SENSING OF ENDOGENOUS NUCLEIC ACIDS BY THE INNATE IMMUNE SYSTEM DURING VIRAL INFECTION

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Immunology and Microbiology Program
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In loving memory of my grandfather Robert Couture
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“The only true wisdom is in knowing you know nothing.” – Socrates

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Abstract

Innate sensing of nucleic acids lies at the heart of antiviral host defense. However, aberrant activation of innate sensors by host nucleic acids can also lead to the development of autoimmune diseases. Such host nucleic acids can also be released from stressed, damaged or dying cells into the tissue microenvironment. It however remains unclear how the extracellular nucleic acids impacts the quality of the host immune responses against viral infections. Using a mouse model of influenza A virus (IAV) infection, we uncovered an important immune-regulatory pathway that tempers the intensity of the host-response to infection. We found that host-derived DNA from necrotic cells accumulates in the lung microenvironment during IAV infection, and is sensed by the DNA receptor Absent in Melanoma 2 (AIM2). AIM2-deficiency resulted in severe immune pathology highlighted by enhanced recruitments of immune cells, and excessive systemic inflammation after IAV challenge, which led to increased morbidity and lethality in IAV-infected mice. Interestingly, these effects of AIM2 were largely independent of its ability to mediate IL-1β maturation through inflammasome complexes. Finally, ablation of accumulated DNA in the lung by transgenic expression of DNaseI in vivo had similar effects. Collectively, our results identify a novel mechanism of cross talk between PRR pathways, where sensing of host-derived nucleic acids limits immune mediated damage to virus infected tissues.
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List of Abbreviations

AAV: Adeno-associated virus
AdV: Adevovirus
AGS: Aicardi-Goutieres syndrome
AIM2: Absent in melanoma 2
AM: alveolar macrophage
ASC: Apoptosis-Associated Speck-Like Protein Containing A CARD
BAL: bronchiole alveolar lavage
BL: Burkitt’s lymphoma
BMDC: Bone marrow-derived dendritic cells
BMDM: Bone marrow-derived macrophages
cAMP: cyclic-adenosine monophosphate
CARD: caspase activation and recruitment domain
Caspase: cysteinyI aspartate protease
CBV: Coxsackie B virus
CD: cluster of differentiation
cDC: conventional dendritic cells
CDN: Cyclic-dinucleotide
cGAMP: cyclic-guanine-adenosine monophosphate
cGAS: cyclic-GMP-AMP synthetase
cGMP: cyclic-guanine monophosphate
CMV: cytomegalovirus
DAI: DNA–dependent activator of IRFs
DAMP: Danger-associated molecular pattern
DC: dendritic cell
DDR: DNA damage response
DDX41: DEAD (Asp-Glu-Ala-Asp) box polypeptide 41
DNA: deoxyribonucleic acid
DNA-PK: DNA-dependent protein kinase
dpi: days post-infection
EAE: experimental autoimmune encephalitis
EBER: Epstein Barr-encoded RNA
EBV: Epstein Barr virus
EMCV: Encephalomyocarditis virus
ER: endoplasmic reticulum
FADD: FAS-associated death domain
HCV: Hepatitis C virus
HHV: human herpesvirus
HIV: Human immunodeficiency virus
HSV: Herpes simplex virus
IAV: Influenza A virus
IFI: Interferon-γ-inducible
IFN: Interferon
IFNAR: IFN-α/β receptor
IL: interleukin
IRAK: IL-1 receptor associated kinases
IRF: Interferon regulatory factor
ISG: Interferon stimulated genes
KSHV: Kaposi’s sarcoma-associated herpesvirus
LANA: latency associated nuclear antigen
LGP-2: laboratory of genetics and physiology 2
LPD: lymphoproliferative disorder
LPS: Lipopolysaccharide
LRR: leucine rich repeat
MAVS: mitochondrial antiviral signal
MCD: multicentric Castleman’s disease
MCMV: murine cytomegalovirus
MDA-5: melanoma differentiation-associated gene 5
MEF: mouse embryonic fibroblasts
MHV-68: mouse gammaherpesvirus 68
MOI: multiplicity of infection
MRE11: meiotic recombination 11 homolog A
MVA: Modified vaccinia virus Ankara
MyD88: myeloid differentiation primary response protein 88
NBD: nucleotide binding domain
NET: neutrophil extracellular traps
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
NLR: Nod-like receptor
NP: nucleoprotein
NTase: nucleotidylyltransferase
PAMP: Pattern-associated molecular pattern
pDC: plasmacytoid dendritic cells
PEL: primary effusion lymphoma
polyI:C: polyriboinosinic:polyribocytidilic acid
PRR: Pattern recognition receptor
PV: Polio virus
PYHIN: Pyrin and HIN domain
RA: rheumatoid arthritis
RIG-I: retinoic acid-inducible gene I
RIP1: receptor interacting protein 1
RIP3: receptor interacting protein 3
RLR: RIG-I-like receptor
RNA: ribonucleic acid
ROS: Reactive oxygen species
SeV: Sendai virus
SLE: systemic lupus erythematosus
STING: Stimulator of interferon genes
TBK1: TANK binding kinase 1
TIR: Toll/Interleukin-1 receptor
TLR: Toll-like receptor
TMEV: Theiler’s murine encephalomyelitis virus
TNFα: Tumor necrosis factor α
TRADD: TNF receptor-associated death domain
TRIF: TIR-domain-containing adapter inducing IFN
TRIM: tripartite motif protein
VACV: Vaccinia virus
VSV: Vesicular stomatitis virus
VZV: varicella zoster virus
Chapter 1

Introduction and Background
Introduction
The immune system of vertebrates is a complex network of cells surveying its host for microbial invasion or damaged self and enacting the mechanisms for elimination of the threat and the return to homeostasis. The immune system can be broadly categorized into the innate and adaptive branches of immunity based on the receptors used in the sensing of “self” and “non-self”. The innate immune system uses a fixed set of receptors able to recognize a breadth of exogenous and endogenous molecules present in all forms of life. On the other hand, the receptors of the adaptive immune system are rearranged to recognize and respond to a specific antigen. The cooperation of these two systems not only protects us from the vast number of microbes in our environment, but it also mediates the removal of dysfunctional cells and repair in damaged tissues. The relatively slow kinetics of generating a targeted response by the adaptive response necessitates a broad antimicrobial and inflammatory response to contain the infection by the innate immune system. The recognition of molecular patterns conserved across all forms of life by the innate pattern recognition receptors is a paradoxical problem for the immune system in its efforts to distinguish between “damaged” versus “undamaged” while maintaining tolerance. While the robust inflammation generated by the innate immune system in response to infection is important for the survival of the host, the continuous draw of sterile inflammation stimulated by endogenous molecules can spur the development of autoimmunity, allergy, cancer, and immune pathology. The ever-
present microflora on our bodies and persistent viruses, lying quietly past every barrier of the cell or even infiltrating our genome, further adds to the complexity of regulating innate immunity and inflammation.

Nucleic acids are present in all forms of life, yet the innate immune system of mammals has developed strategies to recognize abundant and misplaced nucleic acids as viral infection requiring an antiviral response. New insights into the genesis of autoimmunity and autoinflammation have implicated the continuous sensing the host’s own nucleic acids as a driver of pathology and disease, thereby highlighting the complicated balancing act that is maintaining tolerance while providing protection. In this dissertation I focus on the mechanisms utilized by the innate immune system for the sensing of infection with a DNA virus and host DNA in tissues damaged by virus. Understanding the mechanisms of molecular recognition and responses downstream by the innate immune system will not only be useful for developing new and better vaccines against pathogens but also for effective immunotherapies targeting tumors.
Molecular patterns stimulate the innate immune response

The immune response to infection in vertebrates ultimately culminates in the formation of a protective antigen-specific adaptive response. Nonetheless, the detection of the pathogen by the innate immune system must first occur. That is to say, the stimulation of innate immune responses is required for the development of the adaptive response to a particular pathogen. Notification of damage to the immune system similarly requires recognition by the cells of the innate immune branch for the removal and repair of damaged tissue. The innate phase of the immune response is spurred by the sensing of “molecular patterns” broadly conserved across kingdoms of life to react to damage derived from both exogenous and endogenous sources. These immunostimulatory molecules are collectively termed pathogen-associated molecular patterns (PAMPs) when found in the macromolecular structures of the pathogen, while endogenous molecules are referred to as damage-associated molecular patterns (DAMPs). The innate immune system recognizes the molecular signature of PAMPs and DAMPs through a set of germline-encoded proteins called pattern recognition receptors (PRRs). PAMPs and DAMPs take on a wide array of molecular structures including carbohydrates, lipids, proteins, and nucleic acids. The recognition of broad molecular patterns, particularly in the case of nucleic acids, is a means for the immune system to survey for damage, in addition to the detection of microbial invasion. While the carbohydrates, lipids, and proteins of bacteria, viruses, and fungi may only contain small differences in their molecular structure, receptors of
the innate immune system have evolved as such to distinguish between host cell and microbial molecules. On the other hand, nucleic acids are the genetic basis of life for both microbes and host alike, and aside from the order nucleotide sequence, they are largely indistinguishable. Thus, the recognition of both host and microbial nucleic acids must be regulated by the innate immune system to prevent unwanted responses.

Immunostimulatory Activity of Nucleic Acids: A Brief History

The antiviral effect of foreign nucleic acids on cells was appreciated well prior to the discovery of the innate immune system and its function. In 1957, Isaacs and Lindemann reported that infection of eggs with heat-inactivated influenza A virus triggered the release of a factor able to interfere with subsequent infection and replication of live virus (Isaacs and Lindenmann, 1957). They dubbed these unknown molecules “interferons” (IFNs) based on their activity and interference of viral infection. While it was clear even the non-viable virus could stimulate the production of interferon in cells, it was unknown for some time what viral molecules were recognized by the cell as “foreign” and induced interferon. A clue into the identity of the viral molecule required for interference came in a 1958 article by Paucker and Henle reporting that influenza virions lacking viral RNA did not stimulate IFN (Paucker and Henle, 1958). With some insight from these findings, Rotem and colleagues demonstrated in their 1963 Nature paper that the addition of either purified RNA or DNA to cells prior to infection interfered with the
replication of both DNA or RNA viruses (Isaacs and Lindenmann, 1957; Rotem et al., 1963). Importantly, they found nucleic acids induced interferon without regard to the source, as RNA and DNA from chickens, mice, and cows were all able to interfere with virus replication, thus viral and eukaryotic nucleic acids were indiscriminately sensed by the cells for IFN induction. Despite this finding however, much of the efforts in the following few decades focused on defining the molecular signature that marked viral nucleic acids as “foreign” in a sea of cellular nucleic acids so that it might be mimicked for therapeutic uses. Studies by the Hilleman group lead to the discovery of the polymer polyriboinosinic:polyribocytidilic acid (poly I:C), a long double-stranded RNA synthetic, as potent inducer of IFN (Paucker and Henle, 1958; Field et al., 1967). The identification of long dsRNA as the inducer of IFN immediately made clear that there was some level of discrimination by the host in identifying nucleic acids. While long dsRNA genomes and replication species are common during lytic viral replication, eukaryotic cells do not have any long dsRNA species to be recognized (Hilleman, 1970). Since that time, uncapped single stranded RNAs with 5’-phosphates or panhandle secondary structure have been shown to also induce IFN production (Hornung et al., 2006; Pichlmair et al., 2006; Wu and Chen, 2014). Thus, the number of species, sizes, and modifications of RNA provide a mechanism of distinction between “self” versus “non-self” by the innate immune system.
A number of other molecular characteristics required for the immunostimulatory activity of DNA have also been described over the past 30 years. Bacterial DNA and palindromic synthetic oligodeoxynucleotides (ODNs) with unmethylated CpG motifs were found to stimulate B cell proliferation and the antitumor activity of NK cells (Yamamoto et al., 1992; Krieg et al., 1995). Unlike the RNA, the stimulatory activity of DNA can depend greatly on the nucleotide sequence. ODNs containing TTAGGG repeats abundant in telomeric DNA mimics the immunosuppressive effect of self-DNA (Klinman et al., 2008).

These early efforts have highlighted the potent immunostimulatory activity of nucleic acids and garnered much attention for its therapeutic potential since its discovery. It is important to note that in the studies mentioned above, the induction of IFN and inflammatory genes was in response to extracellular nucleic acids confirming either the presence of cell surface receptors able to sense RNA and DNA or, mechanisms directing them to subcellular compartments for recognition. However, work over the last 10 years has found that the delivery of DNA and RNA to the cytosol also induces potent IFN and inflammatory responses through a distinct set of receptors than those sensing extracellular nucleic acids.

**Pattern Recognition Receptors for Nucleic Acids**

**Toll-like Receptors**
With the discovery of the immunostimulatory activity of nucleic acids, the next obvious question became: what receptors were recognizing nucleic acids and inducing a response downstream? The Toll-like receptors (TLRs) were the first family of PRRs in vertebrates to be identified for their ability to recognizing PAMPs and stimulate innate immune responses. First found in fruit flies, the Toll receptor of fruit flies induced expression of antimicrobial peptides in response to bacterial infection (Lemaitre et al., 1996). The TLRs are type I transmembrane proteins consisting of a leucine-rich repeat (LRR) extracellular domain for the recognition and binding of PAMPS, and cytosolic Toll/Interleukin-1 receptor (TIR) domain to initiate intracellular signaling. The work of Medzhitov and Janeway first highlighted the importance of TLR signaling in stimulating early inflammatory cytokine production and the expression of co-stimulatory molecules necessary for activation of the adaptive response in mammalian cells (Medzhitov et al., 1997). The subsequent discovery of TLR4 as the much sought after receptor for the lipopolysaccharide (LPS) from the cell wall of gram-negative bacterium prompted a flurry of work identifying a number of other TLRs in the ensuing years (Poltorak et al., 1998). To date, 13 TLRs in mice and 10 in humans have been identified with TLRs 1-9 being shared by both species. The TLR family can be categorized into two groups based on their location in the cell and the molecular patterns of the ligands they recognize. Located at the plasma membrane are TLRs 1, 2, 4, 5, and 6 with their LRR domains facing the extracellular side for the detection of extracellular PAMPs. The cell surface TLRs recognize PAMPs primarily bacterial
in origin including flagellin, peptidoglycan, lipoproteins, LPS, etc (O’Neill, 2008). In addition to those at the cell surface, the nucleic acid sensing TLR3, 7, 8, and 9 are localized to the endosomal compartment. For the purposes of this dissertation I will focus our discussion on the nucleic acid sensing TLRs.

**Nucleic Acid Sensing TLRs**

Work in the Akira lab identified TLR9 as the receptor for unmethylated CpG DNA, showing it was required for NF-κB activation and inflammatory response to CpG ODN (Hemmi et al., 2000). Soon after TLR3 was found to be the receptor for poly I:C and long dsRNA (Alexopoulou et al., 2001). TLR7 and 8 recognize ssRNA of viral genomes and replication intermediates and small molecule nucleoside analogs (Hemmi et al., 2002; Diebold et al., 2004). Dimerization to the TLRs initiates intracellular signaling by differentially recruiting the adaptor proteins myeloid differentiation primary response protein 88 (MyD88) or TIR-domain-containing adapter inducing IFN (TRIF). Signaling downstream of TRIF and MyD88 proceeds through two distinct pathways, and deletion of both MyD88 and TRIF completely blocks signaling downstream of TLR stimulation. TLR3 recruits TRIF to its cytosolic TIR domain activating the tank binding kinase 1 (TBK1) complex to phosphorylate the transcription factors interferon regulatory factor 3 and 7, which are required for type I IFN induction (Fitzgerald et al., 2003). TRIF also activates receptor interacting protein 1 (RIP1) kinase, which then activates the NF-κB pathway (Meylan et al., 2004). TLR4 signals through both TRIF and
MyD88, whereas MyD88 is required for signaling by the remaining TLRs. Recruitment of MyD88 to TLRs leads to activation of IL-1 receptor associated kinases (IRAK), which activates the NF-κB and the mitogen-activated kinase (MAPK) pathways (Motshwene et al., 2009). NF-κB, IRF, and MAPK activation downstream of TLR stimulation results in upregulation of inflammatory cytokines/chemokines, type I IFNs, co-stimulatory molecules, and antigen presentation to provide the critical input signals necessary for initiating the adaptive response.

The main function of the endolysosomal network is the transport of cargo from the extracellular environment or cell surface for degradation, with the pH of the lumen becoming more acidic as the endosome matures. The specific localization of nucleic acid sensing TLRs suggests the conditions in the lumen of the endosome are somehow important for their recognition of nucleic acids. Acidification of the endolysosome is indeed required for recognition of CpG DNA by TLR9 (Lund et al., 2003). The endosomal location of TLRs is a physical mechanism preventing the unwanted sensing of self-nucleic acids, with DNA confined to the nucleus, and RNA present in both the nucleus and cytoplasm. Mislocalization of TLR9 to the cell surface was unable to respond to viral DNA but acquired the ability to respond to self-DNA (Barton et al., 2005). Thus, the endosomal localization of TLR9 prevents unwanted recognition of self-DNA in the extracellular environment and nucleus. Expression of TLR3, 7, 8, and 9 varies amongst different cell types of the innate immune system suggesting certain cells
might be better specialized for nucleic acid sensing. For example, plasmacytoid dendritic cells (pDC) express high levels of TLR7 and 9 and produce large amounts of IFN-α, when stimulated through these pathways.

**Cytosolic Nucleic Acid Sensors**

The uptake of foreign nucleic acids into the endosome is important for its detection by nucleic acid-sensing TLRs, however many viruses and intracellular bacteria bypass the endosome during entry and enter directly into the cytosol. Regardless of their site of replication in the cell, viral transcripts, genomes, and replication intermediates all gain access to the cytosol at some point during lytic infection. Evidence supporting the existence of cytosolic nucleic acid sensors came in 2003, when it was found that delivery of poly I:C directly to the cytosol by transfection or electroporation induced high levels of type I IFN in a TLR3- and MyD88-independent manner (Diebold et al., 2003). Similarly, delivery of DNA directly to the cytosol induces type I IFN and proinflammatory cytokines independent of TLR signaling but required IRF3 (Stetson and Medzhitov, 2006). The discoveries spurred considerable efforts to identify the cytosolic receptors able to sense cytosolic nucleic acids over the last decade.

**RIG-I-like Receptors**

RIG-I-like receptors (RLRs) were first identified as the receptors for cytosolic RNA. The family consists of three identified members: retinoic acid-inducible
gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA-5), and laboratory of genetics and physiology 2 (LGP-2). All the RLRs belong to the DExD/H box RNA helicase superfamily, containing a DExD/H box domain with ATPase activity and a C-terminal repressor domain (RD). RIG-I and MDA-5 have tandem N-terminal caspase activation and recruitment domains (CARD) required for their signaling upon sensing cytosolic RNA, however LGP-2 lacks any CARD domain.

The RLRs all bind to dsRNA species, but each have more specific requirements in RNA modifications and the length of the RNA due to their distinct mechanisms of recognizing invading dsRNA. RIG-I recognizes short blunt-ended dsRNAs bearing a 5’-triphosphate, a modification not present on host mRNA, or short hairpin ssRNA found in negative-sense viruses such as Influenza A virus (IAV) and vesicular stomatitis virus (VSV) (Schlee et al., 2009; Baum et al., 2010). RNA Polymerase III (Pol III) has also been shown to generate short ssRNA transcripts using AT-rich DNA in the cytosol as template, and these are also recognized by RIG-I (Ablasser et al., 2009). RIG-I is present in the cytosplasm in an auto-inhibited state undergoing a conformational change upon binding to dsRNA, allowing its ubiquitination by tripartite motif protein 25 (TRIM25) (Gack et al., 2007). MDA-5 on the other hand, recognizes long dsRNA found in the picornaviruses encephalomyocarditis virus (EMCV) and polio virus (PV), amongst others. MDA-5 binds and oligmerizes on long dsRNAs forming large filament structures (Bin Wu et al., 2013).
MDA-5 and RIG-I both signal through the adaptor protein mitochondrial antiviral signal (MAVS), also known as VISA and IPS-1, located on the outer membrane of mitochondria and peroxisomes by homotypic interaction of their CARD domains. (Xu et al., 2005; Dixit et al., 2010). MAVS is required for all signaling downstream of RLRs with the exception of LGP-2 due to it lacking a CARD domain to bind to MAVS. Currently, the function of LGP-2 is not clear but is thought to negatively regulate RIG-I and MDA-5 signaling. Activation of the MAVS-dependent pathway occurs through its aggregation on the surface of the mitochondria stimulated by the binding of activated RIG-I or MDA-5 filaments. TNF receptor associated factor (TRAF) 3 and 6, caspase-8, RIP1, FAS-associated death domain (FADD), and TNF receptor-associated death domain (TRADD) are recruited to activated MAVS, forming a signaling complex for activation of the kinases IKKα/β and TBK1, resulting in downstream NF-kB- and IRF3/7-dependent transcription of inflammatory and antiviral genes (Figure 1.1) (Kawasaki et al., 2011).

Unlike TLR3, 7, and 8 which respond to extracellular nucleic acids, the RLRs only recognize cytosolic RNA, suggesting they exist specifically for the detection of incoming and replicating RNA viruses and the stimulation of innate immune responses. Studies using knock-out mouse models have confirmed the importance of MAVS-dependent signaling for stimulating protective innate immune responses to RNA virus infection (Kumar, 2006). RNA viruses can be categorized by their dependency on RIG-I or MDA-5 for their detection. Most
RNA viruses have either positive, negative, or ambisense single-stranded RNA genomes and as such, the majority of RNA viruses activate RIG-I signaling. Some notable examples of viruses recognized by RIG-I include IAV, VSV, Sendai virus (SeV), hepatitis C virus (HCV), and ebola virus (Takeuchi and Akira, 2009). MDA-5 however, detects the long dsRNA genomes and replication intermediates present in a smaller number of RNA virus families including EMCV, PV, coxsackie B virus (CBV), and Theiler’s murine encephalomyelitis virus (TMEV) (Kato et al., 2006).

Cytosolic DNA Sensors for IFN induction
Delivery of dsDNA to the cytosol during DNA virus infection or by transfection induces the type I IFN and inflammatory cytokine responses in a TLR-independent manner (Stetson and Medzhitov, 2006). However, unlike the modifications to RNA required for their recognition by RLRs, any dsDNA longer than 25bp with a sugar-phosphate backbone is able to stimulate innate immune responses when delivered to the cytosol. DNA present in the cytoplasm is clearly an indicator of infection or stress since it is normally confined to the nucleus and mitochondria in eukaryotic cells. Thus, separating ligand and receptor into different subcellular compartments prevents the unwanted activation of the innate immune responses to self-DNA.

The first key finding towards identifying the components necessary for the sensing of cytosolic DNA upstream of the IFN response was the discovery of
adaptor protein stimulator of interferon genes (STING). Similar to MAVS, STING was identified for its ability to drive type I IFN production when overexpressed (Ishikawa and Barber, 2008). STING is required for the stimulation of the innate immune response by cytosolic DNA, regardless of the receptor recognizing the foreign DNA. Four hydrophobic α-helices localize STING to the outer membrane of the endoplasmic reticulum (ER), however it relocalizes to the Golgi forming large aggregates after cytosolic DNA signaling (Ishikawa et al., 2009). The STING signaling complex recruits TBK-1, stimulating its kinase activity and phosphorylation of its substrates IRF3 and IRF7 (Ishikawa et al., 2009). Cytosolic DNA signaling also activates the NF-κB pathway in a STING-dependent manner, but how this occurs is unknown. STING is absolutely required for inducing type I IFN and inflammatory responses to cytosolic DNA and DNA virus infection. Mice deficient for STING have impaired IFN and inflammatory cytokine responses, and are susceptible to HSV-1 infection (Ishikawa et al., 2009). Interestingly, STING-/- mice are also susceptible to VSV infection suggesting it may also have a role in the pathogenesis of RNA viral infection, perhaps through the sensing host DNA released into damaged tissues (Ishikawa et al., 2009).

In addition to serving as an adaptor downstream of cytosolic DNA sensors, the C-terminal domain of STING directly recognizes bacterial cyclic-dinucleotides (CDN) through its C-terminal domain resulting in its dimerization and activation of TBK1 and NF-κB signaling. Bacteria often utilize CDNs as secondary messengers in intracellular signaling pathways. For example, cyclic-adenosine
monophosphate (cAMP) and cyclic-guanine monophosphate (cGMP) from the bacterium *V. cholerae* stimulates type I IFN in a STING-dependent manner (Burdette et al., 2011). In addition to bacterial CDNs, the enzyme, cyclic-GMP-AMP synthetase (cGAS), has recently been identified in vertebrates, with its enzymatic activity producing endogenous cyclic-GMP-AMP (cGAMP) in a DNA-dependent manner upstream of STING activation.

**cGAS**

Though it is clear that STING is a direct sensor of CDNs and an adaptor required for type I IFN responses to cytosolic DNA, the identities of the cytosolic DNA receptors and enzymes upstream of STING have only recently been uncovered. Of the cytosolic DNA receptors identified, the activity of cGAS is the only one thus far with a clear mechanism coupling its activity to STING activation. cGAS is a member of the nucleotidyltransferase (NTase) family of enzymes able link nucleotide monomers into oligomers and other higher order structures. After activation by direct binding to cytosolic DNA, cGAS catalyzes the formation of cGAMP from ATP and GTP as a secondary messenger able to stimulate STING activation (Sun et al., 2013; Li et al., 2013a; Zhang et al., 2013b; Ablasser et al., 2013b). Structural studies have confirmed cGAMP is able to directly bind to the C-terminus of STING, stimulating its dimerization and activation (Shu et al., 2012; Huang et al., 2012). Whether or not cGAMP functions as a secondary messenger exclusively for the response to cytosolic DNA is currently unknown, but it is likely
to activate other signaling pathways yet to be identified. cGAMP appears activate STING signaling in adjacent cells by passing through gap junctions, demonstrating cGAMP also functions as an intercellular signal for inducing type I IFN (Ablasser et al., 2013a). Deletion of cGAS in macrophages, dendritic cells, and fibroblasts completely abrogates IRF3 activation and downstream type I IFN responses to transfected DNA and DNA virus infection (Li et al., 2013b). The generation of cGAS-deficient mice has confirmed its importance in the immune response to DNA virus infection. Mice deficient for cGAS are susceptible to infection with herpes simplex virus-1 (HSV-1) and vaccinia virus (VACV), and had higher viral loads during latent infection with mouse γ-herpesvirus 68 (MHV-68) (Li et al., 2013b) (Schoggins et al., 2014).

DAI
The DNA–dependent activator of IRFs (DAI) was the first potential cytosolic DNA receptor to be identified. DAI binds to Z-form DNA by its N-terminal Z-α and Z-β domains, as well as to B-form DNA through its D3 domain. In the initial report identifying DAI as a receptor for the induction of type I IFN, its overexpression drives type I IFN responses in mouse L929 cells, and its knockdown diminished this response to cytosolic DNA (Takaoka et al., 2007). However, later studies using cells from DAI-deficient mice found normal type I IFN responses to cytosolic DNA in the absence of DAI. Thus it is unclear whether or not DAI is a bona fide DNA receptor for the induction of IFN. Recent work has found DAI
positively regulates programmed necrosis through its interaction with RIP3 during MCMV infection (Upton et al., 2012).

**IFI16/p204**

Interferon-inducible gene 16 (IFI16) is a member of the pyrin and HIN domain (PYHIN) family of proteins, a family of conserved proteins containing a PYRIN domain and at least one or more HIN-200 domain able to bind directly to dsDNA in a sequence-independent manner (Schattgen and Fitzgerald, 2011). IFI16 was first identified as a putative sensor by its affinity to dsDNA in pulldow assays using human cell extracts (Unterholzner et al., 2010). Though mice lack a clear homolog to IFI16, the mouse PYHIN p204 is the closest in secondary structure to IFI16 and thought to be its homolog. The role of IFI16 as a cytosolic DNA sensor is controversial with its requirement for IFN induction varying between studies. Knockdown of IFI16 or p204 in human THP-1 cells and mouse macrophages, respectively, partially inhibited IRF3 activation and IFN responses to transfected DNA and HSV-1 infection (Unterholzner et al., 2010). Similar results were found in response to infection with human immunodeficiency virus (HIV) (Jakobsen et al., 2013). However, other reports have found knockdown of IFI16 had no effect on the response to cytosolic DNA. A confounding detail in determining the function of IFI16 is its localization in the nucleus and cytoplasm varies between cell types (Li et al., 2012). Indeed, IFI16 appears to sense HSV-1 and Kaposi’s sarcoma-associated herpesvirus (KSHV) genomic DNA in the cytosol and
nucleus, respectively (Kerur et al., 2011) (Johnson et al., 2013). Besides its potential role as a redundant DNA sensor for stimulating the IFN response, IFI16 has also been described to positively regulate the transcription of interferon stimulate genes (ISGs) in response to RNA virus infection as well (Thompson et al., 2014). Future studies using p204 knock-out mice and IFI16 knock-out human cell lines will better delineate its role in cytosolic DNA sensing.

**DDX41**

An RNAi screen of DExD/H helicases family members identified DEAD (Asp-Glu-Ala-Asp) box polypeptide 41 (DDX41) as a putative cytosolic DNA sensor. Depletion of DDX41 was found to inhibit the IFN response to cytosolic DNA and HSV-1 infection (Zhang et al., 2011). DDX41 directly binds to DNA by its DEADc domain and has recently been shown to directly bind to CDNs upstream of STING (Parvatiyar et al., 2012). Other studies have found no role for DDX41 in the IFN response to cytosolic DNA however, thus further study in DDX41-deficient mice will clarify its function in vivo.

**DNA-PK and MRE11**

In addition to the newly discovered putative DNA sensors, proteins well described to be involved in the DNA damage response (DDR) have also been implicated in the sensing and signaling by cytosolic DNA. The DNA-dependent protein kinase (DNA-PK) was identified as a potential DNA sensor for cytosolic DNA and DNA
virus infection. DNA-PK is a holoenzyme consisting of three subunits: the DNA binding proteins Ku70/Ku80 and the catalytic subunit DNA-PKcs. Studies in DNA-PK-deficient mice showed attenuation but not abolishment of the IFN response to VACV infection (Ferguson et al., 2012). Recently, the DNA damage sensor meiotic recombination 11 homolog A (MRE11) was reported to be a cytosolic dsDNA sensor that activates the STING pathway. Studies using human cells bearing a MRE11 hypomorph showed it to be required for the type I IFN response to cytosolic DNA but not HSV-1 (Kondo et al., 2013). The identification of DNA-PK and MRE11 as important for the response to cytosolic DNA clearly suggest cross-talk exists between the DDR and cytosolic nucleic acids sensing pathways.
Figure 1.1 Signaling pathways of nucleic acid PRRs. The RLR proteins RIG-I and MDA-5 are cytosolic RNA receptors for the activation of NF-κB and IRF3/7. RIG-I recognizes 5' triphosphate RNA and short dsRNA, while MDA-5 recognizes long dsRNA. RIG-I and MDA-5 both signal through the adaptor protein MAVS on the outer membrane of mitochondria for the recruitment of TRAF3/6, FADD, TRADD, and RIP1 for the activation of IKKα/β and TBK1. The cytosolic DNA sensors cGAS, IFI16, DAI, MRE11, and DNA-PK recognize dsDNA in the cytoplasm for activation of NF-κB and IRF3/7 downstream of the adaptor protein STING. Binding of DNA to cGAS stimulates its enzymatic activity for the creation of cGAMP, which directly binds and activates STING. Dotted arrows indicate unknown mechanisms used by the other cytosolic DNA sensors for the activation of STING-dependent signaling. The endosomal TLRs 3, 7/8, and 9 recognize dsRNA, ssRNA, and CpG DNA for their dimerization and activation. TLR3 signals through the adaptor TRIF. The recruitment of TRAF3/6, TAK1, TRADD, and RIP1 to TRIF stimulates IKKα/β and TBK1 activation. TLR7/8 and TLR9 signal through MyD88, recruiting IRAK1, IRAK2, IRAK4, TRAF6, and TAK1 for the activation of IKKα/β and TBK1. All these pathways culminate with activation and translocation of NF-κB and IRF3/7 to the nucleus for induction of inflammatory cytokines and type IFN.
Activation of the Inflammasome Complex by Cytosolic Nucleic Acids

In addition to the induction of type I IFN, nucleic acids in the cytosol are also sensed by a distinct set of sensors for activation of the inflammasome complex. The inflammasome is a large multimeric complex consisting of a receptor, the adaptor protein Apoptosis-Associated Speck-Like Protein Containing A CARD (ASC), and its effector protein cysteiny1 aspartate proteases-1 (caspase-1) (Davis et al., 2011). Most of the receptors identified for the activation of the inflammasome belong to the Nod-like receptor (NLRs) family of PRRs. Sensing of cytosolic perturbations in the form of PAMPs and DAMPs triggers the assembly of the inflammasome complex resulting in the activation of caspase-1. Two important substrates of caspase-1 are the inactive forms of IL-1β and IL18 in the cytoplasm. Activated caspase-1 cleaves pro-IL-1β and pro-IL18 into their mature, bioactive forms whereupon they are secreted from the cell. Caspase-1 activation also triggers an inflammatory form of cell death dubbed pyroptosis where the intracellular contents of the cell are released into the microenvironment where they stimulate inflammatory responses in cells recognizing DAMPs.

Inflammasome activation, regardless of the activating receptor, requires two signals: (1) TLR-dependent upregulation of inflammasome components and substrates (i.e. IL-1β) and (2) sensing of the activating signal by the receptor and subsequent processing of caspase-1.

A number of inflammasome receptors have been described to sense and respond to a wide variety of cytosolic molecules of microbial and endogenous
origin. Here, I will discuss those receptors shown to sense cytosolic nucleic acids for activation of the inflammasome complex.

**NLRP3**

NLRP3 is the best studied of the inflammasomes sensors. Like most other NLRs, NLRP3 has an N-terminal PYRIN domain, a central nucleotide binding domain (NBD), and a large LRR domain in its C-terminus. Upon assembly of the inflammasome complex, NLRP3 interacts with ASC through homotypic PYRIN domain interactions to form a large multimeric complex, which then recruits caspase-1 to ASC through interaction of their CARD domains, ending with autoproteolytic processing of caspase-1 into its active form. The NLRP3 inflammasome is activated in response to a diverse array of exogenous and endogenous molecules including bacterial pore-forming toxins, uric acid and cholesterol crystals, silica particles, high concentrations of extracellular ATP, and nucleic acids to name just a few (Martinon et al., 2009). Notably, there is no evidence supporting direct binding and recognition of the activating stimuli by NLRP3. Rather the evidence on NLRP3 suggests it is a sensor of the cytosolic state, responding to changes in the intracellular environment. Despite intensive study, there is no unified mechanism for activation of the NLRP3 inflammasome. Reactive oxygen species (ROS), potassium efflux from the cell, and lysosomal rupture leading to the leakage of cathepsin proteases have been implicated as the mechanisms of NLRP3 activation (Davis et al., 2011). Thus, it remains to be
seen whether NLRP3 activation occurs through a common mechanism or more specific means depending on the nature of the stimulus.

In addition to alerting the innate response for induction of IFN and antiviral defenses, nucleic acids are also sensed in the cytosol by the inflammasome. IL-1R signaling plays a key role in defense to bacterial and viral infections, thus their sensing and activation of the inflammasome are important events during the innate immune response. A number of RNA and DNA viruses have been found to activate the inflammasome in an NLRP3-dependent manner including: IAV, SeV, Hepatitis C virus (HCV), VSV for RNA viruses; and HSV-1, Modified vaccinia virus Ankara (MVA) and adenovirus (AdV) for DNA viruses (Kanneganti, 2010). Purified IAV vRNA and poly I:C delivered into the cytosol is sensed by the NLRP3 inflammasome stimulating IL-1β processing and secretion (Allen et al., 2009).

IAV infection has also been shown to activate the NLRP3 inflammasome by disrupting intracellular ion gradients with insertion of the IAV M2 ion pore into the Golgi network (Ichinohe et al., 2010). Studies in mice have shown NLRP3 is dispensable low dose IAV infection but is required for protection to higher doses of virus. ASC, caspase-1, and IL-1R are all required for the development of flu-specific T and B cell responses and protection from IAV infection, regardless of the dose (Allen et al., 2009; Ichinohe et al., 2009; Pang et al., 2013; Thomas et al., 2009). How NLRP3 senses cytosolic RNA is unclear since it does not directly bind to RNA. Recent works have implicated sensing of mitochondrial dysfunction by NLRP3 as the mechanism for RNA recognition. Mitochondrial damage by
RNA virus infection requires activation of the RIP1-RIP3 complex, leading to ROS production and NLRP3 inflammasome activation (Wang et al., 2014). Interestingly, inflammasome activation in response to DNA virus infection does not require RIP3 indicating the sensing of RNA and DNA viruses by NLRP3 does not occur through the same mechanism (Wang et al., 2014). Delivery of dsDNA activates the inflammasome complex independent of NLRP3, making it unclear how NLRP3 senses DNA virus infection. Processing of IL-\(\beta\) in response to transfected DNA as found to require ASC and caspase-1 however, indicating another receptor was able to assemble an inflammasome in the presence of dsDNA.

**AIM2**

Absent in melanoma 2 (AIM2) was discovered to be the receptor necessary for inflammasome activation in response to dsDNA. AIM2 is also a member of the PYHIN family of proteins and as such has an N-terminal PYRIN domain coupled to a HIN-200 domain on the C-terminus required for binding to dsDNA. As with other inflammasome receptors, AIM2 forms an inflammasome complex by binding ASC through interaction of their PYRIN domains in turn recruiting caspase-1. Unlike the wide array of insults indirectly sensed by NLRP3, dsDNA binds directly to AIM2 to trigger inflammasome activation. Structural study shows the positive charge of the HIN-200 domain binds to the negatively charged sugar-phosphate backbone of dsDNA across the major and minor grooves, meaning
ssDNA is unable to form stable complexes with AIM2 (Jin et al., 2012). Binding of DNA to AIM2 is independent of nucleotide sequence, but the dsDNA must be at least 80bp in length. Transfection of synthetic, mammalian, or microbial DNA all equally activates the AIM2 inflammasome.

DNA leaked into the cytosol by invading intracellular bacteria and the DNA viruses activates the AIM2 inflammasome leading to the processing and secretion of IL-1β and IL-18. AIM2 is required for IL-1β production by macrophages and dendritic cells in response to infection with the intracellular bacterium Francisella tularensis and Listeria monocytogenes, as well as the DNA viruses VACV and murine cytomegalovirus (MCMV) (Tsuchiya et al., 2010) (Rathinam et al., 2010; Fernandes-Alnemri et al., 2010; Jones et al., 2010). Unable to control the bacterial burden, mice deficient for AIM2 are susceptible to Francisella tularensis infection (Fernandes-Alnemri et al., 2010). AIM2-deficiency in the case of MCMV infection lead to increased viral titers due decreased NK cell activation, likely a result of decreased IL-18R signaling. (Rathinam et al., 2010). UV-treatment of MCMV severely blunts activation of the AIM2 inflammasome suggesting its replication is somehow required for recognition by AIM2. Despite its clear specificity for dsDNA, one would hypothesize that the AIM2 is required for inflammasome activation in response to all DNA virus infections. Surprisingly, inflammasome activation by infection with the herpesviruses HSV-1 and MHV-68 is independent of AIM2, rather, they require NLRP3. How AIM2 is able to recognize some DNA viruses and not others is
unclear, but future studies will yield insight into the determinants of inflammasome activation by DNA virus infection.

**IFI16**

Recent works have suggested in addition to its role positively regulating IFN induction in response to cytosolic DNA, IFI16 may also function as an inflammasome receptor. IFI16 was found to co-localize with ASC and caspase-1 around the periphery of the nucleus in KSHV infected endothelial cells, and in the cytosol of fibroblasts infected with HSV-1 (Kerur et al., 2011). Knockdown of IFI16 was found to block processing of caspase-1 and IL-1β in response to KSHV and HSV-1 but not VACV infection (Johnson et al., 2013). Abortive infection of CD4+ T cells with HIV triggers cell death in an IFI16- and caspase-1-dependent manner adding further evidence for caspase-1 activation by IFI16 (Monroe et al., 2014). However, other works have reported IFI16 is unable to form an inflammasome in other cell types meaning further work is necessary to define the cellular contexts in which IFI16 associates with ASC and caspase-1 (Hornung et al., 2009).
Figure 1.2 Inflammasomes for nucleic acid and virus sensing. (grey box) Schematic of the inflammasome complex and activation. The inflammasome is comprised of a receptor protein (NLRs or ALRs), the adaptor protein ASC, and the effector protease caspase-1. Sensing of stimuli by the receptor protein triggers assembly of the inflammasome complex leading to caspase-1 activation and subsequent cleavage of its substrates pro-IL-1β and pro-IL-18 into their active forms. (yellow box) NLRP3 recognizes a variety of DAMPs and PAMPs for the activation of the inflammasome. A number of DNA (MVA, AdV, and HSV-1) and RNA (SeV, VSV, and IAV) viruses activate the NLRP3 inflammasome. (purple box) AIM2 recognizes and binds dsDNA for inflammasome activation. The DNA viruses MCMV and VACV activate the inflammasome in an AIM2-dependent manner. (green box) IFI16 associates with ASC and caspase-1 in response to the DNA genome of KSHV.
Herpesviruses

The herpesviruses are a family of large DNA viruses able to establish lifelong latent infection in their host. All herpesviruses have a large, linear dsDNA genome encoding genes categorized as immediate early (IE), early (E), and late (L) genes depending when during the viral life cycle they are expressed. Herpesviruses have been found in a large and growing number of vertebrates species and as a result of co-evolution over millions of years, are often able to naturally infect only their host (Pellet and Roizman, 2007). There are eight human and two murine herpesviruses categorized into α-, β-, and γ-herpesviruses based on DNA sequence similarity, genomic arrangement, and tropism. The α-herpesviruses include herpes simplex virus-1 (HSV-1; HHV-1), herpes simplex virus-2 (HSV-2; HHV-2), and varicella zoster virus (VZV; HHV-3). Defining characteristics of the α-herpesvirus subfamily include their variable host range, short viral replication cycle, and tendency to establish latency in neurons of the trigeminal ganglia. The β-herpesvirus members cytomegalovirus (CMV; HHV-5), roseolovirus (HHV-6), pityriasis rosea (HHV-7) and murine cytomegalovirus (MCMV) are characterized by their restricted host range, long replication cycle, and latent infection of secretory glands. Two γ-herpesviruses have been identified to naturally infect humans and one γ-herpesvirus has been found to naturally infect mice, these include Epstein Barr virus (EBV; HHV-4), Kaposi’s sarcoma-associated herpesvirus (KSHV; HHV-8), and murine γ-herpes virus 68 (MHV-68). γ-herpesviruses are severely restricted to the family or order of their
natural hosts and establish latency in lymphocytes and lymphoid tissues. The $\gamma$-herpesviruses are further subdivided in to lymphocryptoviruses, which includes EBV, and rhadinoviruses, which includes KSHV and MHV-68. For purposes of this dissertation, I will attempt to limit the discussion to the pathophysiology of $\gamma$-herpesvirus infection and its sensing by the innate immune system.

**Epstein Barr Virus and Kaposi’s Sarcoma-associated Herpesvirus**

EBV and KSHV are human $\gamma$-herpesviruses and thus are only able to naturally infect humans. Virions of both EBV and KSHV contain large, linear dsDNA genomes packaged in to an icosahedral protein capsid, which is surrounded by a lipid envelope with tegument proteins positioned between the capsid and envelope. As with all herpesviruses, the envelopes of EBV and KSHV are decorated with glycoproteins (i.e. gB, gH, etc) to mediate virion binding and membrane fusion with the target cell during infection, followed by the releasing viral capsid and tegument proteins into the cytosol or endosome (Kieff and Rickinson, 2007). Viral capsids bearing the dsDNA genome are shuttled to the nucleus along microtubules by dynein motors (Kieff and Rickinson, 2007). Once outside the nucleus, the capsids bind to nuclear pores and are disassembled to allow entry of the genome into the nucleus through the nuclear pore. There, the linear dsDNA genome is circularized, within 8 to 12 hours in the case of EBV, and chromatinized, recruiting host histones and DNA-binding proteins to the genome for early transcription of IE viral genes (Kieff and Rickinson, 2007).
Herpesvirus genomes persist in the nucleus as covalently circularized episomes during latent infection.

EBV is the most widespread of the human herpesviruses with up to 90% of adults being seropositive for virus-specific immunoglobulins. EBV is transmitted by oral contact to the oropharynx and tonsils, where new infection undergoes lytic replication. Acute EBV is asymptomatic in most cases, but it is estimated up to 25% of new EBV infections results in infectious mononucleosis (IM), characterized by fever, rash, swollen tonsils and lymph nodes, and fatigue. EBV transitions to latency after its clearance in the throat, colonizing CD27+ memory B cells in the periphery where it enters its latency program (Rickinson, 2007). Infection of primary human B lymphocytes with EBV in vitro is non-permissive, rather the entry of the virus into latency is sufficient for their conversion into immortalized lymphoblastic cell lines (LCLs) (Rickinson, 2007). Two classes of viral antigens are consistently expressed in latently infected B cells: six nuclear antigens, referred to as Epstein-Barr nuclear antigens (EBNAs), and three membrane proteins, referred to as latency-associated membrane proteins (LMPs), each contributing to the establishment and maintenance of latency. In addition to these proteins, viral non-coding Epstein Barr-encoded RNAs (EBERs) present in the nucleus are also suggested to contribute to latency and cellular transformation (Kieff and Rickinson, 2007). EBV infection is closely associated with the development of Burkitt’s lymphoma (BL), a malignancy commonly found in patients suffering from acquired immunodeficiency syndrome.
(AIDS) and is also the most common childhood cancer in equatorial Africa. Endemic cases of BL are nearly always positive for EBV genome but only 30% of AIDS-related BLs cases are EBV+ (Kieff and Rickinson, 2007). Thus, despite its clear association with the development of BL, it is unclear whether EBV infection of B cells is sufficient for cellular transformation in vivo.

Unlike its better-studied family member EBV, the pathogenesis and infection cycle of KSHV is less well understood. Initially discovered on the basis of its association with the development of Kaposi’s sarcoma (KS), KSHV is the most recent of the human herpesvirus to be identified. KSHV prevalence varies widely by geographic location with an estimated 3% of the population in the United States being seropositive, whereas it rises to near 60% in Africa (Ganem, 2007). Its transmission occurs both vertically and horizontally but the route of infection in which KSHV is passed between humans is unclear. Acute KSHV infection is asymptomatic as the virus quickly establishes latent infection, which can be detected in endothelial, epithelial, B cells, and monocytes (Cai et al., 2010). KSHV is associated with the development of several cancers including KS, primary effusion lymphoma (PEL), and multicentric Castleman’s disease (MCD), most often in immunocompromised adults, but also in children. KSHV is most strongly associated with KS, an indolent systemic malignancy characterized by oligoclonal tumors of mixed endothelial and lymphocyte origin, presenting as papular lesions on the skin. KSHV appears to be required, but not sufficient for
the development of KS as nearly 100% of KS tumors are positive for KSHV latency-associated nuclear antigen (LANA) (Ganem, 2007).

**Murine γ-herpesvirus 68**

The use of cell culture systems has gone a long way towards understanding the molecular mechanisms of replication and latency programs of EBV and KSHV. However, their inability to infect and recapitulate pathogenesis in animal models has limited the understanding of γ-herpesvirus infection in vivo, at least until relatively recently. The isolation of MHV-68 from bank voles and wood mice offered the possibility of a robust small mammal model for the study of γ-herpesvirus pathogenesis (Nash et al., 2001; Collins et al., 2009). Acute MHV-68 infection of laboratory mice is marked by lytic viral replication in the lung and spleen until 9-12 days post infection. This early phase of viral expansion is followed by its entrance into latency primarily in B cells, but also lies dormant in macrophages and dendritic cells, establishing lifelong persistent infection (Weck et al., 1999). The pool of latently infected cells in the spleen is independent of the inoculating dose of MHV-68, suggesting host and viral homeostatic mechanisms regulate the maintenance of latency and reactivation (Tibbetts et al., 2003). MHV-68 uses a number of viral proteins to aid in the establishment of latency including LANA, vCyclin, vGPCR, vBcl-2 (Barton et al., 2011).

The potential for chronic infections to result in chronic inflammation has long been appreciated and has fueled speculation about the roles of herpesvirus
infections in exacerbating inflammatory diseases in humans. The discovery of MHV-68 allows for the study of the role of chronic γ-herpesvirus infection as modulator of inflammatory disease processes. The development of lymphoproliferative disease (LPD) has been observed during long-term infection of Balb/c mice with MHV-68 and systemic immune suppression by cyclosporin A further increases the incidence of LPD (Sunil-Chandra et al., 1994). In addition to driving de novo carcinogenesis, chronic MHV-68 infection has been shown to accelerate and worsen the pathology of other inflammatory conditions in mice. Experimental autoimmune encephalitis (EAE), widely considered a model of multiple sclerosis, is exacerbated in mice by acute and latent MHV-68 infection (Peacock et al., 2003). Development of pulmonary fibrosis is also accelerated by chronic MHV-68 infection (McMillan et al., 2008). MHV-68-infected ApoE-/- mice develop more severe atherosclerotic lesions (Alber et al., 2000). Chronic infection with MHV-68 exacerbated the morbidity, mortality, and colon pathology in the IL-10-deficiency model of spontaneous colitis (Nelson et al., 2009). Despite these finding, little is known about the mechanisms whereby MHV-68 infection modulates these disease processes. These data strongly implicate chronic γ-herpesvirus infection as a modulator of systemic inflammation.

**Innate Immune Recognition of γ-herpesviruses**

Control of viral infection first requires that the virus be recognized by PRRs of the innate immune system for the stimulation of acute inflammatory and
antiviral programs, as well as for the initiation of virus-specific adaptive responses for long-term control of the virus. The mechanisms of \( \gamma \)-herpesvirus recognition have been less well studied compared to the other members of the herpesvirus (e.g. HSV-1 and CMV), this may reflect the assumption that all herpesviruses are recognized through similar mechanisms. The establishment of chronic infection by herpesviruses presents a special case for the innate immune system. Studies on the mechanisms the innate immune system senses and responds to viruses is often studied within the context of acute infection. However, sensing of latent and reactivated virus may stimulate innate responses through other PRRs more than does acute infection, which may have important consequences for the control of persistent virus infection by innate and adaptive effector mechanisms. Nonetheless, the innate immune pathways necessary for detecting and controlling \( \gamma \)-herpesvirus infection are being elucidated.

TLRs 2, 3, and 9 have been implicated in the sensing of EBV, KSHV, and MHV-68 infection for the stimulation of the innate immune response. Infection of human macrophages and monocytes with EBV activates the NF-\( \kappa \)B pathway leading to expression proinflammatory cytokines in a TLR2-dependent manner (Gaudreault et al., 2007; Ariza et al., 2009). Sensing of the EBV-encoded dUTPase by TLR2 has been shown to be sufficient for the expression of luciferase driven by NF-\( \kappa \)B but how TLR2 recognizes dUTPase is unclear (Ariza et al., 2009). Sensing of MHV-68 by TLR2 in mouse embryonic fibroblasts (MEFs) has also been revealed to be important for upregulation inflammatory
cytokines and type I IFNs (Michaud et al., 2010). TLR2-deficient mice have decreased levels of IL-6 and type I IFN and increased viral loads during acute MHV-68 infection (Michaud et al., 2010). TLR3 recognizes the EBER non-coding RNA of EBV to trigger IRF3 phosphorylation and type I IFN production. EBER is present in the sera of patients with active EBV infection and is sufficient for induction of type I IFN and inflammatory cytokines when added to cells (Iwakiri et al., 2009). Recognition of EBV, KSHV, and MHV-68 genomic DNA by TLR9 stimulates production of IFN-α in pDCs, but type I IFN production is TLR9-independent in conventional DCs (cDCs) infected with MHV-68 (Guggemoos et al., 2008; Pezda et al., 2011; Fiola et al., 2010; West et al., 2010). Studies in mice demonstrated TLR9 is dispensable for the control of MHV-68 during the acute phase of infection but required for controlling viral burden during latency (Guggemoos et al., 2008).

Recent studies have found the sensing of γ-herpesvirus infection also occurs through the cytosolic PRRs. In addition to their sensing by TLR3, EBV EBERs are also recognized by RIG-I for inducing IFN (Samanta et al., 2006). IFI16 has been shown to form an inflammasome with ASC and caspase-1 for the processing of IL-1β during de novo KSHV infection of endothelial cells (Kerur et al., 2011). Work by Schoggins et al., described higher viral burden in the spleen and lungs of cGAS-deficient mice infected with MHV-68, but whether or not signaling through cGAS is required for cytokine and IFN upregulation in response to MHV-68 was not tested (Schoggins et al., 2014). Contrary to these findings
however, our work found STING, the downstream adaptor required for cGAS
signaling, was not required for the control of acute or latent MHV-68 (Sun et al.,
2015). Thus, further work is needed to better understand the role of the cGAS-
STING pathway in the immune response to γ-herpesvirus infection.

Figure 1.3 PRR sensing of γ-herpesvirus infection. TLR2 on the cell surface
senses EBV and MHV-68 to signal through the MyD88 pathway for downstream
activation of NF-κB. EBV dUTPase is sufficient for TLR2 activation, but its
unknown if it is required. EBER non-coding RNA from EBV activates TRIF-
dependent signaling through TLR3. EBV, KSHV, and MHV-68 genomic DNA are
recognized by TLR9 for MyD88-dependent signaling. RIG-I recognizes EBERs
leading to MAVS-dependent IFN production. IFI16 associates with ASC and
caspase-1 near the nucleus of cells infected with KSHV for IL-1β processing.
Dissertation Objectives

This dissertation has two main focuses: (1) define the pathways responsible for inflammasome activation by γ-herpesvirus infection and determine its role for *in vivo* for the immune response to MHV-68 infection, and (2) to determine whether DAMPs released into tissues damaged by viral infection modulates the host immune response to the virus.

1.1 *Investigate the innate immune response to MHV-68 infection.* Using primary mouse macrophages and dendritic cells, we compared the induction of innate immune response genes in response to HSV-1, MCMV, and MHV-68 infection. We found MHV-68 infection stimulated a milder innate immune response compared to HSV-1 and MCMV using Nanostring gene expression analysis.

1.2 *Determine the ability of MHV-68 to activate the inflammasome and define the sensor necessary for its recognition.* We compared MHV-68 stimulated IL-1β production by primary mouse macrophages and dendritic cells from mice deficient for the inflammasome proteins NLRP3, AIM2, and ASC. Here, we found MHV-68 infection resulted in the secretion of IL-1β in a manner dependent on ASC and NLRP3.

1.3 *Define the role of the NLRP3 inflammasome for the immune response to and control of MHV-68 infection in vivo.* Cytokine levels (IL-1β and IFNγ) and viral loads during the acute phase, and viral loads during the latent phase were
measured in Nlrp3-/-, Asc-/-, and Aim2-/- mice infected with MHV-68. Here, we observed decreased amounts of IL-1β and IFNγ in Nlrp3-/- and Asc-/- mice during acute MHV-68 infection. NLRP3 and ASC were dispensable for controlling viral burden during the acute phase, but Nlrp3-/- and Asc-/- mice had higher loads of MHV-68 during latent infection.

2.1 Investigate the presence and quantities of DNA in the lung microenvironment during IAV infection. Histological examination of lungs from IAV infected mice indicated the presence of extracellular DNA extruded from necrotic cells. Quantification of DNA in the BAL fluid of IAV infected mice revealed the amount of DNA in the lung increases as the infection progresses to involve more tissue.

2.2 Determine the role of cytosolic DNA sensors in shaping the immune response to IAV infection. First, we found mice deficient for the cytosolic DNA sensor AIM2 were susceptible to IAV infection. AIM2 was not required for IL-1β production by primary mouse dendritic in response to IAV infection in vitro. Inflammatory cytokines level and the number of leukocytes recruited to the lung were increased in Aim2−/− mice during IAV infection in vivo. AIM2 was not required for the control of viral replication or the formation of IAV-specific T cell responses. Using Nanostring and qPCR analysis, we compared the induction of innate immune response genes in WT and Aim2−/− alveolar macrophages to IAV infection ex vivo. Here, we found cell intrinsic effects as the expression of several ISGs and cytokines was increased in Aim2−/−.
macrophages. Taken together, these findings show AIM2 tempers the lethal inflammatory response to IAV infection.

2.3 Determine if DNA released into the lung microenvironment during IAV infection is required for protection and dampening of inflammation. We generated a recombinant adeno-associated virus vector for ectopic expression of DNasel in the lungs of mice and examined its effect on mortality, cytokine responses, and cellular recruitment during IAV infection. Wild-type mice treated with the AAV-DNasel vector had increased numbers of T cells lung and were more susceptible to IAV infection. AAV-DNasel treatment had no effect on cytokine responses and viral titers.
Preface to Chapter 2

Some of the data from this chapter has appeared in the following publication:


*These authors contributed equally to this work.

All experiments in this chapter were done by Stefan A. Schattgen
Chapter 2

Defining the role of inflammasomes in innate immune responses to gammaherpesvirus infection
Abstract

Herpesviruses are a family of dsDNA viruses able to establish lifelong, latent infection in their co-evolved hosts. Infection with herpesviruses stimulates the innate immune response leading to induction of inflammatory molecules and establishment of the antiviral state. An important host response pathway in detecting herpesviruses is the inflammasome complex. Sensing of the virus by cytosolic NLR or PYHIN proteins stimulates assembly of the complex leading to activation of its effector protein caspase-1, which cleaves its downstream substrates IL-1β and IL-18 for their activation and secretion. While the receptors have been identified for inflammasome activation in response to α-herpesvirus (HSV-1) and β-herpesvirus (MCMV), it is unknown whether γ-herpesviruses trigger the inflammasome and if so, what receptor is necessary for their detection. In these studies, we found infection with a mouse γ-herpesvirus (MHV-68) stimulated secretion of IL-1β by APCs in vitro, and this required the cytosolic PRR NLRP3, and inflammasome adaptor ASC, but not AIM2. ASC and NLRP3-deficiency had no effect on controlling viral replication during acute infection, however latent viral loads in the spleen were increased in their absence. Taken together, we highlight previous uncharacterized role for the inflammasome in γ-herpesvirus infection.
Introduction

Herpesviruses are important human pathogens that establish lifelong latent and recurrent infection. In most cases, acute infection with one of the eight human herpesviruses is asymptomatic but can lead to more serious complications and pathologies, particularly in the immunocompromised. Members of the herpeviridae family are classified into the $\alpha$, $\beta$, and $\gamma$ subfamilies based on genome similarity and tropism. Herpesvirus virions consist of a protein capsid containing a large dsDNA genome and tegument proteins, enveloped in a lipid membrane. Infection occurs via fusion of the virion with the plasma membrane or entry through the endosomes which ejects the genome containing capsid into the cytosol where it quickly shuttles to the nucleus to begin early transcription and replication (Pellet and Roizman, 2007). Sensing of viral infection by the innate immune system is the first line of defense in host protection against herpesviruses (Bowie and Unterholzner, 2008). While much work has been done to characterize the innate immune pathways and PRRs important for recognizing and stimulating the immune response to HSV-1 and CMV, the innate mechanisms responsible for sensing $\gamma$-herpesvirus infection are only now being elucidated.

Viral nucleic acids are believed to be the most important class of molecules recognized by PRRs for the stimulation of innate immune and antiviral responses (Barbalat et al., 2011). Two important biological responses to viral
infection are the production of type I IFN and IL-1β maturation downstream of inflammasome activation. In the case of γ-herpesviruses, it has been shown that recognition of viral RNA and DNA leads to the induction of type I IFN and inflammatory cytokines through endosomal TLRs (Fiola et al., 2010; Iwakiri et al., 2009). However it has recently been discovered that recognition of viral DNA by cytosolic and nuclear DNA receptors is also important for stimulating immune responses to herpesvirus infection (Rathinam et al., 2010; Unterholzner et al., 2010; Kerur et al., 2011; Sun et al., 2013). Sensing of cytosolic DNA induces the production of type I IFN and other inflammatory cytokines through the adaptor protein stimulator of IFN genes (STING) (Ishikawa et al., 2009). Recognition of HSV-1 and CMV viral DNA in the cytosol has been shown occur through cyclic-GMP-AMP synthetase (cGAS) and interferon-inducible protein 16 (IFI16) upstream of STING-dependent cytokines responses (Sun et al., 2013; Unterholzner et al., 2010).

In addition to type I IFN production another important response to viral infection is activation of the inflammasome by cytosolic PRRs. The inflammasome is a large multimeric protein complex comprised of a receptor, the CARD and PYRIN domain adaptor protein ASC, and the effector protease caspase-1. Assembly and activation of the inflammasome in response to microbial and environmental stimuli triggers the processing of caspase-1 into its active form which goes on to cleave the inflammatory cytokines IL-1β and IL-18 into their biologically active forms and allows for their exit from the cell (Davis et
al., 2011). Work from our lab has found inflammasome activation by α-herpesvirus (HSV-1) and β-herpesvirus (MCMV) requires the cytosolic PRRs nucleotide-binding oligomerization domain protein-like receptors protein 3 (NLRP3) and absent in melanoma 2 (AIM2), respectively (Kaminski, 2014; Rathinam et al., 2010). AIM2 is a PYHIN protein with a HIN-200 DNA binding domain and PYRIN domain able to complex with ASC and caspase-1 forming an inflammasome in response to dsDNA (Hornung et al., 2009). NLRP3 on the other hand indirectly senses a wide variety of host and microbial molecules. Importantly, it has been shown that NLRP3 indirectly senses viral RNA and disrupted intracellular ion gradients during influenza A infection leading to inflammasome activation (Allen et al., 2009; Ichinohe et al., 2010). It is unclear how NLRP3 senses herpesvirus infection and why different herpesviruses activate different inflammasomes. However, despite the importance of NLRP3 and AIM2 in the response to α- and β-herpesviruses, there have been no studies to date examining their role in innate responses to γ-herpesvirus infection.

The human γ-herpesviruses include Epstein-Barr Virus (EBV; HHV-4) and Kaposi’s sarcoma-associated herpesvirus (KSHV; HHV-8). Detection of EBV and KSHV viral DNA has been shown to occur through TLR9. It has also been reported that IFI16 forms an inflammasome in response to KSHV DNA around the nucleus to stimulate caspase-1 and IL-1β activation in endothelial cells. However IFI16 does not appear to form an inflammasome in other cell types tested (Kerur et al., 2011; Hornung et al., 2009). Nonetheless, a role for
inflammasome activation in shaping the immune response to \( \gamma \)-herpesvirus infection in vivo has not been examined. In these studies, we found mouse \( \gamma \)-herpesvirus-68 (MHV-68) stimulated modest IL-1\( \beta \) secretion by APCs in response to infection in vitro. Furthermore, sensing of MHV-68 infection by NLRP3, but not AIM2, was required for inflammasome activation. NLRP3 and ASC were also required for the control of acute lytic replication during in vivo MHV-68 infection. Interestingly, latent viral loads were significantly increased in the absence of either NLRP3 or ASC. Collectively, these findings show that infection with the \( \gamma \)-herpesvirus MHV-68 activates the NRLP3 inflammasome and that this pathway influences the establishment of lytic-latent viral homeostasis.

**Results**

**Characterization of the innate immune response to MHV-68 infection**

To begin our studies on innate immune recognition and responses to MHV-68, we compared the induced expression of immune response genes in WT (C57BL/6J) mouse bone-marrow derived macrophages (BMDM) and bone-marrow derived dendritic cells (BMDCs) in response to representative viruses from the \( \alpha \)- (HSV-1), \( \beta \)- (MCMV), and \( \gamma \)-herpesvirus (MHV-68) subfamilies. Additionally, we compared the responses between a strain of HSV-1 lacking the immediate-early protein ICP0 (7134) (Cai and Schaffer, 1989), and the revertant WT strain (7134R). ICP0 is a viral E3 ligase able to block the induction of
immune response genes downstream of IRF3/7 and NF-κB activation (Lin et al., 2004; Zhang et al., 2013a). Expression of 94 different immune response genes was measured in total RNA purified from cells at 6 hours post-infection using Nanostring technology, a fluorescent mRNA barcoding technology for multiplexed host-cell transcript quantification. An MOI 10 was used for each virus to better compare the quality of the response to a similar number of infectious virions. Infection of WT BMDMs and BMDCs with all the viruses tested induced most of the genes on our custom codeset of innate immune response, cytokine/chemokines, antiviral genes, and interferon-stimulated genes (ISGs) (Fig 2.1 and Appendix II). Of the viruses tested, infection with ICP0-deficient strain of HSV-1 7134 stimulated the highest level of induction for most genes measured, especially in macrophages (Fig 2.1B). MCMV stimulated higher expression for a number of genes in dendritic cells compared to HSV-1 7134, however (Fig 2.1C). Infection with the ICP0-sufficient HSV-1 7134R elicited significantly weaker responses compared to HSV-1 7134. Interestingly, ICP0 appeared to have a stronger effect on blocking PRR signaling in dendritic cells compared to macrophages (Fig 2.1 B+C). Infection of both macrophages and dendritic cells with MHV-68 stimulated upregulation of most of the genes analyzed (Fig 2.1 B+C) however; the responses to MHV-68 were less than those to HSV-1 7134 and MCMV. The responses in BMDMs infected with MHV-68 were, in particular, lower than the response to HSV-1 and MCMV. In contrast, the responses in dendritic cells were more similar across viruses, with exception
of HSV-1 7134R, suggesting they are more sensitized to detecting herpesvirus infection than macrophages (Fig 2.1 B).

Upon closer inspection, we found the upregulation of a number of cytokines (Fig 2.2A-D) and ISGs (Fig 2.2E-H) to be particularly low in response to MHV-68. Infection of BMDMs and BMDCs with MHV-68 resulted in a dramatically lower level of induction of il6, tnfa, and il12b (Fig 2.2A-C), relative to the response to HSV-1 7134. For example, HSV-1 7134 infection in BMDMs led to a ~12,000-fold induction in il6 compared to ~166-fold seen in MHV-68 (Fig 2.2A). Expression of il10 was limited to BMDMs but again lower in response to MHV-68 infection compared to HSV-1 7134 or MCMV (Fig 2.2D). Expression of type I IFNs (ifnb1 and ifna4) and the IFN-dependent chemokine cxcl10 was similarly lower in response to MHV-68 compared to HSV-1 7134 and MCMV (Fig 2.2D-G). Interestingly, expression of stat1, the transcription factor critical to amplifying the IFN response, was similar in response to all viruses (Fig 2.2H). We further investigated what affect herpesvirus infection had on the expression of PRRs previously demonstrated or implicated in detecting their invasion (Paludan et al., 2011). Infection with all the herpesviruses stimulated upregulation of tlr2, tlr3, tlr9, and ifi204 in both BMDMs and BMDCs (Fig 2.2I-L). Notably, MHV-68 infection stimulated upregulation of tlr2, tlr3, tlr9, and ifi204 to similar levels as the other viruses. These observations suggest that despite similar expression of PRRs with known roles in detecting herpesvirus infection, MHV-68 stimulated a milder innate immune response compared to HSV-1 and MCMV.
Figure 2.1 MHV-68 induces mild innate immune responses in vitro. (A) Heatmap visualization of innate immune response gene expression in BMDMs and BMDCs infected with HSV-1 7134 (MOI 10), HSV-1 7134R (MOI 10), MCMV (MOI 10), MHV-68 (MOI 10) for 6 hours. Total RNA used for Nanostring analysis. Each square represents the normalized transcript count for respective gene and virus. (B and C) Upregulation of immune response genes in (B) BMDMs and (C) BMDCs infected with herpesviruses. Shown as log2 fold-change over mock control. Genes ranked from most to least induced in HSV-1 7134 infected samples.
Figure 2.2 MHV-68 weakly stimulates cytokines and ISGs. (A-L) Normalized transcript counts for selected cytokines (A-D), ISGs (E-H), and PRRs (I-L) in response to herpesvirus infection. Plots are representative of results from values displayed in Figure 2.1A.
Sensing of MHV-68 infection by NLRP3 stimulates inflammasome activation and IL-1β secretion.

Our lab has long been interested in identifying the cytosolic receptors able to activate the inflammasome upon sensing viral infection. We previously identified absent in melanoma 2 (AIM2) as the cytosolic DNA receptor required for inflammasome activation in response to MCMV but not HSV-1 (Rathinam et al., 2010). Interestingly, we found sensing of HSV-1 infection by NLRP3 was required for inflammasome activation (Kaminski, 2014). Clearly different subfamilies of herpesviruses are sensed by different cytosolic PRRs leading to inflammasome activation. The mechanisms that determine whether detection of herpesviruses occurs by one receptor or another are unknown. In these studies, we sought to determine whether γ-herpesvirus infection stimulated inflammasome activation, and which receptor is required for this response.

To better understand the relationship between transcriptional responses of genes for inflammasome components to herpesvirus infection, we examined the expression of nlrp3, aim2, casp1, il1b, and il18 in our Nanostring dataset. Expression of both nlrp3 and aim2 were both strongly induced by HSV-1 7134 infection in BMDCs and BMDMs (Fig 2.3A+B). Here, upregulation of aim2 in response to MHV-68 was comparable to the other viruses (Fig 2.3B) however; induction nlrp3 was low in both BMDMs and BMDCs (Fig 2.3A). Expression of the inflammasome effector protease, caspase-1, was upregulated to similar levels in response to all the viruses tested (Fig 2.3C). pro-IL-1β and pro-IL-18
are important substrates of caspase-1 downstream of inflammasome activation, which are then upon secreted from the cell as inflammatory cytokines. Here, the expression of *il1b* was upregulated to varying levels in response to different viruses. Induction of *il1b* was robust in response to 7134 infection while 7134R, MCMV, and MHV-68 induced its expression at much lower, but detectable, levels (Fig 2.3D). Expression of *il18* was induced to similar levels by infection with all the viruses in BMDMs. In contrast BMDCs appeared to have high basal levels of *il18* and this gene was induced only in response to MCMV (Fig 2.3E). Broadly, infection with all herpesviruses induced the expression of receptors and effectors required for inflammasome activation.

As many of the inflammasome components are induced via common transcriptional pathways it is difficult to draw definite conclusions about their particular roles based solely on relative expression. Thus we sought to determine which sensor was required for IL-1β secretion in response to MHV-68 using primary immune cells from mice genetically deficient for inflammasome proteins. Since both AIM2 and NLRP3 have been previously shown to be required for inflammasome activation in response to herpesviruses, we hypothesized MHV-68 would be likely be sensed by one of these receptors. Here, we measured IL-1β secretion by primary BMDMs and BMDCs from mice deficient in well-described inflammasome receptors in response to MHV-68. Cells were first stimulated with LPS in order to upregulate pro-IL-1β expression prior to viral infection. Here we found MHV-68 stimulated IL-1β secretion in both BMDCs
(MOI 100; Fig 2.4B) and BMDMs (MOI 10; Fig 2.4B), and this required the inflammasome adaptor protein ASC, which is required for the recruitment and activation of caspase-1 downstream of both NLRP3 and AIM2. IL-1β production was low, but detectable, despite the high MOI of MHV-68 used in these experiments. In contrast, we saw robust IL-1β production in response to nigericin (NLRP3), polydA:dT (AIM2) delivered to the cytosol by lipofectamine transfection, and MCMV (AIM2). NIGERICIN, polydA:dT AND MCMV behaved as expected and sensing by their respective receptors was required for IL-1β secretion by both BMDCs (Fig 2.4A) and BMDMs (Fig 2.4B). Production of IL-1β in response to MHV-68 was reduced in both Nlrp3-/- and Asc-/- cells while deletion of AIM2 had not no effect in BMDMs (Fig 2.4A) and BMDCs (Fig 2.4B). Similarly deletion of other inflammasome sensors including: NLRP1b, NLRP6, NLRP12, and IPAF, had no effect on IL-1β secretion in response to MHV-68 (data not shown). Together these findings identify NLRP3 as the receptor required for inflammasome activation in response to MHV-68.
Figure 2.3 MHV-68 infection upregulates the expression of inflammasome components. (A-E) Normalized transcript counts for nlrp3 (A), nlrp3 (B), casp1 (C), il1b (D), and il18 (E) in response to herpesvirus infection. Plots are representative of results from values displayed in Figure 2.1A.
Figure 2.4 NLRP3 is required for inflammasome activation by MHV68. (A) BMDCs and (B) BMDMs from WT (C57BL/6J or Aim2+/+ littermate), Aim2−/−, Nlrp3−/−, and Asc−/− mice were primed for 3 hours with LPS (100 ng/mL) prior to treatment with nigericin (10 μM), poly dA:dT complexed with lipofectamine (1 μg/mL), MCMV (MOI 10), and MHV-68 ((A) MOI 100 and (B) MOI 10). Supernatants were harvested 16 hours later and IL-1β measured by ELISA. Representative of at least three or more independent experiments. **, P ≤ 0.01; ****, P ≤ 0.001
Characterizing the immune response to MHV-68 infection in vivo. A role for the inflammasome.

Upon identifying NLRP3 as the sensor of MHV-68 required for inflammasome activation in vitro, we focused our attention to the affect of inflammasomes on stimulating immune responses and controlling acute MHV-68 infection in vivo. There have been no previous studies investigating what role inflammasomes have in shaping the immune response to MHV-68. Any requirement for IL-1R or IL-18R signaling in controlling MHV-68 infection also has yet to be described. During MHV-68 infection, the virus first undergoes robust replication in mucosal epithelium tissues and spleen prior to entering latency in the spleen after several weeks. Here we tested whether there was a requirement for NLRP3, ASC, and AIM2 in controlling the lytic replication phase of MHV-68 infection. Mice were given $5 \times 10^5$ pfu of MHV-68 by intraperitoneal injection and their spleens and serum were harvested at 6 dpi. Splenomegaly, a result of early B cell proliferation, is a common pathological finding during acute MHV-68 infection (Weck et al., 1996). Spleen weights significantly increased over mock treated controls at 6 dpi with MHV-68 at (Fig 2.5A-C). There was no difference in spleen weight of $Nlrp3^{-/-}$ (Fig 2.5A), $Asc^{-/-}$ (Fig 2.5B) and $Aim2^{-/-}$ (Fig 2.5A) compared to controls at 6 dpi. We measured viral loads in the spleen at 6 dpi by determining the copy number of viral genomes in DNA extracted from the spleen. $Nlrp3^{-/-}$ (Fig 2.5A), $Asc^{-/-}$ (Fig 2.5B) and $Aim2^{-/-}$ (Fig 2.5A) mice had similar
Figure 2.5 AIM2, ASC, and NLRP3 are dispensable in controlling acute MHV-68 infection in vivo. (A-C) Spleen weights of Nlrp3-/- (A), Asc-/- (B), and Aim2-/- (C) mice and respective WT controls at 6 dpi with 5 x 10^5 pfu i.p. (D-F) Viral loads in spleens of Nlrp3-/- (D), Asc-/- (E), Aim2-/- (F) and WT controls at 6 dpi. DNA isolated from spleen tissue was use as template to quantify number of MHV-68 genomes present in reference to a plasmid standard curve. Representative of two independent experiments *, P ≤ 0.05; ***, P ≤ 0.005
viral loads as their WT counterparts, suggesting the inflammasome is not required to control acute MHV-68 infection in the spleen.

Next, we investigated the role of NLRP3, ASC, and AIM2 in regulating IL-1\textbeta production in the spleen during MHV-68 infection. There was no detectable IL-1\textbeta in the serum at this time but there was a modest increase in IL-1\textbeta in the spleen at 6 dpi (Fig 2.6A-C). Despite our in vitro findings, the amount of IL-1\textbeta in Nlrp3-/- spleen was similar to those in WT mice (Fig 2.6A). Asc-/- spleens had a significant reduction in IL-1\textbeta however, suggesting the inflammasome complex is required for IL-1\textbeta responses to MHV-68 (Fig 2.6B). In contrast to response in Asc-/- mice, Aim2-/- mice had a notable increase in IL-1\textbeta in the spleen compared to AIM2 +/+ controls (Fig 2.6C).

IFN\textgamma is an important cytokine in limiting acute MHV-68 infection (Tsai et al., 2011). We have previously shown IFN\textgamma production by NK cells during MCMV infection requires the AIM2 inflammasome (Rathinam et al., 2010). Nlrp3-/- mice had a marked decrease in IFN\textgamma protein in both the spleen and serum compared to WT (Fig 2.6D+G). Surprisingly, IFN\textgamma levels in WT and Asc-/- mice were similar in both spleen and serum (Fig 2.6E+H). In line with the observations for IL-1\textbeta, Aim2-/- mice had increased amounts of IFN\textgamma in the spleen and serum (Fig 2.6F+I).

Once early MHV-68 replication is controlled by the immune system at 14 to 16 dpi, the virus then establishes lifelong latency in germinal center B cells (Collins et al., 2009). In a final set of experiments, we measured the effect of
NLRP3, ASC, and AIM2 on controlling latent viral burden in the spleen. Nlrp3−/− and Asc−/− mice had higher copy numbers of MHV-68 DNA in the spleen compared to WT at 60 dpi, while AIM2 deficiency had no affect on latent viral load (Fig 2.7). These findings suggest a role for the NLRP3 inflammasome in controlling the viral burden during latent MHV-68 infection.
Figure 2.6 Differential roles for AIM2, ASC, and NLRP3 in regulating early IL-1β and IFNγ during MHV-68 infection. Protein Levels of IL-1β (A-C) and IFNγ (D-F) in spleen homogenates of Nlrp3-/- (A+D), Asc-/- (B+E), and Aim2-/- (C+F) mice and respective WT controls at 6 dpi with 5 x 10⁵ pfu i.p. (G-I) IFNγ levels in the serum of Nlrp3-/- (G), Asc-/- (H), Aim2-/- (I) and WT controls at 6 dpi. ELISA was used to quantitate IL-1β and IFNγ. Representative of two independent experiments. P ≤ 0.05; **, P ≤ 0.01
Figure 2.7 NLRP3 and ASC control latent MHV-68 burden. Comparison of MHV-68 genome copy number in spleens of B6, Nlrp3−/−, Asc−/−, and Aim2−/− mice at 50 dpi. DNA isolated from spleen tissue was used as template to quantify number of MHV-68 genomes present in reference to a plasmid standard curve. Experiment performed once. **, P ≤ 0.01; ***, P ≤ 0.005
Discussion and Perspectives

In these studies, we aimed to determine: (1) if MHV-68 stimulates the innate immune response and how these responses compared to those of other herpesviruses, (2) if MHV-68 is sensed by cytosolic PRRs, and (3) how these PRRs affect immune responses in vivo. Infection of antigen-presenting cells (APCs) with MHV-68 induced the expression of innate immune response genes in a modest manner compared to responses elicited by HSV-1 and MCMV. MHV-68 infection triggered IL-1β secretion by APCs, and its recognition required the inflammasome components NLRP3 and ASC but not AIM2. Mice deficient in NLRP3 and ASC had lower IL-1β and IFNγ responses with no effect on viral burden during the lytic-phase of MHV-68 infection, but latent viral loads were higher in mice lacking NLRP3 and ASC. Interestingly, Aim2−/− mice had increased IL-1β and IFNγ responses with no difference in acute or latent viral burden suggesting it may suppress inflammation in response to MHV-68 independently of its function as an inflammasome receptor. These data taken together begin to describe a previous unknown role for the NLRP3 inflammasome in the immune response and control of γ-herpesvirus infection.

We began our studies comparing the transcriptional responses of cytokines/chemokines, ISGs, PRRs, and other immune-related genes to infection with an α- (HSV-1), β- (MCMV), or γ-herpesvirus (MHV-68) in primary mouse dendritic cells and macrophages (Fig 2.1). While MHV-68 induced the expression of many of the genes we analyzed, this response was milder than
those to HSV-1 7134 and MCMV infection at the same MOI. Others have also reported MHV-68 as poorly immunogenic, inhibiting type I IFN production and cDC maturation despite being able to infect, replicate, and establish latency in cDCs (Weslow-Schmidt et al., 2007; Flano et al., 2005). Several mechanisms used by the virus to evade PRR recognition have been highlighted in recent years. Work by Pezda et al., found the number of TLR9-stimulatory CpG motifs to be low in the genome of MHV-68 compared to MCMV and suggested that evolutionary pressure has selected for viral species with less immunogenic genomes (Pezda et al., 2011). Our own work identified the MHV-68 large tegument protein and deubiquitinase (DUB) ORF64 as a suppressor of STING-dependent cytokine responses, mutation of which increased the type I IFN and cytokine response by cDCs (Sun et al., 2015). The conserved herpesvirus kinase ORF36 and the tegument protein ORF11 have similarly been shown to target the type I IFN pathway by blocking activation of IRF3 and TBK1 downstream of STING (Hwang et al., 2009; Kang et al., 2014). Clearly the virus goes to considerable lengths to avoid and inhibit detection of viral nucleic acids by PRRs leading to type I IFNs, highlighting their importance in controlling MHV-68 infection.

Outside the type I IFN response, sensing of viruses by other cytosolic PRRs triggers assembly of the inflammasome complex the subsequent production of IL-1β and IL-18 secretion. Activation of the NLRP3 and AIM2 inflammasome is an important step in mounting the immune response to
herpesvirus infection. Work from our group has determined inflammasome activation in BMDMs and BMDCs by HSV-1 required NLRP3 (Kaminski, 2014), and AIM2 was required to respond to MCMV infection (Rathinam et al., 2010). Here, we found MHV-68 weakly triggered the secretion of IL-1\(\beta\) in vitro and required NLRP3 but not AIM2 for inflammasome activation. It is interesting to note that inflammasome activation by the herpesviruses across subfamilies does not occur through the same receptor. NLRP3 senses a variety of environmental and microbial insults, including a variety of RNA and DNA viruses (Gram et al., 2012). Indirect sensing of viral RNA by NLRP3 has been described as a mechanism of inflammasome activation for RNA viruses (Allen et al., 2009), however the mechanisms of NLRP3 activation in response to DNA viruses is entirely unknown. On the other hand, while it is clear the AIM2 inflammasome is activated by directly binding dsDNA (Hornung et al., 2009), it is unclear why it recognizes MCMV but fails to recognize MHV-68 and HSV-1 infection. One plausible explanation is herpesviruses spend little time in the cytosol after infecting its host cell. The genome, surrounded by viral capsid, shuttles to the nucleus to begin replication and early gene expression, avoiding cytosolic PRRs on the way. Once it is in the nucleus the viral genome is quickly chromatinized, binding up free histones and other host cell DNA-binding proteins thus further masking it from PRRs (Conn and Schang, 2013). This is not a sufficient explanation though given that herpesviruses are clearly sensed by cytosolic and endosomal PRRs, thus there appears to be some leaking of nucleic acids and
other molecules from the capsid en route to the nucleus. Indeed, we found the genome of MHV-68 located in both the cytosol and nucleus early after infection (Sun et al., 2015). Viral DNA packaged in the capsid of HSV-1 virions is naked and forms electrostatic interactions with capsid proteins to compact the genome meaning it should be easily recognized by DNA receptors in the nucleus (Gibson and Roizman, 1971; Cohen et al., 1980). If this is the case then why do we not see activation of the AIM2 inflammasome by all the herpesviruses?

Besides localization and chromatinization of the genome there are perhaps other active mechanisms used by the virus to circumvent AIM2 recognition. Mutation of ORF64 increased the IL-1β response to MHV-68 in cDCs (Sun et al., 2015), but at what step ORF64 blocks inflammasome priming or activation remains to be seen. MHV-68 may have other unidentified proteins blocking the inflammasome as well. Indeed, the tegument protein ORF63 (aka UL37) of KSHV is a viral NLR homolog able to antagonize the NLRP1, NLRP3, and the NOD2 inflammasomes (Gregory et al., 2011). ORF63 of MHV-68 appears to be a homologous to ORF63 of KSHV and share a remarkable similarity to mouse NLRs and is likely to inhibit inflammasome activation as well (unpublished analysis). Viral inhibitors of the inflammasome are likely to be found in other herpesvirus subfamilies as well.

In addition to AIM2, the human PYHIN protein IFI16 has been shown to form an inflammasome complex with ASC and caspase-1 that co-localizes with KSHV DNA around the nucleus (Kerur et al., 2011), however our group could not
demonstrate the formation of an IFI16 inflammasome in THP-1 cells (Hornung et al., 2009). Nonetheless, mice lack an obvious homolog of IFI16 though their genome encodes more PYHINs than humans. The function for most of the mouse PYHIN proteins is unknown and its possible one of these could form an inflammasome in and around the nucleus in response to foreign viral DNA. Work from Brunette et al. demonstrated several of the mouse PYHINS induced aggregation of ASC when overexpressed in HeLa cells (Brunette et al., 2012), thus it is possible several members of the mouse PYHIN proteins may form inflammasomes in different cellular compartments and contexts.

There have been no studies to date looking at the role of inflammasome or IL-1R signaling in the immune response to MHV-68 infection. NLRP3 and ASC were required for IL-1β secretion in vitro, however levels of IL-1β in the spleen were similar in Nlrp3−/− but decreased in Asc−/− mice (Fig 2.6). Interestingly, IFNγ was decreased in the spleen and serum in Nlrp3−/− but not Asc−/− mice. (Fig 2.6). IFNγ is important for controlling viral replication during acute and latent MHV-68 infection (Tsai et al., 2011; Steed et al., 2006), however NLRP3 and ASC were dispensable for controlling acute viral replication suggesting the levels of IFNγ were sufficient in either case (Fig 2.5). AIM2-deficient mice on the other hand had increased amounts of IL-1β and IFNγ during acute infection and a mild increase in viral loads. These observations might indicate an inflammasome-independent function for AIM2 in controlling MHV-68 replication. Whether AIM2 might limit viral replication in a cellular intrinsic or extrinsic manner remains to be
seen. From these studies it appears the inflammasome is not a major pathway in controlling acute MHV-68 infection.

MHV-68 establishes latency in peritoneal macrophages and B cells in the spleen where it ceases to replicate by 16 to 18 dpi. After the establishment of latency by MHV-68, low levels of spontaneous and stimulated reactivation occur in latently infected cells. Despite no clear effect on limiting viral replication early, NLRP3- and ASC-deficient mice had increased loads of latent MHV-68 in the spleen at 50 dpi (Fig 2.7). Viral burden during latent MHV-68 infection is independent of the initial dose, and the pool of latently infected cells is held stable by homeostatic mechanisms in both the host and virus (Tibbetts et al., 2003). Many of the host factors controlling the size of the latency pool are proteins involved in the induction and signaling of type I IFN. Latent viral loads increase in the absence of STING, cGAS, and TLR9 (Sun et al., 2015; Schoggins et al., 2014), strongly implicating IFN-dependent regulation of MHV-68 in latency. In these studies IL-1R and IL-18R signaling was implicated as another input maintaining the balance between latent virus and host, however further studies using mice deficient in IL-1R/IL-18R would support the role of cytokines downstream of inflammasome activation in MV-68 infection.

In these studies we determined MHV-68 is able to activate the NLRP3 inflammasome and this was required in vivo for maintaining a low latent viral burden. It is important to note that MHV-68 was a weak stimulator of IL-1β in vitro, thus even low levels of IL-1R signaling appear to act on latent MHV-68.
Special consideration should be taken as we investigate the innate immune responses to herpesvirus infection since unlike most viral infections; it establishes lifelong latency and parasitism in the host, with periods of reactivation providing new antigen and PAMPs to be sensed by the immune system. We often investigate the role of PRRs for their ability to detect early viral infection and immediate initiation of antiviral and inflammatory programs, however detection of reactivated virus by PRRs is likely to have a profound impact on the quality and magnitude of memory and inflammatory responses. Further work examining the contribution PRRs in the detection reactivating herpesvirus would likely be insightful in understanding the long-term immune control of chronic virus infection.

In addition to understanding the immune response to acute γ-herpesvirus, the discovery of MHV-68 provides a useful model for studies into latent γ-herpesvirus infection as a co-factor modulating inflammatory responses to acute co-infections and chronic inflammatory conditions. Indeed, transcriptional analysis of tissues with latent MHV-68 infection revealed a modest but significant upregulation in inflammatory cytokines and immune responses (Canny et al., 2013). MHV-68 infection protects mice against *L. monocytogenes* and *Y. pestis* infection by increasing the basal immune activity of latently infected macrophages (Barton et al., 2007), and another group reported similar findings for influenza infection (Saito et al., 2013). In addition to modulating immune responses during co-infection, latent MHV-68 infection has been demonstrated to
exacerbate inflammation in models of experimental autoimmune encephalitis and atherosclerosis (Peacock et al., 2003; Alber et al., 2000). Highlighting its affect on immune responses in the gut, MHV-68 infection accelerated the development of colitis with worsened pathology and increased the permeability of the colon epithelium in the IL-10-deficiency model of experimental colitis (Nelson et al., 2009). Notably, the NLRP3 inflammasome and downstream IL-1R signaling have been shown to be important modulators of inflammation in these models (Gris et al., 2010; Duewell et al., 2010; Zaki et al., 2010).

The potential for chronic herpesvirus infections to drive systemic and local inflammation and exacerbate inflammatory disorders in humans long been appreciated and speculated upon. EBV and HHV-6 infection in humans is associated with the development of systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and multiple sclerosis (MS) amongst others (Lossius et al., 2012). Though γ-herpesvirus infection is neither causative nor sufficient to drive the development of autoimmunity in susceptible individuals, it is becoming increasingly evident that responses controlling latent γ-herpesvirus infection may have broader effects on the maintenance of host tolerance. A tempting extension of the immune modulatory effects of latent γ-herpesvirus infection is their potential contribution in tumorgenesis. The idea that chronic inflammation may be a major contributor to tumorgenesis has gained significant support in the research community (Trinchieri, 2012). This low grade inflammation or “para-inflammation” as result of tissue stress or malfunction is commonly found during
tumorgenesis, and provides proliferative signals to the cancerous cell (Medzhitov, 2008). Of the eight human herpesviruses only EBV and KSHV infection are associated with the development of cancer, particularly lymphoproliferative diseases and lymphoma. There has been much effort implicating transformation by EBV and KSHV infection as the causation of lymphomas, but could the systemic modulatory effects of latent γ-herpesvirus infection on the immune response contribute to the development of cancer? Future works investigating para-inflammation surrounding viral latency and the development cancer will be insightful as to whether or not these processes are linked.

While it is clear that latent γ-herpesvirus infection induces para-inflammation, the mechanisms used by the innate immune system to detect latent infection remain unclear. Viral nucleic acids are the most abundant viral product in the cell during latency thus they are likely the main source of signal inciting the innate immune system, but several questions remain. How is the DNA genome detected by cytosolic receptors once it is wrapped in host histones and DNA binding proteins in the nucleus? How is herpesvirus infection sensed by the NLRP3 inflammasome but avoids detection by AIM2? What signaling pathways mediate para-inflammation during latent infection? Are the modulatory effects on the innate immune response by latent γ-herpesvirus infection largely cellular intrinsic or systemic? Future studies into the relationship between latent infection of cells of the immune system and the homeostatic mechanisms of inflammation
and chronic disease would bring more insight into the complicated long-term relationships between us and herpesviruses.
Chapter 3

DNA in the lung microenvironment during influenza virus infection tempers inflammation by engaging the DNA sensor AIM2
Abstract

Innate sensing of nucleic acids lies at the heart of antiviral host defense. However, aberrant activation of innate sensors by host nucleic acids can also lead to the development of autoimmune diseases. Such host nucleic acids can also be released from stressed, damaged or dying cells into the tissue microenvironment. It however remains unclear how the extracellular nucleic acids impacts the quality of the host immune responses against viral infections. Using a mouse model of influenza A virus (IAV) infection, we uncovered an important immune-regulatory pathway that tempers the intensity of the host-response to infection. We found that host-derived DNA from necrotic cells accumulates in the lung microenvironment during IAV infection, and is sensed by the DNA receptor Absent in Melanoma 2 (AIM2). AIM2-deficiency resulted in severe immune pathology highlighted by enhanced recruitments of immune cells, and excessive systemic inflammation after IAV challenge, which led to increased morbidity and lethality in IAV-infected mice. Interestingly, these effects of AIM2 were largely independent of its ability to mediate IL-1\(\beta\) maturation through inflammasome complexes. Finally, ablation of accumulated DNA in the lung by transgenic expression of DNasel in vivo had similar effects. Collectively, our results identify a novel mechanism of cross talk between PRR pathways, where sensing of host-derived nucleic acids limits immune mediated damage to virus infected tissues.
Recognition of conserved microbial molecules is a primary strategy by which the hosts mount defense responses against infections. These molecules, known as pathogen-associated molecular patterns (PAMPs), include bacterial components such as lipopolysaccharide (LPS), peptidoglycan, and flagellin. In contrast to those molecules exclusively found in prokaryotes, nucleic acids are present in both viral and bacterial pathogens, as well as the host cell they infect. All notable species of nucleic acids are recognized by the mammalian innate immune system, which leads to potent inflammatory responses including antiviral immunity. Recognition of nucleic acids by pattern-recognition receptors (PRRs) including RIG-I-like receptors (RIG-I), Nod-like receptors (NLRs), Toll-like receptors (TLRs), and PYHIN proteins represent the diverse strategies employed by host cells to sense viral infections (Barbalat et al., 2011; Schattgen and Fitzgerald, 2011). Sensing of nucleic acids through PRRs triggers signaling pathways leading to the production of type I interferons (IFNs), inflammatory cytokines, and antiviral molecules including IFN-stimulated genes (ISGs).

In general, viral nucleic acids recognized by PRRs are largely indistinguishable from the nucleic acids found in the host cell. The molecular strategies employed by host cells to distinguish self versus non-self nucleic acids, and prevent inappropriate activation of nucleic acid sensing PRRs by are only beginning to be understood. One such strategy is the physical separation of
host nucleic acids and their respective PRRs into distinct subcellular compartments. For example, DNA is packaged within the nucleus and mitochondria, whereas DNA-sensing PRRs such as absent in melanoma 2 (AIM2) and toll-like receptor 9 (TLR9) are sequestered in the cytosol and endosomes, respectively. The presence of DNA in either of these compartments during microbial infections or sterile cellular stress can lead to the activation of the DNA sensing machinery. A growing body of evidence indicates that sensing of endogenous DNA contributes to the development of autoimmune diseases (Kawasaki et al., 2011). Mammalian DNA exonucleases including DNasel, DNasell, and TREX1 prevent accumulation of endogenous DNA in extracellular matrix, phagolysosomes and the cytosol, respectively. Genetic mutations in any of these DNase proteins are linked to a spectrum of autoimmune diseases (Napirei et al., 2000; Stetson et al., 2008; Kawane et al., 2006; 2001).

In addition to the large amounts of viral nucleic acids produced during an infection, endogenous danger signals (e.g. DNA) can also be released from dying cells. It is presently unclear if these danger signals are detected and if they in turn alter the host’s response to the pathogen. We set out to address this question using influenza A virus (IAV). IAV is an orthomyxovirus with an (-) ssRNA genome and the viral life cycle involves the creation RNA species only. IAV is sensed by RIG-I (Rehwinkel et al., 2010), as well as TLR7 (Diebold et al.,
2004), and TLR3 (Le Goffic et al., 2007), all of which mobilize antiviral defenses to curb viral replication, prevent viral spread and activate adaptive immunity.

Here we report the abundant release of endogenous DNA into the lung microenvironment during infection with IAV. We identify an important role for the DNA sensor AIM2 in sensing this host DNA. Mice lacking AIM2 are hyper susceptible to IAV infection. Although AIM2 contributes to the production of IL-1β early during IAV infection, the enhanced susceptibility of AIM2-deficient mice is not due to a failure to engage IL-1R signaling and IAV specific adaptive immunity. Rather, AIM2 appears to function to dampen inflammatory responses that would otherwise lead to excessive immunopathology. Thus our study uncovers important cross talk between PAMP and DAMP sensing in controlling the magnitude of the host response to infection.

Results

**DNA accumulates in the lung microenvironment during IAV infection**

To determine whether host DNA is sensed by the innate immune system during viral infections, we first sought a mouse model of viral infection that was an RNA virus (with no DNA intermediates), and caused extensive tissue damage leading to detectable levels of the host DNA released within the affected tissue. For these reasons, we chose the mouse model of IAV infection using the mouse-adapted PR8 strain since this is an RNA virus capable of causing fatal viral
pneumonia. We first set out to determine whether the host DNA was released in the lung microenvironment during the course of IAV infection. In the lungs of Influenza virus infected mice we detected the extracellular DNA, which was primarily localized to the bronchi (Fig 3.1A). The source of this DNA is likely necrotic bronchiolar epithelial cells or neutrophil extracellular traps (NETs), both of which have previously been identified in IAV infected animals (Capelozzi et al., 2010; Narasaraju et al., 2011). We also quantified the levels of extracellular DNA released within IAV-infected lungs by obtaining bronchiole alveolar lavage (BAL) fluid, which were depleted of host cells. Uninfected mice had low, albeit detectable quantities of dsDNA using PicoGreen assay, fluorescent dye specific for dsDNA (Fig 3.1B). We were able to detect a significant increase in the amount of DNA as early as 1 day post infection (dpi). The amount of DNA in the interstitial space continued to gradually increase at 2, 3, and 6 dpi (Fig 3.1B). Since IAV is a single-stranded RNA virus, and produces only RNA intermediates during replication (Gultyaev et al., 2010), we conclude that the host-derived DNA is the most likely source of DNA in this model. Thus the mouse IAV model fit the necessary criteria needed to test our hypothesis.
Figure 3.1 Extracellular DNA is present in the lung microenvironment during IAV infection. (A) H&E staining of lung tissue from uninfected and infected WT mice at 5 dpi at 100X magnification. Arrows indicate DNA free of intact cells. Scale bar = 10 μm (B) Concentration of DNA in BAL fluid collected from infected WT mice at indicated times. DNA quantified by PicoGreen assay (n = 2 independent experiments). *, P ≤ 0.05; **, P ≤ 0.01; ****, P ≤ 0.001
**AIM2 protects against lethal IAV challenge independently of the inflammasome**

Given the abundance of DNA in the lungs during IAV infection, we next examined whether DNA sensing receptor(s) play a role in mediating host immune responses to IAV. To this end, we tested mice lacking AIM2, the well described DNA receptor required for activation of the inflammasome complex in response to cytosolic dsDNA (Hornung et al., 2009; Rathinam et al., 2010; Fernandes-Alnemri et al., 2009). Previous studies have identified roles for inflammasomes in IAV infection (Pang and Iwasaki, 2011). IAV infection has been shown to regulate IL-1β via the NLRP3 inflammasome by sensing IAV M2 ion channel and viral RNA (Ichinohe et al., 2010; Allen et al., 2009). Further, the inflammasome components including ASC and caspase-1 are also required for protection from IAV infection (Ichinohe et al., 2009; Allen et al., 2009; Thomas et al., 2009). The importance of IL-1 in IAV infection is also supported by the observations that IL-1R-deficient mice have impaired T cell responses to IAV (Pang et al., 2013). Therefore, we first tested whether AIM2 was required for IL-1β production by BMDCs in response to IAV in vitro. Treatment of BMDCs with MCMV and poly dA:dT, which are known to engage the AIM2 inflammasome, stimulated IL-1β secretion in an AIM2-dependent manner, while the NLRP3 dependent stimuli ATP and nigericin did so independent of AIM2 as expected (Fig 3.2). Secretion of IL-1β by IAV infected BMDCs was independent of AIM2 (Fig 3.2).
Fig 3.2 IL-1β responses to IAV in vitro are AIM2-independent. Mouse BMDCs were stimulated with LPS alone (200ng/mL), lipofectamine 2000 alone, poly dA:dT complexed with lipofectamine (1 μg/mL), MCMV (MOI 10), nigericin (10 μM), ATP (5 mM), or IAV PR8 (MOI 2). All samples, excluding media controls, were primed for 3 hours with LPS (200 ng/mL) prior to stimulation. IL-1β secreted into supernatants were measured by ELISA.

To test the contribution of AIM2 during IAV infection in vivo, mice lacking AIM2 were infected with a lethal IAV challenge. While 75% of WT mice survived IAV infection, only 28% of Aim2−/− mice survived at 14 dpi indicating that AIM2-deficient animals are highly susceptible to IAV infection in vivo (Table 3.1). Comparison of survival curves and weight loss as a measure of morbidity revealed that Aim2−/− mice succumb to IAV with quicker kinetics and increased morbidity compared to WT animals (Fig 3.3 A and B). We next tested if the increased mortality of Aim2−/− mice was due to uncontrolled viral replication. To this end, we measured IAV viral RNA copy numbers using RT-qPCR analysis of the total RNA purified from the lungs of WT and Aim2−/− mice at days 3 and 5 post infection. Interestingly, viral loads were comparable between WT and Aim2-deficient animals (Fig 3A). These observations indicate that while required for protection during lethal IAV challenge, AIM2 is dispensable for controlling IAV replication.
Table 3.1 AIM2 is required for protection from lethal IAV infection.

<table>
<thead>
<tr>
<th>Days post Infection</th>
<th>7</th>
<th>10</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>17/20</td>
<td>16/20</td>
<td>15/20</td>
</tr>
<tr>
<td>AIM2 -/-</td>
<td>12/18</td>
<td>5/18</td>
<td>5/18</td>
</tr>
</tbody>
</table>

Table 3.1: Proportion surviving

Figure 3.3 Kinetics of mortality and morbidity following IAV infection. Survival comparison between WT and \( Aim2^{/-} \) mice challenged with \( 4 \times 10^4 \) pfu PR8. (A) Representative survival curve from one experiment (WT, \( n=6; \ Aim2^{/-}; n=5 \)). (B) Representative plot of weight loss from one experiment (WT, \( n=6; \ Aim2^{/-}; n=5 \)).
Figure 3.4 Aim2 is not required to the control of IAV burden. (A) Copy number of PR8 vRNA measured in total lung RNA from WT and Aim2<sup>−/−</sup> mice at 3 and 5 dpi measured by qRT-PCR. (B) PR8 viral titers measured by plaque assay using lung homogenates from WT and Aim2<sup>−/−</sup> mice at 5 dpi.

Given the importance of Aim2 as a regulator of IL-1β maturation, we considered the possibility that Aim2<sup>−/−</sup> mice were susceptible to IAV because they failed to elicit IL-1β production <em>in vivo</em>. We found that levels of IL-1β protein in lung homogenates were significantly lower at 3 dpi (Fig 3.5A). By later time points however (5 dpi), the levels of IL1β were similar between wild type and Aim2− deficient mice (Fig 3.5B). Levels of Iif1b (Fig 3.5B+E) and IL-1R antagonist (Iif1ra)
mRNA (Fig 3.5C+F) were similar between Aim2−/− and WT mice at both time points. These results indicate that there was no defect in Il1b transcription or increased negative regulation of IL-1R signaling, respectively. IL-1R signaling during IAV infection is required for eliciting a protective flu-specific T cell response (Pang et al., 2013). Therefore, we next determined whether AIM2 was required for the induction of flu-specific T cell responses, and whether the noted decrease in IL-1β was similarly sufficient. After infection with a sublethal dose of PR8, we performed tetramer staining using single-cell suspensions from the lung and spleen from IAV-infected animals with class I (NP 366, PA 244, PB1 703) and class II (NP 311) IAV PR8 epitopes to determine the frequency and the total number of flu-specific CD8+ and CD4+ T cells respectively. We found no significant difference in the frequency (Fig 3.6A) or number (Fig 3.6B) of tetramer-positive CD8+ CD44hi T cells for all class 1 epitopes between WT and AIM2-deficient animals in the lungs at 9 dpi. Similar results were also found in the spleen of IAV-infected WT and AIM2-deficient animals for the class I and class II T cell epitopes (Fig 3.6C). Taken together, these data indicate that AIM2 plays a protective role during IAV infection independent of its role on early IL-1β production or the formation of flu-specific T cell responses.
Figure 3.5 Aim2<sup>−/−</sup> mice have intact IL-1β production in vivo during IAV infection. (A, D) Levels of IL-1β protein in lung homogenates at 3 dpi (A) and 5 dpi (D) as determined by ELISA. Relative expression of *il1b* (B, E) and *il1ra* mRNA (C, F) in the lungs at 3 dpi (B, C) and 5 dpi (D, E). Normalized to GAPDH. ****, P ≤ 0.001
Figure 3.6 AIM2 is not required in generating flu-specific T cell responses. (A) Frequency and (B) number of tetramer+ CD8+ T cells in total lung of WT and Aim2−/− mice at 9 dpi. (C) Number of tetramer+ CD8+ and CD4+ T cells in the spleen of WT and Aim2−/− mice at 9 dpi with 10^3 pfu PR8 (n = 2 independent experiments). Populations were gated on CD45+ CD44^hi TCRβ+ cells. *, P ≤ 0.05

AIM2 regulates inflammatory responses in the lung during IAV infection

We next used flow cytometry to examine whether the absence of AIM2 altered leukocyte recruitment to the lungs of IAV infected mice. This analysis revealed a significant increase in both the frequency (Table 3.2) and the total number (Table 3.3) of CD45+ leukocytes in the lungs of Aim2−/− mice compared to WT controls at 5 dpi. We further looked at specific immune cell populations to determine whether the increase in leukocytes was attributed to an increase in a particular population. We found that the increased numbers of CD45+ leukocytes
in the lungs of *Aim2*^{−/−} mice were due to an increase in the total numbers of CD4+ and CD8+ T cells, immature macrophages, and CD11b+ DCs (Table 3.3). We observed no significant difference in the number of neutrophils, B cells, monocytes, or NK cells (Table 3.3). These findings further support the conclusion that the protective effect of AIM2 during IAV infection is independent of its role as an inflammasome sensor since mice deficient in NLRP3, ASC, and caspase-1 have been reported to manifest impaired cellular recruitments during IAV infection (Ichinohe et al., 2009; Allen et al., 2009; Thomas et al., 2009).
### Table 3.2 Frequency of specific immune cell populations in lungs of WT and at 5 dpi with IAV.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>WT (n=6)</th>
<th>Aim2-/- (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45+ leukocytes*</td>
<td>10.3 ± 0.78</td>
<td>16.0 ± 2.01</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>6.5 ± 0.55</td>
<td>8.5 ± 0.67</td>
</tr>
<tr>
<td>CD4+ T cells</td>
<td>2.5 ± 0.25</td>
<td>3.2 ± 0.56</td>
</tr>
<tr>
<td>Alveolar macrophages</td>
<td>0.91 ± 0.20</td>
<td>0.55 ± 0.11</td>
</tr>
<tr>
<td>CD11b+ dendritic cells</td>
<td>21.7 ± 1.40</td>
<td>16.7 ± 1.48</td>
</tr>
<tr>
<td>CD11b- dendritic cells</td>
<td>1.1 ± 0.19</td>
<td>0.28 ± 0.041</td>
</tr>
<tr>
<td>Immature macrophages</td>
<td>17.2 ± 1.07</td>
<td>18.7 ± 0.64</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>34.8 ± 1.99</td>
<td>41.5 ± 3.12</td>
</tr>
<tr>
<td>NK cells</td>
<td>0.23 ± 0.03</td>
<td>0.10 ± 0.021</td>
</tr>
<tr>
<td>monocytes</td>
<td>44.0 ± 1.13</td>
<td>40.0 ± 2.23</td>
</tr>
<tr>
<td>B cells</td>
<td>10.6 ± 0.48</td>
<td>10.1 ± 1.75</td>
</tr>
</tbody>
</table>

Mean ± SEM. Frequency of total cells is shown for CD45+ leukocytes. Frequency of CD45+ cells is shown for specific populations. Data from one of two independent experiments shown *, P ≤ 0.05; **, P ≤ 0.01

### Table 3.3 Total cell numbers for specific immune cell populations in lungs of WT and Aim2-/- at 5 dpi with IAV.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>WT (n=6)</th>
<th>Aim2-/- (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45+ leukocytes*</td>
<td>18.1 ± 0.23</td>
<td>32.9 ± 0.59</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>0.41 ± 0.068</td>
<td>1.157 ± 0.2</td>
</tr>
<tr>
<td>CD4+ T cells</td>
<td>1.1 ± 0.2</td>
<td>3.081 ± 0.24</td>
</tr>
<tr>
<td>Alveolar macrophages</td>
<td>0.13 ± 0.019</td>
<td>0.1997 ± 0.04</td>
</tr>
<tr>
<td>CD11b+ dendritic cells</td>
<td>3.6 ± 0.57</td>
<td>6.040 ± 0.53</td>
</tr>
<tr>
<td>CD11b- dendritic cells</td>
<td>0.18 ± 0.04</td>
<td>0.09994 ± 0.015</td>
</tr>
<tr>
<td>Immature macrophages*</td>
<td>3.0 ± 0.37</td>
<td>6.118 ± 1.0</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>2.0 ± 0.52</td>
<td>4.025 ± 1.1</td>
</tr>
<tr>
<td>NK cells</td>
<td>0.037 ± 0.008</td>
<td>0.03665 ± 0.007</td>
</tr>
<tr>
<td>monocytes</td>
<td>8.0 ± 1.0</td>
<td>13.10 ± 2.5</td>
</tr>
<tr>
<td>B cells</td>
<td>1.9 ± 0.26</td>
<td>3.312 ± 0.88</td>
</tr>
</tbody>
</table>

Mean ± SEM. Data from one of two independent experiments shown *, P ≤ 0.05; **, P ≤ 0.01; ****, P ≤ 0.001
The increased number of leukocytes in the lungs of Aim2<sup>-/-</sup> mice prompted us to determine whether inflammatory cytokine responses were similarly increased. In line with the decreased IL-1β levels during early infection, we found that IL-6 (Fig 3.7A) and TNFα (Fig 3.7B) levels were similarly decreased in lung homogenates of Aim2<sup>-/-</sup> mice at 3 dpi compared to WT controls. However, these cytokines (TNFα and IL-6) were significantly elevated in Aim2<sup>-/-</sup> mice compared to WT animals during later infection (Fig 3.7 A + B). The early effects of AIM2-deficiency on these cytokines may result from decreased IL-1β production and the IL-1R dependent induction of these cytokines. The studies at later time points however, suggest that AIM2 has a broader role in dampening inflammatory response at later stages of IAV infection.

**Figure 3.7** Altered inflammatory cytokine responses in the lungs of Aim2<sup>-/-</sup> mice. (A) IL-6 and (B) TNFα protein in lung homogenates measured by ELISA at 3 and 5 dpi. (n = 3 independent experiments). *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.005
The heightened inflammatory cytokine responses at later time points could be due to increased leukocyte infiltration or cell intrinsic differences in the production of these cytokines, or possibly both. To resolve these possibilities, we purified alveolar macrophages (AMs) from lungs of naïve mice by FACS sorting (SSC^b CD45^+ CD11c^+ Siglec-F^+ autofluorescence^+ cells) as previously described (Schneider et al., 2014). We compared expression and induction of innate immune responses in WT and Aim2^-/- AMs infected ex vivo with IAV PR8 by Nanostring gene expression analysis (Table 3.4). Of the 94 immune response genes analyzed, 12 of these showed a 2-fold or greater increase in IAV-infected Aim2^-/- alveolar macrophages compared to the infected WT sample, while 4 genes were decreased by 2-fold or more within the same samples (Fig 3.8A). Most of the genes showing elevated expression in Aim2^-/- AMs were interferon-stimulated genes (ISGs). Interestingly, Il6 expression was decreased in Aim2^-/- AMs while Tnfa expression was similar between WT and AIM2-deficient cells (Fig 3.8A). Amongst the genes that were upregulated in AIM2-deficient cells, cxcl10, appeared as a potentially important contributor to the inflammatory phenotype observed in Aim2^-/- mice since it functions as a key chemokine driving T cell recruitments to the site of infection (Taub et al., 1993). In addition, high levels of serum CXCL10 positively correlates with worsened morbidity in patients suffering from H5N1 IAV infection (Le Goffic et al., 2006). Expression of cxcl10 in our Nanostring analysis was found to be 9.4-fold higher in Aim2^-/- AMs infected with IAV when compared to WT (Fig 3.8A). Similar results were also obtained by
qPCR (Fig 3.8B). To determine whether the cell intrinsic effects of AIM2 deficiency were specific to AMs or IAV, we looked at the effect of several PRR ligands on cxcl10 expression in WT and Aim2<sup>-/-</sup> BMDMs. Expression of cxcl10 mRNA was significantly higher in Aim2<sup>-/-</sup> BMDMs stimulated with IAV, LPS, MCMV, and Sendai virus compared to WT controls (Fig 3.8C). Another gene implicated as possibly mediating the observed inflammation in Aim2<sup>-/-</sup> mice is tlr3, an endosomal TLR leading to TRIF-dependent inflammatory cytokine and type I IFN induction in response to dsRNA. TLR3 positively regulates inflammatory cytokine responses and CD8+ T cell recruitment during IAV infection, and mice deficient in TLR3 are protected from immune pathology (Napirei et al., 2004). Here we found that infection of WT AMs with IAV led to tlr3 down-regulation (Fig 3.8A + B). In contrast, we noted a significant increase in tlr3 expression in Aim2<sup>-/-</sup> AMs infected with IAV suggesting AIM2 negatively regulates TLR3 signaling (Fig 3.8A + B). Taken together, these results indicate that Aim2<sup>-/-</sup> macrophages have cell intrinsic differences in innate immune responses to IAV that possibly contributes to the heightened inflammation in Aim2<sup>-/-</sup> mice. Whether AIM2-deficiency has a similar effect on the induction of cytokine response in other cell types remains to be determined.
Figure 3.8 *Aim2*^{−/−} macrophages have cellular intrinsic differences in innate immune responses to IAV. (A) Heatmap of Nanostring gene expression analysis of purified alveolar macrophages infected *in vitro* with PR8 (MOI 2) for 6 hours. Selected genes show a 2-fold or greater difference in mRNA counts between infected WT and *Aim2*^{−/−} alveolar macrophages. Data shown as normalized counts of gene-specific probes. (B) qPCR validation of *cxcl10* and *tlr3* mRNA expression in WT and *Aim2*^{−/−} alveolar macrophages infected *in vitro* with PR8 (MOI 2) for 6 hours. (C) qPCR analysis of *cxcl10* mRNA expression in WT and *Aim2*^{−/−} BMDMs stimulated for 6 hours with IAV (MOI 2), LPS (200 ng/mL), MCMV (MOI 10), Pam3CSK4 (1 ug/mL), and SeV (200 HA U). Shown as relative expression normalized to GAPDH. *, P ≤ 0.05; **, P ≤ 0.01
Table 3.4 Normalized probe counts from Nanostring analysis of WT and *Aim2*−/− alveolar macrophages infected *ex vivo* with IAV.

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We next sought to better understand the cell-type specific contributions of AIM2 in mediating protective immunity against lethal IAV challenge. We performed bone marrow transplantations studies to define the role of AIM2 function in hematopoietic versus non-hematopoietic cells. First, we tested whether transplantation of \( \text{Aim2}^{\text{-/-}} \) bone marrow in WT recipients was sufficient to impart susceptibility to IAV infection. Lethally irradiated WT mice were engrafted with either \( \text{Aim2}^{\text{-/-}} \) or control WT bone marrow, and subsequently challenged with a lethal dose of IAV to compare survival between the two groups. Here, we found that mice reconstituted with \( \text{Aim2}^{\text{-/-}} \) bone marrow were hypersusceptible to IAV infection compared to mice receiving WT bone marrow (Fig 3.9A). These results indicate that the expression of AIM2 in the hematopoietic compartment is required for protection from IAV. Since the function of AIM2 as a PRR is best characterized in myeloid cells, we hypothesized that its expression in macrophages and dendritic cells was required for protection. To answer this possibility, we targeted AIM2 deletion in granulocytes/macrophages and dendritic cells by crossing mice harboring \( \text{Aim2}^{\text{lox/lox}} \) alleles with mice expressing Cre recombinase driven by LysM and CD11c, respectively. Surprisingly, we saw no differences on mortality following lethal IAV challenge in both \( \text{LysM-Cre x Aim2}^{\text{fl/fl}} \) (Fig 3.9B) and \( \text{CD11c-Cre x Aim2}^{\text{fl/fl}} \) (Fig 3.9C) mice compared to \( \text{Aim2}^{\text{fl/fl}} \) controls. These results suggest that while AIM2 expression in hematopoietic cells is required for protection against IAV infection, its expression in granulocytes/macrophages and dendritic cells appears dispensable. Further work
is necessary to define the cell-type specific functions of AIM2 during IAV infection. The functional role of AIM2 in non-hematopoietic cells is an attractive avenue for future studies.

Figure 3.9 Expression of AIM2 in hematopoietic cells is required for protection to IAV infection. (A) Survival comparison of lethally irradiated WT mice engrafted with WT and Aim2$^{-/-}$ bone marrow infected with PR8. (B) Survival comparison of LysM-Cre x Aim2fl/fl and (C) CD11c-Cre x Aim2fl/fl in response to IAV infection. Number of mice used indicated next to each experimental group.
**DNA in the lung microenvironment provides protection to IAV infection**

Having found that genetic deletion of AIM2 increased susceptibility and inflammation during IAV infection, we next wanted to determine if the functional role of AIM2 was linked to its ability to sense DNA. In particular, we wanted to determine if clearance of the extracellular DNA from the lung microenvironment in wild type mice would yield a similar effect that was observed in AIM2 deficient animals. We hypothesized that the removal the DNA ligand would uncouple the contributions of DNA-dependent signaling from the cellular-intrinsic effects observed when AIM2 is genetically removed (Fig 3.8). In order to test this hypothesis, we generated a recombinant adeno-associated virus (AAV) to ectopically express mouse DNaseI in the lungs of C57BL/6 mice (Fig 3.10A). DNaseI is a secreted protein, and is normally absent in the lung with respect to its enzymatic activity and expression (Napirei et al., 2005). The use of AAV for transgene expression in these experiments was ideal due to the relatively low immunogenicity of the virus (Mingozzi and High, 2013). The DNaseI transgene was packaged using an AAV9 serotype vector since this has a high tropism for lung tissues (Bell et al., 2011). Intranasal infection with AAV-DNaseI resulted in robust and stable expression of the transgene in the lung two weeks after transduction (Fig 3.10B). The DNaseI transgene expression could be detected until for at least 3 months (data not shown). Importantly, the levels of DNA in the BAL fluid of IAV infected mice were significantly reduced in mice treated with AAV-DNaseI compared to GFP expressing control virus (Fig 3.10C). Upon
confirming stable expression and functional activity of DNasel in the lungs of AAV-DNasel treated mice, we wished to determine whether ablation of DNA in the lung microenvironment during IAV infection impacted the susceptibility of mice to lethality. Following IAV challenge (LD_{50} of PR8), mice treated with AAV-DNasel showed a significant decrease in survival (40%) compared to controls (79.6%) out to 14 dpi (Fig 3.10D). Consistent with our observations in Aim2^{-/-}, there was no difference in viral load when compared between control and AAV-DNasel treated mice at 5 dpi (Fig 3.10E). These results indicate that the presence of DNA in the lung microenvironment is protective against lethal IAV infection.
Figure 3.10 DNA in the lung microenvironment is protective to IAV infection. (A) Schematic of ectopic expression of DNaseI. WT mice were given $10^{10}$ pfu of AAV-DNaseI or AAV-GFP intranasally. (B) $dhase1$ mRNA measured by qPCR with total RNA isolated from lungs of AAV-GFP and AAV-DNaseI treated mice. (C) DNA in cell-free BAL in uninfected controls compared to AAV-GFP and AAV-DNaseI treated mice at 5 dpi with PR8. (n = 2 independent experiments) (D) Survival comparison between AAV-GFP and AAV-DNaseI treated WT mice challenged with $4 \times 10^4$ pfu PR8. Data pooled from two independent experiments (AAV-GFP, n = 17; AAV-DNaseI, n = 15). (E) Copy number of IAV PA in total lung RNA of AAV-GFP and AAV-DNaseI treated mice at 5 dpi. *, $P \leq 0.05$

We also characterized the infiltrating leukocyte populations in the lungs of AAV-DNaseI treated mice at 5 dpi. Consistent with the effects observed in Aim2-deficient animals (Table 3.3), we detected significantly elevated numbers of CD4+ and CD8+ T cells in AAV-DNaseI treated mice compared to controls (Table 3.5). However, the overall number of CD45+ leukocytes was unaffected suggesting that the extracellular DNA may signal specifically to regulate T cell recruitments to the lungs of infected animals (Table 3.5). We further examined the levels of inflammatory cytokines in AAV-DNaseI and control treated mice at 3 and 5 dpi. Levels of IL-1$\beta$, IL-6 and TNF$\alpha$ were comparable between the two groups at both time points tested (Fig 3.11A-C). We also investigated the expression of chemokines involved in T cell recruitments that could account for the increased numbers of T cells in AAV-DNaseI (Table 3.5). Here, we found that the expression of $cxcl9$ was higher in AAV-DNaseI mice compared to controls (Fig 3.11D). The $cxcl10$ levels however were similar between the WT and AIM2-deficient animals at 5 dpi (Fig 3.11E). Together, these results suggest that DNA
present within the lung microenvironment during IAV infection acts as a DAMP to
limit T cell-mediated immune pathology.

Table 3.5 Total cell numbers for specific immune cell populations in lungs
of AAV-GFP and AAV-DNaseI-treated WT mice at 5 dpi with IAV.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>AAV-GFP (n = 8)</th>
<th>AAV-DNaseI (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45+ leukocytes</td>
<td>1.470 ± 0.1789</td>
<td>1.946 ± 0.2357</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>0.05839 ± 0.009699</td>
<td>0.09326 ± 0.01253</td>
</tr>
<tr>
<td>CD4+ T cells</td>
<td>0.02586 ± 0.001970</td>
<td>0.03971 ± 0.005760</td>
</tr>
<tr>
<td>CD11b+ dendritic cells</td>
<td>0.07193 ± 0.006984</td>
<td>0.05964 ± 0.01344</td>
</tr>
<tr>
<td>CD11b- dendritic cells</td>
<td>0.03811 ± 0.004913</td>
<td>0.04121 ± 0.004899</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.05836 ± 0.01331</td>
<td>0.07769 ± 0.01110</td>
</tr>
<tr>
<td>NK cells</td>
<td>0.07606 ± 0.007532</td>
<td>0.1014 ± 0.01257</td>
</tr>
<tr>
<td>monocytes</td>
<td>0.4199 ± 0.06689</td>
<td>0.5513 ± 0.08339</td>
</tr>
</tbody>
</table>

Mean ± SEM. Data from one of two independent experiments shown *, P ≤ 0.05
Figure 3.11 Inflammatory cytokine responses during IAV infection are unaffected by AAV-DNaseI treatment. (A) IL-1β, (B) IL-6, and (C) TNFα protein in lung homogenates measured by ELISA at 3 and 5 dpi with IAV. (n = 2 independent experiments). (D) cxcl9 and (E) cxcl10 mRNA expression in total lung RNA at 5 dpi with IAV. Normalized to GAPDH. Data pooled from two independent experiments. *, P ≤ 0.05
Figure 3.12 Model for the regulation of lung inflammation by AIM2 and extracellular DNA. IAV replication primarily occurs in the epithelium of the bronchioles and alveoli. Necrosis of the epithelial cell as result of IAV infection releases the intracellular contents into the microenvironment. These released molecules, including DNA, act as DAMPs and are recognized by PRRs of the innate immune system. DNA taken up by innate immune cells, possibly by phagocytosis or another unknown mechanism, gains access to the cytosol for its recognition by AIM2. AIM2 represses expression of inflammatory cytokine and chemokine genes, and engagement of AIM2 by DNA may further repress these genes. It is unknown how AIM2 represses the expression of inflammatory genes. Increased expression of T cell chemokines leads to the increased numbers of activated CD4+ helper T cells and CD8+ cytotoxic lymphocytes, which drive inflammation and immune pathology. IFNγ produced by T cells could act as a feed-forward response that further drives inflammation in the lung.
Discussion and Perspectives

Recognition of foreign nucleic acids lies at the heart of the mammalian innate immune surveillance against viral infections. The sensing of viral genomes by host-cell PRRs initiate signaling pathways leading to the production of type I IFNs, inflammatory cytokines, and other antiviral molecules to mediate potent host defense responses. In addition to viral nucleic acids, host-derived nucleic acids released from dying cells can also be sensed by the nucleic acid sensing machinery. We set out to determine the molecular details and the functional consequences of sensing host derived DNA in the broader context of host–pathogen interaction. Our studies indicate that host-derived DNA accumulates in the lungs following IAV infection, which acts to modulate the intensity and the outcomes of the antiviral immune response. The extracellular DNA released into the lung microenvironment during IAV infection in turn engages AIM2 to curb further immune-mediated tissue damage. AIM2 has largely been studied for its role as a dsDNA receptor in the context of the inflammasome complex. Our work highlights a novel function for an intracellular DNA receptor in modulating immune responses against an RNA virus infection.

There is a growing appreciation that the that the immune system cares less about the distinction of “self” versus “non-self”, rather it surveys to distinguish between “healthy” versus “damaged” states (Matzinger, 1994). Host molecules released from stressed and damaged tissues act as DAMPs (or alarmins) to alert the innate immune system to damage for the initiation of
inflammatory responses. A growing number of endogenous molecules have been
described to function as DAMPs that modulate inflammatory responses during
microbial infection and sterile inflammation. Such alarmins include HMGB1, ATP,
uric acid, RNA and DNA. A growing body of evidence suggests that the sensing
of host cell DNA is a major mechanism in the onset of autoimmune diseases
[cite]. Thus, there are considerable efforts by the host to remove unneeded DNA
before it can accumulate. Deficiency of \textit{dnase2a} in mouse embryos leads to
engorgement of liver macrophage phagolysosomes with DNA from the ejected
nuclei of erythroid precursors, triggering TLR-independent type I IFN responses
(Okabe et al., 2005). Indeed, DNase2/-/ mice are embryonic lethal due to IFN-
driven anemia, and further deletion of the IFN-\(\alpha/\beta\) receptor (IFNAR) recues
lethality (Kawane et al., 2001; Yoshida et al., 2004). However, DNase2/IFNAR
double knock-out mice develop chronic polyarthritis driven by IFN-independent
inflammatory cytokines (Kawane et al., 2006). Similarly, loss of function
mutations in the DNA exonuclease Trex1 cause Aicardi-Goutieres syndrome
(AGS) (Crow et al., 2006), a systemic autoimmune disease. Stetson and
Medzhitov showed that ssDNA derived from reverse-transcribed endogenous
retroelements accumulating in the cytoplasm as result of Trex1-deficieny, leading
to cell intrinsic initiation of autoimmune disease (Stetson et al., 2008). Together,
these studies clearly support the idea that preventing the accumulation of host-
derived DNA essential for normal health
In addition to the cell-intrinsic inflammation driven by the DNA accumulation, extrinsic nuclear and mitochondrial DNA provides a danger signal to the immune system. An interesting study by Zhang et al. demonstrated that the traumatic injury releases mitochondrial DAMPs, including mitochondrial DNA, into circulation (Zhang et al., 2010). Mitochondrial DNA sensed by neutrophils stimulates their migration, degranulation, and activation of MAPK signaling all leading to neutrophil-mediated tissue injury. Neutrophil responses were mediated by the TLR9 sensing of DNA, which could be effectively blocked using inhibitory oligodeoxyribonucleotides (iODNs) carrying TTAGGG repeats. Previous work from our lab has further showed that the iODNs can broadly regulate inflammatory responses downstream of AIM2 and IFI16 by directly interacting with these proteins, and competitively binding with activating dsDNA (Kaminski et al., 2013). A recent study demonstrated that DNA released by necrotic hepatocytes during acetaminophen-induced liver injury drives neutrophil recruitment, and liver damage in a TLR9-dependent manner (Marques et al., 2015). These studies collectively implicate that the recognition of DNA by TLR9 acts as a major pathway to regulate the inflammatory responses during sterile inflammation. Whether extracellular DNA can also function as a DAMP to regulate inflammatory processes during microbial infections however remains poorly understood.

Influenza A virus is an orthomyxovirus carrying an RNA genome, which is sensed by the RNA sensors RIG-I (Rehwinkel et al., 2010), TLR7 (Diebold et al.,
2004), and TLR3 (Le Goffic et al., 2007). The only source of DNA during IAV infections in our model is the host-derived DNA from either the mitochondrial or nuclear origin. IAV infection causes extensive pulmonary pathology including extensive necrosis of epithelial cells in the bronchioli (Taubenberger and Morens, 2008), providing DAMPs capable of activating the immune system. Another source of DNA comes in the form of neutrophil extracellular traps (NETs), which are released in response to IAV infection (Narasaraju et al., 2011). Here, we have found the levels of extracellular DNA in the lung microenvironment increased as the IAV infection spread through the tissue (Fig 3.1B). Ablation of extracellular DNA in the lung by the ectopic expression of DNase1 in the lung imparted susceptibility on WT mice to lethal IAV infection (Fig 3.1D), but had no detectable effect on viral burden (Fig 3.1E). Moreover, we observed increased recruitment of CD8+ and CD4+ T cells into the lungs of mice treated with the AAV-DNaseI (Table 3.5) accompanied by a modest increase in expression of the T cell chemoattractant cxcl9 (Fig 3.1D). Whether CXCL9 is itself responsible for the increased number of T cells in the lungs of AAV-DNaseI has not been explored. Notably, we were unable to detect increased levels of inflammatory cytokines in the lungs of treated mice compared to those of controls (Fig 3.1A-C). These findings are in contrast to the increased levels of TNFα and IL-6 in lungs of Aim2−/− mice at the same time points (Fig 3.7). This observation might be explained by one or more possibilities: (1) ectopic expression of DNaseI is unable to remove all of the DNA, thus it able to still provide a signal to PRRs, (2) genetic
deletion of AIM2 is more complete in blocking the regulatory effect of DNA
signaling on inflammatory responses or (3) AIM2 deletion has cell intrinsic effects
regulating the inflammatory response. Further work will determine whether the
observations in the AAV-DNaseI mice is an uncoupling from the cell intrinsic
responses in the absence of AIM2, or DNaseI expression is less effective at
blocking DNA signaling than genetic deletion of AIM2.

Having found an abundance of DNA in the lungs during IAV infection, we
turned our attention to identifying a potential sensor of this DNA able to modulate
immune responses during infection with an RNA virus. Mice deficient in the
cytosolic DNA sensor AIM2 were particularly sensitive to IAV infection without a
detectable difference in viral burden (Fig 3.3 + 4). The best described function of
AIM2 is its role as a dsDNA receptor able to activate the inflammasome in the
presence of cytosolic DNA (Hornung et al., 2009), leading to processing of pro-
IL-1β into its active form by the effector caspase-1. Here, we found AIM2 was not
required for the secretion of IL-1β by dendritic cells in response to IAV in vitro
(Fig 3.2). As we would predict given the lack of DNA in IAV virions, AIM2 does
not appear to play a role in direct sensing of IAV. Indeed, the activation of the
inflammasome by IAV infection has been shown by others to require NLRP3.
Two inclusive models for NLRP3 activation is through the indirect sensing of viral
RNA (Allen et al., 2009), and the disruption of intracellular ion gradients by
insertion of the viral M2 ion pore into the Golgi network (Ichinohe et al., 2010).
Although AIM2 impacted early IL-1β responses to IAV infection in vivo, this
response was indistinguishable from that of wild type mice at later time points (Fig 3.5). Similarly, there was no defect in il1b mRNA expression, or increased negative regulation of the IL-1R pathway by the natural IL-1R antagonist (Fig 3.5). Consistent the largely intact IL-1ß response, Aim2−/− mice were able to mount normal flu-specific T cell responses (Fig 3.6), which are known to lie downstream of IL-1R signaling (Pang et al., 2013), and require caspase-1 and the adaptor protein ASC (Ichinohe et al., 2009). From these results, the ability of AIM2 in mediating protection against IAV infection appears to be independent from its role as an inflammasome sensor.

Second to the complicating bacterial pneumonia, fatality due to infection with highly pathogenic IAV strains is thought to be the result of excessive inflammatory responses dubbed a “cytokine storm” leading to exorbitant inflammatory responses and resulting immune pathology (La Gruta et al., 2007). Often fatal symptoms of IAV pneumonia include pulmonary edema and acute respiratory distress, marked by alveolar hemorrhage and massive numbers of mononuclear infiltration (Taubenberger and Morens, 2008). Patients infected with the highly pathogenic avian H5N1 were found to have higher serum levels of CXCL10, IFNγ, IL-6, and IL-8 than patients infected with a less pathogenic H3N1 strain (de Jong et al., 2006). Similarly, high levels of the chemokines CXCL10, MIG, MCP-1, RANTES and IL-8 positively correlated with disease severity in H5N1 infected patients (Peiris et al., 2004). Despite strong correlations between excessive cytokines/chemokine production and morbidity, a cohesive mechanism
of immune-mediated lung pathology during IAV infection has yet to be described. FACS analysis examining cellular infiltration revealed an increase in the number of leukocytes recruited to the lungs of Aim2-/- mice (Table 3.3). Immune phenotyping of infiltrating leukocytes further demonstrated this was due to increased numbers of immature macrophages, CD11b+ dendritic cells, as well as CD8+ and CD4+ T cells at 5 dpi (Table 3.3). Interestingly, works investigating the role of CD8+ T cells during IAV infection suggest they are an important contributor in the development of immune pathology (Moskophidis and Kioussis, 1998; Wells et al., 1981; Wiley et al., 2001; Small et al., 2001). It is tempting to speculate the cause of mortality in Aim2-/- is attributable to increased numbers of CD8+ and CD4+ T cells exacerbating lung pathology during IAV infection however, further work is need to formally test this possibility. Analysis of levels of the inflammatory cytokines revealed decreased levels of IL-6 and TNFα in the lungs of mice deficient for AIM2 at 3 dpi however, levels of both these cytokines surpassed those of WT mice by 5 dpi (Fig 3.7). The reason behind the observed decrease in IL-6 and TNFα early in infection is unclear and is perhaps related to the noted decrease in IL-1β at this time point (Fig 3.5). However, the heightened levels of these cytokines at the later time point correlated with the increase in leukocytes, thus it is likely there are more cytokine-producing leukocytes in the absence of AIM2 at this time point. These data taken together suggest that AIM2 mice display a “hyper-inflammatory” phenotype during IAV infection and ascribe a
new function for AIM2 in regulating inflammatory responses during an RNA virus infection in vivo.

It is important to note that the phenotype observed in AAV-DNase1 treated mice during IAV infection is less severe than \( \text{Aim2}^{-/-} \) mice. Indeed, while we noted an increase in mortality and T cell infiltration in both groups, mice treated with AAV-DNase1 displayed normal cytokine responses compared to their controls while \( \text{Aim2}^{-/-} \) mice displayed a hyper inflammatory response. As I had alluded to in the paragraphs above, one plausible explanation for these observations is cellular-intrinsic differences in innate immune responses due to the genetic deletion of AIM2. In support of this possibility, previous work in our lab has demonstrated \( \text{Aim2}^{-/-} \) splenocytes and macrophages produce more IFN\(\beta\) than WT controls in response to cytosolic DNA (Rathinam et al., 2010). Similarly, AIM2-deficiency in splenocytes leads to higher expression of several ISGs basally, including \text{stat1}, \text{viperin}, \) and \text{ifi202} (Panchanathan et al., 2010), but decreased expression of the inhibitory IgG receptor, \text{fcgrb2} (Panchanathan et al., 2011). The mechanism in which AIM2 regulates the expression of ISGs is unknown. Differences in host genetic factors between inbred mouse strains have been shown to regulate the inflammatory response to IAV infection. The inbred mouse strain DBA/2J has been shown to have strong innate immune responses, notably increased ISG and proinflammatory cytokines, to IAV infection compared to C57BL/6J mice (Alberts et al., 2010). DBA/2J mice are highly susceptible to IAV infection and display worsened lung pathology compared to C57BL/6J mice.
as a consequence of this excessive inflammation (Srivastava et al., 2009). These reports provide precedence for exploring whether such intrinsic differences may contribute to the underlying pathogenesis to IAV infection we have observed here in \textit{Aim2}\textsuperscript{-/-} mice. To test this possibility, we compared the expression of innate immune-related genes in WT and \textit{Aim2}\textsuperscript{-/-} AMs in response to in vitro IAV infection. Of the 94 innate response genes on our custom Nanostring codeset, expression of 16 genes showed a two-fold or greater difference in \textit{Aim2}\textsuperscript{-/-} AMs responding to IAV over WT (Fig 3.8A). Notably, induction of \textit{il6} was decreased and \textit{tnfa} was unaffected, supporting the possibility the increase we observed for these cytokines in lungs of \textit{Aim2}\textsuperscript{-/-} mice is due to a greater number of leukocytes producing them. In good agreement with previous findings (Panchanathan et al., 2010), the genes we did find expressed at higher levels in \textit{Aim2}\textsuperscript{-/-} AMs were mostly ISGs including \textit{ifi204}, \textit{irf7}, \textit{stat1}, \textit{ifit1}, etc. We found two notable genes, \textit{tlr3} and \textit{cxcl10}, whose increased expression could potentially account for the increased inflammatory responses of AIM2-deficient mice. A previous reports by le Goffic et al. demonstrated IAV is recognized by TLR3, and its deletion in mice alleviated mortality and lung inflammation during IAV infection (Le Goffic et al., 2007; 2006). In our studies, we found the expression of \textit{tlr3} was repressed in AMs infected with IAV and that deletion of AIM2 alleviated this repression (Fig 3.8B). Thus, one possibility to consider is sensing of viral RNA through increased TLR3 expression in \textit{Aim2}\textsuperscript{-/-} mice exacerbates inflammatory responses to IAV in vivo. The other gene of interest from our data is the IFN-inducible CXCL10 (aka
IP-10), a chemokine able to attract activated CD8+ T cells (Taub et al., 1993) and inflammatory monocytes (Lin et al., 2008) expressing the receptor CXCR3. Though thought to be largely important in antiviral immunity, the effector functions of CD8+ T cells have been strongly implicated as the key cell type mediating immune pathology during IAV infection (Moskophidis and Kioussis, 1998; Wells et al., 1981; Wiley et al., 2001; Small et al., 2001). Indeed, CXCR3-deficiency impairs entry of CD8+ T cells into the airways of IAV infected mice (Fadel et al., 2008). Furthermore, genetic deletion or blocking of CXCL10 with monoclonal antibodies during IAV infection improved both survival and lung pathology in mice (Wang et al., 2013). In addition to the cell intrinsic effects of Aim2 deficiency in AMs, we found expression of cxcl10 to be higher in Aim2−/− BMDMs in response to LPS (TLR4), MCMV (cGAS), and SeV (RIG-I), implicating a broad role for AIM2 in suppressing cxcl10 expression downstream of multiple PRRs. Taken together, CXCL10 is an important chemokine contributing to detrimental inflammation during IAV infection. Further work will be insightful in determine CXCL10’s contribution in the inflammatory phenotype observed in Aim2−/− mice.

Overall, these studies suggest that accumulation of DNA in tissues damaged by infection may provide a mechanism for alerting the immune system to the extent of tissue damage and function as a signal to limit excessive immune pathology. Additionally, we have described a novel function for the DNA receptor AIM2 in tempering inflammation during an RNA virus infection. The identification
of AIM2 as a regulator of tissue inflammation adds to our understanding of the cross talk that exists between innate immune pathways. We theorize that host cell DNA signals via AIM2 as a mechanism of sensing tissue damage and adjusting the innate response appropriately to mitigate immune pathology. However, the exact molecular mechanisms extracellular DNA is sensed by AIM2 and how AIM2 is able to modulate inflammation is unknown. Further work will need to be done in order to delineate the cell autonomous effects of AIM2 deficiency from the relative contribution of DNA sensing by the innate immune system.

Also unknown is whether the mechanism we have identified here is specific to viral pneumonia and lung damage brought about by IAV infection, however, we would predict that similar mechanisms may be at play in other viral infections. Further study of endogenous DAMPs and their role in shaping antiviral immunity, repair and regenerative responses will also lead to improved understanding of viral pathogenesis and could also yield new insights for vaccine developments.
Chapter 4

Discussion
The capacity for nucleic acids to stimulate antiviral responses has been appreciated for sometime but the means of their sensing through the innate immune system have only recently begun to be uncovered. Present in microbes and host alike, the presence of nucleic acids outside their normal residence provides a signal of infection and damage requiring the attention of the immune system. The ability for PRRs to indiscriminately sense nucleic acids allows for rapid detection of incoming virus infection to initiate robust antiviral and inflammatory responses. There is a price to be paid in gaining this ability however as the prolonged signaling by the host’s own nucleic acids through PRR pathways can have deleterious effects in the form of autoimmunity and other pathologies. Though we tend to think of microbial-derived molecules as the sole input signal for initiation of the immune response, fact of the matter is the tissues damaged by the pathogen release molecules, including nucleic acids, available for sensing by PRRs. These host-derived signals may have significant effect on modulating both the innate and adaptive immune responses. Understanding the mechanisms for the detection of nucleic acids, as well as how these signals integrate, is important expanding our knowledge of the immune response and how we might better manipulate these pathways to create better therapeutics and vaccines.

The works presented here examine the receptors and pathways involved in the recognition both DNA virus infection and host DNA alike, and how they contribute to host defense. Much to our surprise there appears to be a limited
role for known DNA sensing pathways in responding to gammaherpesvirus infection. In investigating the contribution of the inflammasome in defense against gammaherpesviruses we found both NLRP3 and ASC are required both in vitro and in vivo for the production of IL-1β, however AIM2 appears to not be involved despite MHV-68 being a DNA virus. Though these inflammasome components were dispensable in controlling acute phase MHV-68 infection, the higher viral burden later on suggest they play an important role in regulating the pool of latently infected cells.

Further highlighting the complexity of innate sensing, we found during infection with an RNA virus that host DNA, presumably released for necrotic cells, accumulating in the lung microenvironment provides protection. Consistent with this observation is that the DNA receptor AIM2 is required for protection from influenza infection but not for the control of viral replication. Rather AIM2 appears to dampen the magnitude of the inflammatory response to flu as its genetic ablation resulted in an increase in the number of recruited immune cells and amount of proinflammatory cytokines in the lungs. Interestingly, AIM2 deficiency appears to have cell autonomous effects by negatively regulating responses through other PRR pathways activated by nucleic acids as well as other molecular structures in vitro, however it remains to be determined whether these effects regulate responses in vivo. Thinking about the cause of mortality in the case of DNA ablation or AIM2-deficiency, I hypothesize that the increased number of recruited immune cells leads to exacerbated immune pathology. If this
were to be true, then it would suggest that host nucleic acids are recognized by the innate immune system as a means of surveying the extent of tissue damage during infection to avoid further self-inflicted damage by the immune response. Future efforts will help to determine whether this is truly the case and if it applies to other scenarios.

The evolved ability by vertebrates to detect and respond to nucleic acids gives a big advantage in the defense against viruses, but these pathways must be tightly regulated to prevent their unwarranted activation. While these works will help to further our understanding of the mechanisms for recognizing both foreign and endogenous molecules, they also serve as an example of the complex integration of signals by the innate immune system in order to elicit an appropriate immediate response to infection and damage. The sum of the signals from multiple PRR pathways dictates the immune response, both qualitatively and quantitatively, rather than one molecule or pathway being responsible for driving and regulating the response in each case. Investigation into the cross-regulation of innate sensing pathways should be fruitful and further expand our knowledge of the immune system as a whole.
Chapter 5

Materials and Methods
Chapter 2

Animals

Asc/-/- and Nlrp3/-/- were used in this study with C57BL/6 controls. Aim2+/+ and Aim2/-/ mice were generated in our own lab (Rathinam et al., 2010). All procedures used in this study complied with federal guidelines and were approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee.

Viruses

MHV-68 (WUMS strain) and MCMV (Smith strain) were gifts from R Welsh, (UMass Medical School). HSV-1 7134 and 7134 were gifts from D Knipe (Harvard Medical School). MHV68 was propagated and titrated in Vero cells in Dulbecco’s modified essential medium. MCMV was propagated and titrated in 3T3 fibroblasts in Dulbecco’s modified essential medium. MCMV and MHV-68 was pelleted by ultracentrifugation for 2 h at 4 °C at 24,000 rpm, and the pellets were resuspended in endotoxin-free sterile Dulbecco’s PBS.

Generation and culture of mouse macrophages and dendritic cells from bone marrow

Bone marrow cells were harvested from the hind legs of mice. BMDCs were cultured in RPMI 1640 medium with 10% heat-inactivated FCS, 200 IU/ml penicillin, 100 mg/ml streptomycin and 40 ng/ml GM-CSF (Preprotech). On days 3 and 5, fresh GM-CSF was added and BMDCs used on days 7 and 8. BMDMs were cultured in DMEM containing 10% heat-inactivated FCS, 200 IU/ml penicillin, 100 mg/ml streptomycin, and 20% conditioned L929 supernatant.

Nanostring analysis

RNA was isolated from cells that were mock infected or infected with HSV-1 7134, HSV-1 7134R, MCMV and MHV68, at MOI 10. Cells were harvested into RLT buffer containing 2-ME for subsequent processing with the RNeasy Mini kit (QIAGEN). Each RNA sample was adjusted to contain the same quantity using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific). RNA was hybridized and quantified with the nCounter Analysis System (NanoString Technologies, Seattle, WA) using a customized probe set containing probes for innate immune genes, per the manufacturer’s protocol. The gene expression data were normalized to an internal positive control, then to an internal negative control, and, finally, to seven housekeeping genes: GAPDH, b-glucuronidase, b-actin, hypoxanthine phosphoribosyltransferase 1, tubulin b, phosphoglycerate kinase 1, and clathrin H chain 1. All values were log2 transformed, and a heat map was generated using the open source R-based software at the University of Massachusetts Medical School.
MHV-68 infection

Mice were given $5 \times 10^5$ pfu MHV-68 in 200 uL sterile PBS by intraperitoneal injection.

Quantification of MHV-68 viral loads

For detection of viral genomic material, DNA was isolated from spleens using an ISOLATE II Genomic DNA Kit (Bioline). Viral genome copy numbers were determined by quantification of gB DNA using a plasmid standard curve. For amplification of MHV68 glycoprotein B DNA we used the following primers: forward: 5'-CCGCTCATTACGGCCCAAATTCAA-3' and reverse: 5'-GGCAGCGACAGGCTTTCCATAAAT-3'. The standard plasmid was created by insertion of a 121 bp fragment of glycoprotein B DNA amplified with the primers and inserted into a TOPO-TA cloning vector (Invitrogen). qPCR was performed using iTaq SYBR Green Super Mix (Bio-Rad) and a CFX96 thermocycler (Bio-Rad).

Cytokine quantification

Levels of IL-1β and IFNγ in spleen homogenates was quantified by ELISA (eBioscience). Spleen tissues were weighed prior to homogenization in 500 uL PBS. Homogenates were spun at 3,000g for 10’ and supernatants were collected for analysis.

Statistical Analysis

Data were analyzed using the two-tailed Student’s t test comparing means between groups. A p-value <0.05 was considered statistically significant. Graphing and statistical analyses were done using GraphPad Prism.

Chapter 3

Animals

Both Aim2−/− mice generated in our own lab and AIM2fl/fl mice, a kind gift from Vishva Dixit, crossed to CMV-Cre mice for germline deletion were used in this study (Jones et al., 2010; Rathinam et al., 2010). All knockout mice have been backcrossed twelve generations onto the C57BL/6 background. Age- and sex-matched C57BL/6 (WT) controls were purchased from The Jackson Laboratory (Bar Harbor, ME). Itgax-Cre, LysM-Cre, and Vav-Cre transgenic mice were purchased from The Jackson Laboratory. All procedures used in this study complied with federal guidelines and were approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee.
Influenza virus infection

Influenza virus A/Puerto Rico/8/1934 H1N1 (PR8) grown in chicken eggs was purchased from Charles River Laboratories (Wilmington, MA). Mice were first anesthetized with isofluorane and inoculated via intranasal route with 4 x 10^4 pfu in 30 µL PBS.

Measurement of viral load and gene expression

Total RNA from lung tissue was extracted using Qiazol reagent (Qiagen) and reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad). qPCR assays were performed using iTaq Universal Probes Master Mix or iTaq SYBR Green Super Mix (Bio-Rad) using a CFX96 thermocycler (Bio-Rad). Relative gene expression was determined using the 2ΔCt method with GAPDH as a housekeeping gene. Viral loads were determined by quantification of viral PA copy number by TaqMan probes (Trudeau Institute Molecular Biology Core Facility, Saranac Lake, NY) using a plasmid standard curve as previously described (McKinstry et al., 2009). Dnase I mRNA levels were quantified by qPCR using the following primer sequences: mDnaseI fwd 5’-TCGCTATGACATCGCTGTTAT-3’, mDnaseI rev 5’-GTCAGGTTTGTCGCCGATTGAG-3’, mGAPDH fwd 5’-TGGCAAAAGTGAGATTGTGCC-3’, mGAPDH rev 5’-AAGATGGTGATGGGCTTCCG-3’

Quantification of DNA in BAL

BAL was harvested using 1 mL PBS. Cells and other debris in BALF were pelleted by centrifugation at 5,000 x g, 10 min, at 4°C and supernatants harvested. dsDNA in BAL supernatants was quantitated by PicoGreen assay using dilutions of calf thymus DNA to make a standard curve.

Histological analysis

Lungs were inflated with 1 mL of 10% neutral buffered formalin prior to fixation in 10% neutral buffered formalin at least overnight. Samples were subsequently paraffin-embedded, sectioned for hematoxylin and eosin staining.

Flow cytometry and tetramer staining

Mice were perfused with 10 mL PBS in the right ventricle prior to the removal of lungs. To make single cell suspensions, lungs were minced with a razor blade and strained through a 70 µM cell strainer (BD Biosciences). Prior to staining, Fc receptors were blocked using supernatant from 2.4G2 hybridoma cells (anti-CD16/32). Cells were stained using anti-TCRβ-PerCP-Cy5.5, anti-CD19-PerCP-Cy5.5, anti-B220-AlexaFluor 488, anti-CD11b-APC-eFluor 780, anti-CD11c-eFluor 450, anti-Ly6G-PE-Cy7, anti-Ly6C-APC, anti-NK1.1-APC (eBioscience),
anti-CD45-V500, anti-CD4-PE-Cy7, and anti-CD8α-PE (BD Biosciences). Live/Dead Blue (Life Technologies) was included to determine cell viability determined. Data acquisition was performed using a 4-laser LSRII (BD Biosciences). For tetramer staining, cells were stained using anti-TCRβ-PerCP-Cy5.5, anti-CD8α-PE, anti-CD4-PE-Cy7, anti-CD44-APC-eFluor780 and APC-labeled tetramers Kb/NP 366, Kb/PA 244, Kb/PB1 703, or I-Ab/ NP 311 (Trudeau Institute Molecular Biology Core Facility, Saranac Lake, NY) in 100 uL 2% FCS – PBS. Live cells were gated based on forward and side scatter, and Live/Dead Blue negative staining prior to subsequent gating. Analysis was performed using FlowJo analysis software (TreeStar).

**Cytokine quantification**

Levels of IL-1β, IL-6, and TNFα in lung homogenates was quantified by ELISA (eBioscience). Lung tissues were weighed prior to homogenization in 500 uL RIPA buffer. Homogenates were spun at 3,000g for 10’ and supernatants were collected for analysis.

**Alveolar macrophage sorting and Nanostring analysis**

Lung single-cell suspensions were blocked with anti-CD16/32 prior to being stained with anti-CD45-APC-Cy7 (eBioscience), anti-CD11c-APC (Tonbo Biosciences), and anti-Siglec- F-PE (BD Biosciences). Alveolar macrophages were defined as SSChi CD45+ CD11c+ Siglec-F+ autofluorescence+ and sorted using a BD FACSJazz (Schneider et al., 2014). Sorted alveolar macrophages were plated into a 12 well dish in RPMI containing 10% FCS. Cells were infected with IAV PR8 at MOI 2. Total RNA was purified at 6 hours post-infection using an RNeasy kit (Qiagen). RNA was hybridized and quantified with the nCounter Analysis System (NanoString Technologies, Seattle, WA) using a customized probe set containing probes for innate immune genes, per the manufacturer’s protocol. The gene expression data were normalized to an internal positive control, then to an internal negative control, and, finally, to seven housekeeping genes: GAPDH, b-glucuronidase, b-actin, hypoxanthine phosphoribosyltransferase 1, tubulin b, phosphoglycerate kinase 1, and clathrin H chain 1. All values were log2 transformed, and a heat map was generated using the open source R-based software at the University of Massachusetts Medical School.

**Generation of recombinant adeno-associated virus**

Briefly, mouse DNaseI was cloned into an rAAV vector plasmid carrying a vector genome with the expression cassette driven by CMV enhanced chicken β-actin promoter and flanked by AAV2 ITRs. The AAV-Dnasel plasmid was co-transfected into HEK 293 cells with an AAV9 packaging plasmid and adenovirus helper plasmid. The recombinant virus was purified by standard CsCl gradient
sedimentation method and desalted by dialysis (Gao and Esteves, 2012). Mice were first anesthetized with isofluorane and inoculated via intranasal route with 1010 pfu in 30 μL PBS at least two weeks prior to experiments.

**Bone marrow transplantation**

Recipient mice were lethally irradiated with 600 rad followed by intravenous tail injection with 10^7 donor bone marrow cells. Recipients were put on sulfatrim antibiotic water and allowed to rest for 6 weeks after engraftment.

**Statistical Analysis**

Data were analyzed using the two-tailed Student’s t test comparing means between groups. Kaplan-Meier survival curves were analyzed by Mantel-Cox log-rank test. A p-value <0.05 was considered statistically significant. Graphing and statistical analyses were done using GraphPad Prism.
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