. *J Infect Dis* **207**, 458-468 (2013).

226. Kwant-Mitchell, A., Ashkar, A.A. & Rosenthal, K.L. Mucosal innate and adaptive immune responses against herpes simplex virus type 2 in a humanized mouse model. *J Virol* **83**, 10664-10676 (2009).

227. Kim, M. *et al.* Herpes simplex virus antigens directly activate NK cells via TLR2, thus facilitating their presentation to CD4 T lymphocytes. *J Immunol* **188**, 4158-4170 (2012).

228. Mizutani, T. *et al.* Conditional IFNAR1 ablation reveals distinct requirements of Type I IFN signaling for NK cell maturation and tumor surveillance. *Oncoimmunology* **1**, 1027-1037 (2012).

229. Zhu, J., Huang, X. & Yang, Y. A critical role for type I IFN-dependent NK cell activation in innate immune elimination of adenoviral vectors in vivo. *Mol Ther* **16**, 1300-1307 (2008).

230. Martinez, J., Huang, X. & 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**, 1592-1597 (2008).

231. Swann, J.B. *et al.* Type I IFN contributes to NK cell homeostasis, activation, and antitumor function. *J Immunol* **178**, 7540-7549 (2007).

232. Madera, S. *et al.* Type I IFN promotes NK cell expansion during viral infection by protecting NK cells against fratricide. *J Exp Med* **213**, 225-233 (2016).

233. Mack, E.A., Kallal, L.E., Demers, D.A. & Biron, C.A. Type 1 interferon induction of natural killer cell gamma interferon production for defense during lymphocytic choriomeningitis virus infection. *MBio* **2** (2011).

234. Gibbert, K. *et al.* Interferon-alpha subtype 11 activates NK cells and enables control of retroviral infection. *PLoS Pathog* **8**, e1002868 (2012).

235. Beuneu, H. *et al.* Cutting Edge: A dual role for type I IFNs during polyinosinic-polycytidylic acid-induced NK cell activation. *J Immunol* **187**, 2084-2088 (2011).

236. Schreiner, B. *et al.* Interferon-beta enhances monocyte and dendritic cell expression of B7-H1 (PD-L1), a strong inhibitor of autologous T-cell activation: relevance for the immune modulatory effect in multiple sclerosis. *J Neuroimmunol* **155**, 172-182 (2004).

237. Marshall, J.D., Heeke, D.S., Abbate, C., Yee, P. & Van Nest, G. Induction of interferon-gamma from natural killer cells by immunostimulatory CpG DNA is mediated through plasmacytoid-dendritic-cell-produced interferon-alpha and tumour necrosis factor-alpha. *Immunology* **117**, 38-46 (2006).

238. Pontiroli, F. *et al.* The timing of IFNbeta production affects early innate responses to Listeria monocytogenes and determines the overall outcome of lethal infection. *PLoS One* **7**, e43455 (2012).

239. Ahlenstiel, G. *et al.* Early changes in natural killer cell function indicate virologic response to interferon therapy for hepatitis C. *Gastroenterology* **141**, 1231-1239, 1239 e1231-1232 (2011).

240. Gu, J. *et al.* Multiple organ infection and the pathogenesis of SARS. *J Exp Med* **202**, 415-424 (2005).

241. Peiris, J.S., Hui, K.P. & Yen, H.L. Host response to influenza virus: protection versus immunopathology. *Curr Opin Immunol* **22**, 475-481 (2010).

242. de Jong, M.D. *et al.* Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nat Med* **12**, 1203-1207 (2006).

243. To, K.K. *et al.* Delayed clearance of viral load and marked cytokine activation in severe cases of pandemic H1N1 2009 influenza virus infection. *Clin Infect Dis* **50**, 850-859 (2010).

244. Channappanavar, R. & Perlman, S. Pathogenic human coronavirus infections: causes and consequences of cytokine storm and immunopathology. *Semin Immunopathol* (2017).

245. Peiris, J.S. *et al.* Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. *Lancet* **361**, 1767-1772 (2003).

246. Zhang, Y. *et al.* Analysis of serum cytokines in patients with severe acute respiratory syndrome. *Infect Immun* **72**, 4410-4415 (2004).

247. Chien, J.Y., Hsueh, P.R., Cheng, W.C., Yu, C.J. & Yang, P.C. Temporal changes in cytokine/chemokine profiles and pulmonary involvement in severe acute respiratory syndrome. *Respirology* **11**, 715-722 (2006).

248. Nicholls, J.M. *et al.* Lung pathology of fatal severe acute respiratory syndrome. *Lancet* **361**, 1773-1778 (2003).

249. Channappanavar, R. *et al.* Dysregulated Type I Interferon and Inflammatory Monocyte-Macrophage Responses Cause Lethal Pneumonia in SARS-CoV-Infected Mice. *Cell Host Microbe* **19**, 181-193 (2016).

250. Falzarano, D. *et al.* Treatment with interferon-alpha2b and ribavirin improves outcome in MERS-CoV-infected rhesus macaques. *Nat Med* **19**, 1313-1317 (2013).

251. Screaton, G., Mongkolsapaya, J., Yacoub, S. & Roberts, C. New insights into the immunopathology and control of dengue virus infection. *Nat Rev Immunol* **15**, 745-759 (2015).

252. Zampieri, C.A., Sullivan, N.J. & Nabel, G.J. Immunopathology of highly virulent pathogens: insights from Ebola virus. *Nat Immunol* **8**, 1159-1164 (2007).

253. Geisbert, T.W. *et al.* Pathogenesis of Ebola hemorrhagic fever in primate models: evidence that hemorrhage is not a direct effect of virus-induced cytolysis of endothelial cells. *Am J Pathol* **163**, 2371-2382 (2003).

254. Geisbert, T.W. *et al.* Pathogenesis of Ebola hemorrhagic fever in cynomolgus macaques: evidence that dendritic cells are early and sustained targets of infection. *Am J Pathol* **163**, 2347-2370 (2003).

255. Libraty, D.H. *et al.* Differing influences of virus burden and immune activation on disease severity in secondary dengue-3 virus infections. *J Infect Dis* **185**, 1213-1221 (2002).

256. Green, S. *et al.* Early immune activation in acute dengue illness is related to development of plasma leakage and disease severity. *J Infect Dis* **179**, 755-762 (1999).

257. Zhao, J. *et al.* Pathological functions of interleukin-22 in chronic liver inflammation and fibrosis with hepatitis B virus infection by promoting T helper 17 cell recruitment. *Hepatology* **59**, 1331-1342 (2014).

258. Thimme, R. *et al.* CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. *J Virol* **77**, 68-76 (2003).

259. Shin, E.C., Sung, P.S. & Park, S.H. Immune responses and immunopathology in acute and chronic viral hepatitis. *Nat Rev Immunol* **16**, 509-523 (2016).

260. Niemialtowski, M.G. & Rouse, B.T. Predominance of Th1 cells in ocular tissues during herpetic stromal keratitis. *J Immunol* **149**, 3035-3039 (1992).

261. Xia, L. *et al.* Interleukin-17 enhanced immunoinflammatory lesions in a mouse model of recurrent herpetic keratitis. *Microbes Infect* **15**, 126-139 (2013).

262. Popovic, B. *et al.* IL-33/ST2 pathway drives regulatory T cell dependent suppression of liver damage upon cytomegalovirus infection. *PLoS Pathog* **13**, e1006345 (2017).

263. Kuklin, N.A., Daheshia, M., Chun, S. & Rouse, B.T. Immunomodulation by mucosal gene transfer using TGF-beta DNA. *J Clin Invest* **102**, 438-444 (1998).

264. Sarangi, P.P., Sehrawat, S., Suvas, S. & Rouse, B.T. IL-10 and natural regulatory T cells: two independent anti-inflammatory mechanisms in herpes simplex virus-induced ocular immunopathology. *J Immunol* **180**, 6297-6306 (2008).

265. McNally, B., Ye, F., Willette, M. & Flano, E. Local blockade of epithelial PDL-1 in the airways enhances T cell function and viral clearance during influenza virus infection. *J Virol* **87**, 12916-12924 (2013).

266. Lin, K.L., Suzuki, Y., Nakano, H., Ramsburg, E. & Gunn, M.D. CCR2+ monocyte-derived dendritic cells and exudate macrophages produce influenza-induced pulmonary immune pathology and mortality. *J Immunol* **180**, 2562-2572 (2008).

267. Herold, S. *et al.* Lung epithelial apoptosis in influenza virus pneumonia: the role of macrophage-expressed TNF-related apoptosis-inducing ligand. *J Exp Med* **205**, 3065-3077 (2008).

268. Narasaraju, T. *et al.* Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis. *Am J Pathol* **179**, 199-210 (2011).

269. Li, F., Zhu, H., Sun, R., Wei, H. & Tian, Z. Natural killer cells are involved in acute lung immune injury caused by respiratory syncytial virus infection. *J Virol* **86**, 2251-2258 (2012).

270. Abdul-Careem, M.F. *et al.* Critical role of natural killer cells in lung immunopathology during influenza infection in mice. *J Infect Dis* **206**, 167-177 (2012).

271. Kubo, M. Innate and adaptive type 2 immunity in lung allergic inflammation. *Immunol Rev* **278**, 162-172 (2017).

272. Sigurs, N., Bjarnason, R., Sigurbergsson, F. & Kjellman, B. Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. *Am J Respir Crit Care Med* **161**, 1501-1507 (2000).

273. Sigurs, N. *et al.* Asthma and allergy patterns over 18 years after severe RSV bronchiolitis in the first year of life. *Thorax* **65**, 1045-1052 (2010).

274. Saravia, J. *et al.* Respiratory Syncytial Virus Disease Is Mediated by Age-Variable IL-33. *PLoS Pathog* **11**, e1005217 (2015).

275. Stier, M.T. *et al.* Respiratory syncytial virus infection activates IL-13-producing group 2 innate lymphoid cells through thymic stromal lymphopoietin. *J Allergy Clin Immunol* **138**, 814-824 e811 (2016).

276. Jackson, D.J. *et al.* IL-33-dependent type 2 inflammation during rhinovirus-induced asthma exacerbations in vivo. *Am J Respir Crit Care Med* **190**, 1373-1382 (2014).

277. Davidson, S., Crotta, S., McCabe, T.M. & Wack, A. Pathogenic potential of interferon alphabeta in acute influenza infection. *Nat Commun* **5**, 3864 (2014).

278. Hogner, K. *et al.* Macrophage-expressed IFN-beta contributes to apoptotic alveolar epithelial cell injury in severe influenza virus pneumonia. *PLoS Pathog* **9**, e1003188 (2013).

279. Duerr, C.U. *et al.* Type I interferon restricts type 2 immunopathology through the regulation of group 2 innate lymphoid cells. *Nat Immunol* **17**, 65-75 (2016).

280. Marr, N. *et al.* Attenuation of respiratory syncytial virus-induced and RIG-I-dependent type I IFN responses in human neonates and very young children. *J Immunol* **192**, 948-957 (2014).

281. Cormier, S.A. *et al.* Limited type I interferons and plasmacytoid dendritic cells during neonatal respiratory syncytial virus infection permit immunopathogenesis upon reinfection. *J Virol* **88**, 9350-9360 (2014).

282. Makris, S., Paulsen, M. & Johansson, C. Type I Interferons as Regulators of Lung Inflammation. *Front Immunol* **8**, 259 (2017).

283. Johnson, T.R. *et al.* Role for innate IFNs in determining respiratory syncytial virus immunopathology. *J Immunol* **174**, 7234-7241 (2005).

284. Durbin, J.E. *et al.* The role of IFN in respiratory syncytial virus pathogenesis. *J Immunol* **168**, 2944-2952 (2002).

285. Stifter, S.A. *et al.* Functional Interplay between Type I and II Interferons Is Essential to Limit Influenza A Virus-Induced Tissue Inflammation. *PLoS Pathog* **12**, e1005378 (2016).

286. Liu, F.D. *et al.* Timed action of IL-27 protects from immunopathology while preserving defense in influenza. *PLoS Pathog* **10**, e1004110 (2014).

287. McHedlidze, T. *et al.* IL-27 suppresses type 2 immune responses in vivo via direct effects on group 2 innate lymphoid cells. *Mucosal Immunol* **9**, 1384-1394 (2016).

288. Lee, A.J. *et al.* Inflammatory monocytes require type I interferon receptor signaling to activate NK cells via IL-18 during a mucosal viral infection. *J Exp Med* **214**, 1153-1167 (2017).

289. Moro, K. *et al.* Interferon and IL-27 antagonize the function of group 2 innate lymphoid cells and type 2 innate immune responses. *Nat Immunol* **17**, 76-86 (2016).

290. Sheehan, K.C., Lazear, H.M., Diamond, M.S. & Schreiber, R.D. Selective Blockade of Interferon-alpha and -beta Reveals Their Non-Redundant Functions in a Mouse Model of West Nile Virus Infection. *PLoS One* **10**, e0128636 (2015).

291. Jaitin, D.A. *et al.* Inquiring into the differential action of interferons (IFNs): an IFN-alpha2 mutant with enhanced affinity to IFNAR1 is functionally similar to IFN-beta. *Mol Cell Biol* **26**, 1888-1897 (2006).

292. de Jong, H.J.I. *et al.* Evaluating the safety of beta-interferons in MS: A series of nested case-control studies. *Neurology* **88**, 2310-2320 (2017).

CHAPTER 7: APPENDIX I

Permission to include a JEM article in my PhD Thesis

A.J. Lee **<leea55@mcmaster.ca>** Mar 28

to permissions

Hi Ms. O'Donnell,

I hope this e-mail finds you well.

My name is Amanda Lee, I'm currently a PhD candidate at McMaster University.

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