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<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>AIM2</td>
<td>Absent in Melanoma 2</td>
</tr>
<tr>
<td>ASC</td>
<td>Apoptosis-associated speck-like protein containing a CARD</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase Activation and Recruitment Domain</td>
</tr>
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<td>CD</td>
<td>Cluster of Differentiation</td>
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<td>DC</td>
<td>Dendritic Cell</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IRF</td>
<td>Interferon Regulatory Factor</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon Stimulated Genes</td>
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<tr>
<td>LDL</td>
<td>Low-density Lipoprotein</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine Rich Repeats</td>
</tr>
<tr>
<td>M51R</td>
<td>Methionine to Arginine substitution at position 51 of VSV M</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic Acid Inducible Gene 1</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>TD</td>
<td>Thymus Dependent</td>
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</table>
TI    Thymus Independent

TLR   Toll-like receptor

TNF-α Tumor Necrosis Factor α

VSV   Vesicular Stomatitis Virus
ABSTRACT

Vesicular stomatitis virus (VSV) vaccine vectors have been engineered to express many different antigens. Studies in mice and nonhuman primates have demonstrated efficacy against several pathogens. A novel strategy to enhance VSV as an effective vaccine is to engineer vectors that induce innate immune mechanisms. We have generated an improved VSV vaccine vector that incorporates two enhancing strategies; an M protein mutation (M51R) that prevents the virus from suppressing host responses and the addition of a vaccine adjuvant, bacterial flagellin, expressed intracellularly from the viral genome (M51R-F). The ability of the vectors to induce innate immune responses was tested in murine dendritic cells. The presence of flagellin led to production of IL-1β and an inflammatory form of cell death called pyroptosis that were not induced by control vectors. Flagellin signalling was determined to be through the sensor, NLRC4. Previous studies have shown that expression of flagellin enhanced the antibody response in murine in vivo models. My experiments analyzed the T-cell responses in mice vaccinated intranasally with M51R or M51R-F. Results showed no significant difference between M51R and M51R-F vectors in either CD8+ or CD4+ T-cell responses. To address the memory phase mice were vaccinated with M51R or M51R-F and 40 days later challenged intranasally with vaccinia virus expressing VSV proteins. Mice vaccinated with M51R or M51R-F had significantly more IFN-γ producing CD8 T-cells than mock vaccinated mice. However, there was no statistical difference between M51R and M51R-F vectors.
These results show that VSV vectors are effective vaccines, even with minimal effects of flagellin expression on T cell responses.
INTRODUCTION

The goal of this research project is to determine the response of dendritic cells and T cells to vesicular stomatitis virus (VSV) vectors that express flagellin. VSV is currently in clinical trials as a recombinant vaccine vector against heterologous antigens (1). Incorporating flagellin gene into the VSV genome would lead to a more robust innate immune response triggering an enhanced adaptive response to target antigens. Purified flagellin has proven potential as a vaccine adjuvant and is also currently in clinical trials (2, 3). It has also been incorporated into different viral vaccine vectors including VSV (4-8). The hypothesis is that flagellin will activate pathways not induced by virus alone and is tested in Chapters 1 and 2. Expression of flagellin from the VSV genome increased antibody production which raises the question of whether flagellin would also enhance the T cell response which is tested in Chapter 3.

Infectious diseases have been plaguing mankind for all of time. The use of vaccines however, is a more recent discovery that stems from attempts to control smallpox. The Chinese first practiced inoculation with smallpox in the 1000s although the exact route is unknown. In the 1600s and 1700s a method called variolation was developed. This method was the deliberate infection of healthy individuals with smallpox from infected persons. This would lead to a smallpox infection that was not as severe as a typical infection but could still lead to death. Additionally, it could be spread from inoculated individuals to healthy persons leading to an epidemic. If you survived variolation you would be protected from subsequent exposure to smallpox. Variolation could be done by
blowing dried smallpox scabs into the nose or through skin punctures. In 1796, Edward Jenner advanced the idea that infection with cowpox would lead to prevention of smallpox. He tested this by inoculating an 8 year old boy with cowpox obtained from the sore of a milk maid. Jenner then exposed the boy to smallpox as if he was variolating him. The boy did not show any signs of sickness following this challenge. This is the first demonstrated and tested use of a vaccine (9, 10).

According to the Centers for Disease Control and Prevention there are vaccines targeting 27 different infectious diseases approved for use in the United States; 17 viral and 10 bacterial (11). Several vaccines have performed exceptionally and have reduced cases of the disease in the U.S. to zero; smallpox, polio, diphtheria (12). However, there are still many diseases not on this list and even the vaccines that are approved are rarely 100% effective. Some notable viruses for which there is no current vaccine include HIV, SARS, and Ebola. Additionally, some of the current approved vaccines need improvement. This includes vaccines against influenza, anthrax, and pertussis. The goal of this project is to enhance a viral vaccine vector to provide a better immune response to a target antigen.
VIRUSES AS VACCINES

The purpose of a vaccine is to provide immunity to a target pathogen. How that immunity is developed depends on how the vaccine is designed. One method is to use a live attenuated virus. Current examples would be the measles, mumps, rubella vaccine, the live attenuated influenza vaccine (LAIV), and the oral polio vaccines. These vaccines were generated by passage of the virus in alternative host cells to achieve attenuation by accumulation of multiple mutations. For example, the oral polio vaccine developed by Albert Sabin passaged the polio virus in monkey kidney tissue culture (MKTC). After multiple passages the virus was tested for neurovirulence in non-human primates through direct injection into the spinal cord and brain. Those viruses that showed limited signs of neurovirulence, were still infectious in cell culture and the human intestinal tract, and were able to elicit high levels of antibodies were chosen for licensure (13). Another example is the influenza virus which was passaged to obtain a cold adapted, temperature sensitive mutant. These mutations prevent the virus from replicating in the lung and it is restricted to the upper respiratory tract. Then using reassortment the attenuated influenza virus can be modified to encode the protective antigens derived from the currently circulating influenza virus strains. Typically, six gene segments from the cold adapted virus are combined with the genes for hemagglutinin and neuraminidase of the target influenza virus (14).

Current live, attenuated vaccines elicit a strong immune response engaging most of the normal immune effector mechanisms. There is also the
possibility of mutation to revert to virulence. For example, the oral polio vaccine developed by Albert Sabin has been shown to cause vaccine-associated paralytic poliomyelitis (15). This activity, in conjunction with reduced polio cases in the U.S., led to the sole use of the inactivated polio vaccine in the U.S. in 2000. However, the potential for virulent revertants is very remote and they are still considered very safe for use in humans. Live vaccines also have strict refrigeration requirements making transport difficult especially to remote locations. An alternate method is to design a vaccine based on subunits of the target pathogen or use of killed or inactivated virus. Examples of this method would be the current inactivated polio vaccine (IPV). IPV is generated by killing of live polio viruses with formaldehyde. The vaccines contain several D antigens from all three subtypes of virulent polio (16, 17). The vaccine against hepatitis B virus is a subunit vaccine made from the viral surface protein, HBsAg (18). The proteins are generated in yeast using recombinant DNA strategies (19). The use of killed virus or subunit vaccines is usually the safest as there is no fear of mutation. It is also easier to transport as refrigeration requirements are not as strict. However, a significant drawback is that this method usually elicits a weaker immune response in comparison to live vaccines. This would require multiple booster shots to help maintain immunity (20). Replication of the infectious agent is often the key to eliciting a strong immune response.

In addition to the use of live, attenuated viruses to provide homologous immunity there is development of live, attenuated viruses to provide heterologous immunity. Homologous immunity is the use of the disease causing virus to
provide immunity such as polio or influenza. Heterologous immunity would be the use of one virus containing antigens targeting a different pathogen. This can be accomplished by incorporating proteins of the target pathogen into a virus particle or incorporating genes encoding specific proteins that will be produced upon viral infection. When the virus infects a cell it would then produce the target antigen in conjunction with other viral proteins allowing for an immune response. Many viruses are being developed as vaccine vectors. They span the spectrum of viruses and include DNA viruses such as adenovirus or pox virus and RNA viruses such as Newcastle Disease virus, Sendai virus or VSV (21-24). Several of these are currently in clinical trials including adenovirus and VSV. Adenovirus is in multiple clinical trials targeting HIV. Several interesting developments have stemmed from those trials. The first is that in one trial the risk of contracting HIV actually increased following vaccination. This is thought to be due to prior exposure to adenovirus resulting in a larger T cell pool for HIV to infect (25). This demonstrates the need for a vaccine vector where there is no preexisting immunity. The other interesting development is the role of non-neutralizing antibodies. The study showed that people with nnAB against HIV were better protected from HIV infection (26).
DEVELOPMENT OF VESICULAR STOMATITIS VIRUS VACCINE VECTORS

VSV is a single stranded, negative sense, non-segmented, enveloped, RNA virus that is a member of the *Rhabdoviridae* family. Its genome is approximately 11 kb and encodes for 5 proteins: nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and an RNA dependent RNA polymerase (L) (27). The receptor for VSV has been linked to the LDL receptor family (28). However, this is not the only receptor as cells that did not express LDL receptor can also be infected with VSV. Following VSV attachment to the cell it is taken up through clathrin mediated endocytosis (27). When the endosome acidifies the viral membrane fuses to the endosomal membrane allowing release of the viral contents into the cytosol. All transcription and replication of VSV occurs in the cytoplasm. Transcription of the VSV genome is mediated through the viral polymerase composed of the P and L proteins. The N protein is also involved in encapsulating the genome to protect it from cellular detection. The genome is encapsidated with N during replication and is maintained that way even during transcription and incorporation into virions (29, 30). Transcription is a sequential, start-stop style. Cis-acting elements determine if the polymerase continues or falls off the genome. This leads to a gradient level of expression with genes at the beginning of the genome being transcribed more frequently. Once the required levels of proteins are achieved then viral genome replication begins. As progeny genomes are produced secondary transcription can occur. This leads to a significant amplification of viral protein synthesis.
Gathering of viral components at the cell membrane allow the virus to assemble and bud creating new viral particles. The virus replication begins with primary transcription that starts within 2 hours of entry into the cell. The remaining steps occur continuously for the next 12-24 hours before the cell dies (27).

VSV is typically a pathogen of domestic livestock such as cattle, horses and pigs. Infected animals develop vesicular lesions in the mouth, coronary band, and teats. It is very similar to foot- and-mouth disease (31). Currently, VSV is considered enzootic only in North and South America. It is more of a problem for farmers in South America and causes an economic burden on the livestock industry due to losses (27). Wild-type VSV could also infect humans leading to flu-like symptoms. Infections of humans, however, are exceedingly rare. Because VSV is not encountered frequently by the general population, most humans do not have anti-VSV neutralizing antibodies. VSV is also a potent inducer of innate immune responses that drive adaptive immunity. All of these qualities contribute to the attractiveness of VSV as a vaccine candidate. Additionally, VSV is amenable to insertion of heterologous genes including antigens and immune adjuvants such as flagellin. For example, there are currently several antigens that have been incorporated into VSV including HIV Env and Gag, Influenza HA, and Ebola G protein (21, 22, 32-36). VSV viruses containing HIV antigens are currently in clinical trials.

In 1994, the ability to recover infectious virus from genomic cDNA was established using rabies virus (37). This quickly expanded to other RNA viruses including VSV (38-43). This allowed the manipulation of the viral genome to
express an antigen as a separate gene transcript (44). However, recombinant VSV expressing heterologous antigens retained its wild-type properties and was shown to be neurovirulent in young mice and could be lethal if given to livestock intra-cranially (45-49). This led to research on attenuation methods and neurovirulence testing modeled after the mumps virus vaccine test (50). Attenuation research focused on three main areas; gene order, G protein, and M protein (51-56). Scrambling of the gene order leads to modified levels of gene expression. This leads to reduced levels of viral replication. G protein attenuation focuses on truncation of the cytoplasmic tail that also leads to reduced viral replication. M protein attenuation focuses on mutating M protein to prevent inhibition of host gene expression. This would allow cells to mount an anti-viral response to VSV.

DEVELOPMENT OF M PROTEIN MUTANT (M51R) VSV

The M protein of VSV is involved in many aspects of the viral lifecycle. It forms the layer between the envelope and the nucleocapsid of the virion. During assembly, it helps compact the nucleocapsid into a tightly coiled helix, condensing the nucleocapsid (57). It is involved in virus particle budding and plays a major role in suppression of the host innate immune response (58-61). Its ability to suppress the host innate immune response is directly tied to its ability to
inhibit host gene expression on the levels of transcription, translation and mRNA transport through interaction with Rae1 (62-64).

M proteins assembly and inhibitory functions are genetically separable. The mutation of two nucleotides in the gene for the matrix protein replaces a methionine with an arginine. This mutation prevents M protein from interacting with Rae1 and prevents VSV from inhibiting host gene expression without significantly interfering with its roles in virus assembly and budding (64). This in turn allows cells to mount an anti-viral response to VSV. This mutant VSV is called the M51R vector. VSV containing wild-type M protein suppress the innate response in many cell types. In contrast M51R vectors are potent activators of the innate response in many cell types.

ACTIVATION OF INNATE IMMUNE RESPONSE BY M PROTEIN MUTANT VSV

The M51R vector, although attenuated, is still a potent stimulator of the immune response. There are several ways that innate immune cells can detect and respond to M mutant VSV. Two that have been well-characterized are TLR7 and RIG-I (65-67). TLR7 is a Toll-like receptor that is found in the endocytic compartments and recognizes single-stranded RNA (68). TLR7 is MyD88 dependent and when activated leads to activation of IRF7 and production of Type I IFNs (69). Another sensor of viral RNA is retinoic acid inducible gene I or RIG-I.
RIG-I detects viral RNA during replication when aberrant products are formed such as dsRNA and 5' triphosphate RNA (67). Activation of RIG-I leads to association with IPS-1 (also called MAVS) which can lead to activation of protein kinases leading to transcription factor activation (NF-κB) and IRFs such as IRF7 and IRF3. The pathway that activates IFN has been extensively reviewed and summarized in reference 63. Briefly, production of Type I IFNs, IFN-α and IFN-β, leads to amplification of the signal through both an autocrine and a paracrine manner. Both IFN-α and IFN-β share the same receptor; IFNAR. Activation of IFNAR leads to phosphorylation of STAT1 and STAT2. These proteins form a trimer with IRF9 to form ISGF3. ISGF3 is a transcription factor complex for interferon stimulated genes. Transcription of these genes leads to hundreds of products involved in the anti-viral response such as 2’-5’ Oligoadenylate Synthetase (OAS) and Protein Kinase R (PKR) (68).

Activation of the IFN response is responsible for attenuation of M51R vectors. In addition to Type I IFNs other anti-viral cytokines in response to M51R vectors can stimulate adaptive immune responses. Combination of attenuation of virulence and stimulation of the adaptive response is the basis for using M51R vectors as a vaccine vector.
DEVELOPMENT OF FLAGELLIN AS AN IMMUNE ADJUVANT

Many bacteria utilize flagella for chemotaxis or sensing. The principal component of the flagellum is the protein flagellin. Flagellin is an approximately 50 kDa protein. Its termini are fairly constant regions but the center portion is a hypervariable region among flagellated bacteria. This project focused on the \textit{Salmonella enterica} flagellin produced from the \textit{FliC} gene. This particular flagellin has a size of 53 kDa and was chosen based on its ability to induce higher levels of TNF-\(\alpha\) in U38 cells compared to other bacterial flagellins (70). The conserved regions of flagellin are important in their role for assembly and function of flagella. These conserved regions are important for binding to a leucine rich repeat (LRR) on TLR5 (71).

Flagellin is found in large amounts on flagellated bacteria and is not found in mammals. For this reason the innate immune system has multiple ways to detect flagellin. The innate immune system has ways to identify foreign pathogens through pathogen associated molecular patterns or PAMPs. PAMPs are detected by pattern recognition receptors that recognize specific microbial signatures such as cytosolic DNA or the presence of flagellin. If flagellin is extracellular it can be detected by a toll-like receptor (TLR) called TLR5. TLR5 is an MyD88 dependent receptor that when activated leads to a cascade that involves activation of NF-\(\kappa\)B and production of TNF-\(\alpha\) (among other cytokines) (3). If flagellin is in the cytosol of a cell it can be detected through a cytosolic receptor called NOD-like receptor 4 or NLRC4 (72). Once activated NLRC4
recruits adapter proteins such as ASC along with Caspase-1. Caspase-1 is activated through autoproteolysis leading to production of IL-1β and IL-18 and can also lead to an inflammatory form of cell death called pyroptosis.

There are several approaches to using flagellin as an adjuvant. One method is as a soluble protein mixed with antigen. This method has been shown to enhance both CD8+ and CD4+ T cell responses and antibody responses (73-75). Another method is to utilize a fusion protein. In this method flagellin is fused to the target antigen. This has also been a highly effective use of flagellin as an adjuvant and is currently in clinical trials targeting two different pathogens (76, 77). A final method is to incorporate flagellin into viruses either directly into the virion or as a gene product. This allows activation of additional pathways not induced by virus alone. Several viruses have been developed using this method to include influenza, SV5, rabies, and VSV (4, 5, 8, 78). Based on the available data, the use of flagellin as an adjuvant should enhance the effectiveness of VSV as a vaccine vector.

**ACTIVATION OF INFLAMMASOMES**

Myeloid cells of the innate immune systems have the ability to form inflammasomes. Inflammasome are multiprotein complexes formed within the cell that mediate activation of caspase-1 and 11 in response to danger or pathogen signals (72). Inflammasomes have been linked to both bacterial and
viral pathogens and are necessary for the clearance or suppression of some of these pathogens (79). The three main components to any inflammasome are the platform proteins, adaptor proteins, and effector proteins (72, 79, 80). The majority of platform proteins are NOD-like receptors such as NLRP3 or NLRC4. One that is not is HIN200 (68). This platform protein serves as the basis for the AIM2 inflammasome that responds to cytoplasmic DNA from DNA viruses and some intracellular bacteria such as *Listeria* (81).

Once a platform protein detects a signal it recruits adaptor proteins such as ASC and Naip5 (82). ASC contains a CARD domain to recruit caspase-1 (79). Some platform proteins also contain a CARD domain but may still use ASC as well, as is the case for NLRC4. However, activation of the NLRC4 inflammasome is not always dependent on adapter proteins such as ASC (83). Recruitment of caspase-1 leads to autoproteolysis of caspase-1 and its activation. Activation of caspase-1 leads to cleavage of IL-1β and IL-18 allowing them to be released from the cell (79). Activation of caspase-1 can also lead to a form of cell death called pyroptosis (68, 80). Pyroptosis is an inflammatory type of cell death in contrast to the immunologically silent apoptosis. Pyroptosis ruptures the cell allowing cellular contents to enter the extracellular space. Pyroptosis limits replication of intracellular pathogens and recruits additional immune cells to the site of infection (72). Pyroptosis and apoptosis do share some similarities, they both rely on caspase activity (although actual caspases differ), condensation of the nuclear compartment, and fragmentation of genomic DNA (72). The cytokine
cleavage of IL-18/IL-1β and pyroptotic functions of caspase-1 are not interdependent and can function separately (68, 80).

**ACTIVATION OF DENDRITIC CELLS**

The human innate immune system is part of the first line of defence against pathogens. The innate immune response is non-specific and responds to a wide variety of PAMPs (68). It also does not develop memory cells like those of the adaptive immune system. The innate immune system is composed of many cell types that perform several functions. This involves responding to PAMPs through PRRs, elimination of infected cells, and activation of the adaptive immune response (68). Many pathogens require an adaptive immune response for clearance. The ability of the innate immune system to elicit an adaptive response is based on antigen presenting cells or APCs. One of the most critical APCs of the innate immune system are dendritic cells (84). Dendritic cells in the immature state are very phagocytic and once activated will present antigen on the cell surface. They are excellent activators of T cells through antigen presentation, high expression of co-stimulatory molecules and production of cytokines (84, 85). DCs also play a role in presenting antigen and cytokine production for B cell activation (86).

Dendritic cells are found throught the body. They sample the surrounding environment for PAMPs that can trigger pattern recognition receptors (PRRs)
such as TLR7 or NLRC4 (68). When they encounter a PAMP they can become activated. Activation of a dendritic cell leads to several changes. It is no longer phagocytic in nature and undergoes a morphology change (86). Additionally, it begins producing cytokines, presents the antigen on MHC complexes, and upregulates co-stimulatory molecules (84). These three items are important in activating T cells. DCs also stimulate B cells. This is accomplished by intact antigen presentation by DCs to B cells or through activation of CD4+ T cells which in turn activate B cells. Additionally, cytokine secretion by DCs aids in activating B cells (87).

There are many types of dendritic cells in the human body. They are broken into two major classifications: plasmacytoid DCs and classical (conventional) DCs (84). Plasmacytoid DCs or pDCs, are typically found in the blood and lymphoid tissue and can enter the lymph nodes from circulating blood. They also express a very select few PRRs such as TLR7 and TLR9 (84). When activated they produce large amounts of Type I IFN and can present foreign antigen on their cell surface, although this is not thought to be their primary function. Classical DCs, or cDCs, encompass the remaining DC populations. This subset can be further broken down by location of these DCs; lymphoid versus non-lymphoid, and through cell surface markers such as CD8α, CD11b, and CD103 (84). Classical DCs, due to location, are constantly able to acquire antigens from tissue and blood. They can then process antigen for presentation on the cell surface. They can then migrate with antigen to the T cell zone of secondary lymphoid organs and activate naïve T cells (84, 85).
The focus of this project will be on four different DCs cultured in vitro that resembles some of these DCs mentioned. Bone marrow derived cells will be cultured in the presence of granulocyte macrophage colony stimulating factor, also called GM-CSF, and resemble the monocyte derived cDC subset. These DCs migrate to sites of infection from the blood and bone marrow where they can acquire antigen for processing and migrate to T cell areas of lymphoid tissue to activate naïve T cells.

The other DCs generated for this study resemble the splenic DC population. These DCs are derived from the bone marrow and cultured in the presence of Fms-like tyrosine kinase 3 ligand also called Flt3 ligand (88). This DC population contains three distinct populations of DCs. The first is the plasmacytoid DC. As mentioned previously these DCs are responsible for Type I IFN production. They are identified by the cell surface marker B220. The other two DC subsets are CD8α+ and CD8α− cDC correlates. The CD8α+ DCs reside in the LN and spleen. In the spleen they are located in the marginal zone allowing access to blood antigens. They then migrate to the T cell zone to activate naïve T cells. The CD8α− subset, which is also CD11b+, express a variety of PRRs that differ from the CD8α+ subset. The role of these DCs in vivo is not fully understood although they are capable of priming naïve T cells (84, 89).

Dendritic cells are a critical link between the innate and adaptive immune responses. The ability of a vaccine to activate these cells will lead to a more robust immune response.
Because of the importance of dendritic cells in activating the adaptive immune response my experiments will determine the response of dendritic cells to VSV vectors.

**INDUCTION OF ADAPTIVE IMMUNITY BY VIRAL VACCINES**

The important component of vaccines is the ability to elicit an adaptive immune response. The adaptive immune system responds to specific pathogens and is a more targeted response in contrast to the innate immune response. There are two main components of the adaptive immune system: cellular and humoral.

Activation of the cellular component of the adaptive immune system is triggered by antigen presenting cells (APC) such as dendritic cells. This is accomplished by providing three signals to the T cell (90). Signal 1 is the interaction of the MHC/antigen complex (MHC II for CD4\(^+\) and MHC I for CD8\(^+\)) on the APC with the T cell receptor (TCR). This signal activates the T cell. Signal 2 is provided by the same APC and involves engagement of co-stimulatory molecules such as B7.1 or B7.2 (CD80 and CD86 respectively) binding CD28 on the T cell surface. This signal triggers survival and proliferation of the T cell. Signal 3 is typically provided by cytokines secreted by APCs. These cytokines are involved in differentiation of T cells into their effector functions. Activated T cells then proliferate and acquire effector functions. These effector functions vary.
depending on T cell type. CD8+ T cells are cytolytic (90). When activated CD8+ T cells encounter a cell presenting their cognate antigen on MHC Class I they release perforin, granulysin, and granzyme. Perforin creates pores in the target cell membrane. Granulysin perforates the cell membrane. These actions allow granzyme to enter the target cell trigger apoptosis. The CD8+ T cell can also engage the Fas receptor to trigger apoptosis. Additional effector functions of CD8+ T cells include production of the cytokines IFN-γ and TNF-α. IFN-γ is important in controlling replication of intracellular pathogens and activating macrophages. TNF-α signalling can lead to activation of NF-κB and upregulation of anti-pathogen responses (91).

CD4+ T cells have different effector functions depending on which subset they fall into. This project focuses on three subsets; T_h1, T_h2, and T_h17. T_h1 cells produce IFN-γ and aid in the activation of macrophages and other components of the cellular immune response (90). T_h2 cells produce IL-4 that can aid in the activation of B cells and leads to class switching among other activities (90). T_h1 cells typically respond to intracellular pathogens while T_h2 respond to extracellular pathogens. T_h17 cells also target extracellular pathogens. They produce the cytokine IL-17 and activate neutrophils and B cells that target extracellular pathogens (90, 92, 93).

The humoral response targets pathogens in the extracellular region. There are two main types of humoral responses to antigens; a thymus independent (TI) response and a thymus dependent (TD) response (94). A thymus independent response is generated without the assistance of T cells and typically targets non-
protein antigens such as LPS and molecules with repeating motifs but can also target viral capsids. A TI response requires additional signalling to activate naïve B cells that can be provided by TLR engagement or extensive BCR cross-linking. A major difference between TI and TD is the lack of class switching in a TI response. This is due to the lack of T cell help. In a T cell dependent response naïve B cells are activated through engagement of the B cell antigen receptor (BCR). The BCR internalizes the antigen and presents peptides complexed with MHC II molecules on the B cell surface (94). An activated CD4⁺ T cell targeting the same pathogen can then bind the MHC II/peptide complex. This binding in conjunction with cytokine stimulation leads to proliferation and differentiation of the B cell. B cells can differentiate into plasma cells or memory cells. Plasma cells are short lived cells that secrete antibodies until they die (94). Memory B cells have extended survival mechanisms that allow survival for months or even years (94). Memory B cells can respond to their cognate antigen much more quickly and do not require all the rigorous signalling to become activated. CD4⁺ T cells also induce class switching in B cells. Typically an antibody response begins with IgM. Once class switching is induced B cells will begin to secrete other classes of antibody such as IgG, IgA, and IgE (94). The class induced is dependent on the cytokine environment. For example, although T₉₁ cells are poor inducers of class switching they do produce IFN-γ. IFN-γ focuses class switching to IgG2a and IgG3 (91). T₉₂ cells on the other hand secrete IL-4 and IL-5 which induces IgG1, IgE, and IgA switching (87, 91).
Following any acute infection or vaccination the adaptive immune response must be brought under control. With the removal of the antigen that activated them many T cells will undergo apoptosis. However, some T cells differentiate into memory T cells (90). These memory T cells are able to respond to their cognate antigen without undergoing the rigorous signalling process. This allows for a more rapid adaptive response to previously seen antigens. Memory B cells are also able to respond rapidly to previously encountered pathogens (94). This is demonstrated by the presence of very high levels of IgG early in the response to secondary or tertiary exposure. This is the basis for vaccination, training the adaptive immune system to respond quickly to a target pathogen, thus preventing that pathogen from establishing an infection.

CURRENT PROJECT

Previous experiments with M protein mutants as a vaccine vector have demonstrated its potent ability to induce an adaptive immune response (4, 95, 96). Analysis of anti-VSV antibody response in mice was shown to be induced with the absence of neurovirulence (97). Additionally, M51R engineered to express heterologous antigens from smallpox demonstrated the ability to provide protection from a lethal challenge (95). VSV utilized in a prime boost experiment with rabies virus and Newcastle disease virus (NDV) performed exceptionally well in conjunction with NDV in eliciting CD8+ T cells (96). Furthermore, the
potency of M51R has been shown to be enhanced by the expression of flagellin (4). This vector activated human dendritic cells and increased the antibody response in mice, especially at the lower doses. This sets the stage for experiments in my thesis: determine the pathways activated in dendritic cells and analyze the T cell response to M51R vectors.
References


64. **Lyles DS, McKenzie MO.** 1997. Activity of vesicular stomatitis virus M protein mutants in cell rounding is correlated with the ability to inhibit host gene expression and is not correlated with virus assembly function. Virology 229:77-89.


CHAPTER 1

SIGNALLING PATHWAYS IN INNATE IMMUNE CELLS AND THEIR RESPONSE TO VSV MUTANT VECTORS

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The following manuscript was published in The Journal of Virology, in press, 2014, and is reprinted with permission. Stylistic variations are due to the requirements of the journal. Jason R. Smedberg performed all experiments (with the exception of LPS and purified flagellin responses of DCs), data analysis, and prepared the manuscript. Dr. Maryam Ahmed performed the LPS and purified flagellin experiment. Drs. Marlena W. Westcott and Douglas S. Lyles acted in an advisory and editorial capacity.
Abstract

Vesicular stomatitis virus (VSV) vectors that express heterologous antigens have shown promise as vaccines in pre-clinical studies. The efficacy of VSV-based vaccines can be improved by engineering vectors that enhance innate immune responses. We previously generated a VSV vaccine vector that incorporates two enhancing strategies; an M protein mutation (M51R) that prevents the virus from suppressing host antiviral responses, and a gene encoding bacterial flagellin (M51R-F vector). The rationale was that intracellular expression of flagellin would activate innate immune pathways in addition to those activated by virus alone. This was tested in dendritic cells (DCs) from mice containing deletions in key signaling molecules. Infection of DC with either M51R or M51R-F vector induced the production of IL-12 and IL-6, and increased surface expression of T cell costimulatory molecules. These responses were dramatically reduced in DCs from IPS-1\(^{-/-}\) mice. Infection with M51R-F vector also induced the production of IL-1β. In addition, in approximately half of the DC, M51R-F vector induced pyroptosis, a pro-inflammatory-type cell death. These responses to flagellin were ablated in DCs from NLRC4\(^{-/-}\) mice but not TLR5\(^{-/-}\) mice, indicating that they resulted from inflammasome activation. These results demonstrate that flagellin induces additional innate immune mechanisms over those induced by VSV alone.
Introduction

Activation of innate immune cells is key to the activity of vaccine adjuvants as well as the success of live virus vaccines. Thus a strategy for developing improved vaccines is to enhance their ability to activate the innate immune system. There are currently multiple viruses being developed as live virus vaccine vectors for the delivery of heterologous antigens that are effective activators of the innate immune system. Examples include the DNA viruses adenoviruses and poxviruses, and the RNA viruses Newcastle disease virus, Sendai virus, and vesicular stomatitis virus (VSV) (1-4). In an effort to further stimulate the innate response and trigger a greater adaptive response many viral vectors have also incorporated vaccine adjuvants. For example, influenza virus, SV5, rabies virus, and as we have reported, VSV, have been engineered to express bacterial flagellin (5-8). The purpose of the current study was to test the hypothesis that the expression of flagellin by VSV will lead to the activation of additional pathways not triggered by the virus alone.

VSV is currently being developed as a vaccine vector for the delivery of a wide array of heterologous antigens (1, 2, 9-13). Although laboratory strains of recombinant VSV are not usually pathogenic in humans, the potential for VSV to cause disease in humans has been addressed by attenuation of VSV vectors. Multiple methods of attenuation have been developed, including introducing mutations into the viral M protein. (14-17). Substitution of arginine for methionine at position 51 of M protein prevents VSV from inhibiting host gene expression, thus allowing cells to mount an antiviral response. This leads to an increased
immune response to VSV in the absence of neurovirulence (18). VSV’s ability to elicit an immune response was further enhanced by insertion of the gene for flagellin from *Salmonella enterica* as a separate gene transcript. This allows the expression of full length flagellin from the viral genome (5). We have previously reported that VSV encoding flagellin induces higher antibody titers in mice as compared to a VSV vector without flagellin (5).

The hypothesis that flagellin activates additional pathways in innate immune cells compared to those triggered by the virus alone was tested by analyzing the ability of VSV encoding flagellin to activate dendritic cells (DCs) from mice with deletions in key signaling molecules. DCs provide the critical link between the innate and adaptive immune systems. Previous studies have shown the ability of VSV that expresses flagellin to elicit a higher level of cytokine production in human monocyte-derived DCs (5). VSV has been shown to signal in DCs primarily through the pattern recognition receptors (PRR) TLR7 and RIG-I (19, 20). Not all subsets of DCs express TLR7, and in these cells, the RIG-I pathway is likely the major inducer of antiviral responses (20). The RIG-I response to VSV is mediated through the mitochondrial adapter protein IPS-1 (21). In the case of flagellin, two different pathways have been shown to mediate the response in DCs (22-24). If the flagellin is extracellular, the response is mediated by the toll like receptor TLR5. If the flagellin is intracellular, the NOD-like receptor NLRC4 mediates the response. Depending on which molecule detects flagellin, different outcomes for the cell can result. TLR5 activation leads primarily to NF-κB activation and cytokine production (25). NLRC4 activation
leads to formation of an inflammasome. The inflammasome is a multi-protein complex that activates innate immune response pathways. The composition of an inflammasome and the biological outcome is dependent on the sensor that is activated. In the case of NLRC4 activated by flagellin, the inflammasome is formed with pro-caspase-1 and adapter proteins such as ASC. Formation of the NLRC4 inflammasome leads to processing and secretion of IL-1β and IL-18 and can also lead to a form of cell death called pyroptosis (26-28). Pyroptosis is an inflammatory cell death that can promote enhanced immune responses (28, 29). This form of cell death differs from apoptosis in that it is more rapid and is manifested by lysis of the cell, which leads to release of cellular contents into the surrounding environment. Pyroptosis is detected by other innate immune cells as an inflammatory signal.

In the current report, we found that the expression of flagellin from the viral genome led to the production of IL-1β that was not elicited by virus alone. In addition, expression of flagellin led to pyroptotic cell death in approximately 50% of the DCs. The remaining DCs matured and produced cytokines IL-12 and IL-6, and increased surface expression of the T cell costimulatory molecules, CD86, CD80 and CD40. Both the production of IL-1β and induction of pyroptosis were ablated in DCs from NLRC4−/− mice but not TLR5−/− mice, indicating that they resulted from inflammasome activation. In contrast, production of IL-12 and IL-6 and expression of costimulatory molecules were dependent on IPS-1, and thus resulted primarily from antiviral signaling.
Materials and Methods

Reagents. Recombinant mouse GM-CSF generated from a baculovirus vector was provided by S. Mizel, Wake Forest School of Medicine. Fluorescently labeled antibodies against mouse cell surface antigens (CD11c, clone N418; CD86, clone GL-1; I-A^b, clone AF6-120.1) were purchased from Biolegend. Fluorescently labeled antibodies against CD40 (clone 3/23), and CD80 (clone 16-10A1) were purchased from BD Pharmingen. Caspase-1 p10 (M-20) antibody was purchased from Santa Cruz. IL-1β antibody was purchased from R&D Systems. Flagellin antibody and purified flagellin were generously provided by S. Mizel. Lipopolysaccharide (LPS) from Salmonella enterica serovar Minnesota was purchased from Sigma (St. Louis, MO). Monoclonal anti-VSV N protein antibody 10G4 was produced as described previously (30, 31).

Mice. C57BL/6 mice were purchased from Charles River (Wilmington, MA). With the permission of S. Akira, TLR5^−/− mice were acquired from S. Mizel (Wake Forest School of Medicine), and IPS-1^−/− mice were acquired from M. Schnell (Thomas Jefferson University). NLRC4^−/− mice were obtained from V. Dixit (Genentech). All mice were maintained and bred in the animal facility at Wake Forest School of Medicine, and experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee.

Dendritic cells. GM-CSF derived DCs were cultured as previously described (32). Briefly, bone marrow was obtained from the femurs and tibias of 8 – 24 wk
old mice. Red blood cells were lysed and washed, progenitor cells were plated at a density of 5 X 10^5/mL in RPMI containing 10% fetal calf serum supplemented with 10 ng/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF) and 10 μg/mL of gentamicin. Cells were cultured at 37°C for 6 days and fed with fresh media and GM-CSF on days 2, 4, and 5. On day 6 the nonadherent and loosely-adherent cells were harvested. These cells were 90% (±5%) CD11c^+ and showed low levels of CD40, CD80, CD86, and major histocompatibility complex class II antigens (MHC II), typical of immature DCs (33).

**Virus growth and preparation.** Recombinant M51R and M51R-F viruses were isolated from infectious VSV cDNA clones and virus stocks were prepared and maintained on BHK cell monolayers as previously described (34). The M51R-F was purified by sucrose gradient centrifugation to remove extracellular flagellin.

**Measurement of costimulatory molecule and cytokine expression following viral infection.** DCs were plated in a 48-well plate at a density of 5 X 10^5 cells/mL in antibiotic free medium, and were infected with virus at the indicated MOI for 6, 12, or 24 hours. In a separate set of experiments, cells were treated with purified flagellin or LPS (both at 100ng/ml) as controls. Supernatants were collected and stored at -80C, and cytokine levels (IL-1β, IL-6, and IL-12p40) were measured by ELISA (BD Biosciences). DCs were analyzed by staining for MHC II and T cell costimulatory molecules CD86, CD80, and CD40 using fluorescently
labeled antibodies. Data were acquired using a BD FACSCalibur™ flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc.).

**Immunoblotting.** DCs were infected at an MOI of 10, and at 8 hours postinfection supernatants were collected, and cells were lysed using RIPA buffer without SDS. Protein concentrations of the lysates and supernatant were measured (BioRad) and equivalent amounts of protein per sample were analyzed by SDS-PAGE followed by transfer to 0.45μm PVDF membranes. Membranes were blocked and probed with antibodies against flagellin, caspase-1, IL-1β, or actin followed by the appropriate secondary antibody conjugated to horseradish peroxidase. Chemiluminescent substrate was used for detection (Thermo Scientific, SuperSignal West Dura). Membranes were stripped and re-probed the following day until each protein had been analyzed. Pixel intensities were analyzed utilizing ImageQuant software.

**Cell viability and death.** DCs were infected at an MOI of 10 and at the indicated time point were analyzed for cell viability utilizing an MTT assay (Promega) or were stained with 7-aminoactinomycin D (7AAD) according to the manufacturer's instructions (BD Pharmingen). Data were acquired using a BD FACSCalibur™ flow cytometer and analyzed using FlowJo software.
VSV N-protein detection. DCs were infected at an MOI of 10. At 24 hours postinfection cells were harvested, fixed and permeabilized, and stained with anti-VSV-N antibody followed by a fluorescently labeled secondary antibody as described (35). Data were then acquired by flow cytometry as described above.

Statistical Analysis. Comparisons were tested for statistical significance by calculating the t statistic and the probability (p) of the null hypothesis using Microsoft Excel software. Values of p < 0.05 were considered statistically significant.
Results

Incorporation of a gene encoding bacterial flagellin has been shown to enhance the immune response and DC function for a variety of live virus vectors, including VSV (5-7, 36, 37). The mechanism of enhancement is thought to be due to activation of innate immune pathways in antigen-presenting cells in addition to those activated by virus vectors that lack flagellin. This hypothesis was tested by analyzing the response to VSV vectors in murine myeloid DCs that lack key elements of the signaling pathways that respond to virus and flagellin. Construction of the M protein mutant VSV vector and the vector encoding *Salmonella enterica* flagellin, depicted in Fig. 1A), have been described previously (5). The M51R M protein mutant vector has a single amino acid change, methionine to arginine, at position 51 of the M protein. This mutation prevents the virus from inhibiting host gene expression and allows infected cells to mount an anti-viral response. The gene for flagellin (*fliC*) was inserted into the M51R backbone as a separate transcriptional unit between the M and G genes. These vectors induce potent innate and adaptive immune responses in the absence of viral pathogenicity in mice, whereas vectors with wt M protein retain pathogenicity in mouse models (5, 16, 18). The flagellin gene does not encode a eukaryotic signal sequence, so the flagellin would be expected to be primarily intracellular. The expression of flagellin was tested in myeloid DCs cultured from bone marrow precursors in the presence of GM-CSF. Cells were infected with the flagellin-expressing vector (M51R-F) or control vector (M51R), and flagellin
Fig. 1. VSV genomes and flagellin expression. (A) Diagram of genomes of WT and M51R VSV and M51R-F VSV with the gene for flagellin (fliC) inserted into the M51R backbone as a separate transcriptional unit between the M and G genes. (B) Murine DCs were infected with M51R or M51R-F vectors or mock-infected. At 8 hrs postinfection, cell lysates (20µg of protein) and media (70µg of protein) were analyzed for flagellin expression by immunoblots. The volumes analyzed in the figure shown were Cell lysates: 15.7µL from a total 120µL. Media: 4.3µL from a total 2mL.
expression in cell lysates or culture supernatants was determined by immunoblots (Fig. 1B). As expected, flagellin produced by cells infected with M51R-F vector was present in the cell lysate and also the culture supernatant. When the relative amounts of flagellin in the supernatant and lysate were measured in the immunoblots and corrected for the relative volumes of the two samples, it was estimated that approximately 85% of the flagellin was in the supernatant. This was likely released upon cell lysis.

The expression of flagellin by the M51R-F vector enhanced cytokine production by murine DCs over that induced by M51R vector, similar to published results with human DCs (Fig. 2) \((5)\). DCs were infected with M51R and M51R-F at varying MOIs for 6 or 24 hours. Supernatants were analyzed for the cytokines IL-1β, IL-6 and IL-12 by ELISA. Infection of DCs with M51R vector did not result in detectable levels of IL-1β above the background of the ELISA. In contrast, the M51R-F vector elicited robust production of IL-1β. DCs infected with M51R vector showed a strong IL-6 and IL-12 response, which was increased in cells infected with M51R-F vector at MOIs of 0.1 and 1.0 \((p = 0.005 \text{ for } \text{IL-6 and } p = 0.01 \text{ for } \text{IL-12, respectively})\). This shows that the presence of flagellin can enhance IL-6 and IL-12 production, and is required to elicit production of IL-1β. Treatment of murine DCs with purified flagellin has been reported to elicit low levels of cytokine production compared to other TLR agonists\((22, 25)\). This was confirmed in our experiments by comparing IL-6 and IL-12 production in response to flagellin with that in response to lipopolysaccharide (LPS) (Fig. 2). Cytokine production at 6
Fig. 2. Cytokine production by DCs following treatment with VSV vectors. DCs were treated at varying MOIs for 24 hours. Supernatants were collected and cytokines were measured by ELISA. (A) IL-1β production (B) IL-6 production (C) IL-12 production. Three independent determinations are shown for each treatment. The line indicates the mean. In a separate set of experiments, DCs were treated with purified flagellin or lipopolysaccharide (LPS) as described (1), and analyzed for production of IL-6 and IL-12.
hours post infection had a similar trend to 24 hours with lower levels produced overall.

To determine the signaling pathways that contribute to cytokine production, DCs were cultured from bone marrow of wild-type (WT), TLR5\(^{-/-}\), NLRC4\(^{-/-}\), or IPS-1\(^{-/-}\) mice and were treated with M51R or M51R-F vectors. Supernatants were analyzed for the production of IL-1\(\beta\), IL-6 and IL-12 at 6 or 24 hrs postinfection. The data obtained at 24 hrs is shown in Fig. 3. As in Fig. 2, only the M51R-F vector stimulated production of IL-1\(\beta\) in WT DCs. IL-1 production was reduced slightly in TLR5\(^{-/-}\) DCs, but was completely abolished in NLRC4\(^{-/-}\) DCs (p = 0.001) (Fig. 3A). Production of IL-1\(\beta\) by IPS-1\(^{-/-}\) DCs was similar to that of WT DCs. The same results were obtained at 6 hrs, except that overall cytokine levels were lower (data not shown). These results indicate that NLRC4 is the major PRR for flagellin induction of IL-1\(\beta\) production in these DCs, whereas antiviral signaling through IPS-1 does not play a role.

The effects of the gene deletions on production of IL-6 and IL-12 (Fig. 3B, C) were the opposite of those for IL-1\(\beta\). Both TLR5\(^{-/-}\) and NLRC4\(^{-/-}\) DCs produced IL-6 and IL-12 at levels similar to those produced by WT DCs after infection with M51R and M51R-F. However, IPS-1\(^{-/-}\) DCs treated with M51R vector produced no detectable IL-6 or IL-12 above mock-infected controls. IPS-1\(^{-/-}\) cells treated with M51R-F virus did produce detectable amounts of IL-6 and IL-12, but they were well below the levels produced by cells of the other genotypes. Collectively the data in Fig. 3 indicate that production of IL-1\(\beta\) in response to M51R-F vector
Fig. 3. Cytokine production by DCs with deletions of key signaling molecules at 6 hours post-infection.

Dendritic cells were obtained from bone marrow of wild-type (WT), TLR5⁻/⁻, NLRC4⁻/⁻, or IPS-1⁻/⁻ mice. DCs were infected for 6 hours and cytokines were measured in supernatants by ELISA. (A) IL-1β (B) IL-6 (C) IL-12.

Three independent determinations are shown for each treatment. The line indicates the mean.
primarily involves signaling through NLRC4, whereas production of IL-6 and IL-12 primarily involves signaling through IPS-1.

Production of IL-1β requires stimulation of proIL-1β production, followed by caspase-1-mediated cleavage to generate the mature form of the cytokine. The latter process results from inflammasome activation. To determine the signaling pathways involved in expression of proIL-1β, DCs from WT, NLRC4−/−, or IPS-1−/− mice were treated with M51R or M51R-F vector for 8 hrs, and cell lysates and supernatants were analyzed by immunoblots (Fig. 5). Only uncleaved proIL-1β was detected in cell lysates, and only the cleaved IL-1β was detected in the supernatants. There was a significant increase (p = 0.003 compared to mock) in proIL-1β in the WT cells treated with M51R-F vector, whereas cells treated with M51R vector were similar to mock-treated cells, indicating that virus infection alone in the absence of flagellin does not trigger production of proIL-1β. Cells deficient in NLRC4 and treated with M51R-F had a significant reduction in proIL-1β expression (p = 0.007 compared to WT) indicating that flagellin utilizes NLRC4 to trigger proIL-1β production. DCs deficient in IPS-1 also had a significant reduction in proIL-1β expression (p = 0.04), although secreted levels of IL-1β were similar between WT and IPS-1−/− DCs treated with M51R-F vector, indicating that expression of total IL-1β (proIL-1β + cleaved IL-1β) was higher than mock- or M51R vector-treated cells.

An important indicator of DC maturation is the upregulation of costimulatory molecules and MHC II molecules on the cell surface. To determine the effect of flagellin on expression of these surface markers, DCs were treated
Fig. 4. Cytokine production by DCs with deletions of key signaling molecules at 24 hours post-infection

Dendritic cells were obtained from bone marrow of wild-type (WT), TLR5\(^{-/-}\), NLRC4\(^{-/-}\), or IPS-1\(^{-/-}\) mice. DCs were infected for 24 hours and cytokines were measured in supernatants by ELISA. (A) IL-1\(\beta\) (B) IL-6 (C) IL-12. Three independent determinations are shown for each treatment. The line indicates the mean.
Fig 5. Production of proIL-1β and mature IL-1β following VSV treatment. DCs were cultured from bone marrow of WT, NLRC4−/−, or IPS-1−/− mice and treated for 8 hours with M51R or M51R-F vectors. (A) Immunoblot of cells and media from infected cells, probed with antibodies to detect pro-IL1β, mature IL-1β and actin, as an internal reference. (B) Quantitation of pro-IL1β in immunoblots. Integrated pixel densities were measured using Imagequant software and results are expressed as percent of the maximal response (WT DCs treated with M51R-F vector). Data shown are mean ± sd from 3 experiments.
with M51R or M51R-F vectors for 24 hours, then harvested and stained for CD11c, CD80, CD86, and MHC II (Fig. 6). The cells were analyzed by flow cytometry with gating on CD11c-positive cells. Data are shown as a fold increase in mean fluorescence intensity (MFI) over mock-infected cells. WT cells treated with M51R or M51R-F vector upregulated CD86, CD80 and MHC II. There was no significant enhancement due to the presence of flagellin. DCs from TLR5 and NLRC4 deficient mice showed no difference in comparison to WT cells. However, DCs deficient in IPS-1 failed to respond to either M51R or M51R-F vector, with a statistically significant difference in the CD86 responses (p < 0.04 for WT, NLRC4+/−, and TLR5−/− vs IPS-1−/−, M51R and M51R-F treated). Expression of CD80 and MHC II by IPS-1−/− DCs also trended toward lower values but did not reach statistical significance in these experiments. These results indicate that the upregulation of co-stimulatory molecules, particularly CD86, was primarily dependent on anti-viral signaling through IPS-1.

Activation of the NLRC4 inflammasome can lead to cell death by pyroptosis in some cell types. The observation that DCs treated with M51R-F vector elicit an IL-1β response is an indicator of activation of caspase-1, which is a requirement for pyroptosis. The major characteristics of pyroptosis that distinguish it from other forms of cell death are rapid permeabilization of the plasma membrane, inflammasome activation, and IL-1β release. (28). The release of IL-1β was shown in Fig. 5. Evidence for rapid membrane permeabilization and inflammasome activation is shown in Fig. 7. WT and mutant
Fig. 6. M51R and M51R-F vectors increase co-stimulatory molecule expression. WT, NLRC4−/−, TLR5−/−, and IPS-1−/− DC were treated with M51R and M51R-F vectors. At 24 hours, the cells were harvested, stained for cell surface markers and analyzed by flow cytometry. (A) Representative histograms depicting CD86 expression levels. Solid line, M51R treated; Dotted line, M51R-F treated; shaded histogram, Mock treated. (B) Mean fluorescence intensities (MFI) from multiple experiments indicated as fold-induction in MFI over mock-infected controls. Like symbols represent independent determinations for each treatment. The line represents the mean.
DCs were treated for 6 hours with M51R or M51R-F vectors, and then stained with 7AAD to measure membrane permeabilization. Cells were analyzed by flow cytometry for 7AAD fluorescence and forward light scattering (FSC-H). In the WT cells, approximately 20% of both mock-treated and M51R vector-treated DCs were 7AAD-positive. In contrast, approximately 50% of DCs treated with M51R-F vector were 7AAD-positive. This indicates that DCs treated with flagellin-expressing virus elicited a rapid permeabilization of the plasma membrane that suggests the induction of pyroptosis. When NLRC4−/− cells were treated with either vector, this early membrane permeabilization did not occur, and all treatments resembled levels in mock-infected cells. Consistent with these results, flagellin was not detected in the supernatant of NLRC4−/− DCs (data not shown). Therefore the early cell death triggered by flagellin is dependent on NLRC4. In both the TLR5−/− and IPS-1−/− cells the effect of flagellin expression was similar to WT cells. This shows that signaling through NLRC4, but not IPS-1 or TLR5, plays a role in triggering cell death consistent with induction of pyroptosis in response to M51R-F virus infection.

At later time points, 12 and 24 hours (Fig. 8), the number of 7AAD positive cells treated with M51R-F show similar levels to mock and M51R treated cells. This difference between 6HR and 12HR is most likely due to the fact that cells undergoing pyroptosis at the 6HR time point, and thus 7AAD positive, are no longer represented as intact cells at 12 hours postinfection. However, at 24 hours postinfection IPS-1−/− treated DCs showed significant signs of membrane permeabilization for both treatment types. This is possibly due to the inability of
Fig 7. Membrane permeabilization in DCs at 6 hours following viral vector treatment. DCs from WT, NLRC4−/−, IPS-1−/−, and TLR5−/− mice were treated with either M51R or M51R-F vectors for 6 hours. Cells were stained with 7AAD to measure membrane permeability and analyzed by flow cytometry. (A) Representative dot plots depicting forward scatter (FSC-H) versus 7AAD. Numbers indicate % positive cells in the gate. (B) Results of multiple experiments expressed as % CD11c+ 7AAD+ cells (mean ± sd n = at least 3 experiments per group).
Fig 8. Membrane permeabilization in DCs at later times following viral vector treatment. DCs from WT, NLRC4<sup>-/-</sup>, IPS<sup>1</sup>−/−, and TLR5<sup>-/-</sup> mice were treated with either M51R or M51R-F vectors for 12 (A) or 24 (B) hours. Cells were stained with 7AAD to measure membrane permeability and analyzed by flow cytometry. Results of multiple experiments expressed as % CD11c<sup>+</sup> 7AAD<sup>+</sup> cells (mean ± sd n = at least 3 experiments per group).
the cell to detect the virus and allows the virus to produce higher levels of apoptosis inducers.

The observation that the M51R-F vector induces rapid membrane permeabilization raises the question of how many cells remain viable after treatment with these viruses. DCs were treated with M51R or M51R-F vectors, and cell viability was determined at 6, 12, and 24 hrs after infection using an MTT assay (Fig. 9). There was lower cell viability after treatment with M51R-F versus M51R vector at each time point. However, there was no further decrease in cell viability between 12 and 24 hrs, with M51R vector-treated cells stabilized at approximately 65% viability and M51R-F vector-treated cells at approximately 48% viability.

The cell viability data raises the question of whether the surviving cells express viral antigens. Alternatively, they may be cells that are resistant to infection. To address these questions DCs infected for 24 hours were stained for surface CD11c and CD86, and intracellular VSV N protein. Cells were analyzed by flow cytometry with gating on live, CD11c-positive cells. Fig. 10 shows representative histograms and data from multiple experiments expressed as mean percent of N protein-positive cells. At 24 hours roughly 70% of M51R vector-treated DCs and 55% of M51R-F vector-treated DCs were positive for viral N protein. This result was largely independent of DC genotype. In addition, of those N protein-positive cells, approximately 90% were also positive for CD86 (data not shown). This demonstrates that M51R-flagellin-infected DCs were not only activated to mature, but expressed viral antigen as well.
Fig. 9. Viability of DCs following infection with M51R or M51R-F vectors. WT DCs were infected with M51R or M51R-F vectors for 6, 12, and 24 hours. Cell viability was measured by MTT assay. Each point indicates the mean ± sd from 3 experiments.
Fig 10. Viral N protein expression in DCs following treatment with VSV. WT, NLRC4<sup>−/−</sup>, TLR5<sup>−/−</sup>, and IPS-1<sup>−/−</sup> DCs were treated with M51R and M51R-F vectors for 24 hrs. Cells were labeled for CD11c, then were fixed, permeabilized and stained for VSV N protein. (A) representative histograms depicting N protein expression in infected cells (B) results of multiple experiments expressed as mean ± sd of % CD11c+ cells that were also N protein+ (n= 3 experiments per group).
Discussion

The activity of bacterial flagellin as a vaccine adjuvant is well established both as a purified protein and when expressed in viral vaccine vectors [reviewed in (24, 25)]. There are many cell types in the immune system whose activity is enhanced by flagellin. The experiments presented here address the mechanisms involved in the activity of DCs infected with VSV vectors that express flagellin. Previous experiments demonstrated the enhanced activation of human DCs compared to VSV vectors that do not express flagellin (5). The results presented here extend these results to murine DC, in which we demonstrated that the expression of flagellin in the intracellular compartment leads to the activation of pathways distinct from those activated by virus alone and leads to an increased production of mediators of inflammatory responses. The enhanced release of inflammatory mediators included the production of IL-1β (Figs. 2 - 5) and the triggering of pyroptosis (Figs. 7 and 9). Additionally it was shown that the main pathway that contributes to IL-1β production and pyroptosis is the NLRC4 inflammasome (Figs. 4 and 7).

The pattern of expression of cytokines and costimulatory molecules provided a clear distinction between pathways induced by virus and those induced by flagellin. The production of IL-6, IL-12, and costimulatory molecules was primarily through antiviral signaling as shown by the effects of deletion of IPS-1. In contrast, production of IL-1β and induction of pyroptosis were primarily through inflammasome activation as shown by deletion of NLRC4, which ablated
both responses (Figs. 4 and 7). Deletion of TLR5, the other major PRR for flagellin, had only minor effects in this study. There was certainly the potential for activation of TLR5, since a substantial proportion of the flagellin produced by infected DCs was released into the extracellular environment. In contrast to human DCs, which respond robustly to extracellular flagellin, murine DCs generated in culture respond less well to extracellular flagellin than other TLR agonists, presumably because of their low levels of TLR5 expression (5, 22, 24, 25). Nonetheless, it was important for us to test the role of TLR5, because of the potential for virus infection to stimulate its expression. TLR5 may play a larger role in vivo due to the presence of subsets of DCs that do express TLR5 as well as other cell types that respond effectively to flagellin (22, 24, 25).

Previously published experiments have demonstrated VSV triggering the production of IL-1β through activation of the NLRP3 inflammasome in DCs similar to those studied here (38). In contrast, we detected little if any IL-1β production induced by VSV vectors that do not express flagellin. The discrepancy in our results could be due to minor differences in the culture technique for these DCs. This might lead to a difference in differentiation of DCs prior to infection, which could lead to a different result postinfection. Despite these differences in results, it appears safe to conclude that activation of the NLRC4 inflammasome by flagellin is a much more potent inducer of IL-1β production than activation of the NLRP3 inflammasome by VSV.

In addition to production of IL-1β, the other important effect of activation of the NLRC4 inflammasome in DCs by flagellin was the induction of cell death
consistent with pyroptosis. This form of cell death depends on activation of caspase-1 (which also cleaves pro-IL-1β to generate mature IL-1β) or caspase-11 by NLRs containing Pyrin domains or CARD domains like NLRC4 (28, 29). Evidence for the induction of pyroptosis was the NLRC4-dependent permeabilization of the plasma membrane by 6 hrs postinfection and by the rapid induction of cell death as determined by MTT assay (Figs. 6 and 7). The principal mechanism of cell death induced by VSV in the absence of flagellin expression is apoptosis, which is effectively induced by M51R viruses through activation of both the death receptor and mitochondrial pathways (39-41). Induction of apoptosis in cell culture typically leads to membrane permeabilization and cell lysis in the late stages (39). However, apoptotic cells are usually taken up by phagocytic cells in vivo before their intracellular contents are released. In contrast to induction of cell death by apoptosis, induction of pyroptosis leads to rapid release of intracellular contents both in vivo and in cell culture (28, 29). Many of these intracellular contents represent “danger” signals that are potent activators of innate immune cells. These include metabolites such as ATP and uric acid, and proteins, such as heat shock proteins Hsp70 and Hsp90, and high mobility group box 1 (HMGB1) protein (28, 29). It is also possible that factors released from infected cells induce cell death in adjacent cells. In addition to release of these activators of inflammatory responses, lysis of cells by pyroptosis leads to release of intracellular viral antigens, which can be cross-presented by other classes of DCs, such as CD8+ DCs (42, 43). For example, in the experiments in Fig. 10, the non-viable cells were positive for viral N protein. Cell
death by pyroptosis has been described most often in myeloid cells infected with a variety of pathogens, but it is likely that other non-myeloid cells are also capable of undergoing pyroptosis in response to vectors that express flagellin (28, 29).

The results in Fig. 9 show that approximately 50% of DCs infected with M51R-F virus remain viable at 24 hrs postinfection. This raises the question of why some cells survive the infection while others undergo cell death by pyroptosis. We ruled out the trivial explanation that the surviving cells were resistant to infection by demonstrating the expression of viral antigen in the surviving cells (Fig. 10). These were also the cells that express costimulatory molecules such as CD86, which were assayed at 24 hrs postinfection (Fig. 6). There is a similar dichotomy between cells that die as a result of apoptosis versus those that survive the induction of apoptosis. This is also apparent in Fig. 9 as partial loss of cell viability following infection with M51R vector that does not express flagellin. In the case of the induction of apoptosis, individual cells vary in the extent to which they express both the inducers of apoptosis, such as caspases, as well as inhibitors of apoptosis, such as XIAP. The cells that survive are likely those in which either the inducers are expressed at a lower level or the inhibitors at a higher level or both. Similarly, the cells that survive the induction of pyroptosis are likely to be those in which the balance of regulators favors survival. In addition, there is variation among cells in the level of flagellin expression, so that cells with relatively less flagellin would be expected to survive. One of the key questions about this mechanism of cell death is the
identity of the regulators of pyroptosis, which could have a profound effect on the inflammatory response to signals such as flagellin.

Currently both VSV and flagellin are in clinical trials in humans as vaccine vectors and adjuvants for heterologous antigens (44) (http://clinicaltrials.gov/ct2/show/NCT01859325?spons=profectus&rank=1, http://clinicaltrials.gov/ct2/show/NCT01381744, http://clinicaltrials.gov/show/NCT01628640, 9/18/13). VSV has several qualities that make it an attractive candidate vaccine vector, such as a low seroprevalence in humans and ability to replicate in the cytoplasm without genetic shift or recombination, and thus far the safety profile has been favorable. Flagellin also has many desirable qualities, such as retention of activity in the presence of pre-existing antibody, when used as an adjuvant in the form of intact flagellin or as fusion proteins with heterologous antigens (24). Thus far the safety profile for flagellin has also been favorable, without excessive side effects related to the induction of inflammatory responses. However, there is currently not a vaccine vector that incorporates both VSV and flagellin in clinical trials, although this is an attractive approach for the next generation of VSV vectors. There are other viruses that are also being developed as vectors expressing flagellin (SV5, influenza virus, rabies virus) that may also be subject to future clinical trials.
Acknowledgements

We thank Drs. Matthias Schnell, Thomas Jefferson University School of Medicine, and Vishva Dixit, Genentech Corporation, for providing IPS-1−/− and NLRC4−/− mice, respectively. We thank Dr. Steven Mizel, Wake Forest School of Medicine, for providing TLR5−/− mice, purified flagellin, antibody against flagellin, and GM-CSF. This study was supported by NIH grants R01 AI032983 and P01 AI082325, and has complied with all relevant federal guidelines and institutional policies related to animal care and use.
References


CHAPTER 2

CELLULAR RESPONSES TO WILD-TYPE VSV
Introduction

VSV is being developed by multiple companies and laboratories as a vaccine vector targeting heterologous antigens (1-7). The original vaccine vector was generated by inserting the gene for a target antigen into the VSV genome between the G and L genes. This allows VSV to express the target antigen during transcription of the viral genome. However, this vector was pathogenic in murine and nonhuman primate animal models. Due to the potential for VSV to cause disease in humans, the original vaccine vector had to be attenuated. One method that was developed was to truncate the G protein, and another was to scramble the gene order (8, 9). Both of these methods reduce viral replication and attenuate viral pathogenicity. The vector that is currently in clinical trials with the Profectus Biosciences company targeting HIV incorporates both of these attenuating strategies.

An alternative method to attenuate VSV pathogenicity is to mutate the M protein to induce an innate immune response (10, 11). The M protein mutant virus signals through IPS-1 and activates immune cells leading to upregulation of co-stimulatory molecules and cytokine production (12, 13). To enhance the induction of the immune response the gene for flagellin from Salmonella enterica was inserted between the M and G genes of the VSV genome (14-16). This allows flagellin to be expressed during viral transcription. Flagellin is then able to signal through NLRC4 leading to IL-1β production and pyroptosis (17). Additionally, if flagellin enters the extracellular space following cell death it can
signal through TLR5 expressed on neighboring cells leading to additional cytokine production (18).

VSV vectors with a wild-type M protein suppress the antiviral responses in dendritic cells (DC) and other immune cell types (19). For those vectors with a wild-type M protein the introduction of flagellin may provide the ability to overcome these differences through activation of pathways not induced by the virus alone. The hypothesis that flagellin could lead to an enhanced DC activation for vectors with wt M protein was tested by analyzing the ability of recombinant wild-type (rWT) VSV constructs either expressing or not expressing flagellin, to activate DCs. DCs are an important link between the innate and adaptive immune responses. The activation of DCs and the production of cytokines and co-stimulatory molecules is a vital step in activating the adaptive response. Previous studies showed the ability of M51R vectors expressing flagellin to trigger additional pathways in DCs leading to increased cytokine production and pyroptosis (17). However, rWT vectors inhibit host gene expression and typically prevent these DCs from activating. In the experiments described here the expression of flagellin was able to elicit the production of cytokines, IL-1β, IL-6, and IL-12, but did not lead to upregulation of co-stimulatory molecules.
Materials and Methods

Reagents. Recombinant mouse GM-CSF generated from a baculovirus vector was provided by S. Mizel, Wake Forest School of Medicine. Fluorescently labeled antibodies against mouse cell surface antigens (CD11c, clone N418; CD86, clone GL-1; I-A^b, clone AF6-120.1) were purchased from Biolegend. Fluorescently labeled antibodies against CD40 (clone 3/23), and CD80 (clone 16-1OA1) were purchased from BD Pharmingen. Flagellin antibody and purified flagellin were generously provided by S. Mizel.

Mice. C57BL/6 mice were purchased from Charles River (Wilmington, MA). All mice were maintained and bred in the animal facility at Wake Forest School of Medicine, and experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee.

Dendritic cells. Bone derived DCs were cultured as previously described (20). Briefly, bone marrow was obtained from the femurs and tibias of 8 – 24 wk old mice. Red blood cells were lysed and washed, progenitor cells were plated in 24 well plates (G-DC) or 6-well plates (F-DC) in RPMI containing 10% fetal calf serum supplemented with glutamine, gentamicin, and 2-mercaptoethanol. For G-DCs, cells were seeded at 5x10^5/mL along with 10 ng/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF). Cells were cultured at 37°C for 6 days and fed with fresh media and GM-CSF on days 2, 4, and 5. For F-DCs,
cells were seeded at 1.5x10^6 to 2.0x10^6/mL along with 150 ng/mL recombinant Flt3L (Invitrogen). Cells were cultured at 37°C for 8 or 9 days and fed with fresh additional media and Flt3L on day 3. In both culture systems the nonadherent and loosely-adherent cells were harvested (21).

**Virus growth and preparation.** Recombinant rWT and rWT-F viruses were isolated from infectious VSV cDNA clones and virus stocks were prepared and maintained on BHK cell monolayers as previously described (19). The rWT-F was purified by sucrose gradient centrifugation to remove extracellular flagellin.

**Measurement of costimulatory molecule and cytokine expression following viral infection.** DCs were plated in a 48-well plate at a density of 5 X 10^5 cells/mL in antibiotic free medium, and were infected with virus at the indicated MOI for 24 hours. Supernatants were collected and stored at -80°C, and cytokine levels (IL-1β, IL-6, and IL-12p40) were measured by ELISA (BD Biosciences). DCs were analyzed by staining for T cell costimulatory molecules CD86, CD80, and CD40 using fluorescently labeled antibodies. Data were acquired using a BD FACSCalibur™ flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc.).

**Immunoblotting.** DCs were infected at an MOI of 10, and at 8 hours postinfection supernatants were collected, and cells were lysed using RIPA buffer without SDS. Protein concentrations of the lysates and supernatant were
measured (BioRad) and equivalent amounts of protein per sample were analyzed by SDS-PAGE followed by transfer to 0.45μm PVDF membranes. Membranes were blocked and probed with antibodies against flagellin, caspase-1, IL-1β, or actin followed by the appropriate secondary antibody conjugated to horseradish peroxidase. Chemiluminescent substrate was used for detection (Thermo Scientific, SuperSignal West Dura). Membranes were stripped and re-probed the following day until each protein had been analyzed. Pixel intensities were analyzed utilizing ImageQuant software.

**Cell viability and death.** DCs were infected at an MOI of 10 and at the indicated time point were analyzed for cell viability utilizing an MTT assay according to the manufacturer's instructions (Promega). Absorbance was measured using a microplate reader (BioRad).

**Statistical Analysis.** Comparisons were tested for statistical significance by calculating the t statistic and the probability (p) of the null hypothesis using Microsoft Excel software. Values of p < 0.05 were considered statistically significant.
Results

The original VSV vaccine vector, as well as the vector currently in clinical trials, encode for wild-type M protein. Wild-type M protein inhibits host gene expression in many cell types (11, 22). This includes GM-CSF derived DC’s where infection with rWT virus leads to cell death and no detectable cytokine production or co-stimulatory molecule expression (19). In previous studies the adjuvant flagellin enhanced the activation of DCs treated with M51R vectors (17). Therefore, the hypothesis that flagellin could also enhance the response to vectors with wt M protein was tested in murine GM-CSF derived DCs.

The construction of the rWT virus expressing flagellin (rWT-F) was similar to the M51R-F mutants described previously (Fig. 11A) with the gene for flagellin being inserted as a separate transcriptional unit between the M and G genes. DCs were infected with either rWT-F, the control rWT, or the M51R-F vector for comparison, and flagellin expression was measured in cell lysates and supernatant by immunoblotting (Fig. 11B). As expected mock and rWT vector-treated DCs showed no detectable flagellin in either the cell lysates or the supernatant. DCs treated with rWT-F vector expressed higher levels of flagellin in both cell lysates and supernatant compared to DCs treated with M51R-F vector.

The expression of flagellin by rWT-F vectors enhanced cytokine production by DCs compared to that of rWT vectors (Fig. 12). DCs were treated at varying MOIs for 24 hours. Supernatants were analyzed by ELISA for the
Figure 11. rWT VSV vectors and flagellin expression. (A) Diagram of genomes of rWT and rWT-F with the gene for flagellin (fliC) inserted as a separate gene transcript between the M and G genes. (B) Murine DCs were infected with rWT or rWT-F or M51R-F as control. At 8 hours post infection cell lysates and media were analyzed for flagellin expression by immunoblots.
Figure 12. Cytokine production by GM-CSF derived DCs following treatment with rWT VSV vectors. DCs were treated at varying MOIs for 24 hours. Supernatants were collected and cytokines were measured by ELISA. (A) IL-1β (B) IL-6 (C) IL-12 production. Each bar represents three separate experiments with SEM.
cytokines IL-1β, IL-6, and IL-12. Mock and rWT virus treated DCs produced no significantly measurable levels of any cytokine for any MOI. In contrast, rWT-F vector elicited IL-1β and IL-6 production that increased as MOI increased. rWT-F vector also elicited IL-12 production but had reduced levels at an MOI of 10. These data show that the expression of flagellin in conjunction with viral infection elicits cytokine production whereas infection with rWT virus alone cannot elicit any detectable cytokine production.

The production of mature IL-1β requires stimulation of proIL-1β expression and subsequent cleavage by activated caspase-1. To determine if rWT vector alone could stimulate proIL-1β production DCs were treated with either rWT or rWT-F or M51R-F vector as a control for 8 hours, and cell lysates and supernatants were analyzed by immunoblots (Fig 13). Only uncleaved IL-1β was detected in the cell lysate and cleaved IL-1β in the supernatant. There was a larger stimulation of proIL-1β by rWT-F vector compared to both mock and rWT vector. There was also no detectable mature IL-1β in the supernatant for mock or rWT vector treated DCs, in agreement with the IL-1β ELISA data from Figure 10.

In addition to cytokine production another important indicator of DC maturation is the upregulation of co-stimulatory molecules such as CD86 and CD80. To determine the effect of flagellin on co-stimulatory molecule expression GM-CSF derived DCs were treated with rWT or rWT-F vectors or M51R vector as control for 24 hours, then harvested and stained for CD11c, CD86 and CD80 (Figure 14). The cells were analyzed by flow cytometry with gating on CD11c-positive cells. Figure 14A shows representative histograms of the CD86
Figure 13. Production of proIL-1β and mature IL-1β following rWT VSV treatment. DCs were treated with rWT or rWT-F or M51R-F as control for 8 hours. Cell lysates and supernatants were probed for IL-1β and actin.

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<th>Cells</th>
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Figure 14. rWT VSV vectors do not increase co-stimulatory molecule expression. DCs were treated with rWT or rWT-F or M51R as a control. At 24 hours the cells were harvested, stained for cell surface markers and analyzed by flow cytometry. (A) Representative histogram depicting CD86 expression levels. Solid line, rWT; dashed line rWT-F; shaded histogram, mock treated. (B) Mean fluorescence intensities (MFI) from multiple experiments indicated fold induction in MFI over mock-infected cells. Like symbols represent independent determinations for each treatment. The line represents the mean.
expression levels of DCs following infection. Most mock infected cells (grey) have low levels of CD86. In this experiment a small sub-population had higher CD86 expression. This may reflect activation of some dendritic cells during culture. Infection with rWT or rWT-F vectors led to a slight increase in CD86 expression. Figure 14B shows data from multiple experiments as fold increase in MFI over mock infected cells. DCs treated with either rWT or rWT-F vectors did not increase CD86 expression more than 1.6 fold over mock compared to M51R vectors increase of 3.6 fold over mock. This confirms previously shown data that rWT virus is relatively ineffective in upregulating co-stimulatory molecules and indicates that the expression of flagellin also does not stimulate any further upregulation (19).

The low level of upregulation of co-stimulatory molecules following infection by either rWT vector raises the question, are there any viable cells remaining at 24 hours postinfection. To address this, DCs were infected at an MOI of 10 with either rWT or rWT-F vectors and cell viability was determined at 6, 12, or 24 hrs post infection using an MTT assay (Fig. 15). Both vectors demonstrated an ability to continually reduce cell viability over the course of the experiment. However, DCs treated with rWT-F vector showed a much earlier dramatic loss of cell viability. After the six hour time point both curves were similar suggesting that rWT-F vector utilized a mechanism of rapid killing such as pyroptosis.

The ability of VSV vectors to elicit cell death has made it an attractive oncolytic agent. From the previously shown data the expression of flagellin
Figure 15. Viability of DCs following infection with rWT vectors. DCs were infected with rWT or rWT-F vectors for 6, 12, and 24 hours. Cell viability was measured by MTT assay. Each point indicates the mean ± sd from 3 experiments.
Figure 16. Viability of 4T1 cells following infection with VSV vectors. DCs were infected at an MOI of 0.1 (A) or 10 (B) with rWT, rWT-F, M51R, or M51R-F vectors for 6, 12, 24, 36 or 48 hours. Cell viability was measured by MTT assay. Each point indicates the mean ± sd from 3 experiments.
enhances cell death especially at the earlier time points. The hypothesis that flagellin expression would enhance cell death in tumor cells was tested in a murine tumor cell line, 4T1 (Fig. 16). Tumor cells were treated with either rWT, rWT-F, M51R, or M51R-F vectors for 6, 12, 24, 36, or 48 hours with cell viability measured by MTT assay. rWT and rWT-F vectors showed similar levels of cell viability at all time-points indicating that the expression of flagellin from the rWT vector did not enhance cell death. However, comparison of M51R versus M51R-F vectors showed increased cell death in the MOI of 0.1 at time points 24 hours and later. At the higher MOI the two M51R vectors were similar, indicating that the differences observed at MOI of 0.1 were a response to multiple cycle infection and were not characteristic of a single cycle of virus replication.

GM-CSF derived DCs resemble a subset of inflammatory DCs found in vivo. In contrast, DCs differentiated in the presence of Flt3 ligand resemble DCs typically found in the spleen (F-DC). These DCs are more resistant to rWT virus infection. F-DCs treated with rWT virus survive longer than G-DCs. This would allow the F-DCs more time to respond to both virus and flagellin. These DCs were also tested with all four viral vectors to evaluate their response with and without the presence of flagellin. DC responses were measured by their ability to upregulate co-stimulatory molecules.

To analyze the co-stimulatory response, F-DCs were infected with one of the four viral vectors and 24 hours post infection were stained for co-stimulatory molecules and analyzed by flow cytometry (Fig. 17). All four viral vectors were able to elicit the upregulation of the co-stimulatory molecules CD80
Figure 17. VSV vectors increase co-stimulatory molecule expression in Flt3 ligand derived DCs. DCs were cultured from murine bone marrow in the presence of Flt3 ligand and treated with either rWT, rWT-F, M51R, or M51R-F. At 24 hours cells were harvested, stained for cell surface markers and analyzed by flow cytometry. (A) Representative histograms depicting CD86 expression for rWT vectors or M51R vectors. Solid line is rWT or M51R; dashed line is rWT-F or M51R-F; shaded histogram is mock-treated. (B) Mean fluorescence intensities (MFI) from multiple experiments indicated fold induction in MFI over mock-infected cells. Like symbols represent independent determinations for each treatment. The line represents the mean.
and CD86. However, the M51R viruses were able to elicit higher levels of up-regulation for both CD80 and CD86 in comparison to the rWT vectors. Also, the presence of flagellin was able to generate slightly higher levels of co-stimulatory molecule expression that is more noticeable in the rWT vs rWT-F vector treated DCs.
Discussion

VSV’s M protein is effective at inhibiting host gene expression in many cell types (19, 22). It is very effective in G-DCs as these cells fail to respond to wild-type VSV. However, wild-type VSV vectors that express flagellin were able to elicit a cytokine response from G-DCs. This cytokine production was most likely triggered by flagellin before VSV was able to inhibit host gene expression. Also, expression of flagellin delays the inhibition of host protein synthesis in human DCs (14). However, the presence of flagellin did not prevent inhibition of host gene expression as can be seen by the reduced cytokine levels compared to M51R vector and the low level of upregulation of co-stimulatory molecules. The ability of rWT-F to stimulate cytokines but not costimulatory molecules appears to reflect that cytokines are produced earlier than costimulatory molecules. It has been shown previously that costimulatory molecules require IFN (19).

Additionally, in my pilot experiments I was not able to detect costimulatory molecule expression at 12 hours postinfection even in cells infected with M51R vector.

Although, GM-CSF derived DCs are susceptible to VSV, Flt3L derived DCs are much more resistant (21). These DCs are more resistant to wild-type M protein and its ability to inhibit host gene expression. Early resistance of F-DCs to VSV allows the cells to produce Type I IFN. This early production confers greater resistance among the DC population. With greater resistance to the inhibitory effects of VSV infection, the expression of flagellin from rWT leads to increased
co-stimulatory molecule expression in F-DCs. This demonstrates flagellin’s ability to stimulate improved DC responses in both susceptible and resistant cell types.

Although flagellin elicited a cytokine response it also led to an earlier cell death in G-DC. As noted from the MTT data, G-DCs treated with rWT-F showed a more rapid decline in cell viability than those treated with rWT virus. This cell death is likely due to activation of two different mechanisms. The inhibition of host gene expression induces apoptosis in most cell types that have been examined (23-26). This is also likely to be the cause of cell death in G-DCs infected with rWT virus. In addition, I showed in Chapter 1 that the rapid cell death in DCs is most likely due to pyroptosis triggered by flagellin. Pyroptosis is triggered by signalling of flagellin through NLRC4.

The induction of cell death by VSV is the basis for several different cytolytic therapies. Perhaps the best example is its potential use as an oncolytic virus for cancer therapy. When VSV was tested as an oncolytic agent within a murine tumor cell line, 4T1, it showed great promise at eliminating tumor cells. The observation that VSV vectors that express flagellin induce cell death more rapidly suggested that they may be more effective oncolytic agents. However, the failure of flagellin to significantly enhance tumor killing may be due to the lack of NLRC4 in these cells. This would explain the absence of significant early cell death that would be indicative of pyroptosis.

The current VSV vaccine vector in clinical trials encodes for a wild-type M protein. Data shown here indicate that an immune response to wild-type VSV vectors containing wild-type M protein could be enhanced through the addition of
flagellin. Flagellin leads to enhanced cytokine production when expressed either from the wild-type vector, rWT, or the M51R vector. Additionally, it was shown that the expression of flagellin from the rWT vector increased the antibody response to VSV (14). Thus future improvements to the current vector could include the addition of vaccine adjuvants such as flagellin.
References


CHAPTER 3

ADAPTIVE IMMUNE RESPONSES TO VSV VACCINE VECTORS
Introduction

The ability of a vaccine to elicit an adaptive immune response to the target antigen is essential to conveying immunity. Some antigens require an adjuvant to aid in eliciting an immune response, particularly if they are composed of purified components or inactivated organisms. In the U.S., according to the CDC, there is currently only one licensed vaccine adjuvant; alum. However, there are several others in development and one in particular that is in clinical trials is flagellin (1). Flagellin’s ability to enhance immune responses is through engagement of either the extracellular receptor TLR5 or through the intracellular receptor NLRC4 primarily in innate immune cells (2).

Live viruses are attractive candidates for vaccine vectors, based on the success of live virus vaccines against pathogens such as smallpox, mumps, and measles. Incorporation of adjuvants into viral vectors offers the potential for activation of additional pathways beyond those activated by virus alone leading to an enhanced immune response. VSV vectors have shown promise through their ability to efficiently activate DCs and stimulate the adaptive immune response (3-5). The addition of the vaccine adjuvant flagellin to the VSV vector led to the activation of additional DC pathways not activated by virus alone (5, 6). Those pathways led to production of IL-1β and induction of pyroptosis leading to an inflammatory environment. The expression of flagellin from the VSV genome could lead to enhanced B cell and T cell responses versus virus alone. Flagellin expressed from VSV vectors, either rWT or M51R, is able to increase the antibody response to VSV (5). This was most evident at the lower doses. Other
studies have also shown increases in antibody responses due to the presence of flagellin (2). The increased antibody responses to VSV vectors that express flagellin raise the question of whether there is a corresponding increase in T cell responses, which is addressed in the experiments described here.

Clearance of VSV relies on the involvement of the adaptive immune response. The surface protein of VSV, the G protein, induces a thymus independent response indicated by an initial increase in IgM. However, DCs can transport viral antigens to secondary lymphoid organs activating virus specific T and B cells, leading to switching to other immunoglobulin isotypes. VSV induces both a $T_h1$ and a $T_h2$ response although the $T_h1$ response is dominant. This is demonstrated by the large population of IFN-γ producing CD4$^+$ T cells and the significantly increased levels of IgG2a induced by a $T_h1$ response. At this point there is only one VSV G protein epitope mapped for MHC-II (I-A$^b$) and used for CD4$^+$ VSV specific T cells (7).

VSV is also a potent stimulator of CD8$^+$ T cells. The CD8$^+$ T cell response is focused on conserved sequences in the N and G proteins of VSV and many cytotoxic T cells are cross-reactive among VSV serotypes (8, 9). The immunodominant epitope for MHC-I (H-2D$^b$) is derived from the N protein although in mice from other haplotypes there are MHC-I restricted epitopes from the G protein (10-12).

Flagellin’s ability to elicit an immune response is based on its signalling through the PRRs TLR5 and NLRC4. Activation of these PRRs leads to cytokine production and can induce pyroptosis (2, 6). Flagellin has demonstrated adjuvant
activity on adaptive immune responses when combined with an antigen (13, 14). This effect is greater if flagellin is fused with the antigen allowing both flagellin and antigen to enter the same cell. However, adjuvant effects have also been seen when flagellin is solubilized with antigen.

This study determined the ability of flagellin expressed from VSV vectors to enhance the T cell response. In the acute vaccination phase T cell responses were similar to both M51R and M51R-F vectors. In addition to analyzing the primary T cell response the ability to elicit a memory response was analyzed using a challenge model. During the challenge phase M51R vectors actually provided better protection against challenge with vaccinia virus expressing VSV G protein. However, both vectors provided increased protection in comparison to mock vaccinated mice.
Materials and Methods

Reagents. Fluorescently labeled antibodies against mouse cell surface and intracellular antigens (CD44, clone IM7; CD8, clone 53-6.7; CD4, clone GK1.5; CD107a, clone 1D4B; IL-17a, clone TC11-18H10.1; IL-4, clone 11B11; IFN-γ, clone XMG1.2; TNF-α, clone MP6-XT22) were purchased from Biolegend. GolgiPlug and GolgiStop as well fluorescently labeled antibodies against CD107b (clone ABL-93) were purchased from BD Pharmingen. T cell peptides were purchased from Chi Scientific.

Mice. C57BL/6 mice were purchased from Charles River (Wilmington, MA). All mice were maintained and bred in the animal facility at Wake Forest School of Medicine, and experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee.

Virus growth and preparation. Recombinant M51R and M51R-F viruses were isolated from infectious VSV cDNA clones and virus stocks were prepared and maintained on BHK cell monolayers as previously described (15). The M51R-F was purified by sucrose gradient centrifugation to remove extracellular flagellin. Vaccinia viruses were prepared and grown in HeLa cells as described previously (16).
**Measurement of VSV specific T cells.** To measure VSV specific CD8 and CD4 T cells spleens were harvested from treated mice. Spleens were then disrupted, red blood cells lysed with ACK, and remaining lymphocytes resuspended and counted. Lymphocytes were then plated in 96-well round-bottom plates at a density of 5E5 cells per well. GolgiPlug/Stop, peptide, and CD107a/b were mixed in to the appropriate wells for 5 hours. Control wells received GolgiPlug/Stop and CD107a/b but not peptide. Cells were spun and media removed. Cells were stained for cell surface markers: CD8 or CD4, and CD44. 30 minutes later cells were washed and fixed and permeabilized following kit instructions. Cells were then stained for intracellular cytokines, for CD4⁺ cells: IL-17a, IL-4, and IFN-γ. For CD8⁺ cells: IFN-γ and TNF-α. 30 minutes later cells were washed and resuspended in 2% PFA. Cells were analyzed the following day by flow cytometry using a BD Canto instrument.

**Vaccinia virus challenge.** Mice were treated intranasally with 5E5 pfus (in 10uL) of either M51R or M51R-F or mock vaccinated. 40 days later all mice were challenged with either vaccinia virus expressing VSV-G (VV-G; 5E6 pfu/mouse) or VSV-N (VV-N; 1E7 pfu/mouse). Mice were anesthetized with isoflurane prior to intranasal challenge with vaccinia virus in a volume of 40uL. Challenged mice were monitored daily for weight loss and disease progression. On day 6 post-challenge mice were euthanized and lungs, ovaries, spleens, and serum were collected. Organs were flash frozen in liquid nitrogen and stored at -80°C.
**Plaque assay.** Lungs and ovaries of each challenged mouse were thawed in 1 mL of grinding media (2X DMEM, BSA fraction V, HEPES, Gentamycin). Organs were homogenized, centrifuged and serial dilutions added to mono layers of CV-1 cells. Two hours later media was removed and replaced with fresh growth media. 24 hours later media was removed and cells stained with crystal violet. Plaques were then counted to determine viral titer.

**Statistical Analysis.** Comparisons were tested for statistical significance by calculating the t statistic and one-way ANOVA using Microsoft Excel and GraphPad software. Values of p < 0.05 were considered statistically significant.
Results

It has been shown previously that the expression of flagellin from VSV vectors enhances activation of human (5) and murine (6) DCs *in vitro* and enhances anti-VSV antibody levels in vaccinated mice (5). The goal of the experiments described here was to determine whether expression of flagellin by VSV vectors enhances *in vivo* T cell activation. Wild-type mice were treated intranasally with either M51R or M51R-F vectors for 6, 8, 10, or 12 days. Spleens were harvested, and splenocytes were stimulated with peptides corresponding to the immunodominant N protein epitope for CD8⁺ T cells or G protein epitope for CD4⁺ T cells, then were labeled for intracellular cytokine staining (ICS) and analyzed by flow cytometry. Both CD8⁺ (Fig 18) and CD4⁺ (Fig 19) T cells were analyzed. Data presented are cytokine-producing cells expressed as a percent of cells that express CD8 (or CD4) and are CD44 high following subtraction of the background of cytokine-producing cells observed in mock-infected mice.

The elicitation of CD8⁺ T cells producing IFN-γ (Fig. 18A) or TNF-α (Fig. 18B) was very similar for M51R and M51R-F vectors with maximum levels at days 8 and 10. Only day 10 achieved a statistically significant difference with M51R producing higher percentages of both IFN-γ and TNF-α producing CD8⁺ T cells. Although not statistically significant, there was a trend that the response to M51R-F appeared to peak earlier but was not as substantial as the response to M51R vector.
Figure 18. CD8+ T-cell response following vaccination. Wild-type mice were treated intra-nasally with 5E5 pfu of M51R or M51R-F vector. At 6, 8, 10, and 12 days post treatment spleens were harvested, and splenocytes were treated with VSV N peptide, labeled for ICS and analyzed by flow cytometry. Points shown are percentage of cytokine producing T-cells within the CD8+, CD44 high population for individual mice. The line indicates the mean of nine mice processed per treatment type. (A) IFN-γ producing cells. (B) TNFα-producing cells.
Figure 19. CD4+ T-cell response following vaccination. Wild-type mice were treated intra-nasally with 5E5 pfu of M51R or M51R-F vector. At 6, 8, 10, and 12 days post treatment spleens were harvested and splenocytes were treated with VSV G peptide, labeled for ICS and analyzed by flow cytometry. Points shown are percentage of cytokine producing T-cells within the CD4+, CD44 high population for individual mice. The line indicates the mean of nine mice processed per treatment type. (A) IFN-γ producing cells. (B) IL-4 producing cells. (C) IL-17a producing cells.
The CD4+ T cell response was not statistically different between groups treated with M51R versus M51R-F vectors (Fig. 19). The IL-4 and IL-17 CD4+ T cells showed little if any response above the background of cells in mock-treated mice (Fig. 19 B and C). The IFN-\(\gamma\) producing CD4+ T cells did show a significant response above background (note the difference in y-axis scale in Fig. 19A), but only in some of the treated mice, indicating that the T\(_h\)1 response is not always detectable following treatment with M51R vectors.

Since it was shown that both M51R and M51R-F vectors elicited a CD8+ T cell response to fairly similar levels, the question was raised as to whether elicitation of memory T cells was similar. To address this, wild-type mice were vaccinated intranasally with M51R or M51R-F vectors or mock vaccinated. 40 days post-vaccination mice were challenged with vaccinia viruses expressing either VSV N protein (VV-N), to analyze the CD8+ T cell response or VSV G protein (VV-G) to analyze the CD4+ T cell response. The amount of weight loss following challenge was used as an indicator of the extent of vaccinia virus-induced disease. The data indicates the mice were sick but the weight loss was minor even in mock vaccinated mice indicating this was a sub-lethal vaccinia virus challenge (Fig. 20). Six days post-challenge spleens, lungs, and ovaries were collected. Lymphocytes from the spleen were processed for ICS and lungs and ovaries were processed and titered for vaccinia virus. The splenic T cell response (Fig 21) showed that the vaccinated mice produced a significantly higher level of N peptide-specific cytokine-producing CD8+ T cells compared to
Figure 20. Murine weight loss following challenge by vaccinia virus. Wild-type mice were vaccinated intra-nasally with either M51R or M51R-F. 40 days after vaccination they were challenged with vaccinia virus expressing either VSV N protein (A) or VSV G protein (B). Mice were weighed daily and plotted as a percentage of weight on day of challenge. Each point represents the average of 10 mice from two separate experiments.
Figure 21. T-cell response following challenge by vaccinia virus.

Wild-type mice were vaccinated intra-nasally with either M51R or M51R-F. 40 days after vaccination they were challenged with vaccinia virus expressing either VSV N protein (A) or VSV G protein (B). Spleens were harvested, and splenocytes were incubated with VSV N peptide (A) or G peptide (B), then treated for ICS and analyzed by flow cytometry. Data shown are a percentage of cytokine producing T-cells within the CD8⁺, CD44 high (A) or CD4⁺, CD44 high (B) populations.
mock vaccinated mice. However, there was little difference between M51R or M51R-F treated mice indicating that flagellin did not enhance the memory response of the CD8+ T cells. There was no significant difference in the G peptide-specific CD4+ T cell response between vaccinated mice and mock vaccinated mice indicating that vaccination with M51R vectors did not contribute to a memory response for CD4+ T cells above that of the primary responses against vaccinia G virus in mock vaccinated mice.

Vaccination leads to higher levels of VSV-specific CD8+ T cells following vaccinia virus challenge, but the question remains if this higher level limits viral replication. To test this lungs and ovaries of challenged mice were processed and titered for vaccinia virus (Fig 22). Vaccinia was detected at very low levels in the ovaries of only 4 of the mock treated mice (data not shown). In the lungs, mice challenged with VV-N did not show any significant difference in viral titers across vaccination groups. However, in mice challenged with VV-G the mock vaccinated group showed statistically significant elevated levels within the lung compared to M51R vector vaccinated mice. Although M51R-F vector vaccinated mice did not have significantly lower levels of vaccinia titers compared to mock vaccinated mice they trended lower. Vaccinia virus was still detected in the lungs of all mock vaccinated mice but only some of the vaccinated mice. In addition, vaccinated mice with detectable vaccinia in the lungs had reduced levels of viral titers compared to mock vaccinated. There was also statistical difference between M51R and M51R-F vector vaccinated mice. These data indicate that
M51R vectors provided better protection against challenge with vaccinia virus expressing VSV G protein.
Figure 22. Vaccinia virus titers in lungs of challenged mice. Wild-type mice were vaccinated intra-nasally with either M51R or M51R-F. 40 days after vaccination they were challenged with vaccinia virus expressing either VSV N protein or VSV G protein. Lungs were harvested and homogenized, and vaccinia virus titers were determined. Each point represents data from an individual mouse. The line indicates the geometric mean. LD = limit of detection.
Discussion

VSV vectors expressing flagellin have been shown to induce an increased antibody response in mice (5). Flagellin has also been shown to affect T cell responses in mice (13, 14). Flagellin as a soluble protein has been shown to increase Ag-specific CD4$^+$ T cells when administered i.v. along with antigen (13). Flagellin fusion proteins with OVA have been shown to enhance Ag-specific CD8$^+$ T cell responses (14). Additionally, studies in human CD4$^+$ T cells demonstrated that TLR5 is expressed on those cells and that they respond to extracellular flagellin (17). This raises the question of the effect of flagellin expressed from VSV on T cell activation. I expected that expression of flagellin from a VSV vector would enhance the T cell response above that induced by virus alone. However, in this study I observed little if any effect of flagellin on the anti-VSV T cell response in the acute effector phase and in elicitation of memory T cells utilizing vaccinia virus expressing VSV antigens as a challenge.

During the acute phase there was no significant difference in CD8$^+$ T cell responses between mice vaccinated with M51R versus M51R-F vectors. In the experiments in Fig. 18 only CD8$^+$ T cells in the spleen were analyzed. It is possible there is an effect of flagellin on CD8$^+$ T cells in other compartments. For example, flagellin might enhance the CD8$^+$ T cell response at the site of VSV infection in the upper respiratory tract. However, both viruses elicited a strong enough immune response to allow generation of memory cells as seen during the challenge study. When mice vaccinated with either M51R or M51R-F vectors were challenged with VV-N virus, which contains the major MHC-I epitope,
similar levels of CD8\(^+\) T cells were elicited both of which were above mock vaccinated controls. These results indicate that both M51R and M51R-F vectors lead to similar memory CD8\(^+\) T cell responses.

The CD4\(^+\) T cell response induced by M51R and M51R-F vectors was primarily due to T\(_h\)1 cells as shown by the greater percentage of IFN-\(\gamma\) producing T cells compared to IL-4 or IL-17 producing T cells. This is consistent with anti-VSV antibody being IgG2a isotype in which class switching is promoted by IFN-\(\gamma\) (4). When vaccinated mice were challenged with VV-G the G protein specific CD4\(^+\) T cell response was similar to mock vaccinated mice. The lack of an increased memory CD4\(^+\) T cell response compared to the primary response in mock vaccinated mice could be due to several factors. The first is that the primary CD4\(^+\) T cell response could be equivalent to the memory to VV-G in vaccinated mice. Alternatively, the CD4\(^+\) T cell response may have peaked earlier than the six days postchallenge that was analyzed. A final explanation could be that portions of the responding CD4\(^+\) T cells in vaccinated mice have migrated to tissues other than the spleen.

The ability of flagellin expressing VSV viruses to elicit a better antibody response has been shown previously (5). If T cell levels are similar what is the mechanism for an improved antibody response? One mechanism could be that even if T cell numbers were similar or even elevated in M51R vector treated mice the CD4\(^+\) T cells responding to M51R-F vectors were functionally superior. This study focused only on a select few cytokines to measure CD4\(^+\) T cell responses. One item that could be further examined would be T cell avidity following
treatment with these VSV vectors. Another explanation could be that B cells are more sensitive to the DC changes elicited by the VSV vectors than T cells. Treatment with M51R-F creates a more inflammatory environment which may not affect the T cell response but leads to a more robust B cell response.

One of the unexpected results from this experiment was that mice challenged with VV-G were better protected when vaccinated with M51R vector versus M51R-F vector or mock vaccinated. This is demonstrated in both the weight loss data (Fig. 20) and the vaccinia viral titers (Fig. 22). This may seem a bit perplexing since M51R-F vectors have previously showed increased antibody production versus M51R vectors. There may be several reasons for this discrepancy. Vaccination with VSV vectors would elicit antibodies and CD8+ T cells targeting the VSV G protein and that would be the only antigen previously encountered when challenged with VV-G. Neutralizing antibodies would not play a role in clearance of VV-G. This is because the G protein is not incorporated in the vaccinia virion. Even if G protein was incorporated vaccinia does not utilize G protein so neutralizing it should have a minimal effect on vaccinia replication. However, VV-G-infected cells that have expressed G protein on the cell surface could be targeted through antibodies. Infected cells could then be lysed through the actions of complement and antibody-dependent cell-mediated cytotoxicity (ADCC). Another possible explanation is the CD4+ T cell response. Although there was not a statistical difference in the CD4+ response following challenge, mice vaccinated with M51R vector trended higher. Clearance of a primary vaccinia virus infection relies on CD4+ T cells and antibodies (18-22). However,
in a secondary infection CD8+ memory T cells also play a significant role in preventing and established vaccinia virus infection (18). It may be that in this model the M51R vector was able to elicit better CD8+ T cells targeting VSV G protein that in turn prevented vaccinia virus from establishing an infection in the lung tissue.

The effect of flagellin expressed from VSV on DCs is the additional production of IL-1β and induction of pyroptosis (6). These additional inflammatory signals did not lead to an enhanced protection against VV-G in the current model. As shown in Chapter 1, the ability of M51R and M51R-F vectors to upregulate co-stimulatory molecules and produce cytokines necessary for T cell activation were similar and could explain similarities in T cell responses. The levels of viral protein expression in individual dendritic cells were also similar in DCs infected with either M51R or M51R-F vectors. However, due to the early induction of pyroptosis and rapid cell death in DCs infected with M51R-F vectors there may be overall less viral antigen produced in vivo.

This study focused on T cell responses to VSV in mice following intra-nasal inoculation. Although flagellin did not enhance the T cell response in this model, as has been previously demonstrated, there are other variables that need to be examined. The route of inoculation is an important component in vaccine development. Perhaps flagellin’s adjuvant properties are better exerted following vaccination through a different route. Flagellin’s adjuvant effect on CD4+ T cells was demonstrated through an intra-venous route (13). Flagellin’s adjuvant effect on CD8+ T cells was demonstrated through an intra-muscular route (14).
Although the increased antibody responses to M51R-F vector was demonstrated through an intra-nasal inoculation perhaps enhanced T cell responses require a different route (5).

In addition animal models can vary significantly in their responses. Although flagellin did not enhance the T cell response in this model it is possible that flagellin could enhance the T cell response in other animal models such as non-human primates. Routes of inoculation would most likely play an important role in a non-human primate model.
References


antigen-specific CD8+ T cell response independently of TLR5 and MyD88. 
J Immunol 186:6255-6262.

15. **Ahmed M, Brzoza KL, Hiltbold EM.** 2006. Matrix protein mutant of 
vesicular stomatitis virus stimulates maturation of myeloid dendritic cells. J 
Virol 80:2194-2205.

nucleoprotein is a major target antigen for cross-reactive anti-influenza A 

17. **Crellin NK, Garcia RV, Hadisfar O, Allan SE, Steiner TS, Levings MK.** 
2005. Human CD4+ T cells express TLR5 and its ligand flagellin enhances 
the suppressive capacity and expression of FOXP3 in CD4+CD25+ T 

immunity against vaccinia virus infection of mice. J Immunol 172:6265- 
6271.

19. **Belyakov IM, Earl P, Dzutsev A, Kuznetsov VA, Lemon M, Wyatt LS, 
Shared modes of protection against poxvirus infection by attenuated and 
conventional smallpox vaccine viruses. Proc Natl Acad Sci U S A 
100:9458-9463.

20. **Spriggs MK, Koller BH, Sato T, Morrissey PJ, Fanslow WC, Smithies 
CD8+ T-cell-deficient mice survive inoculation with high doses of vaccinia


SUMMARY

Previous data had demonstrated the ability of VSV vectors expressing flagellin to enhance dendritic cell activation (1). The incorporation of flagellin into viral vaccine vectors was based on the idea of the activation of pathways not induced by virus alone. This hypothesis was tested in Chapter 1 of this thesis. Analysis of activation of DC from transgenic mice defective in key signaling molecules provided a clear distinction between IPS1 and NLRC4. Activation of IPS1 led to the production of cytokines such as IL-6 and IL-12 and up regulation of costimulatory molecules. In contrast, activation of NLRC4, the cytosolic flagellin sensor, led to production of IL-1β and could lead to pyroptosis. However, only approximately 50% of the cells infected with M51R-F underwent pyroptosis with the other 50% becoming activated and containing viral antigen. This indicates flagellin’s ability to induce an innate immune response and trigger pathways not induced by virus alone. This could lead to a more robust adaptive immune response to a vaccine antigen.

Current VSV vectors in clinical trials encode for a wild-type M protein. VSV vectors expressing wild-type M protein inhibits host gene expression in many cell types including GM-CSF derived DCs. The hypothesis that flagellin could also enhance DC responses to wild-type M protein expressing VSV vectors was tested in Chapter 2. The expression of flagellin from rWT vectors led to an increased cytokine production. However, co-stimulatory molecules were not upregulated with either rWT or rWT-F viruses. This was likely due to the ability of
flagellin to trigger an early cytokine response before M protein could inhibit host gene expression. In contrast the expression of costimulatory molecules requires longer times than cytokine production. As a result, wild type M protein produced by rWT-F vector was able to inhibit costimulatory molecule expression. Nonetheless, the enhanced cytokine response provides rationale for the incorporation of flagellin into current VSV vectors in clinical trials.

Flagellin has been shown to enhance both the humoral and cellular immune responses (1-3). However, in this study flagellin did not enhance the T cell response to VSV above that induced by M51R vector without flagellin. It also hindered the protective response M51R vectors elicited in the challenge with vaccinia virus expressing G protein. This raises the possibility that vectors expressing flagellin may be less effective in humans against some agents but more effective against others in which the antibody response, rather than the T cell response is the major protective mechanism. It has been difficult to determine the immune correlates of protection in humans against HIV. However, a recent clinical trial demonstrated that non-neutralizing antibodies appeared to correlate with better outcomes (4, 5). Expression of flagellin by viral vaccine vectors may enhance the production of these antibodies.

Although flagellin did not enhance the T cell response in this model further investigation is required before ruling out the ability of flagellin expressed from the VSV genome to enhance the T cell response. This includes routes of inoculation and animal models. Different routes of inoculation with flagellin led to enhancement of differing T cell populations (2, 3). In addition to the intra-nasal
route used in the current project, intra-muscular and intra-venous routes should also be examined. Also, treatment responses vary from animal to animal. Although flagellin did not enhance the T cell response in the current murine model use of a non-human primate model should be examined as flagellin has shown the ability to increase antibody production in non-human primates as well as mice (1, 6, 7). If flagellin expressing viral vectors induce enhanced immune responses in non-human primates this would point the way for their use in humans.
References


6. Weimer ET, Ervin SE, Wozniak DJ, Mizel SB. 2009. Immunization of young African green monkeys with OprF epitope 8-OprI-type A- and B-
flagellin fusion proteins promotes the production of protective antibodies against nonmucoid Pseudomonas aeruginosa. Vaccine 27:6762-6769.

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EMPLOYMENT:

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Graduate Student, Wake Forest School of Medicine, Winston-Salem, NC
Thesis project focused on the development of vesicular stomatitis virus (VSV) as a vaccine vector. Examined the innate immune response pathways to VSV and the vaccine adjuvant flagellin. Also analyzed the adaptive response (T-cell, antibody) both in the vaccination phase and the memory/challenge phase.

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Assistant Professor of Military Science (USAR), Wake Forest University, Winston-Salem, NC
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Adjunct Faculty, Chemistry Department, High Point University, High Point, NC
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Responsible for curriculum development, implementation, and teaching for physical science, honors chemistry, AP chemistry, and honors physics. Also coached mens and womens cross-country, mens and womens swimming, and womens softball.

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Automations Officer (USAR), 80th Training Command, Richmond, VA
Assisted in development, management, and implementation of information management strategies and policies for the command, three subordinate divisions, 13 brigades, and 63 battalions geographically dispersed across the US.

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Battalion Signal Officer, 1st BN, 3rd Special Forces Group (Airborne), Ft. Bragg, NC
Responsible for installation, operation, and maintenance of all battalion communications equipment to include computer networks, satellite communications, and HF/VHF/UHF radio communication systems. Directly responsible for 15 soldiers while deployed for combat operations in Afghanistan.

11MAY2004 – 16MAY2005
Executive Officer, C Co., 112th Signal Battalion (Airborne), Ft. Bragg, NC
Responsible for all maintenance on equipment and vehicles, all personnel actions, and all supply issues for a 62 person company.

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13MAY2002 – 20SEP2002
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RESEARCH SKILLS:

- Protein purification using FPLC
- Virus infections and plaque assays
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- Gel electrophoresis and immunoblotting
- Molecular cloning and mutagenesis (using E. Coli.)
- Flow cytometry
- Use of radioactive materials ($^{35}$S)
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01APR2010  Wake Forest Biochemistry Department
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*Breast Cancer Therapies Using Vesicular Stomatitis Viruses Engineered to Express Bacterial Flagellin* (Oral)

14OCT2010  Wake Forest Biochemistry Department
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*Genetically Engineered Vesicular Stomatitis Virus Stimulates the Innate Immune System* (Oral)

03NOV2011  Wake Forest Biochemistry Department
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*Determining the Signaling Pathways in Innate Immune Cells that Affect the Immune Response to Vesicular Stomatitis Virus Vectors* (Oral)
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