Acute Pathogenesis of Recombinant Vesicular Stomatitis Virus Vaccine Vectors is Linked to Interleukin-1

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Thesis submitted in partial fulfillment of
the requirements for the degree of Master of Science in the Department of Pathology in the Graduate School
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ABSTRACT

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Abstract

Recombinant vesicular stomatitis virus (rVSV) is a promising candidate viral vaccine vector for use in humans. VSV is highly immunogenic, pre-existing immunity to VSV is rare, and VSV is able to grow to high titers in cell lines approved for vaccine use. Its potential reactogenicity is a barrier to its use in humans, with small laboratory animals developing fever and losing up to 20% of their pre-immunization body weight in the first four days after administration [1, 2], and the one person to date that has received an experimental rVSV vaccine developed headache, fever, and muscle pain within 12 hours and transient VSV viremia was detected [3]. The underlying cause of these reactions has not yet been studied. Here, we have found that IL-1β and/or IL-1α contributes to rVSV pathology after intramuscular immunization in mice and that IL-1 production is not required for control of rVSV replication *in vivo*, or for the generation of protective immune responses to VSV antigens. Suppression of IL-1 may be a safe strategy to reduce vector reactogenicity without affecting immunogenicity. Utilizing mice deficient in either ASC or caspase-1, we have also found that production of mature IL-1β in response to rVSV might be independent of inflammasome activation or caspase-1 cleavage. The exact mechanism is yet to be determined, but might depend upon which cell types secrete mature IL-1β after immunization.

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1. Introduction

1.1 Vesicular Stomatitis Virus

Vesicular stomatitis virus (VSV) is an enveloped non-segmented negative sense RNA virus of the family Rhabdoviridae. It naturally infects livestock, including cattle, horses, and pigs, causing an acute disease that is clinically similar to foot-and-mouth disease (FMD)[4]. Infection in livestock is characterized by fever and vesicles in the oral mucosa and in the skin of the coronary band and teat. The disease is currently considered enzootic only in the Americas [4]. VSV-Indiana (VSV-I) and VSV-New Jersey (VSV-NJ) are the two serotypes most commonly isolated in the Americas[4]. VSV-I was isolated in 1925 from an outbreak in cattle being transported from Kansas City, Missouri to Richmond, Indiana[5]. VSV-NJ was isolated from an outbreak in cattle in New Jersey during 1926, and was determined to be serologically different from VSV-I [5, 6]. Commonly studied laboratory-adapted strains mostly belong to the VSV-I serotype [4]. VSV infection can occur in humans, causing mild flu-like symptoms, but is rare in the world population [7].

1.2 VSV as a vaccine vector

The low prevalence of human infection and the ability of VSV to induce potent systemic and mucosal antibody and cytotoxic T lymphocyte (CTL) responses [8] in infected hosts make VSV a good candidate for a vaccine vector. VSV based vaccines

have been created against such diseases as Ebola and Marburg viruses [9], S/HIV [10], SARS coronavirus [11], Lassa fever [12], measles [13] and influenza [2]. In non-human primates, rVSV vaccines have been demonstrated as protective for Ebola and Marburg viruses, Lassa Fever, and SHIV [9, 10, 12]. Recombinant VSV vaccines are generated by incorporating the DNA sequence of interest into the VSV genome such that the antigenic protein will be produced in addition to viral proteins upon immunization.

Thus far, use of rVSV in humans has been impeded by the concern over vector associated pathology. Unmodified VSV can induce pathology by its neurotropism, entering the central nervous system via the olfactory nerve of olfactory receptor neurons [14-16] , and by its reactogenicity, causing fever, chills, and myalgia in infected humans [17-19]. The recombinant VSV (rVSV) constructs approved for Phase I clinical trials in humans are highly attenuated "genome-scrambled" viruses that are not neurovirulent, even when directly injected into the brain of non-human primates [20]. Methods for attenuation include truncations or deletions of the attachment protein VSV G [21, 22], scrambling of the viral genome such that replication is inhibited [20, 23], and deletions in the matrix protein which reduce its interferon antagonist activity [1]. These strategies are able to eliminate the neurovirulence of rVSV, but can also reduce vector immunogenicity [24] and make it difficult to produce *in vitro* the quantity of virus necessary for vaccine production [23].

To date, one person has been immunized with a single cycle non-replicating experimental VSV vaccine. The recipient developed a headache, fever, and muscle pain within 12 hours and transient viremia was detected [3]. The production of pro-inflammatory cytokines is important to activate the immune system in response to infection, but can also be detrimental to the health of the host. Septic shock is an example of too much of a pro-inflammatory response. IL-1 and TNF-α are two examples of well known pro-inflammatory cytokines. TNF-α is known to induce anorexia and fever after administration to small animals [25-27]. In another study, IL-6 was shown to be necessary for TNF-α-induced fever [28]. Injection of IL-1β into humans has been shown to result in fever, headache, myalgia, and arthralgia [29]. IL-1 has also been demonstrated to cause acute weight loss/cachexia in mice [30] and humans [31, 32].

The yellow fever vaccine, which is a live attenuated virus, has been shown to elicit production of the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 [33]. Side effects associated with vaccination include fever, aches, soreness, and redness or swelling at the injection site. Up to 25% of vaccinees experience these effects for up to a week after administration. More seriously, 1 in 250,000 people receiving the vaccine can experience a life-threatening severe illness with organ failure, with more than half dying [34]. The live virus vaccinia is used to vaccinate people against smallpox. Vaccinia virus has been shown to induce production of the pro-inflammatory cytokines IL-1, TNF-α, and IL-6 [35, 36]. Common side effects include itching, swollen lymph nodes, sore arm,

fever, headache, body ache, mild rash, and fatigue. 1 out of 3 people may feel ill enough to miss work or school, or have trouble sleeping after administration [37]. The majority of the side effects seen after the administration of live-virus vaccines can be attributed to the production of pro-inflammatory cytokines and therefore it is necessary to develop vaccine vectors that minimize these effects.

1.3 VSV Replication

The 11kb negative sense VSV genome consists of five genes: the nucleoprotein N, the phosphoprotein P, which together with the large protein, L, constitutes the viral polymerase activity, the matrix protein M, and the envelope glycoprotein G, which mediates attachment to host cells [4]. VSV is able to infect all known mammalian cell types, though the cell surface receptor to which VSV G binds is unknown. Phosphatidyl serine was thought to act as the cellular receptor for VSV [38], though later experiments have indicated this is not the case [39]. It has been proposed that nonspecific electrostatic and hydrophobic interactions are able to mediate the attachment of VSV to cell membranes [40]. Once attached to the cell membrane, the VSV particle is endocytosed in a clathrin-dependent manner. Acidification of the endosome leads to fusion of the viral and endosomal membranes, and the viral ribonucleoprotein (RNP) core is released into the cytoplasm of the host cell where replication occurs [41]. The mature virus forms rod-shaped particles, approximately 100 to 430nm in length and 45

to 100nm in width. Length of the virion is dictated by the length of the RNA genome; consequently, incorporating new genes into the viral genome causes an increase in virion length [42].

1.4 Immune detection of VSV

Detection of VSV by TLR4 [43], TLR7 [44], and RIG-I [45] induces production of type I interferon. TLR4 present on the cell surface detects VSV G on the viral envelope and signals in a MyD88-independent, CD14-dependent manner [43, 46]. TLR7 detects viral ssRNA released when some virions degrade upon acidification of the endosome and signals in a MyD88 dependent manner [44]. Interferon production through RIG-I is triggered through detection of viral RNA in the cytoplasm. This was demonstrated by infection with VSV or transfection of VSV RNA into cells isolated from WT and RIG-I-/- mice [45, 47]. The production of type I interferons is essential for control of VSV in vivo. Mice lacking type I IFN receptors were extremely susceptible to VSV infection, succumbing to an IV dose of 30 to 50 PFU within 3 to 6 days, whereas the LD_{50} for IV administration in WT mice is in the range of 10^8 PFU [48]. TLR signaling results in production of pro-inflammatory cytokines, which are predicted to contribute to acute pathology after rVSV administration. Pro-inflammatory cytokines are known to be involved in fever, headache, anorexia, and myalgia, side effects that are seen after immunization with rVSV. IL-1β is a common pro-inflammatory cytokine produced after

VSV infection and is known to contribute these side effects. Mature IL-1β is primarily produced after formation of the inflammasome and activation of caspase-1, which cleaves pro-IL-1β to its biologically active form. The disconnect between pro-IL-1β production and IL-1β activity provides multiple targets to reduce its production or signaling.

1.5 Production of biologically active IL-1β is a two step process

The production of active IL-1β and IL-18 consists of two steps. In the first step, signaling through a TLR or other "sensor" molecules upregulates transcription and translation of biologically inactive pro-IL-1β and pro-IL-18. In the second step, activated caspase-1 following inflammasome formation cleaves the inactive precursors to their active forms [49, 50]. Non-caspase-1 cleavage of IL-1β also occurs. Infiltration of short-lived neutrophils can result in the release of pro-IL-1β from their intracellular stores and extracellular cleavage by proteinase-3, a common neutrophil protease [51]. Upon detection of infected cells, CTLs and NKs can release granzyme A, which is able to process pro-IL-1β to IL-1β[52]. Elastase , matrix metalloprotease 9, and a mast cell chymase are also able to process pro-IL-1β extracellularly [49, 50, 53, 54].

The inflammasome is a cytosolic signaling complex that leads to the activation of caspase-1. The inflammasome complex generally consists of a sensor molecule, an adaptor molecule, and caspase-1. Binding of caspase-1 in the inflammasome leads to its

autoactivation and subsequent cleavage of its substrates, pro-IL-18 and pro-IL-1β, to their biologically active forms [55]. The nucleotide-binding domain and leucine-rich repeat containing gene family, pyrin domain containing 3 (NLRP3) inflammasome, which is the best characterized, utilizes the adaptor molecule apoptotic speck-like protein containing a CARD domain (ASC), and its assembly and activation is triggered by a variety of stimuli, including uric acid crystals [56], bacterial toxins [57], and certain DNA and RNA viruses, such as influenza [58] and modified vaccinia virus Ankara (MVA) [55]. The absent in melanoma 2 (AIM2) inflammasome has been shown to form in response to cytosolic dsDNA from the host, to microbes, and to viruses, such as vaccinia [59].

1.6 VSV infection triggers formation of the inflammasome.

There have been conflicting reports of VSV triggering inflammasome formation. Muruve *et al*. were unable to confirm *in vitro* inflammasome formation in after infection with VSV, as measured by Western blot of cell extracts and supernatants for 17kDa cleaved active IL-1β [60]. Recently, Poeck *et al* have reported that RIG-I is required for VSV or 5′-triphosphate RNA (3pRNA) triggered activation of caspase-1 and production of active IL-1β. The authors concluded that RIG-I is able to physically associate with ASC *in vitro* after infection of THP-1 cells with VSV, based on immunoprecipitation of endogenous ASC followed by immunoblotting for the presence of RIG-I [61]. This

indicates that VSV infection is able to cause formation and activation of an inflammasome utilizing RIG-I, ASC, and caspase-1 to produce biologically active IL-1β. It is not stated in the manuscript what cell type Muruve *et al* used to detect IL-1β *in vitro* after infection with VSV, which if it were not THP-1 cells, may explain the discrepancy with the results of Poeck *et al* in THP-1 cells.

Rajan *et al* have also recently reported that VSV activates the NLRP3 inflammasome [62]. BMDCs were primed with the TLR2 agonist Pam_3 and harvested 6 hours after VSV infection. Primed BMDCs were found to produce active caspase-1 and secrete IL-1β while unprimed cells did not. It was determined that NLRP3, caspase-1, and ASC were required for IL-1β secretion in experiments using BMDCs isolated from Nlrp3-/-, Casp1-/-, and Asc-/- mice. In contrast to Poeck *et al*, Rajan *et al* found that RIG-I was not required for induction of IL-1β secretion in response to VSV, through use of Pam_3-primed and unprimed WT and RIG-I-/- BMDCs. The discrepancy between the two papers may be explained in part by the fact that Rajan *et al* primed the cells with a TLR2 agonist, Pam_3, while Poeck *et al* did not conduct any exogenous priming of the cells in their experiments.

1.7 Biological functions of IL-1.

IL-1β is a proinflammatory cytokine which signals through the IL-1 receptor, along with IL-1α. IL-1α and IL-1β only share 25% amino acid sequence homology, but

have similar three dimensional structures [63]; though both bind and signal through the same receptor, they do not have identical biological activities. IL-1α is cleaved by the endogenous protease calpain to its mature form, though its precursor is also biologically active, and it is not generally found in the circulation [64]. IL-1β has been studied extensively due to its role in human autoinflammatory diseases, such as gout [56], Muckle-Wells syndrome [65], and familial cold-induced autoinflammatory syndrome [66]. Autoinflammatory diseases are distinguished from autoimmune diseases by their response to blockade of IL-1β rather than TNF-α, and to caspase-1 inhibition for reduction of symptoms. IL-1β is produced primarily by caspase-1 processing in monocytes, macrophages, and dendritic cells, and less commonly by B cells and NK cells [50]. In addition, it has been reported that IL-1β can be produced from keratinocytes, fibroblasts, endothelial cells, myoblasts, and microglia in an inflammasome-dependent manner [67-73]. Release of IL-1β induces gene expression and synthesis of cyclooxygenase type 2 (COX-2), type 2 phospholipase A, and inducible nitric oxide synthase (iNOS), which increases production of prostaglandin-E2 (PGE_2), platelet activating factor, and nitric oxide (NO). This results in fever, lowered pain threshold, vasodilatation, and hypotension [50]. IL-1β has also been shown to cause acute weight loss/cachexia in mice [30] and humans [31, 32], and to activate the acute phase response [74]. IL-1β is a good target for suppression due to the fact that the majority of its effects are what we are trying to eliminate after administration of rVSV vaccines.

1.8 IL-1 and the adaptive immune response.

The role of IL-1 in the generation of an adaptive immune response is not well understood. In hepatitis C infection, IL-1β has been suggested as having antiviral activity, inhibiting subgenomic RNA replication and viral protein expression [75]. Mice deficient in caspase-1, ASC, Nlrp3, or IL-1R have been described as more susceptible to influenza A infection than wild-type mice, though viral loads were not significantly different[76-79]. The studies agreed that survival of ASC-/-, Casp1-/-, and IL-1R-/- mice was significantly decreased relative to WT. With NLRP3-/- mice, there were conflicting reports on survival. Two papers reported significantly decreased survival of Nlrp3-/- mice infected with 6000-8000 PFU of virus [76, 77], while one reported no difference in survival, though those animals were only infected with 10 PFU [78], which could explain the discrepancy. It seems that although IL-1β mediates the acute pathology observed in the lungs after infection with influenza virus, it is important for survival after infection. Inflammation caused by adenoviral vectors *in vivo* is mediated by IL-1α upon interaction of the viral RGD motif with macrophage β3 integrins, and increased by virus-mediated endosome rupture [80]. All together, these reports do not provide a comprehensive picture of the role of IL-1 in the response to viral infection. Different viral properties may alter the effect that the IL-1 response has. Viral genomes differ in length and segmentation, some are DNA while others are RNA, replication occurs at different rates, cellular receptor attachment and tissue tropism vary from virus to virus, and some are

enveloped while others are enclosed by a capsid. The different mechanisms by which each virus interacts with the host can have an effect on the host response to infection.

Ichinohe *et al* reported defective adaptive immune responses to influenza infection upon disruption of the NLR3 inflammasome. Nasal IgA was significantly decreased in ASC-/-, caspase-1-/-, and IL-1R-/- mice, IgG2c was reduced in ASC-/-, caspase-1-/-, and IL-1R-/- mice, and the IgG3 response was reduced in caspase-1-/- mice. The $CD4^+$ and $CD8^+$ T cell response was reduced in caspase-1-/-, ASC-/-, and IL-1R-/- mice [78]. At the same time, Allen *et al* and Thomas *et al* reported that there was no impact on the generation of an adaptive immune response to influenza infection. There were no differences in antibody secreting cells for IgM, IgG, and IgA in caspase-1-/- mice and the $CD8^+$ T cell response to four dominant epitopes was comparable with WT mice [76]. Allen *et al* did not look in detail at the adaptive response but IFN_γ production was not altered in ASC-/- mice [77]. There were slight differences in the preparation of the influenza virus A/PR/8/34, with Allen *et al* using 0.1% alum along with the virus. Alum has been shown to activate the NLRP3 inflammasome [81], the same inflammasome activated by influenza, thus increasing formation and activity of the inflammasome in the mice, and in turn could boost the generation of the immune response. The challenge dose used to infect the mice by Ichinohe *et al* was much lower than that used by the other two groups, which may explain the difference in results because it might not have been sufficient to activate a more significant response.

1.9 Aims and Hypothesis

The main goal of this project was to identify a strategy by which rVSV-based vaccine vector reactogenicity could be reduced without compromising immunogenicity. Specifically, we aimed to determine: (1) if vector-associated pathology is reduced in mice defective in the production of or response to IL-1β; (2) if the adaptive immune response to rVSV is affected by disruption of the production of or response to IL-1β; (3) if mice defective in the production of or response to IL-1β develop protective immunity after immunization. VSV is a good candidate for a vaccine vector due to its low prevalence of natural infection in humans [7] and its ability to elicit potent systemic and mucosal antibody and cytotoxic T lymphocyte (CTL) responses [8]. The main hurdle to its use as a vaccine vector in humans is its potential reactogenicity, causing fever, chills, and myalgia in infected humans [14-16[3]. Current strategies to address this, such as genome scrambling, can result in reduction of immunogenicity [24] and reduce the ability to produce the high titers of virus *in vitro* that would be necessary to vaccine production [23]. Targeting the host response to rVSV as opposed to the vector itself presents a strategy that could reduce reactogenicity without affecting the production and immunogenicity of rVSV vaccines.

2. Materials and Methods

2.1 Viruses

Vesicular stomatitis virus (Indiana strain) was obtained from Dr. John Rose (Yale University). Virus was propagated in BHK cells and titered using a standard plaque assay.

2.2 Inoculation of mice

Eight to ten-week-old C57BL/6 mice of the indicated genotypes were either obtained from Jackson Laboratories or bred in our animal facility. Mice obtained commercially were housed for at least 1 week before experiments were initiated. Mice were housed in microisolator cages in a biosafety level 2-equipped animal facility. Viral stocks were diluted to appropriate titers in serum-free DMEM. For intramuscular immunization (i.m.), mice were injected with the indicated amount of virus(es) in 50µl total volume. For intranasal (i.n.) vaccination, mice were lightly anesthetized with isoflurane using a vaporizer and administered the indicated amount of virus in 30µl total volume. The Institutional Animal Care and Use Committee of Duke University approved all animal experiments.

2.3 Determination of viral titers by plaque assay

Mice were euthanized via anesthetic overdose and organs removed aseptically. After dissection organs were weighed, and homogenized in sterile buffer (100µl buffer

per 0.1g organ weight). Homogenates were titered by standard plaque assay on BHK-21 cells using a semi-solid overlay to detect infectious VSV. After 48 hours the overlay was removed and the cell layer stained with crystal violet to visualize plaques.

2.4 IL-1β ELISA

Mice were bled and then euthanized via anesthetic overdose and organs removed aseptically. Organs were homogenized in 500μL (100μL for lymph nodes) of buffer containing 137mM NaCl, 20mM Tris-Cl pH 8.0, 5mM EDTA, 0.05% Triton-X 100, and protease inhibitor cocktail (Roche). Serum and organ homogenate supernatants were assayed for IL-1β by ELISA (R&D Systems, SMLB00B). Organ IL-1β amounts were normalized to the amount of protein in the samples, as determined by a Bradford protein assay (Thermo Scientific).

2.5 Assay for neutralizing antibody against VSV

Blood was obtained from mice on days 7, 14, and 28 after vaccination via cheek bleed. Heat inactivated serum was diluted in serum-free DMEM such that the final dilution in the first well of a 96-well plate was 1:10 for day 7 samples and 1:100 for day 14 and 28 samples, with subsequent two-fold dilutions. Samples were assayed in duplicate. 100 PFU of rVSV diluted in serum-free DMEM was added to each well and incubated for one hour at 37°C-5%CO_2, after which 4,000 BHK-21 cells diluted in 5%FBS-DMEM were added to each well. Plates were incubated at 37°C-5%CO_2 for three days,

and cytopathic effect was observed. The neutralizing titer was defined as the highest dilution of serum that gave 100% neutralization of rVSV.

2.6 Tetramer assay

To obtain peripheral blood lymphocytes blood was collected into serum free medium (DMEM) containing heparin. Blood was layered onto a Ficoll gradient and spun, after which lymphocytes were collected from the interface. Cells were washed and resuspended in DMEM containing 5% FCS. Staining was performed on freshly isolated lymphocytes as previously described [82]. Briefly, approximately $5x10^6$ cells were added to the wells of a 96-well V-bottom plate and were blocked with unconjugated streptavidin (Molecular Probes) and F_c block (Pharmingen) for 15 min at room temperature (RT). Following a 5-min centrifugation at 500 x *g*, lymphocytes were labeled with a FITC-conjugated anti-CD62L antibody, (Pharmingen), an allophycocyanin-conjugated anti-CD8 antibody (Pharmingen), and tetramer for 30 min at RT. The tetramer was a PE-conjugated major histocompatibility complex (MHC) class I K^b tetramer (NIH Tetramer Facility) containing the H-$2K^b$ restricted peptide VSV N_{53-59} (N-RGYVYQGL-C). Sham-inoculated control animals were used to determine background levels of tetramer binding. Background was routinely less than 0.1% and was subtracted from all reported percentages.

3. Results

3.1 Mice deficient in the interleukin-1 receptor are protected from weight loss after rVSV immunization while mice deficient in ASC, caspase-1, and TNF-α are only partially protected

Mice immunized with rVSV lose up to 20% of their pre-immunization body weight with maximal weight loss usually occurring on the second day, and return to normal weight by five to seven days after administration [1, 2]. It has previously been published that the pro-inflammatory cytokine, TNF-α, contributes to weight loss after intranasal immunization with rVSV [1]. To determine if TNF-α also contributes to weight loss after intramuscular immunization, WT and TNF-α-/- mice were immunized with a single intramuscular dose of $5x10^8$ PFU of rVSV. TNF-/- mice lost significantly less weight than WT mice on the second through fourth day after immunization (Fig. 1A, p=0.0008 on day two), but did not seem to be as protected from weight loss as in the intranasal model.

IL-1β is a pro-inflammatory known to cause acute weight loss and fever in mice and humans [30-32]. IL-1β is produced as a precursor molecule, pro-IL-1β, which is processed to mature IL-1β by caspase-1 upon activation of the inflammasome, which contains the adaptor molecule ASC [49, 50]. Extracellular non-caspase-1 cleavage of IL-1β also occurs by proteinase-3, which is a common neutrophil protease, granzyme A, elastase , matrix metalloprotease 9, and a mast cell chymase [49-52]. We predict that

blocking generation of or response to IL-1β will reduce pathology after rVSV administration without affecting immunogenicity of the vector.

To determine if IL-1 contributes to the acute pathology observed after rVSV administration, C57BL/6 WT and IL-1 receptor-1 deficient mice (IL-1R-/-) were immunized intramuscularly with a single dose of $5x10^8$ PFU of VSV WT and monitored for weight loss. WT mice (n=4) lost 10% of their initial weight and did not return to their pre-immunization weight until eight days after immunization. IL-1R1-/- mice (n=4) lost only 5% of their body in the first day after immunization with return to initial weight occurring on the second day (Fig. 1B). This result was consistent in two separate experiments. In additional experiments, mice deficient in either ASC or caspase-1, which are both components of the inflammasome, were used to determine if inflammasome processing of IL-1β contributed to pathology. Both ASC-/- and Casp1-/- mice were only partially protected from acute weight loss after immunization, compared to the protection observed in IL-1R-/- mice. ASC-/- mice (n=6) lost ~7% of their body weight, compared to WT mice (n=10) which lost over 10% of their body weight in the first day after immunization (Fig. 1C, $p<0.05$ comparing weight by unpaired t-test on the first two days). Casp1-/- mice (n=5) lost over 10% of their body weight by the second day after immunization, while WT mice lost over 15% (n=5, $p=0.0011$) (Fig. 1D). These results were consistent across four separate experiments with ASC-/- mice and two with Casp1-/- mice. In an experiment with WT (n=5), IL-1R-/-(n=4), and ASC-/- mice (n=5), IL-

1R-/- mice lost significantly less weight in the first two days after immunization than both ASC-/- ($p<0.005$) and WT mice ($p<0.0001$) (Fig. 1E), indicating that lack of IL-1 signaling is more protective than disruption of the inflammasome.

3.2 Mice deficient in IL-1R, ASC, or Caspase-1 are able to control viral replication

To determine if mice with a defective inflammasome or response to IL-1β were able to control rVSV replication, quadriceps were harvested 24 hours after a single intramuscular dose of $5x10^8$ PFU rVSV in order to measure viral loads. 24 hours is the timepoint at which VSV replication *in vivo* reaches its peak. There were no significant differences in viral load for IL-1R-/- (Fig. 2A), ASC-/- (Fig. 2B), or Casp1-/- mice (Fig. 2C) as compared to WT mice, and there was no infectious virus found in the blood of the same mice, which supports the hypothesis that IL-1β production/signaling is not necessary for control of rVSV replication *in vivo*.

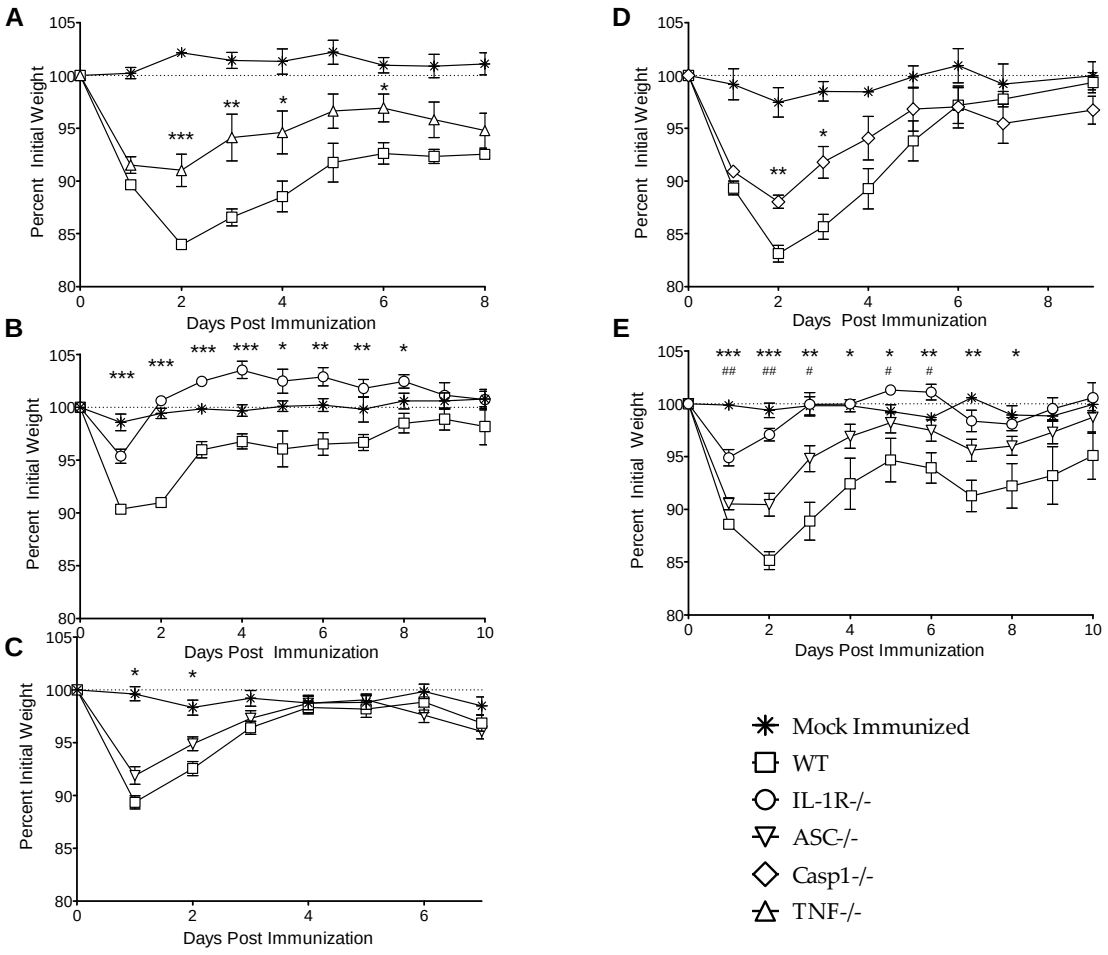

Figure 1: Mice were immunized with a single IM dose of $5x10^8$ PFU VSV in the rear quadriceps and weighed daily. A. TNF-/- mice (n=5) lost significantly less weight than WT mice (n=6). B. IL-1R-/- mice lost significantly less weight than WT mice (n=4) in the first eight days after immunization C. ASC-/- mice (n=6) lost significantly less weight than WT mice (n=10) in the first two days after immunization D. Casp1-/- mice lost significantly less weight than WT mice in the first two days after immunization (n=5) E. IL-1R-/- mice (n=4) lost significantly less weight than WT and ASC-/- mice (n=5) Graphs show average percent of initial weight ± SEM. Unpaired t-test used for statistics. *p<0.05 **p<0.01 ***p<0.001 for comparison to WT mice. #p<0.05 ##p<0.01 IL-1R-/- mice compared to ASC-/- mice.

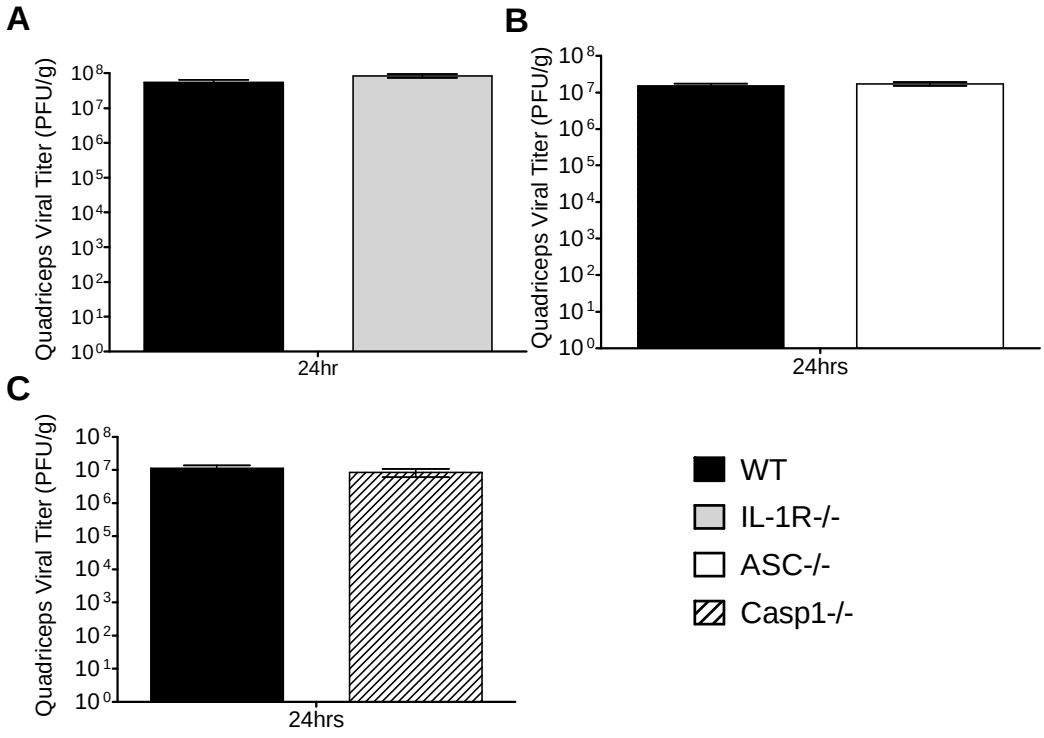

Figure 2: Mice were immunized with a single IM dose of $5x10^8$ PFU VSV in the rear quadriceps. Mice were euthanized after 24 hours and quadriceps homogenized and assayed for viral titer by standard plaque assay. A. WT and IL-1R-/- mice (n=3) B. WT and ASC-/- mice (n=4) C. WT and Casp1-/- mice (n=3). Graphs show average ± SEM. There were no significant differences found using an unpaired t-test.

3.3 IL-1β is production is not significantly different in ASC-/- and Casp1-/- mice compared to WT mice

An ELISA assay was used to determine if IL-1β was being produced after immunization and if its production was dependent upon ASC and/or caspase-1, which are both components of the inflammasome. IL-1β was detected locally in the quadriceps muscle at the site of injection and also in the draining popliteal and inguinal lymph nodes. Systemic production of IL-1β was found in the serum of the same mice. IL-1β production was not significantly disrupted in ASC-/- mice compared to WT mice at 12 and 24 hours after immunization (Fig. 3A). In Casp1-/- mice, IL-1β production was not significantly different in the draining lymph nodes ($p=0.237$) or the quadriceps compared to WT mice (Fig. 3B).

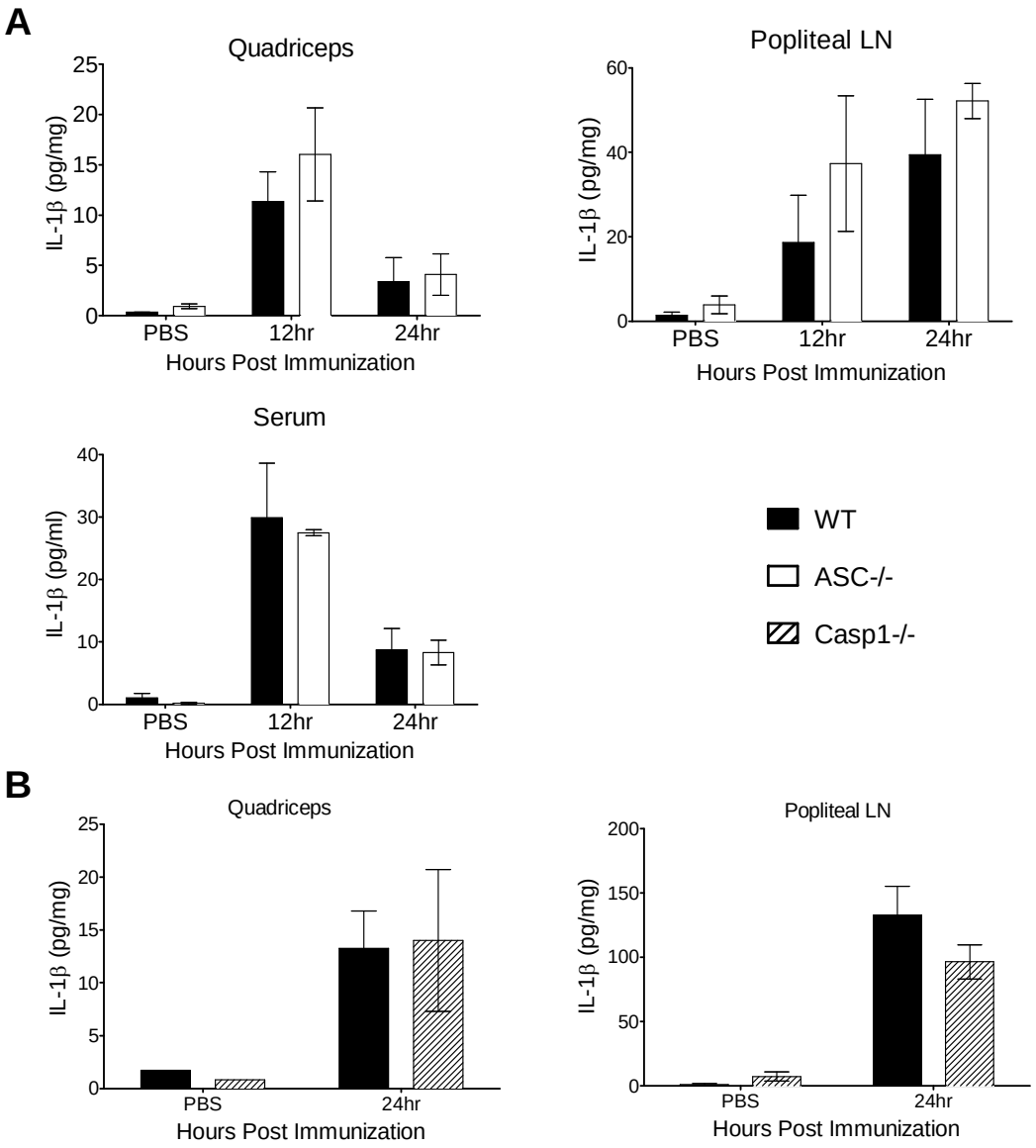

Figure 3: Mice were immunized with an IM dose of $5x10^8$ PFU VSV in each rear quadriceps. Mice were sacrificed and organs harvested at 12 and/or 24 hours after immunization. Organs for IL-1β ELISA were homogenized in a buffer of 137mM NaCl, 20mM Tris-Cl pH 8.0, 5mM EDTA, 0.05% Triton-X 100, and protease inhibitor cocktail (Roche). IL-1β levels were determined by ELISA (R&D Systems) and normalized to protein content as determined by a Bradford Protein Assay (Thermo Scientific). A. IL-1β levels in the quadriceps, lymph node, and serum were not significantly different between ASC-/- and WT mice (n=4) at 12 and 24 hours after immunization. B. IL-1β levels were not significantly different in the quadriceps and lymph node of Casp1-/- mice compared to WT (n=3) at 24 hours after immunization. Graphs show average ± SEM. Unpaired t-test used for statistics.

3.4 The cellular and humoral immune responses are normal in mice deficient in the IL-1R, ASC, or Caspase-1

One study has reported that the antibody and CD8+ T cell response in mice deficient in the IL-1 receptor, ASC, or caspase-1 was significantly decreased after influenza A infection [78], while others have reported no impact on the adaptive response [76, 77]. Because of these reports and the fact that the cytokine response to infection is important in the development of the adaptive immune response, neutralizing antibody titers and VSV-N specific CD8+ T cells levels were evaluated in mice deficient in the IL-1 receptor, ASC, or caspase-1. Mice immunized with a single intramuscular dose of $5x10^8$ PFU of rVSV were bled on days 7, 14, and 28 for evaluation of VSV-N specific CD8+ T cells by flow cytometry. There were no significant differences between IL-1R-/-, ASC-/-, and Casp1-/- mice compared to WT mice, except for day 14 after immunization (Fig. 3A-C). The difference returned to non-significance by day 28 after immunization, except in the case of caspase-1-/- mice. Although statistically the differences are significant, there is probably no biological significance.

Mice were also bled for serum collection on days 7, 14, 28, and 52. The heat inactivated serum was then serially diluted for a microneutralization assay to determine the titer of neutralizing antibody to rVSV. There was no significant difference in neutralizing antibody titers of IL-1R-/-, ASC-/-, and Casp1-/- mice compared to WT mice, and there was no significant difference between IL-1R-/- and ASC-/- mice (Fig 3D-E).

This result indicates that IL-1β signaling is not important for serum neutralizing antibody production after infection with rVSV.

3.5 Mice deficient in IL-1R, ASC, or Caspase-1 are protected from a high dose re-challenge

Although the antibody and CD8+ T cell responses were not significantly altered by a disrupted inflammasome or lack of IL-1 signaling, a high dose secondary challenge with rVSV was done eight weeks after immunization to determine if the primary response was protective. Mice were immunized intranasally with a single dose of $1x10^8$ PFU of rVSV. Previously immunized IL-1R-/- (Fig. 5A), ASC-/- (Fig. 5B), Casp1-/- (Fig. 5C) and WT mice survived the re-challenge while naïve WT mice lost up to 25% of their initial body weight before recovery and at least one succumbed to infection in each experiment. This result indicates that IL-1 receptor signaling, ASC, and caspase-1 are not required for the development of a memory immune response to rVSV.

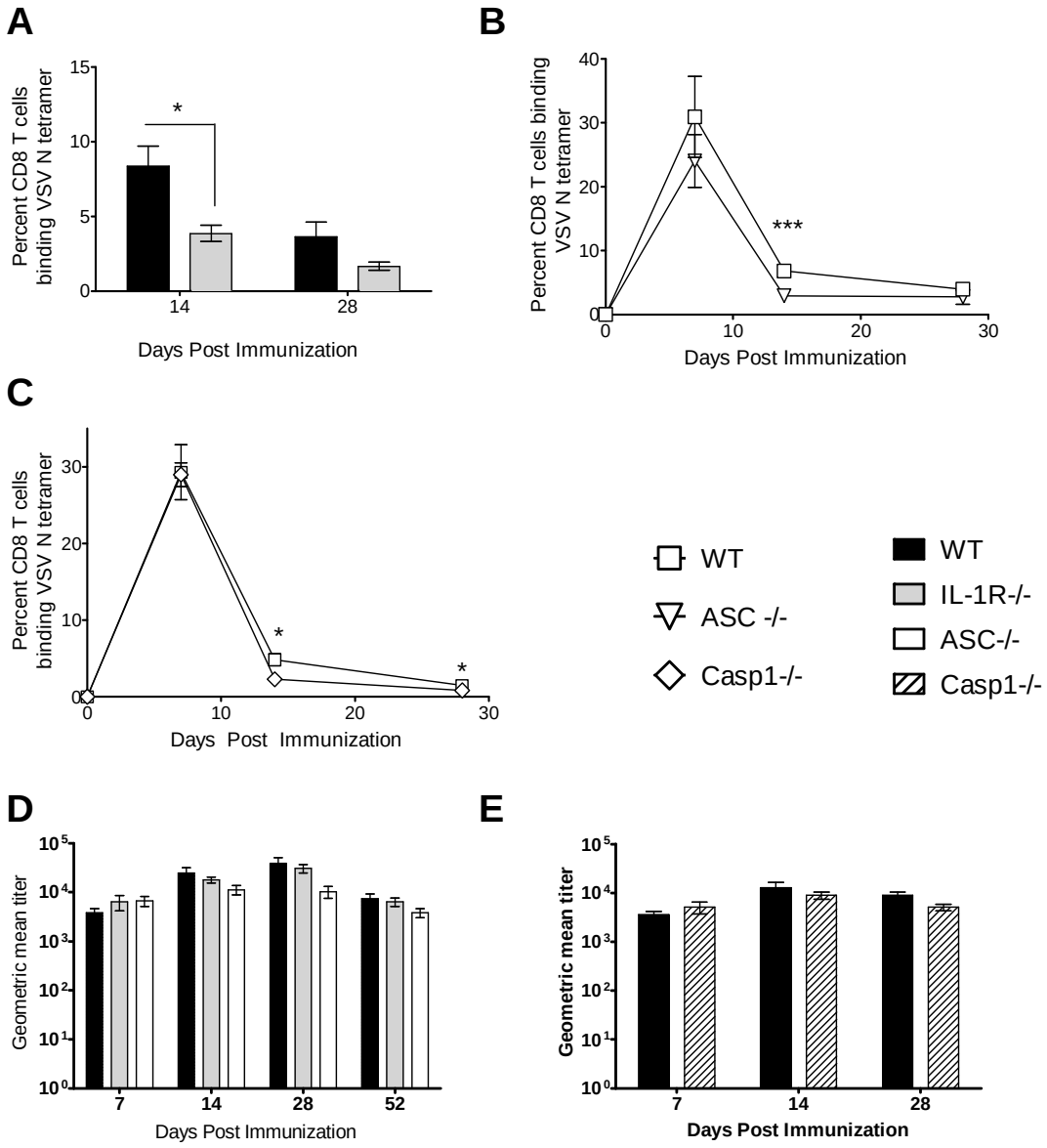

Figure 4: Mice were immunized with a single IM dose of $5x10^8$ PFU VSV in the rear quadriceps. and bled on days 7, 14, 28, and/or 52 after immunization. A-C. Percent of CD8+ T cells specific for the class 1 restricted epitope present in VSV N. IL-1R-/- (n=4), ASC-/- (n=4), and Casp1-/- mice (n=5) had significantly less CD8+ T cells on day 14 after immunization than WT mice (n=5). Graphs show average ± SEM D-E Geometric mean titer of anti-VSV serum neutralizing antibody after immunization. There were no significant differences in antibody titers between IL-1R-/- (n=4), ASC-/- (n=5), and WT mice (n=5) nor between Casp1-/- and WT mice (n=5) after immunization. Graphs show geometric mean titer ± SEM. Unpaired t-test used for statistics *p<0.05 ***p<0.001

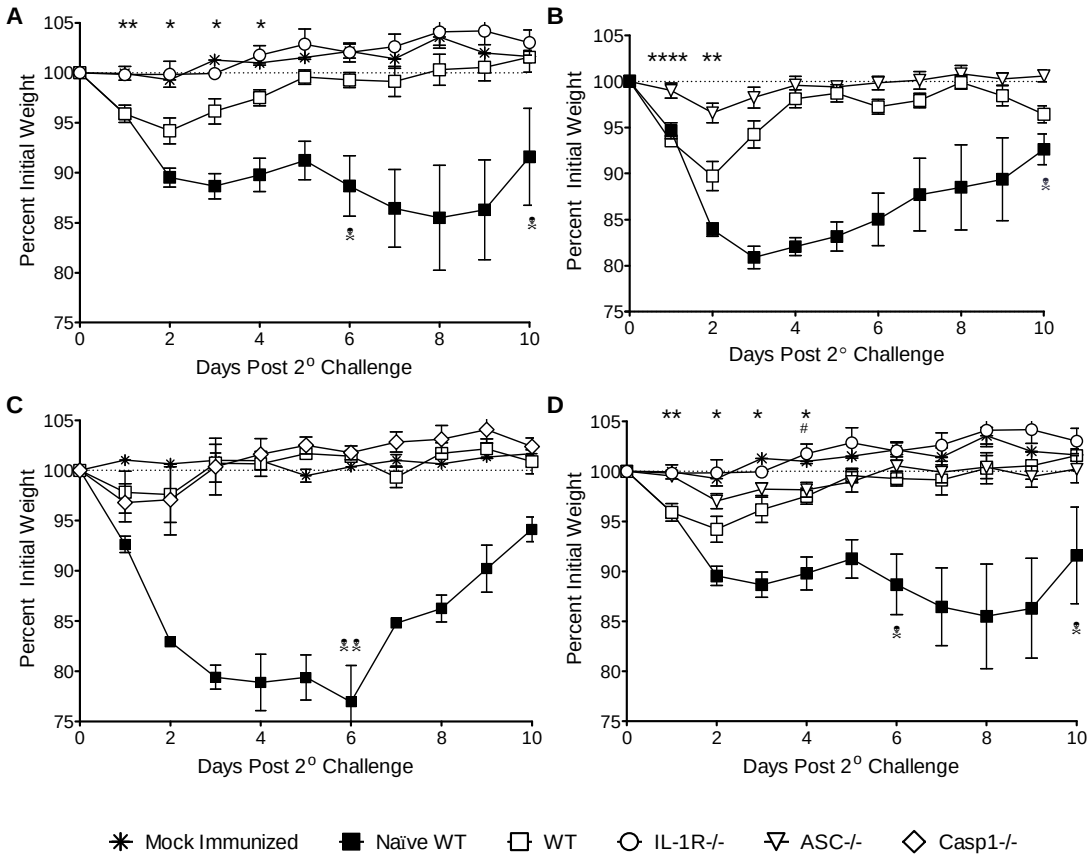

Figure 5: 8 weeks after immunization, mice were challenged with 1x10^8 PFU VSV intranasally. Mice were monitored daily for weight loss. A. IL-1R-/- mice (n=4) lost significantly less weight than WT mice (n=5) in the first four days after challenge B. ASC-/- mice (n=6) lost significantly less weight than WT mice (n=10) in the first two days after challenge C. Casp1-/- and WT mice were protected after challenge. D. IL-1R-/-, ASC-/-, and WT mice were protected from a high dose re-challenge Graphs show average percent of initial weight ± SEM. Unpaired t-test used for statistics *p<0.05 **p<0.01 ****p<0.0001 for knockout mice compared to WT; # p<0.05 IL-1R-/- compared to ASC-/-; .

4. Discussion

The purpose of this project was to identify which cytokines contribute to rVSV-induced pathology after intramuscular immunization. VSV is a good candidate for a vaccine vector due to its low prevalence of natural infection in humans [7] and its ability to elicit potent systemic and mucosal antibody and cytotoxic T lymphocyte (CTL) responses [8]. The main hurdle to its use as a vaccine vector in humans is its potential reactogenicity. The one human to date receiving a single cycle non-replicating experimental rVSV vaccine developed a headache, fever, and muscle pain within 12 hours [3]. Current strategies to address this, such as genome scrambling, can result in reduction of immunogenicity [24] and reduce the ability to produce the high titers of virus *in vitro* that would be necessary to vaccine production [23]. Targeting the host response to rVSV as opposed to the vector itself presents a strategy that could reduce reactogenicity without affecting the production and immunogenicity of rVSV vaccines.

It has previously been reported that TNF-α contributes to rVSV induced pathology after intranasal immunization, with TNF-α-/- mice losing less than 5% of their pre-infection body weight in the first few days after immunization compared to 10% for WT mice [1]. For use as a vaccine vector, it is unlikely that the intranasal route will be used. When administered intranasally, VSV replicates primarily in the olfactory receptor neurons and other cells of the olfactory epithelium and is able to migrate to the brain [14-16]. Strategies to reduce the neurovirulence include scrambling of the genome

and truncations of the envelope protein G, which reduce the ability of rVSV to replicate [23, 83]. Even a slight chance of neurotropic spread makes it unlikely that the intranasal route would be used in people. A safer, more likely route of administration for use in humans is via intramuscular injection.

To determine if TNF-α also contributed to pathology after intramuscular immunization, TNF-α-/- mice were immunized with a single intramuscular dose of $5x10^8$ PFU of rVSV in the rear quadriceps. The mice were partially protected from weight loss, losing up to 10% of their pre-immunization weight compared to 15% for WT mice (Fig. 1A). We also decided to look at IL-1β, which is a pro-inflammatory cytokine known to cause weight loss and fever in mice and humans. IL-1R-/- mice were protected from weight loss after intramuscular immunization, losing only about 5% of their body weight compared to 10% for WT (Fig. 1B), indicating that IL-1 signaling contributes to rVSV-induced pathology. While IL-1β is more commonly associated with the pathologies observed [30-32, 74], it does not exclude IL-1α from also contributing. These results identify also do not exclude some other inflammatory process from contributing to rVSV-induced pathology.

The interleukin-1 receptor has two isoforms, IL-1RI and IL-1RII. IL-1RI contains a long intracellular domain capable of activating signal transduction pathways, while IL-1RII has a truncated intracellular domain and is biologically inert [49]. IL-1RII is present on the cell surface, competing with IL-1RI for ligand binding, and is also secreted,

allowing it to bind IL-1 before it reaches the cell surface [49]. To initiate signaling, IL-1α or IL-1β must bind to IL-1RI and then associate with the IL-1 receptor accessory protein (IL-1RAcP). IL-1α and IL-1β only share 25% amino acid sequence homology, but have similar three dimensional structures [63]. Although both bind and signal through the same receptor, they do not have identical biological activities. The pro-inflammatory cytokine IL-1β has been shown to cause acute weight loss/cachexia and fever in mice [30] and humans [31, 32], activation of the acute phase response [74], and up-regulation of IL-6 along with other inflammatory mediators [84]. It is primarily cleaved by caspase-1 to its active form, but non-caspase-1 cleavage is known to occur [49, 50]. IL-1α is not usually associated with these pathologies. Previous studies have shown that after turpentine injection, IL-1β deficient mice are protected from fever, but not IL-1α deficient mice [30, 63]. Dysregulation of L-1β production, resulting in increased secretion of mature IL-1β, has been shown to be the cause of multiple human autoinflammatory diseases, such as gout [56], Muckle-Wells syndrome [65], and familial cold-induced autoinflammatory syndrome [66]. Autoinflammatory diseases are distinguished from autoimmune diseases by their response to blockade of IL-1β rather than TNF-α, for reduction of symptoms [50].

IL-1α is cleaved by the endogenous protease calpain to its mature form, though its precursor is also biologically active, and it is not generally found in the circulation [64]. IL-1α has been more commonly implicated as a mediator of sterile inflammation

and inflammation due to apoptosis [50, 85], though a recent study has reported that IL-1α is the primary mediator of inflammation *in vivo* after infection with adenovirus [80]. Although we have shown that IL-1β is produced *in vivo* after rVSV immunization, the greatest protection from weight loss was seen in IL-1R-/- mice compared to our experiments with either ASC-/- or Casp1-/- mice. This could indicate that IL-1α plays a role in the inflammatory response to rVSV and/or that pro-IL-1β processing is not dependent upon an ASC inflammasome or caspase-1.

Once we determined that IL-1 contributed to rVSV-induced pathology, it was important to evaluate the effect of suppression of IL-1 signaling on viral replication. There were no significant differences in viral load in the quadriceps (the site of injection), in any of the knockout mice compared to wild-type (Fig. 2), and there was no detectable infectious virus in the serum of the same mice, which indicates that IL-1 is not necessary for control of viral replication *in vivo*. Suppressing IL-1 is therefore unlikely to affect the host's ability to control rVSV replication after intramuscular immunization.

Using rVSV as a vaccine vector, it is important to preserve the immune response after immunization. One previous study reported decreased adaptive immune responses to influenza infection in mice deficient in ASC, caspase-1, or the IL-1R, while two others found either no difference or only evaluated IFN-γ production [76-78]. Our results found no differences in serum neutralizing antibody titers compared to wild type mice for IL-1R-/-, ASC-/-, or Casp1-/- mice. Although there was no difference in the

serum neutralizing antibody titers in our mice, we did not look at specific antibody subtypes as Ichinohe *et al* did. Further experiments could be done using binding ELISAs to determine if there in fact any differences in which subtype of antibodies are produced after rVSV immunization in the IL-1R-/-, ASC-/-, or Casp1-/- mice.

There was a significant difference in the percent of CD8+ T cells which bind to a VSV-N-specific tetramer at day fourteen after immunization, with IL-1R-/-, ASC-/-, and Casp1-/- mice all having decreased percentage relative to WT mice, which was still present at day twenty eight for Casp1-/- mice. This was consistent with Ichinohe *et al*, which found significantly decreased anti-influenza CD8+ T cell responses in IL-1R-/-, ASC-/-, and Casp1-/- mice at day 14 after infection [78]. Although this difference was statistically significant, there is most likely no biological significance. All our knockout mice were protected from a high dose intranasal challenge eight weeks after immunization, with IL-1R-/- and ASC-/- mice losing significantly less weight than WT mice in the first two days after challenge (Fig. 5). These results demonstrate that IL-1 signaling is not required for the generation of a protective immune response to rVSV after intramuscular immunization.

An important next step would be to address what effect suppressing the IL-1 response to rVSV would have on the generation of an immune response to a foreign antigen. It has previously been reported that the relative magnitude of response to rVSV antigens correlates to the relative magnitude of response to a foreign antigen expressed

by rVSV [86]. Our results have shown that there is a robust immune response to rVSV in IL-1R-/- mice and we do not foresee a diminished response to a foreign antigen expressed by rVSV, but it would be necessary to confirm this with further experiments.

To determine a suitable target to reduce reactogenicity of rVSV vectors, it was important to determine the mechanism by which active IL-1β is produced after immunization. There have been conflicting reports of inflammasome formation and active IL-1β production after VSV infection. Muruve *et al* were unable to confirm inflammasome activation and production of mature IL-1β after *in vitro* infection with rVSV, though detailed methods were not provided [60]. Two other recent studies confirmed production of IL-1β after rVSV infection of BMDCs *in vitro.* Poeck *et al* detected IL-1β production *in vitro* and found that it was dependent upon ASC and caspase-1, but not NLRP3. They proposed a model where RIG-I acts as a sensor for inflammasome activation, forming a complex with ASC to activate caspase-1 and IL-1β production [61]. This contrasts with the results of Rajan *et al*, which found that ASC, caspase-1, and NLRP3 were necessary for IL-1β production *in vitro* after infection with rVSV, but RIG-I was not [62]. Differences in experimental techniques may explain the discrepancies between the studies. Different media was used to culture the cells, the ELISA kits used may have differed because the one used Poeck *et al* was not specifically stated, and the multiplicity of infection (MOI) might not have been the same; Rajan *et al* infected cells at an MOI of 5 [62], but Poeck *et al* only states range of 5-10 [61]. The fact

that Rajan *et al* also stimulated the cells with a TLR2 agonist prior to viral infection, while Poeck *et al* did not, may explain the discrepancy.

In contrast to these previous reports, our results demonstrated that IL-1β was produced *in vivo* both locally at the site of injection and draining lymph nodes and systemically in the blood after immunization with rVSV. A lack of ASC or caspase-1 did not have a significant effect on production of IL-1β in immunized mice, but further experiments with Casp1-/- mice could determine whether or not there is a significant difference in IL-1β production in the draining lymph nodes. In two experiments, one showed a significant decrease in IL-1β, though the background IL-1β levels in PBS injected mice were high, while the other did not. It is important to note that the instruction manual for our ELISA kit states that it detects, though considerably underestimates, pro-IL-1β. A Western blot would help to determine if pro-IL-1β was detected in our samples in addition to mature IL-1β, which would affect our results.

The disagreement between our results and the *in vitro* results of previous studies could be due to the complex interactions found *in vivo* that are not replicated in cell culture. Inflammasome formation and caspase-1 processing may not be the primary source of IL-1β production in response to rVSV *in vivo* and/or it may be dependent upon which cell types are secreting active IL-1β. To determine if caspase-1 processing occurs primarily in hematopoietic and/or stromal cells after rVSV immunization, chimeric mice could be generated such that WT mice with Casp1-/- bone marrow, and Casp1-/- mice

with WT bone marrow would be used in experiments. These chimeric mice could then be immunized with rVSV and then evaluated for production of mature IL-1β as in our previous experiments. Neutrophils are a source of extracellular pro-IL-1β release and cleavage and histology could help to determine if there is infiltration at the site of injection [51].

Future plans would be to engineer an rVSV vector capable of reducing its own reactogenicity, of which IL-1 is a causative agent. This allows for targeting of cells directly infected with VSV or cells within the locally infected area, as opposed to a systemically administered agent. ASC and caspase-1 do not seem to be suitable targets for suppression due to the fact that they do not appear to play a significant role in IL-1β production after rVSV immunization. Mice deficient in either ASC or caspase-1 were also not protected from weight loss to the same extent that IL-1R-/- mice were. Engineering an rVSV that suppresses either of these components of the inflammasome will most likely not have much of an effect on the reactogenicity of the vector, based on our results. Targeting the biological response to IL-1 itself may be more effective.

The endogenous soluble IL-1 receptor antagonist (IL-1Ra) binds to the IL-1R and does not allow binding of the IL-1R accessory protein (IL-1RAcP), which is necessary for signaling. This would block the effects of both IL-1β and IL-1α. The B15R gene of the Western Reserve strain of Vaccinia encodes a secretory protein with homology to IL-1RII [87]. While endogenous IL-1RII is able to bind all three IL-1 ligands (IL-1α, IL-1β, and

IL-1Ra) [49], the B15R protein has been demonstrated to be specific for binding of IL-1β [87]. Targeting the IL-1R or IL-1β specifically would also allow for determination of the effect, if any, of IL-1α after VSV infection.

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