SUPPRESSIVE OLIGODEOXYNUCLEOTIDES INHIBIT CYTOSOLIC DNA SENSING PATHWAYS

A Dissertation Presented

By

John Joseph Kaminski, III

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester

In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

April 29th, 2013

MD / PhD Program

Suppressive oligodeoxynucleotides inhibit cytosolic DNA sensing pathways

A Dissertation Presented

By

John Joseph Kaminski, III

The signatures of the Dissertation Defense Committee signify completion and approval as to style and content of the Dissertation

Katherine A. Fitzgerald, Ph.D., Thesis Advisor

Egil Lien, Ph.D., Member of Committee

Eva Szomolanyi-Tsuda, M.D., Member of Committee

Peter Newburger, M.D., Member of Committee

Stefanie Vogel, Ph.D., Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

Evelyn A. Kurt-Jones, Ph.D., Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the school.

Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

MD / PhD Program

April 29th, 2013

Acknowledgements

When I entered graduate school I thought I knew what it was to work hard, to sacrifice and to push on despite defeat. I thought I had the will and the intelligence to accomplish any task. I was wrong. Without your guidance, without your support and without your friendship I would never have made it. Thank you.

I would like to thank my mentor, Dr. Katherine Fitzgerald, for supporting my research throughout my time here at UMass. I have truly appreciated your candid advice and guidance throughout this graduate process. I would also like to thank Dr. Evelyn Kurt-Jones for her suggestions and guidance over the years.

I would like to thank the University of Massachusetts and all of the support staff that have assisted me in various capacities. The core facilities whose expertise was remarkable and the administrative staff who kept me informed, helped me organize and finalize the requirements for graduation. In particular I'd like to thank Gaile Arcouette, Anne Michelson and Heather Montoya for all of their assistance.

I would like to thank the members and former members of the Fitzgerald Lab. Thank you Lisa, Vijay, Shruti, Zhaozhao, Susan and Maninjay, Sandhya, Mikayla, Stefan, Jennie, Soren, Sreya, Numana, Joe and Annie for all your advice, for your help conducting experiments, your support, encouragement and friendship. I would also like to thank members of the Kurt-Jones lab, Anna and Glennice for helping me with *in vivo* experiments and analysis, members of the Golenbock lab, Parisa and Jenny for their assistance with confocal experiments and for sharing cell lines and Tia Bumpus from the Marshak-Rothstein lab for helping me edit this dissertation. I would like to thank my Thesis Research Advisory Committee, Dr. Evelyn Kurt-Jones, Dr. Egil Lien, Dr. Eva

Szomolanyi-Tsuda, and Dr. Peter Newburger for guiding me towards the completion of my graduate studies.

I would also like to thank all my family and friends, my mother, my father and brother. Your endless support, love and encouragement gives me the strength to keep going. You are truly the key to my success. Thank you.

Abstract

The innate immune system provides an essential first line of defense against infection. Innate immune cells detect pathogens through several classes of Pattern Recognition Receptors (PRR) allowing rapid response to a broad spectrum of infectious agents. Activated receptors initiate signaling cascades that lead to the production of cytokines, chemokines and type I interferons all of which are vital for controlling pathogen load and coordinating the adaptive immune response. Detection of nucleic acids by the innate immune system has emerged as a mechanism by which infection is recognized. Recognition of DNA is complex, influenced by sequence, structure, covalent modification and subcellular localization.

Interestingly certain synthetic oligodeoxynucleotides comprised of the TTAGGG motif inhibit proinflammatory responses in a variety of disease models. T hese suppressive oligodeoxynucleotides (sup ODN) have been shown to directly block TLR9 signaling as well as prevent STAT1 and STAT4 phosphorylation. Recently AIM2 has been shown to engage ASC and assemble an inflammasome complex leading to the caspase-1-dependent maturation of IL-1β and IL-18. T he AIM2 inflammasome is activated in response to cytosolic dsDNA and plays an important role in controlling replication of murine cytomegalovirus (MCMV). In the second chapter of this thesis, a novel role for the sup ODN A151 in inhibiting cytosolic nucleic acid sensing pathways is described. Treatment of dendritic cells and macrophages with the A151 abrogated type I IFN, TNF-α and ISG induction in response to cytosolic dsDNA. A151 also reduced INF-β and TNF-α induction in BMDC and BMDM responding to the herpesviruses HSV-1 and MCMV but had no effect on the responses to LPS or Sendai virus. In addition, A151

abrogated caspase-1-dependent IL-1β and IL-18 maturation in dendritic cells stimulated with dsDNA and MCMV. A lthough inhibition of interferon-inducing pathways and inflammasome assembly was dependent on backbone composition, sequence differentially affected these pathways. While A151 more potently suppressed the AIM2 inflammasome, a r elated construct C151, proved to be a more potent inhibitor of interferon induction. A151 suppressed inflammasome signaling by binding to AIM2 and competing with immune-stimulatory DNA. The interaction of A151 and AIM2 prevented recruitment of the adapter ASC and assembly of the macromolecular inflammasome complex. C ollectively, these findings reveal a new route by which suppressive ODNs modulate the immune system and unveil novel applications for suppressive ODNs in the treatment of infectious and autoimmune diseases.

The innate immune response to HSV-1 infection is critical for controlling early viral replication and coordinating the adaptive immune response. The cytokines IL-1β and IL-18 are important effector molecules in the innate response to HSV-1 *in vivo*. However, the PRRs responsible for the production and maturation of these cytokines have not been fully defined. In the third chapter of this thesis, The TLR2-MyD88 pathway is shown to be essential for the induction of pro-IL-1β transcription in dendritic cells and macrophages responding to HSV-1. The HSV-1 immediate-early protein ICP0 has previously been shown to block TLR2 responses and in keeping with this finding, ICP0 blocked pro-IL-1β expression. Following translation, pro-IL-1β exists as an inactive precursor that must be proteolytically cleaved by a multiprotein complex known as the inflammasome to yield its active form. Inflammasomes are composed of cytoplasmic receptors such as NLRP3 or AIM2, the adapter molecule ASC, and pro-

caspase-1. In the present study we found that the NLRP3 inflammasome is important for maturation of IL-1β in macrophages and dendritic cells responding to HSV-1. In contrast the related NLRP12 protein controls IL-1β production in neutrophils. These data indicate that sensing of HSV-1 by TLR2 drives pro-IL-1β transcription and infection activates the inflammasome to mature this cytokine. Moreover, these studies reveal cell type-specific roles for NLRP3 and NLRP12 in inflammasome assembly.

Table of Contents

Title Page……...……i

Signature Page……..……..ii

Acknowledgements……...……iii

Abstract……...……v

Table of Contents……viii

List of Tables……..……x

List of Figures……xi

List of Abbreviations……..……xiii

Preface to Chapter I ……..…xvi

Chapter I

1.1 Introduction……...……...……...……..……..1

1.2 Pattern Recognition Receptors……..……..2

1.2.1 Toll-like receptors…….……..3

1.2.2 Cytosolic sensors……4

1.2.3 RIG-I-like receptors…….……...6

1.2.4 Interferon-inducing cytosolic DNA receptors……9

1.2.5 PYHIN receptors…….……15

1.2.6 NOD-like receptos and the Inflammasome…….……18

1.3 Suppressive Oligodeoxynucleotides……..…..……...24

1.3.1 The Phosphorothioate backbone……...25

1.3.2 Modulation of innate immunity by Suppressive ODNs……....25

1.4 Herpesviruses……...…….……...31

1.4.1 Herpes Simplex Virus Type 1……..……..32

1.4.2 HSV-1, the pathogen and pathophysiology..…….……33

1.3.3 Role of innate immunity in recognizing HSV-1……..……..34

1.5 Dissertation Objectives……...……..……37

Preface to Chapter II ……..39

Chapter II: Suppressive oligodeoxynuclotides inhibit cytsolic DNA sensing

Abstract……40
Introduction……..……41
Results……..……46
Discussion……..……..75

Preface to Chapter III ……..……..82

Chapter III: Cell type-specific activation of the NLRP3 and NLRP12 inflammasomes in response to HSV-1

Abstract……83
Introduction……..……84
Results……..……....87
Discussion…..……100

Chapter IV: Materials and Methods……104

Chapter V: Discussion and Perspectives……..110

Bibliography……...117

List of Tables

Chapter II

Table 2.1 Oligodeoxyribonuclotide Construct Sequences

Table 2.2 Nanostring Normalized mRNA Counts

Table 2.3 Nanostring mRNA Fold Induction Compared to Media

Table 2.4 Nanostring mRNA Fold Reduction by A151 Treatment

Chapter III

Table 3.1 Nanostring Normalized mRNA Counts

Table 3.2 Nanostring mRNA Fold Induction Compared to Media

List of Figures

Chapter I

Figure 1.1 Interferon-inducing cytosolic dsDNA sensing PRRs

Figure 1.2 The human AIM2 and IFI16 proteins

Figure 1.3 AIM2 inflammasome signaling

Chapter II

Figure 2.1 A151 broadly suppresses inflammatory gene induction in response to the dsDNA ligand poly(dA:dT)

Figure 2.2 A151 and C151 suppress IFN-β and TNF-α production in response to poly(dA:dT), HSV-1 and MCMV

Figure 2.3 Sequence and backbone composition affect inhibition of IFN-β production

Figure 2.4 A151 prevents inflammasome activation in response to cytosolic dsDNA

Figure 2.5 Sequence and backbone composition affect suppression of AIM2-mediated cytokine production

Figure 2.6 Sequence and backbone composition affect AIM2-mediated pyroptosis

Figure 2.7 Suppressive ODN inhibit AIM2 inflammasome formation in reconstituted HEK293T cells

Figure 2.8 Suppressive ODN inhibit AIM2 inflammasome formation following infection with MCMV and Listeria

Figure 2.9 A151 prevents ASC recruitment and AIM2 speck formation

Figure 2.10 Suppressive ODN interact with AIM2 and IFI16

Figure 2.11 Schematic model of A151 and C151-mediated inhibition of cytoplasmic receptors

Chapter III

Figure 3.1 Type I IFN, ISG and cytokine responses to HSV-1 depend on STING and MyD88 signaling

Figure 3.2 HSV-1 induces transcription of pro-IL-1β in a TLR2-MyD88 dependent manner

Figure 3.3 HSV-1 induces IL-1β secretion in an NLRP3-dependent manner in PEC, BMDM and BMDC

Figure 3.4 HSV-1 induces IL-1β secretion in an NLRP12-dependent manner in neutrophils

List of Abbreviations

AIM2	Absent in melanoma-2
ALR	AIM2-like receptors
ASC	Apopotosis-associated speck-like protein containing CARD
BMDC	Bone marrow derived dendritic cell
BMDM	Bone marrow derived macrophage
CARD	Caspase activation and recruitment domain
CDC	Conventional dendritic cell
c-di-AMP	Cyclic diadenosine monophosphate
c-di-GMP	Cyclic diguanosine monophosphate
cGAS	Cyclic GMP-AMP Synthase
CVB	Coxsackie B virus
DAI	DNA-dependent activator of IFN-regulatory factors
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DDX	DEAD (Asp-Glu-Ala-Asp) box polypeptide
DHX	DEAH (Asp-Glu-Ala-His) box polypeptide
dsDNA	double-stranded deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid
EBV	Epstein-Barr virus
ECMV	Encephalomyocarditis virus
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FGF	Fibroblast growth factor
HCMV	Human cytomegalovirus
HEK	Human embryonic kidney
HHV	Human herpes virus
HIN-200	Hematopoietic interferon-inducible nuclear antigens with 200 amino acid repeats
HMGB1	High mobility group box 1
HPFS	hereditary periodic fever syndrome
HSE	Herpes simplex encephalitis
HSV	Herpes simplex virus
ICP0	HHV infected cell polypeptide 0
IDO	Indoleamine 2,3-dioxygenase
IFI	Gamma-interferon-inducible protein
IFN	Interferon
IL	Interleukin
IRF	Interferon regulatory factor
ISD	Interferon stimulatory DNA
ISG	Interferon stimulated gene
KSHV	Kaposi sarcoma-associated herpesvirus
LAT	Latency associated transcript
LGP2	laborator of genetics and physiology 2
LPS	Lipopolysaccharide

LRR	Leucine-rich-repeat
LRRFIP1	Leucine-rich repeat flightless-interacting protein 1
Mal	MyD88-adapter-like
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial antiviral signaling protein
MCMV	Murine cytomegalovirus
MDA5	Melanoma differentiation-associated gene-5
MNDA	Myeloid nuclear differentiation antigen
MyD88	Myeloid differentiation primary response gene 88
NALP	NACTH, LRR and PYD domain-containing protein
NDV	Newcastle disease virus
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	NF-κB inducing kinase
NK	Natural Killer
NLRP	NOD like receptors
NO	Nitric oxide
OB	Oligonucleotide/oligosaccharide binding
ODN	Oligodeoxyribonucleic acids
PAMP	Pathogen-associated molecular pattern
PD	Phosphodiester
PDC	Plasmacytoid dendritic cell
PML	Promyelocytic leukemia protein
Poly(dA:dT)	Polydeoxyadenylic acid : polythymidylic acid
poly(dG:dC)	Polydeoxyguanylic acid : polydeoxycytidylic acid
Poly(I:C)	Polyriboinosinic acid : polyribocytidylic acid
PRR	Pattern Recognition Receptor
PS	Phosphorothioate
PYD	Pyrin domain
PYHIN	Pyrin and HIN domain-containing protein
RD	Repressor domain
RIG-I	Retinoic acid-inducible gene-I
RLR	Rig-I-like receptors
ROS	Reactive oxygen species
RPA	Replication protein A
RSV	Respiratory syncytial virus
SLE	Systemic Lupus Erythematosus
ssDNA	single-stranded deoxyribonucleic acid
ssRNA	single-stranded ribonucleic acid
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
Sup ODN	Suppressive oligodeoxyribonucleic acids
SV	Sendai virus
TBK1	TANK-binding kinase 1
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor

TMEV	Theiler's murine encephalomyelitis virus
TNF	Tumor necrosis factor
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adapter molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
VEGF	Vascular endothelial growth factor
VSV	Vesicular stomatitis virus
VACV	Vaccinia Virus
VZV	Varicella-zoster virus

Preface to Chapter I

The chapters of this dissertation have appeared in the following publications/manuscripts:

Mikayla R. Thompson, John J. Kaminski, Evelyn A. Kurt-Jones, Katherine A. Fitzgerald. 2011. *Pattern recognition receptors and the innate immune response to viral infection*. Viruses. 6:920-40.

Chapter I

1.1 Introduction

The human immune system can be divided into two main components, the innate and the adaptive responses. The innate immune response is the first line of defense against pathogens. This response is coordinated by a variety of specialized cells including monocytes, macrophages, dendritic cells (DCs), neutrophils, eosinophils, basophils, natural killer (NK) cells, and NK T cells. These cells are activated by germline-encoded Pattern Recognition Receptors (PRRs). PRRs bind to molecular signatures, often-essential structural or genetic components, which are conserved among pathogens. This allows the innate immune system to respond rapidly to a broad range of infectious agents that have breached the skin and mucous membranes. In contrast, the adaptive immune system is slow to mobilize but provides a highly antigen specific response. This is accomplished through the somatic recombination of T and B cell receptors and provides a mechanism for long-lived specific immunity.

The innate immune system is essential for controlling pathogen load and activating the adaptive immune response. The importance of innate immunity is highlighted by hereditary deficiencies in this response such as that seen in chronic granulomatous disease which leaves the host susceptible to recurrent bouts of infection (1). On the other hand, aberrant or uncontrolled immune responses can cause extensive tissue damage, exacerbate septic shock and contribute to the development of autoimmune diseases (2). Thus, a balance between activation and suppression must be struck to

ensure an appropriate and effective immune response. Understanding how the innate immune system is activated and how this response may be controlled will help in designing safe and effective therapeutic interventions.

1.2 Pattern Recognition Receptors

Innate immune cells detect pathogens through distinct classes of germline-encoded Pattern Recognition Receptors (PRR) including the Toll-like receptors (TLRs), the RIG-I-like receptors (RLRs), the NOD-like receptors (NLRs) and the AIM2-like receptors (ALRs; also known as the PYHIN family of receptors). These PRRs respond to a variety of conserved pathogen- and danger-associated molecular patterns (PAMPs/DAMPs) allowing rapid recognition and response to infectious agents. Activated receptors initiate signaling cascades that lead to the production of cytokines, chemokines and type I interferons, all of which are vital for controlling pathogen load early on and coordinating an effective adaptive immune response. Though the importance of these effectors was first recognized more than half a century ago, only in the past decade have we begun to understand the precise molecular pathways that lead to their production. The Toll-like receptors were the first group of PRRs discovered. TLRs mainly recognize PAMPs and DAMPs in the extracellular and endosomal compartments. More recently a number of PRRs, including the RLRs, NLRs and ALRs have been identified which survey the cytoplasmic and, in some cases, even the nuclear compartments. In the following sections these PRRs will be reviewed in detail with particular emphasis given to the sensing of DNA within the cytosol.

1.2.1 Toll-like receptors

Of the PRRs, the Toll-like receptors (TLRs) are the most extensively studied. TLRs are type 1 transmembrane proteins that traffic between the plasma membrane and endosomal vesicles. Those located on the plasma membrane are usually specific for hydrophobic lipids and proteins while those found in the endosome detect nucleic acids. This segregation allows innate cells to respond to components of the viral envelope and bacterial cell wall at their surface. In contrast, nucleic acids are detected in the endosome where many viruses uncoat their genomes and enter the cytoplasm. To date, 10 TLRs have been identified in humans while 13 have been identified in mice with TLRs 1-9 common to both. TLR1, TLR2, TLR4, TLR5 and TLR6 are located on the plasma membrane while TLR3, TLR7, TLR8, and TLR9 are endosomal. TLR2 forms heterodimers with either TLR1 or TLR6 and can respond to a variety of lipoproteins, peptidoglycan and liptechoic acid (3, 4). TLR4 is activated by LPS, a crucial component of the bacterial cell wall of gram-negative bacteria (5). TLR5 responds to flagellin a component of the flagellum, a highly conserved structure among motile bacteria (6). TLR3 recognizes dsRNA while TLR7 and TLR8 recognize ssRNA, species that are often associated with viral infection and replication (7). TLR9 is activated by unmethylated CpG DNA motifs that are common in bacterial and viral genomes but underrepresented in vertebrate DNA (8).

All TLRs share a common architecture consisting of extracellular leucine-rich repeats and a cytoplasmic Toll/Interleukin-1 Receptor (TIR) domain (9). These receptors

signal as dimers, differentially recruiting the adapter proteins: Myeloid differentiation primary response gene 88 (MyD88) and MyD88 adapter-like (Mal also known as TIRAP) and/or TIR-domain-containing adapter inducing IFN-β (TRIF) and TRIF-related adapter molecule (TRAM). Adapters initiate signal cascades culminating in the activation of nuclear factor kappa b (NF-κB), mitogen-activated protein kinase (MAPK) and interferon regulatory factors 1, 3, 5 and 7 (IRF-1, 3, -5 and -7) (10). Together, these transcription factors not only drive expression of interferons, cytokines and chemokines, but also influence cellular proliferation, maturation and survival.

The specific inflammatory response evoked by PAMPs and DAMPs depends on a variety of factors. First, cellular expression of TLRs differs amongst innate cell types. Human macrophages are known to express high levels of TLR2 and TLR4 while plasmacytoid dendritic cells (PDCs) mainly express TLR7 and TLR9 (9). Expression patterns also vary between species. While TLR9 is restricted to a few cell types in humans, it is widely distributed in mice (11). Furthermore, expression of certain downstream signaling molecules fluctuates between innate cell types. For example, plasmacytoid dendritic cells (PDCs) are unique in that they constitutively express the transcription factor IRF7, allowing them to quickly produce high levels of type I IFNs in response to viral infection while other cell types such as macrophages may respond in a more delayed manner (10, 12). Thus, the response to identical ligands may differ between cell types both in the nature of effector molecules produced and the kinetics of the response.

1.2.2 Cytosolic sensors

All viruses and many bacteria enter the cell's cytoplasmic compartment during their life cycles. Viruses, such as HSV, gain entry via fusion of their envelope with the cell's outer plasma membrane while other viruses, such as influenza, fuse in the endosomal vesicle. During the process of viral replication the host's own cellular machinery is co-opted to produce a large number of virions. This process leads to the accumulation of viral nucleic acids, which are one of, if not the most common, cytosolic PAMP observed in viral infections (13). The cytosolic PRRs responsible for detecting nucleic acids have been intensely investigated over the past few years and our body of knowledge has grown rapidly. Cytosolic nucleic acid sensors can be divided into those that respond to RNA (RLRs) and those that respond to DNA (NLRs, ALRs, and others). Recent research has revealed that cytosolic DNA receptors lead to the activation of two distinct inflammatory pathways. Activation of the first pathway results in the expression of type I interferons, cytokines and chemokines through the activation of IRF-3 and -7 and NF-κB, respectively (13). A growing number of receptors have been identified that activate this pathway though much debate still exists over the relative and specific contributions of each to effector induction. The second pathway is characterized by the assembly of a 'inflammasome complex' and results in the caspase-1-dependent activation and secretion of IL-1β and IL-18. Members of the NLR family of receptors were the first shown to activate this pathway (14, 15). In addition, AIM2 a member of the PYHIN family, has also been shown to assemble an inflammasome (16, 17). Our discussion will begin with the mechanisms of RNA sensing by RLRs and proceed to DNA sensing by NLRs, ALRs and other receptors.

1.2.3 Rig-I-like receptors

The RLR family is comprised of three DExD/H box RNA helicases: retinoic acid-inducible gene (RIG-I), melanoma differentiation-associated gene 5 (MDA-5) and laboratory of genetics and physiology-2 (LGP-2) (18-21). Both RIG-I and MDA-5 are comprised of tandem N-terminal caspase activation and recruitment domains (CARDs) followed by a DExD/H box RNA helicase domain, which has ATPase activity, and a C-terminal repressor domain (RD). Unlike RIG-I and MDA-5, LGP-2 lacks the N-terminal CARD domains and contains only the RNA helicase domain. As such, LGP-2 was postulated to act as a negative regulator of the other RLRs (19, 21). Under resting conditions, RIG-I resides in the cytoplasm in an inactive form that is autoinhibited by its regulatory domain. Upon binding dsRNA, RIG-I undergoes a conformational change and dimerizes in an ATP dependent manner (21). The activated multimeric form of RIG-I or MDA5 interacts with the downstream adapter protein mitochondrial antiviral signaling protein (MAVS), also known as VISA, IPS-1, and CARDIF, via CARD-CARD interactions. MAVS is found on the outer leaflet of the mitochondrial membrane, a localization thought to be essential for downstream signaling (22). Recently, MAVS has also been observed on peroxisomes, where it induces an early antiviral response through the direct induction of a subset of anti-viral genes via the transcription factor IRF1 (23). Upon engagement of RIG-I or MDA5, MAVS activates the IKK-related kinase (TBK1, also known as IKKi), which in turn, activates IRF-3 and IRF-7, resulting in the transcription of type I interferons (24). MAVS also activates NF-κB through recruitment

of TRADD, FADD, caspase-8, and caspase-10 resulting in cytokine and chemokine production (25-27).

The RLRs are critical components of the anti-viral defense pathway in many cell types such as conventional dendritic cells as well as stromal cells (28). Initially, it was thought that both RIG-I and MDA-5 recognized the synthetic dsRNA, polyinosinic acid polyribocytidylic acid (polyI:C). However, studies using RIG-I- and MDA-5-deficient mice determined that MDA-5 alone was responsible for interferon production by polyI:C stimulation, while RIG-I recognized uncapped, 5'-triphosphorylated ssRNA, a common feature in many viral genomes (29). Importantly, RIG-I is unable to recognize the host's 5'-capped ssRNA (30-32). In addition, RIG-I is capable of recognizing short dsRNA, a byproduct of viral replication (33). MDA-5 distinguishes between viral and host RNA, not by its 5' end, but rather, by the length of the RNA sequence. Long dsRNA is not naturally present in host cells but is produced during infection with certain viruses.

RIG-I and MDA-5 recognize different classes of RNA viruses. Studies have implicated RIG-I in the recognition of vesicular stomatitis virus (VSV), rabies virus, Sendai virus (SV), Newcastle disease virus (NDV), respiratory syncytial virus (RSV), measles virus, Influenza A and B viruses, hepatitis C virus (HCV), Japanese encephalitis virus, and Ebola virus (28, 29, 34-36). Studies using MDA-5-deficient mice show that it recognizes encephalomyocarditis virus (EMCV), Theiler's murine encephalomyelitis virus (TMEV), coxsackie B virus (CVB) and polio (30, 35, 37, 38). These viruses do not contain 5'-triphosphate RNA, but produce long dsRNA during replication, providing further evidence that MDA5 discriminates between self and non-self RNA based on

sequence length. Recent studies have shown some viruses, such as dengue, West Nile virus, and reovirus, signal through a combination of both RIG-I and MDA-5 (37, 39, 40).

As mentioned above, LGP-2 lacks N-terminal CARD domains, and was first thought to be a negative regulator of RLR function (19, 21). Initial studies found that overexpression of LGP-2 decreased the capacity of SV and NDV to induce interferon production. Evidence that LGP-2 could associate with RIG-I through mutual RD domains led to the theory that LGP-2 directly prevented RIG-I association and activation. In addition, interferon signaling was found to be increased in LGP-2-deficient mice in response to polyI:C, providing evidence for negative regulation of MDA-5 as well (41). In contrast, a second *in vivo* study using LGP-2 deficient mice and mice harboring a inactivated version of LGP-2 showed that this protein acted as a positive regulator of RIG-I- and MDA-5-mediated signaling after infection. Thus LGP-2 may, in fact, enhance RIG-I- and MDA-5-dependent viral recognition by promoting RNA accessibility.

Another member of the DExD/H box RNA helicase family, DDX3 has been implicated in anti-viral defenses. Schroder *et al*. found that the vaccinia virus protein K7 inhibited IFN-β induction by binding to DDX3, which led to the discovery that DDX3 had a positive role in the RLR signaling pathway (42). A recent study reported that DDX3 binds to both polyI:C and viral RNA introduced into the cytosol and associates with MAVS and TBK-1 to upregulate IFN-β production. These results led the authors to speculate that DDX3 may enhance RNA recognition, forming a complex with RIG-I and MAVS to induce interferon production (43). Further studies are required to determine whether DDX3 is a bona fide RNA sensor or a component of the RLR signaling pathway.

1.2.4 Interferon-inducing cytosolic DNA receptors

The detection of cytosolic DNA has emerged as an important mechanism by which pathogens are recognized and protective immunity is generated. However, as recently as ten years ago, TLR9 was the only known sensor of foreign DNA. Early studies by Dr. Shizuo Akira and colleagues hinted at the existence of additional sensing mechanisms. One such study demonstrated that TLR9-deficient MEFs, which failed to respond to CpG DNA, produced large amounts of IFN in response to transfection with synthetic and viral dsDNA (44). The Medzhitov lab reported similar findings using a 45 bp interferon stimulatory DNA (ISD) derived from the *Listeria monocytogenes* genome (45). Recently, a number of cytosolic sensors have been identified and purported to be essential for the interferon response to dsDNA. These include DNA-dependent activator of IFN-regulatory factors (DAI), RIG-I via RNA polymerase III, Leucine-rich repeat flightless-interacting protein 1 (LRRFIP1), DEAD/H box peptides: DHX9, DHX36 and DDX41, cyclic GMP-AMP Synthase (cGAS) and interferon gamma-inducible protein 16 (IFI16) (16, 17, 46-49).

Like the cytosolic RNA recognition pathways, cytosolic DNA recognition leads ultimately to activation of TBK1 and IRF-3 and the production of type I IFN. However, the signaling pathway(s) linking DNA sensors to TBK1 are poorly characterized. TBK1 associates with DDX3, discussed previously, which regulates IFN-β transcription via IRF-3 (42, 43). In addition, TBK1 interacts with the exocyst protein Sec5 in a complex that includes the recently identified endoplasmic reticulum (ER) adapter stimulator of

interferon genes (STING) (27, 50-52). In both humans and mice, STING is critical in the signaling pathway upstream of TBK1 following HSV-1 infection (27). STING also interacts with the ER translocon components Sec61β and TrapB in a manner essential for regulation of cytosolic DNA-induced type I IFN production, although a mechanistic understanding of this relationship is not yet known (50). In unstimulated cells, STING localizes to the ER and mitochondria (52, 53). Following stimulation with cytosolic DNA and HSV-1, STING translocates to perinuclear foci, via the Golgi. One report suggests STING localizes partially to endosomes, particularly Sec5 positive structures (27), while another report argues that STING localizes to vesicular structures, which are not peroxisomes, mitochondria, endosomes or autophagosomes (54). In contrast, more recent evidence suggests STING can signal from the mitochondrial membrane in response to certain stimuli (53). Moreover, an intact mitochondrial membrane potential is essential for an optimal response. Further work is required to determine the precise subcellular localization of STING. What is evident is the essential role of STING in cytosolic DNA sensing pathways. Much less clear is the mechanisms or receptors which act upstream of STING. A growing number of receptors that utilize the STING-TBK1-IRF-3 pathway have been implicated in cytosolic DNA sensing and will be outlined below.

DAI. DNA-dependent activator of IFN-regulatory factors (DAI) was the first cytosolic DNA sensor discovered. It is composed of two binding domains for left-handed, Z form DNA, although the protein can recognize B form DNA as well (55). When DAI was exogenously expressed in L929 cells, it increased type I IFN production in a dose

dependent manner following stimulation by both B and Z form DNA. Similarly, knockdown of DAI impaired type I IFN production in response to the 45 bp ISD motif and HSV-1 (55). The production of type I interferons by fibroblasts in response to HCMV was also found to be dependent on DAI (56). Surprisingly, studies using DAI-deficient mice found normal immune responses to synthetic and viral dsDNA (57). Moreover DAI-deficient mice had normal responses to a DNA vaccine suggesting that DAI may play a cell-type specific or redundant role in sensing cytoplasmic DNA (58).

RNA polymerase III. Two groups have shown that AT-rich DNA can be transcribed by RNA polymerase III into 5'-ppp RNA, which subsequently activates RIG-I (47, 48). This pathway was reported to be involved in type I IFN induction during EBV infections**.** The RNA Pol III-RIG-I pathway was also reported to be involved in induction of type I IFN following HSV-1 or *Legionella pneumophila* infection (47, 48, 59). However, other groups have cast doubt on these claims suggesting that RIG-I only detects *L. pneumophia* RNA and not DNA (60). Moreover the MAVS signal molecule is not required for cytosolic DNA signaling in macrophages (61). Thus, the RNA Pol III-RIG-I pathway may play a cell-type specific role, but appears to be redundant in macrophages.

LRRFIP1. In addition to DAI and RNA Pol III, Leucine-rich repeat flightless-interacting protein 1 (LRRFIP1) has recently been implicated as a regulator of DNA-driven innate immune signaling. LRRFIP1 was originally identified due to its role in actin organization during drosophila embrogenesis. In a study using *Listeria monocytogenes* to screen for potential cytosolic DNA sensing molecules, knockdown of

LRRFIP1 was found to inhibit the type I IFN response (62). The authors showed that the IFN response to VSV was dampened in these cells as well. Knockdown of LRRFIP1 also inhibited IFN production in response to polyI:C, and the synthetic DNA species, poly(dG:dC) and poly(dA:dT), implicating LRRFIP1 in the recognition of dsRNA and dsDNA. Surprisingly, this function was independent of RNA Pol III. In stark contrast to other DNA sensors, LRRFIP1 did not activate IRF3, but rather, regulated a novel β-catenin-dependent coactivator pathway (63). LRRFIP1 bound RNA or DNA and led to the phosphorylation of β-catenin, which subsequently translocated to the nucleus where it associated with the p300 acetyltransferase at the IFN-β1 promoter, thereby inducing transcription. Further studies are needed in order to determine LRRFIP1's role *in vivo*.

DHX9 and DHX36. DEAH (Asp-Glu-Ala-His) box polypeptides 9 and 36 (DHX9 and DHX36), members of the DEAD/H box helicase family, have recently been shown to bind CpG-B and CpG-A DNA, respectively, in PDCs. Activation of DHX9 leads to IRF-7 activation and IFN-α production, while activation of DHX36 leads to NF-κB activation and IL-6 and TNF-α production (64). Knockdown of DHX9 and DHX36 inhibited cytokine production in response to the DNA virus HSV-1, while the response to the RNA virus influenza A was unaffected.

DDX41. DEAD (Asp-Glu-Ala-Asp) box polypeptide 41 (DDX41), another member of the DEAD/H helicases family, was recently identified as a sensor of cyclic diguanosine monophosphate (c-di-GMP) and cyclic diadenosine monophosphate (c-di-AMP), two secondary messengers used by certain species of bacteria to regulate metabolism, motility

and virulence (65, 66). Knockdown of DDX41 in mouse and human cells reduced IFN-β and IL-6 production in response to c-di-AMP, c-di-GMP, poly(dA:dT) and HSV-1 infection (49, 67). Binding of DNA to DDX41 was mediated by the DEADc domain. DDX41 was then found to associate with STING and TBK1 leading to downstream type I IFN and cytokine induction.

cGAS. Cyclic GMP-AMP Synthase (cGAS) is a member of the nucleotidyltransferase family that has recently been described as a cytosolic sensor of DNA (68). Overexpression of cGAS in HEK293T cells led to IRF3 dimerization and IFN-β induction in a STING-dependent manner. In contrast, knockdown of cGAS in L929 cells inhibited IFN-β induction in response to transfected DNA and HSV-1 infection. cGAS, activated by dsDNA, catalyzed the synthesis of cGAMP from ATP and GTP which then bound to and activated STING leading to IFN-β expression (69).

IFI16. While analyzing immune responses to a dsDNA region derived from the VACV and HSV-1 genomes, Unterholzner *et al.* identified IFI16, a PYHIN protein family member, as a DNA binding receptor that interacted with these dsDNAs (Fig. 1.1) (70). Knockdown of IFI16 or p204 (a member of the murine PYHIN family) led to a reduction in IFN-β responses to these dsDNAs while responses to the RNA virus SV was unaffected. Although IFI16 is primarily nuclear in most cell types, in macrophages, IFI16 is also found in cytosol where it co-localizes with transfected dsDNA. Association of IFI16 with STING was required for the production of IFN-β in response to these DNA

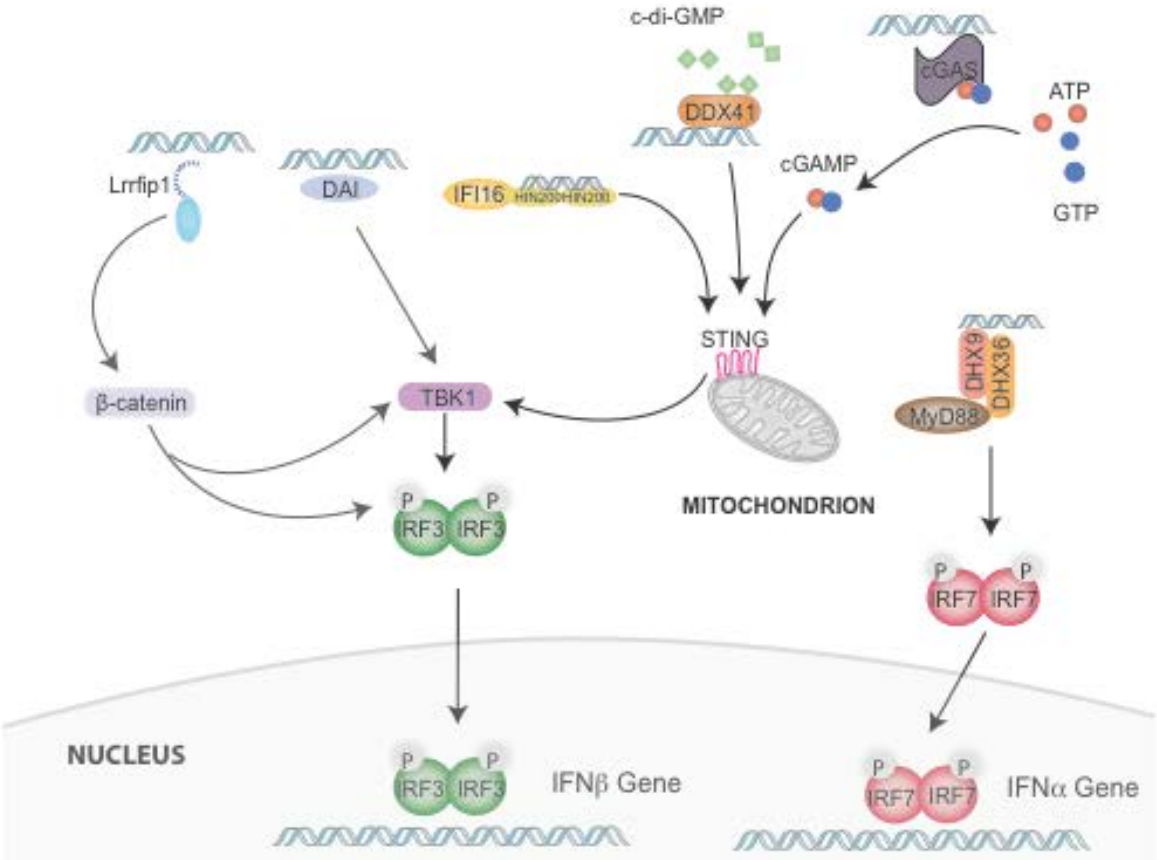


Figure 1.1: Interferon-inducing cytosolic dsDNA sensing PRRs A multitude of DNA sensors, including IFI16, DAI, LRRFIP1, DHX9, DHX36, DDX41 and cGAS recognize dsDNA and drive type I IFN and cytokine production. These receptors signal through the STING-TBK1-IRF3 pathway. Activation of DHX9 leads to IRF7 activation and IFN-α production.

motifs. Knockdown of IFI16, and its mouse ortholog p204 led to a decrease in IRF3 and NF-κB activation and IFN-β gene induction following HSV-1 infection (70). IFI16 and other members of the PYHIN family are discussed in greater detail below.

1.2.5 PYHIN receptors

The founding member of the PYHIN (pyrin and HIN200 domain-containing) family, p202a was first identified over two decades ago. Since then four PYHIN proteins have been reported in humans (IFIX, IFI16, MNDA and AIM2) and six in mice (p202a, p202b, p203, p204, MNDAL and AIM2) though seven additional murine members are predicted. PYHIN receptors are differentially expressed in hematopoietic cells and can be upregulated by type I and II IFN signaling (71). All contain one or more HIN200 domains and, with the exception of p202a and b, all have N-terminal pyrin domains. These HIN200 domains were later categorized into subtypes, A, B and C, according to their sequence similarity. IFI16, and its murine ortholog p204, have both HIN200A and B domains, while AIM2 contains a single HIN200C domain (Fig. 1.2). Each HIN200 domain is comprised of two oligonucleotide/oligosaccharide binding (OB) folds that associate with DNA. These OB folds were first predicted based on modeling of IFI16's HIN200A domain using the human replication protein A (RPA) as a template (72). This study went on to show that IFI16's HIN200A domain had a higher affinity for ssDNA than dsDNA and could wrap and stretch ssDNA. The crystal structures of the IFI16 and AIM2 HIN200 domains revealed that DNA binding was accomplished through



Figure 1.2: The human AIM2 and IFI16 proteins AIM2 and IFI16 both contain an N-terminal pyrin (PYD) domain. While AIM2 has a single HIN200 domain of subtype C, IFI16 contains two HIN200 domains of subtypes A and B separated by a linker sequence.

electrostatic interactions between the positively charged HIN200 residues and the negatively charged dsDNA sugar-phosphate backbone. This observation explains why activation of IFI16 and AIM2 appears to be largely sequence-independent (73). This study suggested unbound AIM2 is maintained in an autoinhibited state by the intramolecular association of its HIN200C and pyrin domain, which is liberated by binding to dsDNA. IFI16, MNDA and p204 contain nuclear localization signals and are mainly found within the nucleus though IFI16 can be observed within the cytoplasm (17, 70). AIM2 and p202 by comparison are localized to the cytoplasm.

The IFI16 mRNA is spliced into three transcripts that differ according to the length of the serine-threonine-proline-rich spacer sequence that separates the HIN200A and B domains (74). Initial biochemical analysis of IFI16 fused to the GAL4DBD revealed it could act as a potent transcriptional repressor though the molecular mechanisms underlying this function are unknown (75). Early *in vitro* experiments found IFI16 associated with p53 and pRb and, when overexpressed, could slow cell growth by delaying G_1 to S phase progression (75). Transcriptional silencing of IFI16 has been implicated in the development of prostate and breast cancer (76, 77). IFI16 has also been

shown to associate with BRCA1. This interaction was found to enhance genotoxic stress-induced cell death through p53-mediated apoptosis, suggesting a role for IFI16 in mediating DNA damage signaling (76, 78). In addition IFI16 has been suggested to induce apoptosis in endothelial cells by activating NF-κB leading to caspase-2-mediated apoptosis independent of p53 (79). The proapoptotic activity of IFI16 has lead to the theory that it may play an etiopathogenetic role in autoimmunity. Indeed, PBMCs isolated from lupus patients have elevated IFI16 mRNA levels and anti-IFI16 antibodies are common in Systemic Lupus Erythematosus (SLE), Systemic Slerosis and Sjogren's Syndrome (80, 81). Thus, a number of genetic and functional studies have linked IFI16 to autoimmunity and cancer. However, the specific pathways through which IFI16 mediates its affects on cellular proliferation and survival are still debated.

Recently, dsDNA sequences derived from the HSV-1 and VACV genomes were shown to bind IFI16, which activated the STING-TBK1-IRF-3 pathway leading to the production of type I IFNs. Knockdown of either IFI16 or its murine ortholog p204 also led to a decrease in IRF3 and NF-κB activation following *in vitro* infection with HSV-1. Intriguingly, a recent study has revealed a role for IFI16 in inflammasome assembly in response to Kaposi sarcoma-associated herpesvirus (KSHV). In endothelial cells, IFI16 in the nucleus can interact with KSHV DNA and the inflammasome adapter molecule ASC leading to the activation of caspase-1 and IL-1β secretion (82). This case of IFI16-mediated inflammasome signaling may be the exception rather than the rule, as AIM2 appears to be the main inflammasome-assembling receptor that responds to cytosolic dsDNA. AIM2 was first identified as a putative tumor suppressor in the human malignant melanoma cell line $UACC_{903}$ (83). Mutations in the *aim2* gene have been

associated with colorectal tumors, gastric and endometrial cancers suggesting like IFI16, AIM2 plays a role in cell cycle progression (84, 85). In support of such a function, restoration of AIM2 expression in colorectal cancer cell lines leads to cell cycle arrest (86). Recently, AIM2 was shown to assemble an inflammasome in response to a variety pathogens, including MCMV, VACV, *Francisella tularensis* and *Listeria monocytogenes*. A IM2 and its role in inflammasome signaling is discussed further below.

1.2.6 NOD-like receptors and the Inflammasome

In contrast to type I IFNs and TNF-α, the production of IL-1β and IL-18 is controlled at the level of transcription, translation, maturation and secretion. IL-1β is a pleiotrophic cytokine that induces fever, activates monocytes, macrophages and neutrophils and drives acute-phase protein synthesis (87). IL-18 increases natural killer (NK) cytolytic activity and IFN-γ production thereby inducing Th1 and Th17 adaptive responses (88, 89). Many cell stimuli including TLR ligands activate the NF-κB-dependent transcription of the pro-forms of IL-1β and IL-18. U nlike most other cytokines, however, these pro-cytokines lack leader sequences and are retained in the cytoplasm rather than loaded into secretory vesicles. Maturation (*i.e.*, the cleavage) of pro-IL-1β and pro-IL-18 is catalyzed by the cysteine protease caspase-1 (formerly known as IL-1 converting enzyme). In resting cells, caspase-1 itself is present as an inactive zymogen pro-caspase-1. A large inflammasome protein complex controls the activity of the inflammatory caspase-1. Several members of the nucleotide-binding oligomerization

domain receptor (NLR) family, including NLRP1, NLRP3, NLCR4 (IPAF) and NLRP12 have been shown to assemble inflammasomes in response to various stimuli.

The NLR family includes 23 genes in humans and 34 in mice (90). They are composed of a C-terminal LRR-rich domain, a central nucleotide-binding NACHT oligomerization domain, and an N-terminal protein–protein interaction pyrin domain (PYD) or in the case of NLRC4 an N-terminal caspase activation and recruitment domain (CARD). NLRs associate with the PYD containing adapter molecule apoptosis-associated speck-like protein (ASC; also termed pycard or TMS1). ASC links the NLRs via its C-terminal CARD domain to the CARD domain of pro-caspase-1. This close association of pro-caspase-1 molecules is then believed to enable self-cleavage into active caspase-1. Active caspase-1 in turn cleaves pro-IL-1β and pro-IL18 into their active forms.

In addition to pro-IL-1β and pro-IL-18, expression of certain NLRs, such as NLRP3, is upregulated in an NF-κB-dependent manner (91). The NLRP3 inflammasome is activated by a wide range of PAMPs and DAMPs such as ATP, the pore forming toxin nigericin, uric acid, silica, cholesterol and asbestos crystals, the vaccine adjuvant alum, chemotherapeutics including gemicitabine and 5-fluorouracil, as well as by fibrillar amyloid-β (92-97). A number of pathogens have been shown to activate NLRP3 including influenza A virus, adenovirus, Sendai virus, *Staphylococcus aureus*, *Listeria monocytogenes* and *Candida albicans* (15, 95, 98-100). In the case of Gram-negative bacteria like enterohermorrhagic *Escherichia coli* and *Citrobacter rodentium*, caspase-11 induction via the TLR4-TRIF-IFN-β pathways is also essential for NLRP3 activation

(101). Despite the identification of these triggers, no ligand has been shown to directly bind to NLRP3 and the mechanism by which NLRP3 is activated remains unclear.

Three models of NLRP3 activation have been proposed. However, no single model can yet account for all activating stimuli. The first posits that disruption of cell membrane ionic gradients may activate NLRP3. This model is supported by evidence that extracellular ATP activates the P_2X_2 receptor causing potassium efflux that results in NLRP3 activation (102, 103). This may explain how bacterial pore-forming toxins such as nigericin activate NLRP3. In addition, studies using influenza A virus revealed that the M2 protein, a proton-specific ion channel, was necessary to trigger NLRP3 activation (104). However certain bacteria are able to activate NLRP3 independently of the P_2X_2 receptor suggesting that alternative pathways exist (102). A second model suggests that destabilization or rupture of the lysosomal membrane by crystals, chemotherapeutics or bacteria leads to the release of enzymes such as cathepsin B or L that activate NLRP3 (96, 97). In support of this theory, pharmalogical inhibition of cathepsin B reduced NLRP3 activation by cholesterol crystals (97). However, another study indicated that NLRP3 activation was independent of cathepsin B or L at higher doses of cholesterol and had no effect on stimulation with other NLRP3 triggers (105). The third model proposes that reactive oxygen species (ROS) generated by DAMPs, such as silica and cholesterol crystals can activate NLRP3 (106, 107). Indeed, studies have shown the addition of hydrogen peroxide can cause NLRP3 inflammasome activation and inhibition of ROS can suppress this activation. However ROS are created in response to a variety of stimuli and not all pathways that induce ROS activate the inflammasome (108). Finally, a study using *Mycobacterium kansasii* reported a role for all three pathways (109). Further

research on the precise molecular events underlying NLRP3 activation are needed to determine if these pathways are, in fact, causal or simply parallel responses and whether they are specific to certain stimuli or act cooperatively.

Unlike NLRP3, NLRC4 can recruit caspase-1 directly through its CARD domain and does not necessarily require ASC (110). However, NLRC4 also signals through an ASC-dependent pathway and ASC is required for maximal activity in certain cases. NLRC4 is activated by the flagellin peptide, in a manner that depends on NAIP5, and by the inner rod component of the bacterial type III secretion system (111). P athogens including *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Shigella flexeri*, *Legionella pneumophila* have been demonstrated to activate NLRC4 (112-115). NLRP1, the first NLR shown to assemble an inflammasome, is activated by anthrax lethal toxin derived from *Bacillus anthracis* (116).

Recently, NLRP12, also known as Monarch-1 and PYPAF7, was shown to assemble an inflammasome in response to *Yersinia pestis* (117). In this study, NLRP12-deficient mice had reduced levels of IL-1β and IL-18 in their serum and were more susceptible to *Y. Pestis* infection. NLRP12 expression has been observed in both human and mouse granulocytes and at lower levels in monocytes and DCs, but is undetectable in resting macrophages and lymphocytes (118). Previous studies have found both pro- and anti-inflammatory roles for NLRP12. One study showed that NLRP12 activated NF-κB and mediated caspase-1-dependent cytokine secretion (118) while another group reported that NLRP12 suppressed noncanonical NF-κB activation by destabilizing NF-κB inducing kinase (NIK) (119-121). Interestingly, in a model of contact hypersensitivity, NLRP12-deficient mice were found to have impaired dendritic cell and neutrophil

migration, but no defect in TNF-α or IL-1β production was observed (122). Another study reported NLRP12 deficiency increased susceptibility to DSS-induced experimental colitis and colitis-associated colon cancer (123). This was attributed to enhanced noncanonical NF-κB and MAPK activation in NLRP12-deficient mice. It is possible that NLRP12 affects multiple inflammatory pathways that are tissue or cell-specific, thus, its activity may manifest itself differently depending on the disease or pathology examined. It is important to note that NLRP12 is highly similar to NLRP3, sharing 58% of its nucleotide sequence (124). In humans, mutations in NLRP12, much like mutations in NLRP3, have been shown to cause hereditary periodic fever syndrome (HPFS) characterized by high fever, arthralgia, myalgia and sensorineural hearing loss (124). These NLRP12-associated HPSFs can be successfully treated with anti-IL-1 therapy, suggesting NLRP12 has a similar role to NLRP3 *in vivo* (124).

Cytosolic dsDNA triggers ASC-dependent activation of caspase-1 and secretion of IL-1β and IL-18. However, analysis of this response in macrophages lacking members of the NLR family revealed normal caspase-1 activation. Subsequent studies from several groups revealed that this response was instead dependent on the Absent in melanoma-2 (AIM2) protein (16, 17, 100, 125, 126). AIM2 recognizes cytosolic dsDNA of self and nonself origin via its HIN200C domain. Upon DNA binding, AIM2 oligomerizes and associates with ASC via its PYD domain. ASC then recruits pro-caspase-1 leading to its activation (Fig. 1.3). AIM2 is essential for inflammasome formation and IL-1β and IL-18 secretion in macrophages and dendritic cells responding to infection with MCMV and VACV (16). AIM2-dependent IL-18 secretion induces NK-cell activation and IFN-γ production that is critical for controlling MCMV

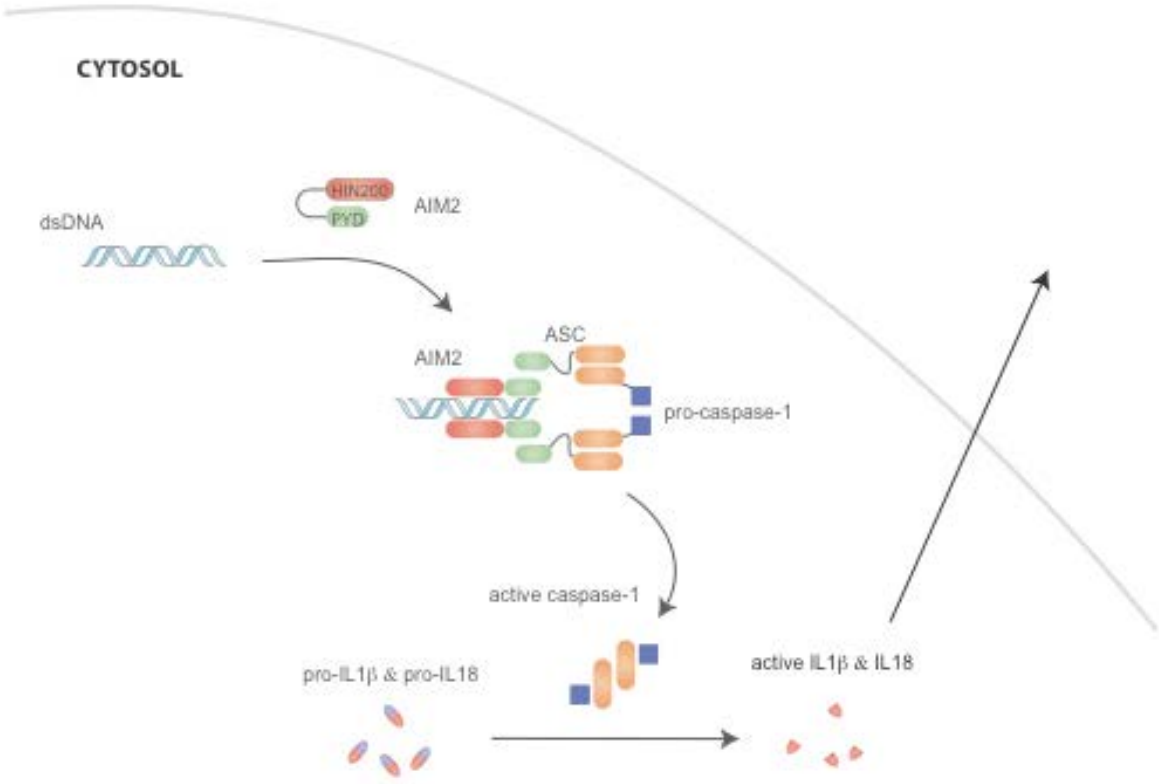


Figure 1.3: AIM2 inflammasome signaling Upon binding to dsDNA, AIM2 oligomerizes and recruits the adapter molecules ASC. ASC, in turn, recruits pro-caspase-1 which cleaves itself into the active caspase-1 subunits. Active caspase-1 then cleaves immature pro-IL-1β and pro-IL-18 into their biologically actives forms. These cytokines are then be secreted into the extracellular space.

replication. AIM2 has also been shown to recognize bacterial pathogens including *Francisella tularensis* and appears to be critical in early control of *F. tularensis* infection *in vivo* (16). A IM2 also synergizes with NLRP3 and IPAF to drive IL-1β and IL-18 secretion in response to *Listeria monocytogenes* (46).

1.3 Suppressive Oligodeoxynucleotides

Since the inhibitory effects of certain DNA sequences on TLR9 signaling were first reported, a variety of different DNA constructs or suppressive oligodeoxynucleotides (sup ODNs) that vary in length, sequence and backbone composition have been examined *in vitro* and *in vivo*. Studies using dissimilar systems have drawn different, seemingly conflicting conclusions. This confusion is compounded by the fact that certain sup ODNs block multiple pathways and cause cell type-specific effects. In order to facilitate characterization, Trieu *et al*. divided sup ODNs into four groups based on their proposed mechanisms of action (127). Class I consists of short G-rich sup ODNs that inhibit TLR9 signaling in a sequence-specific manner. C lass II interfere with STAT1, 3 a nd 4 signaling. Class III prevent cellular uptake by competing for surface receptors, and Class IV consists of long phosphorothioate constructs that inhibit TLR9 in a largely sequence-independent manner. Though this classification system mainly addresses the effects of sup ODNs on TLR9 signaling, it provides a useful framework for interpreting how sup ODNs interfere with other signal pathways. As we will see some sup ODNs display characteristics of multiple classes.

1.3.1 The phosphorothioate backbone

Phosphorothioate (PS) backbones are widely used in stimulatory ODN constructs to activate TLR9, as well as in antisense constructs to mediate transient protein knockdown. These constructs differ from phosphodiester (PD) backbones in that one of the nonbridging oxygens is replaced with a sulfur molecule. This change not only imparts resistance to nuclease degradation, but it also increases cellular uptake (128-131). In addition PS-modified ODNs are known to bind a variety of proteins that do not interact with PD ODNs such as fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) and have been shown to affect cell growth, morphology and viral proliferation independently of sequence (132-138). Unfortunately, how PS constructs mediate their sequence-independent effects is not well understood.

1.3.2 Modulation of innate immunity by Suppressive ODNs

Halpern *et al*. first reported the inhibitory effects of G-rich phosphorothioate DNA on IFN-γ production nearly two decades ago (139). This initial study found dose-dependent inhibition of the IFN-γ response in splenocytes challenged with DNA derived from *E. coli*, Con A or PMA with the calcium ionophore A23187. Inhibition required a PS backbone and though the poly(dG) sequence was optimal, the poly(dC) and poly(dT) constructs could also block activation. Potency correlated positively with ODN length, at 50 μg/ml, a 20 nucleotide poly(dG) sup ODN could achieve 92% inhibition of the IFN-γ response while a 10 nucleotide construct achieved 51% inhibition. Further studies

revealed that these sup ODNs could also block IL-6 and nitric oxide (NO) production by BMDM responding to CpG as well as IL-12 and NO production and CD40, CD86 and MHC II expression by BMDC (140, 141). Again, PS constructs composed of any single base could inhibit CpG activation. These effects were largely sequence-independent, but relied on long PS backbones (class IV). It is possible that these sup ODNs also exerted class III effects as they did interfere with CpG uptake. However, the dose required to inhibit uptake was higher than that required for suppression, arguing against a major contribution from this mechanism (141).

In 1998, Kreig *et al*. reported that DNA derived from serotype 12 adenovirus stimulated TNF-α and IL-6 secretion from human PBMCs while DNA from serotypes 2 and 5 were far less active (142). They found that unlike serotype 12, types 2 and 5 had large numbers of clustered or directly repeating CG sequences within their genome. Treatment of splenocytes with PS hexameric constructs derived from these regions inhibited CpG ODN and *E. coli* DNA-induced cytokine secretion. A follow-up study revealed that these sup ODNs could also block CpG stimulation of mouse B cells and the authors suggested it did so by interfering with CpG binding to TLR9 (143). This exciting report indicates that selective pressure to evade innate immune activation may have led to the enrichment of certain inhibitory motifs in viral genomes. It is tempting to speculate about the role of such adaptations considering that adenovirus serotypes 2 and 5 (which contain the anti-inflammatory sequences) can establish latent infection in lymphocytes while serotype 12 cannot (144). Unfortunately, it is hard to infer sequence specificity from these studies as the hexameric constructs used differed only in a few base positions and all had similar suppressive effects. Moreover, other work using these sup ODN

motifs has shown only a modest role for sequence, again suggesting contributions from a class IV mechanism of action (145-147).

The sup ODN 2114, a 15 bp construct, is one of the most potent, sequence-specific inhibitors of TLR9 activation (class I) (146, 148). This construct has been shown to block IL-6 and IL-12 production by mouse splenocytes in response to CpG. It also prevents IL-6 and IL-10 production and proliferation of human B cells and IFN-α and IL-12 secretion by human PDCs (146). Ashman *et al*. performed a series of experiments, in which each base in the 2114 construct was mutated, to define the sequence requirements for optimal inhibition (148). This study found the optimal inhibitory motif consisted of a CC dinucleotide at the 3' end, a spacer sequence of five nucleotides followed by at least three contiguous G residues (148). Substitutions in this guanine tract rendered the 2114 sup ODN inactive. Some reports indicate a PS backbone is essential for 2114 suppression while others maintain that the PD backbone version can still mediate suppression, but at a significantly reduced potency (127, 146). Whether this difference is the result of increased uptake and retention of the PS version of 2114 leading to a higher intracellular concentration or is due to sequence independent contributions from the PS backbone (class IV) is unclear. However, evidence that simply lengthening the backbone of the 2114 motif by as few as four or five base pairs can increase its inhibitory potency 300-600% may indicate a transition from sequence-specific (class I) to a n onspecific (class IV) mechanism.

Building on p revious evidence that mammalian DNA could suppress CpG-induced immune activation, Gursel *et al*. reported that DNA derived from telomeres was particularly potent compared to whole genome extracts (149). T his report found that

telomeres contained numerous copies of the guanine-rich, hexameric sequence TTAGGG. Although a single repeat was weakly inhibitory, including multiple tandem repeats of the TTAGGG sequence produced a construct $(TTAGGG)_4$, also known as A151, that potently inhibited IL-6, IL-12 and IFN-γ secretion from spleen cells in response to CpG ODN (149). Much like 2114 the presence of guanine residues was thought essential for inhibitory activity. This was the first example of a specific mammalian genomic motif that could suppress immune activation. Its discovery has lead to the hypothesis that this motif, released from dead or dying host cells, may serve to suppress pathological immune responses. Indeed, since this report, the therapeutic potential of A151 has been demonstrated in murine models of CpG and collagen-induced arthritis, toxic shock, systemic lupus erythematosus, atherosclerosis, silica-induced pulmonary inflammation and influenza infection (149-156). D espite the broad applications of A151, there is still debate concerning the precise molecular mechanisms underlying its *in vivo* effects.

Although the initial study by Gursel *et al*. claimed A151 synthesized with either PS or PD backbones could inhibit CpG-induced cytokine production at equimolar concentrations, they showed no da ta. S ubsequent reports have only found inhibitory activity in A151 synthesized with a PS backbone (127, 146). Moreover, this difference cannot be accounted for by variation in cellular uptake. In addition, no role for sequence was reported in a later study, as A151-mediated inhibition was comparable to a poly(dA) PS construct of similar length (127). Initial reports that A151 prevented activation by binding to CpG ODNs through the formation of four-stranded helices (G-tetrads) stabilized by planar Hoogsteen base pairing between guanine residues has not borne out

either (157). As Duramad *et al.* demonstrated, despite containing three contiguous guanines, A151 does not readily form G-tetrads (146). Moreover, when other constructs, such as 2114, that do form G-tetrads, are tested, it is the single-stranded species that mediates inhibition (146). Thus, one early hypothesis that A151 mediated inhibition by binding and sequestering CpG appears to be incorrect. Instead, A151 likely competes with CpG ODN for binding to TLR9 in a manner that depends on its PS backbone more so than sequence.

Importantly, a number of observations suggest that inhibition of TLR9 signaling is not the only mechanism or even the main mechanism by which A151 and other sup ODN block immune activation *in vivo*. For example, Shirota *et al*. demonstrated that A151 prevented Th1 differentiation in wild-type and TLR9-deficient CD4+ cells alike (154). A recent study by Trieu *et al*. found treatment with 2114 suppressed NF-κB-dependent responses to *Salmonella typhimurium* in both wild-type and TLR9-deficient macrophages (158). Moreover, using a panel of sup ODNs, *Ashman et al*. reported optimal sequences for TLR9 inhibition as measured by biological assays did not correlate with their relative affinity for the TLR9 ectodomain (148). Dennis Klinman and collaborators have reported one possible alternative A151-mediated mechanism of suppression. Their studies show that A151 inhibits IFN-γ-induced STAT1 phosphorylation and IL-12-induced STAT3 and STAT4 phosphorylation (class II mechanism) thereby preventing differentiation of naïve CD4+ cells into Th1 effectors (151, 153, 154). Addition of A151 to splenocytes derived from OVA TCR Tg mice significantly inhibited IFN-γ production and Th1 differentiation while enhancing IL-4 in response to Ag-pulsed APCs (154). This effect persisted despite the addition of IL-12 to

culture supernatants, suggesting a role in signal transduction downstream of the IL-12 receptor. A similar effect on IFN-γ signaling was observed *in vivo* when mice were immunized with OVA in the presence or absence of A151. The magnitude of inhibition was dose-dependent and was not observed in mice treated with the C151 control ODN in which A151's guanine triplet had been replaced with adenine residues. A151 specifically blocked phosphorylation of STAT1, 3 and 4 but had no effect on STAT5 or 6. Treatment with A151 also increased survival in a mouse model of LPS-induced endotoxic shock (153). Though A151 had no effect on TNF-α, IL-12 or IFN-β induced directly by LPS, it abrogated subsequent paracrine and autocrine cytokine-induced IFN-γ production. Biotinylated A151 was used to pull down STAT1 and STAT4 from spleens cells stimulated with IFN-β (153). The authors theorized that A151 interacts with the Src homology domain of STAT3, but no e vidence of a direct interaction was provided. Interestingly, the control C151 construct occasionally had an intermediate suppressive effect on the incidence and clinical severity of disease in a collagen-induced arthritis model (159). Though not significant, these findings raise the possibility that some of the anti-inflammatory effects observed in this model are again due to sequence-independent PS backbone effects.

Other mechanisms of action have also been proposed for sup ODNs. A recent report found that inversion of the cytosine and guanine residues in the CpG motif to a GpC configuration also conferred suppressive properties in a model of type I hypersensitivity reaction (160). Interestingly, GpC-mediated suppression was dependent on activation of the TLR7-TRIF signaling pathway leading to noncanonical NF-κB activation and the induction of TGF-β and indoleamine 2,3-dioxygenase (IDO). In

addition, a PS construct composed of poly(dT) was shown to prevent TLR7-mediated signaling, but enhance TLR8 signaling. This study revealed that administration of poly(dT) abolished NF-κB activation in TLR7-expressing HEK 293 cells stimulated with the imidazoquinoline derivative Resiquimod (R-848) (161). However, it enhanced R-848-driven NF-κB activation in TLR8-expressing HEK 293 cells. Moreover, treatment of human PBMCs shifted the cytokine profile from TLR7-mediated IFN-α production, towards TLR8-mediated IL-12 and TNF-α production. Thus, alternative mechanisms to TLR9 inhibition have been demonstrated. These studies have revealed the truly complex and cell-type specific effects sup ODNs can generate. Though the immunoregulatory impact of sup ODNs in experimental models is impressive, and the applications appear broad, how these effects are mediated is not well understood. Investigation into the precise mechanisms of action of sup ODNs is a priority that may one day make tailored therapeutic intervention in human disease possible.

1.4 Herpesviruses

Herpesviruses are a family of enveloped DNA viruses that cause widespread disease in humans and animals. All have large dsDNA genomes, replicate within the host nucleus and can establish both lytic and latent forms of infection. The family *Herpesviridae* is divided into three subfamilies the *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*. The alpha herpesviruses include herpes simplex virus-1 (HSV-1, also known as human herpes virus-1; HHV-1), herpes simplex virus-2 (HSV-2; HHV-2)

and varicella-zoster virus (VZV; HHV-3). Alpha herpesviruses are known for their short replication cycle as well as their ability to latently infect neurons. Though they are only found naturally in humans, alpha herpesviruses can infect a broad range of hosts. The beta herpesviruses include roseolovirus (HHV-6), pityriasis rosea (HHV-7), human cytomelagovirus (HCMV; HHV-5) and murine cytomegalovirus (MCMV). Viruses of the beta subfamily have longer replication cycles than the alphaherpesviruses and establish latency in lymphocytes. In addition, their host range is narrow and often species-specific. The gamma herpesviruses include Epstein-Barr virus (EBV; HHV-4) and Kaposi's sarcoma-associated herpesvirus (KSHV). Most gamma herpesviruses establish latency in B cells and, as such, are associated with the development of a number of lymphoproliferative disorders. The discussion below focuses on HSV-1, it's virion structure, infectious pathophysiology and the innate response.

1.4.1 Herpes Simplex Virus type 1

HSV-1, a member of the alpha herpesviridae subfamily, consists of a large linear double-stranded DNA genome housed within an icosahedral capsid surrounded by a lipid envelope. The tegument, which is composed of as many as 20 different proteins, lies between the capsid and envelope. The HSV-1 genome is 152 kb in length and encodes upwards of 84 proteins. Host RNA polymerase II transcribes genes from the viral genome in three distinct stages resulting in immediate-early (2-4 hrs post-infection), early (5-7 hrs), and late (stage dependent) transcripts (162). The immediate-early genes are involved in host transcription shut-off, immune evasion and regulation of later viral

genes. The early genes function largely to subvert host cellular machinery and replicate the genome but include some minor structural proteins. The late genes encode most of the structural components of the virion. Viral entry is mediated by a group of glycoproteins (gC, gD, gH, gL, gB) found on the viral envelope (162). Upon entry, the capsid is transported along microtubules to the nucleus where the viral genome is injected through a nuclear pore. In the nucleus, the viral genome circularizes and enters the lytic cycle or establishes latent infection. During the lytic cycle, the genome replicates and viral particles are assembled within the nucleus, then bud outward. The final viral envelope is obtained by budding into cytoplasmic vesicles within the host cell. HSV-1 most commonly enters the latent phase in the sensory neurons of the trigeminal and/or olfactory ganglia. During this time the Latency Associated Transcript (LAT) is expressed and acts to silence lytic cycle genes. HSV-1 can be reactivated from its latent state by a variety of stimuli and, therefore, represents a lifelong repository.

1.4.2 HSV-1, the pathogen and pathophysiology

HSV-1 is an old and extremely successful pathogen affecting more than one-third of the world's population. The seroprevalence of HSV-1 increases with age and ranges between 65-90% depending on gender and ethnicity (163). HSV-1 enters the human body through the mucosal membranes or lesions in the skin. Replication takes place initially in epithelial cells resulting in localized infection that can be subclinical or manifest as vesicular lesions. HSV-1-infected cells undergo apoptosis or are lysed by immune cells and the resulting intradermal cellular debris and inflammatory exudate

causes the prototypical vesicles known colloquially as “cold sores”. In certain circumstances, HSV-1 can disseminate and replicate in other anatomical locations including the liver, lung and central nervous system causing severe illness (164, 165). These life-threatening manifestations are rare and more commonly observed in immunocompromised individuals. The key to HSV-1’s success derives from its ability to latently infect neurons. From this location, it is reactivated by stress, UV-light or trauma and transported down axons to the periphery where it re-enters the lytic cycle replicating in epithelial cells (166). B etween outbreaks, HSV-1 is essentially immunologically silent. Even when infectious individuals may display no symptoms resulting in horizontal transmission. In addition to latency HSV-1 has devised a number of strategies to evade the immune system. Certain viral proteins function to inhibit the production of IFN, cytokines and chemokines, downregulate MHC presentation and induce apoptosis in innate immune cells (166-168). HHV infected cell polypeptide 0 (ICP0), an immediate-early protein, disrupts promyelocytic leukemia bodies, inhibits the activity of IRF3 and IRF7, and degrades MyD88 (168-170). Despite this the immune system is usually successful in clearing lytic infections and suppressing reactivation.

1.4.3 Role of innate immunity in recognizing HSV-1

Control of HSV-1 infection requires both innate and adaptive arms of the immune system. Production of cytokines, chemokines and Type I IFNs by the innate immune system is vital for early viral control. These effectors directly block viral replication and reduce cell-to-cell transmission by inducing enzymes such as PKR and RNAse L. They

also recruit additional leukocytes, increase MHC presentation and activate lymphocytes, coordinating the adaptive response that is required for complete viral clearance. Innate immune cells detect HSV-1 infection through several classes of PRRs including the TLRs, RLRs and cytosolic DNA sensors. TLR3, TLR9 and the TLR2/6 heterodimer have all been implicated in the immune response to HSV-1. In plasmacytoid dendritic cells, TLR9 is activated by unmethylated CpG motifs in the HSV-1 genomic DNA and is chiefly responsible for type I IFN induction at early time points (171). Interestingly a second wave of type I IFN is produced by monocytes, macrophages and conventional dendritic cells (CDCs) and is TLR9- and MyD88-independent but requires replication competent virus (171). This second wave is likely due to cytosolic sensors and may explain why survival and viral burdens are unchanged in TLR9 knockout mice challenged with HSV-1 (172, 173). Alternatively TLR9 signaling may be more important in certain manifestations of HSV-1 induced disease. A recent study showed TLR9-deficient mice had higher rates of mortality and viral replication when challenged intranasally with HSV-1 (174). Thus, TLR9's precise role in HSV pathogenesis and the relative contributions of other PRRs requires further investigation

TLR2 is expressed by a wide variety of immune and non-immune cells including monocytes, macrophages, microglia, dendritic cells, neutrophils and epithelial cells (175, 176). T he TLR2/6 heterodimer recognizes an undefined HSV-1 component, which stimulates the production of cytokines and chemokines by microglia (177, 178). However, rather than having a protective role, recent studies have shown TLR2 signaling in microglia contributes to the immunopathology of HSV-1 encephalitis (HSE) (178). TLR3 recognizes double stranded RNA produced during HSV replication (7). T his

appears to be especially important in HSE as patients with TLR3 dominant-negative mutations display increased susceptibility (179).

Double stranded RNA produced during HSV-1 infection has been shown to activate RIG-I and MDA5 (171). Activation of these RNA helicases and recruitment of mitochondrial antiviral-signaling protein (MAVS) results in type I IFN production in macrophages (171). As discussed previously, a number of cytosolic DNA receptors have also been implicated in the Type I IFN response to HSV-1 including DAI, DDX41, IFI16 and cGAS. Our understanding of these cytosolic receptors is rapidly expanding but the precise contribution of each to the HSV-1 response is still debated. Whether they signal collaboratively or function in a cell type-specific manner is still unknown. Interestingly, recent research suggests IFI16 detects HSV-1 DNA within the nucleus and, thus, may be uniquely positioned to detect viral genome replication (180).

The importance of IL-1β in the response to HSV-1 has been clearly established (177). However, receptors responsible for inflammasome formation in response to HSV-1 are poorly understood. A recent study has suggested HSV-1 may assemble an inflammasome through the IFI16 and NLRP3 receptors. This paper demonstrated that after HSV-1 infection, IFI16 and NLRP3 associated with ASC in human foreskin fibroblasts (181). However, this study failed to observe detectable levels of IL-1β secretion following HSV-1 infection. Instead, the authors found that HSV-1 induced the rapid degradation of IFI16 and hypothesized that trapping of caspase-1 in actin clusters prevented cytokine processing downstream of NLRP3 (181). Thus, additional studies are needed to define the contributions of these receptors to inflammasome formation following HSV-1 infection.

1.5 Dissertation Objectives

This dissertation has two main focuses. The first is to explore the role of suppressive oligodeoxynucleotides in cytoplasmic receptor signaling. The second is to define the pathways responsible for IL-1β and IL-18 induction and maturation in response to HSV-1 infection.

Objectives:

1.1) *Investigate the effects of sup ODNs on cytosolic receptor-mediated signaling.* Using primary mouse cell lines and human cell lines, we tested the inhibitory effects of A151 on TLR4, RIG-I, NLRP3, AIM2 and interferon-inducing cytosolic receptors. We demonstrated that A151 mediates specific inhibition of DNA sensing pathways. Using Nanostring analysis, we defined the broad inhibitory potential of A151 on inflammation.

1.2) *Explore construct potency and define the contributions of sequence and backbone.* Using the C151 and A151 and equivalent phosphodiester constructs, we explored the effects of backbone chemistry and sequence on inhibition. We found that the phosphorothioate backbone was essential for inhibition. In addition, we discovered differential sequence requirements for maximal inhibition of IFN-inducing cytosolic signaling versus inflammasome signaling.

1.3) *Identify the mechanisms of inhibition.* Using biotinylated A151 and C151, we explored the association of these constructs with different cytosolic pattern recognition receptors. We found that sup ODN affinity correlated with inhibitory potency, while A151 pulled down more AIM2, C151 pulled down more IFI16. Mechanistically, we discovered that A151 prevented ASC recruitment to AIM2 and assembly of the inflammasome complex.

2.1) *Define the signal pathway(s) responsible for HSV-1 induction of pro-IL-1β and pro-IL-18.* We found that the TLR2-MyD88 signaling pathway was essential for pro-IL-1β expression in macrophages and DCs. In contrast, pro-IL-18 expression was dependent on synergy between MyD88 and STING pathways.

2.2) *Define the receptor responsible for inflammasome assembly in response to HSV-1 infection.* Primary mouse cells deficient in NLRP3, NLRP12, ASC and caspase-1 were used to explore inflammasome formation. We demonstrated that NLRP3 is responsible for IL-1β maturation in dendritic cells and macrophages. However, neutrophils relied on NLRP12 for maturation of IL-1β.

Preface to Chapter II

The chapters of this dissertation have appeared in the following publications/manuscripts:

John J. Kaminski, Stefan A. Schattgen, Christian Bode, Dennis M. Klinman and Katherine A. Fitzgerald. 2013. *Synthetic Oligodeoxynucleotides (ODN) Containing Suppressive TTAGGG Motifs Inhibit AIM2 Inflammasome Activation*. J. Immunol. Submitted, under revision.

John Kaminski performed all experiments, Stefan Schattgen contributed to Fig 2.1

Chapter II

Suppressive oligodeoxynucleotides inhibit cytosolic DNA sensing pathways

Abstract

Synthetic oligodeoxynucleotides comprised of the immunosuppressive motif TTAGGG block TLR9 signaling, prevent STAT1 and STAT4 phosphorylation and attenuate a variety of inflammatory responses *in vivo*. Here, we demonstrate that such suppressive oligodeoxynucleotides (sup ODN) also abrogate activation of cytosolic deoxyribonucleic acid sensing pathways. Pretreatment of dendritic cells and macrophages with the suppressive ODN-A151 abrogated type I IFN, TNF-α and ISG induction in response to cytosolic dsDNA. A151 also reduced IFN-β and TNF-α induction in BMDC and BMDM in response to the herpesviruses HSV-1 and MCMV, but had no effect on the responses to LPS or Sendai virus. In addition, A151 abrogated caspase-1-dependent IL-1β and IL-18 maturation in dendritic cells stimulated with dsDNA and MCMV. Although inhibition of IFN-inducing pathways and inflammasome assembly was dependent on backbone composition, the nucleotide content differentially affected these pathways. While A151 more potently suppressed the AIM2 inflammasome, a related construct, C151 proved to be a more potent inhibitor of IFN induction. A151 suppressed inflammasome signaling by binding to AIM2 and competing with immune-stimulatory DNA. The interaction of A151 and AIM2 prevented recruitment of the adapter ASC and assembly of the macromolecular inflammasome complex. Collectively, these findings reveal a novel mechanism by which suppressive

ODNs modulate the immune system and unveil novel applications for suppressive ODNs in the treatment of infectious and autoimmune diseases.

Introduction

The innate immune system provides an essential first line of defense against infection. Innate immune cells detect pathogens through distinct classes of Pattern Recognition Receptors (PRR) including the Toll-like receptors (TLRs), the C-type lectin receptors (CLRs), the RIG-like helicases (RLRs), the NOD-like receptors (NLRs) and the PYHIN receptors. These PRRs respond to conserved pathogen and danger-associated molecular patterns (PAMPs/DAMPs) allowing rapid recognition and response to infectious agents. Activated receptors initiate signaling cascades that lead to the production of cytokines, chemokines and type I interferons, all of which are vital for controlling pathogen loads directly and coordinating adaptive immune responses. Unrestricted or improper activation of the innate immune system can have dire consequences. Uncontrolled inflammation can cause extensive tissue damage, exacerbate septic shock and contribute to the development of autoimmune diseases (182, 183). Thus, a balance between activation and suppression must be struck to ensure an appropriate and effective innate response.

Detection of DNA by the innate immune system is an important mechanism by which pathogens are recognized in order to activate protective immunity. Recognition of DNA is complex and can be influenced by a variety of factors including sequence, secondary structure, subcellular localization and covalent modification. Hypomethylated

CpG motifs found in bacteria and certain viruses are detected in the endosomal compartment by TLR9 (8, 184). In contrast, a number of DNA sensors have been implicated in cytosolic dsDNA sensing including Gamma-interferon-inducible protein-16 (IFI16) and Absent in melanoma-2 (AIM2): two members of the PYHIN protein family, DDX41; a member of the DEXDc helicase family and cGAS; a recently identified nucleotidyltransferase (16, 17, 46-48, 55).

IFI16 was first identified as a potential intracellular DNA sensor in a screen using a 70 bp DNA motif derived from the Vaccinia virus (VACV) genome to affinity purify binding partners. Unterholzner *et al.* found that IFN-β induction by this VACV 70mer was independent of TLR, DAI and Pol III signaling but was attenuated following IFI16 knockdown (70). Further analysis revealed IFI16 also mediated IFN-β induction following transfection with a 60bp motif derived from the HSV genome as well as by HSV-1 infection. Similarly, targeting the IFI16 murine ortholog p204 attenuated IFN-β and TNF-α production in response to these dsDNA motifs and in HSV-1 infection, suggesting a role in both IRF3 and NF-κB-dependent inflammatory pathways. IFI16 mediated this response by engaging the crucial signaling component STING leading to the activation of TBK1 and nuclear translocation of IRF3 and p65 (70). Both IFI16 and p204 contain a DNA-binding HIN200A and HIN200B domain as well as a pyrin (PYD) domain (71, 72). In macrophages, cytosolic IFI16 may be exposed to HSV-1 genomic DNA following proteasomal degredation of the HSV-1 capsid (185). Recently IFI16 has also been shown to sense HSV-1 DNA within the nucleus and signal via STING through an unknown intermediate (186). In contrast to IFI16, another member of the PYHIN family, AIM2 signals via assembly of an inflammasome.

The inflammasome is a large complex that provides a platform for the activation of caspase-1, an enzyme that cleaves the immature interleukins pro-IL-1β and pro-IL-18 into their active forms. There are distinct types of inflammasomes, differentiated by their protein constituents and activating receptors. In many cases, an inflammasome contains a nucleotide binding and oligomerization leucine-rich repeat (NLR) protein. In addition, our lab and others have recently reported that the cytosolic PYHIN family member, Absent in melanoma-2 (AIM2), directly binds to cytosolic bacterial and viral double-stranded DNA leading to the formation of an AIM2 inflammasome complex (16, 17, 125, 126). The AIM2 inflammasome is activated in response to infection by bacteria such as *Listeria monocytogenes* as well as the viral pathogen murine cytomegalovirus (MCMV) where it p lays an essential role in controlling early viral replication (16). AIM2 is composed of a DNA-binding HIN200C domain and a PYD domain, which recruits caspase-1 via the adapter molecule apoptotic speck protein with CARD domain (ASC) (17, 46, 125, 126, 187).

Certain DNA sequences such as the TTAGGG repeat commonly found in mammalian telomeric DNA have been shown to suppress innate immune activation. The therapeutic potential of these suppressive oligodeoxynucleotides (sup ODN) have been demonstrated in murine models of inflammatory arthritis, toxic shock, systemic lupus erythematosus, atherosclerosis and silica-induced pulmonary inflammation (151-153, 155, 188). Given the known roles for type I IFN and the pro-inflammatory cytokines, IL-1β and IL-18, in the development of many of these diseases, we set out to examine the effect of sup ODNs on cytosolic innate immune sensors (189). Synthetic sup ODNs were first thought to prevent TLR9 activation by binding to unmethylated CpG DNA (149).

However, other studies suggest rather than interacting with CpG ODNs, they likely compete for binding to TLR9 (148). Interestingly, the potency of these sup ODNs was found to be strongly affected by sequence, a phenomenon not explained by their relative avidity to the TLR9 ectodomain (148). In addition, Shirota *et al.* have shown that sup ODNs prevent Th1 differentiation in wild-type and TLR9-deficient CD4+ cells alike, suggesting that their biological activity may be independent of their interaction with TLR9 and, instead, involves as yet undefined receptor(s) (154). Here we demonstrate that treatment with the sup ODN A151, a ssDNA construct composed of four repeats of the hexanucleotide TTAGGG motif, blocks cytosolic DNA-driven IFN and inflammatory cytokine production. A151 specifically inhibited the AIM2 inflammasome and had no effect on NLRP3-mediated inflammasome activation, RIG-I or LPS signaling. The inhibitory effect of A151 was dependent on both its phosphorothioate (PS) backbone and sequence. Substitution of the guanine triplet in A151 for adenine residues (C151) reduced the potency of AIM2 inflammasome suppression by 94% (Table 2.1). Our data indicate that A151 functions as an inhibitor by competing with stimulatory DNA ligands for AIM2 binding. Intriguingly, C151 proved to be a more potent inhibitor of interferon-inducing pathways. In support of a competitive model of inhibition, IFI16 bound more strongly to the C151 construct. Interaction with members of the IFI20X/IFI16 (PYHIN) receptor family may account for many of the previously unexplained anti-inflammatory effects of sup ODNs. Collectively, these observations suggest a novel mechanism for sup ODN-mediated inhibition of the innate immune system.

Table 2.1 : Oligodeoxyribonucleotide Construct Sequences

Name	**Sequence**
A151	5'-TTAGGGTTAGGGTTAGGGTTAGGG-3'
C151	5'-TTCAAATTCAAATTCAAATTCAAA-3'
2114	5'-TCCTGGAGGGGAAGT-3'
4348	5'-TCGTATCCTGGAGGGGAAG-3'
1826	5'-TCCATGACGTTCCTGACGTT-3'
10104	5'-TCGTCGTTTCGTCGTTTTGTCGTT-3'
2336	5'-GGGgacgacgtcgtcgGGGGG-3'

* Capitalized nucleotides are connected by phosphorothioate linkages lower case nucleotides have 5'-phosphdiester linkages

Results

A151 has broad anti-inflammatory activities against cytosolic DNA sensing pathways.

A number of studies have explored the effects of the sup ODN A151 on type I IFN and cytokine production following LPS and CpG ODN stimulation (149, 151, 153). However, these studies often have a limited scope, only reporting A151's effects on a handful of inflammatory molecules. To examine its immunosuppressive potential, we measured the response of bone marrow-derived dendritic cells (BMDC) to A151 treatment alone and explored whether it affected induction of innate response genes following stimulation with synthetic dsDNA poly(dA:dT) and LPS. Total RNA was isolated from cells and mRNA levels of a panel of cytokines, chemokines, PRRs and signal transduction molecules were measured by multiplex gene expression analysis using nCounter (Nanostring) technology (Fig. 2.1 and Table 2.2). Nanostring analysis utilizes fluorescently coded probes that hybridize directly to target mRNA, allowing each individual mRNA to be counted without the need for amplification (190). This allows the detection of many targets simultaneously in a highly sensitive manner particularly when examining mRNAs present at low levels. This analysis revealed, resting BMDC expressed particularly high mRNA levels of TLR2 and TLR4 as well as a number of other receptors including TLR3, TLR7, TLR8, AIM2, IFI204, NLRP3, RIG-I and MDA-5. Molecules involved in signal transduction such as NF-κB, IκBα, MyD88, IRF2, IRF5, STAT3 and STAT1 and the cysteine protease caspase-1 were also expressed at basal levels. Untreated BMDC displayed very low levels of Type I IFN expression. Treatment

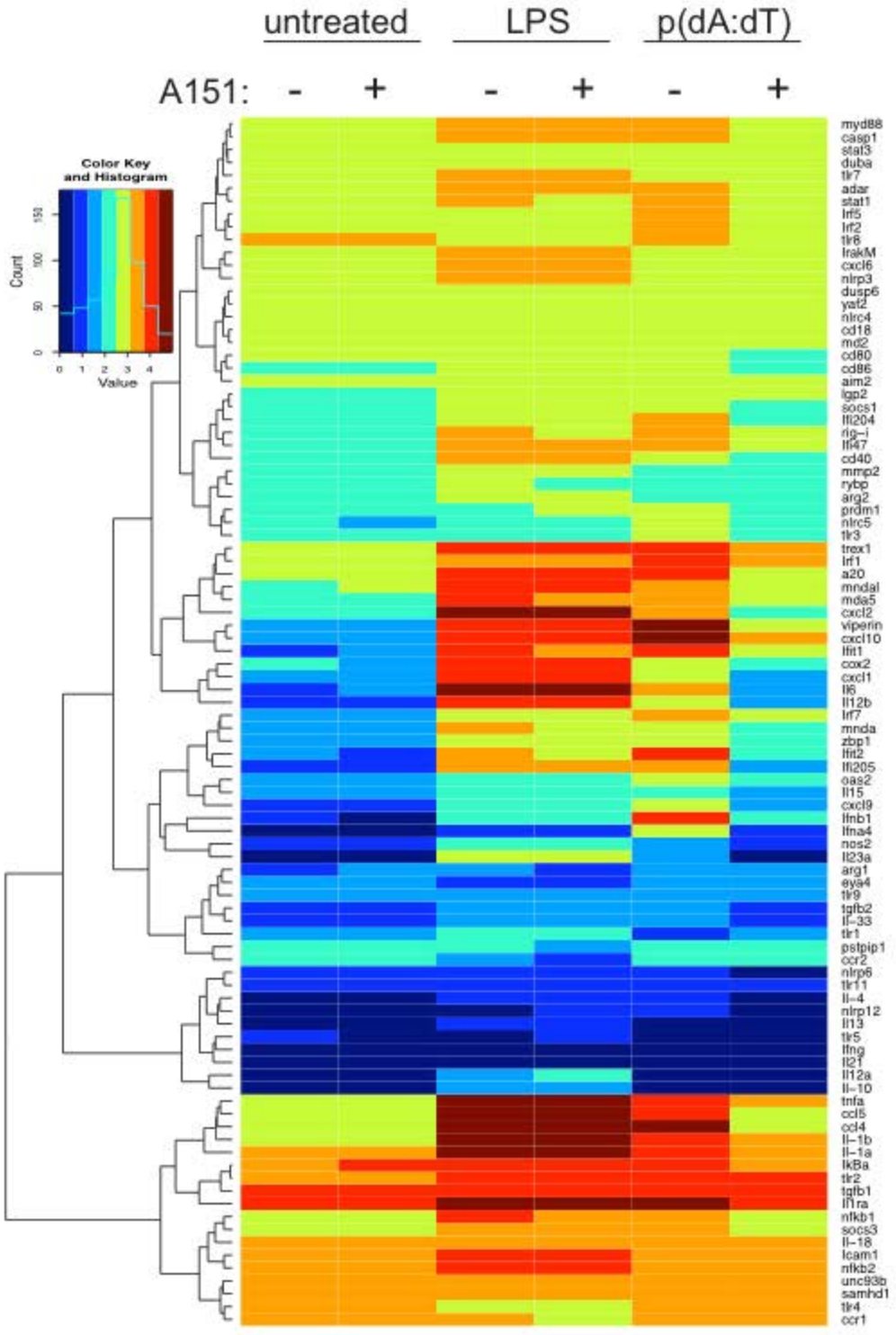


Figure 2.1: A151 broadly suppresses inflammatory gene induction in response to the dsDNA ligand poly(dA:dT) Mouse BMDCs were left untreated, stimulated with LPS (200 ng/ml) or 300 ng of poly(dA:dT) complexed with lipofectamine 2000 in the presence or absence of A151 (3 μM). RNA was extracted and subjected to nCounter Nanostring analysis. Gene expression profiles are displayed as a heat map (log10 transformed) with hierarchical clustering indicated by dendrogram.

Table 2.2 : Nanostring Normalized mRNA Counts

Gene	Stimulation					
	Media	A151	LPS	LPS + A151	p(dA:dT)	p(dA:dT) + A151
Cyokines, chemokines and their receptors						
Ifna4	3	3	14	14	463	15
Ifnb1	5	2	125	121	11674	121
Ifng	2	2	3	1	4	2
Il-1a	2299	3845	90032	85988	22190	3824
Il-1b	513	1190	96483	97982	9166	1610
Il-4	1	2	10	5	7	2
Il-6	12	31	27868	25706	3602	72
Il-10	1	1	18	19	1	1
Il-12a	2	3	73	78	4	1
Il-12b	7	17	9342	8241	360	22
Il-13	1	1	4	10	4	1
Il-15	37	49	283	254	157	49
Il-18	2293	1977	3796	3353	1698	1494
Il-21	2	1	1	2	1	1
Il-23a	2	3	417	478	43	1
Il-33	8	8	30	31	42	11
Il-1ra	18886	20912	74120	69265	49214	22396
ccl4	729	567	59040	55448	31622	1012
ccl5	733	709	41138	36889	6854	758
cxcl1	33	37	13628	12750	756	40
cxcl10	26	36	8505	6490	29161	1336
ccr1	2534	2204	1397	1147	2389	2358
ccr2	169	181	22	13	96	128
cxcl2	158	213	29228	28712	3783	294
cxcl6	440	502	2680	2314	598	413
cxcl9	5	7	182	166	453	23
tgfb1	8108	8788	10811	10902	8657	7987
tgfb2	13	15	22	27	33	13
tnfa	671	1112	30224	29787	15646	1705
PRRs, downstream signaling components						
aim2	439	452	417	387	1058	412
IrakM	612	761	3917	3668	559	586
nlrc4	413	455	650	631	358	402
nlrc5	77	70	227	176	338	93
nlrp12	1	1	3	7	6	4
nlrp3	373	482	5471	5323	1232	498
nlrp6	4	9	7	6	7	4
Ifi204	99	110	1079	762	1910	251
Ifi205	8	10	1903	1520	1541	70
Ifi47	107	117	2374	1477	2716	380
Ifit1	11	20	6927	5165	17556	558
Ifit2	24	15	1573	1129	5953	248
casp1	1014	1022	1454	1354	1654	956

	Media	A151	LPS	LPS + A151	p(dA:dT)	p(dA:dT) + A151
mnda	28	24	1412	1180	894	136
mndal	257	315	12079	11466	5305	515
myd88	883	908	1696	1494	1648	1012
lgp2	166	186	892	683	1042	313
tlr1	25	42	82	131	15	30
tlr11	6	6	4	4	6	4
tlr2	3386	5074	17166	16029	11960	5878
tlr3	148	157	182	132	353	155
tlr4	4290	3557	1062	918	3292	2934
tlr5	5	3	3	4	1	3
tlr7	1044	952	2299	2332	1100	1021
tlr8	1663	1368	931	838	1530	1164
tlr9	33	31	30	18	50	30
rig-i	203	182	1519	1127	1968	423
mda5	264	273	6257	4983	4781	409
IkBa	3773	5889	16694	17159	12996	5520
unc93b	2894	2907	2336	2339	2957	2950
md2	606	589	808	697	485	552
pstpip1	155	163	80	64	148	130
rybp	184	194	348	286	247	178
viperin	50	41	10291	6942	26101	1202
yaf2	573	476	413	364	457	395
zbp1	49	43	728	523	868	110
Transcription factors						
Irf1	492	578	4671	3850	9365	1398
Irf2	959	977	803	659	1445	911
Irf5	944	1129	928	940	1580	1077
Irf7	31	26	988	625	1814	316
nfkb1	932	1147	5666	5262	1772	973
nfkb2	1384	1977	10479	10426	2600	1965
stat1	524	447	1452	1039	2253	770
stat3	1256	1140	1226	1102	1281	983
eya4	21	35	8	13	24	24
Proinflammatory enzymes						
arg1	12	26	22	13	28	29
arg2	123	126	806	734	168	99
cox2	114	53	22628	20632	911	92
nos2	7	7	226	202	39	10
oas2	29	26	268	172	398	93
mmp2	223	229	472	486	247	202
trex1	530	617	6935	6281	7037	1924
adar	590	575	1799	1394	1681	725
samhd1	2132	2288	1693	1415	5494	2122
Cell adhesion and activation markers						
cd18	683	720	633	612	555	562

	Media	A151	LPS	LPS + A151	p(dA:dT)	p(dA:dT) + A151
cd40	100	105	3018	2967	497	110
cd80	363	357	466	438	560	255
cd86	217	229	440	402	625	267
Icam1	1900	2698	9348	9528	4851	3112
Anti-inflammatory signaling components						
socs1	168	142	737	812	1108	304
socs3	516	720	3807	3924	2200	961
duba	866	923	1295	1193	994	809
dusp6	624	591	514	477	446	464
a20	433	750	12997	13020	6293	766
prdm1	90	123	292	332	343	112

Table 2.3 : Nanostring mRNA Fold Induction Compared to Media

Gene	Stimulation		
	A151 / Media	LPS / Media	p(dA:dT) / Media
Ifnb1	0.4	24.2	2253.6
Ifit1	1.8	607.1	1538.7
cxcl10	1.4	328.1	1125.0
viperin	0.8	206.8	524.4
Il6	2.5	2240.2	289.6
Ifit2	0.6	66.0	249.6
Ifi205	1.2	229.3	185.6
Ifna4	1.1	4.4	148.8
cxcl9	1.3	35.2	87.4
Irf7	0.8	31.7	58.3
Il12b	2.3	1286.7	49.6
ccl4	0.8	81.0	43.4
mnda	0.8	50.4	31.9
Ifi47	1.1	22.2	25.4
cxcl2	1.4	185.4	24.0
tnfa	1.7	45.0	23.3
cxcl1	1.1	410.7	22.8
Il23a	1.6	201.4	20.6
mndal	1.2	47.0	20.6
Ifi204	1.1	11.0	19.4
Irf1	1.2	9.5	19.1
mda5	1.0	23.7	18.1
Il-1b	2.3	188.0	17.9
zbp1	0.9	14.9	17.8
a20	1.7	30.0	14.5
oas2	0.9	9.2	13.7
trex1	1.2	13.1	13.3
rig-i	0.9	7.5	9.7
Il-1a	1.7	39.2	9.7
ccl5	1.0	56.1	9.3
cox2	0.5	198.4	8.0
Il-4	2.2	9.2	7.0
socs1	0.8	4.4	6.6
lgp2	1.1	5.4	6.3
nlrp12	1.1	3.1	5.9
nos2	0.9	31.1	5.4
Il-33	1.0	3.6	5.0
cd40	1.1	30.3	5.0
nlrc5	0.9	3.0	4.4
stat1	0.9	2.8	4.3
socs3	1.4	7.4	4.3
Il15	1.3	7.6	4.2
prdm1	1.4	3.2	3.8
tlr2	1.5	5.1	3.5
Il13	1.1	4.1	3.5
IkBa	1.6	4.4	3.4

	A151 / Media	LPS / Media	p(dA:dT) / Media
nlrp3	1.3	14.7	3.3
cd86	1.1	2.0	2.9
adar	1.0	3.0	2.8
Il1ra	1.1	3.9	2.6
samhd1	1.1	0.8	2.6
Icam1	1.4	4.9	2.6
tgfb2	1.1	1.7	2.4
aim2	1.0	1.0	2.4
tlr3	1.1	1.2	2.4
arg1	2.1	1.8	2.3
nfkb1	1.2	6.1	1.9
nfkb2	1.4	7.6	1.9
myd88	1.0	1.9	1.9
Ifng	1.1	1.5	1.8
Il12a	1.6	35.4	1.8
nlrp6	2.2	1.8	1.8
Irf5	1.2	1.0	1.7
casp1	1.0	1.4	1.6
cd80	1.0	1.3	1.5
tlr9	0.9	0.9	1.5
Irf2	1.0	0.8	1.5
arg2	1.0	6.5	1.4
cxcl6	1.1	6.1	1.4
rybp	1.1	1.9	1.3
eya4	1.7	0.4	1.2
Il-10	1.1	17.3	1.2
duba	1.1	1.5	1.1
mmp2	1.0	2.1	1.1
tgfb1	1.1	1.3	1.1
tlr7	0.9	2.2	1.1
unc93b	1.0	0.8	1.0
stat3	0.9	1.0	1.0
tlr11	0.9	0.7	1.0
pstpip1	1.1	0.5	1.0
ccr1	0.9	0.6	0.9
tlr8	0.8	0.6	0.9
IrakM	1.2	6.4	0.9
nlrc4	1.1	1.6	0.9
cd18	1.1	0.9	0.8
md2	1.0	1.3	0.8
yaf2	0.8	0.7	0.8
tlr4	0.8	0.2	0.8
Il-18	0.9	1.7	0.7
dusp6	0.9	0.8	0.7
Il21	0.5	0.5	0.6
tlr1	1.7	3.3	0.6
ccr2	1.1	0.1	0.6
tlr5	0.7	0.6	0.2

Table 2.4 : Nanostring mRNA Fold Reduction by A151 Treatment

Gene	**Stimulation**	
	LPS / (LPS + A151)	p(dA:dT) / p(dA:dT) + A151
Ifnb1	1.0	96.3
Il6	1.1	50.0
Ifit1	1.3	31.5
ccl4	1.1	31.2
Ifna4	1.0	30.0
Il23a	0.9	29.1
Ifit2	1.4	24.0
Ifi205	1.3	22.1
cxcl10	1.3	21.8
viperin	1.5	21.7
cxcl9	1.1	19.9
cxcl1	1.1	18.7
Il12b	1.1	16.3
cxcl2	1.0	12.9
mda5	1.3	11.7
mndal	1.1	10.3
cox2	1.1	9.9
tnfa	1.0	9.2
ccl5	1.1	9.0
a20	1.0	8.2
zbp1	1.4	7.9
Ifi204	1.4	7.6
Ifi47	1.6	7.1
Irf1	1.2	6.7
mnda	1.2	6.6
Il-1a	1.0	5.8
Irf7	1.6	5.7
Il-1b	1.0	5.7
Il12a	0.9	4.9
Il13	0.4	4.9
rig-i	1.3	4.6
cd40	1.0	4.5
oas2	1.6	4.3
nos2	1.1	3.8
Il-33	1.0	3.8
trex1	1.1	3.7
socs1	0.9	3.7
nlrc5	1.3	3.7
lgp2	1.3	3.3
Il-4	1.8	3.3
Il15	1.1	3.2
prdm1	0.9	3.1
stat1	1.4	2.9
samhd1	1.2	2.6
aim2	1.1	2.6
tgfb2	0.8	2.5

	LPS / (LPS + A151)	p(dA:dT) / p(dA:dT) + A151
nlrp3	1.0	2.5
IkBa	1.0	2.4
cd86	1.1	2.3
adar	1.3	2.3
socs3	1.0	2.3
tlr3	1.4	2.3
Il1ra	1.1	2.2
cd80	1.1	2.2
tlr2	1.1	2.0
nlrp6	1.2	2.0
nfkb1	1.1	1.8
casp1	1.1	1.7
arg2	1.1	1.7
tlr9	1.7	1.7
nlrp12	0.4	1.7
Ifng	3.0	1.7
Il21	0.5	1.6
myd88	1.1	1.6
Irf2	1.2	1.6
Icam1	1.0	1.6
Irf5	1.0	1.5
cxcl6	1.2	1.4
rybp	1.2	1.4
tlr11	1.0	1.4
nfkb2	1.0	1.3
tlr8	1.1	1.3
stat3	1.1	1.3
duba	1.1	1.2
mmp2	1.0	1.2
yaf2	1.1	1.2
Il-18	1.1	1.1
pstpip1	1.2	1.1
tlr4	1.2	1.1
tgfb1	1.0	1.1
tlr7	1.0	1.1
eya4	0.7	1.0
ccr1	1.2	1.0
unc93b	1.0	1.0
cd18	1.0	1.0
arg1	1.8	1.0
dusp6	1.1	1.0
IrakM	1.1	1.0
nlrc4	1.0	0.9
md2	1.2	0.9
Il-10	0.9	0.8
ccr2	1.8	0.8
tlr1	0.6	0.5
tlr5	0.8	0.4

with A151 alone had very little effect on the transcriptional profile of BMDC (Fig. 2.1 and Table 2.2). In keeping with previous reports, A151 treatment did not induce anti-inflammatory molecules such as IL-10, TGF-β or SOCS3 (146). By comparison, treatment with the synthetic dsDNA ligand poly(dA:dT) drastically increased mRNA levels of inflammatory cytokines, type I IFNs and ISGs as well as other immune mediators and regulators. IFN-β, the most highly induced gene, was upregulated more than 2000-fold and IL-6 more than 250-fold (Fig. 2.1 and Table 2.3). Importantly, treatment with A151 greatly attenuated the response to poly(dA:dT) (Fig. 2.1 and Table 2.4). Genes such as IFN-β and IL-6, which were among the most highly induced by poly(dA:dT), were also the most potently suppressed by A151. Like poly(dA:dT), LPS stimulated increased mRNA levels of a variety of cytokines and Type I IFN. IL-6, the most highly induced mRNA by LPS, was increased over 2000-fold (Table 2.3). In contrast to A151's effects on poly(dA:dT) signaling, A151 did not mitigate LPS-induced responses (Fig. 2.1 and Table 2.4). Thus, A151 appears to have very little effect on the expression profile of resting cells and cells treated with LPS, but potently and broadly inhibits activation by poly(dA:dT).

A151 and C151 inhibit IFN-β and TNF-α production in response to HSV-1 and MCMV.

To define the specificity of A151-mediated suppression, BMDC and bone marrow-derived macrophages (BMDM) were exposed to the herpesviruses HSV-1 or MCMV and IFN-β expression was measured by qRT-PCR (Fig. 2.2a,b). Pre-treatment with A151 reduced IFN-β induction in response to HSV-1 and MCMV in BMDC and BMDM.

Interestingly pre-treatment with the C151 construct, in which the guanine residues of A151 had been replaced with adenine residues, had a nearly identical suppressive effect. In contrast, the IFN-β response to LPS and Sendai virus, a paramyxovirus that activates the RIG-I pathway, was unchanged by these constructs (Fig. 2.2a,b). Similarly, BMDC pretreated with A151 and C151 reduced TNF-α mRNA induction in response to HSV-1 and MCMV, but had no effect on TNF-α induction in responses to LPS or Sendai virus (Fig 2.2c). These data suggests A151- and C151-mediated inhibition of IRF3- and NF-κB-dependent gene induction is specific to challenge with poly(dA:dT) and the herpesviruses HSV-1 and MCMV.

Both sequence and backbone composition affect inhibitory activity of sup ODN on interferon-inducing pathways.

There is some conflict in the literature regarding the role of sequence in mediating A151's inhibitory effects. Initial reports suggested the guanine residues found within A151's TTAGGG motif played a role in suppression of CpG-induced TLR9 signaling (149). However, other groups demonstrated that inhibition is largely independent of sequence and, instead, relies on the presence of a phosphorothioate backbone (PS) (146). To further explore the role of sequence in suppression, a dose response experiment was performed to establish how potently sup ODN inhibited poly(dA:dT) signaling. Surprisingly, we found the C151 construct more potently suppressed IFN-β secretion than did A151 (Fig 2.3a,b). In BMDC, C151 was approximately 10-times more potent than A151, although at higher concentrations these constructs were equally efficacious. The dose requirements for inhibition also varied between macrophages and dendritic

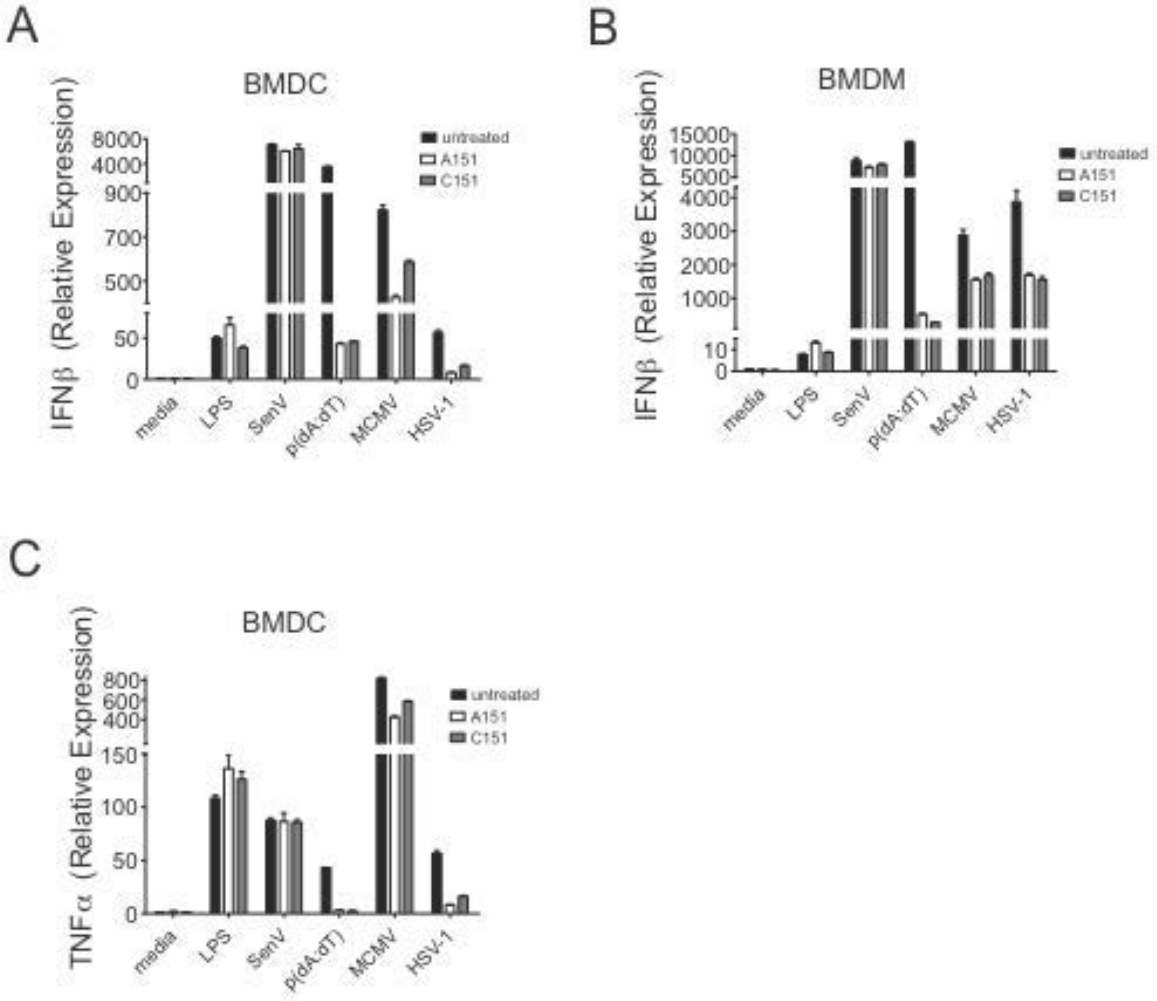


Figure 2.2: A151 and C151 suppress IFNβ and TNFα production in response to poly(dA:dT), HSV-1 and MCMV A, BMDC and B, BMDM were stimulated with LPS (200 ng/ml), 300 ng of poly(dA:dT) complexed with lipofectamine 2000, MCMV (MOI=10), HSV-1 (MOI=10), or SV (200 HAU/ml) in the presence or absence of A151/C151 (3 μM) for 4 h. IFN-β and β-actin mRNA was measured by qPCR. IFN-β induction is represented relative to untreated controls. C, BMDC were stimulated as above. TNF-α and β-actin mRNA was measured by qPCR. TNF-α induction is represented relative to untreated controls.

cells. At a concentration of 3 μM, inhibition of the IFN-β response was near maximal for both A151 and C151. This explains why no differences in suppression were observed in earlier experiments. To assess the effects of the PS backbone on IFN-β secretion BMDM and BMDC were treated with A151, C151 and a phosphodiester (PD) version of C151 (referred to as C151(PD)), followed by transfection with poly(dA:dT) (Fig 2.3c,d). Unlike the two PS constructs, C151(PD) failed to block the IFN-β response. Thus, while a PS backbone is essential for suppression, sequence plays a role only when lower doses of sup ODN are employed. In addition to the A151 construct, a number of sup ODN have been developed to block TLR9 signaling selectively. Ashman *et al*. extensively documented the sequence requirements for optimal inhibition of TLR9 activation and identified a sequence designated 4348 as one of the most potent TLR9 suppressing constructs (148). We explored the relative potency of 4348 on poly(dA:dT)-induced IFN-β secretion using a concentration of 1 μM in BMDC and 0.5 μM in BMDM that provided strong suppression, but allowed observation of sequence specific effects. The 4348 construct mediated suppression comparable to A151 at a similar dose, but did not prove to be as potent as C151 (Fig 2.3e,f).

A151 blocks AIM2 inflammasome activation in response to cytosolic dsDNA.

We next investigated the inhibitory potential of the sup ODN A151 on activation of the inflammasome. BMDC were first stimulated with LPS to drive high levels of pro-IL-1β and pro-IL-18 expression. Cells were then treated with A151 for one hour before exposure to a panel of inflammasome ligands. Pre-treatment with A151 had no effect on IL-1β production in response to the NLRP3 ligands silica, nigericin or ATP, whereas the

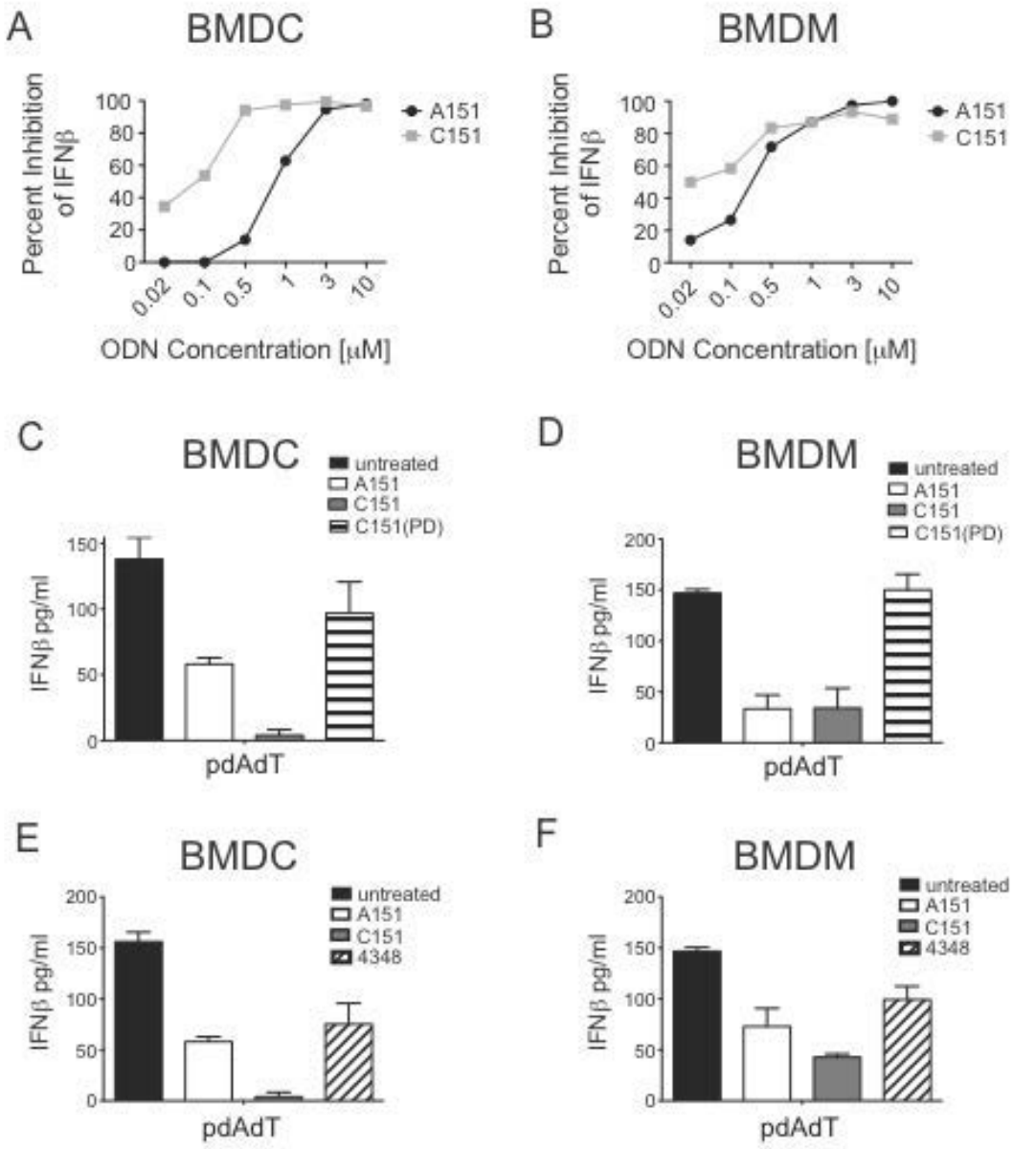


Figure 2.3: Sequence and backbone composition affect inhibition of IFNβ production A, BMDC or B, BMDM were stimlated with 300 ng of poly(dA:dT) complexed with lipofectamine 2000. Cells were pretreated with an increasing amount of A151 or C151 (0.02 μM, 0.1 μM, 0.5 μM, 1 μM, 3 μM, 10 μM) and IFN-β secretion was measured by ELISA. Results are displayed as the percent inhibition of cytokine production compared to poly(dA:dT) challenge alone. C, BMDC or D, BMDM were treated with 300 ng of poly(dA:dT) complexed with lipofectamine 2000. Cells were untreated or pretreated with A151, C151 or the phosphodiester C151(PD) at 3 μM and IL-1β secretion was measured by ELISA. E, BMDC or F, BMDM were treated with 300 ng of poly(dA:dT) complexed with lipofectamine 2000. Cells were untreated or pretreated with A151, C151 or 4348 at 3 μm and IL-1β secretion was measured by ELISA.

response to the AIM2 ligand poly(dA:dT) was significantly reduced (Fig. 2.4a). This pattern of AIM2-specific inhibition was also observed in BMDM and the human THP1 monocytic cell line (Fig. 2.4b, c respectively). A151 also suppressed IL-18 secretion in response to poly(dA:dT), but not nigericin in BMDC (Fig. 2.4d). Western blot analyses confirmed the reduction of cleaved IL-1β in the supernatants of A151-treated BMDC challenged with poly(dA:dT) (Fig. 2.4e). Furthermore, these blots revealed a decrease in caspase-1 cleavage. The reduced levels of the active caspase-1 p10 and p20 subunits following A151 treatment suggests dampened caspase-1 activity. Importantly, exposure to A151 did not diminish levels of pro-IL-1β and pro-caspase-1 in cellular lysates, suggesting A151 treatment blocked the activity of the AIM2 inflammasome rather than affecting expression of the caspase-1 or its substrate. Secretion of the alarmin high mobility group box 1 (HMGB1) also requires caspase-1 activation (191, 192) and, much like IL-1β and IL-18, HMGB1 release into the supernatant was suppressed by A151 (Fig. 2.4e). In contrast to A151, treatment with C151 led to a more modest reduction in IL-1β and IL-18. At 3μM, C151 occasionally reached statistical significance, however its inhibitory effect was difficult to visualize by western blot (Fig. 2.4e,f).

Both sequence and backbone composition effect inhibition of AIM2 inflammasome signaling.

To determine the inhibitory kinetics of A151 and C151 on AIM2 signaling a titration experiment was conducted. BMDC were pre-treated with increasing concentrations of A151 and C151 followed by transfection with poly(dA:dT) and subsequent IL-1β and IL-18 secretion was measured by ELISA. This experiment

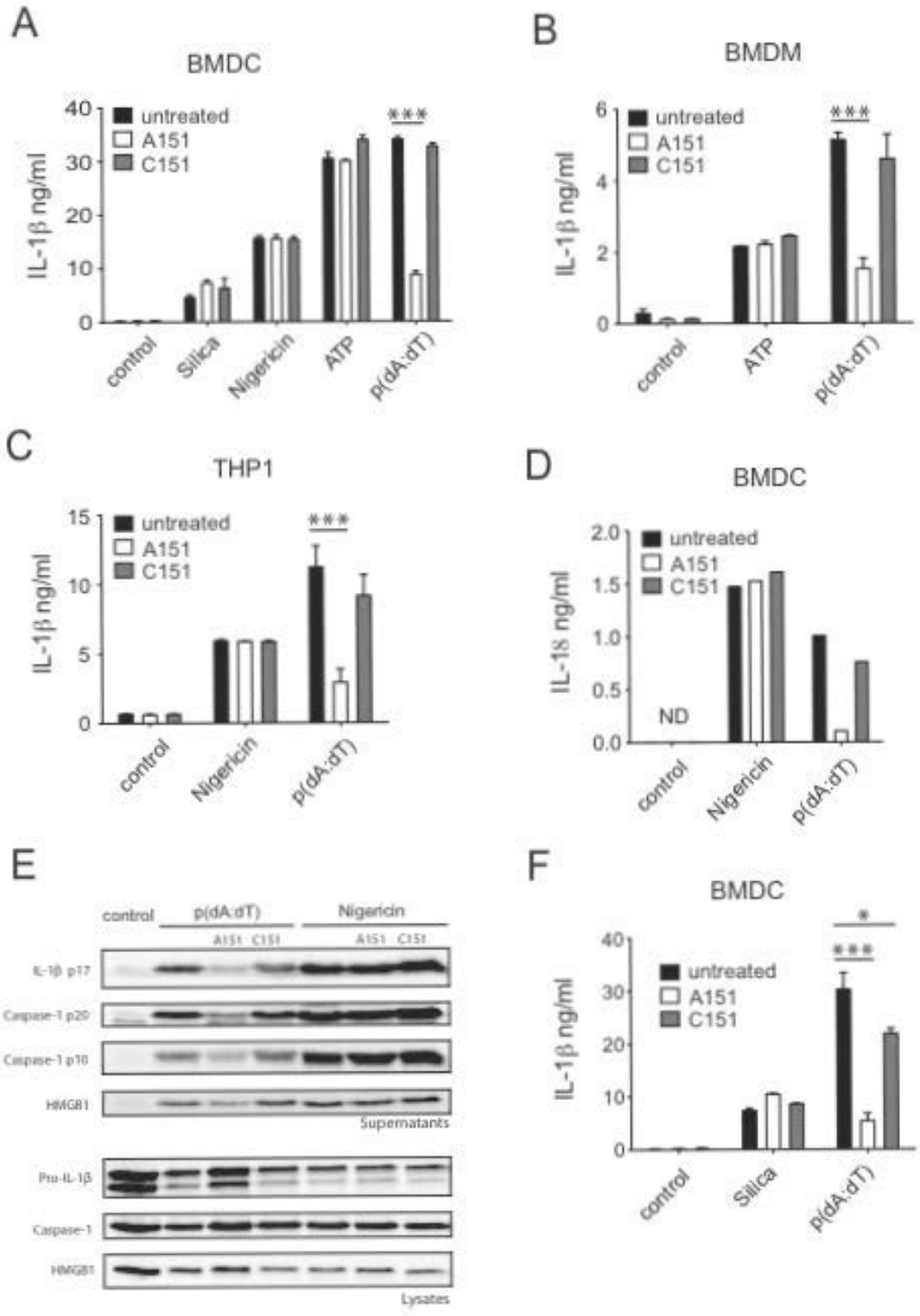

A
BMDC
IL-1β ng/ml
untreated
A151
C151
***
control
Silica
Nigericin
ATP
p(dA:dT)
B
BMDM
C
THP1
D
BMDC
IL-18 ng/ml
ND
E
control
p(dA:dT)
Nigericin
IL-1β p17
Caspase-1 p20
Caspase-1 p10
HMGB1
Supernatants
Pro-IL-1β
Caspase-1
Lysates
F
BMDC
*

Figure 2.4: A151 prevents AIM2 inflammsome activation in response to cytosolic dsDNA A, BMDCs or B, BMDMs were primed with LPS (200 ng/ml; LPS prime alone is control) and challenged with silica (500 μg/ml), nigericin (10 μM), ATP (5 mM) or 300 ng of poly(dA:dT) complexed with lipofectamine 2000 (untreated). Cells were pretreated with A151 or C151 (3 μm) as indicated and IL-1β secretion into the supernatant was measured by ELISA. C, THP1s were differentiated overnight with PMA (0.5 μM), treated as described above and IL-1β was measured by ELISA. D, BMDCs were treated as described and IL-18 was measured by ELISA. E, Immunoblotting of IL-1β, caspase-1 and HMGB1 in the supernatants and lysates from BMDCs. F, BMDC were treated as above and IL-1β secretion into the supernatant was analyzed by ELISA. Data are presented as mean ± SD from three biological replicates representative of three experiments. * $p < 0.01$, ** $p<0.001$, *** $p<0.0001$

revealed that both A151 and C151 were capable of inhibiting AIM2 inflammasome activation (Fig. 2.5a,b). Interestingly, A151 with a half-maximal effective concentration (EC50) of 0.360μm was approximately 20 times more potent than C151 (EC50 = 6.16μm). To determine whether the PS backbone affected A151-mediated inhibition, this sup ODN was synthesized with a phosphodiester backbone A151(PD) and tested in BMDC and BMDM. Pretreatment with A151(PD) had no significant effect on IL-1β release following poly(dA:dT) challenge in either line (Fig. 2.5c,d). As we had observed for IFN-inducing pathways, a PS backbone was essential for inhibition of AIM2 signaling. However, whereas C151 was a more potent inhibitor of the IFN-β response, A151 proved to more potently suppress AIM2 signaling.

Until now we had only tested constructs previously shown to have inhibitory properties. To explore whether the increased inhibitory potency of A151 was due to its specific sequence, or a more general property of guanosine rich PS ODNs, we tested the inhibitory potential of one class A and two class B stimulatory CpG ODNs. The class A ODN 2336 has a backbone composed of PS linkages on either end and 11 interior PD bonds. The two class B ODN, 1826 and 10104, are composed entirely of PS linkages. These ODN contain CpG motifs and, when used alone, activate TLR9 leading to DC maturation and the secretion of cytokines (11, 193). However, much like A151, pre-treatment with 1826 and 10104 inhibited IL-1β secretion by BMDC following poly(dA:dT) challenge. By comparison, the partial PS construct, 2336 had no inhibitory effect, despite having a similar guanosine content to A151 (Fig. 2.5e). These findings underscored the importance of the PS backbone in AIM2 inhibition and revealed a correlation between total guanosine content and potency in PS constructs.

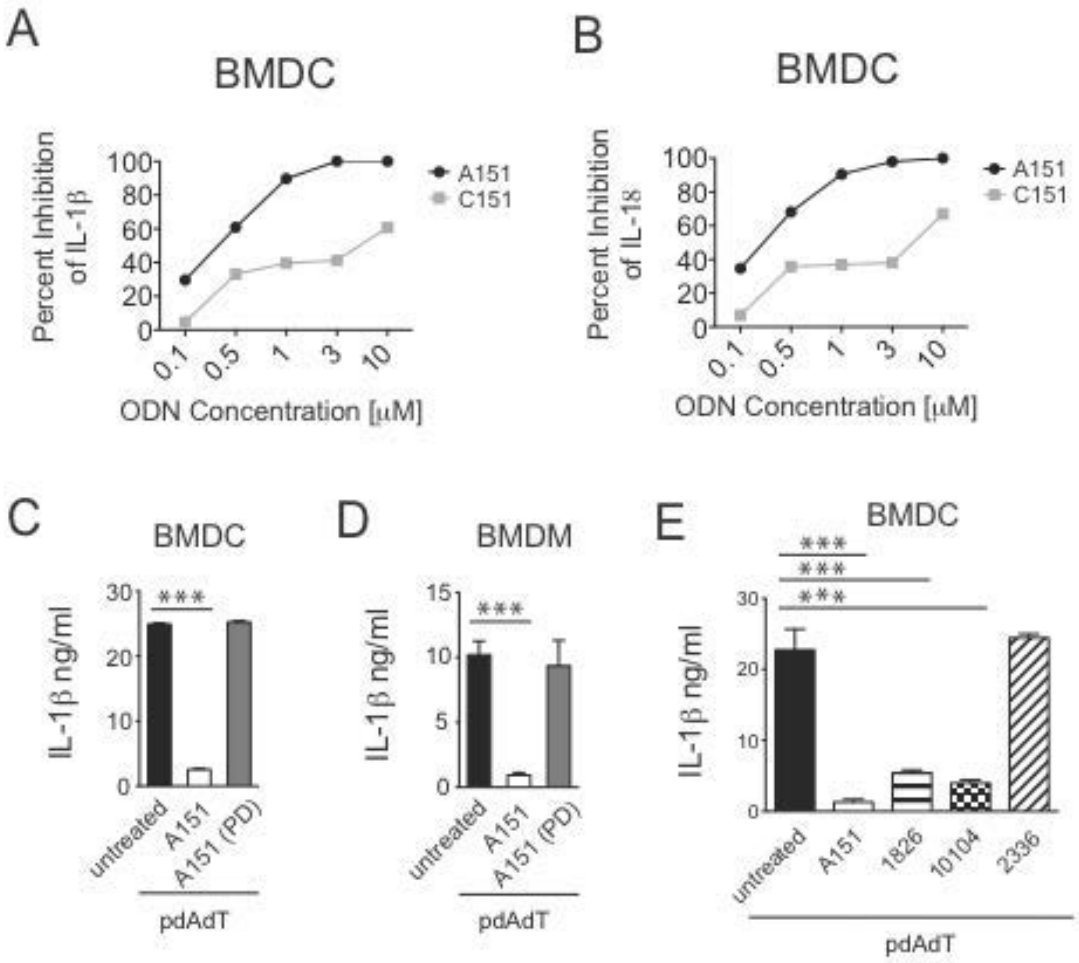


Figure 2.5: Sequence and backbone composition affect suppression of AIM2-mediated cytokine production BMDCs were primed with LPS (200 ng/ml) and challenged with 300 ng of poly(dA:dT) complexed with lipofectamine 2000 (untreated). Cells were pretreated with an increasing amount of A151 or C151 (0.1 μM, 0.5 μM, 1 μM, 3 μM, 10 μM) and (A) IL-1β and (B) IL-18 secretion was measured by ELISA. Results are displayed as the percent inhibition of cytokine production as compared to LPS prime, poly(dA:dT) stimulation alone. C, BMDC or D, BMDM were treated as above in the presence or absence of A151 or the phosphodiester A151 (PD) at 3 μm and IL-1β secretion was measured by ELISA. E, BMDC were treated as above in the presence of A151, 1826, 10104 or 2336 at 3 μm and IL-1β secretion was measured by ELISA. Data are presented as mean ± SD from three biological replicates representative of three experiments. *** $p<0.0001$

A151 prevents AIM2 inflammasome mediated pyroptosis.

In addition to cytokine processing, AIM2 activation leads to a form of programmed cell death known as pyroptosis (16, 194). Pyroptosis is distinct from apoptosis, in that it is caspase-1-dependent, leads to rapid plasma-membrane rupture and the release of pro-inflammatory cytokines; making this form of cell death inherently inflammatory. Using a luminescent cell viability assay, we quantified the survival of BMDC and BMDM following poly(dA:dT) challenge. After 16 hours of exposure, we observed 70% cell death in BMDC and 93% in BMDM. Similarly to its affects on IL-1β and IL-18 secretion, A151 potently inhibited pyroptotic cell death in both macrophages and dendritic cells (Fig. 2.6a,b). In BMDM, A151 was able to restore nearly 100% viability at dose of 1 μM though it was less effective in BMDC. Interestingly, C151 had no effect in BMDC, but was able to inhibit cell death in BMDM, though at a reduced potency and efficacy compared to A151. In keeping with its effects on cytokine production suppression of pyroptotic cell death was dependent on the PS backbone in both lines (Fig. 2.6c,d).

A151 prevents IL-1β cleavage in AIM2-reconstituted HEK293T cells.

The human HEK293T cell line has proven to be a useful tool for studying inflammasome activation. Transient transfection of plasmids encoding Aim2, Asc, caspase-1 and pro-IL-1β leads to the formation of a functional AIM2 inflammasome complex and subsequent IL-1β cleavage (16). Moreover HEK293T are devoid of endogenous TLR expression, allowing us to examine the effects of A151 in a system free of TLR signaling (195). Exposure of AIM2-reconstituted HEK293T cells to 1 μM A151

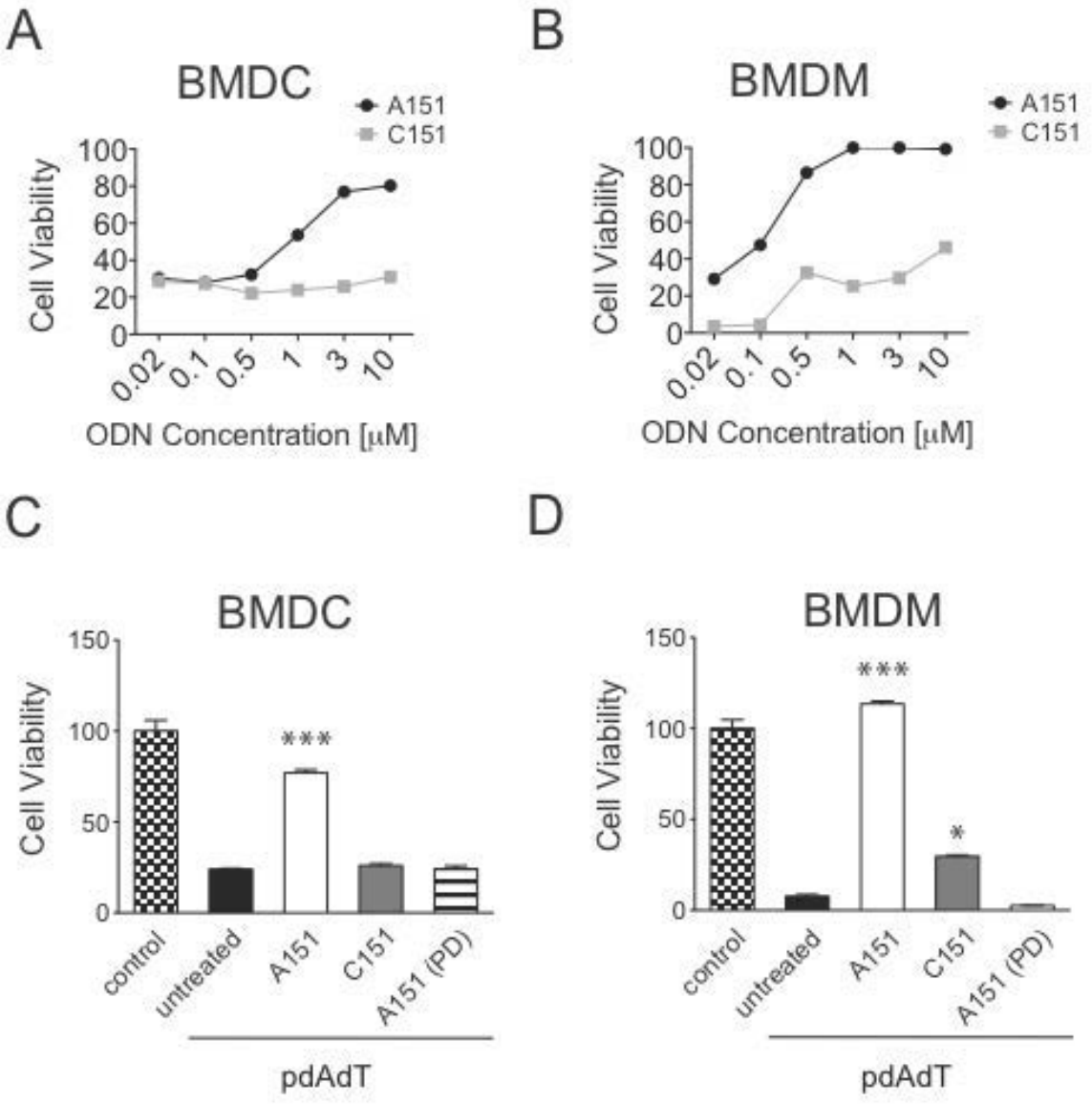


Figure 2.6: Sequence and backbone composition affect AIM2-mediated pyroptosis A, BMDC or B, BMDM were maintained in media or stimulated with 300 ng of poly(dA:dT) complexed with lipofectamine 2000 (untreated) for 16hrs. Stimulated cells were pretreated with an increasing amount of A151 or C151 (0.02 μM, 0.1 μM, 0.5 μM, 1 μM, 3 μM, 10 μM) and cell viability was measured by CellTiter-Glo luminescent viability assay. Results are displayed as the cellular viability compared to media controls. C, BMDC or D, BMDM were maintained in media (control) or stimulated with 300 ng of poly(dA:dT) complexed with lipofectamine 2000 (untreated). Stimulated cells were pretreated with A151, C151 or the phosphdiester A151 (PD) at 3 μM and cell viability was measured by CellTiter-Glo luminescent viability assay. Luminescence levels in media controls were set to 100% viability. Data are presented as mean ± SD from three biological replicates representative of three experiments. * $p<0.05$, *** $p<0.0001$

drastically reduced IL-1β cleavage while C151 had no effect (Fig. 2.7a). Suppression was not observed when IL-1β cleavage was driven by caspase-1 overexpression alone, indicating that A151 inhibits inflammasome activation at a step prior to caspase-1 activation. To determine whether the potency of inhibition in this reconstitution assay were similar to our primary lines, a dose response experiment was performed. This experiment corroborated our previous findings, demonstrating that while both constructs could mediate inhibition at higher doses, A151 was more potent compared to C151 (Fig. 2.7b).

A151 blocks AIM2 activation in BMDC challenged with MCMV and Listeria.

Our lab has previously shown that AIM2 is essential for inflammasome activation in response to the viral pathogen MCMV (16). Secretion of IL-1β by BMDC challenged with MCMV was markedly reduced by A151 pretreatment (Fig. 2.8a). A number of inflammasome receptors including NLRP3, NLRC4 and AIM2 have been implicated in the IL-1β response to *L. monocytogenes* (16, 95, 102, 196, 197). A151 treatment reduced IL-1β production in BMDC responding to *L. monocytogenes*, a reduction proportional to that observed in AIM2-deficient cells (Fig. 2.8b) (16). Collectively, these findings suggest that A151 blocks AIM2-mediated inflammasome signaling in response to the pure dsDNA ligand poly(dA:dT) as well as pathogens such as MCMV and *L. monocytogenes*.

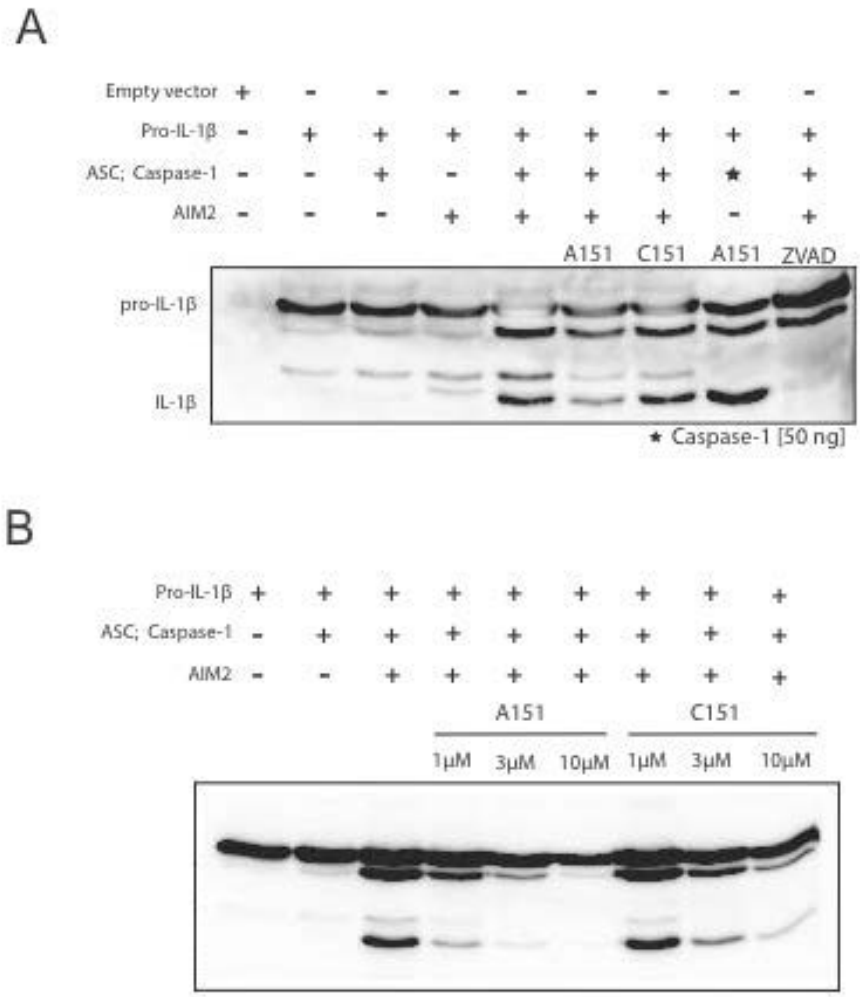


Figure 2.7 : Suppressive ODN inhibit AIM2 inflammasome formation in reconstituted HEK293T cells A, HEK293T cells were transfected with empty vector (pEFBOS) or 50 ng pro-IL-1β-FLAG (pEFBOS) together with 1 ng pro-Caspase-1 (pCI) (★ received 50 ng/well), 1 ng ASC (pCI) and 1 ng AIM2 (pEFBOS) as indicated. A151 or C151 (3 μm) was added 2 hrs post-transfection and 24 hrs later lysates were collected and immunoblotted with anti-Flag antibody. B, HEK293T cells were transfected as described above. A151 or C151 was added in increasing concentrations (1 μm, 3 μm, 10 μm) 2 hrs post-transfection and 24 hrs later lysates were collected and immunoblotted with anti-Flag antibody.

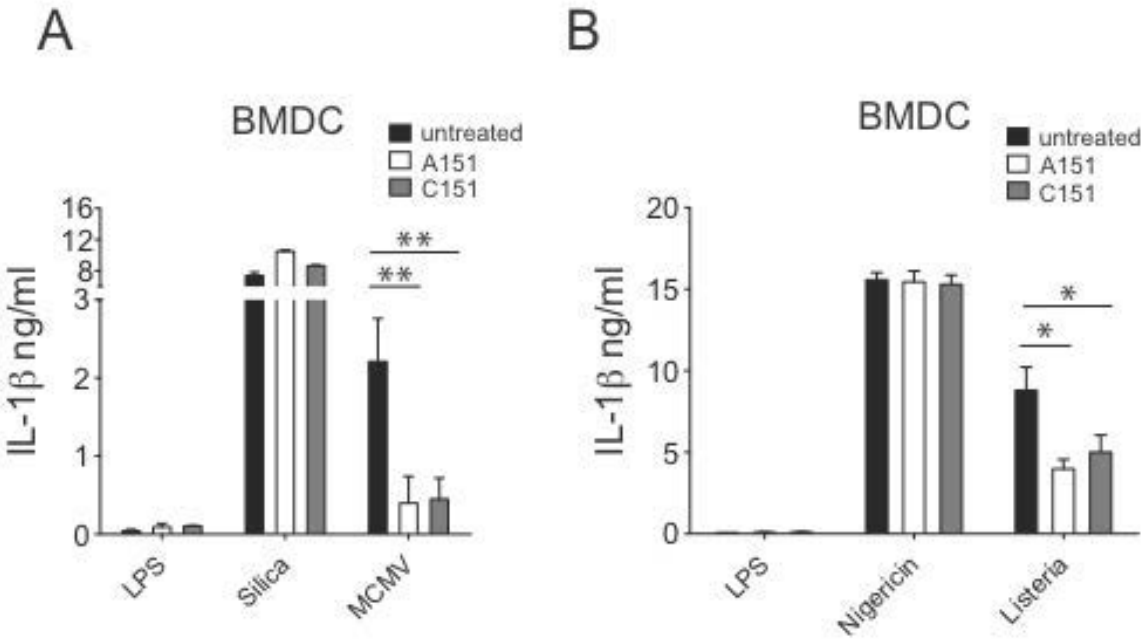


Figure 2.8 : Suppressive ODN inhibit AIM2 inflammasome formation following infection with MCMV and Listeria A, BMDC were primed with LPS (200 ng/ml) and challenged with MCMV (MOI=10) or silica (500 μg/ml) alone or in the presence of A151 or C151 (3 μM). IL-1β secretion into the supernatant was analyzed by ELISA. B, BMDC were primed with LPS (200 ng/ml) and challenged with Listeria (MOI=5) or nigericin (10 μM) alone or in the presence of A151 or C151 (3 μM). Data are presented as mean ± SD from three biological replicates representative of three experiments. * $p < 0.01$, ** $p<0.001$

A151 prevents ASC dimerization.

We next wanted to understand the molecular basis for the suppressive effect of A151 on AIM2 inflammasome activation. We therefore examined the ability of A151 to modulate AIM2-ASC inflammasome complex assembly. To do so BMDM were challenged with poly(dA:dT) and whole cell lysates were cross-linked and fractionated by sequential centrifugation. Following exposure to poly(dA:dT), we observed an increase in the presence of ASC dimers in the macromolecular pellet; a finding consistent with inflammasome activation (Figure 2.9a) (101). Pretreatment with A151 reduced ASC dimer formation to levels observed in media controls whereas C151 led to a modest decrease and A151-PD had no effect. Consistent with these observations, A151-treated cells retained ASC in its soluble, monomeric form, suggesting that A151 blocks recruitment of ASC to AIM2, preventing inflammasome assembly.

A151 blocks inflammasome assembly in an AIM2-citrine reporter cell line.

A defining feature of inflammasome signaling is the formation of a large, multiprotein complex in the cytosol composed of an NLR or ALR sensor, ASC, and caspase-1. This complex can be as large as 2 μm in size and offers a unique opportunity to analyze signaling by tracking the localization of inflammasome components in living cells (194). To visualize the formation of the AIM2 inflammasome, we employed an immortalized murine macrophage cell line stably expressing AIM2-citrine and monitored inflammasome assembly in live cells. In resting macrophages, the AIM2-citrine fusion protein was diffusely cytoplasmic. However, stimulation with poly(dA:dT) caused nearly 50% of these cells to form fluorescent punctate structures or ‘specks’ indicative of AIM2

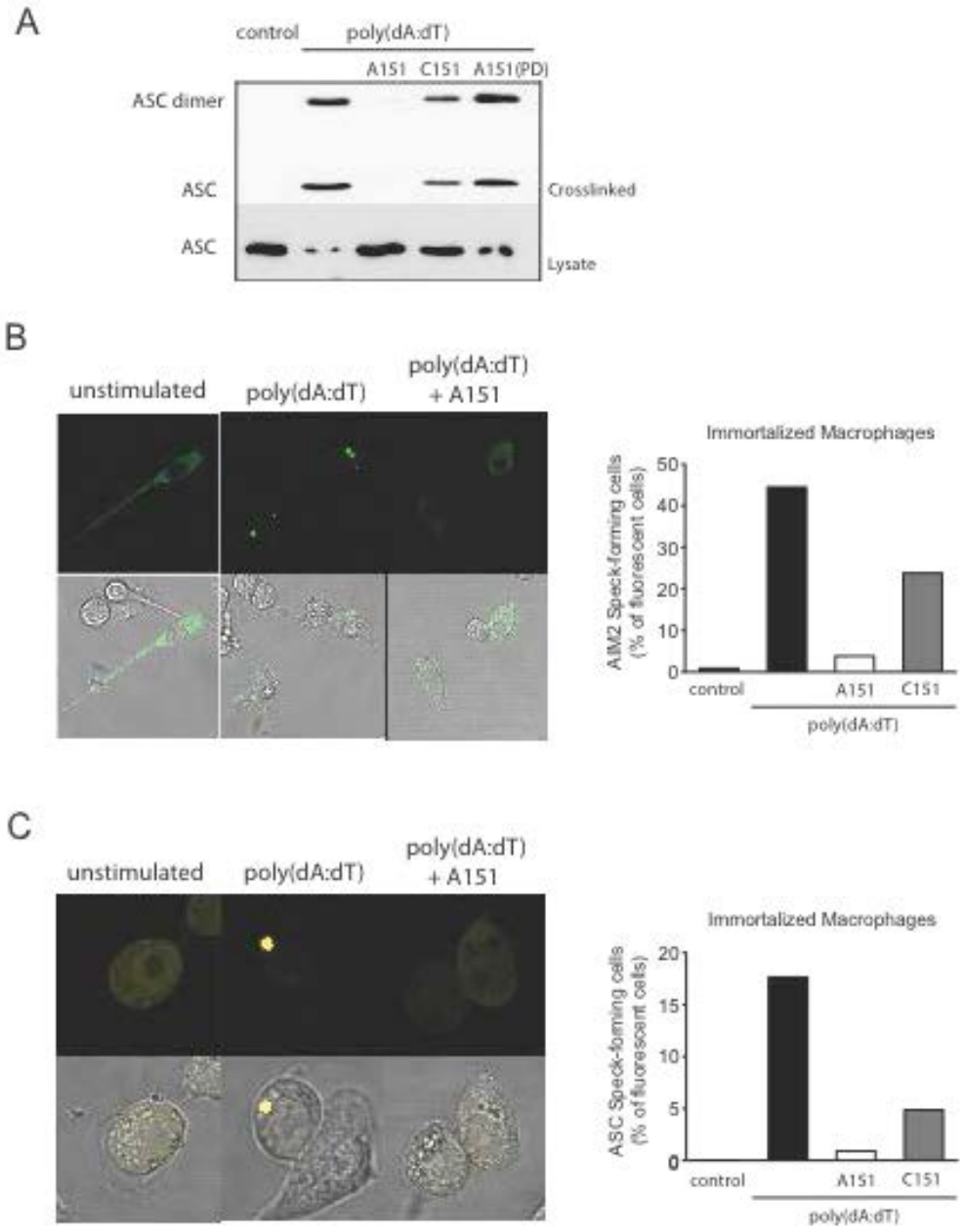


Figure 2.9 : A151 prevents ASC recruitment and AIM2 speck formation A, Oligomerization of ASC in the inflammasome-enriched and cross-linked lysates of BMDMs primed with LPS and challenged with poly(dA:dT) for 3 hrs in the presence or absence of A151 or C151 as described. Immortalized murine macrophages stably expressing (B) AIM2-citrine or (C) ASC-citrine reporter constructs were left unstimulated or challenged with poly(dA:dT) in the presence or absence of A151. Photographs demonstrate typical 'diffuse' and punctate 'speck' fluorescent patterns and are accompanied by graphs quantifying speck formation as a percent of the total fluorescent cells. Graphs are the combine data from three independent experiments.

inflammasome formation (Figure 2.9b). Pre-treatment with A151 strongly inhibited the formation of AIM2-citrine specks in our poly(dA:dT)-treated reporter line. Instead, the AIM2-citirine protein remained dispersed throughout the cytoplasm. In keeping with previous data, C151 demonstrated a more modest inhibitory effect only partially preventing speck formation. A similar pattern of inhibition was observed using macrophages expressing an ASC-citrine construct (Figure 2.9c). T hese results are consistent with our cytokine data and suggest that A151 mediates inhibition by blocking the ability of AIM2 to engage downstream signaling components necessary for aggregation.

A151 binds to AIM2.

A151 has been shown to exert its suppressive effects by association with signal transduction molecules such as STAT1 and STAT4 (149, 153). AIM2 binds stimulatory dsDNA via its C-terminal HIN200 domain thus releasing it from a resting, autoinhibited conformation and allowing inflammasome formation (73). To determine whether our sup ODN can interact with endogenous AIM2, immortalized murine macrophages were treated with with biotinylated A151 or biotiylated C151 for one hour, lysed and incubated with streptavidin beads. These pull-down studies revealed that A151, and to a far less extent, C151 were capable of interacting with AIM2. (Fig. 2.10a) A second pull down with biotinylated A151 was performed in the presence of an increasing concentration of poly(dA:dT). The inclusion of poly(dA:dT) during the binding step led to a proportional decrease in AIM2 recovery, suggesting A151 competes with poly(dA:dT) for AIM2 (Fig 2.10b).

A151 and C151 bind to IFI16.

A number of receptors have been implicated in IFN-β induction in response to HSV-1 infection, including IFI16, DAI, DHX9, DHX36, RNA Pol III, DDX41 and the newly discovered cGAS. The association of AIM2 with A151 suggests other members of the PYHIN family may also associate with A151. Unterholzner *et al*. have previously demonstrated that IFN-β induction and NF-κB activation in response to cytosolic DNA and HSV-1 infection is dependent on the PYHIN protein IFI16 (70). IFI16 is alternatively spliced into three isoforms that differ in the length of the linker region between HIN200A and HIN200B. Using biotinylated A151 and C151 we were able to pull down IFI16 from THP1 lysates (Fig. 2.10c). Interestingly, while A151 associated with a larger, approximately 95kDa isoform of IFI16, C151 interacted more strongly with the smaller 85kDa isoform.

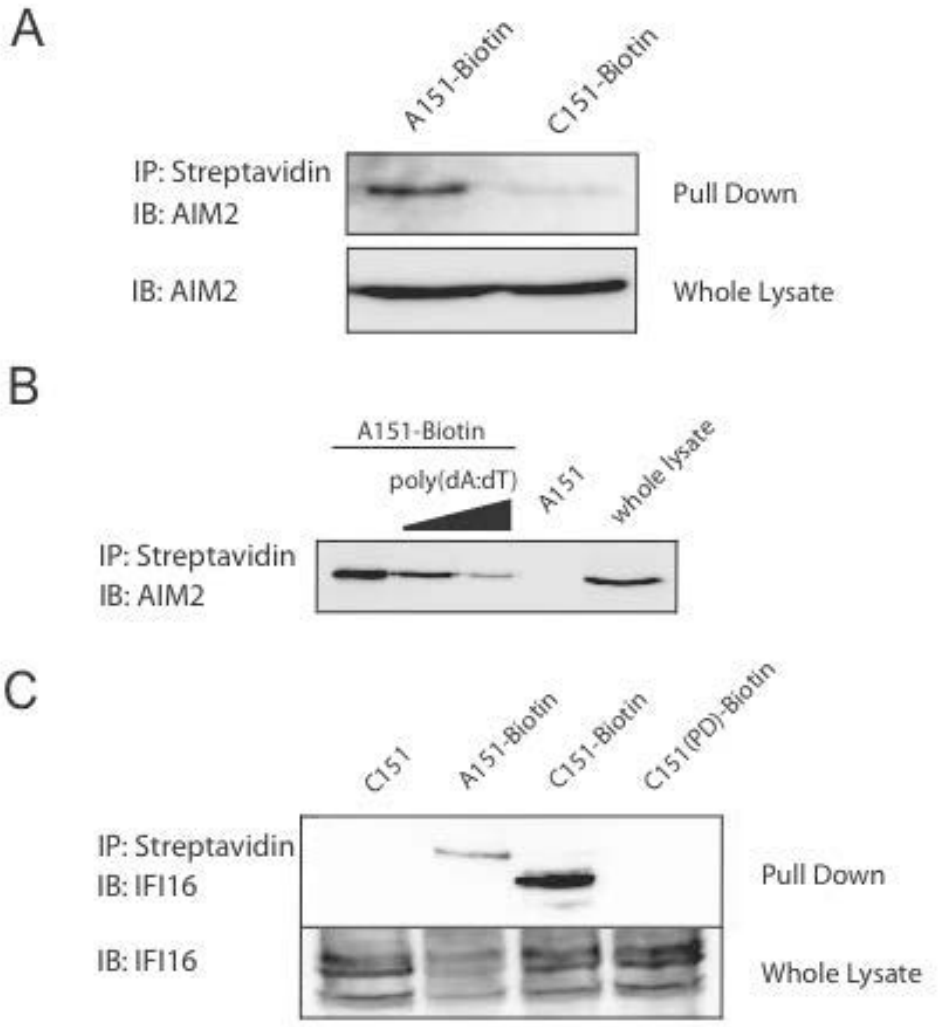


Figure 2.10 : Suppressive ODN interact with AIM2 and IFI16 A, Live immortalized macrophages were pretreated with biotinylated A151 or C151 (3 μM) and challenged with 0.5 ug/ml of poly(dA:dT) complexed with lipofectamine 2000 (untreated) for two hours. Lysates were divided in half, one half subjected to pull-down analysis using streptavidin-agarose beads and the other was run as whole lysate. Western blots were probed for the presence of AIM2. B, Immortalized macrophage lysates were subjected to pull-down analysis using A151 (6 μg) with (lane 1) or without (lane 4) 3'-biotinylation. An increasing amount of poly(dA:dT) was included in lanes 2 (6 μg) and 3 (18 μg) and whole lysate was run in lane 5. Western blots were probed for the presence of AIM2. E, Live THP-1 cells were pretreated with A151 or 3'-biotinylated A151, C151 or A151 (PD) (3 μM) and challenged with 0.5 μg/ml of poly(dA:dT) complexed with lipofectamine 2000 for two hours. Lysates were divided in half, one half subjected to pull-down analysis using streptavidin-agarose beads and the other run as whole lysate. Western blots were probed for the presence of IFI16.

Discussion

Numerous studies have examined the inhibition of TLR9 activation by sup ODN. However, until now no studies have explored the effects of sup ODN on cytosolic sensing pathways. This work identifies DNA species capable of broadly inhibiting cytosolic dsDNA responses and explores the role of DNA backbone chemistry and sequence in mediating suppression. Our data show that A151 added to the media of primary murine dendritic cells and macrophages prevents DNA induced IRF3- and NF-κB-dependent gene induction in response to cytosolic dsDNA as well as infection with HSV-1 and MCMV. As described previously, A151 did not inhibit LPS-driven cytokine production (153), nor did it affect IFN-β induction by Sendai virus, a member of the paramyxovirus subfamily that activates the RIG-I pathway. Notably, Nanostring analysis revealed A151 treatment had little effect on the expression profile of resting cells nor did it increase expression of anti-inflammatory mediators following stimulation. This suggests that it does not induce an anti-inflammatory state but rather blocks activation of dsDNA sensing pathways (Fig. 2.11).

A151 proved to be effective inhibitor of AIM2 inflammasome signaling as well. Treatment with A151 prevented AIM2-mediated caspase-1 activation, IL-1β, IL-18 and HMGB1 secretion and pyroptotic cell death in response to dsDNA challenge. Previously, Sato *et al*. uncovered a role for A151 in the inhibition of silica-induced inflammation (152). However, A151 had no effect on secretion of IL-1β or IL-18 in response to the NLRP3 ligands silica, nigericin, or ATP. Silica treatment leads to significant host cell death *in vitro* and we theorize that the inhibitory effects observed *in vivo* were due to

A151's effect on cytosolic sensing of host DNA released from dead and dying cells, rather than a direct effect on NLRP3 activation. In addition to A151's effects on AIM2 activation by poly(dA:dT), it inhibited IL-1β cleavage in BMDC challenged with the viral pathogen MCMV, which has been shown to be entirely dependent on AIM2 (16). Moreover, A151 partially blocked the response to the bacterial pathogen *L. monocytogenes*.

Dose-response experiments gave insight into the kinetics of inhibition and the role of sequence and backbone composition. Multiple studies by Pisetsky and collaborators using single base ODN constructs have demonstrated that nucleotide composition affects TLR9 suppression (140, 141, 198). Studies of A151 have found that its guanine residues are crucial for inhibition of TLR9, STAT1 and STAT4 signaling (149, 153, 154). Despite this, the role of sequence in A151-mediated inhibition is controversial. We believe that sequence-specific effects are often lost or minimized at higher concentrations, which has led to the misinterpretation that sequence is unimportant. For example, Trieu *et al.* reported TLR9 responses were inhibited by long ODN with PO backbones independent of sequence however they demonstrated differences in construct potency at concentrations below 1 μM (127).

Our experiments revealed that both A151 and C151 inhibit IFN-inducing pathways and AIM2 activation at higher concentrations. However, treatment of cells with lower concentrations revealed stark differences in construct potency. As we demonstrated, A151 proved to be 20-times more potent than C151 in blocking AIM2 activation in BMDC. In contrast, the C151 construct was approximately 10-times more potent than A151 at inhibiting IFN-induction in the same line. Differences in inhibitory

kinetics were also observed between BMDM and BMDC cell lines. Consistent with sup ODN effects on T LR9 activation, conversion of either construct to a PD backbone completely abolished inhibitory activity (127).

Mechanistically, A151 prevented ASC dimerization in macrophages and decreased the formation of cytoplasmic inflammasome specks in both AIM2- and ASC-citrine reporter lines (Fig. 2.11). Interestingly, in pull down experiments, A151 bound to AIM2 more readily than C151 suggesting a possible explanation for the observed differences in suppressive potency. In keeping with this theory, C151 pulled down more IFI16 than did A151. In addition, these experiments indicated that a PS backbone was essential for the interaction between IFI16 or AIM2 and sup ODN. Results were similar whether biotinylated sup ODN was added to the media or directly to lysates suggesting sequence affects affinity rather than cellular uptake. Moreover, the inclusion of increasing amounts of poly(dA:dT) with A151 during the binding step led to a proportional decrease in AIM2 recovery indicating an affinity-driven competition with this stimulatory ligand. Finally, in addition to pulling down cytosolic receptors, the downstream signaling molecule STING also associates with IFN-activating ODNs, such as the AT-rich motif isolated from the Plasmodium falciparum genome (224). However, neither A151 nor C151 pulled down this essential signaling adapter. Thus, we propose a mechanism whereby A151 and C151 compete with stimulatory dsDNA for binding to AIM2 and IFI16 but do not promote activation of downstream signaling events.

Interestingly, Unterholzner *et al.* observed the inhibitory effects of single-stranded phosphorothioate DNA on IFI16 while initially identifying it as an innate immune sensor (70). They found that both single-stranded and double-stranded forms of their VACV

70mer bound to IFI16 but that the single-stranded form acted as a competitive antagonist, inhibiting the dsVACV 70mer from inducing IFN-β. We have extended these observations to other members of the PYHIN family and begun to explore the effects of sequence on inhibitory potency. Ashman *et al*. had previously shown the 4348 construct to be a significantly more potent TLR9 inhibitor than A151's 'TTAGGG' motif. However 4348 and A151 inhibited poly(dA:dT) stimulation of IFN responses comparably and both were less potent than C151. Likewise, the class B stimulatory ODNs and A151 inhibited AIM2 activation similarly, while the class A stimulatory ODN had no effect. This suggests that while a PS backbone is essential for inhibition, total guanine content rather than a specific sequence may influence binding kinetics. Importantly the class B stimulatory constructs maintained their ability activate TLR9-mediate signaling. The inhibition observed was specific for AIM2-mediated inflammasome signaling in response to stimulation with poly(dA:dT).

PS modified constructs are quickly taken up by macrophages and dendritic cells via both scavenger receptor-mediated endocytosis and pinocytosis (138). Multiple wash steps following A151 or C151 pre-treatment had no effect on the construct's ability to inhibit IL-1β production. This suggests the transfection of poly(dA:dT) using lipofectamine did not influence the uptake of A151 and C151. Likewise inhibition was observed following MCMV or *Listeria* infection, stimuli that do not require lipofectamine transfection. Importantly, both the PS backbone and high guanosine content have been shown to increase construct aggregation. Aggregation of A151 could potentially lead to differences in cellular uptake, localization, and receptor avidity compared to C151; which might account for A151's more potent inhibitory effect on

AIM2. Further studies are needed to determine whether these constructs aggregate and how this interaction effects inhibition.

Recently, a crystal structure of the AIM2 HIN200C domain complexed to double-stranded DNA was reported (73). This study indicated that dsDNA recognition was accomplished largely through electrostatic interactions between the HIN domain and the DNA's sugar-phosphate backbone and was therefore independent of sequence. One explanation for the sequence dependence of single-stranded sup ODN is that a single-stranded DNA species may theoretically allow HIN200 residues greater access to its nucleotide bases than a double-stranded construct. Indeed, the interaction between dsDNA and AIM2 was reported to be highly flexible, allowing tilting and sliding (73). In addition to improved access of ssDNA, this inherent flexibility may allow additional residues to participate in the binding of ssDNA. Thus, while data suggests the PS backbone of sup ODN is essential for a strong interaction with members of the PYHIN family it is tempting to speculate that nucleotide residues affect minor contributions to the association thereby influencing relative affinity. In addition to describing the residues responsible for dsDNA binding, docking studies indicate that AIM2's PYD domain interacts with its HIN200 domain leading to autoinhibition at rest (73). This interaction is released upon binding of dsDNA to the HIN domain, freeing the PYD domain to recruit ASC. Studies defining the residues responsible for the interaction of A151 and AIM2 and how the interaction effects AIM2's conformation will help elucidate the mechanism by which A151 associates with, but does not activate AIM2, while dsDNA both associates and activates.

Alternative splicing of the spacer region between IFI16's HIN200 domains leads to the production of three isoforms (74). Interestingly, we found that A151 and C151 associated with different isoforms of IFI16. The functional significance of these isoforms remains unknown, however, it is conceivable that differences observed in inhibitory potency are the result of the selective interaction of A151 and C151 with different IFI16 isoforms. Further analysis, for example mass spectrometry of pulled down proteins, is needed confirm the specificity of this interaction.

Administration of A151 has been used in murine models of shock, lupus, inflammatory arthritis, and atherosclerosis. A growing body of evidence suggests cytoplasmic DNA sensing contributes to the pathogenesis of many of these same diseases. The identification of A151 as an inhibitor of the AIM2 and IFI16 signaling pathways adds to our understanding of how this sup ODN modulates the immune response. We theorize that the interaction of sup ODN with members of the PYHIN family may explain the robust and global anti-inflammatory effects observed in these disease models. These observations invite further investigation into the potential effects of sup ODN on other members of the PYHIN family as well as other cytosolic DNA sensors.

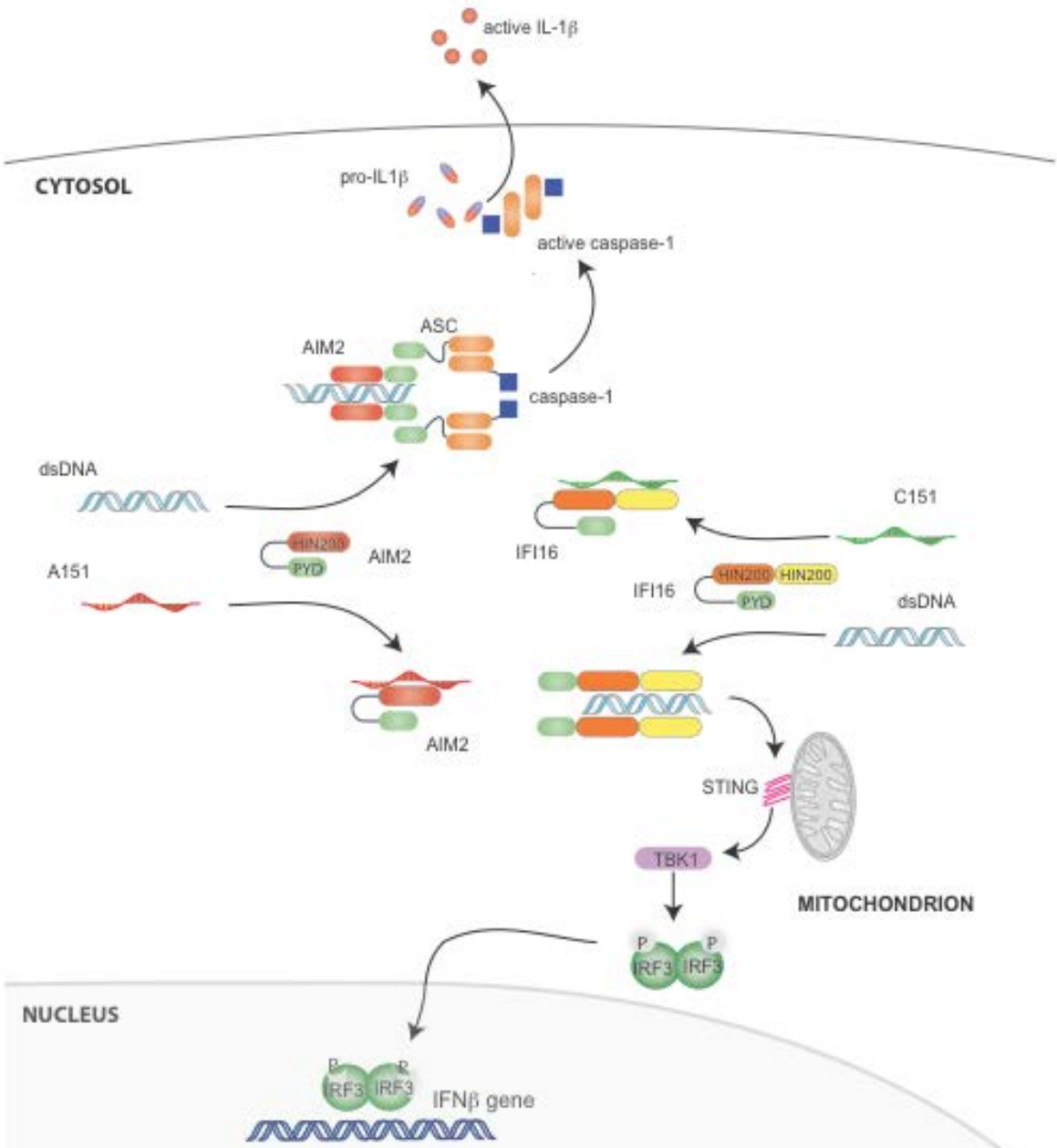


Figure 2.11: Schematic model of A151 and C151-mediated inhibition of cytoplasmic receptors AIM2 binds to dsDNA via its HIN200 domain which induces a conformational change. AIM2's PYD domain recruits ASC which in turn recruits pro-caspase-1. This close association of pro-caspase-1 molecules enables self-cleavage into active caspase-1. Active caspase-1 in turn cleaves pro-IL-1β and pro-IL18 into their active forms. The A151 construct bound to AIM2 but prevented recruitment of the downstream adaptor molecule ASC and subsequent inflammasome complex assembly. IFI16 binds to dsDNA and signals via STING which activates TBK1 leading to the phosphorylation of IRF3. Phosphorylated IRF3 translocates to the nucleus and activates transcription of Type I IFN and ISG. The C151 construct bound to IFI16 but prevented recruitment of STING and subsequent IFN induction

Preface to Chapter III

The chapters of this dissertation have appeared in the following publications/manuscripts:

John J. Kaminski, Stefan A. Schattgen, David M. Knipe and Katherine A. Fitzgerald. 2013. *Cell type-specific activation of the NLRP3 and NLRP12 inflammasomes in response to HSV-1*. Unsubmitted.

John Kaminski performed all experiments, Stefan Schattgen contributed to Fig 3.1

Chapter III

Cell type-specific activation of the NLRP3 and NLRP12 inflammasomes in response to HSV-1

Abstract

The innate immune system responds to HSV-1 infection by producing an array of cytokines, chemokines and type I interferons critical for controlling viral replication and coordinating an adaptive response. The cytokines IL-1β and IL-18 are important effector molecules in the innate response to HSV-1 *in vivo*. However, the pattern recognition receptors responsible for the production and maturation of these cytokines have not been fully defined. We show here that HSV-1 induces IL-1β transcription in dendritic cells and macrophages in a TLR2-MyD88-dependent manner. Following translation, IL-1β and IL-18 exist as inactive precursors that must be proteolytically cleaved by a multiprotein complex known as the 'inflammasome' to yield their active forms. Currently six receptors, NLRP1, NLRP3, NLRC4 (IPAF), NLRP12, AIM2 and IFI16 have been reported to assemble inflammasomes. In the present study, we found that the NLRP3 inflammasome is responsible for maturation of IL-1β in macrophages and dendritic cells in response to HSV-1 while NLRP12 regulates IL-1β production in neutrophils.

Introduction

Herpes simplex virus 1 (HSV-1) is a common and highly contagious viral infection that affects populations worldwide (199). The early innate immune response to HSV-1 infection is essential for controlling HSV-1 viral load and triggering an effective adaptive immune response (200). Innate immune cells detect infection through several classes of pattern recognition receptors (PRRs). A large number of PRRs have been implicated in the response to HSV-1 including TLR2, TLR3, TLR9, RIG-I, MDA-5, DAI, IFI16, DDX41, cGAS and NLRP3. Upon activation, these receptors initiate signal cascades that lead to the production of cytokines, chemokines and Type I interferons - effector molecules that recruit and activate leukocytes and establish an antiviral environment. Recent studies have identified IL-1β and IL-18 as key mediators of resistance to HSV-1 infection. Mice genetically deficient in IL-1β are unable to mount robust immune responses to HSV-1 infection which leads to viral dissemination and lethal encephalitis (177). IL-18 treatment has also been shown to reduce viral titers and increase survival in HSV-1 challenged mice (201). Moreover IL-18-deficient mice are more susceptible to lethal encephalitis likely due to an impaired NK cell response. Despite the importance of IL-1β and IL-18 in the response to HSV-1 infection, the PRRs and downstream signal pathways responsible for the production of these cytokines remain poorly defined.

IL-1β and IL-18 are secreted via an unconventional mechanism that differs from the classical vesicle-mediated pathway employed by many other effector molecules such as TNF-α (202). This nonclassical pathway involves the coordinated action of two PRR.

The first drives NF-κB-dependent transcription and translation of pro-IL-1β and pro-IL-18. Following synthesis, these immature interleukins, which lack leader sequences, are maintained in the cytosol (203). A second signal is then required to mediate assembly of a multi-protein complex termed the inflammasome (204). The inflammasome provides a platform for the activation of the zymogen caspase-1, an enzyme that, in its active form, cleaves pro-IL-1β and pro-IL-18 into mature cytokines (205, 206). Activation of inflammasome receptors results in either direct engagement of caspase-1 or recruitment through the adapter molecule apoptotic speck protein with CARD domain (ASC) that in turn recruits caspase-1. Thus, four members of the nucleotide-binding domain leucine-rich repeat (NLR) superfamily have been shown to assemble inflammasomes. These include NACHT, LRR and PYD domain-containing proteins 1, 3 and 12 (NLRP1, NLRP3 and NLRP12) and NACHT, LRR and CARD domain-containing protein 4 (NLRC4 or IPAF) (15, 95, 98-100, 110-117). NLRP3, the prototypical NLR, is primarily known to respond to crystalline/particulate substances such as silica, alum, asbestos and β-amyloid protein, but has recently been shown to be important in detecting influenza and adenovirus (100, 104). By comparison the precise function of NLRP12 remains controversial. Studies have ascribed seemingly conflicting roles to NLRP12 in the inflammatory response. Wang *et al*. found that NLRP12 can activate the transcription factor NF-κB and caspase-1-mediated IL-1β processing, thus acting in a pro-inflammatory capacity. Elsewhere, NLRP12 is described as antagonizing noncanonical NF-κB activity as well as TLR and TNFR signaling (120). NLRP12 is expressed at high basal levels in granulocytes (118). Recently NLRP12 was shown to assemble an inflammasome in response to *Yersinia pestis* (117).

In addition to members of the NLR family, our lab and others have recently reported the ability of the PYHIN protein absent in melanoma-2 (AIM2) to produce an inflammasome in response to bacterial and viral double-stranded DNA (16, 17). AIM2 dependent IL-18 secretion was found to be critical for controlling MCMV replication in mice through the activation of NK cell-dependent IFN-γ production. Another member of the PYHIN family, interferon gamma-inducible protein 16, (IFI16) has recently been shown to assemble an inflammasome in response to KSHV infection (82). Interestingly, while other inflammasome assembling receptors are localized to the cytosol, IFI16 is thought to bind stimulatory DNA in the nucleus and then relocate to the perinuclear region before assembling an inflammasome structure. However, this function remains controversial as other studies have found IFI16 heterdimerizes with NLRP3 and AIM2 to suppresses activation of the inflammasome (207). Thus the precise role of IFI16 remains to be determined.

As with many viruses, HSV-1 has evolved a number of strategies to evade the host innate immune response. Viral proteins such as ICP27 inhibit Type I IFN signaling while ICP34.5 stimulates dephosphorylation of eIF2α relieving PKR-mediated translational block (208, 209). One such anti-inflammatory protein, ICP0, an immediate-early HSV-1 gene, is a member of the RING finger family of proteins. It is responsible for transactivation of viral genes through ubiquitination of cellular proteins thereby targeting them for degradation (210). In this manner, it reduces the cell's antiviral response by dissolving nuclear promyelocytic leukemia protein (PML) bodies, degrading MyD88, and inhibiting the transcription factors IRF3 and IRF7 (169). While the wild-type KOS strain of HSV-1 was a poor inducer of pro-IL-1β, the 7134 strain of HSV-1,

deficient in Infected Cell Polypeptide 0 (ICP0) strongly induced expression. In order to visualize pro-IL-1β expression and maturation, we utilized this 7134 strain.

In the present study, Nanostring technology was used to characterize the basal expression levels of a panel of cytokines, chemokines and innate immune receptors in BMDC. The same panel was then used to examine mRNA induction in wild-type, STING- and MyD88-deficient cells following HSV-1 challenge. This analysis revealed that the MyD88 adapter was required for pro-IL-1β induction following HSV-1 challenge. In contrast IL-18 induction required contributions from both MyD88- and STING-mediated signaling. Using TLR2- and TLR9-deficient cells, we found the TLR2-MyD88 pathway was necessary for pro-IL-1β induction. We go on to show that the NLRP3 inflammasome is required for IL-1β production in macrophages and dendritic cells. In contrast, we found that neutrophils relied on the related NLRP12 protein to secrete mature IL-1β in response to HSV-1.

Results

Characterization of the innate immune response to HSV-1 in dendritic cells

TLR2, TLR3 and TLR9 have all been implicated in the innate response to HSV-1. Additionally, a growing list of cytoplasmic receptors, have been shown to mediate cytokine and Type I IFN production. Evidence suggests that certain receptors, such as TLR9, may be important for pro-inflammatory responses in specific cell subtypes such as plasmacytoid dendritic cells while TLR2 and various cytoplasmic receptors play a significant role in conventional dendritic cells and macrophages (178, 211). To define

the relative contributions of these receptors to the inflammatory response, we harvested bone marrow from wild-type and mice deficient in MyD88 and STING, two crucial signaling components downstream of TLRs and cytosolic receptors. Using BMDC, we compared the mRNA levels of a panel of Type I IFN, cytokines, chemokines, and PRRs before and after HSV-1 infection by multiplex gene expression analysis using nCounter (Nanostring) technology (Fig. 3.1). As we had observed previously, resting BMDC expressed particularly high mRNA levels of TLR2 and TLR4. Other receptors such as TLR1, TLR9, TLR3, MDA-5, NLRP3, AIM2 and IFI16 were expressed at lower levels basally but were induced following HSV-1 infection (Fig. 3.1 and Table 3.1). Studies have found that TLR signaling not only primes, but also 'licenses' the inflammasome by enhancing expression of inflammasome assembling receptors such as NLRP3 (91). Indeed, NLRP3 expression was increased nearly 20-fold by HSV-1 challenge and this induction was MyD88-dependent. Interestingly, our analysis revealed important differences in the contributions of MyD88 and STING signal pathways to Type I IFN and cytokine responses. In wild-type BMDC, IFN-β and IFN-α were strongly induced by HSV-1 infection. However, this Type I IFN response was completely lost in STING-deficient cells, while MyD88 deficiency had no effect (Fig 3.1 and Table 3.2). Not surprisingly, STING-deficient cells also demonstrated impaired induction of various IFN-stimulated genes including IFI204, IFI205, AIM2, LGP2, MDA-5, RIG-I, IRF7, TREX1 and viperin. Interestingly, STING was also important for the induction of caspase-1, NLRP12 and NLRP6 following HSV-1 infection. In contrast, MyD88 proved to be more important for the induction of cytokines including TNF-α, pro-IL-1β, IL-1α and the IL-

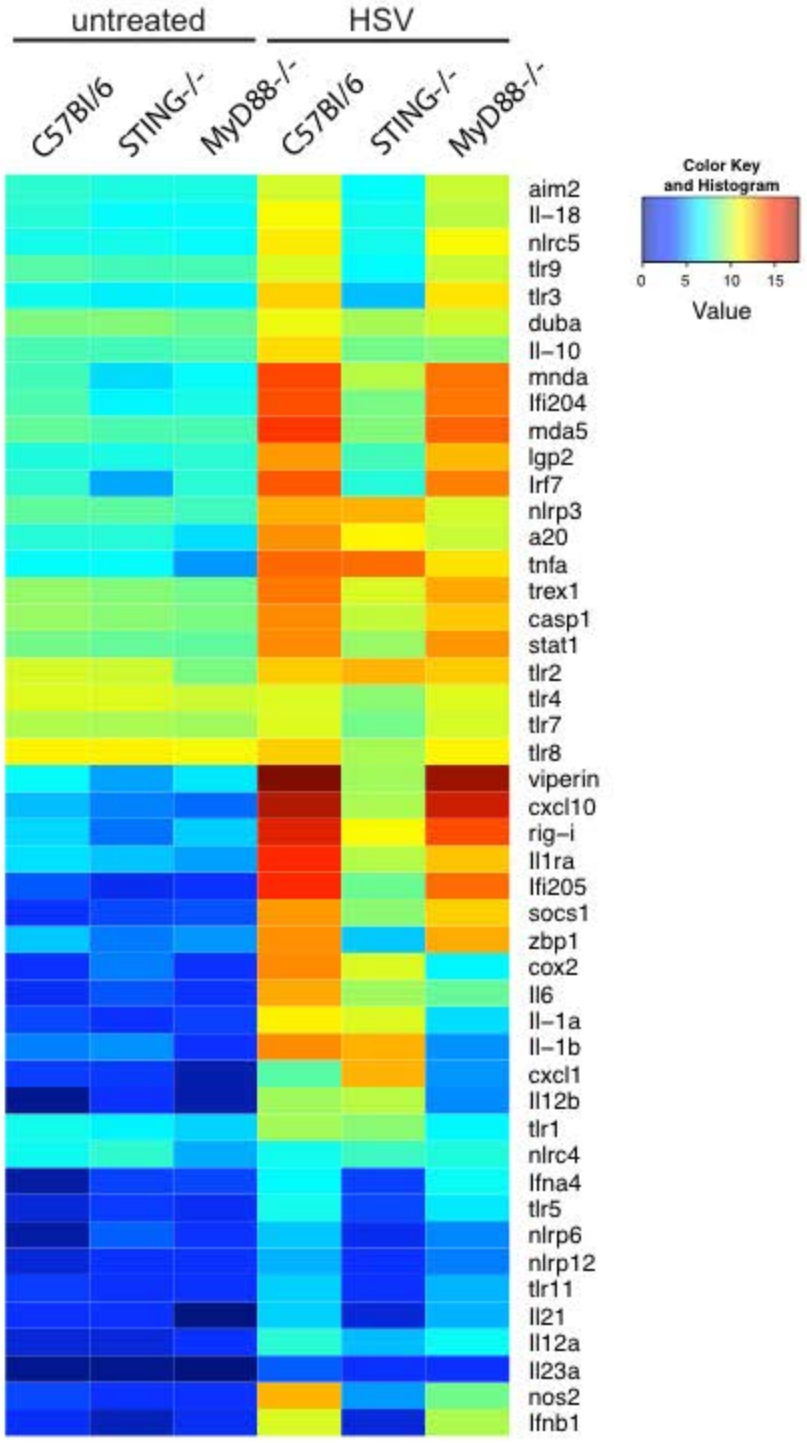


Figure 3.1: Type I IFN, ISG and cytokine responses to HSV-1 depend on STING and MyD88 signaling Mouse BMDCs from C57Bl/6, STING- and MyD88-deficient mice, were left untreated or stimulated with HSV (MOI=10) for 4 hrs. RNA was extracted and subjected to nCounter Nanostring analysis. Gene expression profiles are displayed as a heat map (log10 transformed).

Table 3.1 : Nanostring Normalized mRNA Counts

	Stimulation					
	Media			HSV		
Gene	C57Bl/6	STING-/-	MyD88-/-	C57Bl/6	STING-/-	MyD88-/-
Cyokines, chemokines and their receptors						
Ifna4	1	10	11	101	10	109
Ifnb1	4	2	4	1390	3	830
Il-1a	11	7	10	2541	1459	69
Il-1b	22	28	5	8697	5458	27
Il1ra	71	51	32	38844	904	4305
Il6	4	13	8	5981	705	341
Il-10	247	227	271	3221	406	507
Il12a	3	3	7	164	46	112
Il12b	1	6	2	700	930	25
Il-18	163	104	107	2045	126	976
Il21	8	6	1	59	3	40
Il23a	1	1	1	14	6	8
cxcl1	9	9	2	288	5330	28
cxcl10	46	22	17	117437	804	84494
tnfa	104	103	30	12913	12145	3004
PRRs, downstream signaling components						
aim2	175	141	137	1319	106	1161
Ifi204	254	92	129	17878	437	11149
Ifi205	14	4	6	33916	374	12545
Irf7	175	35	164	16182	154	9994
lgp2	145	133	172	7205	226	4898
mda5	335	252	244	24102	485	13827
mnda	226	67	105	19925	922	11583
nlrc4	115	176	38	119	204	146
nlrc5	126	125	103	2766	123	2001
nlrp12	3	6	7	40	7	21
nlrp3	320	310	220	5561	5342	1282
nlrp6	1	15	8	51	4	24
rig-i	64	18	56	67293	2119	18620
tlr1	127	89	60	744	540	93
tlr11	9	8	5	59	7	42
tlr2	1349	1212	441	3918	5242	3953
tlr3	118	86	89	3794	47	2949
tlr4	1510	1499	1199	1446	542	1492
tlr5	3	9	4	125	11	78
tlr7	863	796	724	1469	408	1332
tlr8	2497	2535	1976	3779	781	2439
tlr9	286	225	238	1469	99	1176
casp1	632	532	440	8608	1061	4127
viperin	106	33	78	218056	710	153290
zbp1	52	20	29	8052	53	5879
Transcription factors						
stat1	411	346	323	8568	632	7554

	Media			HSV		
	C57Bl/6	STING-/-	MyD88-/-	C57Bl/6	STING-/-	MyD88-/-
Proinflammatory enzymes						
cox2	7	21	5	8763	1396	91
trex1	612	503	407	11055	1383	6069
nos2	11	7	5	5164	31	396
Anti-inflammatory signaling components						
socs1	8	11	12	7215	543	3790
duba	469	493	359	1772	781	1201
a20	159	157	69	8020	2384	1159

Table 3.2 : Nanostring mRNA Fold Induction Compared to Media

	HSV infected / Media		
Gene	C567Bl/6	STING-/-	MyD88-/-
cxcl10	2546.7	36.2	4979.7
Ifi205	2500.7	101.1	2033.2
viperin	2061.2	21.3	1967.8
Il6	1469.9	54.5	40.2
cox2	1292.3	65.7	16.9
rig-i	1055.7	114.6	335.3
socs1	886.7	49.0	307.1
Il12b	700.3	167.6	16.2
Il1ra	550.8	17.8	132.9
nos2	475.9	4.2	85.7
Il-1b	400.8	196.7	5.9
Ifnb1	341.6	1.5	215.2
Il-1a	234.2	197.2	6.9
zbp1	156.2	2.6	200.6
tnfa	123.6	118.3	99.9
Irf7	92.5	4.4	60.8
mnda	88.0	13.8	110.4
Ifna4	74.3	1.0	10.1
mda5	71.9	1.9	56.6
Ifi204	70.5	4.8	86.6
Il12a	60.5	16.7	16.1
a20	50.5	15.2	16.7
Igp2	49.6	1.7	28.5
tlr5	46.3	1.2	20.3
nlrp6	37.7	0.2	2.8
tlr3	32.2	0.5	33.2
cxcl1	30.4	576.4	18.5
nlrc5	21.9	1.0	19.4
stat1	20.8	1.8	23.4
trex1	18.1	2.7	14.9
nlrp3	17.4	17.2	5.8
nlrp12	14.9	1.1	3.1
Il23a	13.9	6.4	10.8
casp1	13.6	2.0	9.4
Il-10	13.0	1.8	1.9
Il-18	12.6	1.2	9.1
aim2	7.5	0.8	8.5
Il21	7.2	0.5	52.3
tlr11	6.2	0.9	9.0
tlr1	5.8	6.1	1.5
tlr9	5.1	0.4	5.0
duba	3.8	1.6	3.3
tlr2	2.9	4.3	9.0
tlr7	1.7	0.5	1.8
tlr8	1.5	0.3	1.2
nlrc4	1.0	1.2	3.9
tlr4	1.0	0.4	1.2

12 p40 subunit (IL-12B) (Fig. 3.1 and Table 3.2). In addition, maximal induction of cytokines such as IL-6, pro-IL-18 and IL-10 required both signal pathways to be intact. Thus, HSV-1 infection activates both TLR-MyD88 and cytosolic receptor-STING pathways that function both independently and synergistically.

HSV-1 induces transcription of pro-IL-1β in a TLR2-MyD88-dependent manner

Having broadly defined the inflammatory pathways activated by HSV-1, we focused our investigation on the mechanism of pro-IL-1β induction. We began by comparing the ability of the wild-type KOS HSV-1 strain and the 7134 strain to drive pro-IL-1β transcription. In keeping with ICP0's role as an inhibitor of the inflammatory response, the wild-type KOS strain induced very little pro-IL-1β while infection with the ICP0 knockout strain, 7134 led to a 200-fold increase in pro-IL-1β mRNA in BMDM (Fig. 3.2a). Treatment of the 7134 strain with UV-irradiation further increased the pro-IL-1β mRNA levels, indicating that replication was not required for pro-IL-1β production. We next examined the expression of pro-IL-1β in BMDM and BMDC deficient in MyD88. Induction of pro-IL-1β by HSV-1 and LPS was abrogated in MyD88 knockout lines (Fig 3.2b,c). This confirmed our Nanostring analysis, indicating that TLR recognition of HSV-1 was responsible for pro-IL-1β induction. The MyD88 adapter molecule is important for TLR2 and TLR9 signaling, but unnecessary for TLR3-driven responses. To determine if TLR2 or TLR9 were responsible for pro-IL-1β induction, the response of BMDMs and BMDCs derived from TLR2-, TLR9- and TLR2/9-deficient mice was examined. Transcription of pro-IL-1β was completely abrogated in TLR2 and TLR2/9, but not TLR9 knockouts (Fig. 3.2d,e). Interestingly,

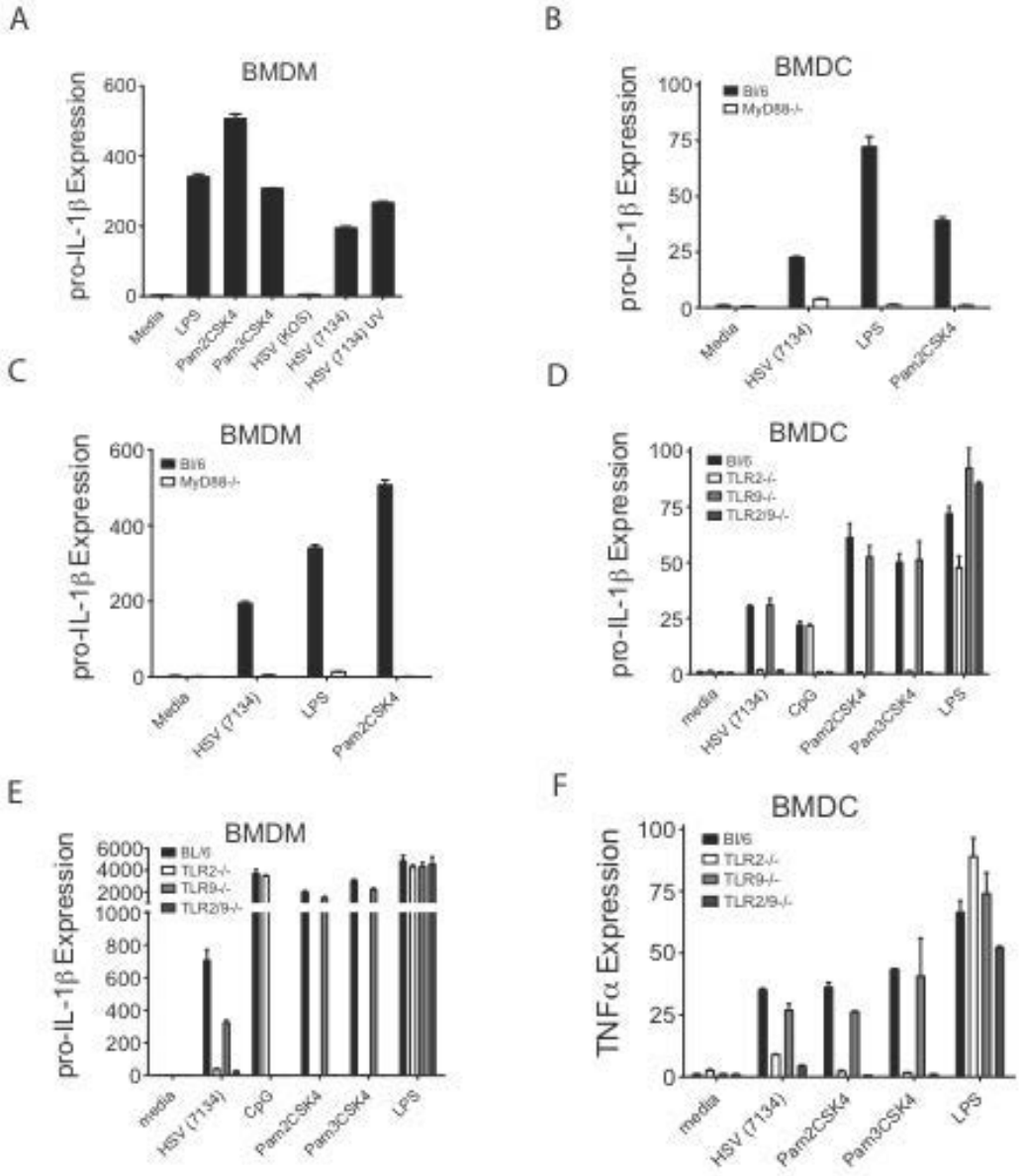


Figure 3.2: HSV-1 induces transcription of pro-IL-1β in a TLR2-MyD88 dependent manner
A, BMDM were stimulated with LPS (200 ng/ml), Pam2CSK4 (1.5 μg/ml), Pam3CSK4 (1.5 μg/ml), HSV-1 (KOS MOI=10), HSV-1 (7134 MOI=10), UV-inactivated HSV (7134 MOI=10) for 4 hrs. Pro-IL-1β and β-actin mRNA were measured by qPCR. Pro-IL-1β induction is represented relative to untreated controls. B, BMDC and C, BMDM derived from C57Bl/6 mice were treated as above and pro-IL-1β and β-actin mRNA were measured by qPCR. D, BMDC and E, BMDM derived from C57Bl/6, TLR2-, TLR9- and TLR2/9-deficient mice were treated as above and pro-IL-1β and β-actin mRNA were measured by qPCR. F, BMDC derived from C57Bl/6, TLR2-, TLR9- and TLR2/9-deficient mice were treated as above and TNFα and β-actin mRNA were measured by qPCR. TNFα induction is represented relative to untreated controls.

augmenting the TLR2-driven response in macrophages. We also examined TNF-α loss of TLR9 in BMDMs led to a partial reduction in IL-1β transcription. Nevertheless, induction remained entirely dependent on TLR2, suggesting a limited role for TLR9 in expression in TLR2, TLR9 and TLR2/9 knockouts and found that like pro-IL-1β, induction was dependent on TLR2 (Fig 3.2f). These findings demonstrate TLR2's central role in dendritic cell and macrophage-dependent cytokine and chemokine responses to HSV-1 (178). Thus the TLR2-MyD88 signaling pathway is necessary for HSV-1-driven 'priming' of the inflammasome in dendritic cells and macrophages through the production of pro-IL-1β and 'licensing' via the upregulation of inflammasome receptors.

HSV-1 induces IL-1β secretion in PEC, BMDM and BMDC

Secretion of mature IL-1β depends on both transcription and translation of the pro-forms of this cytokine as well as activation via inflammasome signaling. Although a number of studies have demonstrated that IL-1β and IL-18 are important for the anti-viral responses *in vivo*, how HSV-1 activates the inflammasome is not well understood (88, 177). An early study found the McIntyre strain of HSV-1 could induce caspase-1 activation and cleavage of IL-1β in THP-1 cells but failed to implicate a specific receptor in inflammasome assembly (100). A recent report by Johnson *et al*. suggested HSV-1 infection induces the transient recruitment of NLRP3 and IFI16 to inflammasome complexes (181). Although the authors were able to visualize IL-1β cleavage in the cellular lysates following HSV-1 infection they could not detect it in the supernatants and suggested that HSV-1 is able to block IL-1β secretion through multiple mechanisms

(181). Other studies using human macrophages have also failed to detect IL-1β secretion following HSV-1 challenge (212). Using an LPS prime prior to HSV-1 challenge enable detection of IL-1β secretion into the supernatant (Fig 3.3a,b). Both the wild-type KOS HSV-1 strain and the 7134 strain were able to induce IL-1β secretion, however the 7134 strain produced approximately twice as much (Fig. 3.3a). HSV-1 infection, in general, did not induce high levels of IL-1β secretion compared to ATP or poly(dA:dT) controls; therefore, we chose to use the 7134 s train moving forward in order to compare differences in IL-1β production in inflammasome-deficient cell lines. Levels of IL-1β secretion increased as the 7134 HSV MOI was raised (Fig. 3.3b). In addition, maturation of IL-1β was blocked by the chemical caspase-1 inhibitor z-VAD-FMK, suggesting secretion was indeed caspase-dependent (Fig 3.3c).

IL-1β maturation is mediated by NLRP3 in dendritic cells and macrophages

To date, two PRRs have been shown to assemble inflammasomes and mediate IL-1β and IL-18 cleavage in response to viral infection. T hese include NLRP3, that has been shown to respond to influenza A virus and adenovirus, and AIM2, that responds to murine cytomegalovirus and vaccinia virus. R athinam *et al*. previously demonstrated that AIM2 is not involved in inflammasome assembly in response to HSV-1 (16). To evaluate the contribution of NLRs to inflammasome activation, BMDMs and BMDCs derived from NLRP3, NLRP12, ASC and capase-1-deficient mice were primed with LPS then challenged with the 7134 s train of HSV-1. We found that while wild-type macrophages and DCs were able to secrete IL-1β in response to HSV-1, those lacking NLRP3, ASC and caspase-1 were unable to respond (Figure 3.3d,e). Importantly,

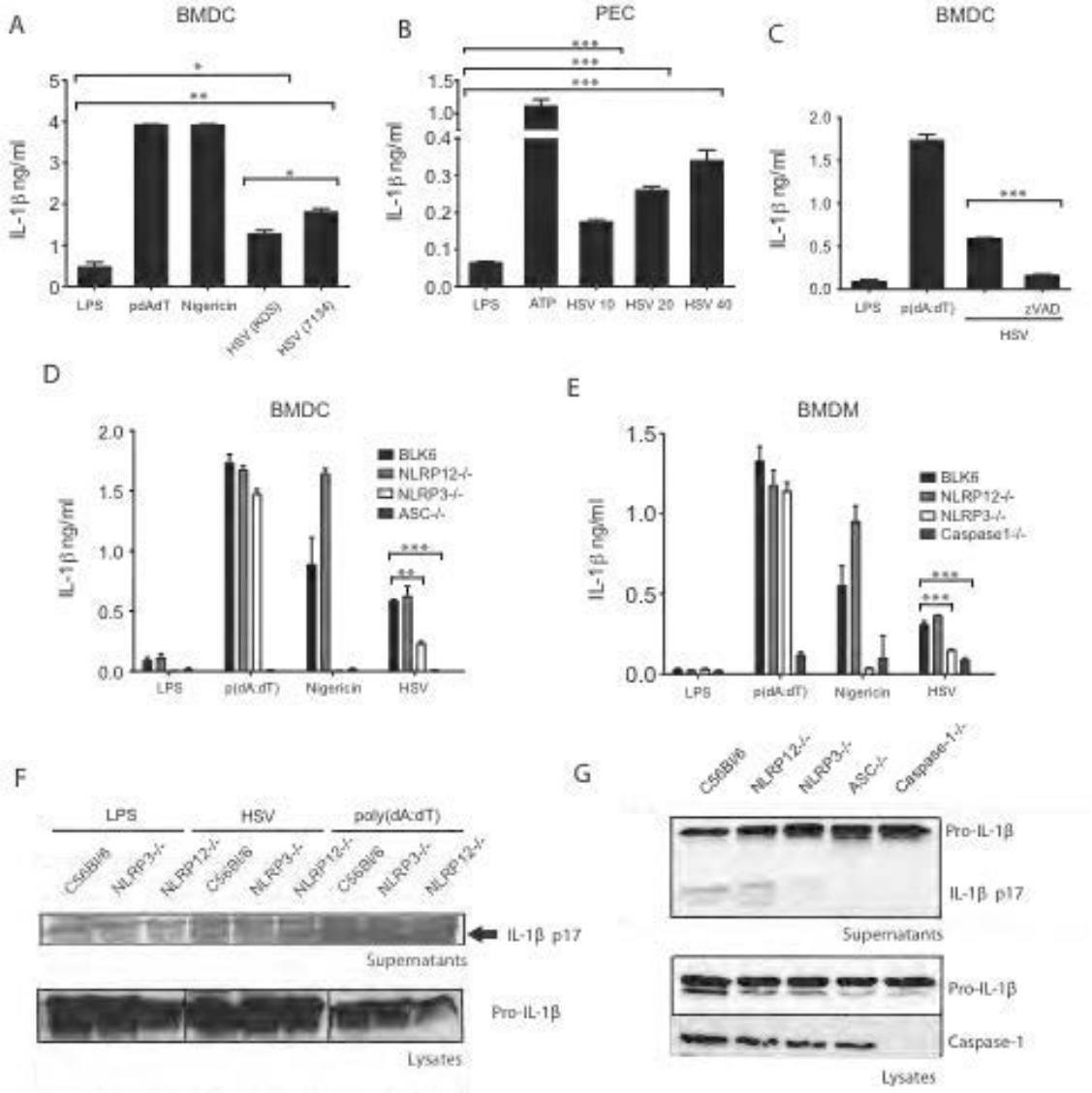


Figure 3.3: HSV-1 induces IL-1β secretion in an NLRP3-dependent manner in PEC, BMDM and BMDC
A, BMDC were primed with LPS (200 ng/ml) for 3 hrs followed by stimulation with poly(dA:dT) complexed with lipofectamine 2000 for 6 hrs, nigericin (10 μM) for 1hr, HSV-1 (KOS MOI=10), HSV-1 (7134 MOI=10) for 16 hrs and IL-1β secretion into the supernatant was measured by ELISA. B, PEC were primed with LPS and challenged with ATP (5 mM), HSV-1 (7134 at increasing MOI=10, 20 or 40) and IL-1β was measured by ELISA. C, BMDCs were primed with LPS and challenged with poly(dA:dT) or HSV-1 (7134 MOI=10) alone or in the presence of zVAD-FMK (10 μM) and IL-1β was measured by ELISA. D, BMDC and E, BMDM were derived from C57Bl/6, NLRP12, NLRP3, ASC or caspase-1-deficient mice were treated as above and IL-1β was measured by ELISA. Data are presented as mean ± SD from three biological replicates representative of three experiments. * $p<0.05$,** $p<0.001$, *** $p<0.0001$ F, BMDC derived from C57Bl/6, NLRP12, NLRP3-deficient mice were primed with LPS followed by stimulation with poly(dA:dT) or HSV-1 (7134 MOI=10). Lysates and Supernatants were immunoblotted for mature IL-1β. F, BMDC from C57Bl/6, NLRP12, NLRP3, ASC and caspase-1-deficient mice were primed and treated with HSV-1 (7134 MOI=10) for 16hrs. Lysates and extracted supernatants were immunoblotted for IL-1β and caspase-1.

NLRP3-deficient cells were still able to respond normally to poly(dA:dT), a synthetic DNA species that activates AIM2 (Fig. 3.3f). To confirm this finding, protein was extracted from the supernatants and IL-1β was visualized by Western blot (Fig. 3.3f,g). In keeping with our ELISA results, NLRP3-deficient BMDC demonstrated reduced production of mature IL-1β in response to HSV-1. In contrast, NLRP12-deficient BMDCs showed no defect in IL-1β production (Fig. 3.3g).

IL-1β maturation is mediated by NLRP12 in neutrophils

During early stages of HSV-1 infection, neutrophils infiltrate areas of viral replication in large numbers (213). To examine the role of these PRRs in neutrophils, we elicited a neutrophil-enriched peritoneal exudate. Although neutrophil migration in NLRP12-deficient mice was found to be impaired in a murine model of contact hypersensitivity (122), no defect in neutrophil recruitment to the peritoneum was observed following thioglycollate injection. Compared to dendritic cells and macrophages, neutrophils express high basal levels of NLRP12 that is increased following stimulation with HSV-1 (Fig 3.4a). In contrast to the macrophage and dendritic lines tested, neutrophils relied on NLRP12 to secrete IL-1β in response to HSV-1 (Fig. 3.4b). Unlike IL-1β, secretion of TNF-α was not reduced by the loss of NRLP12, indicating a specific deficit in inflammasome function rather than a general loss of responsiveness (Fig. 3.4c). These results were confirmed by Western blot (Fig. 3.3c). The smear observed in the media control, ASC-deficient lane is an artifact not observed in subsequent Western blots. Unlike other cell types tested, thioglycollate-elicited neutrophils did not require priming with LPS. Exposure to HSV-1 alone was sufficient to

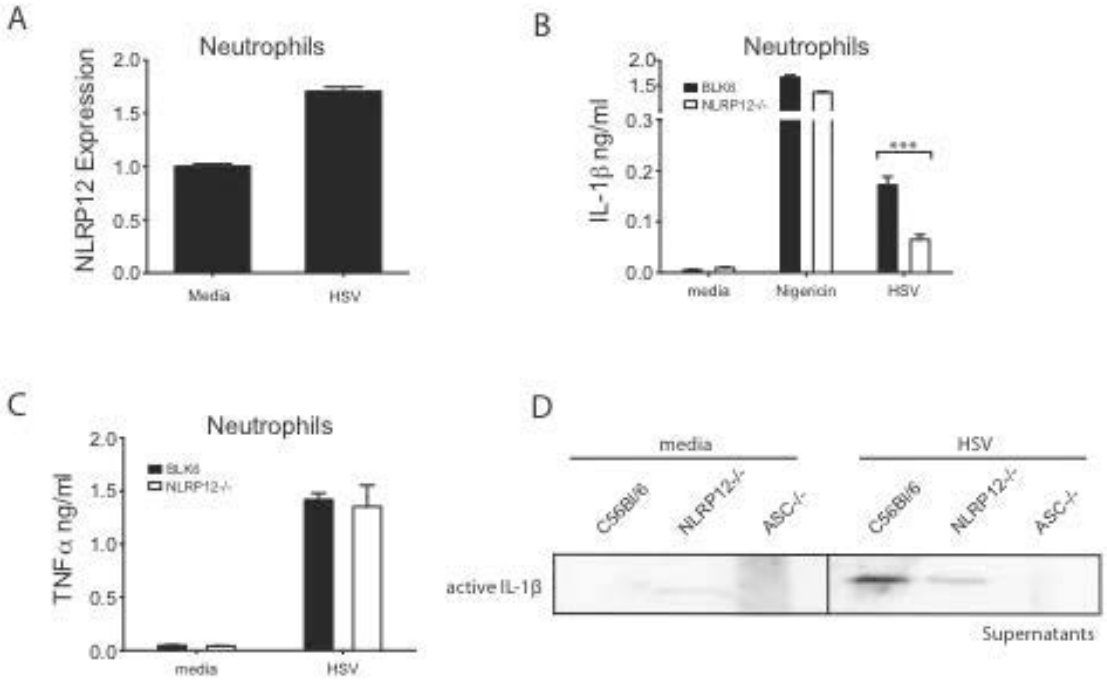


Figure 3.4: HSV-1 induces IL-1β secretion in an NLRP12-dependent manner in neutrophils
A, Neutrophil-enriched peritoneal cells were stimulated with HSV-1 (7134 MOI=10) for 4 hrs and NLRP12 and β-actin mRNA were measured by qPCR. NLRP12 induction is represented relative to media control. B, Neutrophil-enriched peritoneal cells were extracted from C57Bl/6 and NLRP12-deficient mice, stimulated with nigericin (10 μM) for 1hr or HSV-1 (7134 MOI=10) for 6 hrs. (B) IL-1β or (C) TNF secretion into the supernatant was measured by ELISA. D, Neutrophil-enriched peritoneal cells derived from C57BL/6, NLRP12 or ASC-deficient mice were treated with HSV-1 (7134 MOI=10) for 6 hrs. Protein was extracted from supernatants and immunoblotted for mature IL-1β.

induce IL-1β secretion. IL-1β release was also abolished in ASC-deficient mice, suggesting that NLRP12 utilizes ASC to assemble an inflammasome (Fig 3.4d) (214).

Discussion

The cytokines IL-1β and IL-18 play a crucial role in the immune response to HSV-1. Mice genetically deficient in IL-1β fail to mount a robust immune response to HSV-1, which leads to increased viral load, dissemination and death (177). Similarly, genetic deficiency in IL-18 increases susceptibility to HSV-1 infection while administration of IL-18 is protective (88, 201). In this study, we characterized the receptors and pathways responsible for the induction and maturation of IL-1β and IL-18 in dendritic cells, macrophages and neutrophils in response to HSV-1. We began by examining the basal expression levels of cytokines, chemokines and PRRs and their induction following HSV-1 infection. The receptors expressed at rest may be of particular importance during HSV-1 infection as IL-1β production *in vitro* has been reported to be early and brief, perhaps due to subversion of cellular machinery at later time points (181, 215). We found BMDC expressed high basal levels of TLR2 and NLRP3 mRNA, which was further increased following HSV-1 infection. Dendritic cells and macrophages employed the TLR2-MyD88 pathway to drive expression of pro-IL-1β following HSV-1 infection. While expression of pro-IL-1β was negligible following challenge with the wild-type KOS HSV-1 strain, it was robustly induced by the ICP0-deficient 7134 strain. This finding agrees with a previous report demonstrating that ICP0 inhibits TLR2-mediated inflammatory cytokine production (168). Interestingly, in

contrast to pro-IL-1β, expression of pro-IL-18 was dependent on both MyD88 and STING dependent pathways.

Although a number of studies have failed to detect IL-1β secretion by macrophages or fibroblasts infected with HSV-1, we found that with prior LPS stimulation, HSV-1 challenge generated sufficient IL-1β production by cultured macrophages and DCs to allow detection in cellular supernatants (181, 212). Moreover, secretion was dependent on c aspase activity as it was blocked by the chemical pan-caspase inhibitor z-VAD-FMK. Secretion of cleaved IL-1β into supernatant was evident by Western blot. However, the secretion of active caspase-1 subunits was not observed. In a recent study, NLRP3 and IFI16 were found to associate with ASC 4 hours after HSV-1 infection (181). However, at later time points, this association was lost. The authors found that HSV-1 induced the degradation of IFI16 in an ICP0-dependent manner. By comparison, NLRP3 was not degraded, but after early time points no longer colocalized with caspase-1 (181). Thus, the authors hypothesized that HSV-1 had evolved mechanisms to degrade IFI16 and sequester caspase-1 in actin clusters in order to inhibit inflammasome activation. This may explain why they were unable to detect secretion of active IL-1β into the supernatants (181). Importantly, our LPS priming strategy induced high levels of pro-IL-1β expression allowing the visualization of IL-1β secretion in the supernatants. However, sequestration of caspase-1 may account for our inability to detect secretion of the active caspase-1 subunits.

Our findings suggest HSV-1 induces secretion of active IL-1β through activation of the NLRP3 inflammasome receptor in dendritic cells and macrophages. Loss of NLRP3, the adapter ASC, or capase-1 led to a reduced IL-1β response. Notably,

NLRP3-deficient BMDC and BMDC still produced small amounts of IL-1β in response to HSV-1 perhaps indicating other receptors such as IFI16 may contribute to IL-1β production as was suggested by Johnson et al (181). Whether IFI16 is a bona fide inflammasome assembling receptor is still under debate. Evidence for this function is often circumstantial and based on colocalization of IFI16 with ASC, an interaction which others have suggested, in fact, interferes with the activity of NLRP3 and AIM2 inflammasomes (82, 207). In addition, IFI16 has been shown to be important for Type I IFN induction in response to HSV-1 and treatment with IFN strongly augments the cytokine response to HSV-1 (70, 216). Thus IFI16 may potentiate cytokine responses indirectly by inducing IFN rather than by direct inflammasome assembly.

In contrast to dendritic cells and macrophages, IL-1β secretion in neutrophils was dependent on NLRP12. Neutrophils are the first infiltrating leukocytes observed at sites of HSV-1 infection. Although neutrophils are historically thought of as anti-bacterial, recent evidence suggests they play an important role in the antiviral response (217, 218). Vladimer *et al*. recently reported a role for the NLRP12 inflammasome in recognizing the bacterial pathogen *Yersinia pestis* (117). In this study, NLRP12 was important for IL-1β and IL-18 release from neutrophils and macrophages. Moreover, the IL-18 produced by NLRP12 stimulated IFN-γ production that proved to be essential for limiting *Y. Pestis* infection. We found NLRP12 mRNA expression was low in resting BMDC, but could be induced following HSV-1 stimulation. However, NLRP3 was constitutively expressed and, following HSV-1 challenge, induced to levels more than a hundred-fold higher than NLRP12. By comparison we found NLRP12 expression was high basally in neutrophils. While both NLRP3 and NLRP12 can be activated by HSV-1, the kinetics of IL-1β

secretion are known to be rapid and brief and evidence suggests HSV-1 has mechanisms for degrading receptors and sequestering caspase-1 (181). Thus, inflammasome formation may rely heavily on whichever receptor is constitutively expressed at the time of HSV-1 infection. Importantly, the neutrophil-rich peritoneal exudate utilized in these experiments did contain small numbers of other inflammatory cells including macrophages and B cells. It is possible these resident and infiltrating inflammatory cells contributed to the observed phenotype. Additional experiments using negative selection to further purify the neutrophils are necessary to confirm the specific role of NLRP12 in inflammasome formation in neutrophils.

Previous studies have shown that IL-1β and IL-18 are important in the response to HSV-1. This dissertation demonstrates that the TLR2-MyD88 signaling pathway is responsible for IL-1β expression and that MyD88 and STING act cooperatively to induce IL-18. Johnson *et al.* observed the association of NLRP3 and ASC and the cytoplasmic accumulation of cleaved IL-1β following HSV-1 infection. I showed herein that LPS priming of cells prior to HSV-1 challenge results in secretion of mature IL-1β into the supernatant. M oreover, I demonstrated that NLRP3-deficient macrophages and DCs secrete significantly lower amounts of IL-1β. In addition, I have provided evidence that NLRP12 is important for IL-1β secretion in neutrophils. The inhibitory effects of the ICP0 protein on IL-1β secretion and our inability to observe caspase-1 secretion into the supernatants indicate that HSV-1 has evolved multiple strategies to curtail IL-1β production. These studies provide a methodology for further investigation into inflammasome signaling in response to HSV-1, as well as the subversion of this pathway.

Chapter IV Materials and Methods

Reagents and Plasmid Constructs

ATP, LPS, nigericin and poly(dA:dT) were from Sigma-Aldrich (St. Louis, MO). A151 (5'-TTAGGGTTAGGGTTAGGGTTAGGG-3'), C151 (5'-TTCAAATTCAAATTCAAATTCAAA-3'), 4348 (5'-TCGTATCCTGGAGGGGAAG-3'), 1826 (5'-TCCATGACGTTCCTGACGTT-3'), 10104 (5'-TCGTCGTTTCGTCGTTTTGTCGTT-3'), 2336 (5'-GGGgacgacgtccgtGGGGGG-3') constructs were synthesized by IDT technologies (Coralville, IA) (219-221). Residues with phosphorothioate linkages are capitalized. A 3'-biotin tag was added to the sup ODN sequence for pulldowns. mCMV (Smith strain) was a gift from R. Welsh (UMASS Medical School, MA), *L. monocytogenes* (clinical isolate 10403s) was from V. Boyartchuk (UMASS Medical School, MA). HSV-1 (KOS and 7134) was a gift from D. Knipe (Harvard Medical School, MA). Sendai virus (SV, Cantrell strain) was purchased from Charles River Laboratories (Wilmington, MA). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). Genejuice was from Novagen (Madison, WI). ZVAD-FMK was from Calbiochem (San Diego, CA). AIM2, pro-IL-1β, ASC and caspase-1 were as described (16, 17).

Mice

C57BL/6 mice were from Jackson Laboratories (Bar Harbor, ME). Caspase-1 deficient mice were a gift from M. Starnbach (Harvard Medical School, MA). ASC-, NLRP3- and NLRP12-deficient mice were generated by Millennium Pharmaceuticals

(Cambridge, MA) and were backcrossed 8-11 generations to C57BL/6 background. MyD88-, TLR2-, TLR9- and TLR2/9-deficient mice were a gift from S. Akira (Nippon Medical School, Japan). All experiments were conducted with mice maintained under specific pathogen-free conditions in the animal facilities at the UMASS Medical School and were carried out in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee.

Cell Culture, Stimulation and ELISA

HEK293T cells (5 x 10^4 cells/well) in 96-well plates were co-transfected in triplicate using GeneJuice (4 μl/ml) with plasmids encoding pro-IL-1β and the indicated expression plasmids (total DNA, 200 ng). Cultures were incubated for two hours then exposed to sup ODN (3 μM) or left untreated; 24hrs later supernatants were collected and cells were lysed using a 1% NP-40 lysis buffer. PEC, BMDM and BMDC were generated as described (17, 222). Peritoneal exudate neutrophils were harvested 4 hrs after i.p. injection of 1 ml of thioglycolate, as described (117). Cells were plated at $2x10^5$ per well for ELISA, $2\text{-}5x10^6$ per well for immunoblotting. Cells were treated with LPS (200 ng/ml) for 2 hrs prior to the addition sup ODN or CpG ODN then incubated for an additional hour before secondary stimulation. ATP (5 mM) or Nigericin (10 μM) were added one hour before harvesting supernantants and lysates. Poly(dA:dT) was transfected using Lipofectamine 2000 at a concentration of 0.5 μg/ml, 6 hrs before harvesting. Cells were infected with MCMV and HSV-1 at an MOI of 10. Cells were exposed to Sendai virus at 200 HAU/ml. Cells were challenged with *L. monocytogenes* at an MOI of 5 for 1 hr. Cells were then washed twice and media containing gentamicin

(100 μg/ml) was added. All infections were incubated for 16 hrs before harvest. Supernatants from cell culture experiments were assayed for IL-1β (BD Biosciences, Franklin Lakes, NJ) and IL-18 (R&D Systems Piscataway, NJ) by sandwich ELISA.

Nanostring and RT-QPCR experiments

Cells were treated as described above and RNA was purified using an RNeasy Mini Kit (QIAGEN). Total RNA was hybridized to a custom gene expression CodeSet and analyzed on an nCounter Digital Analyzer. Counts were normalized to internal and endogenous controls per Nanostring Technologies' specifications. A pseudocount was ascribed to all values such that the smallest value in the dataset was equal to 1. Values were log-transformed and displayed via heat map (Euclidean clustering) generated using the ggplot package within the open source R software environment. cDNA was synthesized from total RNA and quantitative RT-PCR analysis was performed as previously described (223). Gene expression is shown as a ratio of gene copy number per 100 copies of β-Actin $\pm$ SD.

Western Blotting

Supernatants were harvested and precipitated by methanol chloroform extraction. Cells were washed twice with PBS and lysed using a 1% NP-40 buffer. Immunoblotting was performed as described (16). Anti-Flag antibody (M2) was from Sigma, anti-murine caspase-1 p10 (sc-514) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), anti-murine caspase-1 p20 (5B10) from eBioscience, anti-murine IL-1β from R&D Systems

(Minneapolis, MN), and anti-mouse HMGB1 (3E8) was from BioLegend (San Diego, CA).

ASC Oligomerization Assay

ASC oligomerization assay was performed as described with minor modifications (101). In brief, BMDM (1 x 10^7 cells/condition) were primed with LPS (200 ng/ml) for 2 hrs prior to the addition of A151 or C151 (3 μM). After 30 minutes, 25 μM of zVAD-FMK was added followed 30 minutes later by poly(dA:dT) transfection (0.5 μg/ml) using Lipofectamine 2000. Cells were washed and lysed with 1% NP-40 lysis buffer 3 hrs after poly(dA:dT) challenge. Lysates were cleared by centrifugation at 300 g. Macromolecular structures were then pelleted by centrifugation at 4,500 x g, resuspended in 50 μl CHAPS buffer, and cross-linked with disuccinimidyl suberate (2 μM) (Pierce Thermo Scientific, Rockford, IL). Supernatants from this step were saved and labeled 'lysate' in ASC blots. The pellet was washed, resuspended in Laemmli buffer, incubated overnight with shaking at 4 ^{o}C, then boiled and electrophoresed on a 12% SDS-acrylamide gel as the 'cross-linked' fraction. Blots were probed with anti-ASC antibody (N-15-R, Santa Cruz Biotechnology).

Confocal Microscopy

Confocal microscopy was performed using a Leica SP2 AOBS confocal laser scanning microscope. Immortalized murine macrophages stably expressing AIM2- or ASC-citrine constructs were plated at 2 x 10^6 cells/ml on glass bottom 35 mM culture dishes (MatTek corportation, Ashland, MA) and allowed to adhere. A151 or C151 was

added one hour prior to transfection with poly(dA:dT) or exposure to nigericin. Two hours after poly(dA:dT) challenge or 30 minutes after nigericin exposure cultures were photographed. The total number of fluorescent cells was recorded in more than twenty independent fields representing more than 1000 cells and divided into those displaying diffuse cytoplasmic staining and those exhibiting speck formation. Little variability in the percent of cell exhibiting speck formation was observed between experiments. Graphs quantifying speck formation were calculated by combining data from three independent experiments.

Pull-down Assay

Immortalized murine macrophages (5 x 10^6 cells/condition) were lysed in an ice-cold high salt lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris HCl, pH 7.9, 100 mM EDTA, 10% glycerol, 10 mM NaF, dithiothreitol (DTT) and protease inhibitor cocktail as described previously (224). Cell debris was removed by centrifugation and total lysate was incubated with 6 μg of 3'-biotinylated A151 and pre-washed streptavidin-agarose beads (50% w/v) for 2 hrs at 4 ^{o}C. For competition assays, an increasing amount of poly(dA:dT) was mixed with biotinylated A151 before addition to the lysate. Bead pellets were washed, boiled in Laemmli buffer, and electrophoresed on a 12% SDS-polyacrylamide gel. Blots were probed with polyclonal anti-mouse AIM2 antibody from Genentech (4G9, San Francisco, CA) and anti-mouse β-actin (AC-74, Sigma).

Statistical Analysis

One-way analysis of variance followed by the Bonferroni post-test was performed using Prism 4 Software (GraphPad, San Diego, CA). P values of <0.05 were considered significant.

Chapter V Discussion and Perspectives

Cells of the innate and adaptive immune systems utilize a variety of PRRs to recognize DNA. Endosomal nucleic acid receptors such as TLR7, TLR8, and TLR9 are crucial for host defense, yet can have deleterious functions in immune-mediated diseases. TLR9 responds to ssDNA containing unmethylated CpG motifs (225). In humans TLR9 expression is limited to plamacytoid dendritic cells and B cells while it is more widely expressed in mice. As our understanding of TLR9 has expanded, harnessing TLR-driven cellular responses to modulate inflammation and immunity has become reality. Short synthetic oligodeoxynucleotides (ODN) containing CpG motifs have remarkable immunostimulatory properties such as driving cytokine and IFN production, enhancing APC functional, and lymphocyte maturation (8). These CpG ODNs have tantalizing immunotherapeutic potential. By virtue of their stimulatory properties they can be used to enhance vaccine responses, as anti-cancer agents, and to reduce the transmission of HIV (193, 226, 227). Interestingly, these CpG ODN have been reported to suppress pathological immunity as evidenced by their beneficial effects on allergic diseases such as asthma, and autoimmune diseases such as diabetes (228-230). How CpG treatment can reduce certain inflammatory disease and exacerbate others is still poorly understood (231). It has been suggested that these seemingly paradoxical effects are due to TLR9-dependent induction of the immunosuppressive enzyme IDO and the stimulation of regulatory T cell responses however their mechanisms of action may be more complex than previously thought (230, 232).

In concert with the establishment of synthetic stimulatory CpG ODNs, a number of groups have developed suppressive ODN (sup ODN) sequences that compete with

CpG motifs thereby blocking TLR9 activation. Dr. Klinman and collaborators have pioneered the use of the telomere-derived suppressive A151 construct (149). Other groups have transformed CpG ODNs into suppressive constructs by altering their sequence, for example by replacing the cytosine residue with a guanine (GpG) or reversing the two residues (GpC) (150, 160). That these constructs were first recognized for their ability to prevent TLR9 activation has led many to explain their *in vivo* effects in this context. However more recent studies show the potency of inhibition by sup ODNs is strongly affected by sequence - a phenomenon not explained by their relative avidity to the TLR9 ectodomain (148). In addition suppression of Th1 differentiation is observed even in TLR9-deficient CD4+ T cells suggesting that their biological activity may have more complex mechanisms of action than simply blocking TLR9 (154). Indeed, recent evidence suggests certain GpC motifs can activate TLR7, the well-studied 2114 construct may prevent TLR2 activation and A151 can bind directly to STAT1 and STAT4 thereby interfering with Type I IFN and cytokine signaling (153, 158, 160). Despite our incomplete understanding of their mechanisms of action, the anti-inflammatory range and therapeutic value of sup ODNs is promising. Administration of A151 has been shown to be beneficial in murine models of CpG and collagen-induced arthritis, toxic shock, systemic lupus erythematosus, atherosclerosis, silica-induced pulmonary inflammation and influenza infection (149-156). Likewise GpG has been used to suppress experimental autoimmune encephalomyelitis and lupus nephritis (150, 233).

In recent years, a number of cytosolic DNA sensors have been discovered including IFI16, AIM2, DDX41 and cGAS (16, 49, 68, 70). In the second chapter of this dissertation we explored the role of suppressive oligodeoxynucleotides in inhibiting

cytosolic DNA sensing pathways. Using the synthetic dsDNA ligand poly(dA:dT) and pathogens including HSV-1, MCMV and *Listeria monocytogenes* we found suppressive ODNs were able to block dsDNA-specific cytosolic receptors while having no effect on TLR4, RIG-I or NLRP3 mediated signaling. Moreover backbone chemistry was found to be of central importance to inhibition as only phosphorothioate (PS) DNA was able to bind to receptors and suppress Type I IFN and cytokine production. Interestingly while the guanine-rich A151 construct more potently inhibited AIM2 signaling, the C151 construct was a more potent inhibitor of type I IFN and ISG. In support of an affinity-based model of competitive inhibition, the potency of these constructs correlated with their relative affinity for the AIM2 and IFI16 receptors. Using a number of different constructs we noticed that while total guanine or adenine content affected inhibitory potency the specific sequence did not appear to be of great importance. For example, class B stimulatory ODNs 1826 and 10104 inhibited AIM2 with a similar potency to A151. This off target mechanism of action may contribute to many of the anti-inflammatory effects of suppressive and stimulatory ODN alike.

Evidence that PS backbone ssDNA constructs, even those containing stimulatory CpG motifs, can mediate inhibition of cytosolic receptor signaling has not been previously reported and is an important concept. Particularly in light of the numerous clinical trials in all phases of development using CpG ODN constructs (234). With widespread use as vaccine adjuvants it is possible CpG ODN-mediated suppression of cytosolic sensing pathways may enhance susceptibility to certain pathogenic infections. This was evinced in a study by Trieu *et al*. which demonstrated that treatment with the suppressive 2114 construct increased bacterial loads of the intracellular pathogen

Salmonella typhimurium by suppressing NF-κB-dependent cytokine responses in a TLR9-independent manner (158). In addition to CpG ODN, antisense PS constructs are widely employed in the laboratory to silence expression of target proteins and have recently found traction as therapies in the clinic. Formivirsen - a 21bp PS antisense construct - has already been approved by the FDA for the treatment of cytomegalovirus retinitis in immunocompromised patients (235). However numerous studies report unexplained, non-sequence specific effects of antisense constructs (236, 237). For example, treatment of human leukemia HL-60 cells with PS but not PD antisense constructs has also been reported to inhibit proliferation and induce cell death in a manner that is independent of sequence (131). It is tempting to speculate that interactions between these constructs and members of the PYHIN family, such as IFI16 and p204, known to regulate cell cycle progression and survival, may underlie many of these previously unexplained biological effects (80).

In addition to describing the role of backbone chemistry in ODN potency and affinity for PRRs, this thesis has begun to define the contributions of nucleotide content to inhibition. Our findings indicate that by altering sequences and refining the dose of these constructs it may be possible to selectively block specific pathways allowing examination of the relative contributions of different cytosolic receptors to the inflammatory response. Alternatively, antisense sequences or dosing strategies could be chosen to minimize off target effects. However, more work, defining the dissociation constants of these sup ODN, is needed to further explore the contributions of sequence to inhibitory potency. Finally, we have preliminary evidence that indicates suppressive

ODN can interfere with signaling mediated by other cytoplasmic receptors such as DDX41 and cGAS suggesting additional anti-inflammatory mechanisms exist.

The cytokines IL-1β and IL-18 play a crucial role in the immune response to HSV-1. Mice lacking IL-1β or IL-18 are unable to control viral dissemination and are susceptible to lethal encephalitis (177, 201). In the third chapter of this dissertation the innate pathways responsible for both inducing the pro-forms and ultimately maturing these cytokines were investigated. The TLR2-MyD88 signal axis was found to be essential for induction of pro-IL-1β in macrophages and CDCs. Induction of pro-IL-1β was blocked by the viral ICP0, a protein that has been shown to induce degradation of MyD88 (168). In contrast, optimal pro-IL-18 induction required both MyD88 and STING suggesting both TLR and cytosolic receptor pathways synergistically control expression of this cytokine. Consistent with the need to both 'prime' and 'license' the inflammasome, infection with HSV-1 also stimulated MyD88-dependent expression of NLRP3 in dendritic cells. Where other attempts to detect IL-1β production have failed, our LPS prime, HSV-1 challenge strategy results in secretion of mature IL-1β (181, 212). In macrophages and DCs secretion of IL-1β was dependent on NLRP3. Although we did not identify a specific ligand for NLRP3, inactivation of HSV-1 by UV-irradiation prevented IL-1β production indicating replication competent virus is required for activation. In spite of priming with LPS to produce high levels of pro-IL-1β, treatment with the wild-type KOS strain of HSV-1 induced lower amounts of IL-1β secretion than the ICP0-deficient 7134 strain. It is possible that ICP0 also plays a direct role in blocking inflammasome signaling through the degradation of inflammasome components as has been reported for IFI16 (181). Alternatively, it may indirectly influence cytokine

production by inhibiting Type I IFN signaling, which is known to signal in a paracrine and autocrine manner to induce ISGs and amplify cytokine secretion (238). Additional studies beyond the scope of this dissertation are required to further investigate the functional interaction between the viral protein ICP0 and NLRP3 signaling.

This study also reveals a role for NLRP12 in inflammasome signaling in neutrophils, another cell type that plays a critical role in the acute immune response against HSV-1. Neutrophils rapidly infiltrate sites of HSV-1 infection and have been shown adhere to infected cells and phagocytose antibody coated herpes virions (239-241). Depletion of neutrophils in the bloodstream before corneal infection with HSV-1 caused increased viral replication and dissemination rendering mice significantly more susceptible to lethal encephalitis (242). Inflammasome signaling and IL-1β production have been shown to be important for neutrophil recruitment and neutrophil extracellular trap (NET) formation in cases of bacterial infection and sterile inflammation (243-245). Although other serine proteases have been implicated in IL-1β processing (89), we demonstrate here that IL-1β secretion in neutrophils responding to HSV-1 is largely dependent on the NLRP12 and ASC.

Inhibition of IL-1β signaling is a potential therapeutic strategy in a variety of inflammatory disorders including coronary artery disease and inflammatory arthritis and is beneficial in some manifestations of HSV-1 infection (215, 246). Anakinra, a recombinant IL-1 receptor antagonist is used in the treatment of rheumatoid arthritis but can lead to pain at the injection site and increased risk of infection (247). As demonstrated, different innate cell types rely on different receptors to assemble inflammasomes in response to HSV-1. Thus, rather than general inhibition of IL-1β

signaling, targeted therapy directed against specific receptors may reduce excessive immune responses without impairing the ability to fight infection. For this reason a more comprehensive understanding of the cell type specific mechanisms responsible for IL-1β and IL-18 production is essential for the development of novel and balanced therapeutic interventions.

Bibliography

1. Heyworth, P. G., A. R. Cross, and J. T. Curnutte. 2003. Chronic granulomatous disease. *Curr Opin Immunol* 15:578-584.
2. Bachmann, M. F., and M. Kopf. 2001. On the role of the innate immunity in autoimmune disease. *J Exp Med* 193:F47-50.
3. Murawski, M. R., G. N. Bowen, A. M. Cerny, L. J. Anderson, L. M. Haynes, R. A. Tripp, E. A. Kurt-Jones, and R. W. Finberg. 2009. Respiratory syncytial virus activates innate immunity through Toll-like receptor 2. *J Virol* 83:1492-1500.
4. Bieback, K., E. Lien, I. M. Klagge, E. Avota, J. Schneider-Schaulies, W. P. Duprex, H. Wagner, C. J. Kirschning, V. Ter Meulen, and S. Schneider-Schaulies. 2002. Hemagglutinin protein of wild-type measles virus activates toll-like receptor 2 signaling. *J Virol* 76:8729-8736.
5. Boukhvalova, M. S., G. A. Prince, L. Soroush, D. C. Harrigan, S. N. Vogel, and J. C. Blanco. 2006. The TLR4 agonist, monophosphoryl lipid A, attenuates the cytokine storm associated with respiratory syncytial virus vaccine-enhanced disease. *Vaccine* 24:5027-5035.
6. Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill, and A. Aderem. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 410:1099-1103.
7. Alexopoulou, L., A. C. Holt, R. Medzhitov, and R. A. Flavell. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413:732-738.
8. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408:740-745.
9. Kaisho, T., and S. Akira. 2006. Toll-like receptor function and signaling. *J Allergy Clin Immunol* 117:979-987; quiz 988.
10. Honda, K., H. Yanai, H. Negishi, M. Asagiri, M. Sato, T. Mizutani, N. Shimada, Y. Ohba, A. Takaoka, N. Yoshida, and T. Taniguchi. 2005. IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature* 434:772-777.
11. Guiducci, C., G. Ott, J. H. Chan, E. Damon, C. Calacsan, T. Matray, K. D. Lee, R. L. Coffman, and F. J. Barrat. 2006. Properties regulating the nature of the plasmacytoid dendritic cell response to Toll-like receptor 9 activation. *J Exp Med* 203:1999-2008.
12. Colonna, M., G. Trinchieri, and Y. J. Liu. 2004. Plasmacytoid dendritic cells in immunity. *Nat Immunol* 5:1219-1226.
13. Rathinam, V. A., and K. A. Fitzgerald. 2010. Innate immune sensing of DNA viruses. *Virology* 411:153-162.
14. Chu, J., L. M. Thomas, S. C. Watkins, L. Franchi, G. Nunez, and R. D. Salter. 2009. Cholesterol-dependent cytolysins induce rapid release of mature IL-

1beta from murine macrophages in a NLRP3 inflammasome and cathepsin B-dependent manner. *J Leukoc Biol* 86:1227-1238.

15. Kanneganti, T. D., M. Body-Malapel, A. Amer, J. H. Park, J. Whitfield, L. Franchi, Z. F. Taraporewala, D. Miller, J. T. Patton, N. Inohara, and G. Nunez. 2006. Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. *J Biol Chem* 281:36560-36568.
16. Rathinam, V. A., Z. Jiang, S. N. Waggoner, S. Sharma, L. E. Cole, L. Waggoner, S. K. Vanaja, B. G. Monks, S. Ganesan, E. Latz, V. Hornung, S. N. Vogel, E. Szomolanyi-Tsuda, and K. A. Fitzgerald. 2010. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nat Immunol* 11:395-402.
17. Hornung, V., A. Ablasser, M. Charrel-Dennis, F. Bauernfeind, G. Horvath, D. R. Caffrey, E. Latz, and K. A. Fitzgerald. 2009. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature* 458:514-518.
18. Rothenfusser, S., N. Goutagny, G. DiPerna, M. Gong, B. G. Monks, A. Schoenemeyer, M. Yamamoto, S. Akira, and K. A. Fitzgerald. 2005. The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I. *J Immunol* 175:5260-5268.
19. Yoneyama, M., M. Kikuchi, K. Matsumoto, T. Imaizumi, M. Miyagishi, K. Taira, E. Foy, Y. M. Loo, M. Gale, Jr., S. Akira, S. Yonehara, A. Kato, and T. Fujita. 2005. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J Immunol* 175:2851-2858.
20. Kovacsovics, M., F. Martinon, O. Micheau, J. L. Bodmer, K. Hofmann, and J. Tschopp. 2002. Overexpression of Helicard, a CARD-containing helicase cleaved during apoptosis, accelerates DNA degradation. *Curr Biol* 12:838-843.
21. Yoneyama, M., M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira, and T. Fujita. 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* 5:730-737.
22. Seth, R. B., L. Sun, C. K. Ea, and Z. J. Chen. 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* 122:669-682.
23. Dixit, E., S. Boulant, Y. Zhang, A. S. Lee, C. Odendall, B. Shum, N. Hacohen, Z. J. Chen, S. P. Whelan, M. Fransen, M. L. Nibert, G. Superti-Furga, and J. C. Kagan. 2010. Peroxisomes are signaling platforms for antiviral innate immunity. *Cell* 141:668-681.
24. Kawai, T., K. Takahashi, S. Sato, C. Coban, H. Kumar, H. Kato, K. J. Ishii, O. Takeuchi, and S. Akira. 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat Immunol* 6:981-988.
25. Meylan, E., J. Curran, K. Hofmann, D. Moradpour, M. Binder, R. Bartenschlager, and J. Tschopp. 2005. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 437:1167-1172.

26. Xu, L. G., Y. Y. Wang, K. J. Han, L. Y. Li, Z. Zhai, and H. B. Shu. 2005. VISA is an adapter protein required for virus-triggered IFN-beta signaling. *Mol Cell* 19:727-740.
27. Ishikawa, H., and G. N. Barber. 2008. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature* 455:674-678.
28. Kato, H., S. Sato, M. Yoneyama, M. Yamamoto, S. Uematsu, K. Matsui, T. Tsujimura, K. Takeda, T. Fujita, O. Takeuchi, and S. Akira. 2005. Cell type-specific involvement of RIG-I in antiviral response. *Immunity* 23:19-28.
29. Kato, H., O. Takeuchi, S. Sato, M. Yoneyama, M. Yamamoto, K. Matsui, S. Uematsu, A. Jung, T. Kawai, K. J. Ishii, O. Yamaguchi, K. Otsu, T. Tsujimura, C. S. Koh, C. Reis e Sousa, Y. Matsuura, T. Fujita, and S. Akira. 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 441:101-105.
30. Hornung, V., J. Ellegast, S. Kim, K. Brzozka, A. Jung, H. Kato, H. Poeck, S. Akira, K. K. Conzelmann, M. Schlee, S. Endres, and G. Hartmann. 2006. 5'-Triphosphate RNA is the ligand for RIG-I. *Science* 314:994-997.
31. Pichlmair, A., O. Schulz, C. P. Tan, T. I. Naslund, P. Liljestrom, F. Weber, and C. Reis e Sousa. 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* 314:997-1001.
32. Plumet, S., F. Herschke, J. M. Bourhis, H. Valentin, S. Longhi, and D. Gerlier. 2007. Cytosolic 5'-triphosphate ended viral leader transcript of measles virus as activator of the RIG I-mediated interferon response. *PLoS One* 2:e279.
33. Kato, H., O. Takeuchi, E. Mikamo-Satoh, R. Hirai, T. Kawai, K. Matsushita, A. Hiiragi, T. S. Dermody, T. Fujita, and S. Akira. 2008. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. *J Exp Med* 205:1601-1610.
34. Melchjorsen, J., S. B. Jensen, L. Malmgaard, S. B. Rasmussen, F. Weber, A. G. Bowie, S. Matikainen, and S. R. Paludan. 2005. Activation of innate defense against a paramyxovirus is mediated by RIG-I and TLR7 and TLR8 in a cell-type-specific manner. *J Virol* 79:12944-12951.
35. Gitlin, L., W. Barchet, S. Gilfillan, M. Cella, B. Beutler, R. A. Flavell, M. S. Diamond, and M. Colonna. 2006. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. *Proc Natl Acad Sci U S A* 103:8459-8464.
36. Sumpter, R., Jr., Y. M. Loo, E. Foy, K. Li, M. Yoneyama, T. Fujita, S. M. Lemon, and M. Gale, Jr. 2005. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *J Virol* 79:2689-2699.
37. Barral, P. M., J. M. Morrison, J. Drahos, P. Gupta, D. Sarkar, P. B. Fisher, and V. R. Racaniello. 2007. MDA-5 is cleaved in poliovirus-infected cells. *J Virol* 81:3677-3684.
38. Wang, J. P., A. Cerny, D. R. Asher, E. A. Kurt-Jones, R. T. Bronson, and R. W. Finberg. 2010. MDA5 and MAVS mediate type I interferon responses to coxsackie B virus. *J Virol* 84:254-260.

39. Fredericksen, B. L., B. C. Keller, J. Fornek, M. G. Katze, and M. Gale, Jr. 2008. Establishment and maintenance of the innate antiviral response to West Nile Virus involves both RIG-I and MDA5 signaling through IPS-1. *J Virol* 82:609-616.
40. Shingai, M., T. Ebihara, N. A. Begum, A. Kato, T. Honma, K. Matsumoto, H. Saito, H. Ogura, M. Matsumoto, and T. Seya. 2007. Differential type I IFN-inducing abilities of wild-type versus vaccine strains of measles virus. *J Immunol* 179:6123-6133.
41. Venkataraman, T., M. Valdes, R. Elsby, S. Kakuta, G. Caceres, S. Saijo, Y. Iwakura, and G. N. Barber. 2007. Loss of DExD/H box RNA helicase LGP2 manifests disparate antiviral responses. *J Immunol* 178:6444-6455.
42. Schroder, M., M. Baran, and A. G. Bowie. 2008. Viral targeting of DEAD box protein 3 reveals its role in TBK1/IKKepsilon-mediated IRF activation. *EMBO J* 27:2147-2157.
43. Oshiumi, H., K. Sakai, M. Matsumoto, and T. Seya. 2010. DEAD/H BOX 3 (DDX3) helicase binds the RIG-I adaptor IPS-1 to up-regulate IFN-beta-inducing potential. *Eur J Immunol* 40:940-948.
44. Suzuki, K., A. Mori, K. J. Ishii, J. Saito, D. S. Singer, D. M. Klinman, P. R. Krause, and L. D. Kohn. 1999. Activation of target-tissue immune-recognition molecules by double-stranded polynucleotides. *Proc Natl Acad Sci U S A* 96:2285-2290.
45. Stetson, D. B., and R. Medzhitov. 2006. Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. *Immunity* 24:93-103.
46. Roberts, T. L., A. Idris, J. A. Dunn, G. M. Kelly, C. M. Burnton, S. Hodgson, L. L. Hardy, V. Garceau, M. J. Sweet, I. L. Ross, D. A. Hume, and K. J. Stacey. 2009. HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA. *Science* 323:1057-1060.
47. Chiu, Y. H., J. B. Macmillan, and Z. J. Chen. 2009. RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. *Cell* 138:576-591.
48. Ablasser, A., F. Bauernfeind, G. Hartmann, E. Latz, K. A. Fitzgerald, and V. Hornung. 2009. RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. *Nat Immunol* 10:1065-1072.
49. Parvatiyar, K., Z. Zhang, R. M. Teles, S. Ouyang, Y. Jiang, S. S. Iyer, S. A. Zaver, M. Schenk, S. Zeng, W. Zhong, Z. J. Liu, R. L. Modlin, Y. J. Liu, and G. Cheng. 2012. The helicase DDX41 recognizes the bacterial secondary messengers cyclic di-GMP and cyclic di-AMP to activate a type I interferon immune response. *Nat Immunol* 13:1155-1161.
50. Ishikawa, H., Z. Ma, and G. N. Barber. 2009. STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. *Nature* 461:788-792.
51. Sun, W., Y. Li, L. Chen, H. Chen, F. You, X. Zhou, Y. Zhou, Z. Zhai, D. Chen, and Z. Jiang. 2009. ERIS, an endoplasmic reticulum IFN stimulator, activates innate immune signaling through dimerization. *Proc Natl Acad Sci U S A* 106:8653-8658.

52. Zhong, B., Y. Yang, S. Li, Y. Y. Wang, Y. Li, F. Diao, C. Lei, X. He, L. Zhang, P. Tien, and H. B. Shu. 2008. The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. *Immunity* 29:538-550.
53. Prantner, D., D. J. Perkins, W. Lai, M. S. Williams, S. Sharma, K. A. Fitzgerald, and S. N. Vogel. 2012. 5,6-Dimethylxanthenone-4-acetic acid (DMXAA) activates stimulator of interferon gene (STING)-dependent innate immune pathways and is regulated by mitochondrial membrane potential. *J Biol Chem* 287:39776-39788.
54. Saitoh, T., N. Fujita, T. Hayashi, K. Takahara, T. Satoh, H. Lee, K. Matsunaga, S. Kageyama, H. Omori, T. Noda, N. Yamamoto, T. Kawai, K. Ishii, O. Takeuchi, T. Yoshimori, and S. Akira. 2009. Atg9a controls dsDNA-driven dynamic translocation of STING and the innate immune response. *Proc Natl Acad Sci U S A* 106:20842-20846.
55. Takaoka, A., Z. Wang, M. K. Choi, H. Yanai, H. Negishi, T. Ban, Y. Lu, M. Miyagishi, T. Kodama, K. Honda, Y. Ohba, and T. Taniguchi. 2007. DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature* 448:501-505.
56. DeFilippis, V. R., D. Alvarado, T. Sali, S. Rothenburg, and K. Fruh. 2010. Human cytomegalovirus induces the interferon response via the DNA sensor ZBP1. *J Virol* 84:585-598.
57. Wang, Z., M. K. Choi, T. Ban, H. Yanai, H. Negishi, Y. Lu, T. Tamura, A. Takaoka, K. Nishikura, and T. Taniguchi. 2008. Regulation of innate immune responses by DAI (DLM-1/ZBP1) and other DNA-sensing molecules. *Proc Natl Acad Sci U S A* 105:5477-5482.
58. Ishii, K. J., T. Kawagoe, S. Koyama, K. Matsui, H. Kumar, T. Kawai, S. Uematsu, O. Takeuchi, F. Takeshita, C. Coban, and S. Akira. 2008. TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. *Nature* 451:725-729.
59. Cheng, G., J. Zhong, J. Chung, and F. V. Chisari. 2007. Double-stranded DNA and double-stranded RNA induce a common antiviral signaling pathway in human cells. *Proc Natl Acad Sci U S A* 104:9035-9040.
60. Monroe, K. M., S. M. McWhirter, and R. E. Vance. 2009. Identification of host cytosolic sensors and bacterial factors regulating the type I interferon response to Legionella pneumophila. *PLoS Pathog* 5:e1000665.
61. Ishii, K. J., C. Coban, H. Kato, K. Takahashi, Y. Torii, F. Takeshita, H. Ludwig, G. Sutter, K. Suzuki, H. Hemmi, S. Sato, M. Yamamoto, S. Uematsu, T. Kawai, O. Takeuchi, and S. Akira. 2006. A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. *Nat Immunol* 7:40-48.
62. Bagashev, A., M. C. Fitzgerald, D. F. Larosa, P. P. Rose, S. Cherry, A. C. Johnson, and K. E. Sullivan. 2010. Leucine-rich repeat (in Flightless I) interacting protein-1 regulates a rapid type I interferon response. *J Interferon Cytokine Res* 30:843-852.
63. Yang, P., H. An, X. Liu, M. Wen, Y. Zheng, Y. Rui, and X. Cao. 2010. The cytosolic nucleic acid sensor LRRFIP1 mediates the production of type I interferon via a beta-catenin-dependent pathway. *Nat Immunol* 11:487-494.

64. Kim, T., S. Pazhoor, M. Bao, Z. Zhang, S. Hanabuchi, V. Facchinetti, L. Bover, J. Plumas, L. Chaperot, J. Qin, and Y. J. Liu. 2010. Aspartate-glutamate-alanine-histidine box motif (DEAH)/RNA helicase A helicases sense microbial DNA in human plasmacytoid dendritic cells. *Proc Natl Acad Sci U S A* 107:15181-15186.
65. Pesavento, C., and R. Hengge. 2009. Bacterial nucleotide-based second messengers. *Curr Opin Microbiol* 12:170-176.
66. Mills, E., I. S. Pultz, H. D. Kulasekara, and S. I. Miller. 2011. The bacterial second messenger c-di-GMP: mechanisms of signalling. *Cell Microbiol* 13:1122-1129.
67. Zhang, Z., B. Yuan, M. Bao, N. Lu, T. Kim, and Y. J. Liu. 2011. The helicase DDX41 senses intracellular DNA mediated by the adaptor STING in dendritic cells. *Nat Immunol* 12:959-965.
68. Sun, L., J. Wu, F. Du, X. Chen, and Z. J. Chen. 2013. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science* 339:786-791.
69. Wu, J., L. Sun, X. Chen, F. Du, H. Shi, C. Chen, and Z. J. Chen. 2013. Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. *Science* 339:826-830.
70. Unterholzner, L., S. E. Keating, M. Baran, K. A. Horan, S. B. Jensen, S. Sharma, C. M. Sirois, T. Jin, E. Latz, T. S. Xiao, K. A. Fitzgerald, S. R. Paludan, and A. G. Bowie. 2010. IFI16 is an innate immune sensor for intracellular DNA. *Nat Immunol* 11:997-1004.
71. Johnstone, R. W., and J. A. Trapani. 1999. Transcription and growth regulatory functions of the HIN-200 family of proteins. *Mol Cell Biol* 19:5833-5838.
72. Yan, H., K. Dalal, B. K. Hon, P. Youkharibache, D. Lau, and F. Pio. 2008. RPA nucleic acid-binding properties of IFI16-HIN200. *Biochim Biophys Acta* 1784:1087-1097.
73. Jin, T., A. Perry, J. Jiang, P. Smith, J. A. Curry, L. Unterholzner, Z. Jiang, G. Horvath, V. A. Rathinam, R. W. Johnstone, V. Hornung, E. Latz, A. G. Bowie, K. A. Fitzgerald, and T. S. Xiao. 2012. Structures of the HIN domain:DNA complexes reveal ligand binding and activation mechanisms of the AIM2 inflammasome and IFI16 receptor. *Immunity* 36:561-571.
74. Johnstone, R. W., M. H. Kershaw, and J. A. Trapani. 1998. Isotypic variants of the interferon-inducible transcriptional repressor IFI 16 arise through differential mRNA splicing. *Biochemistry* 37:11924-11931.
75. Johnstone, R. W., J. A. Kerry, and J. A. Trapani. 1998. The human interferon-inducible protein, IFI 16, is a repressor of transcription. *J Biol Chem* 273:17172-17177.
76. Fujiuchi, N., J. A. Aglipay, T. Ohtsuka, N. Maehara, F. Sahin, G. H. Su, S. W. Lee, and T. Ouchi. 2004. Requirement of IFI16 for the maximal activation of p53 induced by ionizing radiation. *J Biol Chem* 279:20339-20344.
77. Alimirah, F., J. Chen, F. J. Davis, and D. Choubey. 2007. IFI16 in human prostate cancer. *Mol Cancer Res* 5:251-259.

78. Aglipay, J. A., S. W. Lee, S. Okada, N. Fujiuchi, T. Ohtsuka, J. C. Kwak, Y. Wang, R. W. Johnstone, C. Deng, J. Qin, and T. Ouchi. 2003. A member of the Pyrin family, IFI16, is a novel BRCA1-associated protein involved in the p53-mediated apoptosis pathway. *Oncogene* 22:8931-8938.
79. Gugliesi, F., M. De Andrea, M. Mondini, P. Cappello, M. Giovarelli, Y. Shoenfeld, P. Meroni, M. Gariglio, and S. Landolfo. 2010. The proapoptotic activity of the Interferon-inducible gene IFI16 provides new insights into its etiopathogenetic role in autoimmunity. *J Autoimmun* 35:114-123.
80. Mondini, M., M. Vidali, P. Airo, M. De Andrea, P. Riboldi, P. L. Meroni, M. Gariglio, and S. Landolfo. 2007. Role of the interferon-inducible gene IFI16 in the etiopathogenesis of systemic autoimmune disorders. *Ann N Y Acad Sci* 1110:47-56.
81. Mandel, M., and A. Achiron. 2006. Gene expression studies in systemic lupus erythematosus. *Lupus* 15:451-456.
82. Kerur, N., M. V. Veettil, N. Sharma-Walia, V. Bottero, S. Sadagopan, P. Otageri, and B. Chandran. 2011. IFI16 Acts as a Nuclear Pathogen Sensor to Induce the Inflammasome in Response to Kaposi Sarcoma-Associated Herpesvirus Infection. *Cell Host Microbe* 9:363-375.
83. Trent, J. M., E. J. Stanbridge, H. L. McBride, E. U. Meese, G. Casey, D. E. Araujo, C. M. Witkowski, and R. B. Nagle. 1990. Tumorigenicity in human melanoma cell lines controlled by introduction of human chromosome 6. *Science* 247:568-571.
84. Woerner, S. M., M. Kloor, Y. Schwitalle, H. Youmans, M. Doeberitz, J. Gebert, and S. Dihlmann. 2007. The putative tumor suppressor AIM2 is frequently affected by different genetic alterations in microsatellite unstable colon cancers. *Genes Chromosomes Cancer* 46:1080-1089.
85. Hasegawa, S., Y. Furukawa, M. Li, S. Satoh, T. Kato, T. Watanabe, T. Katagiri, T. Tsunoda, Y. Yamaoka, and Y. Nakamura. 2002. Genome-wide analysis of gene expression in intestinal-type gastric cancers using a complementary DNA microarray representing 23,040 genes. *Cancer Res* 62:7012-7017.
86. Patsos, G., A. Germann, J. Gebert, and S. Dihlmann. 2010. Restoration of absent in melanoma 2 (AIM2) induces G2/M cell cycle arrest and promotes invasion of colorectal cancer cells. *Int J Cancer* 126:1838-1849.
87. Dinarello, C. A. 1994. The interleukin-1 family: 10 years of discovery. *FASEB J* 8:1314-1325.
88. Reading, P. C., P. G. Whitney, D. P. Barr, M. Wojtasiak, J. D. Mintern, J. Waithman, and A. G. Brooks. 2007. IL-18, but not IL-12, regulates NK cell activity following intranasal herpes simplex virus type 1 infection. *J Immunol* 179:3214-3221.
89. Netea, M. G., A. Simon, F. van de Veerdonk, B. J. Kullberg, J. W. Van der Meer, and L. A. Joosten. 2010. IL-1beta processing in host defense: beyond the inflammasomes. *PLoS Pathog* 6:e1000661.
90. Ting, J. P., R. C. Lovering, E. S. Alnemri, J. Bertin, J. M. Boss, B. K. Davis, R. A. Flavell, S. E. Girardin, A. Godzik, J. A. Harton, H. M. Hoffman, J. P. Hugot, N. Inohara, A. Mackenzie, L. J. Maltais, G. Nunez, Y. Ogura, L. A. Otten, D. Philpott,

J. C. Reed, W. Reith, S. Schreiber, V. Steimle, and P. A. Ward. 2008. The NLR gene family: a standard nomenclature. *Immunity* 28:285-287.

91. Bauernfeind, F. G., G. Horvath, A. Stutz, E. S. Alnemri, K. MacDonald, D. Speert, T. Fernandes-Alnemri, J. Wu, B. G. Monks, K. A. Fitzgerald, V. Hornung, and E. Latz. 2009. Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J Immunol* 183:787-791.
92. Halle, A., V. Hornung, G. C. Petzold, C. R. Stewart, B. G. Monks, T. Reinheckel, K. A. Fitzgerald, E. Latz, K. J. Moore, and D. T. Golenbock. 2008. The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. *Nat Immunol* 9:857-865.
93. Hornung, V., F. Bauernfeind, A. Halle, E. O. Samstad, H. Kono, K. L. Rock, K. A. Fitzgerald, and E. Latz. 2008. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol* 9:847-856.
94. Martinon, F., V. Petrilli, A. Mayor, A. Tardivel, and J. Tschopp. 2006. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 440:237-241.
95. Mariathasan, S., D. S. Weiss, K. Newton, J. McBride, K. O'Rourke, M. Roose-Girma, W. P. Lee, Y. Weinrauch, D. M. Monack, and V. M. Dixit. 2006. Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440:228-232.
96. Bruchard, M., G. Mignot, V. Derangere, F. Chalmin, A. Chevriaux, F. Vegran, W. Boireau, B. Simon, B. Ryffel, J. L. Connat, J. Kanellopoulos, F. Martin, C. Rebe, L. Apetoh, and F. Ghiringhelli. 2013. Chemotherapy-triggered cathepsin B release in myeloid-derived suppressor cells activates the Nlrp3 inflammasome and promotes tumor growth. *Nat Med* 19:57-64.
97. Rajamaki, K., J. Lappalainen, K. Oorni, E. Valimaki, S. Matikainen, P. T. Kovanen, and K. K. Eklund. 2010. Cholesterol crystals activate the NLRP3 inflammasome in human macrophages: a novel link between cholesterol metabolism and inflammation. *PLoS One* 5:e11765.
98. Gross, O., H. Poeck, M. Bscheider, C. Dostert, N. Hannesschlager, S. Endres, G. Hartmann, A. Tardivel, E. Schweighoffer, V. Tybulewicz, A. Mocsai, J. Tschopp, and J. Ruland. 2009. Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature* 459:433-436.
99. Thomas, P. G., P. Dash, J. R. Aldridge, Jr., A. H. Ellebedy, C. Reynolds, A. J. Funk, W. J. Martin, M. Lamkanfi, R. J. Webby, K. L. Boyd, P. C. Doherty, and T. D. Kanneganti. 2009. The intracellular sensor NLRP3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1. *Immunity* 30:566-575.
100. Muruve, D. A., V. Petrilli, A. K. Zaiss, L. R. White, S. A. Clark, P. J. Ross, R. J. Parks, and J. Tschopp. 2008. The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. *Nature* 452:103-107.
101. Rathinam, V. A., S. K. Vanaja, L. Waggoner, A. Sokolovska, C. Becker, L. M. Stuart, J. M. Leong, and K. A. Fitzgerald. 2012. TRIF licenses caspase-11-

dependent NLRP3 inflammasome activation by gram-negative bacteria. *Cell* 150:606-619.
102. Franchi, L., T. D. Kanneganti, G. R. Dubyak, and G. Nunez. 2007. Differential requirement of P2X7 receptor and intracellular K+ for caspase-1 activation induced by intracellular and extracellular bacteria. *J Biol Chem* 282:18810-18818.
103. Petrilli, V., S. Papin, C. Dostert, A. Mayor, F. Martinon, and J. Tschopp. 2007. Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death Differ* 14:1583-1589.
104. Ichinohe, T., I. K. Pang, and A. Iwasaki. 2020. Influenza virus activates inflammasomes via its intracellular M2 ion channel. *Nat Immunol* 11:404-410.
105. Duewell, P., H. Kono, K. J. Rayner, C. M. Sirois, G. Vladimer, F. G. Bauernfeind, G. S. Abela, L. Franchi, G. Nunez, M. Schnurr, T. Espevik, E. Lien, K. A. Fitzgerald, K. L. Rock, K. J. Moore, S. D. Wright, V. Hornung, and E. Latz. 2010. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature* 464:1357-1361.
106. Zhou, R., A. Tardivel, B. Thorens, I. Choi, and J. Tschopp. 2012. Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nat Immunol* 11:136-140.
107. Dostert, C., V. Petrilli, R. Van Bruggen, C. Steele, B. T. Mossman, and J. Tschopp. 2008. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* 320:674-677.
108. Meissner, F., K. Molawi, and A. Zychlinsky. 2008. Superoxide dismutase 1 regulates caspase-1 and endotoxic shock. *Nat Immunol* 9:866-872.
109. Chen, C. C., S. H. Tsai, C. C. Lu, S. T. Hu, T. S. Wu, T. T. Huang, N. Said-Sadier, D. M. Ojcius, and H. C. Lai. 2012. Activation of an NLRP3 inflammasome restricts Mycobacterium kansasii infection. *PLoS One* 7:e36292.
110. Case, C. L., and C. R. Roy. 2011. Asc modulates the function of NLRC4 in response to infection of macrophages by Legionella pneumophila. *MBio* 2.
111. Lightfield, K. L., J. Persson, N. J. Trinidad, S. W. Brubaker, E. M. Kofoed, J. D. Sauer, E. A. Dunipace, S. E. Warren, E. A. Miao, and R. E. Vance. 2011. Differential requirements for NAIP5 in activation of the NLRC4 inflammasome. *Infect Immun* 79:1606-1614.
112. Lara-Tejero, M., F. S. Sutterwala, Y. Ogura, E. P. Grant, J. Bertin, A. J. Coyle, R. A. Flavell, and J. E. Galan. 2006. Role of the caspase-1 inflammasome in Salmonella typhimurium pathogenesis. *J Exp Med* 203:1407-1412.
113. Amer, A., L. Franchi, T. D. Kanneganti, M. Body-Malapel, N. Ozoren, G. Brady, S. Meshinchi, R. Jagirdar, A. Gewirtz, S. Akira, and G. Nunez. 2006. Regulation of Legionella phagosome maturation and infection through flagellin and host Ipaf. *J Biol Chem* 281:35217-35223.
114. Franchi, L., J. Stoolman, T. D. Kanneganti, A. Verma, R. Ramphal, and G. Nunez. 2007. Critical role for Ipaf in Pseudomonas aeruginosa-induced caspase-1 activation. *Eur J Immunol* 37:3030-3039.
115. Franchi, L., A. Amer, M. Body-Malapel, T. D. Kanneganti, N. Ozoren, R. Jagirdar, N. Inohara, P. Vandenabeele, J. Bertin, A. Coyle, E. P. Grant, and G. Nunez.

2006. Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages. *Nat Immunol* 7:576-582.

116. Boyden, E. D., and W. F. Dietrich. 2006. Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. *Nat Genet* 38:240-244.
117. Vladimer, G. I., D. Weng, S. W. Paquette, S. K. Vanaja, V. A. Rathinam, M. H. Aune, J. E. Conlon, J. J. Burbage, M. K. Proulx, Q. Liu, G. Reed, J. C. Mecsas, Y. Iwakura, J. Bertin, J. D. Goguen, K. A. Fitzgerald, and E. Lien. 2012. The NLRP12 inflammasome recognizes Yersinia pestis. *Immunity* 37:96-107.
118. Wang, L., G. A. Manji, J. M. Grenier, A. Al-Garawi, S. Merriam, J. M. Lora, B. J. Geddes, M. Briskin, P. S. DiStefano, and J. Bertin. 2002. PYPAF7, a novel PYRIN-containing Apaf1-like protein that regulates activation of NF-kappa B and caspase-1-dependent cytokine processing. *J Biol Chem* 277:29874-29880.
119. Ye, Z., J. D. Lich, C. B. Moore, J. A. Duncan, K. L. Williams, and J. P. Ting. 2008. ATP binding by monarch-1/NLRP12 is critical for its inhibitory function. *Mol Cell Biol* 28:1841-1850.
120. Williams, K. L., J. D. Lich, J. A. Duncan, W. Reed, P. Rallabhandi, C. Moore, S. Kurtz, V. M. Coffield, M. A. Accavitti-Loper, L. Su, S. N. Vogel, M. Braunstein, and J. P. Ting. 2005. The CATERPILLER protein monarch-1 is an antagonist of toll-like receptor-, tumor necrosis factor alpha-, and Mycobacterium tuberculosis-induced pro-inflammatory signals. *J Biol Chem* 280:39914-39924.
121. Lich, J. D., K. L. Williams, C. B. Moore, J. C. Arthur, B. K. Davis, D. J. Taxman, and J. P. Ting. 2007. Monarch-1 suppresses non-canonical NF-kappaB activation and p52-dependent chemokine expression in monocytes. *J Immunol* 178:1256-1260.
122. Arthur, J. C., J. D. Lich, Z. Ye, I. C. Allen, D. Gris, J. E. Wilson, M. Schneider, K. E. Roney, B. P. O'Connor, C. B. Moore, A. Morrison, F. S. Sutterwala, J. Bertin, B. H. Koller, Z. Liu, and J. P. Ting. 2010. Cutting edge: NLRP12 controls dendritic and myeloid cell migration to affect contact hypersensitivity. *J Immunol* 185:4515-4519.
123. Allen, I. C., J. E. Wilson, M. Schneider, J. D. Lich, R. A. Roberts, J. C. Arthur, R. M. Woodford, B. K. Davis, J. M. Uronis, H. H. Herfarth, C. Jobin, A. B. Rogers, and J. P. Ting. 2012. NLRP12 suppresses colon inflammation and tumorigenesis through the negative regulation of noncanonical NF-kappaB signaling. *Immunity* 36:742-754.
124. Jeru, I., P. Duquesnoy, T. Fernandes-Alnemri, E. Cochet, J. W. Yu, M. Lackmy-Port-Lis, E. Grimprel, J. Landman-Parker, V. Hentgen, S. Marlin, K. McElreavey, T. Sarkisian, G. Grateau, E. S. Alnemri, and S. Amselem. 2008. Mutations in NALP12 cause hereditary periodic fever syndromes. *Proc Natl Acad Sci U S A* 105:1614-1619.
125. Fernandes-Alnemri, T., J. W. Yu, P. Datta, J. Wu, and E. S. Alnemri. 2009. AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature* 458:509-513.

126. Burckstummer, T., C. Baumann, S. Bluml, E. Dixit, G. Durnberger, H. Jahn, M. Planyavsky, M. Bilban, J. Colinge, K. L. Bennett, and G. Superti-Furga. 2009. An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. *Nat Immunol* 10:266-272.
127. Trieu, A., T. L. Roberts, J. A. Dunn, M. J. Sweet, and K. J. Stacey. 2006. DNA motifs suppressing TLR9 responses. *Crit Rev Immunol* 26:527-544.
128. Sester, D. P., S. Naik, S. J. Beasley, D. A. Hume, and K. J. Stacey. 2000. Phosphorothioate backbone modification modulates macrophage activation by CpG DNA. *J Immunol* 165:4165-4173.
129. Roberts, T. L., J. A. Dunn, T. D. Terry, M. P. Jennings, D. A. Hume, M. J. Sweet, and K. J. Stacey. 2005. Differences in macrophage activation by bacterial DNA and CpG-containing oligonucleotides. *J Immunol* 175:3569-3576.
130. Stein, C. A., C. Subasinghe, K. Shinozuka, and J. S. Cohen. 1988. Physicochemical properties of phosphorothioate oligodeoxynucleotides. *Nucleic Acids Res* 16:3209-3221.
131. Ho, P. T., K. Ishiguro, E. Wickstrom, and A. C. Sartorelli. 1991. Non-sequence-specific inhibition of transferrin receptor expression in HL-60 leukemia cells by phosphorothioate oligodeoxynucleotides. *Antisense Res Dev* 1:329-342.
132. Majumdar, C., C. A. Stein, J. S. Cohen, S. Broder, and S. H. Wilson. 1989. Stepwise mechanism of HIV reverse transcriptase: primer function of phosphorothioate oligodeoxynucleotide. *Biochemistry* 28:1340-1346.
133. Vickers, T., B. F. Baker, P. D. Cook, M. Zounes, R. W. Buckheit, Jr., J. Germany, and D. J. Ecker. 1991. Inhibition of HIV-LTR gene expression by oligonucleotides targeted to the TAR element. *Nucleic Acids Res* 19:3359-3368.
134. Ghosh, M. K., K. Ghosh, and J. S. Cohen. 1992. Translation inhibition by phosphorothioate oligodeoxynucleotides in cell-free systems. *Antisense Res Dev* 2:111-118.
135. Gao, W. Y., F. S. Han, C. Storm, W. Egan, and Y. C. Cheng. 1992. Phosphorothioate oligonucleotides are inhibitors of human DNA polymerases and RNase H: implications for antisense technology. *Mol Pharmacol* 41:223-229.
136. Yaswen, P., M. R. Stampfer, K. Ghosh, and J. S. Cohen. 1993. Effects of sequence of thioated oligonucleotides on cultured human mammary epithelial cells. *Antisense Res Dev* 3:67-77.
137. Brown, D. A., S. H. Kang, S. M. Gryaznov, L. DeDionisio, O. Heidenreich, S. Sullivan, X. Xu, and M. I. Nerenberg. 1994. Effect of phosphorothioate modification of oligodeoxynucleotides on specific protein binding. *J Biol Chem* 269:26801-26805.
138. Dias, N., and C. A. Stein. 2002. Antisense oligonucleotides: basic concepts and mechanisms. *Mol Cancer Ther* 1:347-355.
139. Halpern, M. D., Pisetsky, D. S. 1994. In vitro inhibition of murine IFNg production by phosophorothioate deoxyguanosine oligomers. *Immunopharmacology*:47-52.
140. Pisetsky, D. S., and C. F. Reich. 2000. Inhibition of murine macrophage IL-12 production by natural and synthetic DNA. *Clin Immunol* 96:198-204.

141. Zhu, F. G., C. F. Reich, and D. S. Pisetsky. 2002. Inhibition of murine dendritic cell activation by synthetic phosphorothioate oligodeoxynucleotides. *J Leukoc Biol* 72:1154-1163.
142. Krieg, A. M., T. Wu, R. Weeratna, S. M. Efler, L. Love-Homan, L. Yang, A. K. Yi, D. Short, and H. L. Davis. 1998. Sequence motifs in adenoviral DNA block immune activation by stimulatory CpG motifs. *Proc Natl Acad Sci U S A* 95:12631-12636.
143. Lenert, P., L. Stunz, A. K. Yi, A. M. Krieg, and R. F. Ashman. 2001. CpG stimulation of primary mouse B cells is blocked by inhibitory oligodeoxyribonucleotides at a site proximal to NF-kappaB activation. *Antisense Nucleic Acid Drug Dev* 11:247-256.
144. Horvath, J., L. Palkonyay, and J. Weber. 1986. Group C adenovirus DNA sequences in human lymphoid cells. *J Virol* 59:189-192.
145. Stacey, K. J., G. R. Young, F. Clark, D. P. Sester, T. L. Roberts, S. Naik, M. J. Sweet, and D. A. Hume. 2003. The molecular basis for the lack of immunostimulatory activity of vertebrate DNA. *J Immunol* 170:3614-3620.
146. Duramad, O., K. L. Fearon, B. Chang, J. H. Chan, J. Gregorio, R. L. Coffman, and F. J. Barrat. 2005. Inhibitors of TLR-9 act on multiple cell subsets in mouse and man in vitro and prevent death in vivo from systemic inflammation. *J Immunol* 174:5193-5200.
147. Zhao, H., S. H. Cheng, and N. S. Yew. 2000. Requirements for effective inhibition of immunostimulatory CpG motifs by neutralizing motifs. *Antisense Nucleic Acid Drug Dev* 10:381-389.
148. Ashman, R. F., J. A. Goeken, E. Latz, and P. Lenert. 2011. Optimal oligonucleotide sequences for TLR9 inhibitory activity in human cells: lack of correlation with TLR9 binding. *Int Immunol* 23:203-214.
149. Gursel, I., M. Gursel, H. Yamada, K. J. Ishii, F. Takeshita, and D. M. Klinman. 2003. Repetitive elements in mammalian telomeres suppress bacterial DNA-induced immune activation. *J Immunol* 171:1393-1400.
150. Ho, P. P., P. Fontoura, P. J. Ruiz, L. Steinman, and H. Garren. 2003. An immunomodulatory GpG oligonucleotide for the treatment of autoimmunity via the innate and adaptive immune systems. *J Immunol* 171:4920-4926.
151. Klinman, D. M., I. Gursel, S. Klaschik, L. Dong, D. Currie, and H. Shirota. 2005. Therapeutic potential of oligonucleotides expressing immunosuppressive TTAGGG motifs. *Ann N Y Acad Sci* 1058:87-95.
152. Sato, T., T. Shimosato, W. G. Alvord, and D. M. Klinman. 2008. Suppressive oligodeoxynucleotides inhibit silica-induced pulmonary inflammation. *J Immunol* 180:7648-7654.
153. Shirota, H., I. Gursel, M. Gursel, and D. M. Klinman. 2005. Suppressive oligodeoxynucleotides protect mice from lethal endotoxic shock. *J Immunol* 174:4579-4583.
154. Shirota, H., M. Gursel, and D. M. Klinman. 2004. Suppressive oligodeoxynucleotides inhibit Th1 differentiation by blocking IFN-gamma- and IL-12-mediated signaling. *J Immunol* 173:5002-5007.

155. Zeuner, R. A., D. Verthelyi, M. Gursel, K. J. Ishii, and D. M. Klinman. 2003. Influence of stimulatory and suppressive DNA motifs on host susceptibility to inflammatory arthritis. *Arthritis Rheum* 48:1701-1707.
156. Fang, M., M. Wan, S. Guo, R. Sun, M. Yang, T. Zhao, Y. Yan, Y. Zhang, W. Huang, X. Wu, Y. Yu, L. Wang, and S. Hua. 2011. An oligodeoxynucleotide capable of lessening acute lung inflammatory injury in mice infected by influenza virus. *Biochem Biophys Res Commun* 415:342-347.
157. Sen, D., and W. Gilbert. 1988. Formation of parallel four-stranded complexes by guanine-rich motifs in DNA and its implications for meiosis. *Nature* 334:364-366.
158. Trieu, A., N. Bokil, J. A. Dunn, T. L. Roberts, D. Xu, F. Y. Liew, D. A. Hume, K. J. Stacey, and M. J. Sweet. 2009. TLR9-independent effects of inhibitory oligonucleotides on macrophage responses to S. typhimurium. *Immunol Cell Biol* 87:218-225.
159. Dong, L., S. Ito, K. J. Ishii, and D. M. Klinman. 2004. Suppressive oligonucleotides protect against collagen-induced arthritis in mice. *Arthritis Rheum* 50:1686-1689.
160. Volpi, C., F. Fallarino, R. Bianchi, C. Orabona, A. De Luca, C. Vacca, L. Romani, B. Gran, U. Grohmann, P. Puccetti, and M. L. Belladonna. 2012. A GpC-rich oligonucleotide acts on plasmacytoid dendritic cells to promote immune suppression. *J Immunol* 189:2283-2289.
161. Jurk, M., A. Kritzler, B. Schulte, S. Tluk, C. Schetter, A. M. Krieg, and J. Vollmer. 2006. Modulating responsiveness of human TLR7 and 8 to small molecule ligands with T-rich phosphorothiate oligodeoxynucleotides. *Eur J Immunol* 36:1815-1826.
162. Fields, B. N., D. M. Knipe, and P. M. Howley. 2007. *Fields virology*. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia.
163. Chayavichitsilp, P., J. V. Buckwalter, A. C. Krakowski, and S. F. Friedlander. 2009. Herpes simplex. *Pediatr Rev* 30:119-129; quiz 130.
164. Corey, L., and P. G. Spear. 1986. Infections with herpes simplex viruses (1). *N Engl J Med* 314:686-691.
165. Corey, L., and P. G. Spear. 1986. Infections with herpes simplex viruses (2). *N Engl J Med* 314:749-757.
166. Novak, N., and W. M. Peng. 2005. Dancing with the enemy: the interplay of herpes simplex virus with dendritic cells. *Clin Exp Immunol* 142:405-410.
167. Harle, P., B. Sainz, Jr., D. J. Carr, and W. P. Halford. 2002. The immediate-early protein, ICP0, is essential for the resistance of herpes simplex virus to interferon-alpha/beta. *Virology* 293:295-304.
168. van Lint, A. L., M. R. Murawski, R. E. Goodbody, M. Severa, K. A. Fitzgerald, R. W. Finberg, D. M. Knipe, and E. A. Kurt-Jones. 2010. Herpes simplex virus immediate-early ICP0 protein inhibits Toll-like receptor 2-dependent inflammatory responses and NF-kappaB signaling. *J Virol* 84:10802-10811.
169. Lin, R., R. S. Noyce, S. E. Collins, R. D. Everett, and K. L. Mossman. 2004. The herpes simplex virus ICP0 RING finger domain inhibits IRF3- and IRF7-mediated activation of interferon-stimulated genes. *J Virol* 78:1675-1684.

170. Everett, R. D., S. Rechter, P. Papior, N. Tavalai, T. Stamminger, and A. Orr. 2006. PML contributes to a cellular mechanism of repression of herpes simplex virus type 1 infection that is inactivated by ICP0. *J Virol* 80:7995-8005.
171. Rasmussen, S. B., L. N. Sorensen, L. Malmgaard, N. Ank, J. D. Baines, Z. J. Chen, and S. R. Paludan. 2007. Type I interferon production during herpes simplex virus infection is controlled by cell-type-specific viral recognition through Toll-like receptor 9, the mitochondrial antiviral signaling protein pathway, and novel recognition systems. *J Virol* 81:13315-13324.
172. Krug, A., G. D. Luker, W. Barchet, D. A. Leib, S. Akira, and M. Colonna. 2004. Herpes simplex virus type 1 activates murine natural interferon-producing cells through toll-like receptor 9. *Blood* 103:1433-1437.
173. Wuest, T., B. A. Austin, S. Uematsu, M. Thapa, S. Akira, and D. J. Carr. 2006. Intact TRL 9 and type I interferon signaling pathways are required to augment HSV-1 induced corneal CXCL9 and CXCL10. *J Neuroimmunol* 179:46-52.
174. Lima, G. K., G. P. Zolini, D. S. Mansur, B. H. Freire Lima, U. Wischhoff, R. G. Astigarraga, M. F. Dias, M. das Gracas Almeida Silva, S. R. Bela, L. R. do Valle Antonelli, R. M. Arantes, R. T. Gazzinelli, A. Bafica, E. G. Kroon, and M. A. Campos. 2010. Toll-like receptor (TLR) 2 and TLR9 expressed in trigeminal ganglia are critical to viral control during herpes simplex virus 1 infection. *Am J Pathol* 177:2433-2445.
175. Aderem, A., and R. J. Ulevitch. 2000. Toll-like receptors in the induction of the innate immune response. *Nature* 406:782-787.
176. Iwasaki, A. 2010. Antiviral immune responses in the genital tract: clues for vaccines. *Nat Rev Immunol* 10:699-711.
177. Sergerie, Y., S. Rivest, and G. Boivin. 2007. Tumor necrosis factor-alpha and interleukin-1 beta play a critical role in the resistance against lethal herpes simplex virus encephalitis. *J Infect Dis* 196:853-860.
178. Kurt-Jones, E. A., M. Chan, S. Zhou, J. Wang, G. Reed, R. Bronson, M. M. Arnold, D. M. Knipe, and R. W. Finberg. 2004. Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis. *Proc Natl Acad Sci U S A* 101:1315-1320.
179. Zhang, S. Y., E. Jouanguy, S. Ugolini, A. Smahi, G. Elain, P. Romero, D. Segal, V. Sancho-Shimizu, L. Lorenzo, A. Puel, C. Picard, A. Chapgier, S. Plancoulaine, M. Titeux, C. Cognet, H. von Bernuth, C. L. Ku, A. Casrouge, X. X. Zhang, L. Barreiro, J. Leonard, C. Hamilton, P. Lebon, B. Heron, L. Vallee, L. Quintana-Murci, A. Hovnanian, F. Rozenberg, E. Vivier, F. Geissmann, M. Tardieu, L. Abel, and J. L. Casanova. 2007. TLR3 deficiency in patients with herpes simplex encephalitis. *Science* 317:1522-1527.
180. Li, T., B. A. Diner, J. Chen, and I. M. Cristea. 2012. Acetylation modulates cellular distribution and DNA sensing ability of interferon-inducible protein IFI16. *Proc Natl Acad Sci U S A* 109:10558-10563.
181. Johnson, K. E., L. Chikoti, and B. Chandran. 2013. HSV-1 Infection Induces Activation and Subsequent Inhibition of the IFI16 and NLRP3 Inflammasomes. *J Virol*.

182. Sparwasser, T., T. Miethke, G. Lipford, K. Borschert, H. Hacker, K. Heeg, and H. Wagner. 1997. Bacterial DNA causes septic shock. *Nature* 386:336-337.
183. Pisetsky, D. S. 2008. The role of innate immunity in the induction of autoimmunity. *Autoimmun Rev* 8:69-72.
184. Tabeta, K., P. Georgel, E. Janssen, X. Du, K. Hoebe, K. Crozat, S. Mudd, L. Shamel, S. Sovath, J. Goode, L. Alexopoulou, R. A. Flavell, and B. Beutler. 2004. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proc Natl Acad Sci U S A* 101:3516-3521.
185. Horan, K. A., K. Hansen, M. R. Jakobsen, C. K. Holm, S. Soby, L. Unterholzner, M. Thompson, J. A. West, M. B. Iversen, S. B. Rasmussen, S. Ellermann-Eriksen, E. Kurt-Jones, S. Landolfo, B. Damania, J. Melchjorsen, A. G. Bowie, K. A. Fitzgerald, and S. R. Paludan. 2013. Proteasomal degradation of herpes simplex virus capsids in macrophages releases DNA to the cytosol for recognition by DNA sensors. *J Immunol* 190:2311-2319.
186. Orzalli, M. H., N. A. DeLuca, and D. M. Knipe. 2012. Nuclear IFI16 induction of IRF-3 signaling during herpesviral infection and degradation of IFI16 by the viral ICP0 protein. *Proc Natl Acad Sci U S A* 109:E3008-3017.
187. Ludlow, L. E., R. W. Johnstone, and C. J. Clarke. 2005. The HIN-200 family: more than interferon-inducible genes? *Exp Cell Res* 308:1-17.
188. Cheng, X., Y. Chen, J. J. Xie, R. Yao, X. Yu, M. Y. Liao, Y. J. Ding, T. T. Tang, Y. H. Liao, and Y. Cheng. 2008. Suppressive oligodeoxynucleotides inhibit atherosclerosis in ApoE(-/-) mice through modulation of Th1/Th2 balance. *J Mol Cell Cardiol* 45:168-175.
189. Kahlenberg, J. M., S. G. Thacker, C. C. Berthier, C. D. Cohen, M. Kretzler, and M. J. Kaplan. 2011. Inflammasome activation of IL-18 results in endothelial progenitor cell dysfunction in systemic lupus erythematosus. *J Immunol* 187:6143-6156.
190. Geiss, G. K., R. E. Bumgarner, B. Birditt, T. Dahl, N. Dowidar, D. L. Dunaway, H. P. Fell, S. Ferree, R. D. George, T. Grogan, J. J. James, M. Maysuria, J. D. Mitton, P. Oliveri, J. L. Osborn, T. Peng, A. L. Ratcliffe, P. J. Webster, E. H. Davidson, L. Hood, and K. Dimitrov. 2008. Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nat Biotechnol* 26:317-325.
191. Lamkanfi, M., A. Sarkar, L. Vande Walle, A. C. Vitari, A. O. Amer, M. D. Wewers, K. J. Tracey, T. D. Kanneganti, and V. M. Dixit. 2010. Inflammasome-dependent release of the alarmin HMGB1 in endotoxemia. *J Immunol* 185:4385-4392.
192. Yang, D., Y. V. Postnikov, Y. Li, P. Tewary, G. de la Rosa, F. Wei, D. Klinman, T. Gioannini, J. P. Weiss, T. Furusawa, M. Bustin, and J. J. Oppenheim. 2012. High-mobility group nucleosome-binding protein 1 acts as an alarmin and is critical for lipopolysaccharide-induced immune responses. *J Exp Med* 209:157-171.
193. Krieg, A. M. 2006. Therapeutic potential of Toll-like receptor 9 activation. *Nat Rev Drug Discov* 5:471-484.
194. Fernandes-Alnemri, T., J. Wu, J. W. Yu, P. Datta, B. Miller, W. Jankowski, S. Rosenberg, J. Zhang, and E. S. Alnemri. 2007. The pyroptosome: a

supramolecular assembly of ASC dimers mediating inflammatory cell death via caspase-1 activation. *Cell Death Differ* 14:1590-1604.
195. Hornung, V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdorfer, T. Giese, S. Endres, and G. Hartmann. 2002. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol* 168:4531-4537.
196. Meixenberger, K., F. Pache, J. Eitel, B. Schmeck, S. Hippenstiel, H. Slevogt, P. N'Guessan, M. Witzenrath, M. G. Netea, T. Chakraborty, N. Suttorp, and B. Opitz. 2010. Listeria monocytogenes-infected human peripheral blood mononuclear cells produce IL-1beta, depending on listeriolysin O and NLRP3. *J Immunol* 184:922-930.
197. Warren, S. E., D. P. Mao, A. E. Rodriguez, E. A. Miao, and A. Aderem. 2008. Multiple Nod-like receptors activate caspase 1 during Listeria monocytogenes infection. *J Immunol* 180:7558-7564.
198. Halpern, M. D., Pisetsky, D. S. 1994. In vitro inhibition of murine IFNg production by phosphorothioate deoxyguanosine oligomers. *Immunopharmacology* 29:47-52.
199. Smith, J. S., and N. J. Robinson. 2002. Age-specific prevalence of infection with herpes simplex virus types 2 and 1: a global review. *J Infect Dis* 186 Suppl 1:S3-28.
200. Kodukula, P., T. Liu, N. V. Rooijen, M. J. Jager, and R. L. Hendricks. 1999. Macrophage control of herpes simplex virus type 1 replication in the peripheral nervous system. *J Immunol* 162:2895-2905.
201. Fujioka, N., R. Akazawa, K. Ohashi, M. Fujii, M. Ikeda, and M. Kurimoto. 1999. Interleukin-18 protects mice against acute herpes simplex virus type 1 infection. *J Virol* 73:2401-2409.
202. Nickel, W., and M. Seedorf. 2008. Unconventional mechanisms of protein transport to the cell surface of eukaryotic cells. *Annu Rev Cell Dev Biol* 24:287-308.
203. Nickel, W., and C. Rabouille. 2009. Mechanisms of regulated unconventional protein secretion. *Nat Rev Mol Cell Biol* 10:148-155.
204. Bryant, C., and K. A. Fitzgerald. 2009. Molecular mechanisms involved in inflammasome activation. *Trends Cell Biol* 19:455-464.
205. Martinon, F., K. Burns, and J. Tschopp. 2002. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* 10:417-426.
206. Dinarello, C. A. 2009. Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol* 27:519-550.
207. Veeranki, S., X. Duan, R. Panchanathan, H. Liu, and D. Choubey. 2011. IFI16 protein mediates the anti-inflammatory actions of the type-I interferons through suppression of activation of caspase-1 by inflammasomes. *PLoS One* 6:e27040.
208. Chou, J., and B. Roizman. 1992. The gamma 1(34.5) gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programed cell death in neuronal cells. *Proc Natl Acad Sci U S A* 89:3266-3270.

209. Melroe, G. T., N. A. DeLuca, and D. M. Knipe. 2004. Herpes simplex virus 1 has multiple mechanisms for blocking virus-induced interferon production. *J Virol* 78:8411-8420.
210. Freemont, P. S. 2000. RING for destruction? *Curr Biol* 10:R84-87.
211. Hochrein, H., B. Schlatter, M. O'Keeffe, C. Wagner, F. Schmitz, M. Schiemann, S. Bauer, M. Suter, and H. Wagner. 2004. Herpes simplex virus type-1 induces IFN-alpha production via Toll-like receptor 9-dependent and -independent pathways. *Proc Natl Acad Sci U S A* 101:11416-11421.
212. Miettinen, J. J., S. Matikainen, and T. A. Nyman. 2012. Global secretome characterization of herpes simplex virus 1-infected human primary macrophages. *J Virol* 86:12770-12778.
213. Lundberg, P., C. Ramakrishna, J. Brown, J. M. Tyszka, M. Hamamura, D. R. Hinton, S. Kovats, O. Nalcioglu, K. Weinberg, H. Openshaw, and E. M. Cantin. 2008. The immune response to herpes simplex virus type 1 infection in susceptible mice is a major cause of central nervous system pathology resulting in fatal encephalitis. *J Virol* 82:7078-7088.
214. Manji, G. A., L. Wang, B. J. Geddes, M. Brown, S. Merriam, A. Al-Garawi, S. Mak, J. M. Lora, M. Briskin, M. Jurman, J. Cao, P. S. DiStefano, and J. Bertin. 2002. PYPAF1, a PYRIN-containing Apaf1-like protein that assembles with ASC and regulates activation of NF-kappa B. *J Biol Chem* 277:11570-11575.
215. Biswas, P. S., K. Banerjee, B. Kim, and B. T. Rouse. 2004. Mice transgenic for IL-1 receptor antagonist protein are resistant to herpetic stromal keratitis: possible role for IL-1 in herpetic stromal keratitis pathogenesis. *J Immunol* 172:3736-3744.
216. Melchjorsen, J., J. Siren, I. Julkunen, S. R. Paludan, and S. Matikainen. 2006. Induction of cytokine expression by herpes simplex virus in human monocyte-derived macrophages and dendritic cells is dependent on virus replication and is counteracted by ICP27 targeting NF-kappaB and IRF-3. *J Gen Virol* 87:1099-1108.
217. Suresh, P. S., and A. B. Tullo. 1999. Herpes simplex keratitis. *Indian J Ophthalmol* 47:155-165.
218. Carr, D. J., P. Harle, and B. M. Gebhardt. 2001. The immune response to ocular herpes simplex virus type 1 infection. *Exp Biol Med (Maywood)* 226:353-366.
219. Brazolot Millan, C. L., R. Weeratna, A. M. Krieg, C. A. Siegrist, and H. L. Davis. 1998. CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. *Proc Natl Acad Sci U S A* 95:15553-15558.
220. Davis, H. L., R. Weeratna, T. J. Waldschmidt, L. Tygrett, J. Schorr, and A. M. Krieg. 1998. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. *J Immunol* 160:870-876.
221. McCluskie, M. J., J. L. Cartier, A. J. Patrick, D. Sajic, R. D. Weeratna, K. L. Rosenthal, and H. L. Davis. 2006. Treatment of intravaginal HSV-2 infection in mice: a comparison of CpG oligodeoxynucleotides and resiquimod (R-848). *Antiviral Res* 69:77-85.

222. Severa, M., E. M. Coccia, and K. A. Fitzgerald. 2006. Toll-like receptor-dependent and -independent viperin gene expression and counter-regulation by PRDI-binding factor-1/BLIMP1. *J Biol Chem* 281:26188-26195.
223. Charrel-Dennis, M., E. Latz, K. A. Halmen, P. Trieu-Cuot, K. A. Fitzgerald, D. L. Kasper, and D. T. Golenbock. 2008. TLR-independent type I interferon induction in response to an extracellular bacterial pathogen via intracellular recognition of its DNA. *Cell Host Microbe* 4:543-554.
224. Sharma, S., R. B. DeOliveira, P. Kalantari, P. Parroche, N. Goutagny, Z. Jiang, J. Chan, D. C. Bartholomeu, F. Lauw, J. P. Hall, G. N. Barber, R. T. Gazzinelli, K. A. Fitzgerald, and D. T. Golenbock. 2011. Innate immune recognition of an AT-rich stem-loop DNA motif in the Plasmodium falciparum genome. *Immunity* 35:194-207.
225. Krieg, A. M., A. K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky, and D. M. Klinman. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374:546-549.
226. Murad, Y. M., and T. M. Clay. 2009. CpG oligodeoxynucleotides as TLR9 agonists: therapeutic applications in cancer. *BioDrugs* 23:361-375.
227. Becker, Y. 2005. CpG ODNs treatments of HIV-1 infected patients may cause the decline of transmission in high risk populations - a review, hypothesis and implications. *Virus Genes* 30:251-266.
228. Kline, J. N., and A. M. Krieg. 2008. Toll-like receptor 9 activation with CpG oligodeoxynucleotides for asthma therapy. *Drug News Perspect* 21:434-439.
229. Kline, J. N. 2007. Immunotherapy of asthma using CpG oligodeoxynucleotides. *Immunol Res* 39:279-286.
230. Fallarino, F., C. Volpi, T. Zelante, C. Vacca, M. Calvitti, M. C. Fioretti, P. Puccetti, L. Romani, and U. Grohmann. 2009. IDO mediates TLR9-driven protection from experimental autoimmune diabetes. *J Immunol* 183:6303-6312.
231. Krieg, A. M., and J. Vollmer. 2007. Toll-like receptors 7, 8, and 9: linking innate immunity to autoimmunity. *Immunol Rev* 220:251-269.
232. Gupta, G. K., and D. K. Agrawal. 2010. CpG oligodeoxynucleotides as TLR9 agonists: therapeutic application in allergy and asthma. *BioDrugs* 24:225-235.
233. Graham, K. L., L. Y. Lee, J. P. Higgins, L. Steinman, P. J. Utz, and P. P. Ho. 2010. Treatment with a toll-like receptor inhibitory GpG oligonucleotide delays and attenuates lupus nephritis in NZB/W mice. *Autoimmunity* 43:140-155.
234. Krieg, A. M. CpG still rocks! 2012. Update on an accidental drug. *Nucleic Acid Ther* 22:77-89.
235. Roush, W. 1997. Antisense aims for a renaissance. *Science* 276:1192-1193.
236. Stein, C. A., J. L. Tonkinson, and L. Yakubov. 1991. Phosphorothioate oligodeoxynucleotides--anti-sense inhibitors of gene expression? *Pharmacol Ther* 52:365-384.
237. Stein, C. A. 1992. Anti-sense oligodeoxynucleotides--promises and pitfalls. *Leukemia* 6:967-974.
238. Tailor, P., T. Tamura, and K. Ozato. 2006. IRF family proteins and type I interferon induction in dendritic cells. *Cell Res* 16:134-140.

239. van Strijp, J. A., K. P. van Kessel, L. A. Miltenburg, A. C. Fluit, and J. Verhoef. 1988. Attachment of human polymorphonuclear leukocytes to herpes simplex virus-infected fibroblasts mediated by antibody-independent complement activation. *J Virol* 62:847-850.
240. Van Strijp, J. A., K. P. Van Kessel, M. E. van der Tol, and J. Verhoef. 1989. Complement-mediated phagocytosis of herpes simplex virus by granulocytes. Binding or ingestion. *J Clin Invest* 84:107-112.
241. Van Strijp, J. A., K. P. Van Kessel, M. E. van der Tol, A. C. Fluit, H. Snippe, and J. Verhoef. 1989. Phagocytosis of herpes simplex virus by human granulocytes and monocytes. *Arch Virol* 104:287-298.
242. Tumpey, T. M., S. H. Chen, J. E. Oakes, and R. N. Lausch. 1996. Neutrophil-mediated suppression of virus replication after herpes simplex virus type 1 infection of the murine cornea. *J Virol* 70:898-904.
243. Miller, L. S., E. M. Pietras, L. H. Uricchio, K. Hirano, S. Rao, H. Lin, R. M. O'Connell, Y. Iwakura, A. L. Cheung, G. Cheng, and R. L. Modlin. 2007. Inflammasome-mediated production of IL-1beta is required for neutrophil recruitment against Staphylococcus aureus in vivo. *J Immunol* 179:6933-6942.
244. Rider, P., Y. Carmi, O. Guttman, A. Braiman, I. Cohen, E. Voronov, M. R. White, C. A. Dinarello, and R. N. Apte. 2011. IL-1alpha and IL-1beta recruit different myeloid cells and promote different stages of sterile inflammation. *J Immunol* 187:4835-4843.
245. Mitroulis, I., K. Kambas, A. Chrysanthopoulou, P. Skendros, E. Apostolidou, I. Kourtzelis, G. I. Drosos, D. T. Boumpas, and K. Ritis. 2013. Neutrophil extracellular trap formation is associated with IL-1beta and autophagy-related signaling in gout. *PLoS One* 6:e29318.
246. Qamar, A., and D. J. Rader. 2012. Effect of interleukin 1beta inhibition in cardiovascular disease. *Curr Opin Lipidol* 23:548-553.
247. Genovese, M. C., S. Cohen, L. Moreland, D. Lium, S. Robbins, R. Newmark, and P. Bekker. 2004. Combination therapy with etanercept and anakinra in the treatment of patients with rheumatoid arthritis who have been treated unsuccessfully with methotrexate. *Arthritis Rheum* 50:1412-1419.