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Characterization of Innate Immune Pathways in DNA Vaccine-Induced, Antigen-Specific Immune Responses: A Dissertation

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CHARACTERIZATION OF INNATE IMMUNE PATHWAYS IN DNA VACCINE-
INDUCED, ANTIGEN-SPECIFIC IMMUNE RESPONSES

A Dissertation Presented

By

John Joseph Suschak III

Submitted to the Faculty of the University of Massachusetts Graduate School of
Biomedical Sciences, Worcester in partial fulfillment of the requirement for the degree of

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December 8, 2014

Immunology and Microbiology Program

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Immunology and Microbiology Program

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Abstract

A major advantage of DNA vaccination is the ability to induce both humoral and cellular immune responses. DNA vaccines are currently used in veterinary medicine, but their tendency to display low immunogenicity in humans has hindered their usage, despite excellent tolerability and safety profiles. Various approaches have been used to improve the immunogenicity of DNA vaccines. Recent human study data re-established the value of DNA vaccines, especially in priming high-level antigen-specific antibody responses. Data suggests that innate immune responses to the DNA vaccine plasmid itself contribute to the immunogenicity of DNA vaccines, however the underlying mechanisms responsible remain unclear. In this dissertation, we investigate the role of innate immunity in shaping antigen-specific adaptive immune responses following DNA vaccination.

The current belief is that the cytosolic DNA sensing pathways govern DNA vaccine immunogenicity. To date, only the type I interferon inducing STING/TBK1 regulatory pathway has been identified as required for DNA vaccine immunogenicity. Surprisingly, neither the upstream receptor nor the downstream signaling molecules in this pathway have been characterized. I therefore investigated a candidate cytosolic DNA receptor, as well as the downstream transcription factors required for generation of antigen-specific immune responses. Additionally, the effects of pro-inflammatory signaling on DNA vaccine immunogenicity have yet to be comprehensively studied. Previous studies have only provided indirect evidence for the role of inflammatory

signaling in DNA vaccination. As such, I also investigated the role of the DNA sensing AIM2 inflammasome in DNA vaccination. My data indicates that AIM2 is a key modulator in DNA vaccination via a previously unrecognized connection to type I interferon. Importantly, this marks the first time a DNA vaccine sensor has been identified.

Of note, this dissertation represents a departure from many published works in the field. Whereas previous studies have mostly utilized model antigens and only focused on the adaptive immune responses generated, I analyzed the effects on innate immunity as well. Using various innate gene knockout murine models, I quantified antigen-specific humoral and T cell responses, as well as serum cytokine and chemokines following immunization with a clinically relevant DNA vaccine. Overall, this data provides a basis for understanding the mechanisms of DNA vaccination, allowing for the design of more effective vaccines.

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List of Abbreviations

AIM2	Absent in melanoma 2
ALOH3	Aluminum hydroxide gel
APC	Antigen-presenting cell
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine 5'triphosphate
BMDC	Bone marrow-derived dendritic cells
BMDM	Bone marrow-derived macrophage cells
CARD	Caspase activation and recruitment domain
CBA	Cytometric bead array
cGAS	Cyclic guanosine monophosphate–adenosine monophosphate synthase
cGAMP	Cyclic guanosine monophosphate–adenosine monophosphate
CMI	Cell-mediated immunity
CMV	cytomegalovirus
CpG	Deoxycytidylate-phosphate-deoxyguanylate
CTL	Cytotoxic T Lymphocyte
DAI	DNA-dependent activator of IFN-regulatory factors
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot assay

EP	Electroporation
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine system
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GTP	Guanosine 5'-triphosphate
HIV-1	Human Immunodeficiency virus type 1
HMGB1	high mobility group box 1 protein
IFI16	Gamma-interferon-inducible-protein 16
IFN- $\alpha\beta$	Type I Interferon
IL	Interleukin
IFNAR	Type I interferon receptor
IM	Intramuscular injection
IRF	Interferon regulatory transcription factor
KC	Keratinocyte chemoattractant
LPS	Lipopolysaccharide
MEF	Mouse embryonic fibroblast
MHC	Major histocompatibility complex
MIP-1 α	Macrophage inflammatory protein 1 α
MIP-1 β	Macrophage inflammatory protein 1 β
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response 88

NaSCN	Sodium thiocyanate
NFκB	Nuclear factor kappa B
NK	Natural killer cell
NLR	Nucleotide-binding oligomerization domain (NOD) like receptors
NLRP3	NOD-like receptor 3
OAS	2'-5'-oligoadenylate synthase
PAMP	Pathogen-associated molecular patterns
pDC	Plasmacytoid dendritic cell
PYHIN	Pyrin and HIN domain-containing protein family
PRR	Pattern recognition receptor
RANTES	Regulated on activation, normal T cell expressed and secreted
STING	Stimulator of interferon genes
TBK1	TANK-binding kinase 1
TIV	Trivalent Inactivated Vaccine
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRIF	TIR-domain-containing adaptor-inducing interferon
VSV	Vesicular stomatitis virus

Chapter I

1. Principles of Vaccination and Current Vaccine Classifications.

Vaccines induce protective immune responses that prevent or control an infection. They represent one of the great achievements in public health. Edward Jenner's demonstration of vaccination to protect humans from small pox over 200 years ago marked a turning point in the war against infection by a pathogen. Today, the use of safe and effective vaccines to prevent infection is a fundamental aspect of modern medicine. While many vaccination campaigns have been successful, emerging pathogens constantly challenge scientists to develop new vaccination technologies for improved safety and efficacy.

The goals of each vaccine are dependent on the immune response required for protection against a given pathogen. Previously, the effectiveness of a vaccine was predominantly evaluated by its ability to induce protective antibodies, but recent studies suggest that the induction of T cell responses, especially T helper 1 (T_h1) and $CD8^+$ T cells, may be desirable for protection against intracellular pathogens [1-3]. Ideally, the most efficient vaccines will stimulate both cellular and humoral immune responses [4]. Therefore, many investigators are striving to identify new vaccine formulations capable of generating balanced immunity.

While the principles and effectiveness of vaccination are firmly established, many of the underlying immunological mechanisms remain unclear. Intensified research in the field of vaccine immunology suggests that triggering of the innate immune system enhances the adaptive immune response generated by vaccination [5-7]. This appears to

be a common trait of many efficient vaccines. However, as the study of innate immunity is still in its nascent stages, the signals required for conditioning adaptive immune responses are not completely understood. Increased insight into the effect of innate immune signals on the adaptive response will provide a basis for the design of future vaccines.

1.1 Live Attenuated Vaccines.

Several types of vaccines are currently licensed for clinical use. The first, and historically most protective, are live-attenuated vaccines. Live-attenuated vaccines are derived from disease causing viruses or bacteria that have been weakened, usually by repeated culture passage. Live-attenuated vaccines replicate within the host, resulting in low levels of infection without disease. They elicit strong cellular and antibody responses, as well as sustained immunity after one or two doses. As they are living, pathogenic organisms, live-attenuated vaccines do have some inherent safety concerns. Immunocompromised individuals may not be indicated for live attenuated vaccines. To certain high-risk pathogens, such as human immunodeficiency virus 1 (HIV-1), it is difficult to produce a live attenuated vaccine with a sufficient safety margin. Furthermore, although extremely rare, the possibility for reversion to a pathogenic form exists [8]. As such, although live-attenuated vaccines have been extremely successful in the past, they can present challenges for use in future vaccine development.

1.2 Killed or Inactivated Vaccines.

A second type of vaccine encompasses killed or inactivated vaccines (often toxoid vaccines against several bacterial pathogens are included in this group as well). These vaccines represent an alternative to live-attenuated vaccines, as they are not live and

cannot replicate. Typically, inactivation is accomplished through either physical methods such as heating or ultraviolet light treatment, or chemical means such as formaldehyde or formalin. While inactivated vaccines are considered safer than live-attenuated vaccines, they are generally less immunogenic, often requiring multiple doses for inducing protective immunity. Inactivated vaccines tend to induce only humoral responses, with limited ability to elicit high-level cellular immunity. Antibody titers elicited by inactivated vaccines often diminish with time, requiring periodic boosting doses in future years. Additionally, inactivation may also have the unwanted side effect of altering the antigen's structure, thereby inhibiting the development of antibodies against the critical conformational antigens.

1.3 Subunit Vaccines.

A third type of vaccine are the subunit vaccines, which like inactivated vaccines do not contain live components, but instead contain only the antigenic parts of the pathogen. Subunit vaccines can be further categorized depending on the nature of the antigen i.e. recombinant protein, polysaccharide, or conjugate vaccine wherein the sugar antigens are attached to a carrier protein. As with inactivated vaccines, subunit vaccines generally induce protection for a much shorter duration than live-attenuated vaccines and require a series of vaccinations to even establish the initial protective immunity. Recombinant subunit protein vaccines often require the addition of an adjuvant as part of the formulation to increase immunogenicity. Also, subunit vaccines have not proven especially effective in generating strong CD8⁺ T cell responses.

The conventional vaccine approaches described above have proven effective in preventing disease against a range of pathogens. However, they have had only varying degrees of success against such major infections in the world as malaria and HIV. In this regard, subunit HIV vaccines alone have failed to protect in phase III efficacy trials [9, 10] and the concern about the safety and efficacy of live-attenuated and inactivated vaccines has prevented their use. These factors have illustrated the need for additional vaccine technology, preferably one that marries the immunogenicity of live attenuated vaccines with the safety of subunit/inactivated vaccines. DNA vaccines share these characteristics, and represent a novel vaccine strategy for inducing protective immune responses for the reasons outlined below.

2. DNA Vaccination.

2.1 DNA Vaccine Plasmid.

DNA immunization refers to the induction of an immune response to a protein antigen expressed *in vivo* following the introduction of plasmid DNA encoding the polypeptide sequence of candidate antigens [11]. The encoded antigen is expressed under a strong promoter; the most common choice being the human cytomegalovirus (CMV) immediate early promoter, as it yields high levels of transgene expression [12]. Inclusion of transcriptional enhancers such as Intron A enhances the rate of polyadenylation and nuclear transport of messenger RNA (mRNA) [13]. The vaccine plasmids are generally produced in bacterial culture, purified, and then used to inoculate the host.

2.2 Benefits of DNA Vaccine Platform.

DNA vaccination provides several advantages over other traditional vaccination

strategies. DNA vaccines represent a safe, non-live vaccine approach to inducing immunity. Importantly, DNA vaccines are capable of eliciting both antigen specific antibodies [14] and cytotoxic T lymphocyte responses (CTL) [15], something that remains elusive in most non-live vaccines. The antigen of interest delivered by DNA vaccination is produced endogenously and presented to the immune system without concern of safety, a common concern with live-attenuated vaccines.

In vivo production of antigen allows for presentation by both class I and class II major histocompatibility complex (MHC) molecules. As a result, both CD4⁺ and CD8⁺ T cell responses are generated. The generation of T helper populations is critical for the induction of high quality antibody responses via the induction of antigen-specific B cell responses. Furthermore, the relative ease of DNA vaccine design and production allows for the quick and efficient development of immunogens via recombinant DNA technology. DNA vaccines are also stable and can be used with multiple DNA vaccines.

2.3 Progress of DNA vaccination.

The concept of DNA vaccination arose in the early 1990s when Wolff *et al.* showed that intramuscular (IM) administration of naked DNA induced plasmid-encoded reporter genes in muscle cells [16]. The discovery that DNA immunization induces adaptive immune responses in small animals dramatically shifted the vaccine paradigm. One of the first demonstrations of DNA vaccine immunogenicity centered on mice immunized with DNA encoding the influenza A nucleoprotein (NP) [11]. Immunized mice developed NP-specific antibodies and CTLs, suggesting successful antigen presentation to both MHC class I and II molecules. Immunized mice were protected against viral challenge and demonstrated accelerated viral clearance of both homologous

and heterologous viral strains [11]. Restimulation of CTL populations allowed for target cell lysing, proving that DNA vaccination could generate antigen-specific memory responses. These results were shown to be reproducible in a variety of animal models against a wide range of pathogens [17-19].

The success of small animal studies led to several human clinical trials. However, the protective immunity observed in small animals and non-human primates was not observed in human studies when DNA vaccines were used alone and delivered by conventional needle injection. Although DNA vaccines were safe and well tolerated, they proved to be poorly immunogenic. Antibody titers were either low or nonexistent, and CTL responses were inconsistent [20]. Also, no changes in viral load or lymphocyte counts were observed. Some success was obtained with highly immunogenic antigens, especially when a gene gun approach was used. For example, human subjects immunized with a DNA vaccine expressing hemagglutinin antigen of influenza H1 developed measurable anti-HA antibodies by gene gun inoculation, establishing that DNA vaccination can induce immune responses in human subjects [21, 22]. The reasons for lower immunogenicity in humans remain unclear, but several advances in current trials have shown that DNA vaccination can be an attractive platform either alone or as part of a prime-boost platform.

In an effort to increase immunogenicity, several improvements were made to the vaccine delivery method. An important approach to overcoming low immunogenicity is to increase the amount of DNA plasmid delivered to cells by external physical force. The traditional route of DNA immunization is IM injection of DNA diluted in saline, which has the benefit of requiring no special delivery system and can be delivered without

specific tools or instruments. However, standard IM injection is associated with low transfection efficiency. Therefore, many immunization regimens utilize intramuscular electroporation to optimize vaccine uptake by cells at the site of injection.

Electroporation has long been known to increase transfection efficiency *in vitro*, but evidence suggests it also has positive effects *in vivo*. Injection of naked plasmid followed by application of an electrical pulse induces transient enhancement of cell permeability, allowing DNA to traverse the lipid bilayer down the concentration gradient [23, 24]. It is difficult to quantify directly the enhancement of plasmid delivery, but indirect measurements have shown both increased gene expression and immune responses following administration of an electrical pulse [25-29]. Electroporation also has the added advantage of enhancing the influx of antigen presenting cells (APCs) to the site of injection, promoting antigen presentation [30].

Another popular technique involves propelling DNA plasmid-coated gold beads into the skin using a gene gun. The Helios gene gun (Bio-Rad Laboratories, Irvine, CA) uses high-pressure, helium gas-powered particle bombardment to directly transfect target cells or tissues. Studies have demonstrated the ability of gene gun immunization to induce protective immunity in both mice [31] and humans [32, 33]. Several differences exist between IM and gene gun immunization. As the gene gun delivery system is more efficient in delivering vaccine into cells, significantly less plasmid DNA is required for generating protective immunity by gene gun immunization in mice [34, 35]. It is a potentially important issue that only a small amount of DNA can be coated onto the gold beads. However, the main reason preventing wider use of gene gun technology is the limited access to this technology.

Perhaps the major novel immunization method to have emerged from early human trials is the heterologous DNA prime-protein boost vaccine regimen. The heterologous prime-boost platform consists of an initial DNA-priming immunization followed by a boosting immunization of recombinant protein [36, 37]. DNA prime-protein boost immunization has been extensively examined in the context of multiple pathogens. Both preclinical [38-42] and human HIV studies [17] have shown promise in generating protective adaptive responses. Likewise, influenza A [43-46] and malaria [15, 47-49] trials have resulted in generation of protective immunity. Not only did these studies prove that DNA vaccination is feasible in humans, but they also demonstrated that DNA priming dramatically alters the adaptive response; with subjects receiving the DNA prime generating significantly increased CTL and humoral responses when compared to those receiving two doses of protein alone [43, 45]. Moreover, DNA immunization appears to improve the breadth and length of the adaptive immune response. In particular, the memory B cell response generated by DNA immunization is significantly enhanced, with sustained titers [35, 50] and improved B cell development within the germinal center, possibly a result of increased follicular helper T cell generation [51]. These results suggest that DNA vaccination provides an intrinsic adjuvant effect that alters the adaptive immune response through processes that remain unclear. One possible explanation for this outcome is the triggering of innate immune systems by DNA vaccine plasmids, resulting in signals that guide both the humoral and CTL responses.

2.4 Mechanism of DNA Vaccination.

The mechanism by which DNA vaccines induce adaptive immune responses has been well studied. The gene of interest is delivered either intradermally, subcutaneously,

or directly into muscle by one of the previously described delivery methods. It is believed that local myocytes and keratinocytes, including resident APC populations, are directly transfected by DNA vaccines via phago- or pinocytosis [52]. Upon entering the host nucleus, encoded genes are transcribed and translated by host cellular machinery [53], resulting in expression of antigenic peptides. The host-synthesized antigens mimic infection by either being presented as endogenous antigen on MHC class I molecules, or the antigen is shed exogenously, allowing for presentation on MHC class II molecules. Furthermore, the engulfment of apoptotic, transfected cells by APCs also allows for the cross-presentation of exogenous antigen. Antigen-loaded APCs travel to the draining lymph nodes where they present antigenic peptide–MHC complexes in combination with signalling by costimulatory molecules to naive T cells [52]. This interaction provides the necessary secondary signals to initiate an immune response, driving T cell activation, or alternatively, to activate B cell and antibody production cascades (**Figure 1.1**). In this way, both humoral and cellular immune responses are generated.

The *in vivo* production of antigen is a key component in generating protective immunity following DNA vaccination. As antigen is produced directly by host cells, antigenic proteins undergo well-regulated translation processes, allowing for preservation of native protein confirmation, as well as normal post-translational modifications, such as glycosylation. This allows for accurate mimicking of live-attenuated vaccines without the inherent safety risks [54, 55].

In addition to the *in vivo* production of antigen, it is hypothesized that the use of plasmid DNA itself plays a significant role in DNA vaccine immunogenicity. Plasmid DNA contains unmethylated deoxycytidylate-phosphate-deoxyguanylate (CpG) motifs,

which are known to prime APC populations, increasing their ability to stimulate cognate antigen specific CD4⁺ and CD8⁺ T cells. Furthermore, recent evidence has demonstrated that cytosolic DNA itself is a potent trigger of innate immune responses through a variety of pathways [56-58]. How each of these factors impact the immune response induced by DNA immunization will be addressed in the following sections.

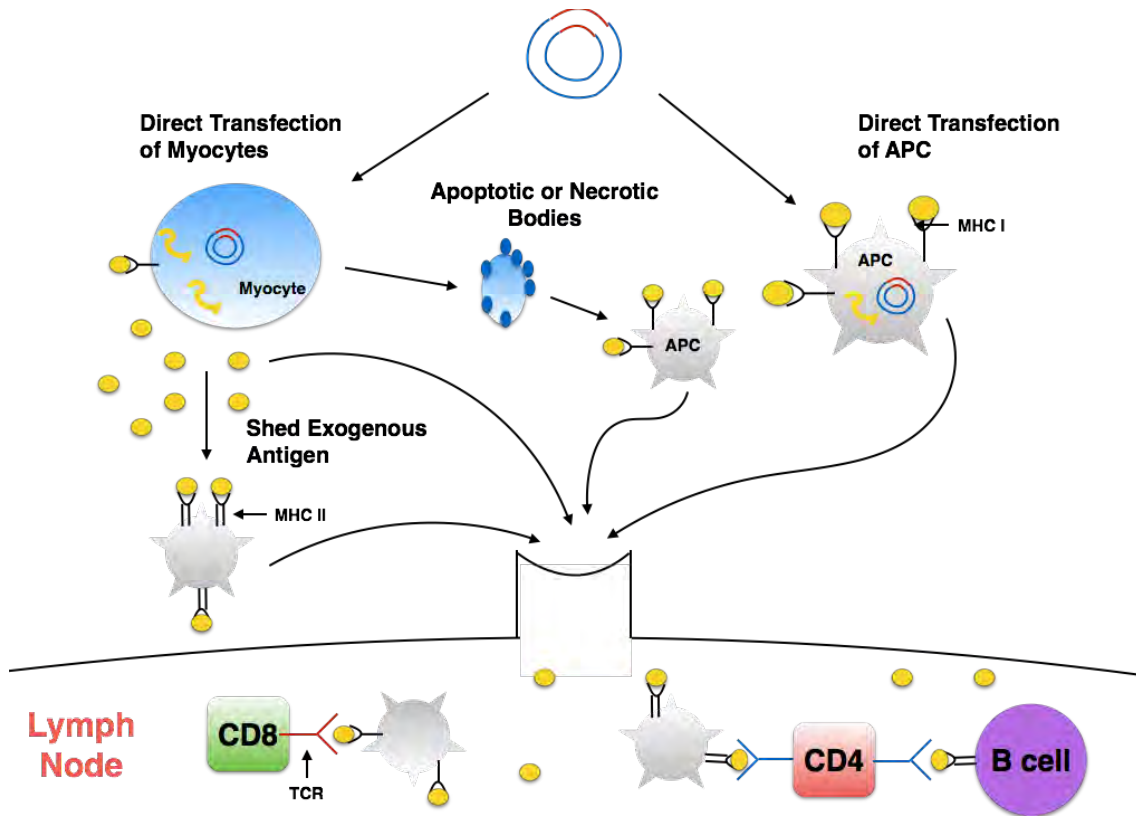


Figure 1.1: Induction of antigen-specific, adaptive immunity by DNA vaccination.

Optimized gene sequences are inserted into a plasmid backbone and then delivered to the host via one of several delivery methods. Vaccine plasmid enters the nucleus of host myocytes and antigen presenting cells by using host cellular machinery. The plasmid components are transcribed and protein is produced. The cell provides endogenous post-translational modifications to antigens, producing native protein conformations. Vaccine-derived endogenous peptides are presented on MHC class I molecules. Engulfment of apoptotic or necrotic cells by APCs also allows for cross-presentation of cell-associated exogenous antigens. Secreted antigen is captured and processed by antigen presenting cells, and presented on MHC class II. Antigen experienced APCs migrate to the draining lymph node to stimulate CD4⁺ and CD8⁺ T cell populations. In addition, shed antigen can be captured by antigen-specific high affinity immunoglobulins on the B cell surface for presentation to CD4⁺ T cells, driving B cell responses.

3. Innate Immune Signaling.

3.1 Pattern Recognition Receptors.

The immune system can be divided into two broad categories: the innate and the adaptive immune responses. In contrast to the adaptive immune system, which develops a broad repertoire of antigen-specific receptors, the innate immune system represents the first line of defense against invading microbes. The innate system's primary role is to initiate an immediate response designed to contain infection until adaptive responses can clear the pathogen. It responds to a large array of pathogens via a limited repertoire of germline-encoded receptors known as pattern-recognition receptors (PRRs) that tend to function at the APC level.

PRRs primarily recognize three types of immune triggers. The first trigger types are pathogen-associated molecular patterns (PAMPs). Microbial PAMPs include lipids such as lipopolysaccharide (LPS), bacterial flagellin, peptidoglycans, and nucleic acid variants normally associated with viruses or bacteria. Microbial nucleic acids, in particular, are extremely effective in stimulating PRRs. The strict specificity of PRRs allows for the differentiation of self nucleic acids from microbial nucleic acids (double-stranded RNA or unmethylated CpG motifs) based on differences in their structure, molecular modifications, or localization [59-61]. The second type of trigger involves the detection of endogenous molecules released upon necrotic or pyroptotic cell death. These so-called danger-associated molecular patterns (DAMPs) include many nuclear or cytosolic proteins, that upon exposure to the extracellular space, move from a reducing to oxidizing milieu, resulting in their denaturation [62]. Mislocalized self-nucleic acids also function as DAMPs [59, 63]. For example, RNA stimulates PRRs should it be present in

the extracellular space. Similarly, extranuclear DNA is an exceedingly powerful DAMP capable of activating a variety of PRRs. The final signal type, alarmins, are inhibitory signals expressed on healthy cells, but not found on infected cells or non-self pathogens [64]. In general, alarmins are not thought to play a significant role in vaccination.

3.2. Type I IFN and Regulation by PRRs.

3.2.1 Role of Type I IFN in Immune Responses.

Type I interferons (IFN- $\alpha\beta$) are the principal cytokines induced during viral and bacterial infection, and represent a vital component of the immune response. IFN- $\alpha\beta$ induces resistance to viral replication and directly activates natural killer (NK) cells. Evidence has also shown that IFN- $\alpha\beta$ production by natural interferon-producing cells promotes APC maturation [65, 66]. Notably, the effect of IFN- $\alpha\beta$ on antigen presentation is multi-faceted. IFN- $\alpha\beta$ secreted by antigen-experienced APCs stimulates bystander APCs, resulting in increased MHC presentation and up-regulation of co-stimulatory molecules, which drive the T cell, and subsequent B cell, response [65, 66]. IFN- $\alpha\beta$ also amplifies the sensitivity of the B cell receptor, boosting the ability of naïve B cells to produce antibodies upon antigen recognition [67]. IFN- $\alpha\beta$ receptor knockout (*Ifnar*^{-/-}) mice exhibit enhanced susceptibility to viral infection [68, 69]. The broad stimulatory effect of IFN- $\alpha\beta$ on the immune system illustrates its importance not only in pathogen immunity studies, but also vaccination as well [70, 71].

3.2.2. Interferon Regulatory Factors.

IFN- $\alpha\beta$ production is transcriptionally regulated by the interferon regulatory factor (IRF) family of proteins. The IRF family contains 9 members (IRF1 to IRF9) and modulates innate and adaptive immune responses as well as immune cell development.

Perhaps the best-characterized IRFs are IRF3 and IRF7. IRF3 and IRF7 share significant sequence homology, and are the central IRFs regulating type I IFN production during viral infection. The triggering of certain PRRs results in IRF3 phosphorylation, which alleviates the auto-inhibitory domain of IRF3, releasing its transactivation domain, and permitting IRF3 dimerization and nuclear translocation. IRF7 undergoes a similar procedure, homodimerizing with itself for IFN- α production, or forming heterodimers with IRF3 leading to IFN- β transcription (**Figure 1.2**).

IRF3 and 7 are crucial components in the anti-viral response generated by PRR activation [72]. The induction of IFN- $\alpha\beta$ in most cell types differs due to the varying endogenous expression levels of IRF3 and IRF7. IRF3 expression is constitutively high in most cells, thus IFN- β is strongly induced early following infection. Contrarily, IRF7 remains at low levels in resting cells. The initial burst of IFN- β triggers type I IFN signaling through the IFN- $\alpha\beta$ receptor to induce IRF7 expression via a positive feedback loop, which then acts on type I IFN genes for the production of high levels of IFN- α . There are many upstream regulators capable of inducing IRF3 and IRF7 activation, some of which will be described in the following sections.

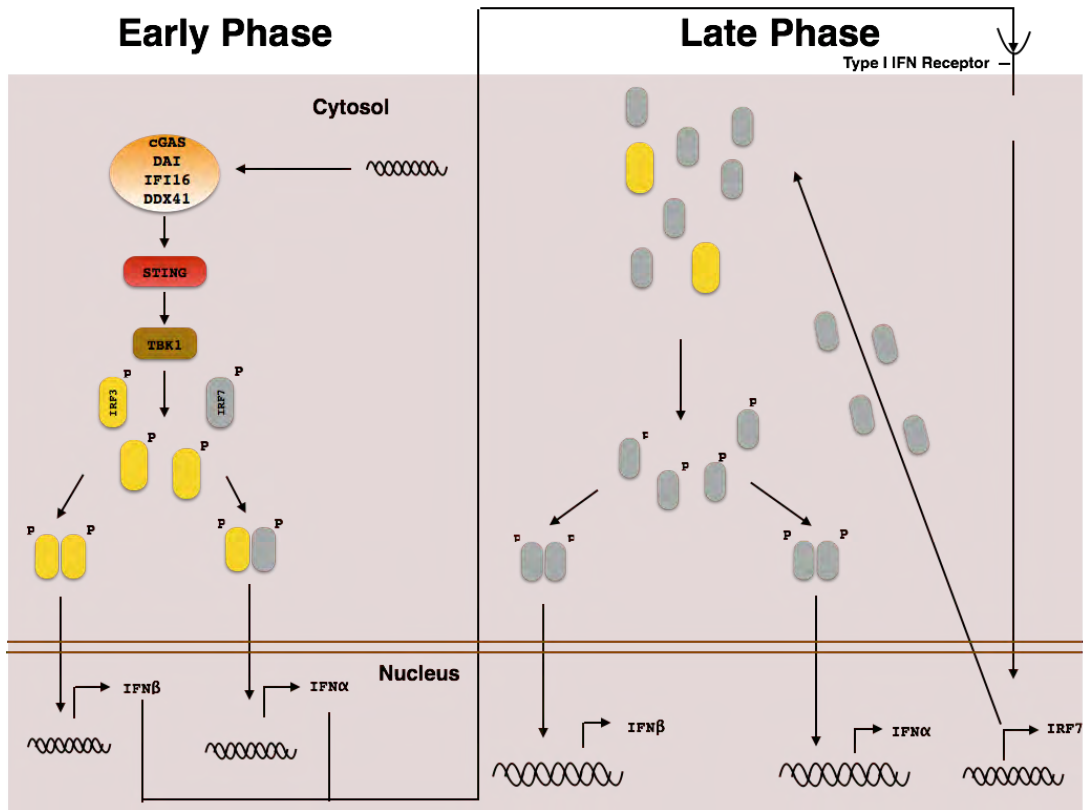


Figure 1.2: Interferon-regulatory factors 3 and 7 function as a positive-feedback loop for regulation of type I interferon genes.

Following detection of cytosolic DNA by one or multiple sensors, interferon regulatory factor 3 (IRF3) and IRF7 are phosphorylated on specific serine residues, resulting in the homodimerization or heterodimerization of IRF3 and IRF7. IRF dimers then translocate to the nucleus and induce small amounts of IFN- $\alpha\beta$. IRF7 is required for the induction of type I IFN genes. IRF3 also contributes to the induction of type I IFN genes (albeit to a lesser extent). In the late stages of IFN- $\alpha\beta$ production, secreted IFNs bind and activate the type I IFN receptor in an autocrine or paracrine manner, leading to the induction and transcription of the IRF7 gene. Activation of newly synthesized IRF7, leads to the expression of large amounts of I IFN- $\alpha\beta$ and many of the IFN- $\alpha\beta$ stimulated proteins, further propagating the positive feed back loop.

3.3 Nucleic Acid Sensing PRRs.

3.3.1 IFN Inducing PRRs.

3.3.1.1 Toll Like Receptors.

Toll-like Receptors (TLRs) are the most thoroughly studied PRRs. The Toll protein was initially identified in *Drosophila* as being necessary for dorsal-ventral patterning, but subsequent investigation established its importance in fly immunity. The TLRs, so named because of their homology to *Drosophila* Toll, play a similar role in mammalian immunity. TLRs detect a diverse array of PAMPs in the extracellular environment. Plasma membrane anchored TLRs typically detect hydrophobic lipids and proteins, while the endosomal receptors detect nucleic acids. 13 TLRs have been identified in mice, but only 10 have been identified in humans. TLRs 1-9 are common in both organisms. TLR1, TLR2, TLR4, TLR5 and TLR6 are found on the surface of the plasma membrane, while TLR3, TLR7, TLR8, and TLR9 are located within the endosome.

TLRs are potent producers of both IFN- $\alpha\beta$ and pro-inflammatory signals. Upon ligand binding, they initiate a variety of immune signaling cascades, resulting in the activation of nuclear factor kappa b (NF κ B) and IRF 1, 3, 5, and 7 [73]. The endosomal TLRs 3, 7, 8, and 9 are powerful receptors for nucleic acids, and therefore, a great deal of light has been shed on their ability to induce type I IFN in response to infection. While all four receptors are stimulated by nucleic acids, they each require specific ligands. Both TLR7 and 8 recognize long, single-stranded RNAs, but each leads to a distinct cytokine profile [74]. In contrast to TLR7 and 8, TLR3 is activated by dsRNA, a common viral PAMP [75]. Finally, TLR9 recognizes the unmethylated CpG motifs in viral and

bacterial DNA [76].

The TLR response evoked by PAMPs and DAMPs depends on a mixture of factors. Most notably, TLR expression varies amongst innate cell types. For instance, human macrophages express high levels of TLR2 and TLR4 while plasmacytoid dendritic cells (pDCs) mainly express TLR7 and TLR9 [73]. Furthermore, variations in TLR expression are seen between mammalian species. TLR9 expression is limited to a few cell types in humans, but widely distributed in mice [77]. The expression of certain downstream signaling molecules also fluctuates between innate cell types. pDCs are unique in that they constitutively express the transcription factor IRF7, allowing for quick production of type I IFN in response to viral infection while other cell types such as conventional macrophages may respond in a delayed manner [78, 79]. 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.

3.3.1.2 Cytosolic DNA Receptors.

TLRs detect viral PAMPs in the extracellular space, but a unique subset of innate sensors patrols the intracellular spaces as well (**Figure 1.3**). These sensors were discovered within the last decade during investigations of TLR-deficiency on viral infection and have yielded several classes of DNA sensors required for clearance of cytosolic replicating viruses.

3.3.1.2.1 STING/TBK1/IFN Pathway.

It has been known for many years that pathogen-derived DNA stimulates IFN- $\alpha\beta$ in TLR-deficient fibroblasts. Medzhitov *et al.* demonstrated that *Tlr9*^{-/-} mouse embryonic fibroblasts (MEFs) produce large amounts of IFN- $\alpha\beta$ when transfected with either B-

form dsDNA or genomic DNA isolated from bacteria, viruses, or mammals [58]. Further work has identified the key components of this pathway, namely the non-canonical I κ B kinase, TANK-binding kinase 1 (TBK1). Independent of TLR signaling, TBK1 directly phosphorylates IRF3 in response to intracellular, cytosolic DNA [56-58]. TBK1-deficient MEFs do not produce cytokines in response to B-form DNA treatment, establishing its role in pathogen infection. Another important molecule is the endoplasmic reticulum (ER) adaptor stimulator of interferon genes (STING) [80-82]. In unstimulated cells, STING localizes to the ER, but traffics to perinuclear vesicles upon cytosolic DNA detection [80]. STING appears to interact directly with TBK1 to induce IRF3 phosphorylation [83]. Additionally, *Sting*^{-/-} mice are highly susceptible to Herpes simplex virus 1 (HSV-1) and *Listeria monocytogenes* infection due to impaired production of IFN- $\alpha\beta$ [81]. While the requirement for STING in IFN- $\alpha\beta$ signaling in response to cytosolic DNA is well known, the upstream regulators of STING activation are still being identified.

3.3.1.2.2 Cytosolic STING Dependent DNA Receptors.

With the identification of STING and TBK1, the list of upstream cytosolic DNA sensors has grown considerably. The first DNA sensor identified was the DNA-dependent activator of IFN-regulatory factors (DAI, also known as DLM-1 or ZBP1). DAI binds both the left-handed, Z form DNA as well as the more naturally relevant B form DNA via two N-terminus Z-DNA binding domains [84]. Type I IFN production by fibroblasts in response to HCMV, HSV-1, and *Listeria monocytogenes* is DAI dependent. However, *DAI*^{-/-} mice respond normally to viral dsDNA challenge, suggesting that DAI is cell type specific and subject to redundancy.

The ubiquitously expressed nucleotidyltransferase cyclic GMP-AMP synthase (cGAS) was recently identified as another DNA sensor upstream of STING/TBK1/IRF3 [85-87]. cGAS synthesizes cyclic GMP-AMP (cGAMP) from ATP and GTP upon recognition of dsDNA. cGAMP functions as a ligand for STING activation, leading to high levels of IFN- $\alpha\beta$ expression.

Crystal structure analysis of cGAS has provided key insights into its mechanism of function. cGAS binds to DNA through electrostatic and hydrogen bonding interactions between the positively charged cGAS surface residues and the sugar-phosphate backbone of DNA. Conformational changes in the cGAS catalytic pocket have confirmed its role as a DNA receptor and a dinucleotide cyclase. Moreover, RNA binding does not result in sufficient widening of the catalytic pocket, suggesting that cGAS is specific for DNA [88-90].

The immunological significance of cGAS has been characterized in several studies. *cGas*^{-/-} fibroblasts and bone marrow-derived macrophages (BMDM) failed to produce IFN- β in response to bacterial DNA or DNA viruses, but responded normally to RNA viruses [86, 91]. Surprisingly, studies in both conventional and plasmacytoid DCs demonstrated that cGAS is the dominant DNA sensor in these cells as well [86]. In vivo studies utilizing HSV-1 revealed significant decreases in serum IFN- $\alpha\beta$ levels in *cGas*^{-/-} mice when compared to *cGas*^{+/+} mice [86]. Importantly, the presence of cGas reduced DNA virus lethality. While all *cGas*^{-/-} mice succumbed to infection, 40% and 70% of *cGas*^{+/+} mice recovered following HSV-1 and Vaccinia virus infections, respectively [86, 91]. Such data demonstrates that cGAS is a general cytosolic DNA sensor.

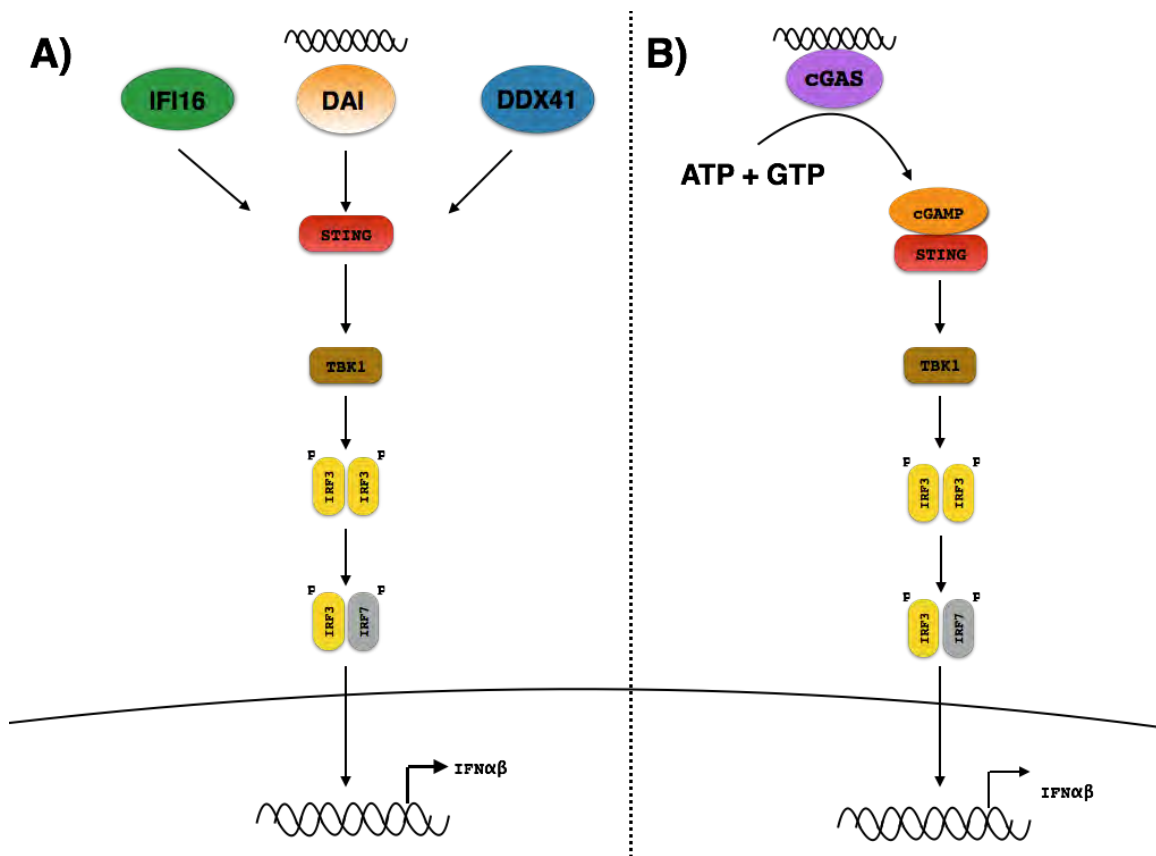


Figure 1.3: IFN- $\alpha\beta$ inducing, cytosolic DNA sensors.

A) The presence of cytosolic DNA triggers innate immune responses through several possible pattern-recognition systems. Activation of cytosolic PRRs results in STING activation and the recruitment of TBK1. TBK1 phosphorylates and activates IRF3. IRF3 subsequently dimerizes and translocates into the nucleus to up-regulate the expression of IRF7, leading to the production of type I IFN and other cytokines. B) Cytosolic DNA activates cGAS to synthesize 2' 3' - cGAMP from ATP and GTP. cGAMP, as a high affinity ligand for STING, binds and activates STING through a series of structural changes. STING activation then drives type I IFN production through the TBK1 pathway as described in part A.

3.3.1.3 Requirement for Type I IFN in DNA Vaccination.

Tudor *et al.* were the first to report the requirement for IFN- $\alpha\beta$ in DNA vaccine immunogenicity [92]. Mice lacking the IFN- $\alpha\beta$ receptor (*Ifnar*^{-/-}) had impaired production of antigen-specific antibodies and CD8⁺ T cells in response to DNA immunization. Several innate sensing pathways generate IFN- $\alpha\beta$, but their requirement for DNA vaccination remains unknown. For example, it was previously believed that the nucleic acid-sensing TLRs 3, 7, and 9 would be essential in generation of adaptive immune populations. TLR9 in particular was thought to regulate DNA vaccine immunogenicity due to the expression of unmethylated CpG motifs within the plasmid backbone. Plasmid-induced cytokine production was completely TLR9-dependent in vitro. Yet, while TLR9 stimulation can prime APCs, deletion does not dramatically affect the T and B cell response in vivo. In fact, TLR-deficient mice mount a comparable immune response to wild-type mice [93, 94]. Therefore, the currently characterized extracellular nucleic acid sensing pathways fail to explain the immunogenicity of DNA vaccination.

Ishii *et al.* established that TBK1 is essential for DNA vaccine immunogenicity [58, 95]. *Tbkl*^{-/-} mice lack antigen-specific CD8⁺ T and B cell responses, consistent with *Ifnar*^{-/-} mice, proving the necessity of both factors in immunization [95]. More recently, Ishikawa *et al.* showed that STING is also critical DNA for vaccine immunogenicity [80, 81], further implying that cytoplasmic DNA receptors play a more prominent role than the endosomal TLRs in mediating the effect of DNA vaccines. In an attempt to identify the upstream sensor for STING/TBK1 activation, Ishii *et al.* characterized the necessity of DAI in DNA vaccination. In accordance with infection data, DAI deletion did not

limit vaccine immunogenicity [95], which is most likely attributable to its limited cellular expression. The role of cGAS in DNA vaccine immunogenicity has yet to be addressed.

3.3.2 Pro-inflammatory PRRs.

3.3.2.1 The Inflammasome.

Cytosolic DNA triggers not only the transcriptional induction of type I IFN, but also the maturation of pro-inflammatory cytokines interleukin-1 β (IL-1 β) and interleukin-18 (IL-18) from their inactive forms. IL-1 β and IL-18 are transcriptionally regulated, requiring an initial microbial stimulus through PRRs for production of both cytokines in their immature pro-forms [96]. Maturation of pro-IL-1 β and pro-IL-18 is regulated by the cysteine protease caspase-1, which in turn is present as an inactive zymogen. The inflammasome complex controls the activity of caspase-1 by initiating self-cleavage into its active form via the apoptosis-associated speck-like protein containing a CARD (ASC) protein.

Inflammatory signaling is required for most pathogen infections. IL-1 β is involved in immune cell recruitment and trafficking, T-lymphocyte activation, and induction of fever. IL-18 boosts the cytolytic activity and IFN- γ production of NK cells [97]. It also increases neutrophil recruitment and activation. Perhaps most importantly for vaccination, IL-18 in the appropriate cytokine milieu directs CD4⁺ T cells towards either a T_h1 or T_h2 humoral response [98-100]. Mice lacking either cytokine have shown a clear susceptibility to viral infection, signifying that both cytokines are required for optimal anti-viral responses [101, 102].

The final aspect of inflammasome activation is an inflammatory form of cell death known as pyroptosis [103]. Cell death is an exceedingly effective method for

limiting intracellular infection by eliminating the host cell, thereby preventing spread of infection. A range of microbial and viral infections, as well as non-infectious host factors, can initiate pyroptosis [104]. Pyroptotic cell death is morphologically and mechanistically distinct from apoptotic and necrotic cell death. Pyroptosis is caspase-1 dependent, but unlike apoptosis, caspase-3, 6, and 8 independent and entails the rupturing of the plasma membrane via caspase-1 dependent ion channels, allowing for osmotic cell lysis. Pyroptotic lysis releases pro-inflammatory intracellular contents (DAMPs) into the extracellular milieu [105]. Similar to apoptosis, pyroptotic cleavage and liberation of nuclear DNA occurs [105-107]. Bystander cells detect the released intracellular, pro-inflammatory DAMPs, further propagating immune signals via multiple PRRs.

3.3.2.2 AIM2 Inflammasome.

The interferon inducible inflammasome AIM2 (absent in melanoma 2) is known to detect cytosolic DNA. AIM2 functions as a dimer to directly bind the sugar phosphate backbone of cytosolic DNA via a HIN200 domain independent of nucleotide sequence [108-113]. Instead dsDNA recognition by AIM2 is dependent on DNA length as fragments less than 80 base pairs are poor triggers of the AIM2 inflammasome [108]. Like all inflammasome receptors, AIM2 contains a pyrin domain that dimerizes with the pyrin domain of ASC, allowing for the recruitment and activation of caspase-1. Prior to dsDNA binding, AIM2 remains in an auto-inhibited state via an intramolecular association between its HIN200 and pyrin domains.

AIM2 is an integral cog in the innate immune response to certain DNA viruses and cytosolic bacteria. It is required for pro-IL-1 β /pro-IL-18 cleavage in response to Vaccinia virus and murine cytomegalovirus, as well as *Francisella tularensis*. *Aim2*^{-/-}

mice fail to activate caspase-1 or generate mature IL-1 β , resulting in poor NK cell activation and high pathogen loads [114-117]. Surprisingly however, AIM2 is not required for all DNA viruses, as HSV-1 escapes detection, despite being a potent activator of IL-1 β . Evidence also exists for AIM2 playing a role in autoimmunity by initiating an inflammatory response to self-DNA [118-120]. Yet, it is not entirely clear as to what cell lineages require AIM2 for control of infection. Current data suggests that AIM2 is necessary in hematopoietic populations, but its role in non-hematopoietic cells remains uncharacterized.

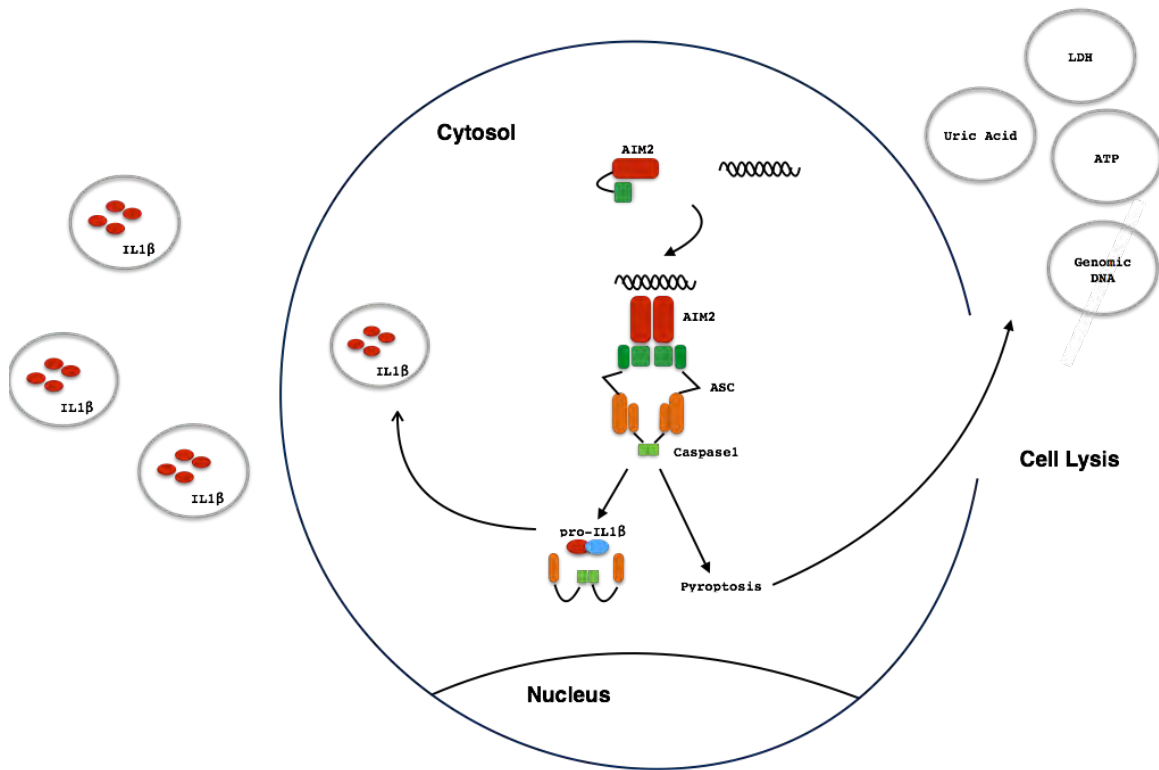


Figure 1.4: AIM2 detects cytosolic DNA and triggers a pro-inflammatory response.

Cytosolic DNA from invading viruses and bacteria engages and activates absent in melanoma 2 (AIM2), resulting in binding to the adapter molecule ASC. ASC mediates caspase-1-dependent pro-interleukin-1 β (pro-IL-1 β)/pro-IL-18 cleavage and secretion into their bioactive forms. IL-1 β and IL-18 are significant mediators of inflammatory responses to infection. Caspase-1 activation also results in pyroptotic cell death, yielding the release of pro-inflammatory DAMPs and PAMPs, thereby propagating the immune response to bystander cells.

3.3.2.3 The Inflammasome and DNA Vaccination.

Pro-inflammatory signaling is necessary for combating the vast majority of pathogen infections; yet, its role in vaccination has been largely unexplored. As such, the requirement for IL-1 β and IL-18 in DNA vaccination has yet to be definitively addressed. The current belief is that inflammasome signaling is dispensable for DNA vaccine immunogenicity based on studies utilizing *MyD88*^{-/-} mice [95]. Nonetheless, it has been established that inclusion of caspase-1, IL-1 β , or IL-18 encoding plasmids within vaccine formulations augments the adaptive response [121-123]. Moreover, immunological memory is heavily dependent on the production of select cytokines including IL-1 β and IL-18 [124-127]. Hence, many vaccine formulations are supplemented with inflammatory cytokines to bolster the expansion and survival of memory T cell populations, suggesting that inflammatory signaling is at least partially required for DNA vaccine immunogenicity [125, 126]. Additionally, recent studies have identified the importance of cell death in vaccine immunogenicity, implying a possible role for pyroptosis in driving the immune response to DNA vaccination [128-130]. Therefore, study of the cytosolic DNA sensing AIM2 inflammasome would provide greater insight into the effects of inflammatory signaling on DNA vaccination.

Research Framework and Objectives

The data described above has laid the foundation for the original body of work described herein. While it has become increasingly accepted that DNA vaccines contain an intrinsic adjuvant effect that mediates their immunogenicity, the mechanisms governing such action remain largely unknown. The aim of this dissertation is to provide a more complete understanding of the effects of the innate immune system on the

development of adaptive immune responses following DNA immunization. In this regard, I will attempt to identify a common innate immune signaling pathway that regulates DNA vaccine immunogenicity using a pandemic influenza HA vaccine model (**Figure 1.5**). Specifically, I will characterize the requirement of the IFN- $\alpha\beta$ inducing cytosolic DNA sensor cGAS, as well as the downstream signaling molecules IRF3 and IRF7. As little work has been done with regard to the pro-inflammatory machinery, I will also investigate the role of AIM2 in DNA vaccination. This will be accomplished by utilizing novel multiplex technology to characterize serum cytokine and chemokine profiles, as well as more traditional immunological assays to quantify the adaptive immune response generated in various innate immune pathway knockout mice. Altogether, this work will provide a strong basis for understanding the mechanisms of action mediating DNA immunization.

As a secondary objective, in the final chapter of this dissertation, I will explore the role of innate immunity in aluminum adjuvants. Several theories currently exist to explain the adjuvant effect of alum, all of which are incomplete. The goal of these studies is to elucidate the importance of innate signaling on the antigen-specific antibody response elicited by inclusion of alum adjuvants, thereby providing a clearer understanding of the immunological mechanisms of alum and allowing for more scientifically informed decisions pertaining to future inclusion of vaccine adjuvants.

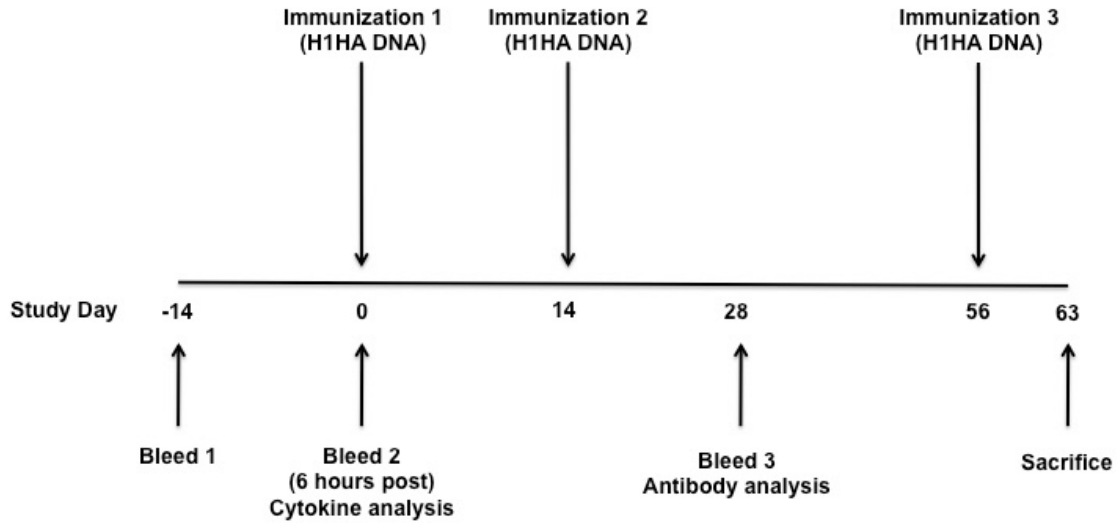


Figure 1.5: DNA vaccine study design and immunization schedule.

Mice were immunized intramuscularly with 100 µg codon optimized plasmid DNA encoding the full-length wild type HA protein from the pandemic 2009 H1N1 influenza (A/Texas/04/09). Immunizations were divided between quadriceps, at 2 and 4 weeks. A third boosting immunization was delivered 1 week prior to sacrifice.

Prefix to Chapter II

This chapter of the dissertation is in preparation for the following manuscript:

John Suschak, Shixia Wang, Katherine Fitzgerald, Shan Lu. *IRF7 is the master regulator of DNA vaccine immunogenicity.* (In Preparation)

John Joseph Suschak III performed and analyzed all experiments.

Chapter II

IRF7 mediates DNA vaccine induced adaptive immune responses

Introduction

The most successful vaccines induce balanced, life long, protective immunity. Unlike many traditional inactivated/subunit protein based vaccines, DNA vaccines elicit not only humoral immunity (Yang, Kong et al. 2004), but also cellular immunity [49]. Mechanistically, DNA plasmid uptake by cells allows for endogenous production and processing of encoded antigen, mimicking the benefits of live attenuated vaccines without the inherent risks. Recent human trials have shown that DNA vaccines have the added benefit of generating elevated antibody responses directed against HIV-1 and pandemic influenza [17, 35, 43-45, 131] when compared to protein alone. This may be attributed to reports that DNA vaccination is particularly effective in shaping germinal center B cell development [51], possibly through increased generation of T follicular helper cells.

Many vaccine formulations contain multiple components that influence their immunogenicity. In addition to the antigen of interest, an adjuvant element is usually included to augment the adaptive immune response [6, 132, 133]. Evidence hints that most clinically utilized adjuvants stimulate the innate immune system, and that these signals regulate the quality and longevity of the adaptive immune response [6, 133]. The ability of DNA vaccines alone to improve the humoral response suggests they contain an intrinsic adjuvant effect, the nature of which remains unclear.

Whereas the majority of adjuvants are TLR ligands, DNA vaccines appear to function independently of TLR signaling [81, 86, 108, 134]. Initial theories posited that the unmethylated CpG motifs encoded within the DNA vaccine plasmid would mediate immune responses via the endosomal DNA receptor, TLR9 [135]. However, both TLR9 and MyD88-deficient mice mount immune responses comparable to wild-type mice, suggesting that multiple innate signaling pathways regulate DNA vaccination [94, 95]. Current evidence suggests that the priming ability stems from the immunostimulatory double-stranded nature of the DNA plasmid itself, as cytosolic DNA is a potent inducer of type I interferon (IFN- $\alpha\beta$) via the stimulator of interferon gene (Sting) and the noncanonical I κ B kinase, TANK binding kinase-1 (Tbk1) [56, 81]. Sting/Tbk1 activation triggers translocation of the interferon regulatory factor 3 (Irf3) and interferon regulatory factor 7 (Irf7) transcription factors into the nucleus, driving IFN- $\alpha\beta$ production through a positive feedback loop.

Studies have shown that Sting/Tbk1 mediated IFN- $\alpha\beta$ production is required for DNA vaccine immunogenicity [81, 92, 95], however, the exact requirements of this pathway remain ambiguous as it has been reported that Irf3 deletion diminishes T cell immunity, but has little impact on B cell responses [136]. Furthermore, Shirota *et al.* reported no significant role for IFN- $\alpha\beta$ in generating high-level antibody titers following DNA vaccination: in stark contradiction to both Tudor *et al.* and Ishii *et al.*'s findings [92, 95]. Additionally, the upstream DNA vaccine sensor has yet to be described, although multiple reports have identified the ubiquitously expressed cyclic GMP-AMP synthase (cGas) as a robust inducer of IFN- $\alpha\beta$ capable of directly binding cytosolic dsDNA [86]. As cGas is vital for immunity to cytosolic DNA viruses and bacterial

infections [86, 91], we hypothesized that it would be essential for DNA vaccine immunogenicity.

Here we investigate the role of Irf signaling in DNA vaccine immunogenicity. We quantified the effects of Irf3 and Irf7 deletion on both the innate and adaptive immune response. Interestingly, we identify Irf7 as being a key modulator in the generation of antigen-specific immune responses. Furthermore, we examine the role of cGas in sensing DNA vaccine plasmid, as well as its necessity in DNA vaccination.

Results

Sting is required for induction of both innate and adaptive immune responses to DNA vaccination.

It was recently reported, using a model antigen, that Sting-deficient mice exhibited severe defects in the adaptive immune responses generated by DNA vaccines [81]. To confirm these results with a clinically utilized vaccine, we immunized mice with plasmid DNA expressing the HA antigen (pH1HA) of the influenza A virus which was responsible for the H1N1 pandemic in 2009. As HA is highly immunogenic and the major protective antigen in clinically licensed inactivated and live-attenuated influenza vaccines, we reasoned that immune responses generated in the current study would be correlative of protective immunity [137-142].

To gain a more complete understanding of the requirement for Sting signaling in innate immune responses, wild-type C57BL/6 (WT) and *Sting*^{-/-} mice were immunized with the pH1HA vaccine and serum cytokines were measured six hours post immunization. Quantification of innate cytokine levels revealed that *Sting*^{-/-} mice had a marked decrease in TNF- α and IL-6 production, as previously described (**Figure 2.1A,B**) [143]. In agreement with the defect in innate signaling, Sting-deletion also negatively impacted the generation of antigen-specific adaptive immunity. While WT mice yielded high levels of HA-specific IFN- γ secreting CD8⁺ T cells following stimulation with an MHC I peptide encoded within the pH1HA vaccine, *Sting*^{-/-} splenocytes failed to respond to peptide stimulation (**Figure 2.1C**). Furthermore, humoral responses were also impaired as immunized WT, but not *Sting*^{-/-}, mice elicited robust anti-HA IgG responses (**Figure 2.2A-B**). Correspondingly, the HA-specific B cell population was decreased in

both the circulating (spleen) and memory (bone marrow) compartments of *Sting*^{-/-} mice, as was the anti-HA binding avidity (**Figure 2.2C-E**).

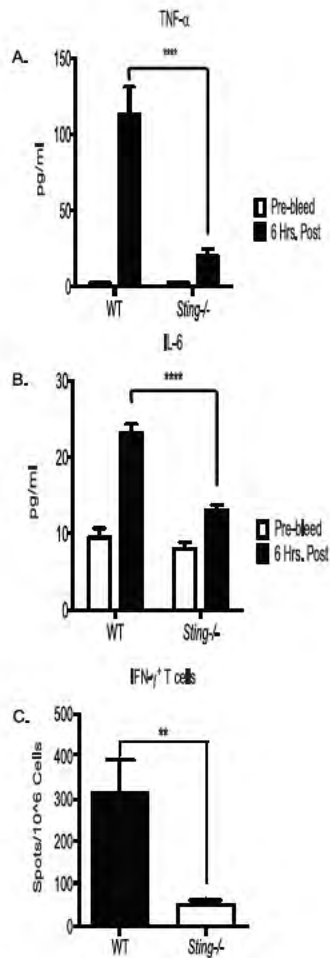


Figure 2.1: *Sting* is required for DNA vaccine immunogenicity.

Sera were collected pre-immunization and 6 hours post DNA vaccination. Cytokines were quantified in the serum of individual mice at a 1:2 dilution using a BD T_h1/T_h2/T_h17 cytokine kit. Shown are significant decreases in cytokines after immunization with pH1HA encoding DNA vaccine: (A) TNF- α and (B) IL-6. WT and *Sting*^{-/-} mice were immunized intramuscularly with a pH1HA encoding DNA vaccine at weeks 2 and 4. (C) Frequency of HA peptide-specific IFN- γ ⁺ T cells in mice immunized with pH1HA. Splenocytes were stimulated with the CD8⁺ cell-restricted HA peptide (IYSTVASSL). Data are the averages \pm SEM of 5 mice per group. **p<0.01, ****p<0.0001 versus control group.

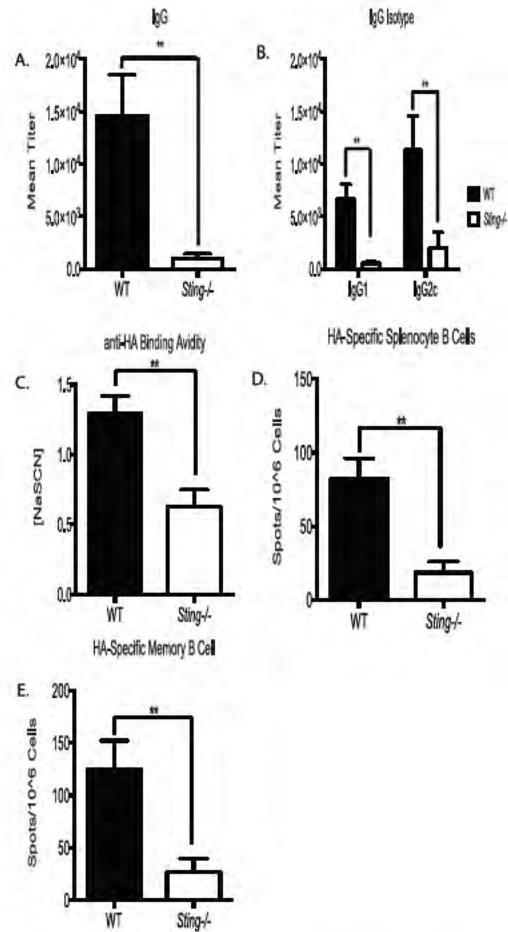


Figure 2.2: DNA vaccine induced antibody responses are Sting dependent.

HA-specific IgG titers (A), including their (B) isotypes, were analyzed fourteen days post second immunization. Anti-HA binding avidity was quantified via ELISA and reported as molar concentration of sodium thiocyanate required to displace anti-HA serum antibodies to 2x pre-bleed levels (C). Splenocytes and bone marrow were harvested at termination 7 days following a 3rd boosting immunization. (D) Splenocyte B cells were plated immediately following isolation, while (E) bone marrow cells were plated after 5 days of culturing in non-specific stimulation to promote clonal expansion. Data are the averages ± SEM of 5 mice per group. **p<0.01 versus control group.

cGas does not mediate the immune response to DNA vaccination.

Previous attempts to identify the upstream sensor for DNA vaccination have been unsuccessful [94, 95]. It is widely believed that a cytosolic DNA sensor regulates IFN- $\alpha\beta$ production in response to DNA vaccination, and therefore vaccine immunogenicity. Several reports have identified cGas as a powerful activator of the Sting/Tbk1 pathway, providing a strong candidate for the unknown DNA vaccine sensor. Hence, we evaluated its necessity in DNA immunization. Initial in vitro studies utilizing bone marrow derived dendritic cells (BMDC) showed a marked decrease in IFN- β production upon transfection of pH1HA into the cytosol of *cGas*^{-/-} cells (**Figure 2.3**). Contrary to expectations, we did not observe an effect of cGas deletion in vivo in pH1HA immunized mice. *cGas*^{-/-} mice did not exhibit the characteristic decrease in pro-inflammatory cytokine production seen in *Sting*^{-/-} mice (**Figure 2.4**). Similarly, no significant change in the magnitude of the adaptive response was seen in immunized *cGas*^{-/-} mice, as they had comparable humoral and cytotoxic T cell levels to WT mice (**Figure 2.5A-D**). The ability of *cGas*^{-/-} mice to generate high-level adaptive immune responses following DNA immunization suggested that IFN- $\alpha\beta$ production was not inhibited by cGas deletion. We therefore quantified local IFN- $\alpha\beta$ production at the site of injection by taking punch biopsies at 6 and 12 hours post immunization. This ensured that we only measured pH1HA-induced IFN- $\alpha\beta$. Surprisingly, IFN- $\alpha\beta$ production was attenuated in *cGas*^{-/-} mice at the initial 6 hour time point. However, the low levels of IFN- $\alpha\beta$ in immunized *cGas*^{-/-} mice recovered by the 12 hour time point, approaching those seen in wild type controls (**Figure 2.6**). These findings indicate that a secondary DNA sensing, IFN- $\alpha\beta$ inducing pathway functions in *cGas*^{-/-} mice, limiting the effects of cGas deletion on DNA vaccine immunogenicity.

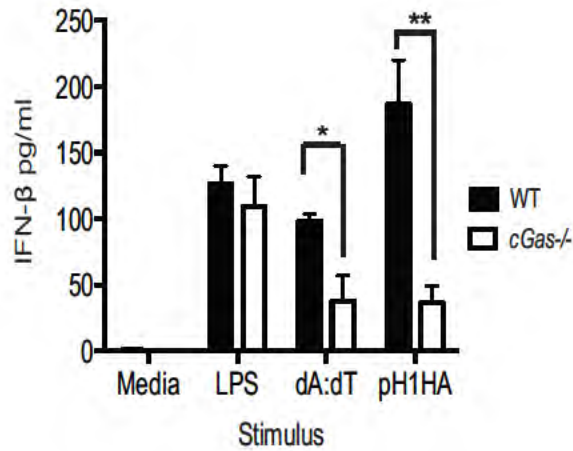


Figure 2.3: *cGas* is required for IFN- $\alpha\beta$ production in vitro.

WT and *cGas*^{-/-} BMDC were transfected with poly(dA-dT) or pH1HA plasmid for 18 hours. Secreted IFN- β in the culture supernatants was analyzed by ELISA. Data are presented as mean \pm SEM from 3 independent experiments. * $p < 0.05$, ** $p < 0.01$ versus control group.

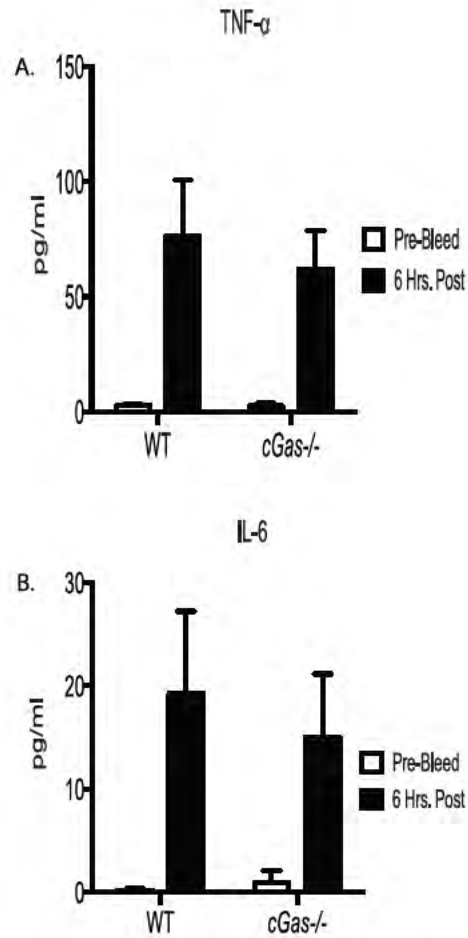


Figure 2.4: cGas deletion does not limit cytokine production.

Sera were collected pre-immunization and 6 hours post DNA vaccination. Cytokines were quantified in the serum of individual mice at a 1:2 dilution using a BD T_h1/T_h2/T_h17 cytokine kit. Shown are cytokine levels after immunization with pH1HA encoding DNA vaccine: (A) TNF- α and (B) IL-6. Data are the averages \pm SEM of 5 mice per group.

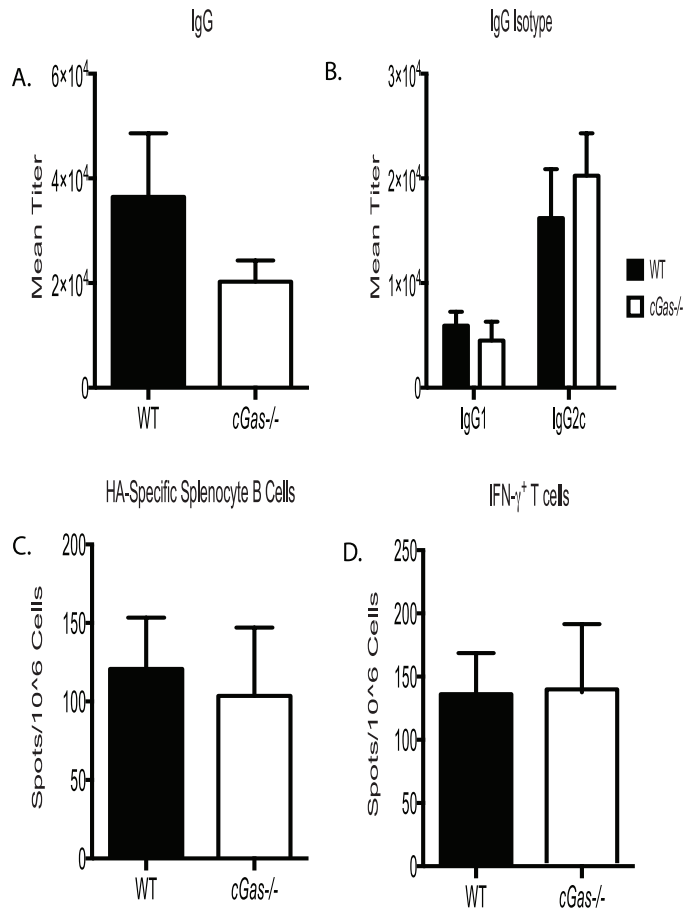


Figure 2.5: DNA vaccine immunogenicity is *cGas* independent.

WT and *cGas*^{-/-} mice were immunized intramuscularly with a pH1HA encoding DNA vaccine at weeks 0 and 2. (A) HA-specific total IgG and (B) IgG isotype titers were analyzed fourteen days post second immunization. Either (C) anti-HA antibody secreting cells or (D) the frequency of HA peptide-specific IFN- γ ⁺ T were quantified cells in mice immunized with pH1HA 1week post third immunization. Splenocytes were stimulated with the CD8⁺ cell-restricted HA peptide (IYSTVASSL). Data are the averages \pm SEM of 5 mice.

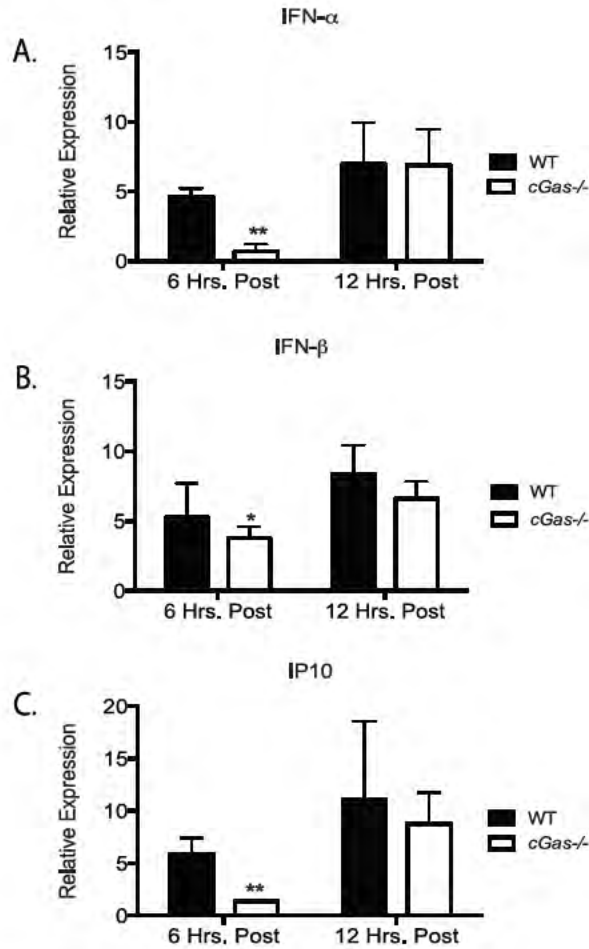


Figure 2.6: IFN- $\alpha\beta$ production is delayed in *cGas*^{-/-} mice.

WT and *cGas*^{-/-} mice were immunized intramuscularly with pH1HA vaccine, and the site of injection was harvested at either 6 or 12 hours post immunization. Total RNA was isolated from tissue biopsies and subjected to rt-PCR for (A) IFN- α , (B) IFN- β , and (C) IP10. Reported expression levels are relative to naïve WT expression. Data are the averages \pm SEM of 4 mice per group. * $p < 0.05$, ** $p < 0.01$ versus control group.

DNA vaccine induced adaptive immune responses are Irf3 independent.

Although the effects of Sting deletion on DNA vaccine immunogenicity are clear, the mechanism by which Sting promotes IFN- $\alpha\beta$ production is not. Therefore, we dissected the downstream signaling molecules to elucidate the IFN- $\alpha\beta$ pathway. As Irf3 is endogenously expressed at high levels and is required for initiating the IFN- $\alpha\beta$ cascade, we analyzed its effects on DNA vaccine immunogenicity. In vitro transfection of immortalized BMDM cultures showed a clear, negative effect of Irf3 deletion on IFN- β production (**Figure 2.7A-C**). As expected, the synthetic B-form dsDNA poly(deoxyadenylic-deoxythymidylic) and pH1HA induced robust IFN- β levels in *Irf3*^{+/+} BMDM as measured by both rt-PCR and cell culture ELISA. Conversely, IFN- β production was limited in *Irf3*^{-/-} and, to a greater extent, Irf3/Irf7 double knockout (DKO) BMDM. However, immunization of *Irf3*^{-/-} mice did not result in impaired adaptive immunity. WT and *Irf3*^{-/-} mice had similar levels of IFN- γ producing CD8⁺ T cells, in contradiction to previously reported results (**Figure 2.8A**) [136]. Furthermore, *Irf3*^{-/-} mice exhibited high levels of anti-HA IgG titers, a result not seen in DKO mice (**Figure 2.8B-D**). Overall, while IFN- $\alpha\beta$ is required for DNA vaccine immunogenicity, Irf3 does not play a substantial role in generating adaptive immunity.

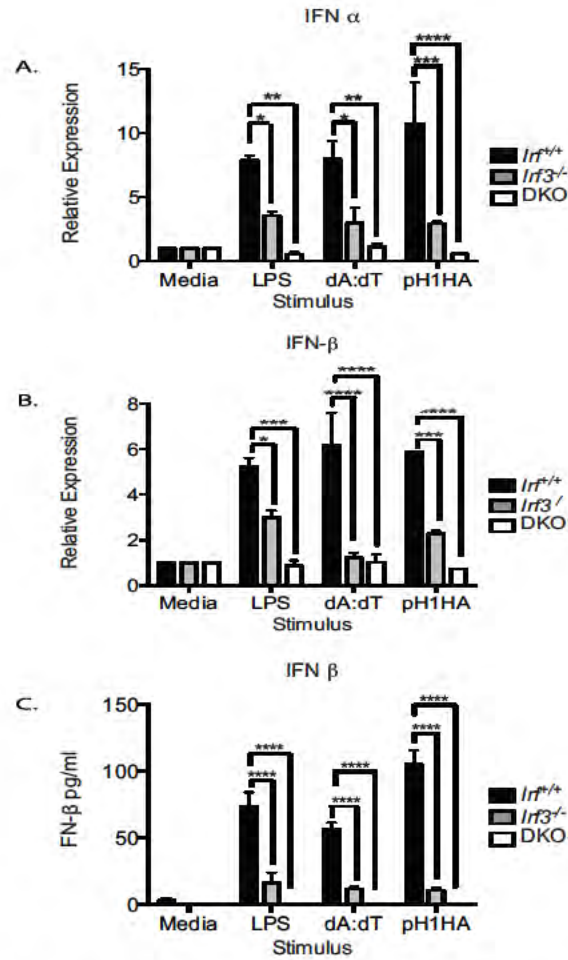


Figure 2.7: *Irf3* is required for DNA vaccine-induced IFN- α β production in vitro.

Irf^{+/+}, *Irf3*^{-/-}, and *Irf3*^{-/-}/*Irf7*^{-/-} (DKO) BMDM were transfected with poly(dA-dT) or pH1HA plasmid for 18 hours. (A) IFN- α and (B) IFN- β levels in transfected BMDM were quantified by rt-PCR. (C) Secreted IFN- β in the culture supernatants was analyzed by ELISA. Data are presented as mean \pm SEM from 3 independent experiments. * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001 versus control group.

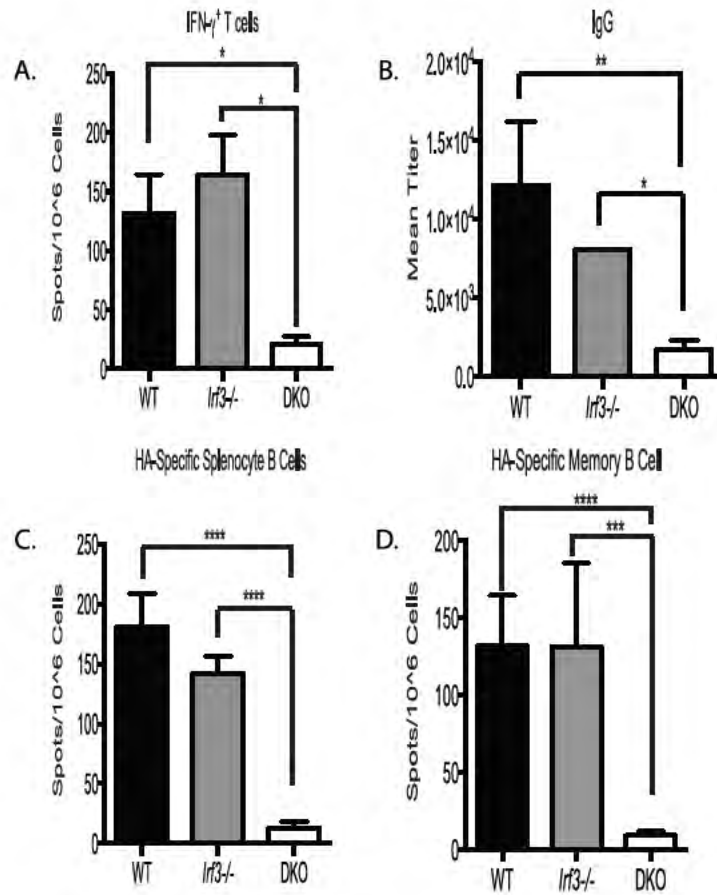


Figure 2.8: *Irf3* is dispensable for DNA vaccine immunogenicity.

WT, *Irf3*^{-/-}, and *Irf3*^{-/-}/*Irf7*^{-/-} (DKO) mice were immunized intramuscularly with a pH1HA encoding DNA vaccine at weeks 0 and 2. Splenocytes were harvested at termination 7 days following a 3rd boosting immunization. (A) Frequency of HA peptide-specific IFN- γ ⁺ T cells in mice immunized with pH1HA. Splenocytes were stimulated with the CD8⁺ cell-restricted HA peptide (YSTVASSL). (B) HA-specific IgG titers were analyzed fourteen days post second immunization. (C) HA-specific splenocyte B cells were plated immediately following isolation. (D) Memory B cells were isolated from bone marrow and plated after 5 days of culturing in non-specific stimulation to promote clonal expansion. Data are the averages \pm SEM of 5 mice. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001 versus control group.

Irf7 regulates DNA vaccine-induced innate cell signaling.

The failure of DKO mice to produce anti-HA adaptive immune responses following DNA vaccination suggests that Irf signaling is required for DNA vaccine immunogenicity. Because *Irf3*^{-/-} mice were similar to WT controls, we next investigated Irf7 in the context of DNA vaccination. As shown in **Figure 2.9**, both WT and *Irf3*^{-/-} mice yielded commensurate levels of serum cytokines six hours post immunization. However, *Irf7*^{-/-} and DKO mice showed clear defects in TNF- α and IL-6 production, yielding comparable levels to *Sting*^{-/-} mice.

The similar cytokine profiles seen in *Sting*^{-/-} and *Irf7*^{-/-} mice suggest that both are required for innate signaling following DNA vaccination. Additionally, both molecules have been identified as key regulators of IFN- $\alpha\beta$ production following DNA virus infection [79, 81]. As DNA vaccine immunogenicity is IFN- $\alpha\beta$ dependent [95], we performed a thorough analysis of the effect of *Sting* and *Irf7* deletion on IFN- $\alpha\beta$ expression. WT, *Sting*^{-/-}, *Irf7*^{-/-}, and DKO mice were immunized with the pH1HA vaccine, and punch biopsies were harvested as above. rt-PCR analysis plainly illustrates that *Sting*^{-/-} and DKO mice lack significant IFN- α and IFN- β expression compared to WT controls (**Figure 2.10A,B**). Interestingly, *Irf7*^{-/-} mice exhibited a similar decrease in IFN- $\alpha\beta$ production, although to a lesser degree. Consistent with impaired IFN- $\alpha\beta$ production, both *Sting*^{-/-} and *Irf7*^{-/-} mice had a corresponding decrease in the IFN stimulated gene, IP10 illustrating the wide-ranging effect of *Sting* and *Irf7* deletion on the innate immune response (**Figure 2.10C**).

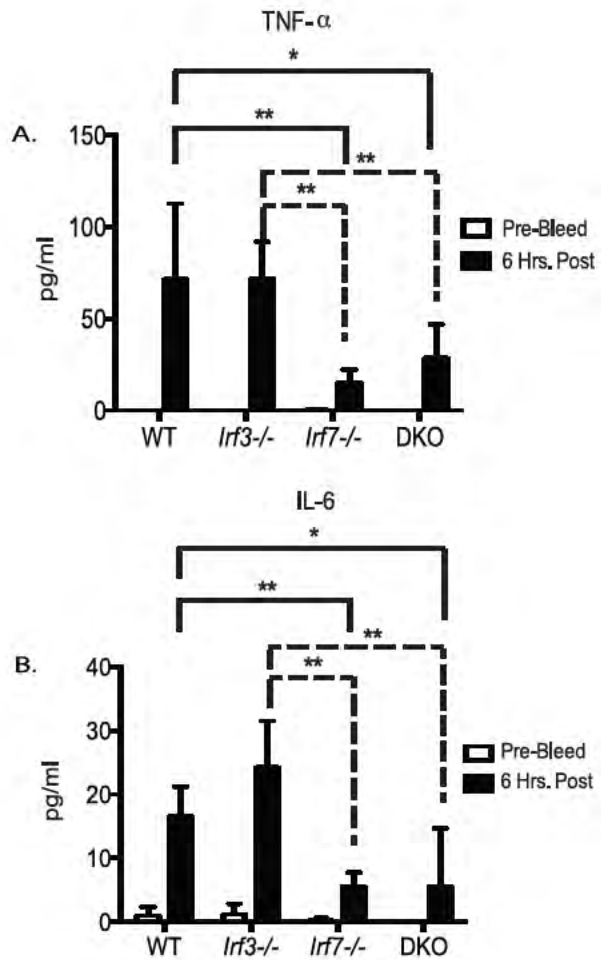


Figure 2.9: *Irf7* is required for immune cytokine production.

WT, *Irf3*^{-/-}, *Irf7*^{-/-}, and *Irf3*^{-/-}/*Irf7*^{-/-} (DKO) sera were collected pre-immunization and 6 hours post DNA vaccination. Serum cytokines were quantified from individual mice at a 1:2 dilution using a BD T_h1/T_h2/T_h17 cytokine kit. Shown are significant decreases in cytokines after immunization with pH1HA encoding DNA vaccine: (A) TNF- α and (B) IL-6. Statistical significance was determined by Student's t test (* p < 0.05). Data are the averages \pm SEM of 5 mice per group. *p<0.05, **p<0.01 versus control group.

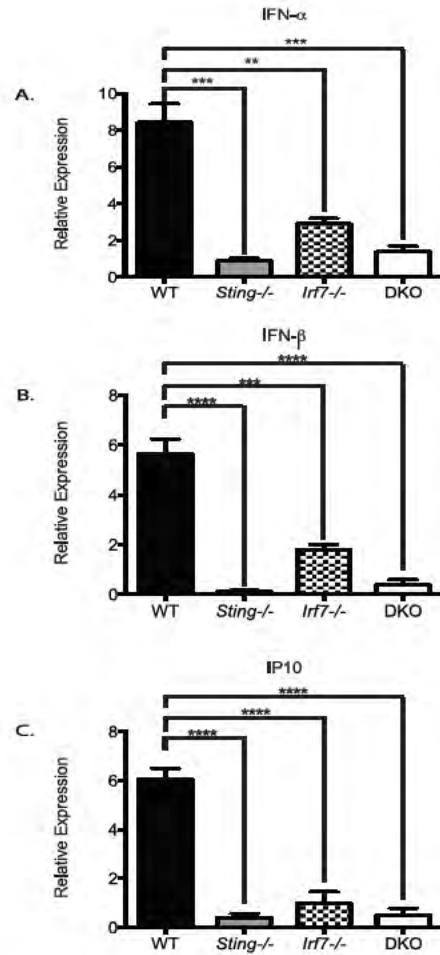


Figure 2.10: IFN- α β production following DNA vaccination is *Irf7* dependent.

WT, *Sting*^{-/-}, *Irf7*^{-/-}, and *Irf3*^{-/-}/*Irf7*^{-/-} (DKO) mice were immunized intramuscularly with pH1HA vaccine, and the site of injection was harvested 12 hours later. Total RNA was isolated from tissue biopsies and subjected to rt-PCR for (A) IFN- α , (B) IFN- β , and (C) IP10. Data are the averages \pm SEM of 5 mice per group. **p<0.01, ***p<0.001, ****p<0.0001 versus control group.

Irf7 is required for generation of antigen-specific immune responses following DNA vaccination.

Because *Irf7*^{-/-} mice failed to produce high levels of pro-inflammatory cytokines and IFN- $\alpha\beta$, we reasoned that *Irf7* deletion would inhibit development of antigen-specific immunity. pH1HA vaccination of WT mice elicited strong antigen-specific humoral responses to the encoded HA antigen, whereas *Irf7*^{-/-} mice failed to generate substantial levels of anti-HA IgG after two immunizations (**Figure 2.11A,B**). Likewise, antibody-binding avidity was decreased approximately 3 fold compared to WT controls (**Figure 2.11C**), consistent with the lack of HA-specific B cells in both the spleen and bone marrow (**Figure 2.11D,E**). Immunization of *Irf7*^{-/-} mice also failed to induce significant numbers of IFN- γ ⁺ CD8⁺ T splenocytes (**Figure 2.11F**). Altogether, these results indicate that *Irf7* plays a broad role in the activation of both T and B cell subsets following DNA vaccination.

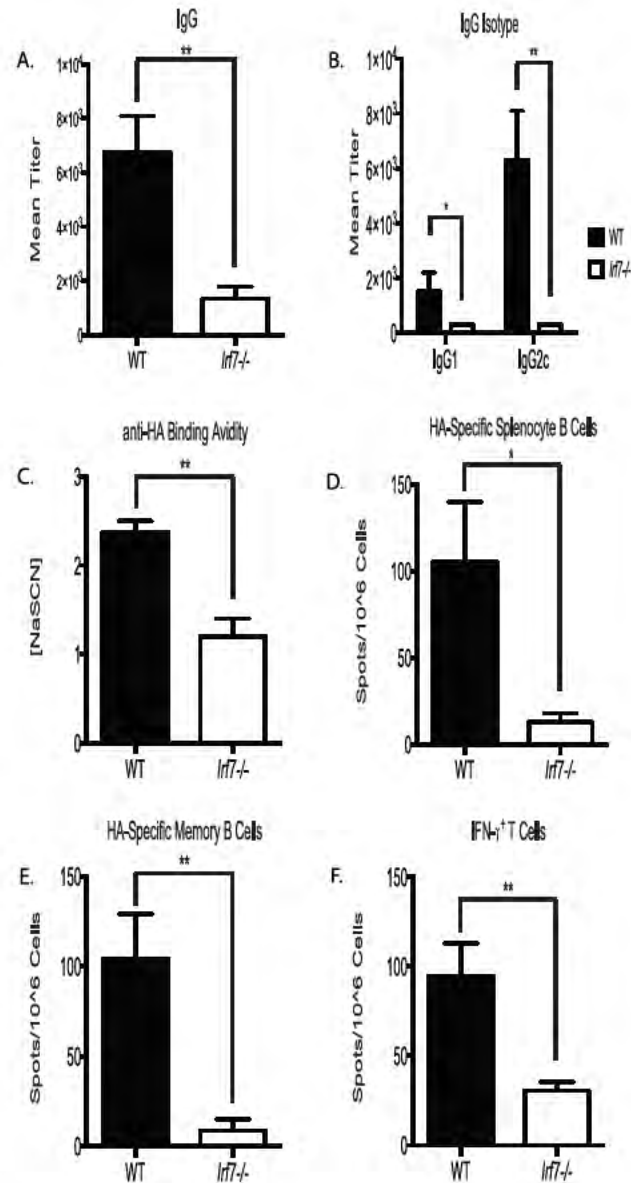


Figure 2.11: *Irf7* is required for generation of anti-pH1HA adaptive immune responses.

WT and *Irf7*^{-/-} mice were immunized intramuscularly with pH1HA. (A) HA-specific IgG titers, including their isotypes (B), were analyzed fourteen days post second immunization. Anti-HA binding avidity was quantified via ELISA and reported as molar concentration of sodium thiocyanate required to displace anti-HA serum antibodies to 2x pre-bleed levels (C). Splenocytes and bone marrow were harvested at termination 7 days

following a 3rd boosting immunization. (*D*) Splenocyte B cells were plated immediately following isolation, while (*E*) bone marrow cells were plated after 5 days of culturing in non-specific stimulation to promote clonal expansion. (*F*) Frequency of HA peptide-specific IFN- γ ⁺ T cells in mice immunized with pH1HA. Splenocytes were stimulated with the CD8⁺ cell-restricted HA peptide (IYSTVASSL). Data are the averages \pm SEM of 5 mice per group. * $p < 0.05$, ** $p < 0.01$ versus control group.

Discussion

The complicated interplay between DNA vaccination and the innate immune system is just beginning to be elucidated, as the canonical TLR pathways seem to have little influence on DNA vaccine immunogenicity. Instead, current findings have shown that sensing of intracytoplasmic DNA plasmid governs DNA vaccine immunogenicity via the non-canonical Sting/Tbk1/IFN- $\alpha\beta$ pathway. However, the processes involved are the subjects of much disagreement. In particular, the requirement for IFN- $\alpha\beta$ in generating high-level antibody responses has yielded contradictory results. Similarly, the necessary transcription factors downstream of Sting and Tbk1 remain uncertain. This report provides a systematic investigation of these factors and gives a clearer understanding of the requirement for each in DNA vaccination.

Shirota *et al.* previously demonstrated the necessity for Irf3 in cellular mediated immune responses [136]. Supporting Shirota *et al.*'s findings, multiple reports have shown that Irf3-dependent IFN- β can augment the production of T_h1 and T_h2 cytokines both in vitro and in vivo [144, 145]. However, our results show no such requirement, with reductions in IFN- γ^+ T cell numbers seen only in DKO mice. Still, in agreement with Shirota *et al.*'s report, we did not see a substantial effect on humoral immunity, confirming that Irf3 is not required for B cell activation. The discrepancy in CD8⁺ T cell responses may be attributed to the inclusion of repeated immunizations in our study. Another possible explanation may be our choice of a more clinically relevant vaccine, as our immunogen has been optimized for development of antigen-specific responses. Regardless, our data suggests that Irf3 plays a limited role in DNA vaccination.

In contrast to Irf3, our results did identify an unexpected role for Irf7 in DNA

vaccine immunogenicity. *Irf7*^{-/-} mice exhibited a similar immune phenotype to *Sting*^{-/-} mice in that *Irf7* deficiency resulted in significantly diminished T and B cell responses, indicating a broad contribution by *Irf7* in the induction of adaptive immunity. Moreover, *Irf7*^{-/-} mice had impaired TNF- α and IL-6 production, characteristic of *Sting* dependent signaling. The lack of IFN- $\alpha\beta$ production in *Irf7*^{-/-} mice further suggests that the defects in DNA vaccination seen in *Sting*^{-/-} mice are due to a failure to initiate the *Irf7*-dependent IFN- $\alpha\beta$ feedback loop. This is in accordance with previous reports that *Irf7* is the main regulator of immunity with regards to DNA virus infection [79, 146]. Altogether, our results indicate that *Irf7* is the driving force behind sustained DNA vaccine-induced IFN- $\alpha\beta$ production, implying that the temporary defect in immune priming provided by *Irf3* deletion is overcome by the subsequent induction of *Irf7*, allowing for rescue of vaccine immunogenicity.

Our attempt to identify the upstream DNA vaccine sensor yielded unforeseen results, as *cGas* deletion did not limit DNA vaccine immunogenicity. In particular, the ability of *cGas*^{-/-} mice to generate wild type IFN- $\alpha\beta$ levels was unexpected. Our results suggest that while *cGas* is required for the early induction of IFN- $\alpha\beta$, it is not necessary for sustained IFN- $\alpha\beta$ production in response to DNA vaccine. Previous studies have suggested that *cGas* is a non-redundant cytosolic DNA sensor [86, 147], but our data implies that at least one other sensor functions in parallel. This provides a possible explanation for the ability of *cGas*^{-/-} mice to generate adaptive immune responses following DNA vaccination.

In conclusion, our results demonstrate that while *Irf3* is not required for DNA vaccine immunogenicity, *Irf7* is a key signaling molecule in DNA vaccination. *Irf7*^{-/-}

mice failed to generate broad vaccine-induced immune responses, exhibiting decreased serum IgG levels and T cell activity. Unexpectedly, deletion of cGas did not dramatically impact the immune response, perhaps evidencing the redundant nature of cytosolic DNA receptors. Overall, our results provide a deeper understanding of the cellular mechanisms through which DNA vaccines stimulate both the innate and adaptive immune pathways to promote immune responses directed against the encoded antigen.

Prefix to Chapter III

This chapter of the dissertation has appeared in the following publication:

John Suschak, Shixia Wang, Katherine Fitzgerald, Shan Lu. 2014. *Identification of Aim2 as a sensor for DNA vaccines*. J. Immunology (In Press)

John Joseph Suschak III performed and analyzed all experiments.

Chapter III

Identification of Aim2 as a sensor for DNA vaccines

Introduction

The discovery of DNA vaccine technology in the early 1990s was a major event in the history of vaccinology due to the many unique features of DNA immunization, including its ability to elicit balanced antibody and T cell immunity [14, 15, 17, 137, 148, 149]. However, in early clinical studies, the immunogenicity of DNA vaccines in humans was low when such vaccines were used alone. More recent human trials have demonstrated that DNA vaccines are actually extremely powerful in priming the host's immune system to develop high level protective antibody responses against HIV-1 and pandemic influenza viruses [17, 35, 43-45, 131]. Animal studies have further demonstrated that DNA immunization is effective in eliciting higher levels of antigen-specific B cell responses [50]. One mechanism to achieve such an outcome is that DNA vaccination is effective in eliciting higher germinal center (GC) B cell development via enhanced follicular helper T (T_{fh}) cells for the production of high quality antibody responses [51].

DNA vaccines produce immunogens *in vivo*, which are then presented to the immune system via the endogenous antigen processing pathways. At the same time, the DNA plasmid itself confers an intrinsic adjuvant effect that enhances the immune response generated towards the vaccine-encoded immunogens [51, 150], but the intracellular processes involved remain to be fully elucidated. Several pattern-recognition receptors (PRRs) have been identified which respond to DNA molecules [59,

151]. One of the best-characterized DNA sensing PRRs is the endosomal TLR9, which is essential for the recognition of unmethylated CpG containing oligodeoxynucleotide (ODN) motifs commonly found in bacterial plasmids [76]. As DNA vaccine plasmid backbones contain certain CpG:ODN motifs, it was initially thought that TLR9 would be critical for DNA vaccine immunogenicity. However, while DNA plasmid activates dendritic cells via TLR9, TLR9-deficient mice were able to mount immune responses comparable to wild-type mice [93, 94]. Likewise, DNA immunization of MyD88 and TRIF-deficient mice yields robust immune responses, further suggesting that TLR signaling may be dispensable for DNA vaccine induced immunogenicity [95].

In recent years, it has become increasingly evident that the double-stranded nature of DNA itself functions as a potent activator of innate immune signals [56, 81, 86, 108, 134]. Cytosolic DNA is a powerful initiator of type I IFN (IFN- $\alpha\beta$) in both immune and non-immune cells, functioning through a STING/TBK1/IFN- $\alpha\beta$ dependent pathway that is independent of CpG motifs and TLRs. At the same time, it is not clear whether other components of the innate immune system beyond the STING/TBK1/IFN- $\alpha\beta$ pathway are involved in the immunogenicity of DNA vaccines [81, 95]. This is especially true in the case of inflammasome pathways. Inflammasomes regulate caspase-1 activity, ultimately resulting in cleavage of the pro-inflammatory cytokines pro-IL-1 β and pro-IL-18 into their active forms. Inflammasome activation also results in pyroptotic cell death; a suicidal form of cell death characterized by the release of damage associated molecular patterns (DAMPs) that further propagates innate immune signaling to surrounding bystander cells [152]. One flavor of inflammasome contains *absent in melanoma 2* (Aim2), which is a direct sensor of cytosolic DNA and a member of the PYHIN family

[108]. Aim2 contains a DNA binding HIN200 domain, as well as a pyrin domain. While Aim2's role in orchestrating immune responses to both viral and bacterial pathogens is well characterized [115, 117, 153], the role of Aim2 in DNA vaccination is unknown.

Here we found that Aim2 and the adapter molecule Asc were required for the generation of optimal immunogen-specific antibody responses to a DNA vaccine expressing influenza HA immunogen in a mouse model. DNA vaccination leads to transcription of key components of the inflammasome. Importantly, the efficacy of DNA vaccination was independent of IL-1 β and IL-18. Surprisingly, *Aim2*-deficient mice were unable to elicit a type I IFN response at the site of injection. Our data therefore establish a novel role for Aim2 as a key player in the regulation of DNA vaccination.

Results

DNA vaccine plasmid induces expression of Aim2, caspase-1 and the inflammasome

While previous studies have mainly used non-coding DNA plasmid or DNA vaccines coding for marker proteins to study DNA-elicited innate immune responses, the current study tested a DNA vaccine (pH1HA) expressing the HA antigen of the type A influenza virus subtype H1N1 virus which was responsible for a pandemic influenza in 2009. HA is the major protective antigen in clinically licensed inactivated and live-attenuated influenza vaccines. DNA vaccines expressing HA have been shown to be immunogenic in eliciting HA-specific antibodies in both animal and human studies [137-142]. The expression of HA antigen by pH1HA used in the current study was confirmed by Western blot and its immunogenicity to elicit HA-specific antibody response was verified in a pilot mouse study (data not shown).

We first wanted to profile key immune response genes following DNA vaccine pH1HA using the Nanostring nCounter gene expression system, which includes a custom array encoding 50 innate immunity targets. Gene induction was quantified from wild-type C57BL/6 mice immunized with the pH1HA DNA vaccine. Messenger RNA was isolated and the expression of innate immune genes profiled using the Nanostring nCounter, and changes in gene induction quantified. Notably, Aim2 was induced ~6 fold within 12 hours of immunization when compared to naïve samples. Aim2 is a type I IFN inducible gene suggesting a potent ability of cells at the site of vaccination to recognize cytosolic plasmid vaccines (**Figure 3.1A**). In accordance with the induction of Aim2, caspase-1 was also highly upregulated. (**Figure 3.1B**). Most striking were the high levels

of the inflammatory cytokines IL-1 α and IL-1 β (**Figure 3.1C,D**). These observations indicate that inflammasome components were present at the site of vaccination. To test if the inflammasome pathway was active at the site of vaccination we utilized a caspase-1 specific FAM/FLICA fluorescent stain to covalently label catalytically active caspase-1. Mature caspase-1 became apparent within 6 hours of immunization and reached a peak at 12 hours (**Figure 3.2**). Collectively, these results led us to examine the role of the Aim2 inflammasome pathway in antigen specific immune responses elicited by pH1HA DNA vaccination.

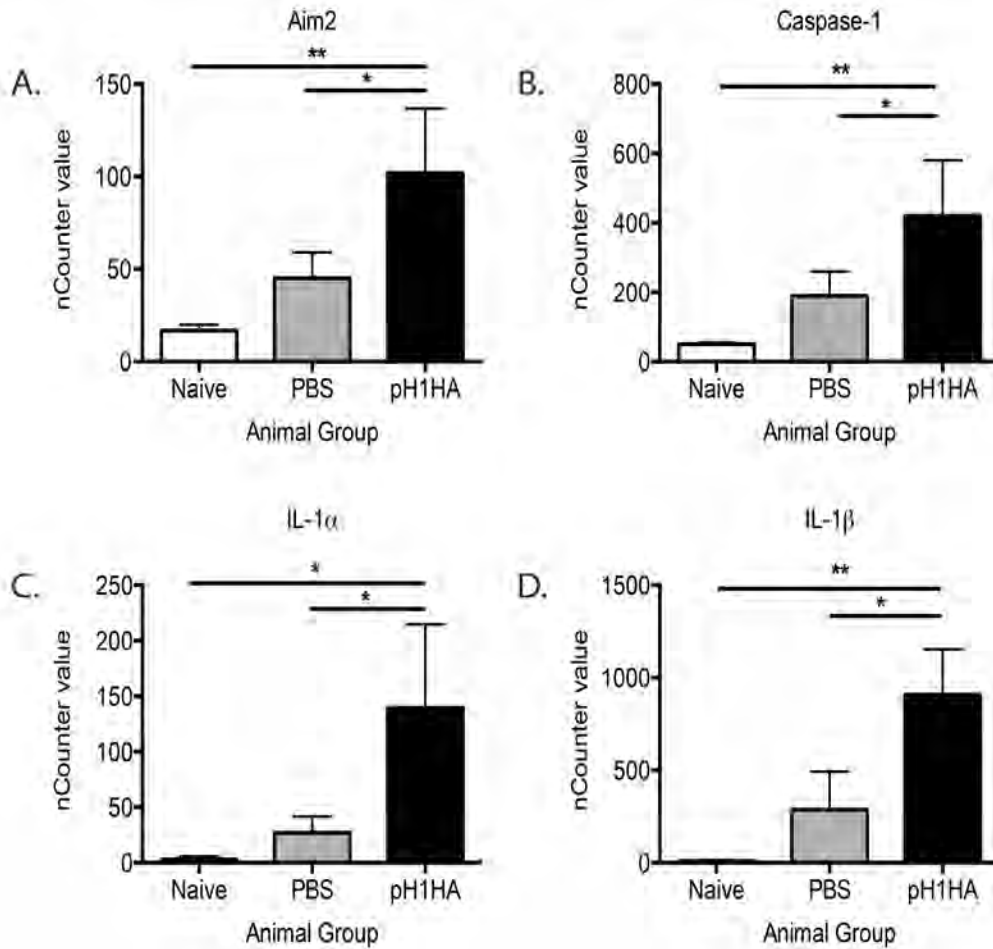


Figure 3.1: Plasmid DNA vaccination induces the inflammasome.

Inflammasome activation at the site of immunization was quantified by Nanostring nCounter analysis 12 hours post DNA immunization. The site of injection was harvested and mRNA was isolated and expression levels were quantified for (A) Aim2, (B) caspase-1, (C) IL-1 α , (D) IL-1 β . Data are the averages \pm SEM of 5 mice per group. *p<0.05, **p<0.01 versus control group.

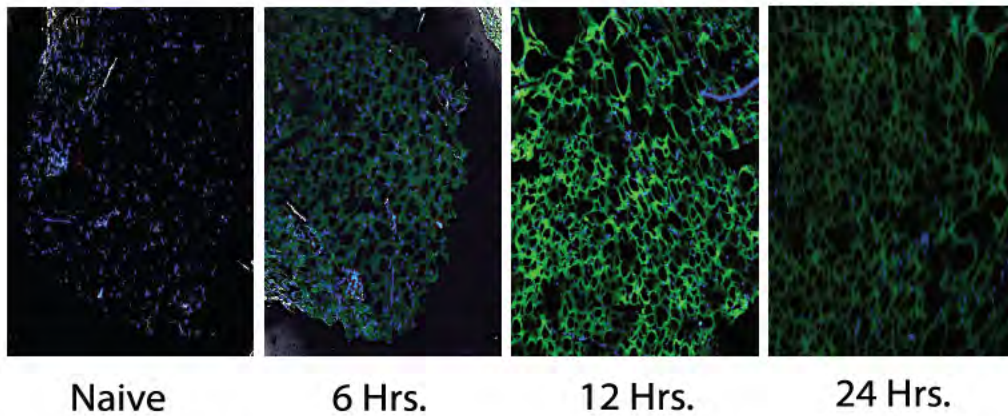


Figure 3.2: pH1HA immunization generates catalytically active caspase-1.

Aim2^{+/+} mice were immunized intramuscularly with pH1HA vaccine and caspase-1 activation was quantified by FAM/FLICA staining. The site of injection was harvested and cryopreserved for tissue sectioning. Sections from PBS injected mice (Naïve) served as background control, while pH1HA immunized samples were harvested at post immunization time points shown. FAM/FLICA staining was visualized by confocal microscopy and is representative of 3 mice per group. Sections of 10 µm were stained with green fluorescent FLICA caspase-1 inhibitor. Nuclei were counterstained with DAPI. Low power resolution presented (Original magnification x 16).

Involvement of Aim2 in cellular responses to DNA vaccine plasmid

We first evaluated the ability of macrophages and dendritic cells to recognize pH1HA DNA vaccine by performing in vitro experiments. IL-1 β production in response to pH1HA DNA vaccine was evaluated in BMDM collected from either *Aim2*^{+/+} or *Aim2*^{-/-} mice (**Figure 3.3A**). As expected, both the synthetic B-form dsDNA poly(deoxyadenylic-deoxythymidylic) and pH1HA DNA vaccine induced a robust IL-1 β response in *Aim2*^{+/+} BMDM as measured by ELISA. However, IL-1 β production was abolished in *Aim2*^{-/-} BMDM. Similar results were seen in BMDC (data not shown). Next, the pan-caspase inhibitor Z-VAD-FMK was included in BMDM cultures prior to adding pH1HA DNA vaccine (**Figure 3.3B**). This resulted in inhibited IL-1 β production in *Aim2*^{+/+} macrophages, yielding IL-1 β levels comparable to *Aim2*^{-/-} wells, supporting the role of Aim2 in DNA vaccine mediated IL-1 β maturation. Finally, pH1HA DNA vaccine induced an inflammatory form of cell death (pyroptosis) in *Aim2*^{+/+} macrophages, as measured by lactate dehydrogenase release (**Figure 3.3C**). This response was attenuated in *Aim2*^{-/-} cells. Collectively, these data indicate that Aim2 acts as a sensor of DNA vaccine plasmid and regulates caspase-1 dependent IL-1 β production and pyroptotic cell death in response to pH1HA DNA vaccines in vitro.

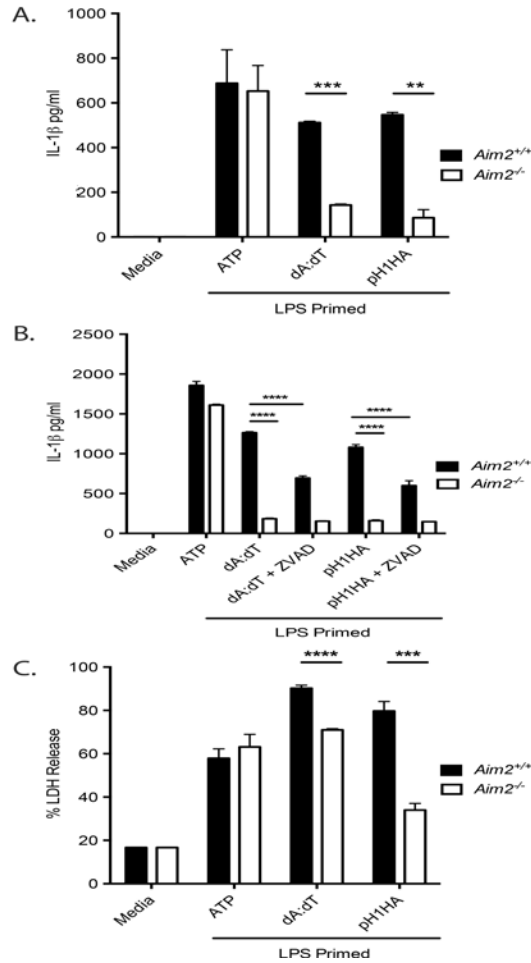


Figure 3.3: Aim2 is required for IL-1 β production in response to DNA vaccines.

LPS (200 ng/ml) primed *Aim2*^{+/+} and *Aim2*^{-/-} BMDM were transfected with poly(dA-dT) or DNA vaccine plasmid for 18 hrs. (A) Secreted IL-1 β in the culture supernatants was analyzed by ELISA. (B) *Aim2*^{+/+} and *Aim2*^{-/-} BMDM were treated as above with the addition of the pan-caspase inhibitor Z-VAD-FMK and secreted IL-1 β was quantified by ELISA. (C) *Aim2*^{+/+} and *Aim2*^{-/-} BMDM were treated as above, and culture LDH amounts were reported as a percentage of lysed cellular controls. Data are presented as mean \pm SEM from 3 independent experiments. **p<0.01, ***p<0.001, ****p<0.0001 versus control group.

Aim2 deletion impairs cytokine production following pH1HA immunization.

As it is evident that the Aim2 inflammasome recognizes and responds to pH1HA DNA vaccine in cultured cells, the role of Aim2 in pH1HA DNA vaccination was next examined in *Aim2*-deficient (*Aim2*^{-/-}) and wild-type *Aim2*^{+/+} mice. We first evaluated the effect of Aim2 deletion on innate cytokine signaling and the pro-inflammatory response. Following pH1HA immunization, *Aim2*^{-/-} mice demonstrated a marked lack of the inflammatory cytokine IL-6 and the chemokines G-CSF, KC, and Rantes compared to *Aim2*^{+/+} controls, suggesting an inability of *Aim2*^{-/-} to properly prime immune cell populations (**Figure 3.4**).

Effects of Aim2 deletion on pH1HA induced HA-specific immune responses.

The marked decrease in cytokine production in *Aim2*^{-/-} mice suggested a possible defect in the adaptive immune response to pH1HA vaccine. Therefore, we next quantified the antigen-specific immune responses generated in *Aim2*^{-/-} mice. The pH1HA DNA vaccine induced high-level HA-specific antibody responses in *Aim2*^{+/+} mice, but significantly lower antibody titers in *Aim2*^{-/-} mice (**Figure 3.5A**). This reduction is isotype-independent as *Aim2*^{-/-} mice exhibited significantly lower levels of HA-specific IgG1, IgG2b, and IgG2c responses (data not shown). Likewise, *Aim2*^{-/-} mice exhibited significantly reduced HA-specific circulating B cells as well as IFN- γ secreting CD8⁺ T cells in the spleen (**Figure 3.5C,D**). The role of Aim2 in regulating the maturation process of pH1HA-induced antibody responses was further confirmed by measuring the avidity of serum HA-specific antibodies in these mice (**Figure 3.5B**). *Aim2*^{+/+} mice required high concentrations of the chaotropic agent NaSCN to disrupt antigen/antibody complexes, while much lower concentrations of NaSCN were required for disassociation

in *Aim2*^{-/-} mice. To confirm the requirement for inflammasome signaling in DNA vaccine immunogenicity, we also quantified the adaptive response in *Asc*^{-/-} mice. *Asc*-deletion similarly inhibited the generation of optimal HA-specific immune responses **(Figure 3.6)**.

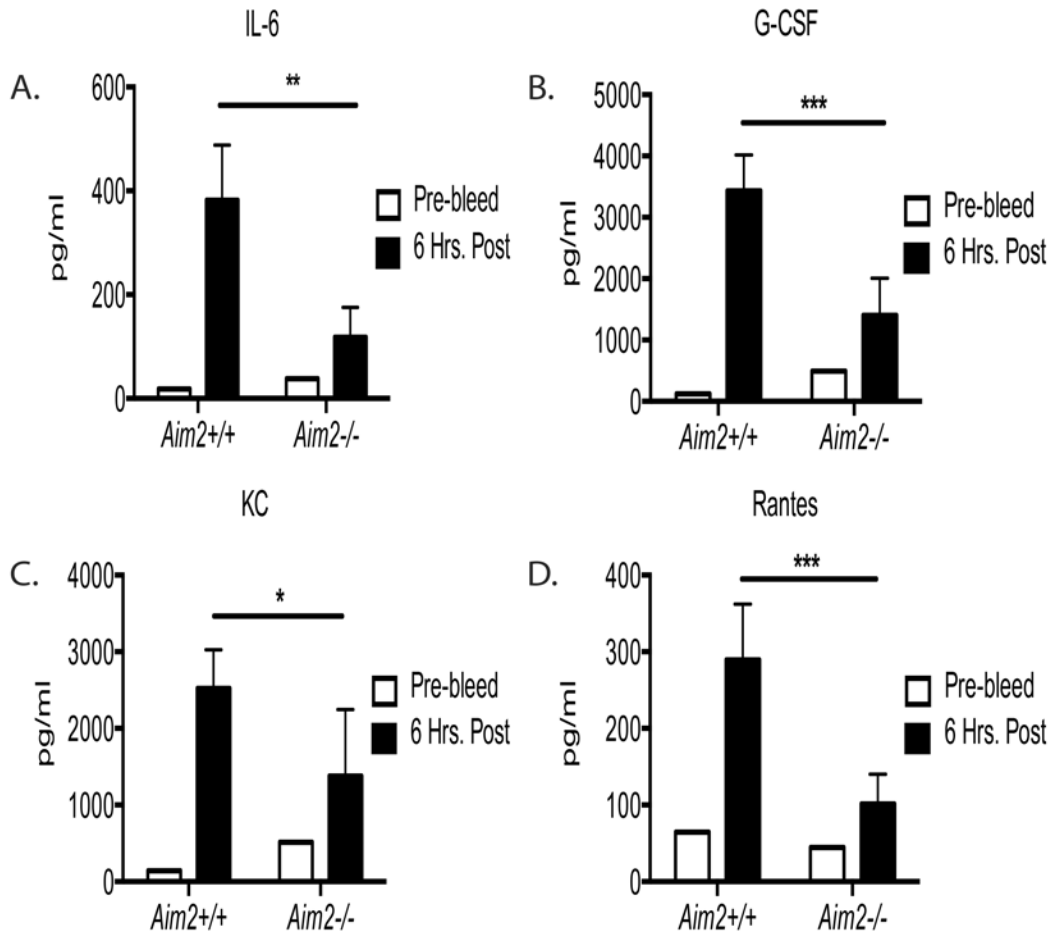


Figure 3.4: Aim2 is required for pro-inflammatory cytokine production in response to DNA vaccines.

Serum chemokine levels of pH1HA immunized mice were measured 2 weeks prior to immunization and 6 hours post primary immunization via Luminex assay. (A) IL-6, (B) G-CSF, (C) KC, and (D) Rantes. Data are the averages \pm SEM of 5 mice per group.

*p < 0.05, **p < 0.01, ***p < 0.001 versus control group.

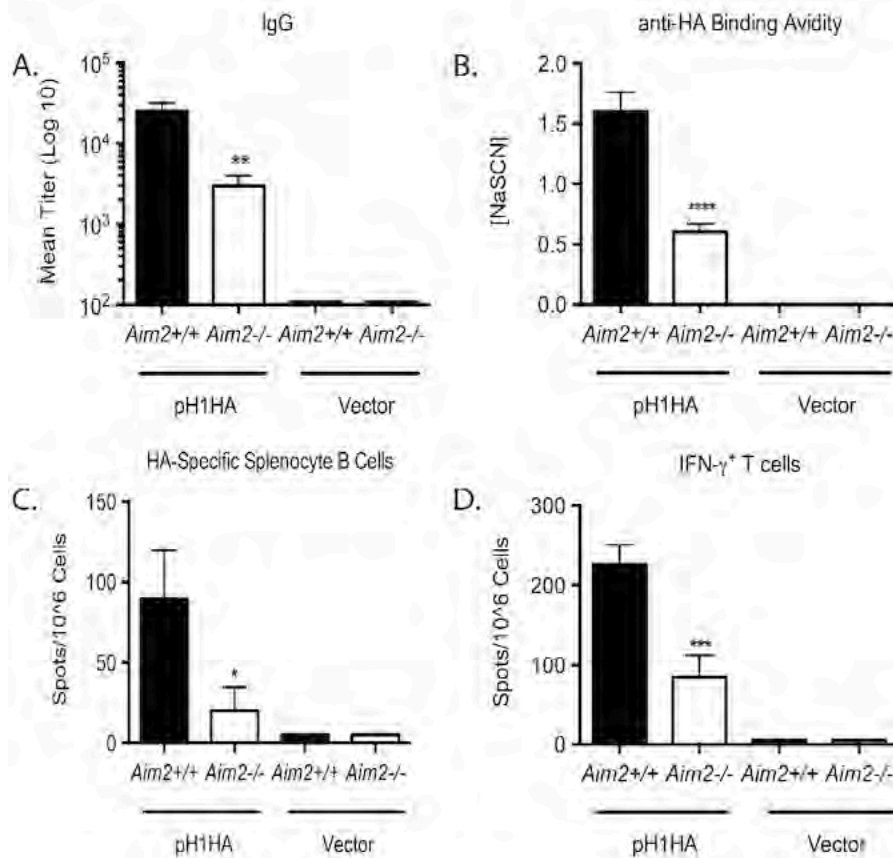


Figure 3.5: Optimal DNA vaccine immunogenicity requires *Aim2*.

Wild-type *Aim2*^{+/+} and *Aim2*^{-/-} mice were immunized intramuscularly with a pH1HA encoding DNA vaccine at weeks 0 and 2. (A) HA-specific IgG titers were analyzed fourteen days post second immunization. Anti-HA binding avidity was quantified via ELISA and reported as molar concentration of sodium thiocyanate required to displace anti-HA serum antibodies to 2x pre-bleed levels (B). For ELISPOT, spleens were harvested at termination 7 days following a 3rd boosting immunization. HA-specific antibody secreting B cells (C) or IFN-γ secreting T cells (D) in mice immunized with either pH1HA or empty vector. Splenocytes were stimulated with the CD8⁺ cell-restricted HA peptide (IYSTVASSL). Data are the averages ± SEM of 5 mice per group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus control group.

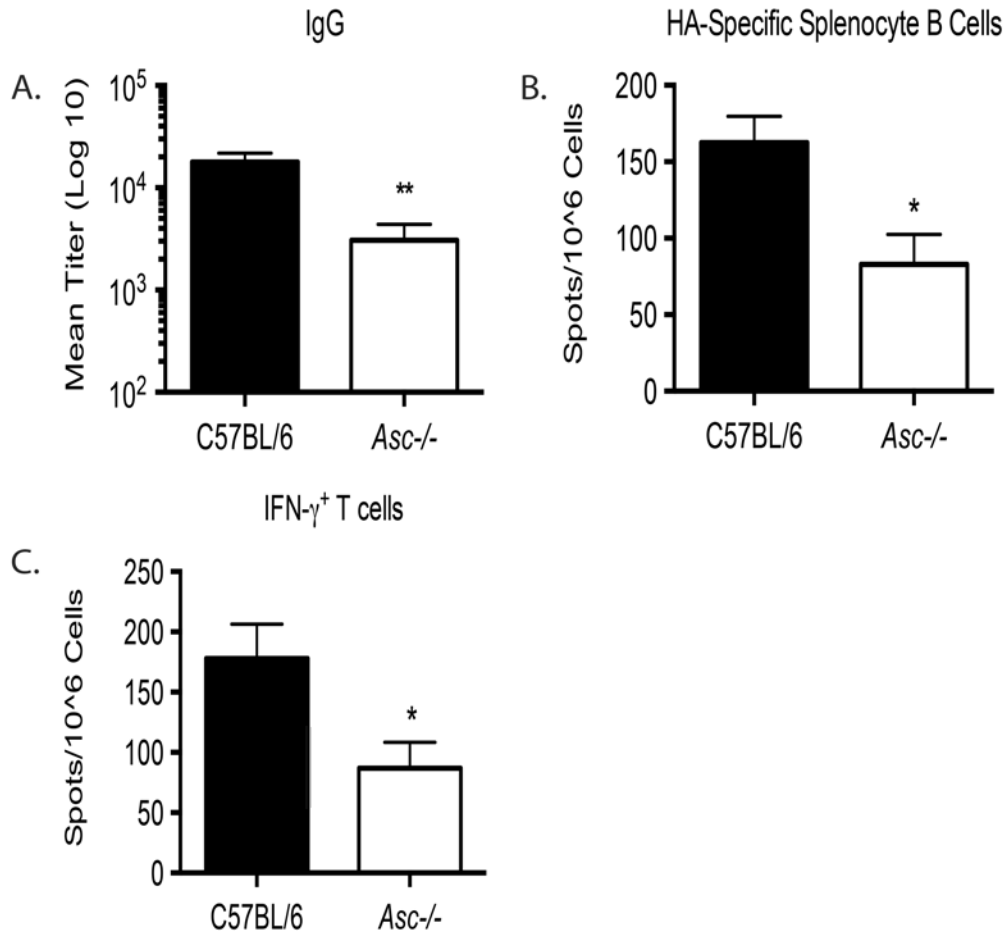


Figure 3.6: *Asc* is required for DNA vaccine immunogenicity.

Wild-type C57BL/6 or *Asc*^{-/-} mice were immunized intramuscularly with a pH1HA encoding DNA vaccine at weeks 0 and 2. (A) HA-specific IgG titers were analyzed fourteen days post second immunization. Spleens were harvested at termination 7 days following a 3rd immunization. Frequency of HA-specific B cells (B) or IFN-γ⁺ T cells (C) were reported as spots per million splenocytes in mice immunized with pH1HA vaccine. Splenocytes were stimulated with the CD8⁺ cell-restricted HA peptide (IYSTVASSL). Data are the averages ± SEM of 5 mice per group. *p<0.05, **p<0.01 versus control group.

***Aim2*-deficient mice fail to cleave caspase-1 into its active form.**

Since DNA vaccination resulted in high levels of caspase-1 activation in *Aim2*^{+/+} mice (**Figure 3.2**), we analyzed *Aim2*^{-/-} mice for their ability to generate catalytically active caspase-1 using the FAM/FLICA assay (**Figure 3.7**). *Aim2*^{-/-} mice demonstrated a clear reduction in caspase-1 activation at the 12-hour peak time point when compared to *Aim2*^{+/+} controls.

Effects of IL-1 and IL-18 deletion on vaccine induced HA-specific immune responses.

As inflammasome signaling ultimately results in the downstream cleavage of pro-IL-1 β and pro-IL18 into their respective active forms, the role of IL-1 β and IL-18 signaling in DNA vaccine was next investigated (**Figure 3.8**). Surprisingly, both of these cytokines were dispensable for the DNA vaccine response as mice lacking the IL-1r or the IL-18r mounted normal vaccine responses. Total serum HA-specific IgG titers were similar to wild-type C57BL/6 mice in both *Il-1r*^{-/-} and *Il-18r*^{-/-} mice. Likewise, no significant difference was seen in total HA-specific B or CD8⁺ T cell numbers as measured by ELISPOT. This data is in line with previously published reports demonstrating little impact on DNA vaccination following MyD88 deletion [95].

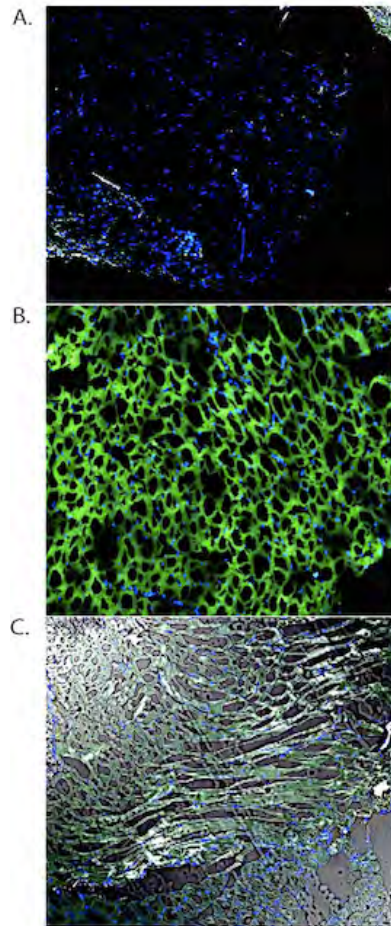


Figure 3.7: *Aim2*-deficient mice exhibit diminished caspase-1 activation at the site of immunization.

(B) Wild-type *Aim2*^{+/+} and (C) *Aim2*^{-/-} mice were immunized intramuscularly with pH1HA vaccine and caspase-1 activation was quantified by FAM/FLICA staining 12 hours post immunization. (A) PBS injected controls were utilized for comparison. The site of injection was harvested and cryopreserved for tissue sectioning. FAM/FLICA staining was visualized by confocal microscopy and is representative of 3 mice per group. Sections of 10 μm were stained with green fluorescent FLICA caspase-1 inhibitor. Nuclei were counterstained with DAPI. Low power resolution presented (Original magnification x 16).

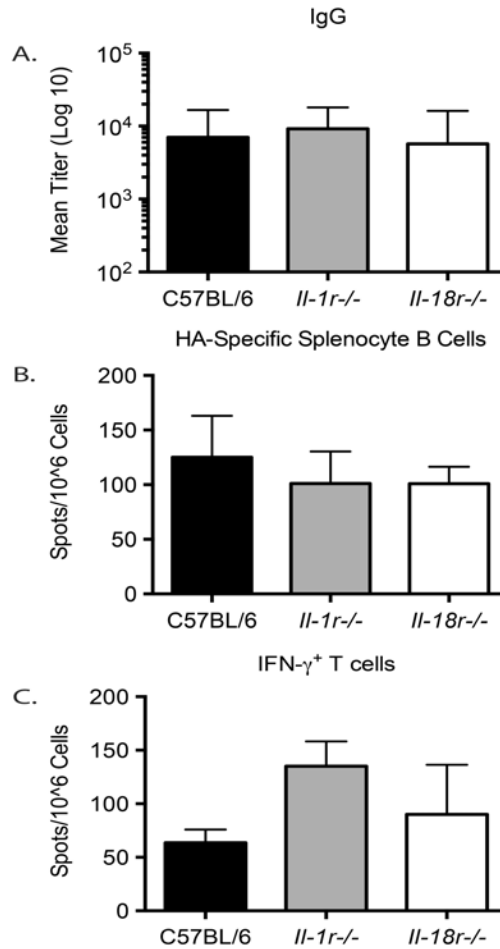


Figure 3.8: IL-1 β and IL-18 are dispensable for DNA vaccine immunogenicity.

Wild-type C57BL/6, *Il-1r^{-/-}*, and *Il-18r^{-/-}* mice were immunized intramuscularly with a pH1HA encoding DNA vaccine at weeks 0 and 2. (A) HA-specific IgG titers were analyzed fourteen days post second immunization. Spleens were harvested at termination 7 days following a 3rd immunization. Frequency of HA-specific B cells (B) or IFN- γ ⁺ T cells (C) were reported as spots per million splenocytes in mice immunized with pH1HA vaccine. Splenocytes were stimulated with the CD8⁺ cell-restricted HA peptide (IYSTVASSL). Data are the averages \pm SEM of 5 mice per group.

The immune response is lineage dependent.

Previously published data has demonstrated the requirement for both hematopoietic and non-hematopoietic cell lineages in DNA vaccination [95]. To further elucidate the role of Aim2, bone marrow chimeric mice were generated by transferring bone marrow from *Aim2*^{+/+} mice into *Aim2*^{-/-} mice, or vice versa (**Figure 3.9**). *Aim2*^{+/+} and *Aim2*^{-/-} mice reconstituted with *Aim2*^{-/-} bone marrow exhibited strong defects in both the T cell response and HA-specific IgG production. Interestingly, transfer of *Aim2*^{+/+} bone marrow into *Aim2*^{-/-} rescued the T cell response, but only partially rescued the humoral response. While HA-specific IgG levels were impaired compared to *Aim2*^{+/+} mice reconstituted with *Aim2*^{+/+} bone marrow, they were significantly higher than *Aim2*^{-/-} bone marrow reconstituted mice. This would support that Aim2 is required in both the hematopoietic and non-hematopoietic lineages for optimal humoral responses, but deficiency in non-hematopoietic lineages does not affect CD8⁺ T cell responses.

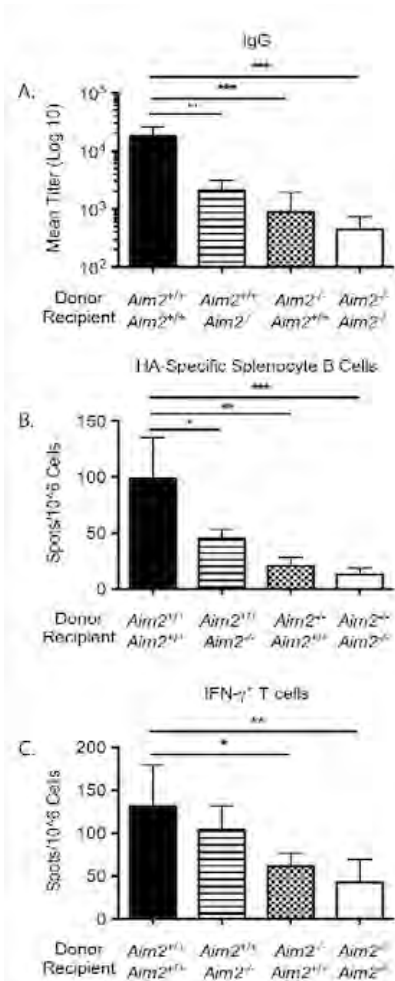


Figure 3.9: Contribution of hematopoietic and non-hematopoietic cells to DNA vaccine induced immunogenicity.

Bone marrow chimeric mice were immunized with a pH1HA vaccine as described in Figure 2. Fourteen days post second immunization, sera from chimeric mice were analyzed for HA-specific IgG titers (A). Spleens were harvested at termination 7 days following the 3rd immunization. Frequency of HA-specific B cells (B) or IFN- γ ⁺ T cells (C) were reported as spots per million splenocytes in mice immunized with pH1HA vaccine. Splenocytes were stimulated with the CD8⁺ cell-restricted HA peptide (IYSTVASSL). Data are the averages \pm SEM of 5 mice per group. *p<0.05, **p<0.01, ***p<0.001 versus control group.

***Aim2*^{-/-} mice lack IFN- $\alpha\beta$ signaling.**

The failure of *Aim2*^{-/-} mice to generate optimal adaptive immune responses implies a defect in immune priming at the site of injection. While the major function of the Aim2 inflammasome is to regulate caspase-1 activation, resulting in IL-1 β , IL-18 and cell death pathways, our data indicate that IL-1 β and IL-18 are not responsible for the Aim2 dependent effects we observed. We therefore endeavored to quantify IFN- $\alpha\beta$ as it has been reported to play a key role in the immune response to B-DNA [56-58, 154]. In addition, it has been established that IFN- $\alpha\beta$ signaling is required for DNA vaccination [81, 95]. Aim2 does not control DNA induced IFN- $\alpha\beta$ production directly. Rather the STING pathway mediates these effects. Since the IFN- $\alpha\beta$ response is so critical for DNA vaccination, we performed a detailed kinetic analysis measuring IFN- $\alpha\beta$ expression in *Aim2*^{+/+} and *Aim2*^{-/-} mice. To ensure we only detect DNA vaccine-induced IFN- $\alpha\beta$, we limited our measurements to the site of immunization. *Aim2*^{+/+} and *Aim2*^{-/-} mice were immunized with the pH1HA DNA vaccine, and punch biopsies were collected from the site of injection. Quantitative rt-PCR analysis of mRNA clearly shows that *Aim2*^{-/-} mice have reduced IFN- α and IFN- β expression compared to *Aim2*^{+/+} controls, with expression peaking at 12 hours post immunization in wild-type mice (**Figure 3.10**). Intriguingly, IFN- $\alpha\beta$ expression in *Aim2*^{-/-} mice peaked at 6 hours post immunization and remained static throughout the time course. Consistent with the decrease in IFN- $\alpha\beta$, there was a corresponding decrease in the IFN stimulated gene, IP10. We also noticed a significant decrease in TNF. As the development of cellular and humoral immunity was cell lineage dependent, we quantified IFN- $\alpha\beta$ levels in Aim2 bone marrow chimeric mice to determine the requirement for Aim2 in cellular lineages with regards to IFN- $\alpha\beta$

production (**Figure 3.11**). Notably, *Aim2*-deletion in both the hematopoietic and non-hematopoietic populations resulted in impaired local IFN- α/β production following DNA vaccination. Reconstitution with *Aim2*^{+/+} bone marrow failed to rescue IFN- α/β production, and no significant difference in IFN- α/β levels was seen between mice with wild-type *Aim2* in the hematopoietic or non-hematopoietic populations, suggesting that both cellular lineages regulate innate immune responses.

Asc^{-/-} mice had similar levels of IFN- α/β , further confirming the requirement for inflammasome signaling (**Figure 3.12**). These data suggest a previously unreported role for *Aim2* in regulating local IFN- α/β levels following DNA vaccination. As *Aim2* controls cell death at the site of infection, it is likely that *Aim2* dependent cell death liberates endogenous DAMP danger signals, which might in turn elicit IFN- α/β via the *Aim2*-independent STING/TBK1 pathways. This broad defect in IFN- α/β signaling likely explains the defects we observed in *Aim2*-deficient mice treated with DNA vaccines.

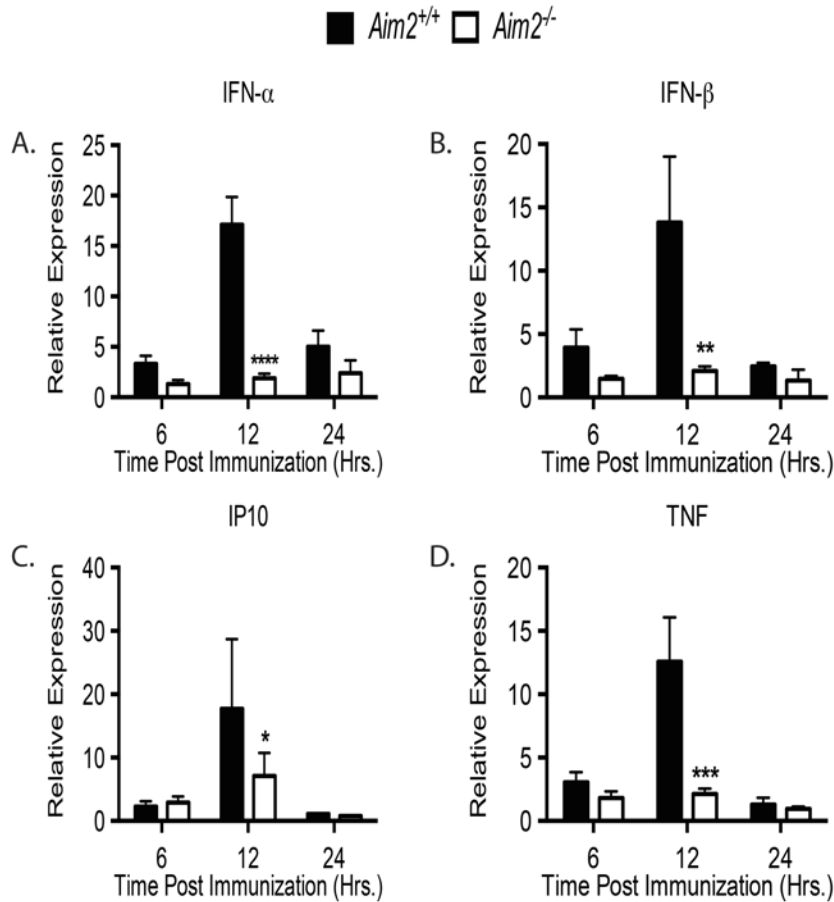


Figure 3.10: *Aim2*-deficiency limits IFN- $\alpha\beta$ production at the site of injection.

Aim2^{+/+} and *Aim2*^{-/-} mice were immunized intramuscularly with pH1HA vaccine, and the site of injection was harvested at various time points. Total RNA was isolated from tissue biopsies and subjected to rt-PCR for (A) IFN- α , (B) IFN- β , (C) IP10, and (D) TNF. Reported expression levels are relative to expression in naïve *Aim2*^{+/+} mice. Data are the averages \pm SEM of 5 mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus control group.

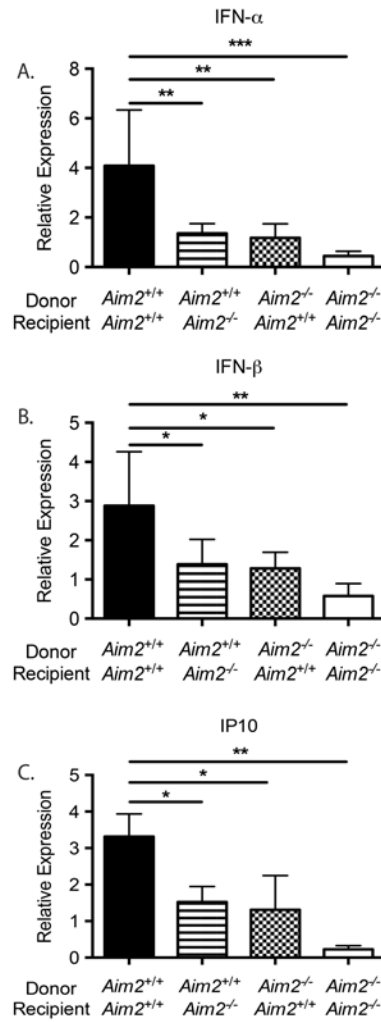


Figure 3.11: *Aim2* is required in both hematopoietic and non-hematopoietic populations for IFN- $\alpha\beta$ production at the site of injection.

Aim2^{+/+} and *Aim2*^{-/-} mice were immunized intramuscularly with pH1HA vaccine, and the site of injection was harvested at various time points. Total RNA was isolated from tissue biopsies and subjected to rt-PCR for (A) IFN- α , (B) IFN- β , and (C) IP10. Reported expression levels are relative to expression in naïve *Aim2*^{+/+}/*Aim2*^{+/+} mice. Data are the averages \pm SEM of 5 mice per group. * p <0.05, ** p <0.01, *** p <0.001 versus control group.

12 Hrs. Post Immunization

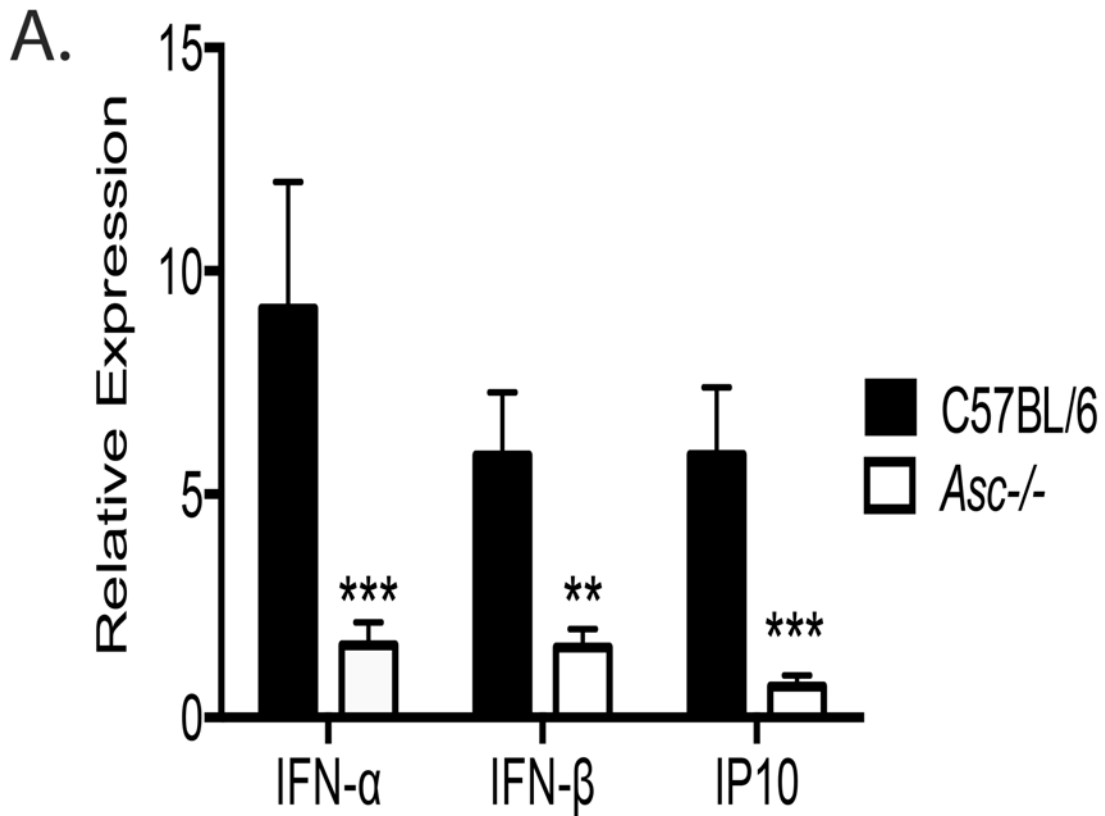


Figure 3.12: *Asc* is required for DNA vaccine induced IFN- $\alpha\beta$ production.

WT C57BL/6 and *Asc*^{-/-} mice were immunized intramuscularly with pH1HA vaccine, and the site of injection was harvested 12 hours later. Total RNA was isolated from tissue biopsies and subjected to rt-PCR for IFN- α , IFN- β , IP10. Data are the averages \pm SEM of 5 mice per group. ** $p < 0.01$, *** $p < 0.001$ versus control group.

Discussion

The innate immune pathways governing DNA vaccination remain to be fully characterized. Recent reports have established the STING/TBK1/IFN- $\alpha\beta$ axis as required for DNA vaccine immunogenicity [81, 95]; however, the PRR(s) required for IFN- $\alpha\beta$ production remain to be identified in this context. Likewise, the involvement of other innate immune signaling pathways is unclear. In particular, the requirement for the inflammasome signaling machinery in DNA vaccine elicited antigen-specific immune responses has not been examined. Here, we identified the Aim2 inflammasome as a DNA vaccine sensor with the ability to regulate the antigen-specific adaptive immune response. Whereas previous reports have focused on downstream signaling molecules, this is the first report to identify a DNA sensor that is required for DNA vaccine immunogenicity.

The failure of *Aim2*^{-/-} mice to generate optimal adaptive immune responses implies a defect in immune priming at the site of injection. While the function of Aim2 has been well characterized, how and where Aim2 interacts with the vaccine plasmid remains unclear. Interestingly, immunization of bone marrow chimeras revealed varying degrees of necessity for Aim2 signaling in both the hematopoietic and non-hematopoietic lineages. Aim2 is required in both cell lineages for optimal humoral responses, as chimeric mice lacked high levels of anti-HA antibodies. This may be attributed to impaired IL-6 production in *Aim2*^{-/-} mice possibly limiting B-cell survival and CD4⁺ T cell expansion as has been reported in several studies (**Figure 3.4**) [155]. In addition, recent reports have described the effect of DNA priming on T follicular helper cell generation [51]. Even more surprising, Aim2-deletion in non-hematopoietic cells did not

impact CD8⁺ T cell responses, as mice reconstituted with *Aim2*^{+/+} bone marrow presented similar levels of IFN- γ to *Aim2*^{+/+}. This provides further evidence for a defect in immune cell priming.

Of note, deletion of IL-1 β and IL-18 signaling in DNA vaccination did not impact DNA vaccine immunogenicity, confirming previous reports describing minimal effect of MyD88 deletion on DNA vaccine immunogenicity [156]. This stands in stark contrast to the importance of IL-1 β and IL-18 inflammatory signaling in the early stages of pathogen infection. Why IL-1 β and IL-18 signaling are dispensable remains unclear, but one possible reason is the highly immunostimulatory nature of the DNA vaccine plasmid itself, as DNA is a potent inducer of both IFN- α/β and several NF κ B regulated immune genes. In addition, the level of several chemokines, such as MIP-1 α/β and MCP-1, remained unchanged (data not shown), allowing for recruitment of monocyte and lymphocyte populations.

Most intriguingly, the reduction of IFN- α/β levels at the site of immunization in *Aim2*^{-/-} suggests a previously unknown relationship between Aim2 and local IFN- α/β production. IFN- α/β induction also appears to be lineage independent, as deletion of Aim2 in either cell lineage attenuated IFN- α/β production. Aim2 is not known to mediate IFN- α/β production directly, hinting at an indirect link between these two divergent pathways. We propose that decreased pyroptotic cell death in *Aim2*^{-/-} mice results in diminished DAMP release, limiting cellular signaling and bystander cell activation. The release of cellular DNA by pyroptotic cells may augment IFN- α/β production by surrounding cells, possibly through the STING/TBK1 signaling axis, further propagating the immune response.

In conclusion, our results indicate that Aim2 plays a significant role in DNA vaccination. *Aim2*^{-/-} mice failed to generate optimal immune responses upon DNA vaccination, exhibiting decreased serum IgG levels and T cell cytokine production, demonstrating the necessity for Aim2 signaling in DNA vaccine immunogenicity. In addition, we report a previously unknown function for Aim2 in augmenting IFN- α/β production at the site of immunization. Our results provide a deeper understanding of the cellular mechanisms through which DNA vaccines stimulate both the innate and adaptive immune pathways to enhance the immune responses targeting the encoded antigen.

Preface to Chapter IV

John Joseph Suschak III performed and analyzed all experiments in this chapter.

Chapter IV

The role of AIM2 in alum adjuvanticity

4.1 Role of Adjuvants in Vaccination.

As discussed, vaccination is a key aspect of public health. Unfortunately, it is sometimes necessary to sacrifice immunogenicity for safety. For instance, subunit and inactivated vaccines tend to induce lower levels of immunity for a significantly shorter term than live-attenuated vaccines, but they do not have the safety risks associated with immunocompromised individuals. To combat this issue, many vaccine formulations include adjuvants to enhance humoral and effector T cell functions. Adjuvants represent a diverse group of compounds that can both vastly improve the immunogenicity of a vaccine and modulate the immune response. While adjuvants have traditionally been used to amplify the adaptive response, another aspect has become increasingly important: adjuvants can shape the immune response to one that is most effective for a given pathogen. Adjuvants have been used to: (1) skew the immune response towards the appropriate type (e.g. T_h1 versus T_h2); (2) increase breadth and specificity [157, 158]; and (3) facilitate the generation of a memory repertoire [159]. Indeed, adjuvant choice is a crucial component of vaccine formulation research and development. Despite the widespread inclusion of adjuvants in vaccine formulations, understanding of their immunological mechanisms remains incomplete.

4.1.1 Vaccine Formulations with Aluminum Salt Adjuvants.

Alexander Glenny first reported the ability of aluminum salts to significantly enhance the antibody response in guinea pigs immunized with soluble toxoid [160]. Since then, aluminum salts (colloquially known as “alum”) have become the most clinically relevant and widely used adjuvant in licensed vaccines. Although the search continues for alternative adjuvants, aluminum adjuvants continue to be used due to their excellent track record of tolerability and adjuvanticity with a variety of antigens. Several alum formulations exist, but the most popular is aluminum phosphate or aluminum hydroxide gel, also known as Alhydrogel®. Another commonly used formulation is Imject® alum, a gel formulation of aluminum hydroxide and magnesium hydroxide. Other proprietary formulations exist, but they are considerably less common.

Aluminum adjuvants are utilized in several vaccine formulations, particularly those administered to children. The diphtheria-tetanus-acellular pertussis vaccine (commonly referred to as DTaP), Hepatitis B, and human papillomavirus vaccines all contain aluminum salts. Aluminum hydroxide gel has also been employed in two successful HIV-1 vaccine clinical trials, VaxGen and RV144. RV144 is especially noteworthy in that it was the first HIV-1 vaccine to demonstrate protection in high-risk individuals [28, 161].

4.2. Cellular Mechanism for Alum Adjuvanticity.

The goal of vaccination is to induce long term immunity while minimizing side effects. The choice of adjuvants has traditionally been made empirically due to their effects on the adaptive immune response. This holds especially true for alum, which is generally chosen for its ability to boost humoral responses with little reactogenicity. Yet, the mechanism of alum’s adjuvant effect remains unclear, owing to inconsistent study

results. Several models exist, but none have been conclusively proven. The disagreement between study data may be attributed to differences in alum formulation and route of immunization. Therefore, further investigation is required.

4.2.1. Antigen Depot Effect.

Originally, it was postulated that alum provided an antigen depot effect, whereby alum absorbed antigen is slowly released, improving uptake by APCs. Intraperitoneal injection of alum results in aluminum depots that can last up to one month. Dendritic cells and antigen specific T cells accumulate around this depot, suggesting that the antigen depot is involved in maintenance of the memory pool [162]. However, this effect is site of immunization dependent, as both intramuscular and subcutaneous injection result in rapid alum/antigen complex disassociation [163]. Furthermore, it has been demonstrated that adsorption of antigen onto alum is not required for adjuvanticity [164, 165]. In fact, characterization studies of HIV-1 gp120 antigen formulated with a variety of aluminum hydroxide compounds showed that gp120 is rapidly desorbed from the adjuvant following immunization of guinea pigs, rabbits, and baboons [164]. These data suggest a fundamental difference in alum depot functionality depending on the site of injection and the local environment.

4.2.2 Aluminum Adjuvants and the Inflammasome.

The cellular signaling pathways governing APC activation and humoral immunity following alum immunization have only recently begun to be addressed. A hallmark of alum immunization is the release of high levels of the pro-inflammatory cytokines IL-1 β and IL-18. This has led several groups to explore the effect of the pro-inflammatory NOD like receptors on alum adjuvanticity. The NOD like receptors trigger

inflammasome assembly in response to microbial pathogens as well as DAMPs. The NOD like receptor 3 (NLRP3) is activated by a wide range of PAMPs as well as DAMPs, including ATP, nigericin, cholesterol, uric acid, silica, and amyloid- β [166-169]. As with all inflammasome receptors, NLRP3 forms a molecular platform with ASC and caspase-1, resulting in IL-1 β and IL-18 activation. NLRP3 also results in caspase-1 dependent pyroptosis.

It has been reported that alum adjuvant signals through NLRP3 inflammasome to stimulate inflammatory dendritic cells in vitro [170-172]. However, in vivo studies have yielded contradictory results. In vivo studies in Asc, caspase-1, and Nlrp3-deficient mice failed to elicit antigen-specific antibody responses, suggesting that inflammasome signaling is critical for adaptive immunity raised by aluminum salt adjuvants. In an in vivo immunization model utilizing OVA antigen, the induction of OVA-specific IgG1 by alum required assembly and activation of the NLRP3 inflammasome. Meanwhile, concurrent studies confirmed that alum activates IL-1 β and IL-18 via the NLRP3 pathway, but argue that NLRP3 is of little importance to the adaptive response. Studies by Li *et al.* supported the above evidence [173], but independent groups reported that while alum activation of the NLRP3 inflammasome was critical for OVA-specific T cell responses, it was unessential for humoral immunity [174-176]. Still other studies reported no impact of either caspase-1 or NLRP3 activation on the specific CD8⁺ T cell, CD4⁺ T cell, antibody, or T_h2-biased responses after alum-adjuvanted vaccination with model antigens [177].

Interestingly, the question remains if aluminum salts act directly or indirectly to trigger innate sensors. Perhaps the most compelling argument for alum's adjuvant effect

centers on its cytotoxic effects [178]. Apoptotic cells release a plethora of endogenous danger-associated molecular pattern (DAMPs), which prime the immune system via innate PRR signaling [63]. Early evidence supported a direct mechanism involving particulate phagocytosis and subsequent lysosomal disruption releasing DAMPs into the extracellular space [167, 171, 176]. More recent studies by Marichal *et al*, using a novel approach, show that alum injection results in the cytotoxic release of host DNA, which mediates the adjuvant activity of alum via a TBK1-dependent IFN pathway [128, 129, 177]. It remains to be seen if the cytotoxic release of cellular DNA also stimulates the pro-inflammatory response, and to what extent this impacts adjuvanticity. Specifically, as several reports have identified the requirement for the inflammasome and IL-1 β , it stands to reason that the cytosolic DNA sensor AIM2 may play a role in alum adjuvanted immune responses, but this has yet to be addressed. Here, we report that alum treatment triggers the AIM2 inflammasome, and the subsequent inflammatory signaling is required for optimal vaccine-induced immunity. Furthermore, AIM2 at least partially regulates the induction of IFN- $\alpha\beta$ at the site of alum immunization. Therefore, we have established a novel role for AIM2 as a key player in alum adjuvanticity.

Results

Alum adjuvant activates the Aim2 inflammasome.

Marichal *et al.* reported that the cytotoxic release of cellular DNA mediated the adjuvant effects of alum through bystander cells [129]. We therefore hypothesized that the release of endogenous DNA by alum treated cells would stimulate the pro-inflammatory cytosolic DNA receptor, AIM2. We first evaluated the ability of BMDM harvested from either *Aim2*^{+/+} or *Aim2*^{-/-} mice to produce IL-1 β following alum treatment in vitro (**Figure 4.1**). As expected, both the synthetic B-form dsDNA poly(deoxyadenylic-deoxythymidylic) and alum induced a robust IL-1 β response in *Aim2*^{+/+} macrophages. Yet, IL-1 β was almost completely absent from *Aim2*^{-/-} cultures. To ensure that IL-1 β cleavage was inflammasome dependent, the pan-caspase inhibitor Z-VAD-FMK was added to BMDM cultures prior to alum stimulation. The addition of Z-VAD-FMK resulted in significantly decreased IL-1 β activation in *Aim2*^{+/+} macrophages, to levels comparable to *Aim2*^{-/-} wells, indicating that the inflammasome regulates caspase-1 dependent IL-1 β maturation in response to alum adjuvant.

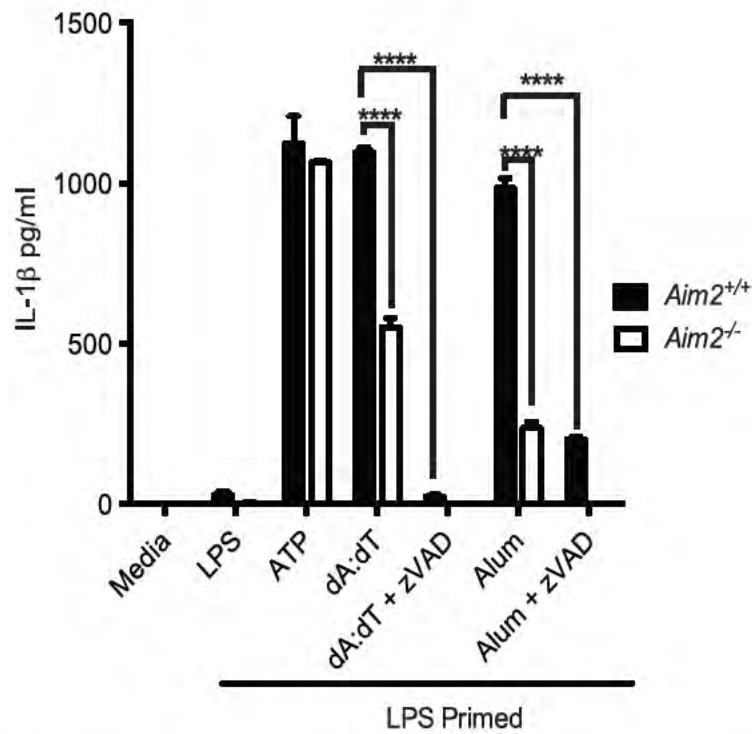


Figure 4.1: *Aim2* is required for IL-1 β production in response to alum treatment.

LPS (200 ng/ml) primed *Aim2*^{+/+} and *Aim2*^{-/-} BMDM were transfected with poly(dA-dT) or treated with alum alone for 18 hours in the presence or absence of the pan-caspase inhibitor Z-VAD-FMK and secreted IL-1 β was quantified by ELISA. Data are presented as mean \pm SEM from 3 independent experiments. ****p<0.0001 versus control group.

Aim2 is required for optimal B cell responses in TIV + alum immunized mice.

As it is evident that the Aim2 inflammasome recognizes and responds to the release of endogenous DNA by alum treated cells in culture, we tested the ability of alum to adjuvant the adaptive immune response generated by the seasonal trivalent inactivated influenza vaccine (TIV). Previous studies have mainly used model antigens absorbed onto alum to study adjuvanticity. We chose to utilize the clinically relevant TIV as it has been shown to be immunogenic in eliciting HA-specific antibodies in both animal and human studies. HA is the major protective antigen in clinically licensed inactivated and live-attenuated influenza vaccines, and serves as a clear target for immunological assays. *Aim2*^{+/+} and *Aim2*^{-/-} mice were immunized with either the seasonal TIV alone or TIV absorbed onto alum according to the schedule shown in **Figure 4.2**. Anti-HA antibodies were detected in both *Aim2*^{+/+} and *Aim2*^{-/-} mice immunized with TIV alone. HA-specific IgG and IgG1 levels were significantly boosted in wild-type *Aim2*^{+/+} mice immunized with alum absorbed TIV. In stark contrast, *Aim2*^{-/-} mice failed to respond to alum inclusion, yielding antibody titers similar to those seen in TIV alone mice (**Figure 4.3A-C**). Further confirming a defect in B cell activation, TIV + alum immunized *Aim2*^{-/-} mice had significantly fewer HA-specific B cells in both the spleen and bone marrow. Aim2 also appears to play a role in regulating the antibody maturation process as *Aim2*^{-/-} mice demonstrated impaired antibody avidity (**Figure 4.3D-F**). *Aim2*^{+/+} mice required high concentrations of the chaotropic agent NaSCN to disrupt antigen/antibody complexes, while much lower concentrations of NaSCN were required for disassociation of *Aim2*^{-/-} sera. All together, this data suggests that Aim2 deficiency results in impaired quality of antigen-specific, alum-adjuvanted antibody responses.

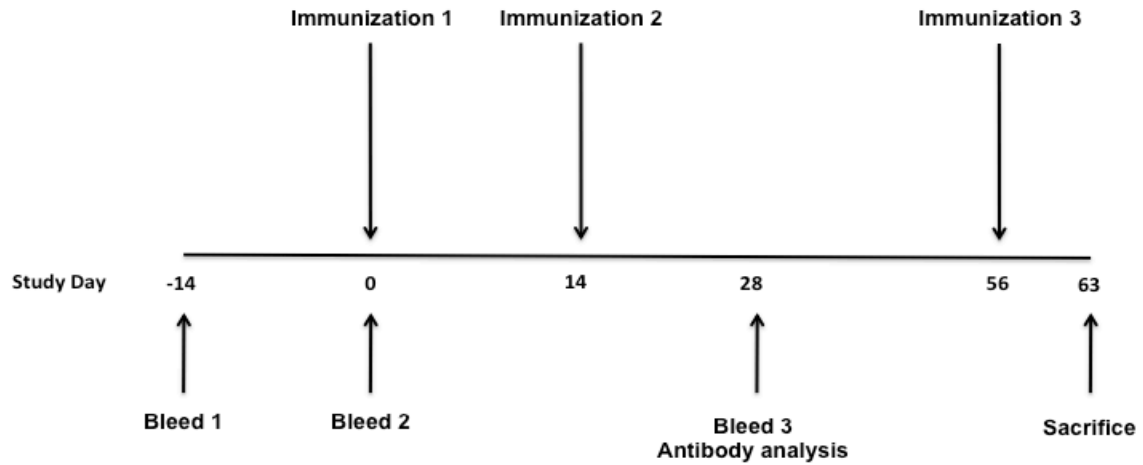


Figure 4.2: Study design and immunization schedule.

Mice received either 1.5 ug Aventis Pasteur Fluzone 2004-2005 trivalent inactivated vaccine (TIV) alone or absorbed onto 65 ug Alhydrogel®, divided between quadriceps, at 2 and 4 weeks. A third boosting immunization was delivered 1-week prior to sacrifice.

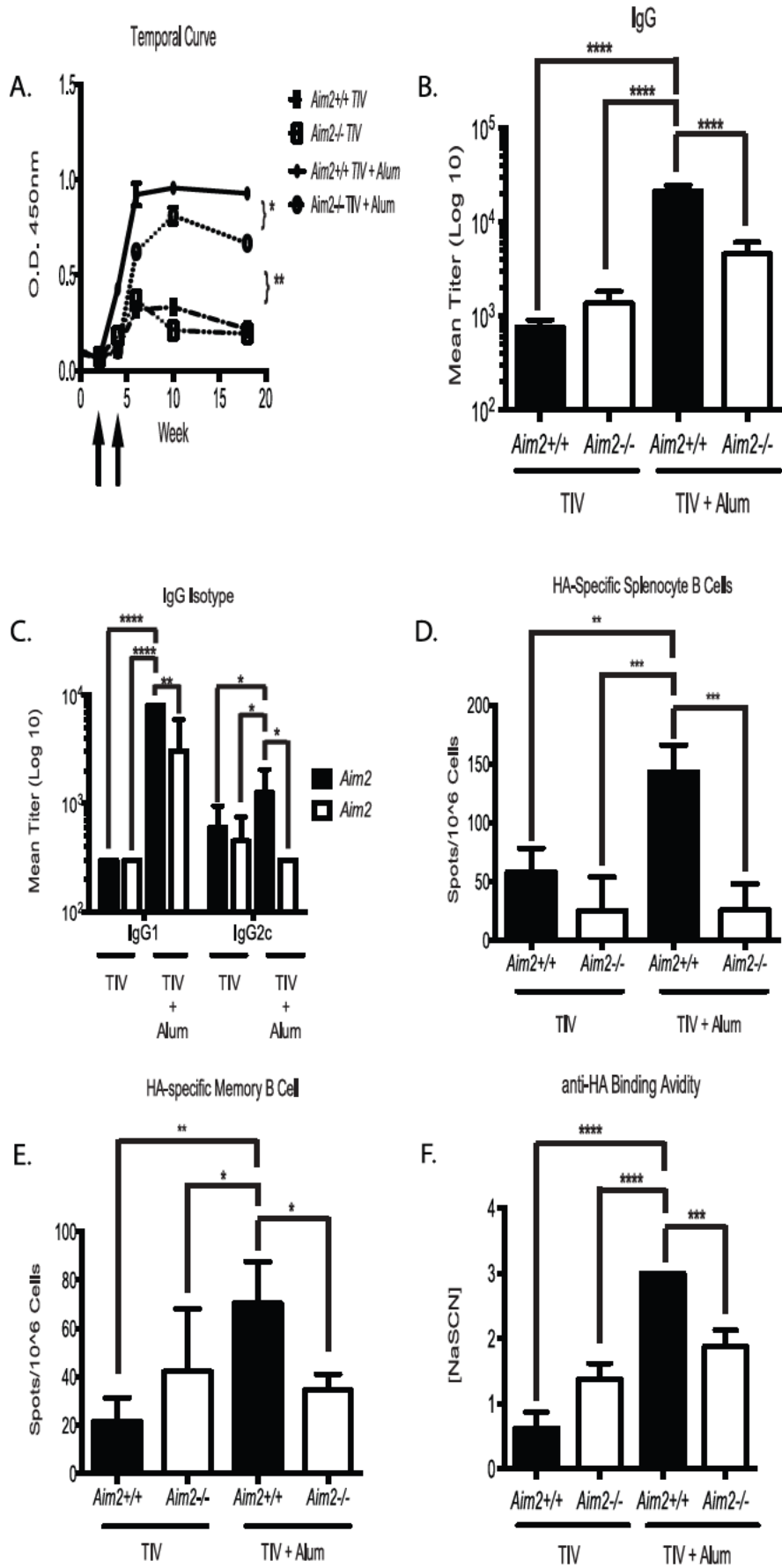


Figure 4.3: Alum adjuvanted humoral responses require Aim2.

Wild-type *Aim2*^{+/+} and *Aim2*^{-/-} mice were immunized intramuscularly with either TIV alone or TIV absorbed onto alum at weeks 0 and 2. (A) Peak level of pooled sera (5 mice per group). Arrows represent points of immunization. HA-specific IgG titers (B), including their (C) isotypes, were analyzed fourteen days post second immunization. Splenocytes and bone marrow were harvested at termination 7 days following a 3rd boosting immunization. (D) Splenocyte B cells were plated immediately following isolation, while (E) bone marrow cells were plated after 5 days of culturing in non-specific stimulation to promote clonal expansion. Anti-HA binding avidity was quantified via ELISA and reported as molar concentration of sodium thiocyanate required to displace anti-HA serum antibodies to 2x pre-bleed levels (F). Data are the averages \pm SEM of 5 mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus control group.

Aim2-mediated alum signaling is lineage dependent.

The necessity of Aim2 inflammasome signaling in diverse cell lineages remains to be determined. As such, we generated bone marrow chimeric mice by transferring bone marrow from *Aim2*^{+/+} mice into *Aim2*^{-/-} mice, or vice versa (**Figure 4.4**). Both *Aim2*^{+/+} and *Aim2*^{-/-} mice reconstituted with *Aim2*^{-/-} bone marrow exhibited strong defects in antigen-specific IgG production. Interestingly, reconstitution with wild-type *Aim2*^{+/+} bone marrow fully rescued the anti-HA IgG response, an effect not seen in mice reconstituted with *Aim2*^{-/-} bone marrow. Moreover, total HA-specific B cell numbers were decreased only in mice receiving *Aim2*^{-/-} bone marrow, providing further evidence that inflammasome-dependent APC priming is an integral component of alum adjuvants.

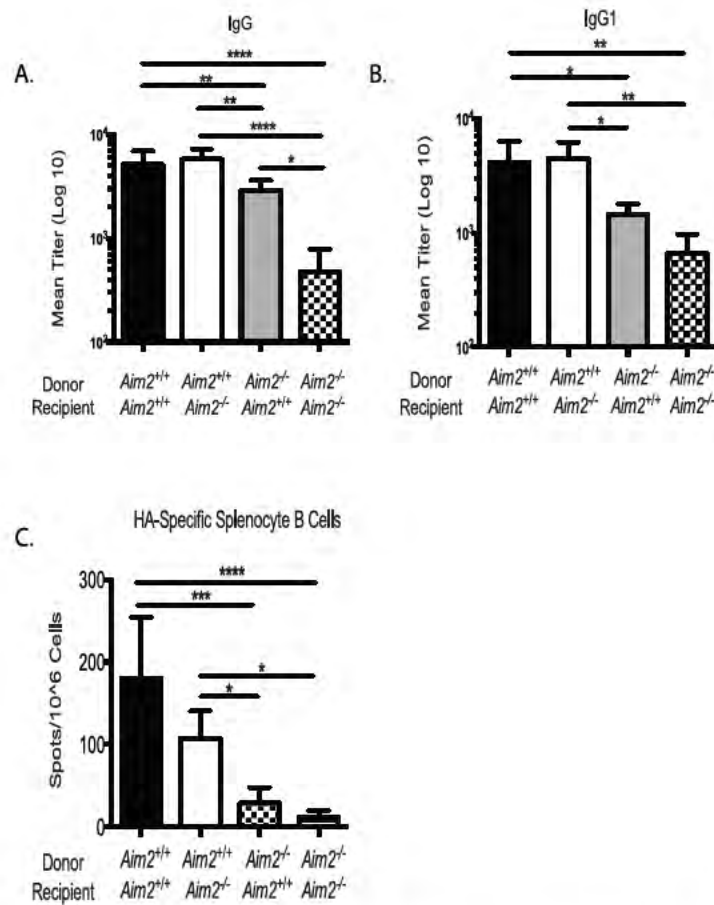


Figure 4.4: Contribution of hematopoietic and non-hematopoietic cells to alum adjuvanticity.

Bone marrow chimeric mice were immunized with a pH1HA vaccine as described in Figure 4.3. Fourteen days post second immunization, sera from chimeric mice were analyzed for HA-specific IgG titers (A) or IgG1 subtype (B). Spleens were harvested at termination 7 days following the 3rd immunization. Frequency of HA-specific B cells (C) are reported as spots per million splenocytes in mice immunized with pH1HA vaccine. Data are the averages \pm SEM of 5 mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus control group.

***Aim2*^{-/-} mice lack IFN- $\alpha\beta$ signaling.**

TBK1 mediated IFN- $\alpha\beta$ production has been reported to play a central role in alum adjuvanticity [129]. The Aim2 inflammasome regulates IL-1 β , IL18, and pyroptosis via caspase-1 activation. As IFN- $\alpha\beta$ is required for alum-mediated adaptive immunity, we quantified IFN- $\alpha\beta$ expression in *Aim2*^{+/+} and *Aim2*^{-/-} mice. To ensure we detect only alum/TIV-induced IFN- $\alpha\beta$, we limited our measurements to the site of immunization. *Aim2*^{+/+} and *Aim2*^{-/-} mice were immunized with the alum/TIV complex, and punch biopsies were collected from the site of injection. rt-PCR analysis of *Aim2*^{-/-} mRNA shows a clear reduction in IFN- α and IFN- β expression at 12 hours when compared to *Aim2*^{+/+} controls (**Figure 4.5**), suggesting a previously unreported role for Aim2 in regulating local IFN- $\alpha\beta$ levels following alum administration. Since Aim2 controls cell death at the site of infection, it is possible that Aim2 dependent cell death releases endogenous danger signals, which might in turn elicit IFN- α/β via the Aim2-independent STING/TBK1 pathways. This likely explains the defects we observed in *Aim2*-deficient mice treated with alum adjuvant.

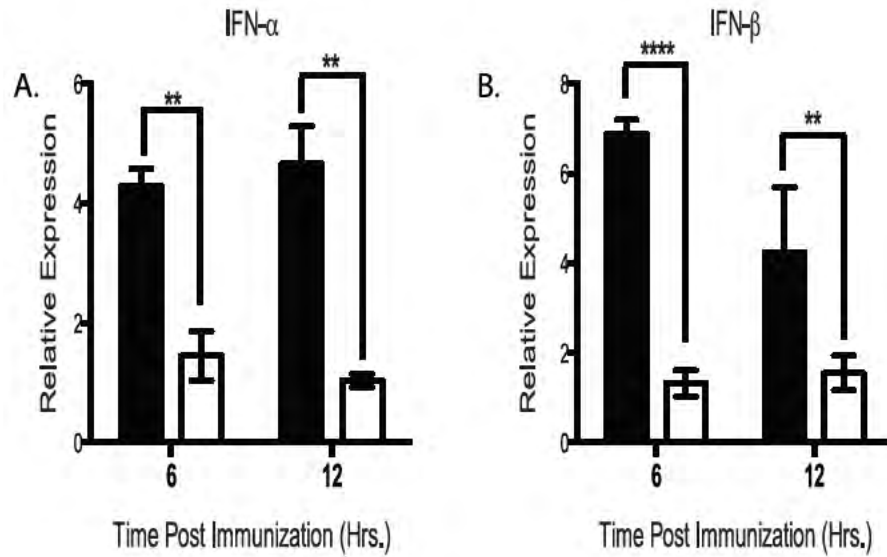


Figure 4.5: *Aim2*-deficiency limits IFN- $\alpha\beta$ production at the site of injection.

Aim2^{+/+} and *Aim2*^{-/-} mice were immunized intramuscularly with alum absorbed TIV, and the site of injection was harvested 12 hours later. Total RNA was isolated from tissue biopsies and subjected to rt-PCR for (A) IFN- α or (B) IFN- β . Reported expression levels are relative to naïve *Aim2*^{+/+} expression. Data are the averages \pm SEM of 4 mice per group. **p<0.01, ****p<0.0001 versus control group.

Discussion

The role of innate immune signaling in aluminum adjuvanticity remains unclear. Specifically, the impact of the inflammasome on elicitation of antigen-specific adaptive immune responses following alum immunization is poorly defined. Whereas previous reports have attributed alum's adjuvant effects to the NLRP3 inflammasome, our results indicate that alum also triggers Aim2-dependent IL-1 β release, suggesting that Aim2 is at least partially required for APC activation.

We found that the Aim2 inflammasome is required for generation of high-level antibody responses following TIV + alum immunization. *Aim2*^{-/-} mice exhibited antibody levels that were similar to those of animals immunized with TIV alone. The failure of *Aim2*^{-/-} reconstituted chimeric mice to generate robust antigen-specific humoral responses implies the defect is primarily in hematopoietic cells. The mechanism of action remains unknown, but as alum rapidly associates with free host DNA [179], it can efficiently deliver DNA to the cytosol where it is detected by Aim2, driving the inflammatory response required for generation of immunological memory [124-127].

Most intriguingly, the reduction of IFN- $\alpha\beta$ levels at the site of immunization in *Aim2*^{-/-} mice suggests a relationship between Aim2 and local IFN- $\alpha\beta$ production. We propose that decreased pyroptotic cell death in *Aim2*^{-/-} mice results in diminished DAMP release, limiting cellular signaling and bystander cell activation. The release of cellular DNA by pyroptotic cells may augment IFN- $\alpha\beta$ production by surrounding cells, propagating the immune response. Marichal *et al* have previously proposed a model in which self-DNA induces high levels of IgG1 via highly active T_h effectors [129]. Aim2 pyroptotic DAMP release may contribute to this pathway, elevating the IgG response

even further.

In conclusion, our results indicate that Aim2 plays a significant role in alum adjuvanticity. *Aim2*^{-/-} mice failed to generate high-level antibody responses following TIV + alum immunization. In addition, we report a previously unknown function for Aim2 in augmenting IFN- α/β production at the site of alum immunization. Of note, our results are in disagreement with Kool *et al* and Franchi *et al*, who reported a limited role for the inflammasome in IgG production, which is most likely attributed to our use of IM injection as opposed to intraperitoneal injection. Furthermore, while we have identified Aim2 as a player in alum adjuvanticity, we cannot discount the role of NLRP3. The impact of each inflammasome may be route of immunization dependent. It is probable that both pathways work in concert to drive the pro-inflammatory cascade. DAMP release contributes not only to the TBK1/IFN- $\alpha\beta$ pathway, but also to memory cell generation via inflammatory signals. Our results provide a deeper understanding of the cellular mechanisms through which alum stimulates both the innate and adaptive immune pathways to enhance humoral responses.

Chapter V

DNA Vaccine Materials and Methods

Cell Culture and Stimulation

Irf^{+/+}, *Irf3*^{-/-}, and *Irf3*^{-/-}/*Irf7*^{-/-} (DKO) immortalized BMDM were generated in house (UMMS). LPS was treated at a concentration of 200 ng/ml. Poly (dA:dT) (Sigma Aldrich) DNA and H1-TX04-09.tPA DNA vaccine plasmid were transfected using Lipofectamine 2000 at a concentration of 1.5 µg/ml. Cultures were incubated 16-18 hours at 37 ° C, and supernatants were harvested. Murine IFNβ sandwich ELISA was used as previously described. IFN-α, IFN-β, rt-PCR was performed on RNA was isolated from immortalized BMDM. cDNA was then used for rt-PCR reactions on a Bio-Rad CFX-96 cycler. Primers sequences are available upon request.

Mouse BMDC were generated from *Aim2*^{+/+} or *Aim2*^{-/-} mice by culturing fresh bone marrow in R10 medium containing GM-CSF for 8 days at 37 ° C. *Aim2*^{+/+} and *Aim2*^{-/-} immortalized BMDM were produced in house. Cells were first primed with 200 ng/ml LPS (Sigma Aldrich) for 4-5 h prior to treatment with appropriate stimuli. All media was removed from the cells, and the appropriate stimulus was added. Poly (dA:dT) (Sigma Aldrich) DNA and H1-TX04-09.tPA DNA vaccine plasmid were transfected using Lipofectamine 2000 at a concentration of 1.5 µg/ml. ATP was added at a concentration of 1.25 µg /ml. Cultures were incubated 16-18 hours at 37 ° C, and supernatants were harvested.

Cell Culture Cytokine ELISA

Irf^{+/+}, *Irf3*^{-/-}, and DKO immortalized BMDM were generated in house (UMMS).

LPS was treated at a concentration of 200 ng/ml. Poly (dA:dT) (Sigma Aldrich) DNA and H1-TX04-09.tPA DNA vaccine plasmid were transfected using Lipofectamine 2000 at a concentration of 1.5 µg/ml. Cultures were incubated 16-18 hours at 37 ° C, and supernatants were harvested. Murine IFNβ sandwich ELISA was used as previously described. IFN-α, IFN-β, rt-PCR was performed on RNA was isolated from immortalized BMDM. cDNA was then used for rt-PCR reactions on a Bio-Rad CFX-96 cyclor. Primers sequences are available upon request.

Cell culture supernatants were assayed for IL-1β (BD Biosciences, Franklin Lakes, NJ) by ELISA. Lactate dehydrogenase (LDH) release was used to measure pyroptotic cell death. LDH assays were performed using the Promega CytoTox96 Non-radioactive Cytotoxicity Kit according to manufacturer's directions (Promega, Madison, WI)

Mice

C57BL/6 mice were obtained from Taconic Laboratories. *Sting*^{-/-} mice were a gift from G. Barber (University of Miami). *Irf3*^{-/-}, *Irf7*^{-/-}, *Irf3*^{-/-}/*Irf7*^{-/-} (DKO), and *cGas*^{-/-} mice were generated in house by K. Fitzgerald group at the University of Massachusetts Medical School (UMMS). All mice were maintained in the Department of Animal Medicine at UMass Medical School according to IACUC-approved protocols. Mice received 100 µg of codon optimized H1HA DNA vaccine expressing the full-length wild type HA protein from A/Texas/04/09 (pH1HA), divided between quadriceps, at 2 and 4 weeks. A third boosting immunization was delivered 1-week prior to sacrifice.

C57BL/6 mice were obtained from Taconic Laboratories. *Aim2*^{-/-} mice were

generated in house by K. Fitzgerald's group at the University of Massachusetts Medical School (UMMS) as previously described [117]. *Aim2*^{-/-} mice were on a mixed B6/129 background and therefore B6x129 mice were utilized as controls. B6.129 (hereafter referred to as *Aim2*^{+/+}) mice were obtained from Jackson Laboratories (Bar Harbor, ME) and were bred at UMMS. IL-1 receptor (Il-1r), IL-18 receptor (Il-18r), and *Asc*-deficient mice were produced in house. All mice were maintained in the Department of Animal Medicine at UMMS according to IACUC-approved protocols. Mice received 100 µg of codon optimized H1HA DNA vaccine expressing the full-length wild type HA protein from A/Texas/04/09 (pH1HA), divided between quadriceps, at 2 and 4 weeks. A third boosting immunization was delivered 1-week prior to sacrifice.

Enzyme Linked Immunosorbent Assay (ELISA)

Transiently expressed H1HA antigen was coated onto 96 well microtiter plates (Costar #3369) at ~1 µg/mL in 100 µL of PBS for 1 hour at room temperature. Plates were washed 5 times in PBS containing 0.1% Triton-X (EWB) and blocked overnight at 4° in PBS containing 4% whey and 5% powdered milk. The following morning, plates were washed 5 times in EWB, serially diluted mouse sera, collected at 2 weeks following either the secondary DNA immunization, was added to the wells in a volume of 100 µL. Plates were washed 5 times in EWB and 100 µL of biotinylated anti-mouse secondary antibody (Vector Labs BA-1000) at 1.5 µg/mL was incubated on the plate for 1 hour at room temperature. Plates were washed 5 times with EWB and incubated with 100 µL of streptavidin horseradish peroxidase (Vector Labs SA-5004) at 500 ng/mL. Plates were washed a final 5 times with EWB and developed for 3 min in 100 µL of a 3,3',5,5'-tetramethylbenzidine substrate solution (Sigma T3405). The reaction was stopped with

addition of 25 μL of 2N H_2SO_4 . Endpoint titers as reported are defined as the last dilution of a serially diluted serum sample with greater than twice the background optical density of a pre-immune serum sample. For temporal antibody time courses, pooled mouse sera dilutions of 1:100 were generated. For mouse IgG isotyping, Biotin-conjugated IgG2b or IgG2c detection antibody (Southern Biotech) was applied at 1.0 $\mu\text{g}/\text{ml}$.

NaSCN Displacement

Transiently expressed H1HA antigen was coated onto 96 well microtiter plates (Costar #3369) at 1 $\mu\text{g}/\text{mL}$ in 100 μL of PBS for 1 hr at room temperature. Plates were washed 5 times in PBS containing 0.1% Triton-X (EWB) and blocked overnight at 4° in PBS containing 4% whey and 5% powdered milk. Mouse sera were then added to the plate at a dilution of 1:100 and incubated at room temperature for 1 hour. Plates were again washed 5 times in EWB. NaSCN was then added at various (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 M) concentrations in PBS for 15 min followed by 5 washes in EWB. Bound IgG was detected as described above. Data is reported as the NaSCN concentration required to yield an optical density twice that of a pre-immune serum sample.

Splenocyte preparation

Spleens were harvested 7 days following the third DNA immunization. Spleens were homogenized in complete RPMI media, with 10% heat-inactivated FBS (HyClone, Logan, UT), and 1% Penicillin-Streptomycin. Single-cell suspensions were generated by homogenization and then draining each spleen through a screen, and washing with media. Red blood cells were lysed with Red Blood Cell Lysis Buffer (Sigma Aldrich). Cells were washed, counted, and diluted to a final concentration of 1×10^7 cells/ml.

Assays for HA-specific antibody secreting cells

B-cell ELISpot reagents were obtained from Mabtech (Mariemont, OH). H1HA-specific antibody secreting cells in immune mouse splenocytes and bone marrow were detected. MAIPSWU plates, (Millipore, Billerica, MA, USA) were coated with 100 μ l (~1.0 μ g)/well of transiently expressed H1HA antigen produced from human embryonic 293T cells and incubated at 4°C overnight. The plates were washed 5 times with PBS, and then blocked by the addition of 200 μ l of complete RPMI in each well for 1 hour at room temperature. Freshly isolated splenocytes (100 μ l/well, 5×10^5 cells/well) in complete RPMI medium with 0.1% β -ME were incubated in triplicate wells for 18 h at 37°C. The plates were then washed with PBS and incubated with 100 μ l of biotinylated goat-anti- mouse IgG 1 μ g/ml in PBS with 0.5% fetal bovine serum (FBS) at room temperature for 2 hours. After additional washes, 100 μ l of HRP-conjugated Streptavidin complex diluted at 1:1000 in PBS with 0.5% FBS was added to each well and incubated at room temperature for 1 hour, then spots were developed by a 3 minute color reaction using 100 μ L of a 3,3',5,5'-tetramethylbenzidine substrate solution. The number of H1HA specific ASCs was counted and calculated.

T Cell ELISpot

ELISpot reagents ($\text{IFN}\gamma$) were obtained from Mabtech (Mariemont, OH). ELISpots were performed according to manufacturer's instructions. Pre-coated MSIP PVDF-plates (Millipore, Billerica, MA) were seeded with splenocytes from immunized mice (prepared as above) at a 2.5×10^5 cells/well. Positive controls were stimulated with 20 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO) and

500 ng/ml ionomycin (Sigma-Aldrich). The H1HA relevant peptide used was a CD8⁺ cell-restricted HA peptide (IYSTVASSL). Antigen-specific stimulation was performed with a concentration of 15 µg/ml. Mock stimulated wells received media only. Plates were incubated 18-20 hr at 37° C. Plates were developed according to manufacturers instructions. Positive spots were visualized on a CTL Imager and counting was performed with Immunospot software (Cellular Technology Ltd., Shaker Heights, OH)

Serum Cytokine Levels

Type I IFN study cytokine levels were quantified in sera collected from individual mice prior to immunization at week 0 and 6 hours post primary DNA using the Mouse T_h1/T_h2/T_h17 CBA kit (BD Biosciences, Franklin Lakes, NJ) according to manufacturer's instructions. The panel of cytokines included: IFN-γ, IL-2, IL-4, IL-6, TNF, IL-10, and IL-17. After collection, serum samples were stored at -80° C until the conclusion of the study, and all serum samples from each time point of interest were run in a single CBA experiment. Prior to assay, serum samples were diluted 1:2 in sample diluent. Samples were read on a LSRII flow cytometer and analyzed with FCAP Array Software version 3.0 (BD Biosciences).

Inflammasome study cytokine and chemokine levels were quantified in serum collected from individual mice prior to immunization at week 0, and 6 hours post primary DNA using a custom Bio-Plex cytokine assay (Bio-Rad, Hercules, CA) according to manufacturer's instructions. The panel of cytokines included: IFNγ, IL-1β, IL-2, IL-4, IL-6, Eotaxin, G-CSF, KC, MIP-1α, MIP-1β, MCP-1, and RANTES. After collection, serum samples were stored at -80° C until the conclusion of the study, and all serum samples from each time point of interest were run in a single Luminex experiment. Prior to assay,

serum samples were diluted 1:4 in sample diluent. Samples were read on a Bio-Plex 200 system with Bio-Plex Manager software (Bio-Rad).

Quantitative Real Time-Polymerase Chain Reaction

Mice were shaved and immunized with 100 µg H1-TX04-09.tPA DNA vaccine plasmid intramuscularly injected into the hind quad muscle. Punch biopsies were harvested from the site of immunization at 6 hours, 12 hours, and 24 hours post immunization and snap frozen. RNA was isolated from tissues biopsies using TRIzol Reagent (Life Technologies #15596-026), and cDNA was generated using the Bio Rad iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). cDNA was then used for RT-PCR reactions on a Bio-Rad CFX-96 cyclor. Primers sequences are available upon request.

In Vivo Caspase-1 activation

Aim2^{+/+} and *Aim2*^{-/-} mice were shaved and immunized with 100 µg H1-TX04-09.tPA DNA vaccine plasmid intramuscularly injected into the hind quad muscle. Punch biopsies were harvested from the site of immunization at 6 hours, 12 hours, and 24 hours post immunization and snap frozen. Cryopreserved tissue sections were generated and adhered to glass slides. Samples were then stained with a caspase-1 FAM/FLICA kit according to manufacturer's instructions (ImmunoChemistry Technologies, Bloomington, MN). Stained slides were visualized on a confocal microscope. Sixteen independent fields were analyzed for fluorescence.

Gene Induction Analysis

C57BL/6 mice were shaved and immunized with 100 µg H1-TX04-09.tPA DNA vaccine plasmid intramuscularly injected into the hind quad muscle. Punch biopsies were harvested from the site of immunization at 6 hours, 12 hours, and 24 hours post

immunization and snap frozen. RNA was isolated from tissue biopsies using TRIzol Reagent (Life Technologies). We analyzed gene expression using the Nanostring nCounter Analysis system (Nanostring Technologies). Each reaction contained 100 ng RNA in a 5 μ l aliquot, plus reporter and capture probes. We also included 6 pairs of positive control and 8 pairs of negative control probes. Gene induction analysis and normalization was conducted using nSolver Analysis Software v1.1. Raw counts were normalized to naïve mice using 3 reference genes: Gapdh, Gusb, and Hprt1.

Aluminum Adjuvant Study Materials and Methods

Mice

Aim2^{-/-} mice were from K. Fitzgerald (UMass Medical School) and were generated as previously described [117]. *Aim2*^{-/-} mice were on a mixed B6/129 background and therefore B6x129 mice were utilized as controls. B6.129 mice (hereafter referred to as *Aim2*^{+/+}) were obtained from Jackson Laboratories (Bar Harbor, ME) and were bred at UMass Medical School. All mice were maintained in the Department of Animal Medicine at UMass Medical School according to IACUC-approved protocols. Mice received either 1.5 ug Aventis Pasteur Fluzone 2004-2005 trivalent inactivated vaccine (TIV) alone or absorbed onto 65 ug Alhydrogel®, divided between quadriceps, at 2 and 4 weeks. A third boosting immunization was delivered 1-week prior to sacrifice.

Cell Culture and Stimulation and IL-1 β Measurement

Aim2^{+/+} and *Aim2*^{-/-} immortalized BMDM were a gift from Katherine Fitzgerald (UMass Medical School, MA). Cells were first primed with 200 ng/ml LPS (Sigma Aldrich) for 4-5 h prior to treatment with appropriate stimulus. All media was removed from the cells, and the appropriate stimulus was added. Poly (dA:dT) (Sigma Aldrich) was transfected using Lipofectamine 2000 at a concentration of 1.5 μ g/well. ATP was added at a concentration of 1.25 μ g/ml. Alhydrogel® was added to cultures at a concentration of 100 μ g/ml. Cultures were incubated 16-18 hours at 37 ° C, and supernatants were harvested. Cell culture supernatants were assayed for IL-1 β (BD Biosciences, Franklin Lakes, NJ) by ELISA.

Enzyme Linked Immunosorbent Assay (ELISA)

Transiently expressed New Caledonia H1HA antigen was coated onto microtiter plates (Costar #3369) at ~1 µg/mL in 100 µL of PBS for 1 hour at room temperature and assayed as previously described [142]. NaSCN displacement was performed at a serum dilution of 1:100. After washing of serum samples, NaSCN was added at various (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 M) concentrations in PBS for 15 min followed by 5 washes in EWB. The assay was then completed as above.

Quantification of HA specific T and B Cells

Splenocyte T and B cell ELISPOT reagents were obtained from Mabtech (Mariemont, OH). H1HA specific T cells were quantified per manufactures instructions. Positive controls were stimulated with 20 ng/ml PMA (Sigma-Aldrich, St. Louis, MO) and 500 ng/ml ionomycin (Sigma-Aldrich). The H1HA relevant peptide used was a CD8⁺ cell-restricted HA peptide (IYSTVASSL). New Caledonia H1HA-specific antibody secreting cells were detected by coating of MAIPSWU (Millipore, Billerica, MA, USA) plates with the transiently expressed H1HA antigen utilized for ELISA (~1.0 µg/well). Positive spots were visualized on a CTL Imager and counting was performed with Immunospot software (Cellular Technology Ltd., Shaker Heights, OH)

Quantitative Real Time-Polymerase Chain Reaction

Aim2^{+/+} and *Aim2*^{-/-} mice were shaved and immunized with 1.5 ug Aventis Pasteur Fluzone 2004-2005 trivalent inactivated vaccine (TIV) onto 65 ug alum via intramuscular injection into the hind quad muscle. Punch biopsies were harvested from the site of immunization at 6 hours, 12 hours, and 24 hours post immunization and snap frozen. RNA was isolated from tissues biopsies using TRIzol Reagent (Life Technologies

#15596-026), and cDNA was generated using the Bio Rad iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). cDNA was then used for rt-PCR reactions on a Bio-Rad CFX-96 cyclor. Primers sequences are available upon request.

Reagents

ATP, LPS, and poly(dA-dT) were from Sigma-Aldrich.

Statistical analysis

All data is presented as the mean of individual mice +/- standard error of the mean (SEM). Statistical analysis was performed using a Student's *t* test, a one-way ANOVA followed by a Tukey post-test, or a two-way ANOVA followed by a Bonferonni post-test.

Chapter VI

Final Comments and Conclusions

The future of DNA vaccination is bright. Advances in plasmid design, and vaccine delivery systems have overcome initial setbacks related to low immunogenicity in humans. DNA vaccination has proven successful even in the face of notoriously difficult pathogens such as HIV and pandemic influenza. The utility of DNA vaccination can be further seen in the heterologous prime-boost strategy, wherein DNA vaccines boost the adaptive immune response generated by more conventional protein-based vaccine modalities. DNA priming of hosts yields higher level cellular and humoral immunity than two doses of protein alone. The mechanism behind the increased DNA vaccine-induced adaptive immunity is not completely understood, but it is imperative researchers begin to understand the extracellular and intracellular processes governing DNA vaccination in order to design safer, more effective vaccines.

Many questions remain about the nature of DNA vaccines and their interactions with the immune system. The immunostimulatory nature of DNA is well known. It is believed that the bacterial plasmid backbone itself functions as an inherent adjuvant in DNA vaccination; however, the beneficial effect of vaccine plasmid immunization has not been truly addressed. Clarification of the ability of the empty vaccine plasmid to boost both innate and adaptive immune responses by co-immunization with a protein based vaccine such as the TIV utilized here would help to define the plasmid's immunostimulatory nature. Such a study may prove that DNA vaccine plasmids themselves, independent of plasmid encoded antigen, are immunogenic and are at least partially responsible for the increase in adaptive immune responses seen in DNA prime-

protein boost patients. This study is crucial gaining a more complete understanding of the underlying mechanism of DNA vaccination.

In Chapter II, I explored the non-canonical IFN- $\alpha\beta$ inducing STING pathway. Evidence suggests that cytosolic DNA sensing pathways regulate DNA vaccine immunogenicity [81, 95]. Currently, only the dsDNA-induced, non-canonical STING/TBK1/IFN- $\alpha\beta$ signaling cascade has been shown to mediate the induction of adaptive immune responses by DNA vaccination. Though several candidates exist, the cytosolic DNA sensor has yet to be identified. Unfortunately, my attempt to identify the sole upstream regulator of STING-dependent IFN- $\alpha\beta$ production was unsuccessful. As cGAS is required for IFN- $\alpha\beta$ production in response to both DNA virus and bacterial infection [85, 86, 91, 147], I hypothesized that it would also be necessary for DNA vaccine immunogenicity. However, despite its importance in pathogen infection, the induction of adaptive immune responses following DNA vaccination appears to be cGAS independent. While *cGas*^{-/-} mice did exhibit an initial impairment in IFN- $\alpha\beta$ production immediately following immunization, wild-type levels were reached within 12 hours. One possible interpretation of these findings is that cGAS is the dominant cytosolic DNA sensor in the early stages of DNA vaccination, but that at least one other cytosolic sensor is able to compensate for cGAS deletion, albeit in a delayed manner. The incongruity between infection and vaccination studies is most likely due to the fact that DNA vaccines are non-replicative and incapable of causing disease. Where a delay in innate immune signaling may yield high viral or bacterial loads resulting in illness or death, DNA vaccines pose no such risk. Therefore, the delay in IFN- $\alpha\beta$ induction by redundant sensors does not appear to have a detrimental effect on the overall immune response.

The question still remains of what cytosolic DNA sensors are required for DNA vaccine immunogenicity. The most obvious approach to confirming the hypothesis that multiple DNA sensors activate STING following DNA immunization is to cross *cGas*^{-/-} mice with other candidate receptors, although this may prove difficult, as multiple IFN- α/β inducing sensors may act in parallel and in different cell types. An alternative possibility is that cGAMP, the endogenous activator of STING, is produced by a second sensor, independent of cGAS. It would be informative to quantify cGAMP levels in *cGas*^{-/-} mice at the site of injection by either rt-PCR or Nanostring to address this possibility. Similarly, it is possible that a molecule sharing a similar conformational structure to the cyclic dinucleotide cGAMP may bind to STING and drive IFN- α/β production. cGAS belongs to a family of cytosolic nucleic acid sensors that also includes the dsRNA sensing 2'-5'-oligoadenylate synthase (OAS) proteins. OAS proteins produce 2'-5'-oligoadenylate in response to both cellular and viral RNA, although structural analysis of these receptors suggests that they do not readily bind to DNA [90, 180, 181]. In vitro STING pull down experiments in DNA vaccine transfected *cGas*^{-/-} cells may be useful in identifying any bound cyclic dinucleotides. In vivo analysis is more complicated as it is necessary to generate cGAS/OAS1 or cGAMP/2'-5'-oligoadenylate double knockout mice to confirm that OAS proteins do not have a role in STING-dependent DNA vaccine immunogenicity.

With regards to the necessity of the IRF3 and IRF7 transcription factors in inducing adaptive immune responses, several questions still remain. Perhaps the most pressing question is the lack of requirement for IRF3 in DNA vaccination. This suggests that IRF3 is not required for sustained IFN- α/β production following DNA vaccination.

Honda *et al.* have proposed that IRF3 contributes little to immune responses in the absence of IRF7 because interaction with IRF7 is required for IRF3 to function completely [79]. Furthermore, it has been established that that IRF7 is crucial for the cytosolic pathway of IFN- $\alpha\beta$ induction [79, 182]. Therefore, it is necessary to quantify IFN- α/β levels in *Irf3*^{-/-} mice using the punch biopsy rt-PCR assay. Unfortunately, a lack of available *Irf3*^{-/-} mice precluded these assays from being performed. Conversely, it is possible that IFN- β is dispensable for DNA vaccination and that the primary interferon signal is provided by IFN- α . The requirement for either IFN- α or IFN- β in DNA vaccine immunogenicity can be confirmed by treating *Irf7*^{-/-} mice with recombinant murine IFN- α and/or IFN- β protein prior to DNA immunization in an attempt to rescue the innate and adaptive immune responses generated.

In addition to the anti-viral STING/TBK1/IRF7 pathway, I also investigated the previously unexplored pro-inflammatory pathway in Chapter III. Both the humoral and cellular antigen-specific adaptive responses were significantly reduced in *Aim2*^{-/-} mice in an IL-1 β /IL-18 independent manner after DNA vaccination. Surprisingly, *Aim2*^{-/-} mice also exhibited significantly lower levels of IFN- α/β at the site of injection. I therefore propose that the defect in IFN- α/β induction is related to impaired pyroptotic cell death by DNA plasmid transfected cells. My current hypothesis is that genomic DNA released into the extracellular milieu by pyroptotic cell death triggers the activation of the STING/TBK1 pathway in bystander cells via cGAS or another of the putative cytosolic DNA sensors. However, other possibilities do exist. The initial reports on STING activity demonstrated that *Sting*-deficient mice are susceptible to infection with RNA viruses such as vesicular stomatitis virus (VSV) or Sendai virus [80]. This suggests that

RNA is capable of inducing a STING-dependent IFN- α/β response through a pathway that has yet to be thoroughly investigated. However, as the deletion of MAVS does not limit DNA vaccine immunogenicity, STING signaling in this manner would function through a previously undescribed mechanism. Furthermore, the release of other intracellular DAMPs such as high mobility group box 1 protein (HMGB1) or heat shock proteins have been shown to induce IFN- α/β production via multiple TLR pathways [183], but the ability of these DAMPs to serve as a secondary signal for DNA vaccine immunogenicity is unlikely, as TLR deletion has no effect on the adaptive immune responses generated [95].

Notably, the role of AIM2-dependent IFN- α/β production in regulating the adaptive immune response still remains to be definitively addressed. It is conceivable that while *Aim2*^{-/-} mice exhibit impaired IFN- α/β production locally, this is not the only mechanism limiting the development of antigen-specific immune responses. One experiment to confirm my hypothesis is the injection of recombinant IFN- α/β into *Aim2*^{-/-} mice following DNA vaccination. If my hypothesis is correct, introduction of exogenous IFN- α/β into the system will rescue the impaired adaptive immune responses seen in *Aim2*^{-/-} mice. It would be particularly interesting if a partial rescue of the immune phenotype following recombinant IFN- α/β injection were seen. This would suggest that at least one other factor in addition to IFN- α/β is limiting the adaptive response.

Importantly, it is also necessary for subsequent studies to focus on the necessity of caspase-1 directly. While the data presented here proves that ASC is required for optimal DNA vaccine immunogenicity, it does not eliminate the possibility of other caspases playing a role in DNA vaccine immunogenicity. Recent evidence has shown that cells

deficient in caspase-1 may revert to AIM2/ASC/caspase-8 dependent cellular apoptosis upon cytosolic DNA sensing [184]. This leaves open the possibility that AIM2-dependent apoptotic cell death may also influence the adaptive immune responses generated following DNA vaccination. Likewise, caspase-11 has been reported to induce pyroptotic cell death independently of caspase-1, suggesting at least one alternative pathway for release of pro-inflammatory signals [185, 186].

The data presented in this thesis represents a series of novel findings that further elucidates the role of innate immunity in influencing the adaptive immune response to DNA vaccination. I have shown that both the anti-viral IFN- α/β and the pro-inflammatory pathways are required for optimal DNA vaccine immunogenicity. One of the goals of this thesis was to identify a common innate immune signaling pathway involved in DNA vaccine immunogenicity. I propose that the pro-inflammatory and anti-viral pathways are linked via DAMP release from pyroptotic cells. These DAMPs, most likely genomic DNA, are detected by nearby bystander cells, and amplify the IFN- α/β response, thereby boosting both the humoral and CMI responses (**Figure 6.1**).

Collectively, these findings provide a firm basis for understanding the relationship between innate immune signals and adaptive immunity, allowing for the design of more effective DNA vaccines. For instance, it may be possible to alter the vaccine plasmid backbone so as to include immunostimulatory motifs specific for particular DNA receptors. Likewise, knowledge of the transcription factors involved in regulating the immune response, such as IRF7, allows for the encoding of highly immunogenic genes within the vaccine plasmid, providing an additional adjuvant effect. It would therefore be

possible to tailor the vaccine plasmid to produce those conditions that are optimal for the desired immune response or a given pathogen.

As a final note, I began preliminary work drawing on recent literature exploring roles for inflammasome signaling in aluminum salt adjuvants. Multiple reports have suggested that alum adjuvanticity is dependent on the NLRP3 inflammasome [170-172]. However, several other groups have presented contrasting data demonstrating that NLRP3 signaling is dispensable for alum's adjuvant effects in vivo. More recent reports have argued that cytotoxic release of host DNA mediates the adjuvant activity of alum via a TBK1-dependent IFN- α/β pathway [128, 129, 177]. As such, I hypothesized that the AIM2-dependent pyroptotic release of genomic DNA is required for alum adjuvanticity. Alum + TIV immunized *Aim2*^{-/-} mice exhibited impaired humoral immune responses compared to *Aim2*^{+/+} mice. This impairment seems to be mediated in part by decreased IFN- $\alpha\beta$ at the site of immunization. I propose that alum adjuvant signals through AIM2 in a manner analogous to the one proposed for DNA vaccination, whereby pyroptotic cell death enhances both anti-viral and pro-inflammatory signaling, most likely via the STING/TBK1 pathway. Therefore, it would be prudent to conduct the studies suggested above for AIM2 and DNA vaccination within this model as well. Of particular importance are those involving the release of pro-inflammatory signaling and IFN- $\alpha\beta$ production. Of note, a key control missing from these studies is the use of a type I IFN-independent adjuvant such as the squalene based MF59 to confirm the requirement for AIM2-dependent IFN- $\alpha\beta$ in alum adjuvanticity. At the very least, the results obtained in *Aim2*^{-/-} mice may partially explain the discrepancies seen in NLRP3 deletion studies, as AIM2 allows for the continued induction of IL-1 β . While these experiments were

preliminary in nature, they lay the groundwork for future study and establish a previously unreported role for AIM2 in alum adjuvanticity.

In closing, as most successful vaccines and adjuvants stimulate innate immune pathways, my data would suggest that the pathways outlined herein may play a role in traditional vaccine modalities as well. While the pro-inflammatory and anti-viral pathways are generally regarded as divergent, the work presented here illustrates how complex their interactions truly are, and demonstrates that both pathways play essential roles in shaping the adaptive immune responses produced by vaccination.

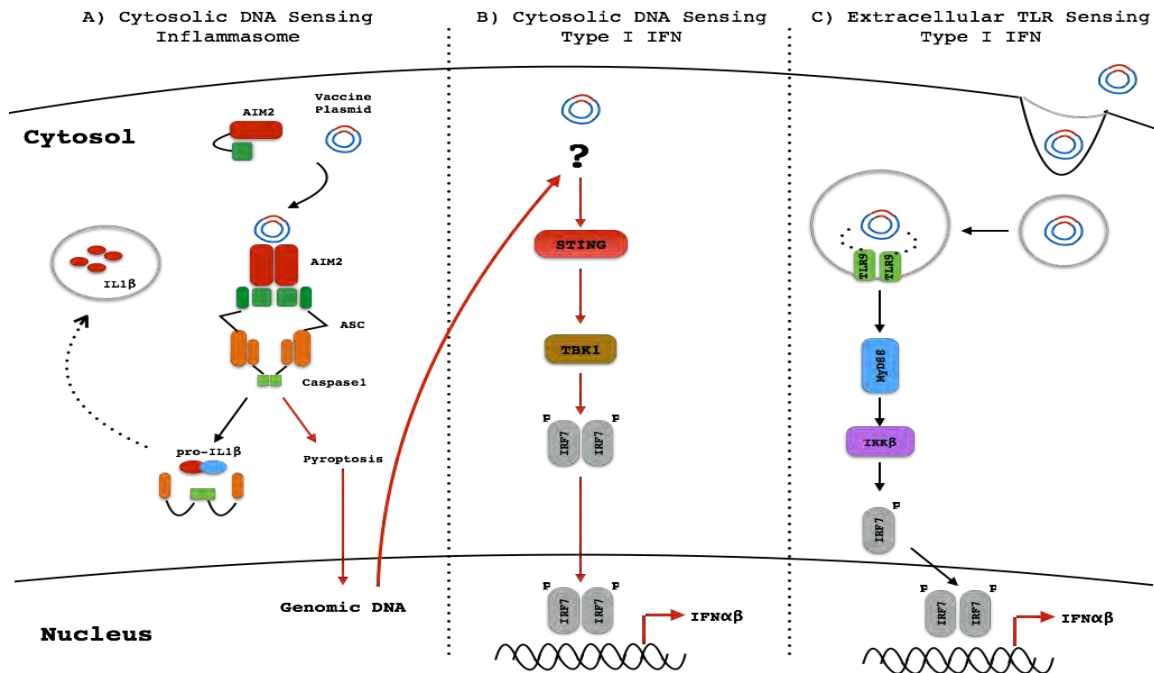


Figure 6.1: Proposed model for innate immune recognition of DNA vaccines.

Three distinct pathways detect DNA vaccine plasmid. (A) Cytosolic vaccine plasmid engages and activates the AIM2 inflammasome, resulting in binding to the adaptor ASC. ASC mediates caspase-1-dependent pro-IL-1 β /pro-IL-18 cleavage and secretion of their bioactive forms. Caspase-1 activation also results in pyroptotic cell death and the release of pro-inflammatory DAMPs, including genomic DNA. Released genomic DNA may then be detected by surrounding bystander cells, inducing type I IFN production through the STING/TBK1/IFN- $\alpha\beta$ pathway. (B) An unknown cytosolic DNA sensor detects DNA vaccine plasmid, activating the non-canonical STING/TBK1/IFN- $\alpha\beta$ inducing pathway. STING/TBK1 activation results in IRF7 phosphorylation and dimerization, generating high levels of IFN- $\alpha\beta$. (C) Extracellular CpG-rich DNA plasmid is transported to TLR9-positive endosomal compartments. TLR9 recognition of DNA plasmid signals via the MyD88/IKK β /IRF7 pathway to induce IFN- $\alpha\beta$ production. Red arrows represent pathways mediating DNA vaccine immunogenicity.

Chapter VII

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