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EDUCATION

- 2006-Feb 2012 **Ph.D., Molecular Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD**
Thesis: Pathogenic Old World Arenaviruses Attenuate TLR2-Dependent Pro-Inflammatory Cytokine Response
- 2000 **B.S., Biotechnology, Worcester Polytechnic Institute, Worcester, MA**
Concentration in Biochemistry
- Major Qualifying Project, Mentor David S. Adams, Ph.D.
“Cloning and Expression of Salmonella Secreted Virulence Protease, SipB” Characterization of this protease will be used in developing a rapid identification assay for contaminated food products.

RESEARCH EXPERIENCE

- Summer 2007 **Rotation, Department of Surgery, University of Maryland School of Medicine, Baltimore, MD (Mentor: Dr. Donna Farber)**
- *In vivo* immune modulation of T cell homeostasis with anti-thymoglobulin
 - Suppressive effects of anti-CTLA4 treated regulatory T cells
- Fall 2007 **Rotation, Applied Immunology Group, Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, MD, (Mentor: Dr. Marcela Pasetti)**
- Determine IgG and IgA antibody titers and avidity of vaccinated elderly patients for Influenza as part of an ongoing clinical trial
- Fall 2006 **Rotation, Arena Virus Laboratory, Institute of Human Virology, University of Maryland School of Medicine, Baltimore, MD, (Mentor: Dr. Maria Salvato)**
- *In vitro* assays with Lymphocytic Choriomeningitis Virus Armstrong and Cox-2 inhibitors, celecoxib and rofecoxib,

to determine the role of Cox-2 down regulation in virulent infection

2001-2006

Senior Research Associate- Department of Research and Development, EXACT Sciences Corporation, Marlborough, MA

- Conducted research in genomics and DNA diagnostics in the continued development of a stool-based mutation detection assay for colorectal cancer, PreGen Plus™
- Optimized current chemistries to improve sensitivity and specificity and to decrease cost of current assay
- Adapted assay to automated nanoliter thermocycler/liquid handler as means in achieving corporate goal of reducing cost and increasing throughput
- Key team member in development of novel platform for target DNA extraction from heterogeneous background
- Manufactured, maintained, and supplied target DNA binding membranes for use in all ongoing research, projected needs of future validations
- Assisted in management of and conducted studies to validate new chemistries within current assay
- Presented data in written reports and oral presentations

1999-2001

Research Associate, Expressive Constructs, Inc., Worcester, MA

- Identified and cloned target genes from bacteria and mammals, optimized expression and purification in E. coli systems
- Development of bacterial detection systems through molecular biology, enzyme expression and characterization
- Maintained bacterial cell lines and mammalian tissue lines for use in model based *in vitro* assays

1996-1999

Laboratory Assistant, Department of Biology and Biotechnology, Worcester Polytechnic Institute, Worcester, MA

- Prepared media, buffer, and materials; collected and disposed of biohazard and laboratory wastes; collected and cleaned glassware from class labs
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- Developed laboratory safety skills; participated in monthly seminars
- Assisted professors in research, maintenance of laboratory, preparation for class labs

Summer 1998

Laboratory Assistant/Summer Internship, Drosophila Sequencing Department, Lawrence Berkeley National Laboratory, Berkeley, CA

- Created a physical map of the homeotic region of *D.virilis* as the first step in sequencing the genome
- Techniques used included library screening, end sequencing, restriction mapping, and fingerprinting

INSTITUTIONAL LEADERSHIP & SERVICE

University of Maryland School of Medicine

2011	Co-Chair, Graduate Student Presentations Committee
2007-2009	Chair, Qualifying Exam Preparation Committee <ul style="list-style-type: none"> • Organized practice sessions for students preparing to sit for Qualifying exam, provided guidance, advice for study strategy
2008-2009	Treasurer, Graduate Student Association (GSA) <ul style="list-style-type: none"> • Managed financials for association, created budget for 2008-2009, managed review and judging of award applications, organized significant campus events including New Student Orientation, Graduate Research Conference, and Doctoral Hooding Ceremony
2008-2009	Program in Molecular Microbiology and Immunology representative to Graduate Student Association
2007-2009	Editor Microscoop, newsletter for Department of Microbiology and Immunology
2008	Student Committee Member for Molecular Microbiology and Immunology and Graduate Program in Life Sciences sponsored “Careers in Microbiology and Immunology”
2008-2009	Student Representative to Molecular Microbiology and Immunology Faculty meetings

LABORATORY SKILLS

DNA/RNA: Cloning, sequence analysis, purification, PCR, real-time and quantitative PCR, capillary electrophoresis, single-base extension analysis

Protein: SDS-PAGE, Western Blot, column purification

Tissue Culture: Growth and maintenance of mammalian and bacterial cell lines (HeLa, BHK-21, Huh7, Vero, HEK293, THP-1, immortalized BMDM; *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli*), cell viability, stable/transient transfection

Immunology: ELISA, ELISPOT, surface/intracellular staining, MACS separation, 4-color Flow Cytometry

Virology: Expertise with arenaviruses (LCMV, Mopeia, Tacaribe, Junin), growth and maintenance of virus stocks, plaque assay, virus purification,

Animal Handling: retro-orbital bleeding, bronchiolar lavage, intraperitoneal injection, organ harvesting, isolation of lymphoid cells

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Hayes MW, Carrion R, Medvedev AE, Salvato MS, Lukashevich IS. Pathogenic Old World arenaviruses fail to stimulate TLR2-dependent proinflammatory cytokine/chemokine responses *in vitro*. Submitted to Journal of Virology (revised manuscript)

PRESENTATIONS

Oral Data Presentations

Hayes MW, Medvedev AE, Salvato MS, Lukashevich IS. July 17, 2011: ASV 30th Annual Meeting Workshop Session “Pathogenic Old World arenaviruses failed to induce pro-inflammatory cyto/chemokine responses *in vitro*”

Hayes MW, Lukashevich IS. November 1, 2010: Young Scientist Day, “Pathogenic Old World Arenaviruses Attenuate TLR2-Dependent Pro-Inflammatory Cytokine Response”

Hayes MW. October 12, 2010: Student Seminar Series, “Pathogenic Old World Arenaviruses Attenuate TLR2-Dependent Pro-Inflammatory Cytokine Response”

Hayes MW, Zapata JC, Medvedev AE, Salvato MS, Lukashevich IS. July 13, 2009: ASV 28th Annual Meeting Poster Session, “Pathogenic Old World arenaviruses failed to induce pro-inflammatory cyto/chemokine responses *in vitro*”

Immunology Journal Club

October 26, 2010: Immunology Journal Club, “Oxidative stress induces angiogenesis by activating TLR2 with novel endogenous ligands”, XZ West et al. Nature 000, 1-5 (2010)

March 9, 2010: Immunology Journal Club, “Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands”, Barbalat R et al. Nature Immunology 10 (11) 2009

ABSTRACTS

Chen WH, Bowen S, Cross AS, Edelman R, **Hayes M**, Lim Y, Reymann M, Ruslanova I, Wu S, Szein MB, Pasetti MF "Cellular and Humoral Immune Responses of Elderly Adults Who Received a High-dose or Standard-dose Influenza Vaccine" 11th Annual Conference on Vaccine Research, May 5-7, 2008 at the Baltimore Marriott Waterfront Hotel in Baltimore, Maryland; sponsored by the National Foundation for Infectious Disease

Hayes MW, Zapata JC, Medvedev A, Salvato M, Lukashevich I “Lassa virus S RNA-encoded gene products inhibit human TLR2-dependent IL-8 response in HEK293 cells” 28th Annual Meeting for the American Society for Virology, July 11-15, 2009 at The University of British Columbia in Vancouver, British Columbia

Hayes MW, Medvedev A, Salvato M, Lukashevich I “Pathogenic Old World arenaviruses failed to induce pro-inflammatory cyto/chemokine responses *in vitro*” 30th Annual Meeting for the American Society for Virology, July 16-20, 2011 at University of Minnesota, Twin Cities, Minneapolis

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Travel Award Grant, “Lassa virus S RNA-encoded gene products inhibit human TLR2-dependent IL-8 response in HEK293 cells” 28th Annual Meeting for the American Society for Virology, July 11-15, 2009 at The University of British Columbia in Vancouver, British Columbia

Travel Award Grant “Pathogenic Old World arenaviruses failed to induce pro-inflammatory cyto/chemokine responses *in vitro*” 30th Annual Meeting for the American Society for Virology, July 16-20, 2011 at University of Minnesota, Twin Cities, Minneapolis

MEMBERSHIPS

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American Association for the Advancement of Science

2008-present

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American Society for Virology- Sponsored by Dr. Maria Salvato

Title: Pathogenic Old World Arenaviruses inhibit the TLR2-dependent activation of innate immune responses *in vitro*

Melissa Ann Wright Hayes, Doctor of Philosophy, 2012

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Abstract

Lymphocytic choriomeningitis virus (LCMV), the prototype arenavirus, and Lassa virus (LASV), causative agent of Lassa fever (LF), have extensive strain diversity and significant variations in pathogenicity for man and experimental animals. LCMV strain WE, but not the Armstrong (ARM) strain, induces a fatal LF-like disease in rhesus macaques. LASV infection of human macrophages and endothelial cells results in reduced levels of proinflammatory cytokines. LCMV was recently shown to be detected by TLR2, but it was unclear whether this was a general innate sensor mechanism to elicit host defense against arenaviruses with different degrees of pathogenicity. Doing pairwise comparisons of virus strains with different degrees of pathogenicity, we have shown that non-pathogenic, but not pathogenic strains of Old World arenaviruses elicit TLR2/6-dependent proinflammatory responses. In contrast to infection with non-pathogenic

strains, cells infected with LASV or with LCMV-WE demonstrated attenuated TLR2/6-dependent proinflammatory cytokine responses. Immunosuppressive LCMV Clone 13 also failed to stimulate IL-6 in macrophages. In contrast, non-pathogenic Mopeia virus (MOPV), a genetic relative of LASV, and LCMV-ARM, induced robust responses that were TLR2/Mal-dependent, required virus replication, and were enhanced by CD14. LCMV-ARM, but not LCMV-WE or Clone 13 strains, induced activation of NF κ B, and super-infection experiments revealed that the WE strain inhibited the ARM-mediated IL-8 response during the early stage of infection while allowing exogenous signaling via TLR2. The results suggest that pathogenic arenaviruses suppress NF κ B-mediated proinflammatory cytokine responses in infected cells. Thus, arenavirus-induced TLR2/6-dependent proinflammatory responses are elicited by non-pathogenic strains and the ability to inhibit these responses could contribute to pathogenicity.

**Pathogenic Old World Arenaviruses inhibit the TLR2-dependent activation of
innate immune responses *in vitro***

By
Melissa Ann Wright Hayes

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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Chapter One

Innate Immunity

The innate immune system provides immediate protection against invading pathogens by means of soluble pattern recognition receptors (PRRs) that mediate opsonization and activation of the complement system, phagocytosis and destruction of microbes, initiation of inflammation and priming of the adaptive immune response. The innate immune response is able to exert protection quickly via pathogen recognition by germ-line encoded PRRs. PRRs are able to efficiently discriminate between self and non-self by recognizing conserved pathogen-associated molecular patterns (PAMPs) that are specific to microbes and cannot be easily mutated because of their importance for microbial virulence and survival (285). PAMPs include microbial lipids such as lipopolysaccharide (LPS) from Gram negative bacteria, lipoproteins from Gram positive bacteria, bacterial proteins such as flagellin, and microbial RNA and DNA. PRRs include membrane associated Toll-like receptors (TLRs) expressed on the cell surface (TLR2, TLR4-6, TLR10-11), or intracellular endosomes (TLR3, TLR7-9), cytosolic nucleotide binding and leucine-rich repeat-containing receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs), and sensors of cytoplasmic microbial DNA, e.g. absent in melanoma-2 (AIM-2) (257). PRRs are expressed by non-immune (epithelial, endothelial cells) and immune cells (macrophages, dendritic cells, neutrophils). All cells of a particular type express the same assortment of PRRs and the collection varies between cell types.

In this study, we focus on PRRs that are able to identify viral infections and the responses elicited. Generally, these receptors recognize nucleic acid motifs that differ from the host, though there is some overlap in the patterns recognized, requiring strict regulation of the innate immune response. However, there is a growing body of research in which non-nucleic acid viral components are being recognized by innate PRRs.

Anti-viral Pattern Recognition Receptors

Multiple PRRs are involved in viral sensing, including TLRs, NLR, and RLHs. TLRs, initially identified in *Drosophila*, were subsequently found in a plethora of other species, including invertebrates and vertebrate mammals. All TLRs are composed of an ectodomain containing multiple leucine-rich repeats (LRRs) involved in agonist recognition and coreceptor interactions, a transmembrane region and an intracellular Toll/IL-1 receptor (TIR) domain (40). Each TLR senses a specific set of PAMPs and endogenous “danger” molecules that are normally sequestered in PRR-inaccessible compartments, but become exposed upon cell damage induced by inflammation, infection or stress (166).

PAMP binding, via TLR ectodomain, initiates hetero- or homo-dimerization that brings TIR domains into close proximity, creating a docking platform to enable recruitment of adaptor proteins (42). All TLRs utilize the adaptor protein myeloid differentiation primary response gene (MyD88), except for TLR3 which solely uses TIR-domain-containing adaptor-inducing interferon (TRIF) (375). A bridging adaptor protein, MyD88-adaptor like (Mal), also called TIR domain containing adaptor protein (TIRAP), is required to recruit the signaling adaptor MyD88 to TLR2 and TLR4 (374),

whereas TLR5, TLR7-8 can recruit MyD88 directly (323). The TLR2 or TLR4 elicited MyD88 signaling pathway is initiated from the cell surface via recruitment of Mal/MyD88 to their TIR domain, promoting recruitment of IL-1 receptor-associated kinase (IRAK)-4, IRAK-1, and IRAK-2. IRAK-4 phosphorylation of IRAK1/2, promotes association with a downstream adaptor TNF receptor-associated factor (TRAF)-6 and TGF- β -activated kinase (TAK)-1 (237). TAK-1 activates I κ B kinases and mitogen activated protein kinases that ultimately lead to the activation of transcription factors NF κ B, and AP-1, leading the expression of inflammatory cytokines and upregulation of major histocompatibility complexes (MHC) and costimulatory factors (164).

TLR-ligand binding generates a protective response by changing the environment of the infection through induction of chemokines (to recruit effector cells), and cytokines to activate the infected and nearby cells, upregulation of costimulatory markers, and activation of antigen presenting cells (APC) for efficient induction of the adaptive immune response (266). TLR induction of chemokines acts to recruit effector cells, such as neutrophils and macrophages, which engulf infected cells and then present antigen, as well as establishing the homing signal for activated lymphocytes (34). Induction of cytokines also activates macrophages and DCs, providing T- and B-lymphocyte activation, via up-regulation of MHC and co-stimulatory molecules and secretion of various polarizing cytokines, instructing cells of the immune system for the proper adaptive immune response to clear the infection (266).

TLR3, TLR7, and TLR9 are major inducers of antiviral immunity. Detecting dsRNA, ssRNA, and unmethylated CpG DNA respectively, these TLRs are found on the

endosome with the LRRs located in the endosomal lumen and the TIR domain located in the cytoplasm (237, 345). Thus, these TLRs are best suited for detecting “captured” viruses or viral nucleic acid while they are less likely to recognize viral infections actively replicating in the cytoplasm or nucleus. Nevertheless, TLRs have an important role in the antiviral response. In macrophages, TLR3 is mainly responsible for initiating a pro-inflammatory response to viral infection through activation of the TRIF-dependent signaling pathway resulting in activation of NF κ B and phosphatidylinositol-3 kinase (PI3K)-dependent Akt (375). These transcription factors are responsible for initiating an inflammatory response which can be either beneficial or detrimental to the host. For example, TLR3^{-/-} mice are more susceptible to mouse cytomegalovirus yet they are more resistant to West Nile virus, and influenza virus (184, 342, 367).

TLR7 and TLR9 appear to play a major role in type I interferon (IFN-I) induction in plasmacytoid DCs (pDC) (273). pDCs are robust producers of IFN-I because of the high level of TLR7 and TLR9 expression (130). Mice deficient in TLR7 and TLR9 do not produce IFN-I or proinflammatory cytokines in response to challenge with ssRNA or CpG DNA (392).

Cytosolic PRRs recognizing intracellular pathogens include the RLHs, such as RIG-I and MDA5; the NLRs, such as NOD-1, NOD-2, and NLRP3; and cytosolic sensors of DNA, such as AIM-2, LRR flightless interacting protein (LRRFIP)-1, and IFN-inducible protein (IFI)16 (96, 161, 165). NLRs detect intracellular bacterial infections via recognition of: meso-diaminopemelic acid and muramyl dipeptide, the main constituents of bacterial peptidoglycan, by NOD-1 and NOD-2 respectively; microbial toxins or RNA, including viral RNA from encephalomyocarditis virus and vesicular

stomatitis virus, through NLRP3; cytoplasmic flagellin by NLRC4 or NAIP5; and type 3 secretion systems by NLRP3 and NAIP2 (289). RLHs detect cytoplasmic ssRNA or dsRNA and are viral sensors. The best understood of the RLHs are retinoic acid-inducible gene (RIG)-I and melanoma differentiation-associated gene (MDA) 5. These sensors contain one or two N-terminal caspase-1 activation and recruitment domains (CARD), a DExD/H helicase domain and a C-terminal repressor domain (162, 344). RIG-I detects uncapped 5'-triphosphate ssRNA/dsRNA or short ssRNA from paramyxoviridae, orthomyxoviridae, rhabdoviridae, and flaviviridae while MDA5 detects long dsRNA from picornaviridae and poly inosinic acid-cytidylic acid (I:C) (149, 162). Another sensor in this category includes laboratory of genetics and physiology (LGP) 2 which lacks the CARD domains and acts as a RIG-I/MDA5 regulator (195, 312, 391). Signaling occurs through CARD-CARD interactions with the common adaptor IFN β promoter stimulator (IPS)-1 which lacks the helicase and repressor domain, instead encoding a transmembrane domain (320). The subsequent signaling pathway through the IKK kinases induces proinflammatory cytokines, while signaling via the TRAF3-TBK1-IRF3 pathway results in expression of type I IFN.

While the above mentioned PRRs are widely accepted as viral sensors, another receptor has been identified. TLR2 is typically known as a receptor for Gram positive bacteria, detecting di- or tri-acylated lipopeptides, such as lipoteichoic acid from the bacterial cell wall (317). However, a number of viruses are now linked to TLR2 induction of proinflammatory immune responses, though the exact mechanisms have not been worked out and vary for each virus (see Table 1). These viruses belong to distinct families from the paramyxoviridae, to herpesviridae, to the arenaviridae. Their genomes

vary from complex dsDNA with dozens of open reading frames, to simpler, negative sense, single stranded RNA genomes comprising a handful of genes. All of the viruses linked to TLR2 are enveloped (Table 1).

Virus	Family	Genome	Ligand	Response	References
Varicella-Zoster Virus	Poxviridae	dsDNA	unknown	Proinflammatory cytokines	(365)
Human Cytomegalovirus	Herpesviridae	dsDNA	Envelope glycoproteins B and H	Proinflammatory cytokines	(69), (36)
Herpes simplex virus-1	Herpesviridae	dsDNA	unknown	Proinflammatory cytokines	(359), (4)
Epstein-Barr virus	Herpesviridae	dsDNA	unknown	Proinflammatory cytokines	(119)
Hepatitis C virus	Flaviviridae	+ssRNA	Core protein, Nonstructural 3 protein	Proinflammatory cytokines	(90), (62)
Respiratory syncytial virus	Paramyxoviridae	-ssRNA	unknown	Proinflammatory cytokines, T cell polarizing cytokines	(243), (28), (300), (171)
Measles virus	Paramyxoviridae	-ssRNA	Hemagglutinin protein	Proinflammatory cytokines, upregulation of virus-host cell receptor CD150	(29), (242), (121)
LCMV	Arenaviridae	-ssRNA	unknown	Proinflammatory cytokines	(155), (388), (386), (387), (108), (22), (286)

Table 1: Summary of TLR2-virus interaction. All viruses shown to elicit a TLR2-dependent response are listed, along with virus family, genome type, TLR2 ligand (if known), and the responses elicited.

Ligand recognition initiates TLR2 heterodimerization with either TLR1 (triacylated lipopeptides) or TLR6 (diacylated lipopeptides), which may lend itself to its broad ligand repertoire including bacterial, viral, fungal, parasitic and endogenous ligands (351). TLR2 may also use CD14 or CD36 as co-receptors (251). The interaction of CD14 with TLR2 is ligand independent, but ligand bound TLR2/6-CD14 complexes

move into lipid rafts to enhance signaling (313). TLR2/6 signaling is partially dependent on CD14 (141). In addition to cell surface interactions with TLR-ligand binding, CD14 may also act in intracellular TLR-ligand signaling, as a microbial ligand transporter, and as a signal amplifier (109, 186).

Typically, TLR2-ligand binding initiates hetero- or homo-dimerization, creating docking platforms within the TIR domain to enable recruitment of a series of adaptor molecules, such as Mal (TIRAP), a bridging adaptor required for TLR2, and MyD88, a signaling adaptor. MyD88 recruitment creates a molecular scaffold to recruit IRAK-4 and IRAK-1, leading to activation of the TRAF6-TAK1-MAPK/IKK axis and induction of transcription factors such as NF κ B, to elicit transcription of proinflammatory molecules such as IL-8 (see Figure 1) (163). Recently, TLR2 was shown to induce IFN-I in response to viral ligands from Vaccinia virus and mouse cytomegalovirus from the endosome of inflammatory monocytes (see Figure 2) (18). The TLR2-induced IFN-I response requires TLR2 trafficking to the endosome and leads to activation of interferon regulatory factor (IRF)3 and IRF7.

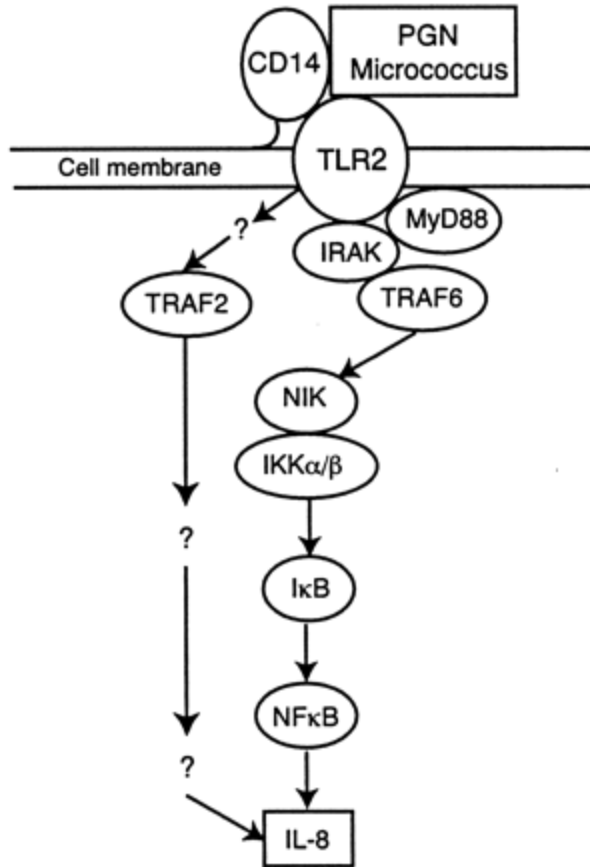


Figure 1: TLR2 Signal Transduction

Signal transduction pathway from bacterial protein stimulation of TLR2 to induction of IL-8 through NFκB. Agonist binding to TLR2, along with co-receptor, TLR1 or TLR6, leads to activation of the NFκB transcription factor and induction of proinflammatory cytokines, such as IL-8. Reprinted with permission from the American Society for Microbiology (366).

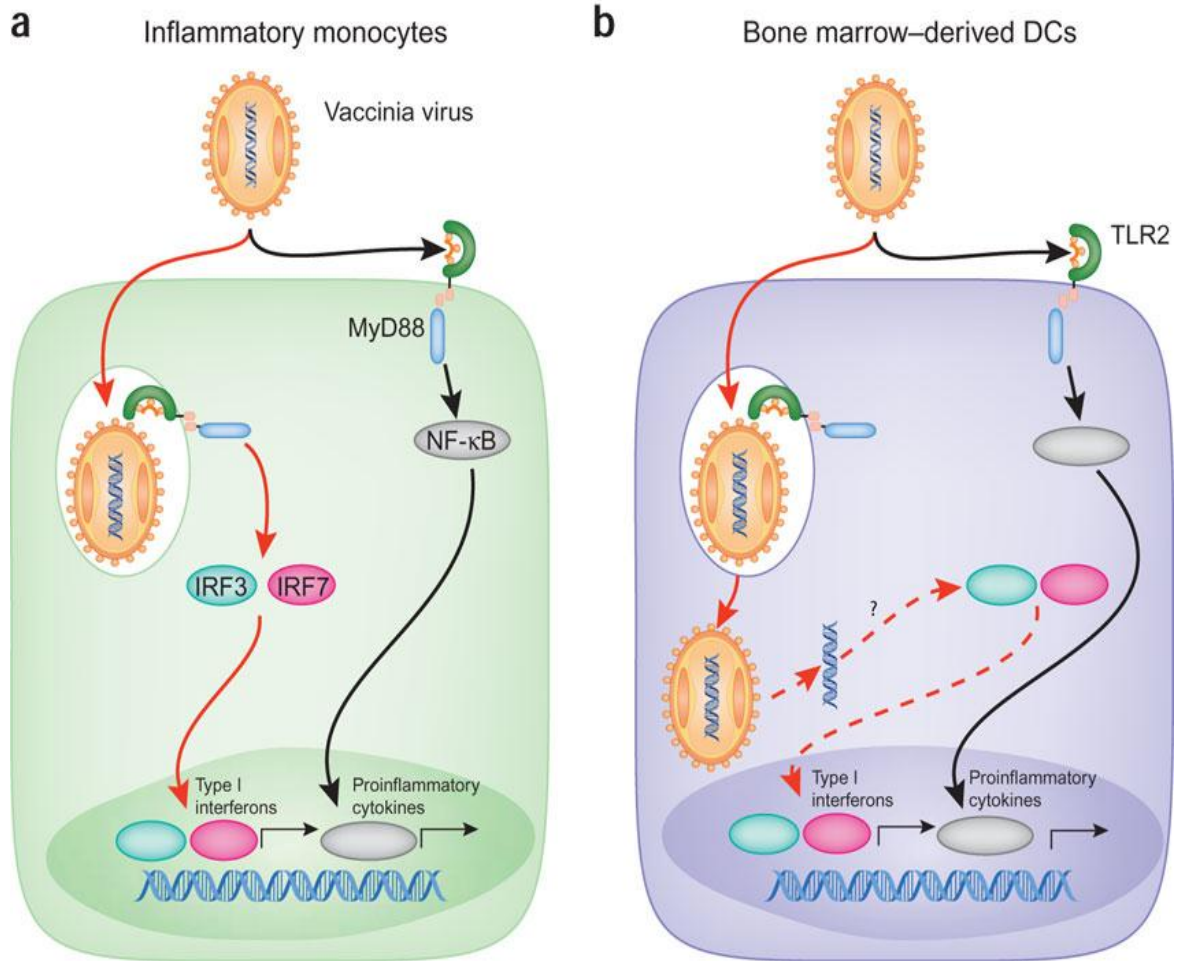


Figure 2: TLR2-induced IFN-I in inflammatory monocytes

Vaccinia virus stimulates activation of NFκB from the plasma membrane to induce proinflammatory cytokines as well as IRF3 and IRF7 from the endosome to induce IFN-I via TLR2 in inflammatory monocytes (a). In bone marrow-derived dendritic cells, Vaccinia virus stimulates TLR2 at the plasma membrane to activate NFκB, but induction of IFN-I is independent of TLR2 (b). Reprinted with permission from Nature Publishing Group (24).

Anti-viral Responses

Upon detection of a viral infection, the host cell immediately initiates a process of creating an antiviral state, both in the infected cell and in neighboring uninfected cells. In the infected cell, mechanisms occur that limit viral replication and release, while the

uninfected nearby cells are alerted to the neighboring infection and are primed to fight off the infection. Ultimately, some cells are lost due to cell death in this process, either as a result of damage from the infection, or as a necessary means of eliminating a source of new virus. These processes are carried out mainly by the production of IFN-I, inflammation, and apoptosis.

Interferons (IFN), identified in the 1950s, are cytokines ranging from 154-208 amino acids in humans and are known for being able to “interfere” with viral replication (146). IFNs are categorized into three groups based on amino acid sequence and receptor usage, type I, type II, and type III. Type I interferons include IFN α , β , ϵ , κ , τ , ω , and ζ , with α and β being the predominant members (107). Within hours of a viral infection, IFN α/β are induced (6, 8, 80, 393). IFN-I then activates a signaling pathway in an autocrine and paracrine manner via IFN- α/β receptor 1 (IFNAR1) and IFNAR2, influencing the response of both infected and uninfected neighboring cells (5). Cytokine-receptor binding leads to the induction of approximately 2000 interferon stimulated genes (ISG) via activation of a JAK/STAT signaling pathway (326). Receptor dimerization, as a result of cytokine binding, results in activation of the constitutively associated Janus kinase (JAK) tyrosine kinases. JAKs then phosphorylate specific tyrosine residues on the receptor, providing a docking platform for signal transducer and activator of transcription (STAT) molecules. STATs are then phosphorylated by JAKs, which then dimerize, leave the receptor complex, bind interferon regulatory factor (IRF)-9 and translocate to the nucleus where they bind the interferon-stimulated response element (ISRE) in the promoter of ISGs (107, 274). Some ISGs have broad activity against many viruses, while others have virus-specific functions or are redundant. Several of the ISGs have been well

characterized, including protein kinase R (PKR), 2',5'-oligoadenylate synthetase (OAS)-directed ribonuclease (RNase) L, and the Mx GTPase. Others are less understood but appear to have significant roles in the antiviral response, including Viperin, ISG15, and ISG20. The roles of these proteins are to limit viral replication and infectious particle release at every step of the virus life cycle from receptor binding and entry, transcription, translation, assembly and budding.

While present at low levels in unstimulated cells, the expression of PKR is induced by IFN-I. PKR is activated by binding to dsRNA, normally of viral origin, dimerization and autophosphorylation. Activated PKR then phosphorylates the translation initiation factor 2 α (eIF2 α), thereby inhibiting both viral and cellular translation (8, 107). PKR is best known for inhibition of RNA viruses and was identified as a mechanism in which rotavirus translation was inhibited (296). Additionally, PKR is activated in the presence of Dengue virus (339), respiratory syncytial virus (193), and influenza (372) to name a few.

2'-5' oligoadenylate synthetases (OAS) are also induced by virus infection and IFN-I stimulation. OAS are latent enzymes that are found cytoplasmically, or can be secreted, and are activated by binding to dsRNA to produce 2'-5' oligoadenylate from AMP (215). Active 2'-5' OAS bind and activate RNase L, leading to degradation of dsRNA, both cellular and viral (8, 107, 172, 173). This degradation protects against infection in two ways. First, it depletes the message and genomic viral RNAs, preventing replication. Second, it increases the level of substrate that is required for activating cytosolic dsRNA sensors, RIG-I and MDA5, increasing the induction of IFN-I and the sensitivity of the cell to infection (215). The requirement for both OAS and PKR to bind

dsRNA for activation provides a level of protection for the cell against non-specific damage. Only in the presence of active virus infection or cellular stress, are OAS and PKR initiated. OAS/RNaseL play a protective role against infection by Dengue virus (192), West Nile virus (311), and Herpes simplex virus-1 (13), while influenza (136, 234) and Vaccinia virus (293) have developed inhibitory mechanisms.

The human myxovirus resistance protein 1 (MxA) GTPase proteins are localized at the cellular and nuclear membrane and are highly similar to the dynamin superfamily of GTPases (137). MxA can self-assemble into highly ordered ring-like structures that form around liposomes, targeting nucleoproteins on MxA-sensitive viruses. The rings may form around nucleoprotein tubules, limiting viral cellular trafficking, and thus preventing efficient assembly of virus particles. The MxA proteins are critical in defending against influenza, measles, and hantavirus infections (138, 159, 316, 390).

Other less understood IFN-I induced antiviral proteins include Viperin, ISG15, and ISG20. Viperin inhibits budding of enveloped viruses as well as disrupts lipid rafts in the cell plasma membrane. ISG15, a ubiquitin-like protein, conjugates to and can stabilize IRF3 expression, a transcription factor critical for IFN-I induction (197). ISG20 is a 3'-5' exonuclease, preferring single stranded RNA, that restricts viral replication by targeting viral RNA directly, a cellular component required for transcription, or by recognizing viral RNA bound to the replication complex (389). Other ISGs are known for activating effector cells, such as macrophages, dendritic cells, and NK cells, as well as initiating maturation of antigen-presenting cells.

Inflammation

Inflammation is an induced innate host response, to recruit phagocytes and other immune cell types to the site of infection, to provide accumulation of soluble PRRs and inflammatory mediators, to activate the complement and clotting cascades for efficient host defense, and to prevent systemic spread of infection. Inflammation provides a physical barrier to prevent the spread of an infectious agent, delivers effector cells to clear the infectious agent or infected cells, and promotes tissue repair. The acute phase response of inflammation is mediated by PRRs, and is the immediate response against infection. Acute phase proteins rapidly increase in concentration and provide protection against pathogens and regulate inflammatory responses (336). Cytokines and chemokines (chemoattractant cytokines) are key mediators in inflammation and are secreted by activated macrophages. These proteins cause changes in the endothelium of the surrounding tissues, such as dilation and increased permeability of blood vessels, upregulation of adhesion molecules on the endothelium resulting in migration of cells into tissues, and recruitment of effector cells such as neutrophils, monocytes, and eventually lymphocytes.

As macrophages and dendritic cells take up pathogens and process them for antigen presentation, these cells are stimulated to secrete cytokines that are effective in promoting cellular infiltration, effector maturation, and damage to the local tissue environment. Some of the classic cytokines produced by these cells are TNF- α , IL-1 α/β , IL-2, IL-4, IL-10, IL-18, IL-12, and IL-8. In addition to inducing inside-out signaling upon binding to its receptors, leading to increased affinity of leukocyte LFA-1 and CR3 for endothelial integrins, IL-8 (CXCL8) acts as a chemokine to recruit neutrophils in

inflammatory loci. Many of these cytokines can also be produced by endothelial cells as well.

The inflammasome is a large protein complex composed of members of the Nod-like receptor (NLR) family. Activation of the inflammasome has been linked with immunity to viral infection. There are several variations of inflammasomes, most that are pathogen specific, but the NLRP3 inflammasome is known to recognize a broad range of agonists: pathogens, such as fungi, bacteria, and viruses; PAMPs, such as bacterial pore-forming toxins, RNA, and rod-like proteins forming type 3 and 4 secretion systems; environmental cues, such as crystals of silica and asbestos; and endogenous “alarmins” such as extracellular ATP, monosodium urate, beta-amyloid protein, and cholesterol crystals (289). NLRP3 inflammasome activation requires two distinct steps: first, TLR or NLR ligand-mediated activation of NF κ B to drive transcription of *Nlrp3*; second, assembly of the NLRP3 inflammasome in response to microbial products or endogenous alarmins (180).

The inflammasome is responsible for activating the proforms of IL-1 β and IL-18 into active forms, via activation of caspase-1 through proximity-mediated cleavage of pro-caspase-1 zymogen. These potent cytokines are induced by binding of a viral ligand with the innate immune receptor and induction of NF κ B, and are often present at basal levels in their inactive forms. Activation of the inflammasome provides the second signal necessary to activate caspase-1 and post-transcriptionally process these cytokines into their mature forms. IL-1 β and IL-18 both play a crucial role in inflammation and instructing the adaptive immune response. IL-1 β contributes by inducing cytokines, adhesion molecules, inflammation and fever, and by activating lymphocytes and

promoting leukocyte infiltration (88, 160, 262). IL-18 lacks the pyrogenic effects of IL-1 β , but induces NK cells and activated T cells to produce IFN- γ and contribute to T cell polarization (122, 255). The two-step regulation of these cytokines is critical given that, while they are instrumental in the antiviral response in clearing virus and protecting against mortality, they also cause significant effects on the host, including fever, vasodilation, hypotension and acute injury to tissue (278, 314).

Several viruses have been associated with activation of the inflammasome. The AIM2 inflammasome recognizes cytosolic dsDNA from vaccinia virus and murine cytomegalovirus (142, 291). RNA viruses have been linked to the NLRP3 inflammasome, including influenza A, encephalomyocarditis virus, and vesicular stomatitis virus (144, 289, 353).

The RIG-I inflammasome, in which RIG-I binds to the adaptor ASC and initiates inflammasome activation via caspase-1, has also been identified to recognize vesicular stomatitis virus, indicating that there may be some overlap in recognition of RNA viruses for the inflammatory response (277).

Ultimately, a final method of clearing a viral infection is to destroy the source of new virus particles, namely, the cells that they infect. This can be mediated either by macrophage engulfment or more extensively by neutrophils, directed cell killing via NK cells, NKT cells or $\gamma\delta$ T cells, or apoptosis. Directed killing of a virus-infected cell by NK cells occurs in two ways. First, NK cells recognize antibody-coated viral antigens via Fc receptors (activation-dependent cytotoxicity, ADCC) (32). Secondly, NK cells are known for recognizing the absence of major histocompatibility complex (MHC) class I receptors. Inhibitory receptors on NK cells recognize MHC I molecules on nearby cells

which prevent them from initiating the killing mechanism. However, when MHC I is down-regulated in response to viral infection, this inhibition is removed and NK cells are able to direct killing of these infected cells (8, 19). Additionally, NK stimulatory receptors can be upregulated in response to viral infection through PRRs (182, 328).

The mechanism of killing commonly utilized by NK cells upon recognition of virally infected cells is either initiated through death receptor pathways or, predominantly through granule exocytosis, namely perforin and granzyme (8, 156, 355). Binding of activating receptors between an NK cell and an infected cell create an immunological synapse, an area of space between the cells that is sealed off from the surrounding tissues (258). Perforin monomers are released into this space which embed into the membrane of the infected cell and oligomerize in a calcium dependent manner, creating pores in the membrane (17). Granzymes, a family of serine proteases, are also released and can traffic through the pores into the infected cell (8). Each member of the granzyme family has a unique cleavage specificity, such as after aromatic amino acid residues (Granzyme H), or after aspartate residues (Granzyme B). Cleavage of cellular substrates by granzymes may activate different effector pathways. Granzymes A, B, C, F, H, K, and M have been shown to induce targeted cell death. In fact, Granzyme B, with its specificity for aspartate residues, is able to cleave pro-caspases to initiate an apoptotic mechanism of cell death and is a major effector mechanism of NK-mediated killing (214). Granzyme A cleaves substrates with consensus sequences at arginine or lysine residues, causing a caspase-independent mechanism of cell death.

As mentioned above, the cleavage of cellular substrates by granzymes can initiate the apoptotic cell death response, however several ISGs have a role in apoptosis as well.

As previously described, IFN-I can up-regulate PKR which becomes activated in the presence of dsRNA. However, *in vitro* studies with functionally non-active or absent PKR demonstrated a resistance to apoptosis indicating that PKR is a pro-apoptotic protein (81, 346, 377). Overexpression of RNaseL in murine fibroblasts induces apoptosis, while cells in which RNase L was knocked down by siRNA were resistant to induced apoptosis (85, 246).

Induction of Adaptive Immunity

Beyond the innate antiviral response, the adaptive antiviral response allows for specific control of a viral infection and ultimately, protection against future infections via immunological memory. While the adaptive immune response provides specific and targeted recognition of pathogens, the type and magnitude of the response is dictated by the innate immune response (225). Recognition of invading pathogens by the innate immune system results in production of a specific cytokine environment, which is responsible for activation of effectors, maturation of antigen-presenting cells (APC), and recruitment of activated lymphocytes to the site of infection. These steps all lead to the ability of the adaptive immune response, mediated by T and B lymphocytes, to mete out the actions that will ultimately rid the host of the infection and establish a long-term memory of the pathogen.

T and B lymphocytes are stimulated by a two signal method involving recognition of antigen peptide-MHC complexes via T or B cell receptors and a second signal through costimulatory molecules. The receptors on lymphocytes recognizing peptide-MHC complexes are randomly generated and clonally distributed. The random generation of

receptors allows for unlimited possibilities in specifically recognizing invading pathogens. The clonal distribution of these receptors increases the possibility that in a lymphocyte pool, one of those lymphocytes will recognize the pathogen. The antigen peptide that is recognized by these receptors is presented in complex with MHC molecules on APC, predominantly dendritic cells. Immature APC recognize a pathogen through innate PRR, process and present that pathogen in the form of peptide-MHC complexes. The type of pathogen determines how it is presented. For example, extracellular pathogens are typically presented in MHC-II, while intracellular pathogens (i.e. viruses, some bacteria) are presented on MHC-I. The MHC determines the type of lymphocyte receptor that will recognize the pathogen. MHC-I-peptide stimulates cytotoxic CD8⁺ T cells, while MHC-II-peptide stimulates helper CD4⁺ T cells. The cytokine environment dictated by the innate immune response enriches this interaction by promoting maturation of monocytes and dendritic cells and up-regulating the expression of MHC in the presence of IFN- α/β , as well as directing the migration of APC and activated lymphocytes in the presence of IL-1 and TNF- α , or chemotaxis in the presence of chemokines (244, 265, 310, 319, 324).

Unregulated activation through MHC-peptide recognition by randomly generated lymphocyte receptors could result in recognition and actions against self-molecules. Thus the activation of the adaptive immune response requires the second signal through costimulatory molecules. Costimulatory molecules are up-regulated on innate effector cells in response to invading pathogen recognized by PRR. Cytokines elicited through this recognition have been shown to potently up-regulate expression of costimulatory molecules. For example, dendritic cells in the presence of IFN- α/β express high levels of

CD83, CD40, CD80, and CD86 (147, 198, 310). Thus, only in the presence of an infection is the second signal delivered to activate the potent adaptive immune response. Additionally, the cytokines elicited during the innate immune response also determine the type of adaptive immune response by directing the maturation of lymphocytes (225). Type 1 cytokines, such as IFN- γ and IL-12, typically produced in response to viral pathogens, induce maturation of CD4⁺ T cells into T_H1 effector cells, enabling a cytotoxic response and contribute to antibody isotype switching to IgG2. Type 2 cytokines, such as IL-4 and IL-10, induce maturation into T_H2 effector cells, inducing more of a humoral response and contribute to isotype switching to IgG1 and IgE.

Immune Evasion Strategies

The many strategies that vertebrates employ to detect and combat viral infections are matched by the number of ways viruses evade, inhibit, or manipulate the immune response. As viruses and vertebrates have evolved together, so have the mechanisms for survival and proliferation. For nearly every step of the antiviral immune response, viruses have evolved a mechanism of evasion, from sensing, to gene expression, to cell death.

The earliest seemingly most effective method of immune evasion is to avoid detection or to shut down the ensuing signaling pathway. Several viruses have developed just such a strategy. For example, the Z protein of New World arenaviruses can bind to RIG-I and prevent subsequent interaction with IPS-1, thus preventing any downstream signaling (103). The NS1 protein of Influenza A and B virus also antagonize detection by RIG-I by interacting with the coil-coil domain of TRIM25, an E3 ubiquitin ligase.

This interaction prevents RIG-I activation and IFN induction by complexing NS1, RIG-I and viral RNA (116, 232, 315). MDA5 is the other best studied cytosolic sensor of the RLH family. Paramyxovirus V proteins bind MDA5 and prevent IFN induction through an unknown mechanism (11, 247, 248). The E5 protein of human and animal papilloma virus interferes with TLR signaling by preventing acidification of endosomes, where binding of CpG motifs to TLR9 occurs optimally at pH of 5 or 6 (301). The E5 protein prevents this acidification and maintains neutral pH where DNA ligands can only weakly bind (334). Several viruses have been shown to inhibit processing of pro-caspase-1 into caspase-1 via the inflammasome, including Kaposi sarcoma-associated herpes virus, Influenza, Baculovirus, and Poxvirus (131, 347). Poxvirus employs a second mechanism in which it prevents assembly of the inflammasome (347).

Another virus employed strategy to down-regulate innate host defense strategy is to shut down signaling at the post-receptor level, whether immediately or by inhibiting the function of signaling molecules. Large DNA viruses have captured host genes and manipulated them for their benefit. Vaccinia virus encodes a protein that is capable of shutting down signaling directly from the sensing molecule. Its A46R protein contains a TIR-domain that is able to suppress signaling from multiple TLRs by interfering with several signaling components downstream, including MyD88, Mal, TRIF, and TRAM, providing for an effective inhibition (60, 331, 332). RNA viruses, however, tend to have much smaller genomes, with many virally encoded proteins having multiple functions. Hepatitis C virus (HCV) encodes a protease, NS3/4a that cleaves the signaling adaptors for TLRs, TRIF, and for RIG-I and MAVS, thus shutting down the ability to induce type I IFN (190, 191).

Several viruses are capable of shutting down the signaling mediators that transmit the signal from detection to antiviral action. Flaviviruses are known for their ability to modulate Jak-STAT signaling, the molecules that carry the amplifying signal from IFN-I/IFNAR1/2. Japanese encephalitis virus NS5 protein blocks Jak-STAT signaling by removing phosphates from protein-tyrosine motifs (191). The NS5 encoded by Tick-borne encephalitis virus can antagonize Jak-STAT signaling by associating with a membrane protein Scribble (27, 183, 368). The NS5 protein of West Nile Virus has also been shown to inhibit Jak-STAT signaling (183). Some viruses are capable of targeting the transcription factors, STAT1 and or STAT2 directly. Dengue virus NS5 blocks phosphorylation of STAT2 (221). Some target the STAT molecules for proteasomal degradation. For example, Simian virus 5 induces proteasomal degradation of STAT1 (86). Paramyxoviridae V protein targets STAT1 molecules for degradation (52, 174). SARS-Coronavirus prevents nuclear translocation of STAT1 via ORF6 by binding to karyopherin $\alpha 2$, a nuclear transport protein (115). RSV targets STAT2 for proteasomal degradation by forming an E3 ligase complex with the cellular proteins, Cul2 and Rbx, and the viral protein NS1 (97). Finally, some viruses have taken advantage of endogenous regulators of Jak-STAT signaling. HCV core protein induces suppressors of cytokine signaling (SOCS) which has been shown to correlate with inhibition of IFN- α induced STAT1 dimerization (92). Influenza, RSV, and HSV also induce SOCS 1 and 3 expression, negatively regulating the Jak-STAT pathway (113, 260, 268).

Ultimately, Jak-STAT signaling leads to transcription factor activation to induce antiviral gene expression. Here, viruses have evolved evasive strategies as well.

Poxvirus encodes two proteins that target NF κ B signaling. The N terminal of CP77 binds

the p65 NF κ B subunit to prevent activation (63). The M013 of poxviruses bind RelA/p65 and prevents localization to the nucleus (152, 288). Additionally, this molecule prevents activation of the ASC-1 inflammasome. HCV NS3/4a cleaves IKK to inactivate IRF3 (209). Pestiviruses, Bovine viral diarrhea virus (BVDV) and CSFV Npro proteins target IRF3 for proteasomal degradation (66, 341). Rotavirus targets IRF3 for degradation via NSP1 (21, 129). HIV-1 Vif and Vif also direct ubiquitination of IRF3 for proteasomal degradation (256). Instead of directed degradation, Rabies P protein and Hantavirus G1 protein prevent phosphorylation of IRF3 (50, 280).

At this stage of antiviral responses, viruses have also evolved to express negative regulators that mimic cellular molecules or upregulate endogenous cellular regulators. African swine fever virus encodes a phosphorylation resistant I κ B homologue that binds NF κ B, thus permanently preventing its activation (343). The P protein of measles virus prematurely upregulates the cellular negative regulator of NF κ B, A20 (378). SUMOylation of IRF 7 is induced by Ebola virus VP35 through interaction with the host E3 ligase protein in a process similar to ubiquitination (64).

Viruses have most extensively acquired the ability to inhibit the expression of cellular genes. RNA viruses generally block antiviral gene expression while DNA viruses modulate their own expression to avoid detection (210, 329). Arena-, phlebo-, and orthobunyaviruses antagonize IFN induction (33, 217-219, 272, 358). Some viruses target nascent cellular transcripts while others interfere with transport of transcripts from the nucleus to the cytoplasm. Coronavirus Nsp1 degrades cellular mRNA through modifications, targeting them for rapid turnover, preventing expression of antiviral genes (157, 158). The Erns glycoprotein of Pestiviruses and Classical Swine Fever Virus

(CSFV) sequesters dsRNA (208, 211). Influenza A virus NS1 interferes with 3'-polyadenylation of nascent cellular mRNA transcripts, while its polymerase cap-snatches the 5'-m7G-cap from nascent transcripts to prime for synthesis of its own viral mRNA (84, 250). Poliovirus and VSV have acquired mechanisms that interfere with transcript trafficking to the cytoplasm. The 3D protein of poliovirus degrades the nuclear pore complex, while the M protein of VSV modifies the nuclear transport channel, both preventing transport of nascent transcripts (99, 124, 134). Other viruses have also taken advantage of endogenous transcriptional regulation. Influenza, TMV, and TEV upregulate p58IPK, a cellular PKR inhibitor (30, 127).

If the antiviral response has been initiated, some viruses are able to shut this response down. Influenza A and B NS1 prevents activation of PKR and OAS by binding to the N-terminal region of PKR and preventing dsRNA binding (136, 189, 235). Some large DNA viruses are able to neutralize secreted mediators through molecular mimicry by encoding "viroceptors" (224, 318). Other viruses are able to shield themselves from detection and ultimate killing of the infected cell or target the host effector cells that would eliminate them. Human cytomegalovirus employs a number of strategies, inducing selective downregulation of MHC I, while preserving the presence of MHC that is unable to elicit an interaction from CD8⁺ T cells and thus is able to avoid detection by NK cells (20). HLA-E MHC I molecules are unable to stimulate an effector response from NK cells. This is used to the advantage of hCMV through its gpUL40 protein which provides the leader sequence for loading into HLA-E that is identical to the leader sequence of classical MHC I (354, 356). Meanwhile, the US6 protein prevents peptide loading by binding to TAP, US2 and UL11 to target HLA-A but not HLA-E for degradation, and

US3 and US10 retain HLA in the ER (279). Remarkably, the US2 and US3 proteins coordinate their efforts for successful down regulation of specific MHC I (252). Human cytomegalovirus also expresses MHC surrogates that bind inhibitory NK receptors (73, 292). Other viruses have been shown to demonstrate similar abilities in modulating the expression of MHC I molecules while some target NK cells directly. HIV-I increases the expression of HLA-E, preventing killing by NK cells (220). Influenza infects and induces apoptosis in NK cells, preventing them from being able to lyse infected cells (133, 216). MicroRNA is also employed by DNA viruses to target cellular transcripts. MICB, a stress induced NKG2D ligand, is targeted by hCMV miR-UL112-1, KSHV miR-K12-7, and EBV miR-BART2-5p to prevent NK killing (245, 333). KSHV microRNA also targets C/EBP β , a transcription factor, resulting in increased production of IL-6 and IL-10, cytokines that inhibit dendritic cell maturation and antigen presentation (68, 282). Some viruses encode inhibitors of effector molecules, such as granzyme B. Adenovirus encodes a specific granzyme B inhibitor, while HCV induces an endogenous granzyme B inhibitor, proteinase inhibitor 9 (9, 338, 370).

In order to proliferate and spread, a virus needs to keep its host cell alive through prevention of directed cytotoxic killing and apoptosis. MicroRNAs from EBV target proapoptotic factors to promote cell proliferation and survival, diverting the cell from the apoptotic pathway (67, 194, 207). The transcriptional regulator, tumor suppressor, and inducer of apoptosis, p53, is a likely target for prevention of apoptosis. Adenoviruses encode early region 1B (E1B) protein which can inhibit p53 (299, 348, 349). KSHV LANA protein can also inhibit p53 and prevent cell death (114).

Recognition of an invading virus by the innate immune system creates an antiviral environment through production of cytokines/chemokines, activation of effector cells, maturation of antigen presenting cells, and ultimately, production of virus-specific antibodies and recruitment of virus-specific T-lymphocytes. In this manner, the innate immune response directs the type and magnitude of the adaptive immune response to clear an infection and generate long term immunity. As vertebrates and viruses have co-evolved, so have viruses developed a myriad of ways to thwart our immune responses. Understanding how a specific pathogen can inhibit the immune response intended to clear an infection can provide a wealth of information in regards to development of therapeutics and vaccines.

Chapter Two

The Old World Arenaviruses

The Arenaviridae family is separated into two groups based on serology, phylogeny, and geography: the Old World group and the New World group (360). The focus of this work is the Old World (OW) Arenavirus group, notably Lassa virus (LASV) and lymphocytic choriomeningitis virus (LCMV). Arenaviruses are single-stranded RNA viruses with an ambisense coding strategy. Their genome is composed of two segments, a long segment and a short segment. Within each segment, two genes are encoded in opposite orientations with a short intergenic region in between, which is predicted to have a stable stem-loop structure. The 3' and 5' 19 nucleotide ends of each segment are complementary to each other and highly conserved. The L segment encodes the RNA-dependent-RNA polymerase (L) and the matrix protein (Z). The S segment encodes structural proteins, the glycoprotein precursor (GPC), which is post-translationally processed into the stable signal peptide (SSP), and the glycoprotein (GP)1 and GP2, and the nucleoprotein (NP). There is considerable variability between arenaviruses within this group, and even between virus strains (360). The nucleotide and amino acid sequence similarity between LASV and LCMV ranges from 50-65% for all four genes. Among strains of LASV, the similarity of nucleotide and amino acid sequence for Z is 74% and 78% respectively and for L is 74% and 81% respectively. The conservation among structural proteins in LASV strains is considerably higher with about 80% similarity for NP and GPC nucleotide sequences and 92% for amino acid sequences. Genetic diversity among LASV strains appears to be more closely related to geographical distances than time and is sorted into four phylogenetic lineages (41).

The polymerase is a large, multifunctional protein (~250kDa) that is predicted to have four distinct domains connected by highly variable sequences (49, 201). L has been shown to interact directly with the matrix protein, Z, with two binding sites, one in the first domain and one in the third domain (369). The polymerase carries out both replication and transcription of the viral genome (229). Beginning at the 3' end of either strand, the polymerase reads through to produce full length genomic or anti-genomic copies. From the 5' end, it reads through an open reading frame, pausing at the intergenic region to produce mRNA. The presence of endonuclease activity and the non-templated sequences at the 5' end of viral mRNA suggest that the first domain carries out a cap snatching function (230, 239, 290). This mechanism has been well described for influenza viruses in which the PA subunit of the influenza polymerase removes caps from cellular mRNA and uses them to prime viral mRNA. (275, 276). The third domain contains highly conserved sequences similar to other RNA-dependent-RNA-polymerases (201, 360). L proteins oligomerize via two sites, one in the N terminus and one the C terminus, allowing homo- and heterodimerization between the domains (49, 309).

Z is a small 11kDa zinc binding Really Interesting New Gene (RING) protein with implications in multiple and sometimes conflicting functions (308). Z protein contains late domain motifs, PPPY in both LCMV and LASV, and PTAP in LASV, responsible for binding to members of the cellular protein sorting pathway and drives virus budding from the cellular membrane (56, 95, 270, 335). The myristoyl modification of Z is required for virus budding as well as for interactions with the viral glycoprotein and efficient homo-oligomerization (56, 271). As mentioned above, Z can interact with L and this binding activity has been mapped to the region surrounding the

zinc-coordination site I, between amino acid residues 37 and 57 (196). Z can also interact with NP, leaving no arenavirus protein untouched by the Z protein (259). Given that Z protein contains the proline rich late domains similar to other matrix proteins and has multiple interactions with viral proteins, one can identify Z as the matrix protein for arenaviruses. Besides its structural functions, Z also has a regulatory role in transcription and replication. The majority of the amino acid sequence in Z comprises the RING motif. This RING motif has been shown to interact directly with eukaryotic translation initiation factor 4E that binds the 7-methyl-guanosine (m^7G) cap and is the rate limiting step in eukaryotic translation (53, 123, 167, 361). Z binds to unique regions on eIF4E leading to conformational changes in the binding pocket for the m^7G cap, potentially antagonizing the ability of eIF4E to initiate transcription (167, 361). This negative regulation affects both host cellular and viral protein production. Z interaction with L has also been implicated in regulating viral RNA transcription and replication. In a New World Arenavirus, Tacaribe, depletion of available Z inhibited genome replication and to a lesser extent, mRNA transcription (118). However, Z was shown not to be required for replication or transcription *in vitro* of LCMV-ARM S segment minigenome, but showed a dose dependent inhibition (71, 72). These conflicting results may be explained in part by the regulation of expression of Z. Z is a structural protein carried into the host cell by the virion (307). Low levels of Z during the early infection may play a role in reducing transcription, thus reducing the recognition of viral infection by cellular sensors. Z is not readily detectable by experimental methods until 24 hours post infection. At this point in the infection, high levels of Z may function to shut down replication and transcription, as well as cellular and viral transcription, but also to carry out its assembly and budding

functions. Z is a highly unique protein, similar to a matrix protein, which acts universally to inhibit transcription.

The glycoprotein precursor (GPC) is synthesized as a single polypeptide that undergoes two cleavage events. The first cleavage event occurs in the endoplasmic reticulum (ER), directed by its stable signal peptide (SSP), carried out by a cellular signal peptidase that releases the 58 amino acid SSP from the precursor (94). Unlike other signal peptides, the SSP is stable and remains associated with the glycoprotein as part of the final glycoprotein complex (93). The SSP is also unique in that it has two transmembrane domains with both the N and C terminus in the cytosol. The SSP functions as a maturation factor even when expressed in trans (93). Post cleavage, the SSP directs the glycoprotein precursor to the Golgi apparatus by masking dibasic ER retrieval motifs (3). The second cleavage event for GPC occurs in the Golgi where the cellular site-1-protease/subtilisin kexin isozyme 1 protease (S1P/SKI-1) cleaves the precursor into GP1 and GP2. The GP1 protein is necessary for binding to α -dystroglycan for all Old World arenaviruses and clade C New World arenaviruses. GP2 is a transmembrane protein, anchoring the complex into the membrane as well as containing the fusion peptide necessary for fusion with the cellular endosomal membrane after uptake. The SSP may also play a role in supporting the pH-induced membrane fusion (379). Together the three components, SSP, GP1, and GP2 form a complex that trimerizes and is the only surface protein found on the arenavirus virion. SSP associates with GP2 through cytoplasmic domain interactions, while GP1 and GP2 are non-covalently associated.

The nucleoprotein (NP) is the most abundant viral protein in infected cells. NP is a structural protein that encapsidates the viral genome and forms the ribonucleoprotein complex along with L. NP also interacts with Z in assembly and budding. NP contains two functional domains, the C terminal domain that interacts with Z and the N terminal domain that oligomerizes with self (188). The C terminus of NP is also known for antagonizing the type I IFN response, though this appears to be non-specific to virulence of the viral strain (218, 219). The critical residues for IFN inhibition, D382 and G385 in the C terminal region of NP are found in the same region that is responsible for binding Z, although the specific residues for each are distinct (217, 259). Surprisingly, additional functions have been identified for NP. Structural data suggests that the C terminus of NP is a 3'-5' exonuclease, similar to those in the DEDD 3'-5' exonuclease superfamily (139, 281). The WT NP in its trimeric or hexameric conformation was able to digest DNA and RNA substrates, confirming this activity (281). This function may also contribute to IFN-I antagonism which seems to be critical in establishing a chronic infection in the rodent host. Structural analysis shows that NP also has a unique cap binding pocket in which the entire m7GpppN structure is inserted through a gate and embedded into the NP domain (281). The protein structure observed is unlike other cap-binding proteins in which only the m7G portion of the cap is bound. Function of this binding pocket was verified using alanine substitutions for residues within the pocket and the gate that are conserved for arenaviruses. These mutations lost transcription ability while the type I IFN antagonism function of NP was unaffected, demonstrating that the cap-binding function is distinct.

Old World arenaviruses, with few exceptions, bind to host cell via α -dystroglycan (α -DG), the peripheral protein, cleaved from dystroglycan that binds the transmembrane β -dystroglycan. α -DG, composed of an N- and C-terminal globular domain and a central mucin-type domain, is critical for assembly of basement membranes (43, 44, 55, 143). Extracellular matrix proteins are the endogenous ligands for α -DG, including laminin, percelan, and agrin (100, 101, 120). α -DG is non-covalently bound to β -DG, which associates with the actin cytoskeleton and signaling mediators, ERK and MEK, among others (61, 330). Extensive posttranslational modification is necessary for α -DG to function properly. Modification by like-acetylglucosaminyltransferase (LARGE), a glycosyltransferase, in the N-terminal part of the mucin-type domain has been shown to be required for binding arenaviruses (176). Interestingly, infection with arenaviruses down-regulates expression of LARGE (297). It is probable that this plays a role in allowing for virus release and spread, similar to cleavage of sialic acid moieties by influenza neuraminidase allowing for virus release (75). Specific strains of OW arenaviruses, LASV, LCMV-WE54 and Clone13 bind α -DG with nearly irreversible high affinity and negligible off rates at neutral pH that is likely followed by receptor internalization (177, 178).

Alternative host cellular receptors have been proposed for LASV and LCMV. Laminin, the endogenous ligand for α -DG, is able to block LASV GP1 binding to α -DG, but it is unable to block LASV entry (176). In mice lacking the enzymes necessary for α -DG modification that enable LCMV entry, replication was similar to WT mice (145). This strong evidence of an alternative cell receptor for OW arenaviruses led to the discovery of four molecules that each enable α -DG-independent LASV entry, Axl and

Tyro3 from the TAM family, and dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) and liver and lymph node sinusoidal endothelial calcium-dependent lectin (LSECtin) from the C-type lectin family (325).

After binding the host cellular receptor, OW arenaviruses employ a unique entry pathway that is cholesterol-dependent, but caveolin-, clathrin-, dynamin-, and lipid-raft-independent (284). However, receptor-mediated endocytosis occurs rapidly, with the virion fusing and releasing its contents within 20 minutes of uptake at low pH, indicating late endosome involvement (284, 298). Recently, arenavirus entry was linked to the multivesicular body and endosomal sorting complex (see Figure 3) (267). This unusual virus entry pathway that requires functional microtubules, phosphatidylinositol-3-kinase (PI3K) activity, components of the endosomal sorting complex required for transport (ESCRT) complex and lysobisphosphatidic acid (LBPA), a lipid involved in formation of intraluminal vesicle may also be utilized for normal degradation of α -DG and thus would be triggered by irreversible ligand binding, such as that with high affinity glycoproteins.

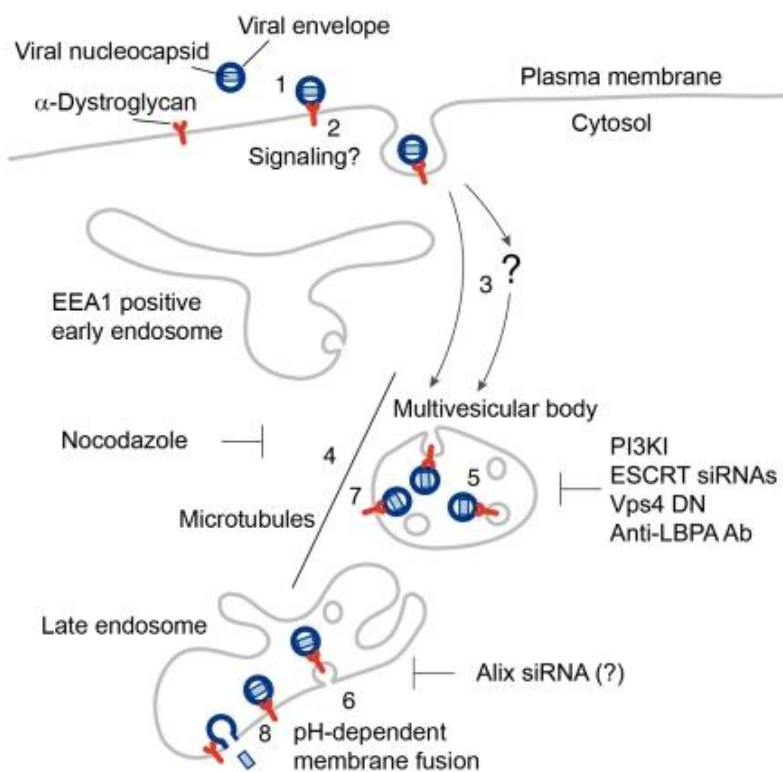


Figure 3: Working model for the cell entry of Old World arenaviruses

1. Old World arenavirus binds to its cellular receptor, α -DG, or Axl, Tyro3, DC-SIGN, or LSECtin. 2. Virus is internalized into smooth vesicles in a manner that is independent of clathrin, caveolin, dynamin, or lipid rafts, but does require cholesterol. 3. Virus-containing vesicles are delivered to the multivesicular body, which is distinct from the early endosomes, via Rab5. 4. Transport to the late endosomes possible requires microtubules, but not Rab7. 5. The ESCRT complex may mediate sorting into the intraluminal vesicles. 6. Intraluminal vesicle-containing receptor-virus complexes fuse with the late endosome. 7. Receptor-virus complexes may also remain associated with multivesicular body membranes. 8. Genomic contents are released to the cytosol when the virus membrane fuses with the endosome in a pH-dependent manner. Reprinted from an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited (267).

Cell entry rapidly directs the invading arenavirus to a late endosome within 20 minutes. Viral membrane fusion with the endosome is low pH dependent, with some strains of arenaviruses having an optimum fusion between pH 3 and 4 (74, 169). Low pH causes the dissociation of GP1, exposing the fusion peptide of GP2 and the

destabilization of GP2, allowing the viral membrane to fuse with the cellular endosomal membrane in a typical Class I fusion protein mechanism (82, 83, 102). Fusion of these membranes creates a pore through which the contents of the virion are then released into the cytoplasm. Inhibition of fusion by treatment with lysosomotropic reagents confirms the requirement for low pH-dependent fusion (125).

Upon release of the genomic material into the cytoplasm, the polymerase starts to transcribe mRNA for NP and L and starts to replicate the full genome by first creating an anti-genome. From the anti-genome, full-length genomic strands are made as well as mRNA for GPC and Z. NP associates with viral nucleic acid and complexes with L to translate viral proteins. GPC is translated into the ER where it undergoes maturation and glycosylation. Z becomes myristoylated and embedded in the cellular membrane, where it co-localizes with GPC. As viral proteins collect at the membrane interface, Z directs assemble and budding of newly formed virions. The multivesicular body pathway is again involved in arenavirus cellular trafficking as Vps4A, Vps4B, and Tsg101 are all critical for virus budding and belong to the multivesicular body pathway (357).

Pathogenesis of disease caused by the Old World Arenaviruses

The most notable of OW arenaviruses is Lassa virus. Endemic to West Africa, it is carried by the rodent *Mastomys* sp., and is often transmitted to humans through contact with contaminated rodent excreta or inoculation with infected bodily fluids (51). Several other OW arenaviruses are found on the African continent, including Ippy virus, Mobala virus and Mopeia virus, which are not known to cause human disease, and the recently identified, highly virulent Lujo virus, isolated in South Africa (45, 126, 151, 340, 373).

The prototypical OW arenavirus, LCMV, is carried by *Mus musculus*, and is found world-wide. LCMV is a powerful tool for understanding immune mechanisms.

Additionally, it has been used as a tractable model for Lassa fever pathogenesis in the non-human primate model, with some strains causing a self-limiting, mild infection, while others cause significant disease with similarities to LASV infection in humans (89, 200, 204, 205, 383). Comparison of these strains with different pathogenic potential can provide insight into the mechanisms involved in pathogenesis. LCMV is also a significant human pathogen in its own right. Human infections with LCMV range from asymptomatic to meningitis and rarely death. LCMV infection poses a significant threat to the immunocompromised and the developing fetus (1, 23).

Lassa Hemorrhagic Fever

Lassa virus (LASV), the causative agent of Lassa hemorrhagic fever (LHF), affects an estimated two million people each year with 5000-10000 deaths (15, 168, 200, 254). In endemic areas of West Africa, such as Sierra Leone, Liberia, Nigeria, and Guinea, up to 50% of the population is sero-positive for past Lassa infection, indicating that this virus is a severe and neglected human pathogen with limited treatment and no commercial vaccine (168, 294). Symptoms of LASV infection range from mild, flu-like febrile illness to severe multisystem disease with lethal outcomes. Of these infections, ~10-20% are symptomatic and 1-2% are fatal. During nosocomial outbreaks, fatality rates can be as high as 15-30% and even higher for certain groups of people (pregnant women, children under five years of age, and newborn infants). Health care workers in these areas are at significant risk, with high fatality rates in nosocomially transmitted disease (91).

While there are several vaccine candidates for LASV based on replication competent or replication deficient strategies and DNA immunization, currently there are no licensed vaccines available for LASV. Off-label treatment with the broad spectrum antiviral, Ribavirin, has been shown to reduce morbidity and mortality in LASV infected patients, but only when administered early in the course of infection (10, 79). Ribavirin treatment is complicated by the high rate of side effects, in which over 40% of patients develop haemolytic anemia. Ribavirin is contraindicated for pregnant women as it has been associated with congenital disorders. Finally, it is most effective when given intravenously, a logistically challenging method in the areas where the virus is endemic (175).

LASV is pantropic, and upon infection, the virus disseminates to multiple organs via the blood stream, lymphatics, respiratory tract, and digestive tract (254). The virus replicates, particularly in cells of the reticulo-endothelial system, causing dysregulation of proinflammatory cytokine and chemokine production, and resulting in increased capillary permeability eventually leading to hemorrhage. Dysfunction in the coagulation system also contributes to hemorrhagic manifestations. However, disseminated intravascular coagulation is not involved in LHF pathogenesis. In many cases, LASV infection has no hemorrhagic manifestations at all. The incubation period of the virus is 7-18 days before the patient begins to develop symptoms (238). Disease begins with non-specific flu-like symptoms: fever, malaise, headache, backache, and myalgia as well as cough, severe headache, and sore throat. These symptoms make it particularly difficult to diagnose LASV infection, without virus-specific assays, early on when treatment with ribavirin is most effective. Constant fever for ten days indicates profound infection and

can lead to weakness, pharyngitis, muscle pains, and mental disorder as well as nausea, vomiting and pain around the hepatic region as the liver and kidneys are generally enlarged. Increased vascular permeability and edema occur in a minority of patients, but is a sign of poor prognosis (112). Some LASV specific features may occur, such as ulcerative lesions in the oral cavity and bleeding from the gums. Internal bleeding occurs in less than a third of patients but is an indicator of severe LHF and probable death. In fatal cases, patients undergo rapid deterioration between six and ten days after onset of symptoms. Recovery from Lassa infection generally occurs two to three weeks after development of symptoms, with some patients developing sensorineural deafness (78). The strongest indicator for fatal outcome is the level of viremia. Patients with viral titers higher than $10^{3.3}$ TCID₅₀/ml were significantly more likely to succumb to infection. Additionally, elevated liver aminotransferase (AST) is also an indicator for fatal outcome when levels at admission begin at 120 international units (IU)/liter. These combined indicators are associated with 78% fatality in hospitalized patients (150). Blood samples from patients in the 1999 Sierra Leone LASV outbreak identified new indicators for poor prognosis and gave insight into the pathogenesis of Lassa infection. Blood samples from those who succumbed to infection during this outbreak presented with an absence of proinflammatory cytokines, IL-8, IP-10, and IL-1 β , while these were elevated in survivors of LASV infection. Fatally infected patients also demonstrated an absence of IFN- γ , IL-12, TNF- α , IL-6, and IL-10, while these cytokines were upregulated in survivors of LASV infection as well as those suffering from non-Lassa febrile illness. These results demonstrate an intriguing glimpse of the human *in vivo* response to LASV infection (212).

The actual cause of death in LASV infection is poorly understood. While there is widespread infection and development of shock, there is little cellular damage and modest infiltration of immune effectors (363). Liver failure is an unlikely explanation as there is usually only minimal hepatic tissue damage and high viral titers are also found in heart, lungs, kidney, spleen and the adrenal gland (223). Blood loss does not account for fatal outcomes, especially where hemorrhage is only a symptom of a minority of Lassa patients (222). LASV-induced death may more likely be a result of global systemic dysregulation and failure to establish an anti-viral state, with the virus infecting a wide range of cell types.

LCMV

LCMV infection in humans results in a range of outcomes from asymptomatic infection to death (148). The typical LCMV disease in the immunocompetent person is generally self-limiting and characterized by mild illness with myalgia, leukopenia, and thrombocytopenia, possibly accompanied by auditory symptoms or arthritis. Often, a second wave of illness occurs with nervous system involvement and aseptic meningitis. Rarely, this progresses to severe or fatal encephalitis. These types of illness are usually due to transmission from the natural host to the infected person through direct contact with fluids or droppings. Infections have been documented in laboratory settings as well as urban and rural environments. A study of aseptic meningitis among military personnel by Adair et al. concluded that in nearly 10% of cases, LCMV was the cause with similar findings in another study by Meyer et al (2, 231). In both of these studies, incidence of LCMV infection peaked in the winter months correlating to rodents moving into living

spaces to escape the cold weather. A well-documented case demonstrated that a five year old boy acquired aseptic meningitis from LCMV infected mice in his home (98).

Trapping of mice in the home revealed 12 isolates of LCMV that were identical to those infecting the boy.

Just as mice transmit the virus vertically, vertical transmission occurs in humans infections during pregnancy. LCMV infection of pregnant women may cause neurological abnormalities in the fetus ranging from microencephaly to hydrocephalus resulting in mental retardation, epilepsy and cerebral palsy as well as chorioretinitis resulting in blindness (38, 352). The diverse outcomes from fetal infection with LCMV may be due to the age of the fetus during infection (37). Using a rat pup model inoculated with LCMV at a series of postnatal ages, it appeared that host age at the time of infection correlated with development of symptoms.

More recently, there is growing concern for horizontal transmission between humans of an artificial nature. In 2003, 2005, and 2008, there were documented cases of solid organ transplantation resulting in LCMV infection (1, 110). LCMV was not directly detected in either of the 2003 or 2005 donors, although the 2005 donor's pet hamster was found to be infected with the same strains in the organ recipients. Samples from both the donor and recipients in 2008 revealed acute LCMV infection. Possibly due to the immunosuppressed state of the organ recipients, these patients develop a hemorrhagic-like disease, similar to that caused by LASV. Sequence analysis of the viral strain showed that of the known LCMV strain sequences, it was most closely related to LCMV-WE and this may be a more likely explanation for the development of hemorrhagic-like symptoms (7). Of the 11 documented cases where LCMV was transmitted through solid organ

transplantation, 10 patients died of multisystem organ failure (1). Compounding the issue of solid-organ transplant transmission of LCMV is the lack of screening for LCMV infection and the lack of correlation between donor history and contact with rodents.

The wide range of outcomes from LCMV infection demonstrate the pan-tropic nature of the virus as well as its importance as a human pathogen, elevating this virus from a laboratory tool or model to a virus of significant human importance. This is further substantiated with the fact that in a non-human primate (NHP) model, rhesus macaques, infected with LCMV-WE, developed a LASV-like hemorrhagic fever with weakened proinflammatory responses, elevated liver enzymes and indication of liver regeneration (58, 205). Thus, LCMV is a relevant virus for study, both as a human pathogen and as a model for arenavirus-induced hemorrhagic fever (128).

Immune Response to the Old World Arenaviruses and Immune Evasion

The innate response to infection by an Old World arenavirus is initiated and mediated by extracellular and intracellular receptors, and effector molecules. In response, the virus has evolved to inhibit these responses and create the best environment possible for replication and spread. Here, we will discuss both the host's response to infection with data from human infection and lessons learned from animal models, as well as the multitude of way the virus combats the resulting immune response.

Innate Immune Response

Infection with an OW arenavirus generates an initial rapid induction of IFN-I, which declines to basal levels by day 5 post infection (31, 89, 227, 295). However,

LASV is a poor inducer of IFN-I, is relatively resistant to IFN, and IFN sensitivity of LASV isolates has not been shown to correlate with disease progression (12). It has been demonstrated experimentally that purified LASV RNA can stimulate IFN β in a 5'triphosphate dependent manner via RIG-I, however, this result has not been confirmed in a live infection, either *in vitro* or *in vivo* (135). In addition to purified RNA, NP associates with MDA5 and RIG-I to induce IFN-I, in conventional DC but not plasmacytoid DC (385). *In vivo* analysis of these results in the serum of mice showed RIG-I and MDA5 is involved in sustained IFN-I responses, but not the earliest induction of IFN-I that is observed in human infection. It is possible that macrophages may play a role in IFN-I production as MOPV infection of macrophages *in vitro* produced significant amounts of IFN-I (263). However, in IPS-1 deficient mice, IFN- β production was similar to WT in LCMV-infected mice and only modest impairment of IFN- α was observed, indicating that RLH are unlikely to be the primary PRR responsible for innate antiviral defenses (155). Tetherin, a membrane-associated protein that has been shown to inhibit release of enveloped viruses is also an interferon stimulated gene (35, 179, 249). Using LASV-virus like particles expressing GPC and Z proteins or Z alone, there was a decrease in VLP release when tetherin was expressed (304). However, the relevance of any IFN-I activity is still in question as *in vitro* treatment of LCMV and LASV with IFN α and IFN γ only reduced viral replication by one log (12). IFN responses to LASV in rodents likely contributes more to establishing and maintaining a persistent infection in the natural host (54).

The induction of innate proinflammatory cytokines and chemokines may play an interesting role in the control of arenavirus infection as there appears to be a correlation

between fatal LASV infection and an absence of proinflammatory cytokine/chemokine responses in patient serum, whereas these cytokines are detected in survivors (212). The absence of the prototypic proinflammatory CXC chemokine, IL-8 (CXCL8), was most strongly correlated with fatal outcome. IL-8 enhances leukocyte adherence to endothelium cells, and directs extravasation and migration of leukocytes into virus infected tissues (25). *In vitro*, it was demonstrated that MOPV, but not LASV was able to induce expression of IL-8, as well as other cytokines, in monocyte-derived macrophages and endothelial HUVEC cells (203). The divergent induction of these cytokines may occur as a result of differences in transcription factor activation. Using a New World arenavirus model for LASV pathogenesis, guinea pigs infected with a virulent strain of Pichinde virus (PICV) induced the suppressive NF κ B homodimer, p50/p50, while the non-virulent PICV strain induced the activating heterodimer p65(RelA)/p50 (105). Further, a thioaptamer decoy of AP-1 proteins was able to induce cytokine expression during virulent PICV infection and reduced virus-mediated mortality *in vivo*, validating the importance of proinflammatory cytokines in protection against arenavirus infection (106).

In vitro and *in vivo* studies have identified TLR2 and MyD88 as being necessary for the proinflammatory response to LCMV, while only MyD88 was required for IFN-I induction (155, 386, 388). Impaired CD8⁺ T cell responses were observed in the MyD88KO mouse model which may have led to uncontrolled viral replication. CD4⁺ T cells also demonstrated functional impairment in the absence of MyD88 (387). These and other studies indicated an intrinsic T cell requirement of MyD88 for proper immune responses to LCMV. It was determined that MyD88 was necessary for the sustained

expansion of LCMV-specific CD8⁺ T cells, but not for the initial clonal burst or for the recall of memory T cells. The impaired support for CD8⁺ T cells likely contributes to the lower magnitude of the clonal burst. It is unlikely that defects in T cell proliferation and function is due to impaired APC maturation as WT CD4⁺ T cells were functional whether APCs expressed MyD88 or not (387). MyD88^{-/-} cDCs were able to efficiently present antigen to WT T cells (155, 387).

TLR2 has been shown to respond to LCMV infection. *In vitro* (IL-8), and *in vivo*, (IL-6 and MCP-1) cytokine responses to LCMV infection were found to be TLR2- and MyD88-dependent (388). Unexpectedly, IFN-I production in mice was also found to require TLR2, but not MyD88. However, recent studies have shown that some viral ligands are able to induce IFN-I production through TLR2 on the endosome, while bacterial ligands could not, demonstrating the plasticity and specificity of TLR2 functions (18). Further, the proinflammatory responses observed during LCMV infection of CNS glial cells was determined to be TLR2, MyD88, and Mal dependent (386). Beyond innate immune responses, MyD88 has been identified as a critical component of the adaptive T cell response to LCMV infection. MyD88 deficient mice are unable to control viral replication or spread in a manner that does not involve IL-1R1, IL-18R, or IPS-1 (22, 155, 387, 388). While WT CD8⁺ and CD4⁺ T cells expand and function in a MyD88^{-/-} host, MyD88^{-/-} CD8⁺ and CD4⁺ T cells have impaired functionality when transferred into a WT host, leading to a reduced cytotoxic T cell response to LCMV infection (155, 286, 387). While T cells are able to be activated and expand, the sustained expansion and accumulation of LCMV-specific T cells is defective in the absence of MyD88 signaling. This appears to be an intrinsic requirement of T cells, as

MyD88 deficient APCs are able to effectively activate WT T cells and adoptive transfer of MyD88 deficient T cells into a WT background are defective as described above (22, 286, 387). Though fewer LCMV-specific T cells develop in the absence of MyD88, memory cells are generated, although at a decreased frequency (286). Experienced LCMV-specific memory T cells do not require the presence of MyD88 to maintain homeostasis or mount an effect response to secondary infection (22, 287).

Adaptive Immune Response

For the OW arenavirus clearance, it is well accepted that a cytotoxic T-cell response is the primary mechanism (111). Patients recovering from LASV infection have low and delayed humoral responses. Proliferative CD4⁺ T cell responses have been detected in seropositive people to epitopes mapped to the nucleoprotein and glycoprotein. The NP-specific responses have been documented in persons having survived LASV infection at least six years prior, demonstrating that long term memory is possible (228, 350). Notably, MyD88 has been described as an important T-cell intrinsic factor to support expansion and survival of LCMV-specific CD8 T cells (286). WT mice with adoptively transferred MyD88^{-/-} T cells and infected with LCMV showed earlier T cell contraction than those with WT T cells (22). However, this requirement for MyD88 may be more important in chronic infection for prolonged expansion, not initial activation, when T cells are exhausted.

Antibody responses generally are not thought to assist in viral clearance. Early and brief IgG and IgM responses have been documented early in illness, but do not correlate with viral clearance as high viremia and high antibody titers can co-exist (111,

150). Neutralizing antibodies are undetected in patients during early convalescence and are only observed in low titers in a minority of surviving patients several months after clearance of infection (150).

Immune Evasion

Since viremia is strongly linked to disease manifestation and fatal outcomes, viral evasion of immune mechanisms that limit replication would be beneficial to the virus. As discussed above, treatment with IFN α/β , but not IFN γ , limited arenavirus replication 10-fold in DC, and 10-100 fold in macrophages (16). In accordance with the minimal effect of IFN-I on viral replication, it has been well documented that the nucleoprotein of all arenaviruses, with the exception of Tacaribe, functions as an IFN antagonist (217-219). It is possible that the regulation of IFN-I by NP provides for chronic infection of the virus in its natural host as both virulent and non-virulent arenaviruses antagonized IFN-I in a dose-dependent manner (218).

Additionally, arenaviruses have a specific tropism for APCs, in which they are able to infect without activating these cells (14, 263, 264). With 99% of the host cellular receptor, α -DG, being expressed in the spleen, the virus is able to infect large numbers of APCs without initiating hallmark activation signals (14, 16, 321). Infected DCs do not undergo migration or upregulation of costimulatory molecules while allowing for efficient viral replication. These infected DCs may provide a reservoir of virus particles that can hide from immune surveillance. Infected macrophages lack phagocytic abilities and do not upregulate cytokines or chemokines that would recruit additional effector cells. Taken together, it is likely that the lack of immune responses from these cells

contributes to defective adaptive immune responses. The reduced expression of co-stimulatory molecules, CD40, CD80 and CD86, as well as down regulation of MHC class I and II molecules likely contributes to the observed lack of activated CD8⁺ and CD4⁺ T cells during infection in non-human primates (NHPs) and absence of immunological killing of infected cells in mice (15, 39, 213, 321, 322). While APCs fail to mature during arenavirus activation, they are still able to respond to external stimuli. Cytokines and costimulatory markers were upregulated even in infected cells when treated with LPS or poly:IC. Such treatment even inhibited viral replication, indicating that the cellular anti-viral mechanisms weren't irreversibly damaged during infection (16).

Infection with rapidly replicating viruses, such LCMV-Clone13 (CL13), often result in clonal T cell exhaustion, where T cells are robustly activated and expanded followed by inactivation, anergy or depletion (241, 261, 380). This often results in chronic infection. Contributing to this, CL13 infection also appears to upregulate host cell negative regulators, such as IL-10 and PD-L1. Antibodies blocking these molecules were able to rescue T cells from exhaustion, allowing for viral clearance (46-48). Clone13 has also been shown to up-regulate a suppressor of cytokine signaling (SOCS)3, a negative regulator of IL-6, as early as one day post infection IL-6 (76, 181, 269).

In summary, while a robust CMI response is observed *ex vivo* from patients surviving LASV infection, lack of efficient maturation of APCs and initiation of the adaptive CTL response may contribute to fatal LASV infections. Early responses to LASV infection are likely key to establishing a successful immune response. Given that IFN-I is poorly induced by LASV and that, with the exception of TACV, all arenaviruses encode a IFN-I antagonist, the proinflammatory immune response is likely necessary for

establishing the ideal environment to combat arenavirus infection and limit viral replication. TLR2 is necessary for the proinflammatory response to LCMV *in vitro* and *in vivo*, however, the induction of proinflammatory responses by arenaviruses of different pathogenic potential has not been thoroughly examined.

Chapter Three

Hypothesis

TLR2-dependent signaling is involved in induction of innate immunity in cells infected with the Old World arenaviruses. Manipulation of this response by pathogenic Old World arenaviruses may contribute to immunosuppressive phenotype of arenaviral disease caused by pathogenic arenaviruses.

Rationale

An immunosuppressive state has been documented in fatal LASV infection in humans and NHPs. In LASV infection, uncontrolled viral replication is the strongest indicator of a poor outcome. Delayed or absent cytotoxic T cell responses have been observed in fatal LASV infection of NHPs and LCMV-CL13 infection of mice (15, 39, 321). LASV can infect APCs without induction of maturation, affecting the ability of these cells to present antigen and to activate T-lymphocytes (14, 16). Serum of patient samples that succumbed to LHF had reduced levels of cytokines, including IL-8, IP-10, IL-6, TNF- α , and IL-1 β (212). Taken together, the immunosuppression observed in fatal LASV infection indicates that an early response is likely inhibited which then has multiple downstream effects.

Efforts to identify the innate sensors to arenaviruses describe TLR2 as being required for LCMV-mediated cytokine induction *in vitro* as well as in mice (388). However, this work did not decipher how TLR2 activation relates to the pathogenic potential of LCMV strains, ARM and WE, used in non-human primates to model a

benign and pathogenic infection, respectively. I hypothesize that TLR2 is required for an efficient innate response to infection with OW arenaviruses, but infection with pathogenic strains fails to induce TLR2-dependent cytokine responses, through evasion of detection or inhibition of TLR2 signaling. This study demonstrates that the OW arenaviruses differentially induce TLR2-dependent cytokine responses, depending on the pathogenic potential of the virus strain in humans and NHPs. Additionally, we will explore how cellular factors, such as TLR2 signaling complex molecules and transcription factor activation, as well as viral factors, such as replication, multiplicity of infection, and superinfection, may contribute to this differential response.

Specific Aims

Specific Aim 1: Determine the impact of pathogenic and non-pathogenic Old World arenavirus infection, *in vitro*, on TLR2-dependent induction of cytokines and chemokines.

1.1 Determine if cytokine and chemokine responses are TLR2-dependent and if these responses differ between viral strains with different pathogenic potential

1.2 Determine if TLR2 expression is altered *in vitro* during infection with Old World Arenaviruses.

Specific Aim 2: Determine whether cellular and viral factors influence the TLR2-dependent cytokine and chemokine responses observed in the Old World arenavirus infection, *in vitro*.

1. Determine how members of the TLR2 signaling complex influence the TLR2-dependent proinflammatory response, and whether this affects activation of NF κ B.
2. Determine how viral factors, such as replication and multiplicity of infection, affect the TLR2-dependent proinflammatory response. Determine if pathogenic virus is able to inhibit TLR2-dependent responses.

Chapter Four

Materials and Methods

Cells and Viruses

THP-1, HEK293T, HEK293-TLR2, and HEK293-TLR4 cells were provided by Dr. A. Medvedev and cultivated as previously described (283). Immortalized bone marrow derived macrophages (iBMDM) from C57BL6/J wild-type mice (NR-9456), TLR2 KO mice (NR-9457), CD14 KO mice (NR-9570), and MAL KO mice (NR-9459) were obtained through the NIH Biodefense and Emerging Infections Research Resources Repository (BEI Resources). HEK293T, Vero, and BHK21 cells were maintained in MEM Medium, supplemented with 10% FBS in a humidified chamber at 37°C, 5% CO₂. THP-1 and iBMDM cells were maintained in complete RPMI, supplemented with 10% FBS in a humidified chamber at 37°C, 5% CO₂. Cells were infected for one hour at 37°C, with LCMV-ARM strain 53b, LCMV-WE strain 54, LCMV-ARM Clone 13, MOPV (AN20410), MOPV ML29 reassortant virus (Lukashevich et al., 2005), or LASV (Josiah), at a multiplicity of infection (MOI) equivalent to 1 PFU/cell. Virus stocks ($\geq 5 \times 10^6$ PFU/ml) were harvested in cell-free medium at kept at -80°C before use. The stocks did not contain detectable levels of cytokines as measured by multiplex immunoassay (Bio-Rad). Simultaneously, control cells were treated with serum-free media as a mock infection control, or with 100 µg/ml of Pam₃CSK4 or 10ug/ml of Lipoteichoic acid (Sigma-Aldrich, source *S. aureus*) as a positive control. At 24, 48, and 72 hours post infection (hpi), unless otherwise stated, media was removed and stored at -80°C for analysis. Viral titers were determined by standard plaque assay. Briefly, a monolayer of Vero E6 cells was inoculated with 10⁻⁶-fold diluted virus in a 6-well culture plate for 1

hr in a humidified chamber at 37°C, 5% CO₂ with periodic rocking. 1xMEM, 4% FBS, 1% agarose was applied to each well and incubated for 5 days. Cells were fixed with 12% paraformaldehyde and the agarose was removed. Cells were stained with crystal violet. LCMV-ARM and LCMV-WE was inactivated by incubation under a UV light source for 30 min on ice. Alternatively, LCMV-ARM and LCMV-WE was inactivated by heating at 56°C for 30 min and then cooled on ice. Residual infectivity of inactivated virus was assessed by standard plaque assay. No plaques or cytopathic effects were observed.

Cytokine Assays

Cytokine secretion was analyzed using BD OptEIA human ELISA kits according to manufacturer's instructions (BD Biosciences). Briefly, EIA flat bottom plates were coated with primary capture antibody specific for IL-8 or IP-10 in Carbonate-Bicarbonate buffer overnight. Plates were blocked in 10% non-fat dry milk (NFDM) for one hour at 4°C. Samples were diluted with 1x PBS, containing 10% FBS to reach linear range of ELISA assay standards. Samples were incubated in plates for two hours followed by extensive washing. Plates were then incubated with secondary antibody specific for IL-8 or IP-10 and streptavidin conjugate. After washing, plates were incubated for 30 minutes with Peroxidase substrate and reactions were stopped by the addition of 1% HCl solution. Within 15 min, plates were analyzed spectrophotometrically by analysis of optical density at 450 nm with subtraction of background values obtained by measuring absorbance at 570 nm. Alternatively, multiplex cytokine analysis was performed using a customized BioPlex assay, a fluorescent, magnetic bead-based, multiplex immunoassay

(Bio-Rad). Culture supernatants were analyzed 24 hpi for IL-8, IL-6, IP-10, TNF- α , and IL-1 β on a Luminex system and quantified using Bio-Plex Manager™ software. The limits of detection for each cytokine were as follows: IL-8, 1.0 pg/ml; IL-6, 2.6 pg/ml; IP-10, 6.1 pg/ml, TNF- α , 6.0 pg/ml; IL-1 β , 0.6 pg/ml.

qRT-PCR

RNA was isolated from cells using Trizol reagent (Invitrogen), as recommended by the manufacturer, reverse transcribed to obtain cDNA using Reverse Transcription System (Promega) and random primers, and subjected to real-time quantitative (q) PCR with Maxima™ SYBR Green/Fluorescein qPCR Master Mix (Fermentas). IL-8 values were normalized to β -actin values. Quantitative PCR was used to verify virus replication in co-infection studies using primers/probes specific for divergent sequences on the S segment for LCMV-ARM and LCMV-WE. Viral RNA Ct values were normalized to β -actin values. The following primers and probes were used: human IL-8: probe 5' /56-FAM/AAGACATACTCCAAACCTTTCCACCCC/36-TAMSp/-3', primer 1 5'-TCCTGATTTCTGCAGCTCTG-3', primer 2 5'-GTCCACTCTCAATCACTCTCAG-3'. β -actin: probe 5' /56-FAM/ATCTGGGTCATCTTCTCGCGGTTG/36-TAMSp/-3', primer 1 5'-ACCTTCTACAATGAGCTGCG-3', primer 2 5'-CCTGGATAGCAACGTACATGG-3'. ARM: probe 5' /56-FAM/AGCAAGATTCCAAGTACTCACACGGC/36-TAMSp/3', primer 1 5'-TTTACAGGGAACCCGTTGATC-3', primer 2 5'-GAGGTCGGCAAGATCCATG-3'. WE: probe 5' /56-FAM/CCACATGGATTGACATTGAGGCAGG/36-TAMSp/3',

primer 1 5'-TTGAAAGACTTGATGGGAGGG-3', primer 2 5'-TCTGGTCCGTAGGTTCTCTG-3'.

Function blocking antibody assay

HEK293-TLR2 were seeded in a 12-well culture plate and grown overnight to 2.5×10^5 cells. Infected, mock-infected, or control cells were pretreated for one hour with medium or functional blocking anti-TLR2 antibody, TL2.1 (Santa Cruz Biotechnology), anti-TLR1 (H2G2), anti-TLR6 (C5C8) (Invivogen), media or isotype control antibody for 1 hr. After washing with warmed 1xPBS, cells were then infected with LCMV or stimulated with Pam₃CSK4 (positive control) as described above.

CD14 transfection and FACS

pcDNA3-CD14 was obtained from Dr. A. Medvedev and was transfected into HEK293T and HEK293-TLR2 cells. In brief, cells were seeded at 1×10^6 in a T25 culture flask, grown overnight, and transfected with 3 μ g of pcDNA3-CD14 or pcDNA3 plasmids using FuGENE HD Transfection Reagent (Roche), as recommended by the manufacturer. Cells were recovered for 48 hr, and CD14 cell surface expression was analyzed by flow cytometry after staining with human anti-CD14 antibodies conjugated to APC. Samples were gated on live cells and fluorescence was measured compared to corresponding isotype control.

Co-infection assay

2.5×10^5 THP-1 cells were seeded into each well of a 12-well culture plate. Cells infected with LCMV-ARM were super-infected at different time points with WE strain for 1 hr. After final infection, virus inoculum was removed, cells were washed and incubated for additional 24 hours. As controls, single infections, as well as mock and incubation with Pam₃CSK4 were conducted. Cell-free culture supernatant samples were collected at 36 hrs and analyzed for IL-8 production by ELISA. For UV-WE inhibition, cells infected with LCMV-ARM or treated with 100ng/ml of Pam₃CSK4 were exposed to UV-inactivated LCMV-WE one hour prior, concurrently, or post LCMV-ARM infection, as indicated in the figures. After final exposure, cells were washed and incubated for an additional 24 hours. For LTA inhibition, cells were infected with LCMV-ARM or LCMV-WE and were then treated with 10ug/ml of LTA at the indicated times post infection. Control cells were treated with 10ug/ml LTA alone for the same intervals. IL-8 production was analyzed by ELISA and normalized to cytokine production induced in response to cell stimulation with LTA alone.

NFκB Luciferase Reporter

2×10^6 HEK293-TLR2 cells were transfected with 0.8ug of pNFκB-MetLuc2-Reporter vector and 0.2ug constitutive pSEAP vector (Clontech) using the Amaxa Nucleofector Kit V (Lonza), program Q-001 and the Nucleofector II device. Cells were rested for 24 hours and then infected as described with LCMV-ARM, LCMV-WE, or LCMV-CL13 at MOI=1. Control cells were treated with 100ng/ml Pam₃CSK4, 10ug/ml LTA, or mock infection. Secreted luciferase was analyzed in culture medium samples at 24 hours post

infection, and normalized to SEAP secretion, using the Ready-to-Glow Luciferase Assay (Clontech) on a Veritas luminometer (Turner Biosystems). Values are expressed as Relative Luciferase Units (RLU).

Data Analysis

Data from a representative experiment are presented as mean, \pm SD, and analyzed by the Student's two tailed unpaired t test with significance level set forth at 95%, using GraphPad Prism 5 statistical package (GraphPad Software). Each experiment was repeated three separate times with similar results.

Chapter Five

Specific Aim 1: Determine the impact of pathogenic and non-pathogenic Old World arenavirus infection, *in vitro*, on TLR2-dependent induction of cytokines and chemokines.

1.1 The OW Arenaviruses with differing pathogenic potential induce differential TLR2-dependent cytokine responses *in vitro*

Virus strains used for comparison

Several virus strains of different pathogenic potential for humans and non-human primates were used to perform side-by-side comparisons: i) non-pathogenic MOPV (AN20410) was compared to human pathogenic virus LASV (Josiah); ii) LCMV-ARM(53b), capable of inducing brain pathology in mice but attenuated in primates, was compared to LCMV-WE, which induces pantropic fatal infection in rhesus macaques and/or iii) LCMV-ARM(53b) was compared to LCMV-CL13, an immunosuppressive derivative of ARM(53b).

MOPV and LASV are closely related and are carried by the same rodent host, *Mastomys* spp. However, MOPV has not been shown to cause disease in humans and can confer cross-protection against LASV challenge in a NHP LHF disease model (362). In human DC infected *ex vivo*, MOPV infected DC induced greater CD4⁺ and CD8⁺ T cell responses than LASV (264).

The MOPV-ML29 is a reassortant virus composed of the L segment of MOPV (~70% of the ML29 genome) and the S segment of LASV (~30% of the ML29 genome)

(240). This reassortant virus contains the major immunogens of LASV, the glycoprotein and the nucleoprotein, as well as the polymerase and zinc protein from MOPV. The MOPV-ML29 is currently being analyzed as a vaccine candidate for LASV as it provides protection against LASV challenge in all tested animal models including NHPs and is more attenuated than MOPV *in vivo* (59, 199).

In our *in vitro* experiments, we used MOPV-ML29 virus as a LASV surrogate mimicking cell-virus interaction because it contains the GPC and NP which are the first viral proteins to encounter a host cell. The MOPV-ML29 also provides a useful model as it can be used in BSL3 containment, whereas LASV must be used in BSL4 containment laboratories which are not readily accessible. Nevertheless, some crucial experiments to confirm our proof-of-concept studies were performed with wild-type LASV (Josiah) in a BSL4 lab.

LCMV-WE is a human isolate that can cause manifested infection in humans and fatal disease in NHPs (205, 384), and is classified as a risk group 3 pathogen (BSL3). LCMV-ARM and LCMV-CL13 are mouse isolates from the brain and spleen respectively. LCMV-WE has been used to model LHF-like disease in a NHP LHF disease model, whereas LCMV-ARM does not cause disease in this model and can confer protection against challenge with WE. LCMV-CL13 is an immunosuppressive strain of LCMV that differs from ARM by only a few amino acids (26, 305, 306, 337). LCMV-CL13 has been studied for its ability to cause persistent chronic infections. Though very similar to LCMV-ARM genetically and isolated from the same host, CL13 causes a persistent and chronic infection in mice whereas ARM leads to an acute and rapidly cleared infection (39).

Differential induction of proinflammatory cytokines

Previous studies in Dr. Lukashevich's lab showed (203) that replication of LASV, but not MOPV, in human monocyte-derived macrophages and endothelial cells failed to stimulate TNF- α and IL-8 gene expression and even down-regulated LPS-inducible cytokine responses. Furthermore, LCMV-WE infection of rhesus macaques resulted in a fatal LHF-like hepatitis with reduced IL-8 in infected tissues, compared to healthy monkeys (202). Thus, we sought to determine if the pathogenicity of OW arenaviruses correlates with their potential to elicit proinflammatory responses *in vitro*.

In experiments presented in Figure 4A, HEK293T untransfected cells and HEK293-TLR2 or HEK293-TLR4/MD2 stable transfectants were infected with MOPV, ML29, LCMV-ARM, and LCMV-WE (MOI=1). Production of IL-8 was measured 24 hours post infection by ELISA. As seen from presented results, no IL-8 production was observed in untransfected HEK293T cells. In cells expressing TLR2, but not TLR4/MD2, MOPV and LCMV-ARM were able to induce a strong IL-8 response. In contrast, LCMV-WE failed to induce a cytokine response, even in the TLR2 expressing cells, while MOPV-ML29 only weakly induced IL-8 production. Virus-induced IL-8 secretion in HEK293-TLR2 cells was comparable to the levels elicited by Pam₃CSK4, a TLR2/1 agonist used as a positive control. In Figure 4B, HEK293T cells or HEK293-TLR2 stable transfectants were infected with MOPV or LASV (MOI=1) and were analyzed for cytokine production by ELISA. Cell infection with MOPV, but not LASV, induced IL-8 production. These results indicate that arenaviruses with different

pathogenic potential have different capacities to induce IL-8. Non-pathogenic MOPV and LCMV-ARM induce levels of IL-8 comparable to the TLR2/1 ligand, Pam₃CSK4. In contrast, LCMV-WE, LASV, and ML29 failed to induce robust levels of IL-8. LCMV-ARM and LCMV-WE infection led to secretion of distinct levels of IL-8, correlating with different abilities of these viruses to cause disease: LCMV-WE has been used to model a LHF-like disease in NHPs, while LCMV-ARM causes a self-limiting infection. The similar patterns of chemokine induction between LASV:MOPV and LCMV-WE:LCMV-ARM allow for further investigation, *in vitro*, into this differential phenotype depending on pathogenic potential.

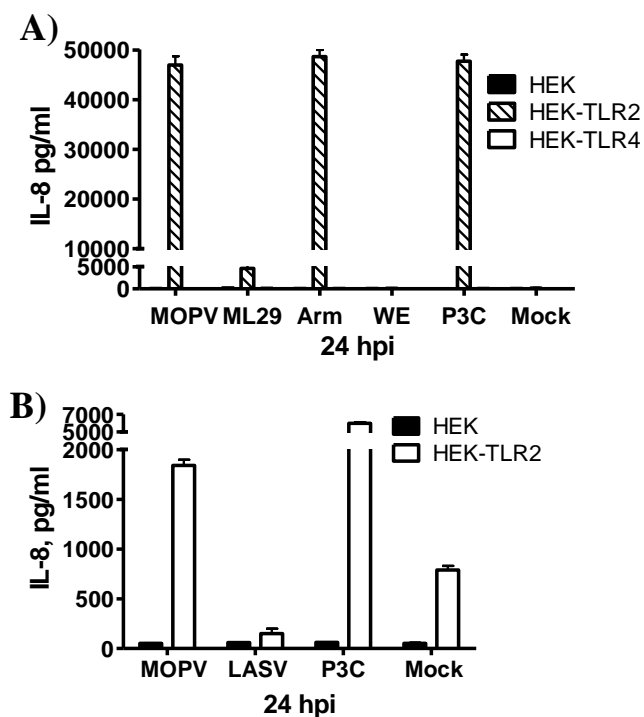


Figure 4: TLR2-mediated signaling is required for induction of IL-8 responses in cells infected with the OW non-pathogenic arenaviruses

Wild-type HEK293T cells or HEK293 cells stably expressing TLR2 or TLR4/MD2 were infected (MOI=1) with MOPV, ML29, LCMV-ARM, and LCMV-WE (A), or with MOPV and LASV (B). Control cells were treated with media (mock infection) and with Pam₃CSK4 (P3C) as a positive TLR2 control. At 24 hpi culture supernatants were removed and analyzed by IL-8 ELISA. Equivalent viral replication was determined by plaque assay (data not shown, see Figures 15 and 16 for analysis of viral replication). Results of a representative experiment (n=3) are shown. MOPV and LCMV-ARM were able to induce robust secretion of IL-8 in the presence of TLR2, while MOPV-ML29, LCMV-WE, and LASV failed to induce IL-8.

Several cytokines/chemokines are known to be differentially induced during fatal vs. non-fatal arenavirus infection, including IP-10, IL-6, TNF- α , and IL-1 β (212).

Therefore, we next determined if these cytokines are induced *in vitro* in response to arenavirus infection, using a customized, multiplex assay to evaluate cytokine production (Bio-Rad). As seen in Figure 5A, this assay confirmed the differential induction of IL-8 by arenaviruses of different pathogenic potential shown in Figure 4. Additionally, IP-10

and IL-6 were also differentially induced, depending on the pathogenic potential of the virus. For non-pathogenic viruses, MOPV and LCMV-ARM, induction of cytokine was significantly higher than levels induced by pathogenic virus and were similar to induction by Pam₃CSK4. While overall induction of IL-6 appears low, values determined were above the limit of detection of the assay at 2.6pg/ml. Levels of TNF- α and IL-1 β cytokines were lower than detection limits in all samples tested (data not shown).

Presented *in vitro* data are in good correlation with clinical results. In patient serum samples from the 1999 Sierra Leone LASV outbreak in which the absence of IL-8 and IP-10 were most strongly correlated with fatal LASV infection outcomes. This is in line with our above results, demonstrating differential induction of cytokine in HEK293-TLR2 cells. To corroborate our results, we next sought to determine if this pattern of cytokine induction would occur in a more natural target for arenaviruses by using the monocytic THP-1 cell line.

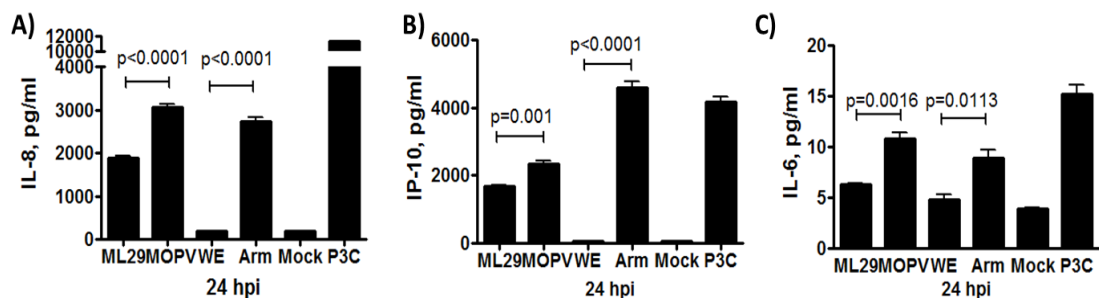


Figure 5: Multiplex cytokine analysis in OW arenavirus infected HEK293-TLR2 cells

A customized BioPlex assay (Bio-Rad) was used to evaluate multiple cytokines in response to LCMV infection. Culture supernatants from LCMV-ARM- and LCMV-WE-infected cells were analyzed for production of IL-8 (A), IP-10 (B), and IL-6 (C) in triplicate. In infected cells, levels of TNF- α and IL-1 β were lower than detection limits in all samples tested. Results of a representative experiment (n=3) are shown. MOPV and LCMV-ARM induced significantly higher secretion of IL-8, IP-10, and IL-6 than MOPV-ML29 or LCMV-WE.

To determine if the OW arenaviruses of differing pathogenic potential induced different proinflammatory responses in cells with a monocyte phenotype, a natural target of these viruses, THP-1 cells, a human monocytic cell line, were infected with LCMV-WE and LCMV-ARM (MOI=1). Cell permissibility was assayed by infectious particle production post infection as well as production of IL-8 and IP-10 by qPCR and ELISA. THP-1 cells were equally permissive to LCMV-WE and LCMV-ARM (Figure 6A). It is important to note that viral replication was similar in both infections, indicating that differences observed in activation of the innate immune response are unlikely attributable to differences in viral replication. Production of IL-8 and IP-10 secreted into the culture media over 72 hours was analyzed by ELISA (Figure 6B, D). These results support those observed in HEK293-TLR2 cells in that LCMV-ARM, but not LCMV-WE, induced a robust cytokine response. To further confirm these results, qPCR for IL-8 (Figure 6C)

and IP-10 (not shown) demonstrated a steady state transcription in LCMV-ARM, but not LCMV-WE infection compared to mock infection control.

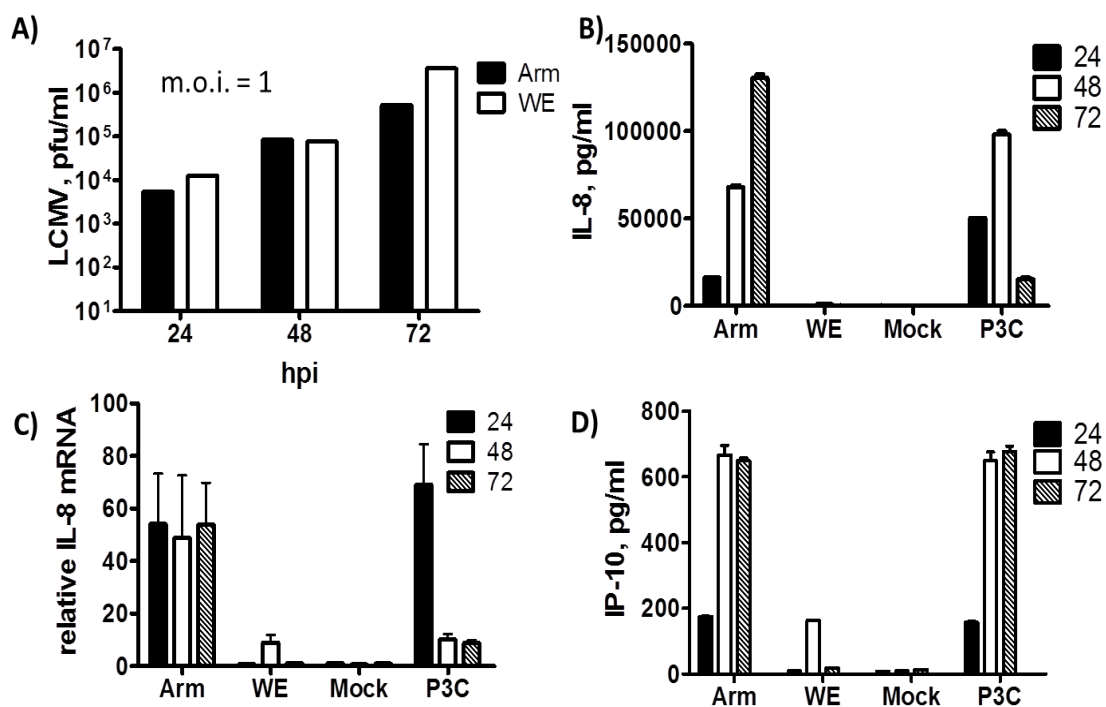


Figure 6: Replication of LCMV in THP-1 cells and induction of pro-inflammatory chemokines

(A), Undifferentiated THP-1 cells were infected with LCMV-ARM53b or LCMV-WE54 (MOI=1) for 1 hr. In all experiments plaque-purified virus, harvested in cell-free media stocks with undetectable levels of pro-inflammatory cytokines were used. Control cells were treated with serum-free media as a mock infection, or with 100 ng/ml of Pam₃CSK4 (P3C) as a positive control for induction of IL-8 and IP-10. At 24, 48, and 72 hpi, culture supernatants were collected and analyzed by plaque assay using a monolayer of Vero E6 cells. (B, D), production of IL-8 (B) and IP-10 (D) was assayed by OptEIA human ELISA (BD Biosciences). (C), real-time qPCR analyses of IL-8 gene expression. Results (mean, +SD) of a representative experiment (n=3) are shown. While LCMV-ARM and LCMV-WE replicated equivalently in THP-1 monocytes, LCMV-ARM, but not LCMV-WE, was able to induce robust secretion of IL-8 and IP-10. qPCR analysis demonstrates a steady state transcription of IL-8 in LCMV-ARM, but not LCMV-WE infected THP-1 cells.

TLR2/6 is required for arenavirus-induced cytokine production

To verify the requirement for TLR2 in virus-induced cytokine induction, HEK293 cells stably expressing human TLR2 were treated with blocking anti-TLR2 antibody (TL2.1) to block ligand binding to TLR2, or with antibodies against endogenous TLR1 or TLR6. HEK293 cells endogenously express TLR1 and TLR6, as evidenced by the ability of Pam₃CSK₄, a TLR2/1 ligand, and LTA, a TLR2/6 ligand, to induce a response in the presence of TLR2 (see Figures 4 and 12) (104). Cells were pretreated with the indicated antibody for 1 hour prior to infection and then infected with MOPV (Figure 7A) or LCMV-ARM and LCMV-WE (Figure 7 B-D). As a positive control, cells were stimulated with Pam₃CSK₄. Pretreatment of HEK293-TLR2 cells with anti-TLR2 Ab led to 5-fold and 2-fold decreased in MOPV-induced induction of IL-8 and IP-10, respectively, and decreased Pam₃CSK₄-mediated IP-10 secretion 1.4-fold. In THP-1 cells, LCMV-ARM-induced IL-8 production was blocked 2.6 fold and 6 fold by anti-TLR2 Ab and anti-TLR6 Ab, respectively. Pretreatment with anti-TLR2 and anti-TLR6 resulted in the greatest inhibition of LCMV-ARM-induced cytokine production by ~15-fold. Even though anti-TLR6 antibody has not been reported as specifically preventing ligand binding to the concave interface of the TLR6, or as blocking responses of TLR2/6 agonists, our data suggest the feasibility of using this anti-TLR6 antibody to decipher the involvement of TLR6 in responses of TLR2-reactive PAMPs. In contrast, addition of anti-TLR1 Ab alone had no statistically significant effects on cytokine production in LCMV-ARM infected cells, compared to isotype control Ab-pretreated cells (Figure 7B). Further experiments with TLR6 gene ablation or the use of TLR6 vs. TLR1 ^{-/-} macrophages are required to unambiguously demonstrate the involvement of TLR1 and

TLR6 co-receptors in viral responses studied herein. Nevertheless, my results indicate that TLR2 along with TLR6 mediate the induction of proinflammatory cytokine in response to LCMV-ARM infection. Additionally, it is important to note that pretreatment with either of the blocking antibodies did not affect production of infectious viruses (Figure 7D-E). Thus, TLR2, 1, and 6 are not likely to play a role in arenavirus uptake.

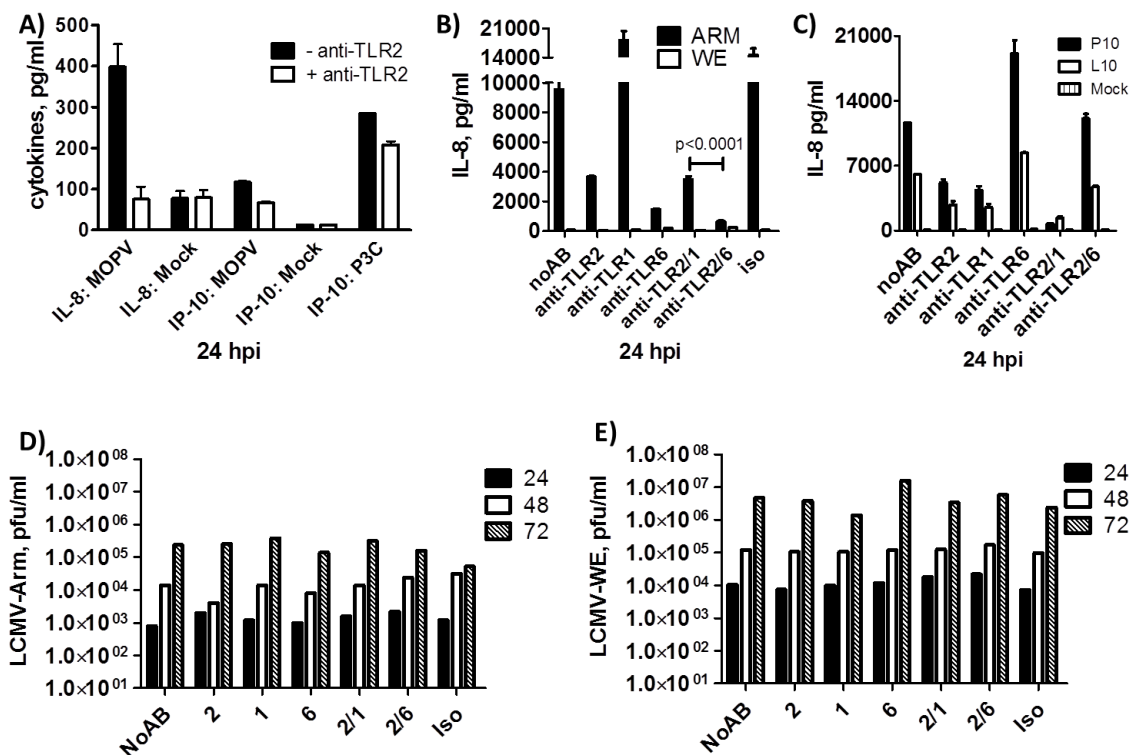


Figure 7: LCMV-ARM induction of cytokines is TLR2 and TLR6 dependent

(A) HEK293-TLR2 cells were pretreated with a function blocking antibody for TLR2 (TL2.1) for one hour prior to infection with MOPV (MOI=1). Culture medium was analyzed at 24 hours post infection for IL-8 and IP-10 production. (B-D) THP-1 cells were pretreated with function blocking antibodies for TLR2, TLR1, TLR6, or a combination of TLR2/1 or TLR2/6 for one hour prior to infection with LCMV-ARM or LCMV-WE (B) or control treatments with 10ng/ml Pam3CSK4 (P10), 10 μ g/ml LTA (L10), or serum-free media as a mock infection control (C). Culture medium was analyzed at 24 hours post infection for IL-8 production by ELISA. (D-E) Production of infectious particles was analyzed by plaque assay at 24, 48 and 72 hours post infection for LCMV-ARM (D) and LCMV-WE (E). Function blocking anti-TLR2 antibody (TL2.1) reduced the ability of MOPV or LCMV-ARM to induce IL-8 or IP-10, confirming the requirement for TLR2 in arenavirus-induced production of proinflammatory cytokines. LCMV-ARM infected cells, pretreated with antibodies against TLR2, TLR6, or TLR2/6 also demonstrated reduced production of cytokine 2.6-, 6-, and 15-fold, respectively. Antibody treatment against TLR2, TLR1, or TLR6 did not affect the infectivity of either LCMV-ARM, nor LCMV-WE, indicating that these receptors do not play a role in virus uptake.

1.2 Changes in TLR2 surface expression are comparable during infection with arenaviruses of differing pathogenicity

Changes in TLR2 surface expression vary depending on the virus infecting the cell. Sendai virus infection resulted in enhanced expression of TLR2 in primary human macrophages, while Influenza increased TLR2 expression on both macrophages and neutrophils (187, 233). Virus replicon particles of Venezuelan equine encephalitis virus were also reported to increase TLR2 expression, but only in NK cells (303). HSV-2 infection down regulated TLR2 expression (376). Infection with Hepatitis C did not affect TLR2 expression in monocytes while chronic infections with Hepatitis B virus resulted in reduced expression of TLR2 in human peripheral blood mononuclear cells (PBMCs) (65, 90). Exogenously added IFN- α enhanced TLR2 expression in epithelial and endothelial cells, while IFN- γ increased expression on macrophages and epithelia (70, 371). The ability of a virus to up-regulate TLR2 levels may depend on the ability to increase either type I or II IFN production in response to infection. Given that arenaviruses encode a IFN-I antagonist and that LASV is a poor inducer of IFN, it is not likely that TLR2 expression would be enhanced during infection. To determine if LCMV infection affects cell surface expression of TLR2, THP-1 cells infected with LCMV-ARM, LCMV-WE, or LCMV-CL13 were analyzed by flow cytometry after staining with anti-TLR2 or isotype control antibodies conjugated to APC.

TLR2 fluorescent levels were normalized to isotype control values and then to mock infection for each time point studied. Representative results of how viral infection affects TLR2 surface expression, as evidenced by FACS, are shown in Figure 8A, which demonstrates specific detection of TLR2 with fluorescent TL2.1-APC antibody,

significantly exceeding fluorescence intensity detected for isotype control IgG-APC. LCMV infection induced modest reductions in TLR2 expression through 24 hours (Figure 8B). LCMV-ARM did not affect TLR2 expression at 3 hours post infection, but induced a 10% reduction at 8 and 24 hours post infection. LCMV-CL13 had similar effects on TLR2 expression compared to LCMV-ARM but reduced TLR2 expression at 24 hours post infection to 20%. WE had an initial brief reduction of 25% at 3 hours post infection that returned to nearly baseline levels by 8 and 24 hours. Pam₃CSK3 increased TLR2 expression slightly at 3 and 8 hours post treatment and returned to baseline levels by 24 hours. With the exception of WE at 3 hours post infection, LCMV strains induced similar effects on TLR2 expression through 24 hours. Differences observed between strains are not sufficient to explain differences in cytokine production.

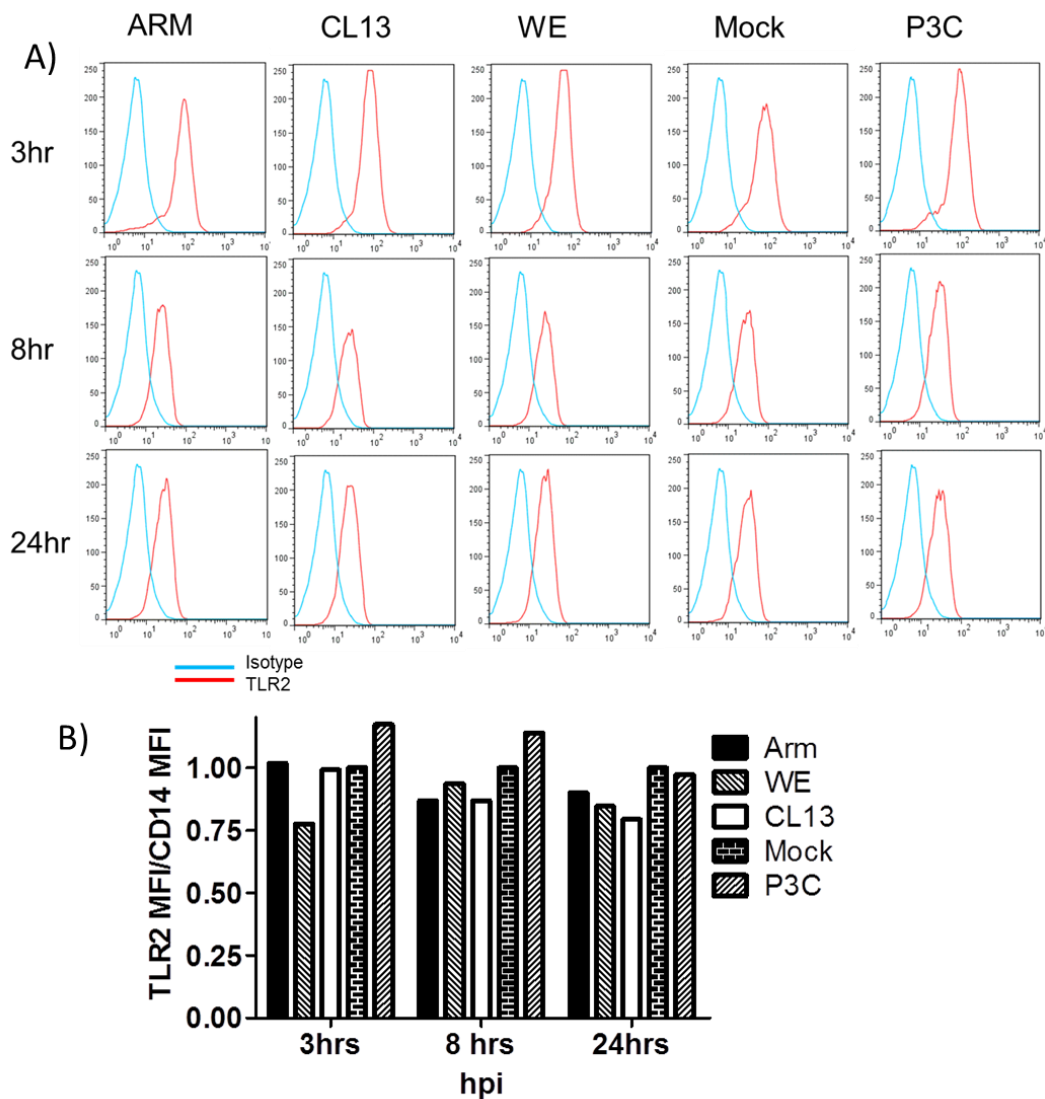


Figure 8: LCMV strains induced similar effects on TLR2 surface expression in THP-1 cells

THP-1 cells were infected with LCMV-ARM, LCMV-WE or LCMV-CL13 at MOI=1. Pam₃CSK4 treatment, 100ug/ml, was used as a positive control. Serum-free media was used as a mock infection control. Infected cells were stained for surface expression of TLR2 using TL2.1-APC. (A) Samples were gated on live cells and were analyzed for mean fluorescent intensity for TLR2. (B) Mean fluorescent intensity for TLR2 was normalized to isotype control and then to mock infection control for the time points analyzed. With the exception of LCMV-WE, surface expression of TLR2 was unchanged at 3 hours post infection. Minimal decreases in TLR2 surface expression were observed at 8 and 24 hours post infection and were similar for all infections.

Conclusions

With this specific aim, we sought to determine if OW arenaviruses of differing pathogenic potential for humans (LASV vs. MOPV) and NHPs (LCMV-WE or LCMV-CL13 vs. LCMV-ARM) induced different TLR2-dependent proinflammatory responses. MOPV is not known to cause human disease and is the closest related arenavirus to LASV. MOPV-ML29 was also used in some experiments as it contains LASV GP and NP, the first two viral proteins that are likely to be detected by the host cell. Additionally, we compared LCMV-ARM with LCMV-CL13 or LCMV-WE. LCMV-CL13 differs from LCMV-ARM by only a few amino acids but is known for being immunosuppressive, rapidly replicating in monocytes and DCs, and causing chronic infections in mice. Genetically, LCMV-WE is also highly similar to LCMV-ARM. However, LCMV-WE has been used to model a LASV-like hemorrhagic fever in NHPs whereas LCMV-ARM causes an acute, self-limiting infection. To determine the level of innate immune activation *in vitro*, proinflammatory cytokines were measured by ELISA, multiplex cytokine analysis (BioPlex), or real time qPCR. To determine the requirement for TLR2, HEK239T cells, which do not express TLR2, or HEK293 cells stably transfected with TLR2 or TLR4/MD2 were infected with the above named viruses. To confirm our results in cells with a monocyte-like background, we used undifferentiated human pro-myelomonocytic THP-1 cells. Blocking anti-TLR2 antibodies were used to prevent signaling through TLR2. Antibodies against TLR2 co-receptors TLR1 and TLR6 were also used to explore their potential to block signaling and to decipher their involvement.

Clear and significant differences in TLR2-dependent cytokine induction have been found during infection with OW arenaviruses of differing pathogenic potential. In both cell types analyzed, HEK293-TLR2 and THP-1 cells, non-pathogenic strains of the OW arenaviruses were able to induce cytokine responses, while pathogenic strains did not. LCMV-ARM and MOPV were able to induce robust production of IL-8, as well as IP-10 and IL-6. Infection with LCMV-WE and LASV did not result in significant production of any cytokine analyzed. Cytokine production, a measure of the activation of the innate immune system, required the presence of TLR2, as demonstrated in the HEK239T cell system and in THP-1 cells with function blocking antibodies, in response to the OW arenavirus infection. Infection with various strains of LCMV modestly reduced the surface expression of TLR2 similarly, despite differences in pathogenic potential.

Specific Aim 2: Determine how viral and host cellular factors influence the cytokine/chemokine responses observed in Old World arenavirus infection.

2.1 Signaling components involved in TLR2-elicited cytokine production in response to OW arenavirus infection.

Ligand binding-induced signaling through TLR2 is mediated by a receptor complex and several adaptor molecules. Upon recognition of tri- vs. di-acylated microbial lipoproteins at the cell surface, TLR2 heterodimerizes with TLR1 or TLR6 respectively. In Aim 1, we demonstrated that TLR2 and TLR6 are required for arenavirus induced cytokine production. Because of the reported role of CD14 in modulating TLR

signaling cascades, we sought to determine if CD14 was required for LCMV-WE-induced TLR2-dependent cytokine responses. To this end, HEK293T or HEK293-TLR2 cells were transfected with pcDNA3-hCD14 expression vector.

As seen in Figure 9, transfection of HEK293T cells with pcDNA3-hCD14 resulted in robust cell surface expression, as demonstrated by FACS analysis of hCD14 expression after staining with the corresponding anti-CD14 Ab, with nearly 40% (HEK293T) and 80% (HEK293-TLR2) CD14 positive cells. These cells were then infected with LCMV-ARM and LCMV-WE as described above. Culture medium was analyzed at 24 hours for IL-8 production by ELISA. In the presence of hCD14, the TLR2-dependent cytokine response to LCMV-ARM was found to be increased nearly five-fold (Figure 10). However, LCMV-WE is not able to induce cytokine production, even in the presence of TLR2 and hCD14. Despite the ability of CD14 to carry signal transduction via LPS and activate DCs, neither virus is able to induce cytokine in the presence of hCD14, but in the absence of TLR2, confirming the requirement for TLR2 in proinflammatory cytokine responses to arenavirus infection (381).

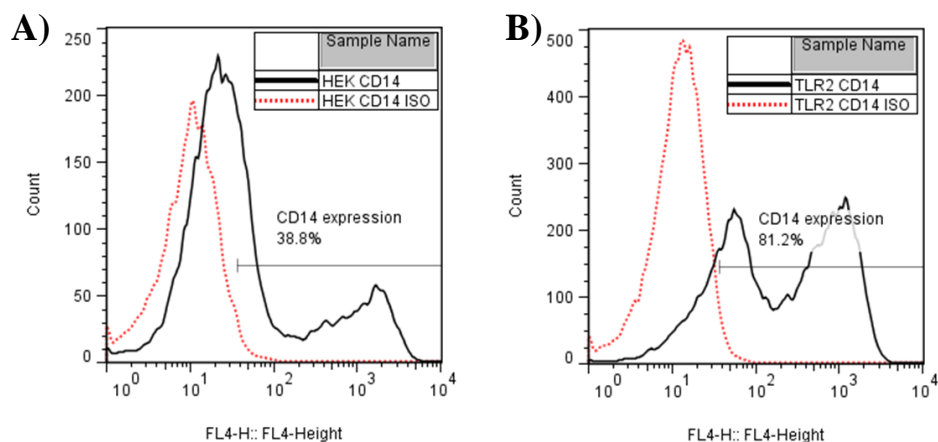


Figure 9: CD14 cell surface expression on HEK293T and HEK293-TLR2 transfectants

HEK293T (A) or HEK293-TLR2 (B) cells transfected with pcDNA3-hCD14, using FuGENE HD Transfection Reagent (Roche). After recovery for 48 hours cells were stained with anti-CD14 (black solid line) or isotype control (red dashed line) antibodies conjugated with APC. Samples were gated for live cells and analyzed for mean fluorescent intensity.

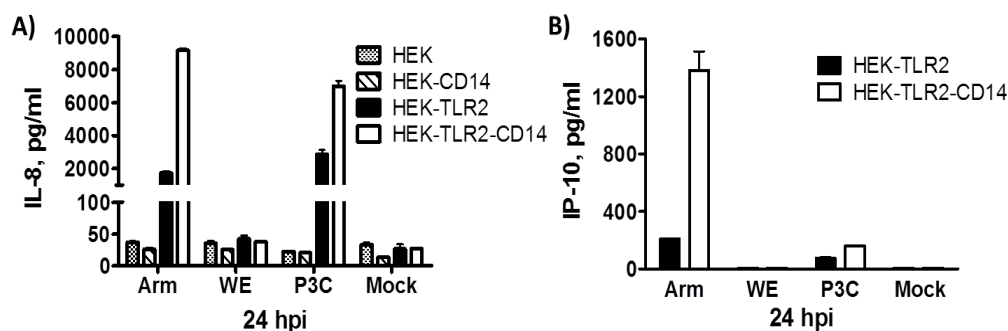


Figure 10: CD14 enhances cytokine production in HEK-TLR2 cells

HEK293T or HEK293-TLR2 cells were left untransfected or transfected with pcDNA3-hCD14 using FuGENE HD Transfection Reagent (Roche). Efficiency of CD14 expression (~40% and 80%, in HEK293T and HEK293-TLR2 cells, respectively) was confirmed by flow cytometry using an anti-CD14 antibody conjugated to APC (PharMingen) (see Figure 9). Untransfected or transfected cells were infected with LCMV-ARM or with LCMV-WE (MOI=1). Culture supernatants were collected at 24 hpi and assayed for IL-8 (A) and IP-10 (B) and virus titers (not shown). Results of a representative experiment (n=3) are shown. hCD14 enhanced the ability of LCMV-ARM to induce TLR2-dependent production of IL-8 and IP-10. In the absence of TLR2, hCD14 did not allow for LCMV-ARM induction of cytokine. LCMV-WE was not able to induce IL-8 or IP-10, even in the presence of TLR2 and hCD14.

Upon ligand recognition, TLR2 hetero-dimerizes with TLR1 or TLR6 co-receptors, creating docking platforms within their TIR domains to recruit a bridging adaptor, Mal (TIRAP), that in turn, associates with a signaling adaptor MyD88 via TIR-TIR domain homotypic interactions (253). To further decipher molecular details of TLR2-mediated sensing of arenavirus infections, leading to induction of proinflammatory cytokine responses, we sought to determine the involvement of Mal. We utilized immortalized bone marrow-derived macrophages (iBMDM) in which TLR2, CD14, or Mal were knocked out. These cells retain signaling characteristics of normal WT or knockout primary macrophages (117). Importantly, these cells provide a more “natural” physiological target, as LASV and LCMV primarily target monocytes, as well as DCs, during infection (203). Using these cells extends our finding to another cell type for TLR2, as well as CD14, and also provided the means to evaluate the requirement for Mal.

In the WT iBMDM, LCMV-ARM, but not LCMV-WE or LCMV-CL13, we observed a marked induction of IL-6 cytokine by ELISA. In contrast, we observed no significant production of keratinocyte chemokine (KC), the mouse equivalent of human IL-8, suggesting the specificity of type of cytokines produced in response to arenavirus infection. The induction kinetics of IL-6 by LCMV-ARM in WT iBMDM also appears to be delayed compared to HEK293-TLR2 or THP-1 cells, demonstrating the cell type-specific responses to arenavirus infection (Figure 11A). The synthetic TLR2/1 agonist, Pam₃CSK4, used as a positive control, failed to induce the robust cytokine response observed in the systems described above. However, analysis of cytokine induction at earlier time points in iBMDM cells, 4 and 8 hours post infection, demonstrate a robust induction of cytokine production by Pam₃CSK4 that peaks at 8 hours post infection,

while virus-induced cytokine responses aren't measurable until after 20 hours post infection. We observed robust cytokine production upon stimulation of WT iBMDMs with LPS, which was used to ensure that the TLR signaling machinery was intact, even in the absence of TLR2, whereas deficiency in Mal or CD14 significantly down-regulated LPS-mediated IL-6 secretion, consistent with the literature (253).

Compared to WT iBMDM, LCMV-ARM is still able to induce an equivalent cytokine response at 48 hours in the absence of TLR2 (Figure 11B). However, this response is reduced 4-fold at 72 hours post infection and indicates that TLR2 is required for sustained cytokine responses to arenavirus infection. In the absence of Mal, the bridging adaptor required by TLR2, the cytokine response to LCMV-ARM infection is reduced, confirming the role of TLR2 signaling in cytokine responses to arenaviruses (Figure 11C). The cytokine response to Pam₃CSK4 is also reduced, while LPS is still able to elicit a response, indicating that the signaling machinery is intact. Finally, the absence of CD14 doesn't negatively affect the cytokine response to LCMV-ARM infection in iBMDM cells, indicating that the ability of CD14 to enhance cytokine production in response to arenavirus infection may be cell and/or host dependent (Figure 11D). It is also possible that another co-receptor, such as CD36, is providing amplification of the LCMV-ARM induced signal and that the absence of CD14 allows for more efficient interaction between TLR2 and CD36. In all iBMDMs tested, LCMV-WE and LCMV-CL13 fail to induce a cytokine response. This is especially noteworthy as LCMV-CL13 differs from LCMV-ARM by only three amino acids. Taken together, these results solidify the requirement of TLR2 and its signaling adaptors in the cytokine response to nonpathogenic arenavirus infection. The inability of pathogenic arenaviruses

to induce a cytokine response is well established now in three cell types, originating from two different hosts.

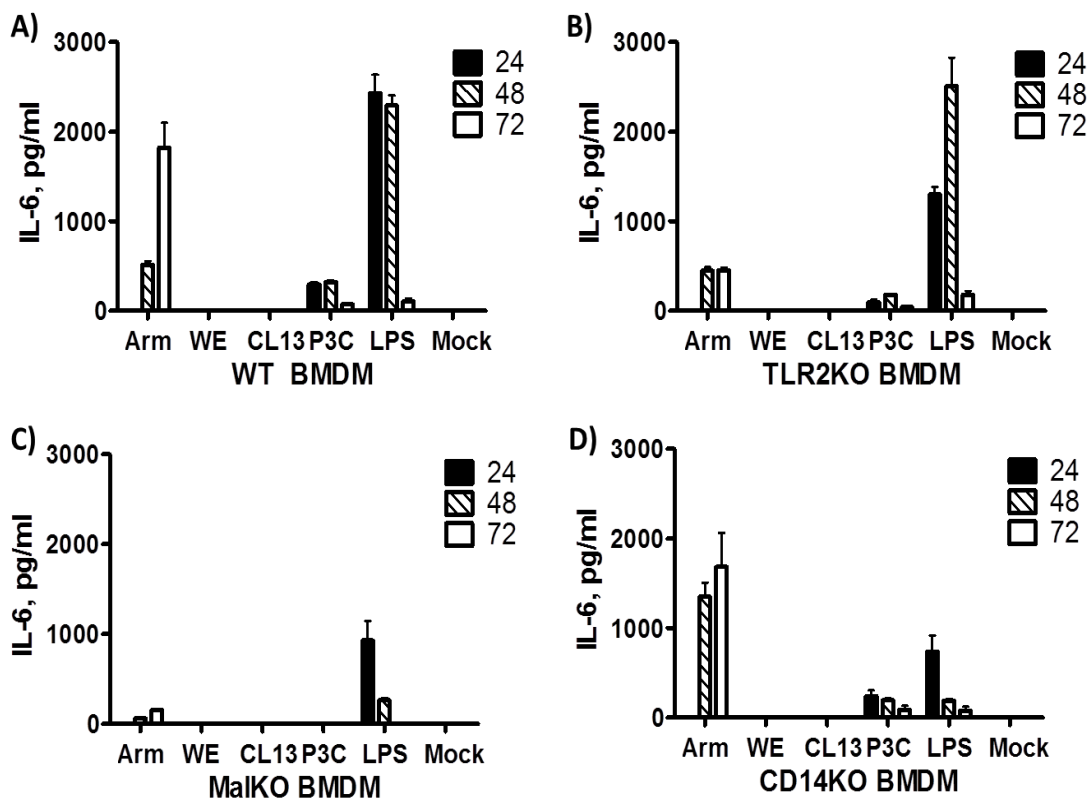


Figure 11: TLR2 and Mal are required for induction of IL-6 in LCMV-ARM infected cells

Immortalized bone marrow-derived macrophages (iBMDM) from C57BL6/J mice (wild-type), and from TLR2- (NR-9457), CD14- (NR-9570), and Mal-knockout mice (NR-9459) (BEI Resources) were infected with LCMV-ARM or LCMV-WE (MOI=1), or were treated with media (mock-infection), 100ng/ml Pam3CSK4, or 100ng/ml K:12 LPS (induction control) for 1 hr. Culture supernatants from WT (A), TLR2 KO (B), Mal KO (C) and CD14 KO (D) iBMDM cultures were collected at 24, 48, and 72 hpi and analyzed for IL-6 by ELISA (BioLegend). Results (mean +SD) of a representative experiment (n=3) are shown. Compared to WT iBMDM, LCMV-ARM induction of IL-6 is not sustained over 72 hours in TLR2 KO iBMDM. LCMV-ARM induction of IL-6 is completely ablated in MalKO iBMDM, but is enhanced in CD14KO iBMDM. In all cells, LCMV-WE and LCMV-CL13 are unable to induce IL-6.

Differential induction of NF κ B

It is well established that the TLR signaling pathway leads to activation of transcription factors to initiate the antiviral response. TLR2 typically activates NF κ B, among other transcription factors to induce production of proinflammatory cytokines and chemokines. To determine if NF κ B is differentially activated by arenaviruses with differing pathogenic potential, HEK293-TLR2 cells were co-transfected with pNF κ B-MetLuc2 (Clontech), a plasmid containing the NF κ B promoter and the luciferase gene with a secretory signal and a plasmid encoding SEAP, a secreted alkaline phosphatase, under a constitutive promoter. Cells were then infected with LCMV-ARM, LCMV-WE or LCMV-CL13. Additionally, cells were treated with Pam₃CSK4 or LTA as positive TLR2 controls, or media as a mock infection control. Culture medium was analyzed at 24 and 48 hours post infection for secreted luciferase, normalized to SEAP. Results reported in Figure 12 demonstrate that LCMV-ARM induced secretion of luciferase was significantly higher than infection with LCMV-WE or LCMV-CL13, indicating that NF κ B is differentially activated by these viruses. These results indicate that TLR2-mediated NF κ B activation is significantly ablated in response to pathogenic arenavirus infection. Future studies will be required to determine the molecular mechanisms of this ablation.

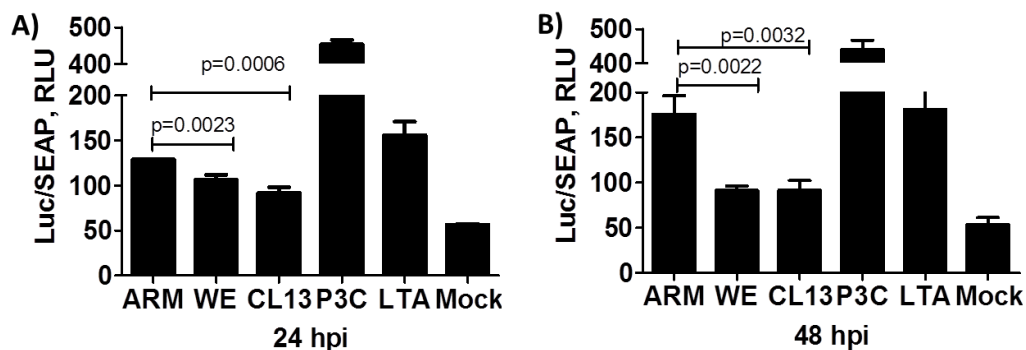


Figure 12: LCMV-ARM, but not LCMV-WE or LCMV-CL13 activates NF κ B

HEK293-TLR2 cells were transfected with pNF κ B-MetLuc2 and a constitutive SEAP reporter (Clontech) using the Amaxa Cell Line Nucleofector Kit V (Lonza). After recovery for 48 hours, cells were infected at MOI=1 with LCMV-ARM, LCMV-WE, or LCMV-CL13. For controls, cells were stimulated with Pam₃CSK4 treatment (100ng/ml), LTA treatment (10ug/ml) or treated with serum-free medium as a mock infection control. Luciferase values were normalized to SEAP values and are expressed as Relative Light Units (RLU). Twenty-four (A) and 48 (B) hours post infection, culture media samples were collected and analyzed for secreted luciferase in triplicate wells. Significance was determined using an unpaired, two tailed, Student's t-test. Data (mean +SD) are shown. LCMV-ARM induced significantly higher activation of NF κ B than LCMV-WE or LCMV-CL13, as evidenced by the increase in luciferase activity at both 24 and 48 hours post infection.

2.2 Viral factors influence cytokine production in response to OW arenavirus infection.

Viral Stocks

Viruses used in this study were plaque-to-plaque purified three times with the final amplification achieving titers at 10⁸ pfu/ml. To ensure that the differences observed between arenaviruses of differing pathogenic potential were not due to contamination of our viral stocks, we evaluated all virus stocks used in this study for proinflammatory

cytokines by multiplex assay. Data in Figure 13 shows that the level of these cytokines is consistent between arenavirus stocks.

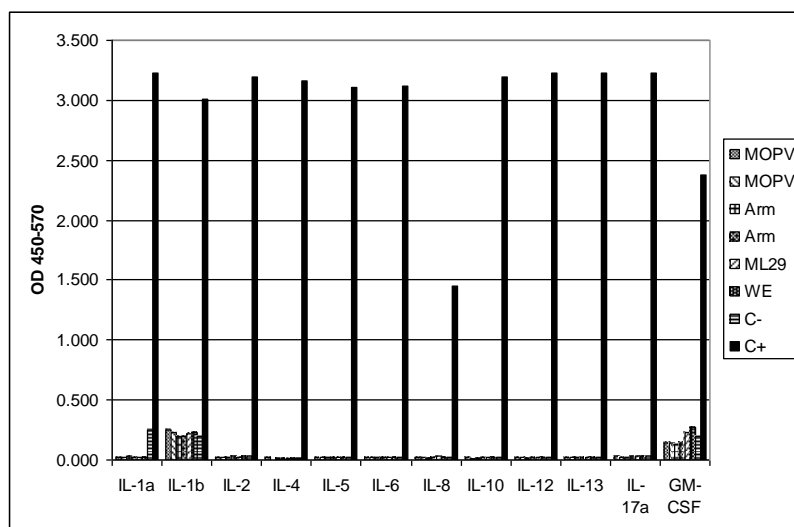


Figure 13: Multiplex cytokine analysis of arenavirus stocks for MOPV, ML29, LCMV-ARM, and LCMV-WE

Viral stocks were analyzed for the presence of proinflammatory cytokines using a multiplex, ELISA based assay. C- and C+ indicate negative and positive controls for each cytokine assayed. Cytokine levels were at or below negative controls and equivalent for each virus stock analyzed. For all cytokines analyzed, the level of cytokine in virus stocks was equivalent or less than the level detected in the negative control.

Viral replication is necessary and determines the magnitude of the proinflammatory cytokine response *in vitro*

A possible explanation for differences observed in cytokine production in response to infection with different viruses could be due to differences in cell permissibility or replication kinetics. To determine if this causes the differences in cytokine production with pathogenic and non-pathogenic arenaviruses, HEK293-TLR2 and THP-1 cells were infected with LCMV-ARM or LCMV-WE at MOI 1, 0.1, 0.01, and

0.001. Production of infectious particles was measured by plaque assay to determine the replication kinetics for each virus every twelve hours. Culture medium was analyzed for production of IL-8 by ELISA. Figure 14 C-D and Figure 15 A-B show that both viruses replicated equivalently at each MOI tested. It should be noted that THP-1 cells infected with LCMV-ARM or LCMV-WE at MOI = 0.001 produced infectious particles below the level of detection for this assay. Despite the lack of significant differences in virus replication, only LCMV-ARM and not LCMV-WE was able to induce the production of IL-8 in either cell type at any MOI (Figure 14 A-B, Figure 15 C-D). The level of cytokine induction appears to be associated with the level of replication as more cytokine is produced in cells infected with the higher MOI of LCMV-ARM.

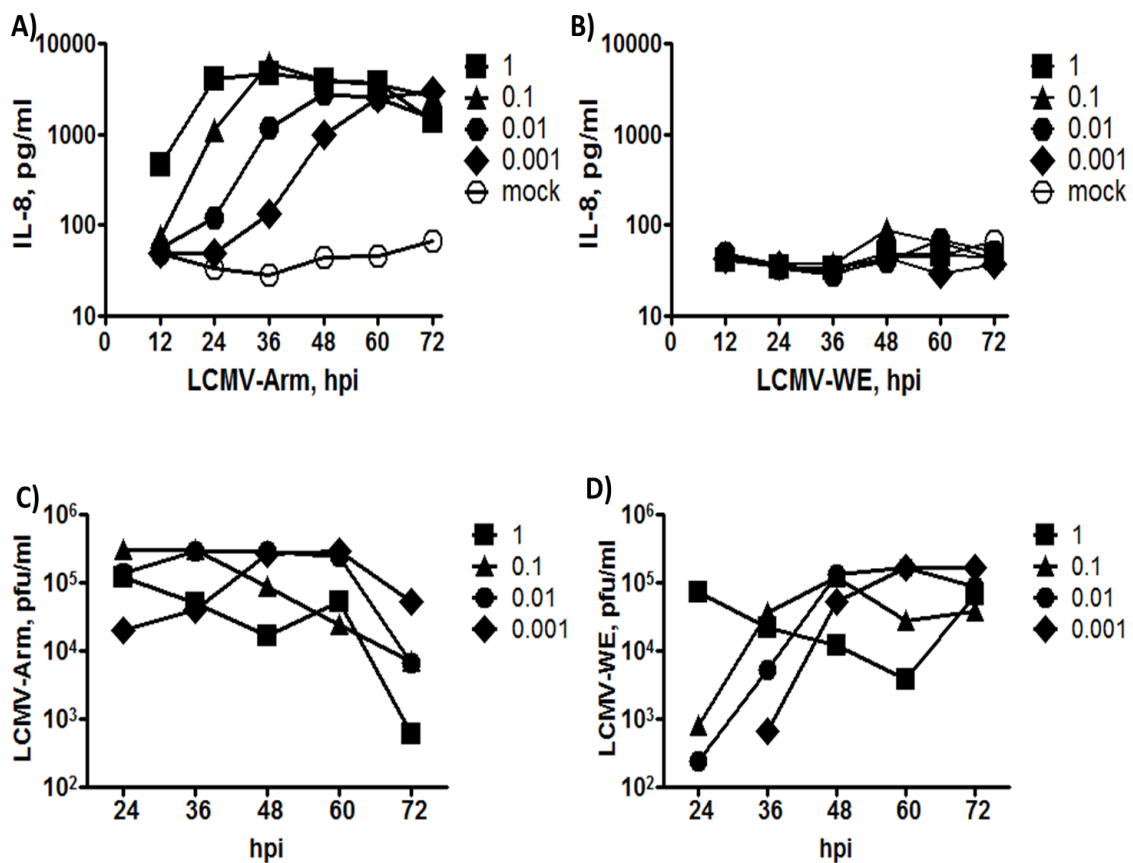


Figure 14: Effects of MOI on IL-8 production in HEK293-TLR2 cells

HEK293-TLR2 cells were infected with LCMV-ARM (A and C) or LCMV-WE (B and D) at different MOI or treated with serum-free media as a mock infection control. At the time points indicated, culture supernatants were collected and analyzed for IL-8 secretion (A and B) and infectious virus production (C and D). Results of a representative experiment (n=3) are shown. Despite differences in ability to induce cytokine in HEK293-TLR2 cells, replication kinetics are similar between LCMV-ARM and LCMV-WE. By 48 hours post infection, both viruses have achieved the same level of replication, although there was a delay in LCMV-WE replication at earlier time points.

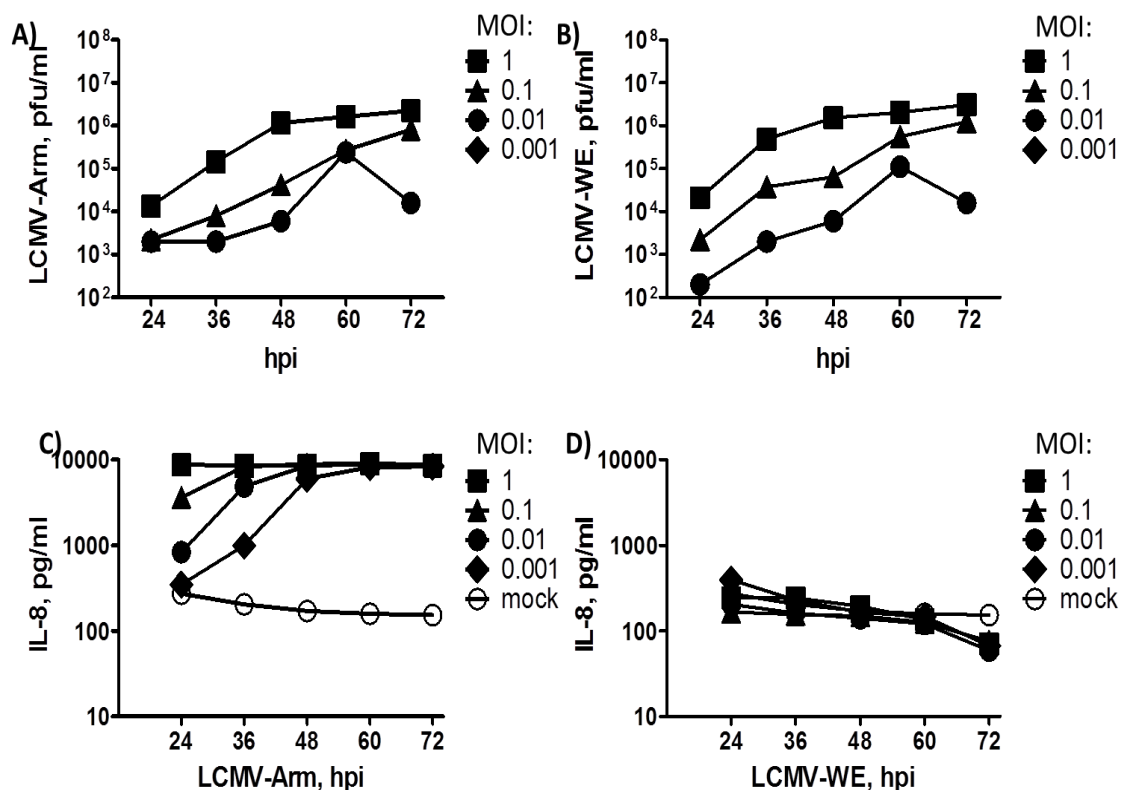


Figure 15: Effects of MOI on IL-8 production in THP-1 cells

THP-1 cells were infected with LCMV-ARM (A and C) or LCMV-WE (B and D) or treated with serum-free media as a mock infection control as described previously at different MOI and culture supernatants were analyzed for IL-8 secretion (C and D) and infectious virus production (A and B). At the MOI=0.001 the virus production was less than $2\log_{10}$ PFU/ml. Results of a representative experiment ($n=3$) are shown. Replication kinetics of LCMV-ARM and LCMV-WE in THP-1 cells appear to be nearly identical. However, only LCMV-ARM is able to induce IL-8 in a manner that is responsive to increasing multiplicities of infection and peaks during the highest level of replication.

The results in Figures 14 and 15 indicate that replication correlates with TLR2-dependent cytokine production in response to infection. To further confirm that viral replication is necessary for cytokine induction, LCMV-ARM and LCMV-WE were UV-inactivated or heat-inactivated and tested for their ability to induce cytokine. Inactivated virus was exposed to Vero cells to determine if residual infectivity remained. No infectious particles were observed up to 72 hours post exposure. HEK293-TLR2 cells

were then infected with LCMV-ARM or LCMV-WE, or exposed to inactivated virus and assayed for cytokine production. UV-inactivated (Figure 16A,B) and heat-inactivated (Figure 16C) LCMV-ARM failed to induce production of IL-8 or IP-10 indicating that live virus is necessary for the induction of TLR2-dependent cytokine responses.

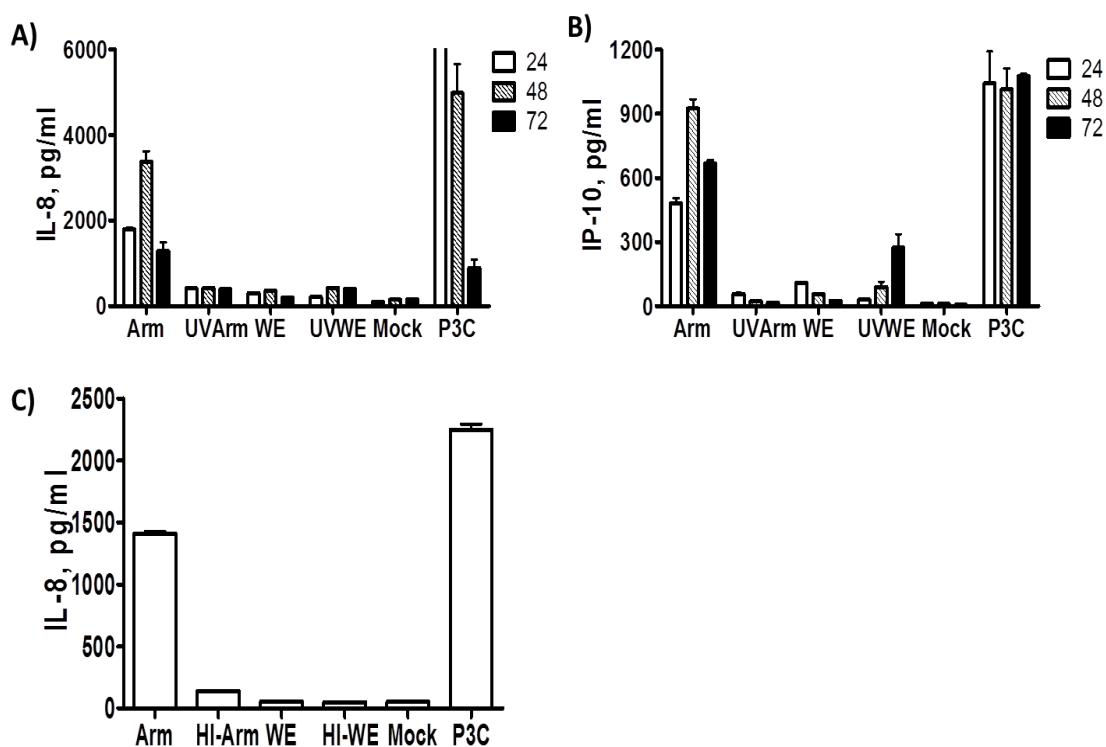


Figure 16: Live replicating virus is required to induce TLR2-dependent chemokine induction

LCMV-ARM and LCMV-WE were inactivated by incubation under UV light for 30 min on ice, or by heating at 56°C for 30 min. Residual infectivity of inactivated virus was assessed by plaque assay. HEK-TLR2 cells were infected (MOI=1) or treated with inactivated virus for 1 hr. At 24, 48, and 72 hpi, culture supernatants were collected and analyzed for IL-8 (A, C) and IP-10 (B) production by ELISA and for infectivity (by plaque assay). All tested samples did not show infectious activities. UVARM, UVWE, HI-ARM, and HI-WE indicate viruses inactivated by UV or by heating, respectively. Results (mean SD)⁺ of a representative experiment (n=3) are shown. UV-inactivated or heat-inactivated LCMV-ARM is no longer able to induce IL-8 or IP-10.

Membrane fusion inhibition

As presented above, replicating virus is required for robust induction of proinflammatory cytokine responses to non-pathogenic arenavirus infection whereas inactivated virus was unable to elicit a cytokine response. Generally, the live virus binds to the host cellular receptor and is taken up by receptor mediated endocytosis. The virus then enters the endosomal sorting complex and within 20 minutes has released its contents into the cytoplasm through pH-dependent membrane fusion, optimally at pH 5.3 (82). After genomic content release into the cytoplasm, replication begins. The eclipse time for arenaviruses is 9-15 hours after internalization (226). During this time, no release of newly assembled virus is observed. The release of virus particles from the host cell precedes the earliest time point in which arenavirus-induced cytokine production occurs. The earliest observation of robust cytokine production in response to LCMV-ARM infection was 20 hours post infection (results not shown). However, to rule out the possibility that arenaviruses are stimulating the TLR2-dependent response through the endosome after cell entry, Bafilomycin A1 (BAFA1), a specific inhibitor of vacuolar type H^+ -ATPase, was used to prevent acidification of the endosome and thus membrane fusion. Endosomal TLR3, 7/8, and 9 require acidification for ligand binding and signaling. Treatment with BAFA1 has been used to demonstrate the involvement of these endosomal TLRs in recognition of various ligands. BAFA1 treatment prevented TLR3 recognition of U1-snRNA, TLR7/8 recognition of imidazoquinoline resiquimod, and TLR9 recognition of bacterial CpG-DNA and HSV-2 (140, 153, 206, 302). However, the impact of BAFA1 treatment on TLR2 signaling is less clear. BAFA1 treatment during influenza infection had no effect on IL-8 production (364). Unlike

arenaviruses though, heat-inactivated influenza can elicit a cytokine response. BAFA1 treatment had no effect on hCMV-induced accumulation of IL-6 transcripts, but similar to influenza, live virus was not necessary (154). Encouragingly, it was recently demonstrated that blocking internalization of TLR2 or preventing acidification with BAFA1 could inhibit TLR2-dependent IFN-I induction by Pam₂CSK4 and Pam₃CSK4, but not proinflammatory responses (87).

As seen in Figure 17A, BAFA1 treatment demonstrated concentration-dependent inhibition of membrane fusion for LCMV-ARM with complete inhibition at 1 μ M. LCMV-WE was more resistant to BAFA1 fusion inhibition, but achieved roughly 50% inhibition at 1 μ M BAFA1. This is in good correlation with previous studies comparing LASV and MOPV low pH dependence in which MOPV was more sensitive to membrane fusion inhibition by NH₄Cl than LASV (125). As expected, LCMV-WE did not induce IL-8 at any concentration of BAFA1 (Figure 17C). Unexpectedly, significantly higher levels of IL-8 were observed in LCMV-ARM infected cells at concentrations of BAFA1 that caused the greatest fusion inhibition (Figure 17B). To account for non-specific increases in IL-8 production in the presence of BAFA1, LCMV-ARM induced IL-8 levels were normalized to zero-BAFA1 LCMV-ARM infection controls (Figure 17D). Pam₃CSK4 and Mock controls at 1 μ M were normalized to their respective zero-BAFA1 controls as well. While there appears to be a modest increase of IL-8 in the presence of high concentrations of BAFA1 for Pam₃CSK4 and mock controls (1.2 and 1.5-fold respectively), LCMV-ARM induced 2-fold and 4.5-fold increases in IL-8 production at 500nM and 1000nM respectively. Total protein levels were determined for each sample using a BCA Protein Assay Kit (Thermo Scientific). Normalization of cytokine levels to

total protein levels didn't alter the pattern of induction and likely doesn't account for differences observed in increasing concentrations of BAFA1 (data not shown).

To explain these results, we postulate that LCMV-ARM signals from the endosome in a replication-independent manner. Pretreatment with BAFA1 followed by exposure to inactivated virus may provide experimental results to address this hypothesis, unless there are unknown requirements of a live virus to achieve this step in the virus life cycle. Another possibility is that by retaining virus particles in the endosome, being unable to fuse and release its contents, the particle would be degraded into components that could be activating other innate immune sensors. To determine if this is the case, gene ablation strategies for other known innate sensors could be employed to determine their impact on cytokine production upon viral infection.

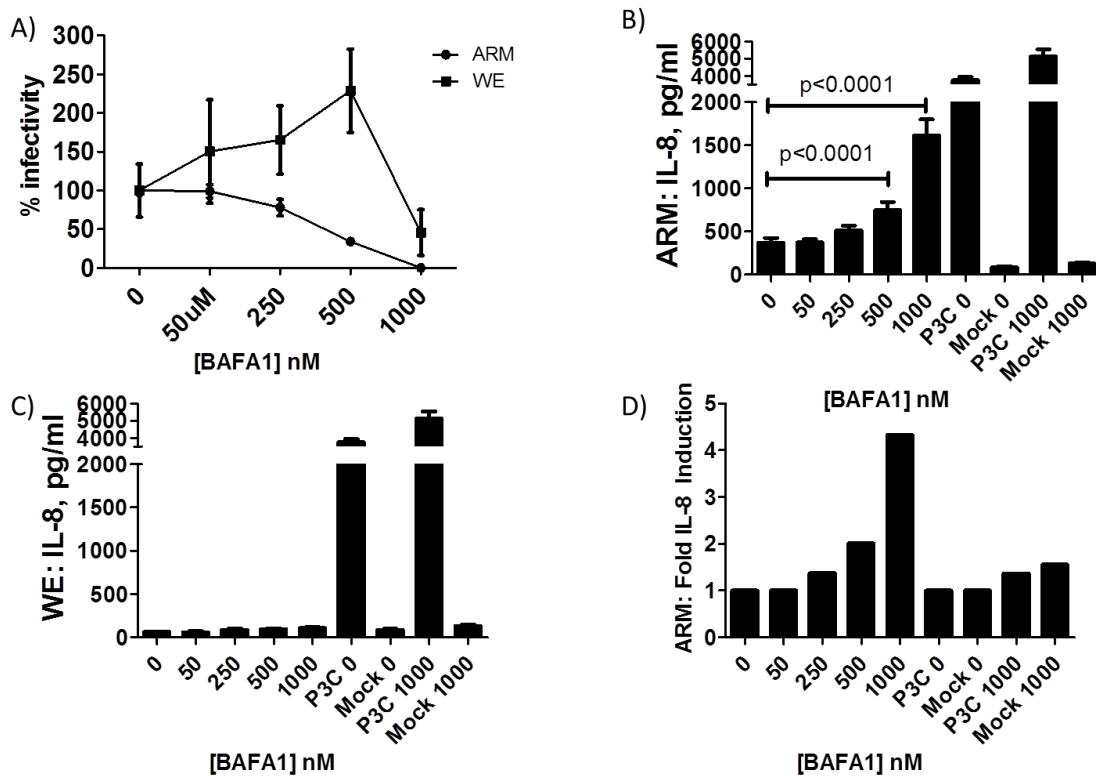


Figure 17: BAFA1 inhibition of membrane fusion results in increased LCMV-ARM induced IL-8 production

HEK293-TLR2 cells were treated with increasing concentrations of BAFA1 from *Streptomyces griseus* for 30 minutes. Cells were then infected with LCMV-ARM or LCMV-WE in the presence of BAFA1 as described previously. To determine if BAFA1 was successful in inhibiting membrane fusion, viral replication was determined by plaque assay (A) and is shown as percent infectivity based on no treatment. At 24 hours post infection, culture medium was assayed by ELISA for IL-8 production for LCMV-ARM (B) and LCMV-WE (C) infected cells. LCMV-ARM fold induction of IL-8 is shown for treated cells compared to no treatment control (D). Data shown is mean +SD. BAFA1 treatment effectively inhibited LCMV-ARM replication at the highest concentrations tested. LCMV-WE was more resistant to treatment with BAFA1, but replication was inhibited by 50% at 1 μ M BAFA1. While LCMV-WE did not induce cytokine at any concentration of BAFA1, as expected, LCMV-ARM induced higher levels of IL-8 with higher concentration of BAFA1. Fold induction of LCMV-ARM induced IL-8 in the presence of higher concentrations of BAFA1 were greater than non-specific increases in Pam₃CSK4 or Mock induction of cytokine at higher concentrations of BAFA1.

LCMV-WE inhibition of LCMV-ARM-induced proinflammatory response

Lack of cytokine induction by pathogenic arenaviruses could be due to evasion of TLR2 recognition or inhibition of TLR2 signaling. To determine if LCMV-WE actively inhibits cytokine production, a series of co-infections were conducted using LCMV-ARM and LCMV-WE. If LCMV-WE is evading induction of a cytokine response, co-infection with both viruses should yield cytokine production similar to LCMV-ARM single infection. If LCMV-WE is actively inhibiting cytokine production, then co-infection with both viruses should yield less cytokine than LCMV-ARM single infection. THP-1 cells were infected with LCMV-ARM at T_0 . These cells were then co-infected one hour prior to, concurrently, or 1-12 hours post LCMV-ARM infection (Figure 18A). Control single LCMV-ARM and LCMV-WE infections, as well as Pam₃CSK4 treatment and Mock infection, were done at T_0 . LCMV-WE co-infection up to six hours post LCMV-ARM infection is able to drastically reduce the level of cytokine production. Even at LCMV-WE co-infection 12 hours post LCMV-ARM infection, the level of cytokine production is significantly reduced ($p=0.0137$). Quantitative PCR-based quantification of viral mRNA, specific for LCMV-ARM or LCMV-WE, demonstrated that each virus replicates in co-infected cells (Figure 18B). Amplified product from qPCR analysis was separated by electrophoresis on a 4% agarose gel to verify amplification and specificity of primers (Figure 18C). To determine if live LCMV-WE is necessary for inhibition of LCMV-ARM or Pam₃CSK4-induced cytokine production, THP-1 cells infected with LCMV-ARM or treated with Pam₃CSK4 at T_0 were treated with UV-inactivated LCMV-WE one hour prior, concurrently, or 1-12 hours post LCMV-ARM infection. Except for a slight decrease at T_3 , UV-WE is unable to inhibit

LCMV-ARM induced IL-8 production (Figure 18D). This indicates that, in conjunction with live virus being necessary for cytokine induction, live virus is also required for inhibition. At first glance, it appears that UV-inactivated LCMV-WE is also able to inhibit Pam₃CSK4-induced IL-8 (Figure 18E). However, the induction of cytokine by this synthetic ligand is very quick and robust. After the initial exposure to Pam₃CSK4, unlike in viral infection, the source of stimulation is removed. Viral inoculums are removed one hour after initial cell culture exposure. Thus, the later the time point in which UV-LCMV-WE is added to Pam₃CSK4 pretreated cells, the more IL-8 is removed with the inoculum and thus appears to be inhibited. This is not true inhibition of cytokine induction, however, as these TLR2 ligands are very different in their cytokine induction and presence of ligand. Additionally, Pam₃CSK4 is a TLR2/1 ligand, while the results reported above (Figure 7) indicate that LCMV-ARM induced a TLR2/6-dependent response. Thus, Pam₃CSK4 is not the most appropriate ligand to evaluate the ability of LCMV-WE in inhibit exogenous signaling. For these purposes, we used Lipoteichoic acid (LTA), a TLR2/6 ligand to evaluate the ability of LCMV-WE to inhibit exogenous signaling.

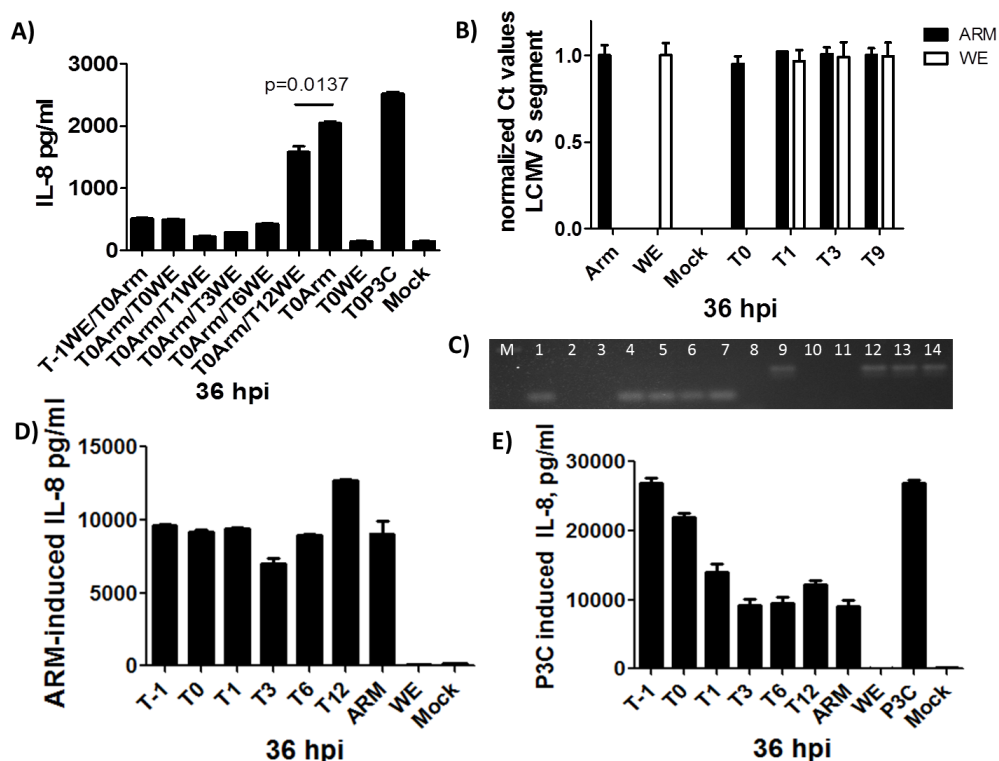


Figure 18: LCMV-WE inhibits LCMV-ARM-induced IL-8 response

(A-B) THP-1 cells were infected with LCMV-WE one hour prior to, simultaneously, or 1-12 hours post LCMV-ARM infection. All time points are based on LCMV-ARM infection at time zero (T₀). T₋₁WE/T₀ARM indicates WE infection one hour prior to ARM infection. T₀ARM/T₀WE indicates simultaneous co-infection with both viruses. T₀ARM/T₁WE, and subsequent time points (T₃, T₆, T₉, T₁₂) indicate WE infection after ARM infection. Single infection controls, mock, and Pam₃CSK4 controls were conducted at T₀. At 13 hours post T₀ infection, culture medium for all conditions was replaced with fresh media. (A) Final culture supernatant samples were collected at 36 hours and analyzed for IL-8 production by ELISA as described previously. (B) RNA was extracted, reverse transcribed, and analyzed by qPCR using primers and probes specific for divergent sequences on the S segment (see Methods and Materials). All Ct values were normalized to β -actin control and then normalized to single infection control. (C) qPCR products were run on a 4% agarose gel in 1xTAE to verify results. Lanes are assigned as follows: M, marker lane; 1-7, ARM-specific amplification: 1, ARM infection; 2, WE infection; 3, mock infection; 4-7, T₀, T₁, T₃, and T₉ infections, respectively; 8-14, WE specific amplification: 8, ARM infection; 9, WE infection; 10-14, mock, T₀, T₁, T₃, and T₉, respectively. Results of a representative experiment (n=3) are shown. (C-D) UV-inactivated WE was used to treat ARM-infected (D) or P3C treated (E) cells as described above. LCMV-WE is able to inhibit LCMV-ARM-induced IL-8 production when co-infected with WE one hour prior to ARM infection, or up to 12 hours post ARM infection. Both viruses are able to replicate similarly in co-infected cells. UV-inactivated LCMV-WE is unable to inhibit LCMV-ARM induce IL-8 production.

LCMV-WE does not inhibit exogenous TLR2 stimulation

To more accurately determine if LCMV-ARM or LCMV-WE can inhibit cytokine induction by exogenous TLR2 ligands, LTA, a TLR2/6 agonist, was added to LCMV-ARM-infected or LCMV-WE-infected THP-1 cells concurrently, or 1-12 hours post infection. LCMV-ARM induced robust production of IL-8 while LTA induced more modest levels (Figure 19A). However, the combination of LCMV-ARM and LTA resulted in IL-8 levels that were equivalent to the additive value of either LCMV-ARM infection or LTA treatment alone, indicating a lack of interference or competition between LCMV-ARM and LTA for TLR2-dependent induction of cytokine. As expected, LCMV-WE failed to induce production of IL-8 (Figure 19B). However, LTA treatment after LCMV-WE infection led to the induced secretion of IL-8 similar to LTA treatment alone. When LTA and LCMV-WE were added simultaneously, it appears that IL-8 levels were higher than in LTA treatment alone. LTA-induced production of IL-8 in LCMV-ARM or LCMV-WE infected cells was normalized to IL-8 levels in LTA treated THP-1 cells. When normalized to LTA treatment alone (Figure 19C) or LCMV-ARM single infection (Figure 19E), there was no apparent interference or inhibition in cytokine induction between LTA and LCMV-ARM. In LCMV-WE infected cells, co-treatment with LTA at T_0 results in an increase of IL-8 production, 2.5-fold higher than LTA treatment alone (Figure 19D). At later time points, LTA treatment of LCMV-WE infected cells induced similar amounts of cytokine as LTA treatment in uninfected cells, indicating that LCMV-WE does not inhibit exogenously induced TLR2-dependent cytokine responses. Taken all together, the mechanism by which LCMV-WE inhibits LCMV-ARM-induced cytokine responses may be virus, or even arenavirus, specific.

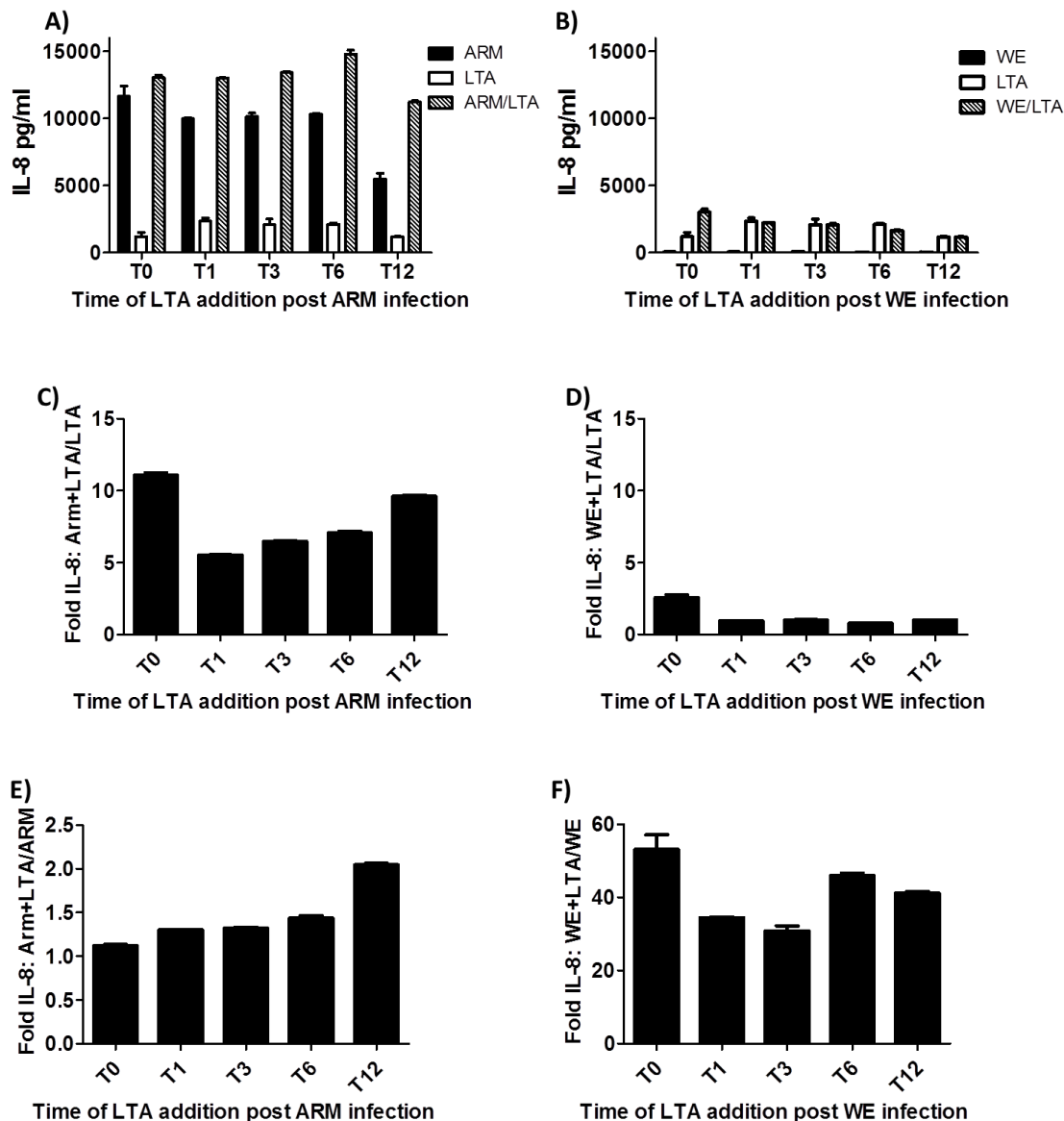


Figure 19: LCMV does not inhibit exogenous stimulation of TLR2/6

THP-1 cells were infected with LCMV-ARM (A,C,E) or LCMV-WE (B,D,F) and were treated with 10 μ g/ml LTA concurrently, or 1, 3, 6, or 12 hours post infection. Culture medium was collected at 36 hours post infection and analyzed by ELISA for IL-8 (A-B). To normalize for differences in cytokine induction kinetics, IL-8 values were normalized to levels determined by LTA treatment alone at each time point (C-D) or to single virus infection at each timepoint (E-F). The addition of LTA to LCMV-ARM infected cells resulted in IL-8 production that was equivalent to the additive values of LCMV-ARM and LTA alone. LCMV-WE was unable to inhibit LTA induction of cytokine, indicating that LCMV-WE is not able to inhibit exogenous TLR2/6-dependent signaling.

Conclusions

In this aim, I addressed both cellular and viral factors that may contribute to the induction of proinflammatory cytokines by arenaviruses of differing pathogenic potential. For cellular contributions, having identified TLR6 as the necessary co-receptor for arenavirus induced TLR2 responses, I addressed the requirement for CD14 and the TLR2 bridging adaptor Mal. Additionally, activation of NF κ B was analyzed for different strains of LCMV. For viral contributions, having established the quality of our viral stocks, I evaluated the contribution of multiplicity of infection, replication and membrane fusion for LCMV-ARM induced TLR2-dependent responses. Additionally, the ability of LCMV-WE to inhibit LCMV-ARM or LTA induced TLR2-dependent cytokine responses was analyzed.

TLR1 or TLR6 are necessary co-receptors for TLR2 signaling in response to stimulation with tri- or di-acylated lipoproteins. Additionally, CD14 may be required or may enhance the response (185, 236). In LCMV-ARM induced TLR2-dependent production of proinflammatory cytokines, hCD14 was not found to be required for production of IL-8 in HEK293-TLR2 cells. However, the presence of hCD14 enhanced IL-8 and IP-10 production 5-fold for LCMV-ARM infection of HEK293-TLR2 cells. In the absence of TLR2, hCD14 could not rescue the cytokine response, further confirming the requirement for TLR2 and demonstrating that hCD14 alone is not sufficient for cytokine production in response to LCMV-ARM infection. LCMV-WE was not able to elicit a cytokine response, even in the presence of hCD14. While the expression of hCD14 enhanced signaling in HEK293-TLR2 cells, its absence did not reduce cytokine production in iBMDM of a murine host. In this case, other co-receptors may be playing a

role in TLR2 signaling, such as CD36. The requirement for TLR2 was confirmed in iBMDM cells, as well as the requirement for Mal, the bridging adaptor for TLR2.

Signaling through TLR2 results in activation of transcription factors to induce cytokine production. NF κ B is the major transcription factor activated by TLR2 and the pathway from TLR2 stimulation to IL-8 production is well documented (see Figure 1). In the differential induction of IL-8 in response to arenavirus infection, the signaling interruption may occur upstream of NF κ B signaling as there are significant differences in LCMV-ARM vs. LCMV-WE or LCMV-CL13 activation of NF κ B. Interruption of signaling could occur as early as TLR2 ligand binding or by inhibiting the phosphorylation of the intermediate molecules involved in activating NF κ B. It is also possible that replicating LCMV-WE may induce a negative regulator of NF κ B activation which would achieve the same results. Determining the point at which signaling is disrupted will be a future area of study.

Having established the quality of viral stocks by plaque-to-plaque purification and cytokine analysis, several viral factors contributed to the activation of the innate immune response through TLR2. Multiplicity of infection dictated the level of cytokine induction in that the higher the MOI used, the higher the level of cytokine produced. Replication was also strongly involved in cytokine production as peak levels of cytokine production occurred at peak replication. Additionally, replication incompetent virus, rendered so through UV or heat inactivation, failed to elicit a cytokine response. While high replication of virulent virus is the strongest indicator of a poor outcome in LHF, LCMV-WE was unable to elicit a cytokine response at any MOI used or at any point during replication. Unfortunately, efforts to determine whether membrane fusion was required

for proinflammatory responses were inconclusive and will require further investigation. In addition to the possible explanations for increased IL-8 production in the absence of membrane fusion, a TLR2 ligand control that signals only from the endosome would help clarify these results.

The lack of cytokine induction by LCMV-WE is due to an active inhibition of the arenavirus-specific cytokine response, as demonstrated by co-infection results. LCMV-WE was able to inhibit cytokine production by LCMV-ARM up to 12 hours after initial infection. Viruses were able to replicate in co-infected cells, indicating that failure to induce cytokine was not due to replicative dominance by one virus over another. However, UV-inactivated LCMV-WE was not able to inhibit LCMV-ARM induced cytokine, indicating that live virus is necessary for inhibition as well as induction. Finally, LCMV-WE failed to inhibit LTA induction of cytokine, demonstrating that the inhibitory nature of LCMV-WE is specific to the manner in which arenaviruses induce cytokine and not exogenous ligands. Whether LCMV-WE is able to inhibit other viruses is undetermined and will be an interesting future study.

Chapter Six

Conclusions and Final Statements

The striking immunological differences between the natural rodent host of arenaviruses (with the exception of TCRV) and human hosts likely accounts for the distinct infection outcomes. While the rodent host has a lifelong, asymptomatic, persistent infection, humans either clear an acute infection with non-pathogenic arenaviruses, or develop severe disease, possibly hemorrhagic fever, that may be fatal. In the rodent host, IFN-I likely plays a critical role in resistance and/or maintenance of arenavirus infection. LASV infection of Stat1 knock-out, but not B, CD4, or CD8 T cell knock-out mice resulted in 67% lethality, indicating the importance of IFN-I in resistance to LASV infection (54). Additionally, all strains of arenaviruses, with the exception of bat-borne TCRV, encode an antagonistic function in their nucleoprotein. The NP was shown to block IRF3, a major regulator of RIG-I-inducible responses (218, 219). This function was specifically mapped in LASV NP, where amino acid residues D389 and G392 were critical in suppression of IFN in murine macrophages and DCs (57). Thus it is likely that regulation of the IFN-I response contributes to the balance between innate antiviral measures to regulate viral replication while allowing for persistent infection in the rodent host.

In humans, LASV is a poor IFN-I inducer, is relatively resistant to IFN, and IFN sensitivity of LASV isolates does not correlate with disease progression (12). Severe LASV infection is characterized by unchecked viremia, functional liver damage, and immunosuppression (222, 238). Recovery and protection is dependent on T-cell responses and is not associated with production of specific

IgG. In human DC-T-cell co-cultures, LASV induced only weak memory phenotype markers, while MOPV strongly stimulated CD8⁺ and CD4⁺ T cells, activation markers, proliferative responses, and CTL activities (264). We can speculate that TLR2-mediated stimulation of cytokines in MOPV-infected cells [Figures 4 and 5, see also (203)] contributes to development of strong adaptive immune responses. Similarly, in LCMV-ARM infected mice, TLR2/Mal/MyD88 signaling played an essential role in antiviral CD8⁺ T cell responses (388). In the absence of MyD88, naive CD4⁺ T cells failed to differentiate to LCMV specific CD4⁺ T cells (387). Whether the inhibited induction of cytokines is a direct or indirect outcome of virulent virus infection, the lack of innate stimulation likely positions the host to have a delayed adaptive immune response that would effectively clear the infection. Additionally, suppression of cytokine responses, whether IFN-I or proinflammatory might be necessary but insufficient contributions to manifestation of disease and may rely on other factors, such as viral replication, though replication was equivalent *in vitro* [Figures 14 and 15, see also (203)].

The immunosuppressive phenotype of LASV infection in humans or non-human primates is generally well accepted (15, 58, 238, 264, 384). A lack of cytokine induction has been documented with pathogenic LASV and LCMV-WE (203-205). Exposure of PBMC from healthy donors to LASV demonstrated that IFN-related and apoptotic genes, as well as NFκB and coagulation pathways were the most highly affected in gene expression analysis (382). In patients succumbing to LASV infection, the absence of IL-8 and IP-10, among a few other

cytokines, was highly correlated to fatal outcome (212). MOPV, but not LASV, infection of human DCs resulted in the activation of APCs, strong T cells responses and activation of memory T cell markers (264). However, the contribution of TLR2 in LASV pathogenesis of this remains a question.

With the identification of TLR2 as a mediator of proinflammatory responses to arenavirus infection, a closer investigation into the role of TLR2 in arenavirus pathogenesis is warranted (155, 386, 388). During preparation of this thesis, a recent publication demonstrated that the vaccine strain of JUNV, Candid 1, elicited TLR2-dependent proinflammatory cytokines in murine macrophages (77). Another group comparing a pathogenic strain of JUNV to closely related, but non-pathogenic, TCRV, demonstrated that TCRV, but not pathogenic JUNV, induced high levels of proinflammatory cytokines in mice (132). Thus, the increasing body of evidence suggests that an early induction of cytokines enhances innate immune responses and promotes effective adaptive immune responses to prevent the development of hemorrhagic fever during arenavirus infection.

The mechanism by which TLR2-dependent cytokine responses are induced during arenavirus infection, and conversely, how those responses are inhibited are unclear. Here, we have examined the contribution of cellular and viral factors to gain insight into these mechanisms. Importantly, It is not yet known which arenaviral gene or genome segment is essential for TLR2 signaling.

Using cytokine production as a measure of TLR2 stimulation, we have demonstrated that TLR6 is the co-receptor for TLR2 in response to arenavirus

infection (Figure 7). CD14, a component of the TLR2 signaling complex was not necessary for arenavirus-induced cytokine production, but was able to enhance the level of cytokine depending on the cell type (Figures 10 and 11). Another member of the signaling complex, Mal, was determined to be necessary for cytokine production (Figure 11). Infection with strains of differing pathogenic potential had similar effects on TLR2 expression at the cell surface, ruling out the possibility that inhibition or absence of cytokine responses were a result of differences in TLR2 expression (Figure 8). Finally, strains of LCMV differentially activated NF κ B, with LCMV-ARM eliciting significantly higher levels of NF κ B reporter activation than LCMV-WE or LCMV CL13 (Figure 12). Striking differences between LCMV-ARM and LCMV-CL13 are notable, given that there are only a few amino acid differences in their consensus sequences. Future studies into possible differential activation of TLR signaling mediators involved in NF κ B activation are expected to determine the point in the signaling cascade at which cytokine induction is inhibited.

TLR2-dependent proinflammatory responses are induced at the plasma membrane. However, we have demonstrated that UV or heat inactivated-LCMV is no longer able to elicit such a response (Figure 16). Additionally, multiplicity of infection, as well as replication, was strongly linked to the level of cytokine induction (Figures 14 and 15). Taken together, this data indicates that live replicating virus is necessary for efficient induction of proinflammatory immune responses. Further, because inactive virus was unable to induce TLR2-dependent responses, the possibility exists that arenaviruses stimulate secretion

of a danger-associated molecule capable of stimulating TLR2. Knowing that live replicating virus is necessary for TLR2-dependent cytokine induction, it is probable that TLR2 stimulation occurs after membrane fusion and release of genomic material into the host cytoplasm. Unfortunately, experiments with membrane fusion inhibitors were unable to definitively answer this question and will remain an area of future study (Figure 17).

Co-infection experiments demonstrated the ability of pathogenic LCMV-WE to actively inhibit the cytokine response elicited by LCMV-ARM (Figure 18A). LCMV-WE was found to inhibit the cytokine responses most effectively up to 6 hours post infection, and partially up to 12 hours post LCMV-ARM infection. By this point in the virus life cycle, nucleoproteins, glycoproteins, and zinc proteins, as well as genomic and mRNAs are being synthesized. Any one of these components may be involved in suppressing the production of cytokines. Of the known viral TLR2 ligands, glycoproteins, core proteins, and nonstructural proteins are capable of stimulating TLR2 (29, 36, 62, 90). Just as live virus was necessary for TLR2-dependent cytokine induction, it was also necessary for inhibition, as UV-inactivated LCMV-WE failed to inhibit LCMV-ARM induced cytokine production (Figure 18C). Similar to TLR2 stimulation, viral proteins or RNAs produced during replication may interrupt the NF κ B activation pathway.

LCMV-WE also could not inhibit exogenous stimulation of TLR2-induced cytokine responses. The addition of LTA in the presence of LCMV-ARM or LCMV-WE infection elicited cytokine production equivalent or greater than LTA treatment alone (Figure 19). This implies that the manner in which LCMV-WE

inhibits the TLR2-dependent cytokine response is arenavirus-specific. Further investigation with other known TLR2-stimulating viruses will answer the specificity of LCMV-WE inhibition.

Two important questions remain: how does TLR2 signaling contribute to resistance/recovery from arenavirus infection and how could the virus inhibit TLR2 signaling? To determine the importance of TLR2 signaling in protection against arenavirus infection, the correct animal model must be used. Mouse models of arenavirus infection rely on IFN-I rather than inflammatory cytokines and cytotoxic T cell responses. These mouse models provide more information for chronic infections rather than severe acute infections, such as that caused by LASV in humans. The non-human primate model most closely resembles the disease progression seen in humans. However, methods to decipher the contribution of TLR2 are more difficult to pursue in such models. The use of function blocking antibody treatment may provide some insight. Studies could then be directed toward the activation of APC, T cells, viral load, etc. in the absence or reduction of TLR2 signaling. These types of studies could help elucidate the contribution of TLR2 signaling in the model presented below (Figure 20), in which pathogenic arenavirus fails to create an antiviral environment during the early stages of infection broadly affecting the ability to elicit an efficient adaptive immune response.

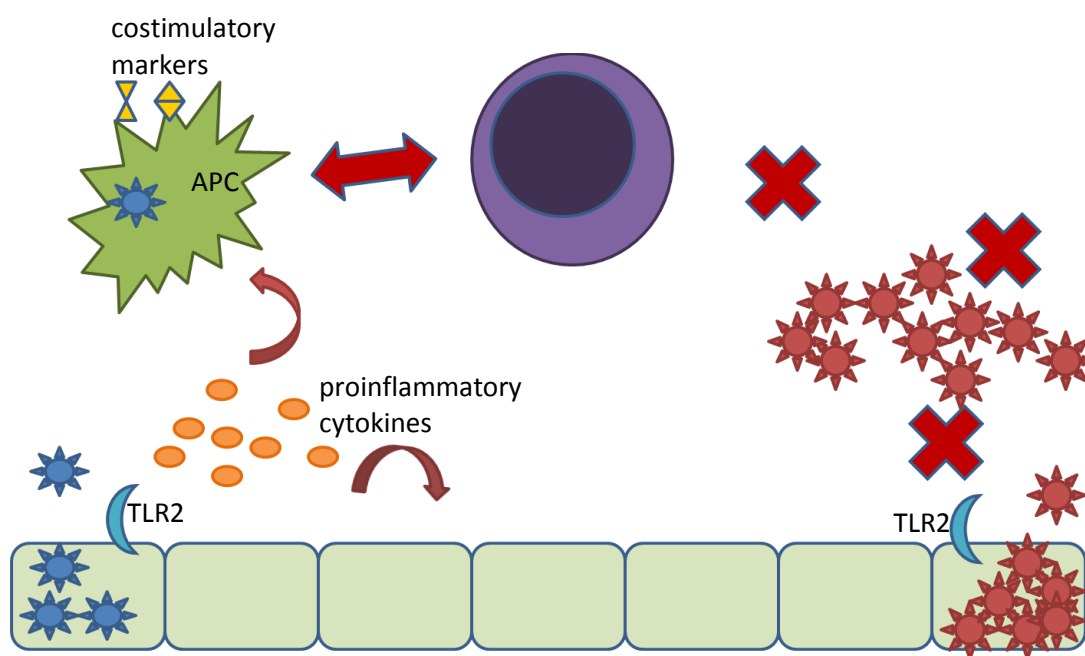


Figure 20: Proposed model for contribution of TLR2 signaling in protection against arenavirus infection

Non-pathogenic arenavirus (blue) infects host cells and begins to replicate, stimulating TLR2. TLR2-dependent signaling induced the production of cytokines creating an anti-viral environment, recruiting innate effector cells, and inducing the activation of APC. APC are then able to efficiently activate an adaptive cytotoxic T cell response, by presenting viral antigen and upregulating costimulatory molecules, which is able to clear the infection. Pathogenic arenaviruses (red) fail to stimulate TLR2, allowing for unrecognized infection and

uncontrolled replication. APCs are not induced towards maturation and cannot effectively activate an adaptive cytotoxic response.

Along with the global effects of TLR2 signaling in arenavirus infection, it is still necessary to determine how arenaviruses stimulate the TLR2-dependent response and how this is inhibited during pathogenic arenavirus infection. The results presented in this thesis demonstrate the requirement for live replicating virus and the differential activation of NF κ B. From these results, we hypothesize that stimulation of TLR2 occurs during the replication cycle of the virus, whether via a protein-derived ligand, or through viral RNA recognition (Figure 21). It is also possible that during the replicative cycle of the virus, the stress induced on the host cell results in release of danger signals that could then stimulate TLR2 (327). While results with fusion inhibitors were inconclusive, demonstrating the requirement for genomic content release is still an important and necessary step to support this theory. One possible method to determine the contribution of intracellular TLR2 signaling would be the use of intrabodies directed towards TLR2. Intrabodies are intracellular antibodies that are expressed and retained in the endoplasmic reticulum (170). Recently, an intrabody directed towards TLR2 was reported that was derived from an antagonistic mAb towards human and murine TLR2. This intrabody was able to retain TLR2 from the plasma membrane effectively and prevent NF κ B activation from the cell surface. Additionally, the use of cross-linking studies and molecular imaging would also be useful in determining how arenaviruses are stimulating TLR2. Ideally, one would like to singly express protein components of arenaviruses to determine if

they stimulate TLR2. However, the requirement for live replicating virus confounds these efforts. The use of virus replicon particles (VRP) in which a replication defective virus particle encodes a gene under a separate promoter may help shed some light on this issue. VRPs could be used to express a particular gene of interest from either pathogenic or non-pathogenic arenaviruses. Additionally, using reverse genetics, proteins of pathogenic arenaviruses could be interchanged with the non-pathogenic protein, providing the replication necessary while singly expressing the pathogenic component.

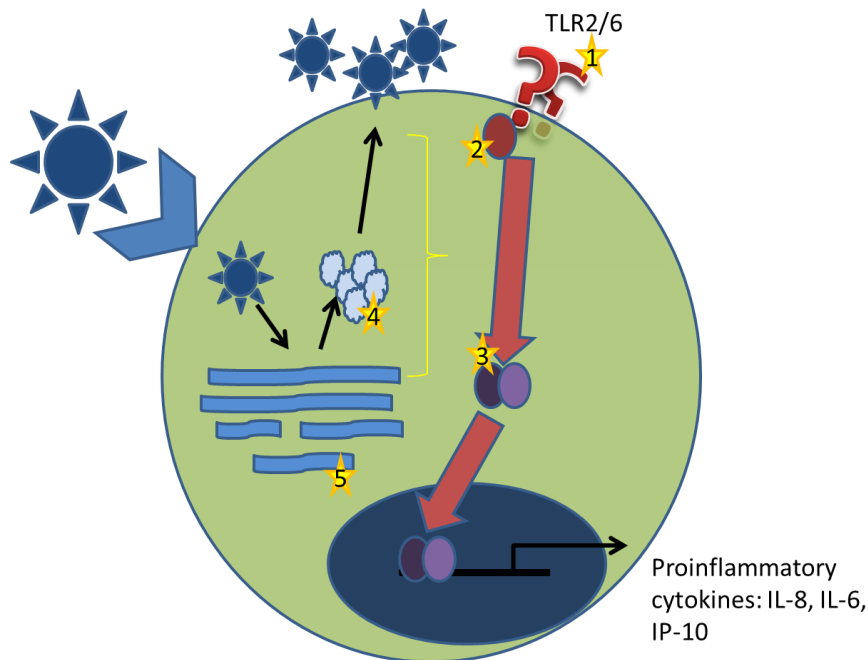


Figure 21: Working model for arenavirus induced TLR2 signaling

Arenaviruses bind the host cellular receptor and enter a host cell via receptor-mediated endocytosis. Through a unique pathway, arenaviruses enter the endosomal sorting complex and have released their genomic contents within 20

minutes. Replication of genomic and mRNAs as well as protein production begins. Virus particles are assembled at the plasma membrane and are released by budding. (1) In arenavirus-TLR2 stimulation, it is unlikely that the virus stimulated TLR2 from the plasma membrane as replication defective virus particles were unable to elicit a TLR2-dependent response. (2) Just as MyD88, a TLR adaptor molecule, can have intrinsic TLR-independent signaling functions, it is enticing to speculate that perhaps arenaviruses induce a response inside the plasma membrane by interacting with TLR2/6 and Mal. (3) NF κ B is differentially activated during infection with pathogenic and non-pathogenic arenaviruses. This indicates that the inhibition observed with LCMV-WE infection occurs upstream of NF κ B activation. Given that viral replication was necessary for both induction of TLR2-dependent responses and inhibition, it is possible that a viral protein (4), or viral RNA (5) is exerting these effects.

The experimental work presented herein significantly contributes to the field of arenavirus pathogenesis and vaccine development. By demonstrating the differences in TLR2-dependent responses during infection by arenaviruses of differing pathogenic potential for humans and NHPs *in vitro*, we have provided a possible explanation for the development of severe disease in LASV infection. Further work in this area could entail looking for differences in TLR2 stimulation, TLR2 mutations, and TLR2-related SNPs to determine why some, but not all infected patients, succumb to LASV infection. Additionally, having identified the differences in TLR2-dependent cytokine responses between pathogenic and non-pathogenic virus infection, this work provides an avenue for development of therapeutics. With limited treatments available to use against LASV infection, use of TLR2 agonists may provide activation of the type of immune response necessary to clear virus more effectively and increase chances for survival. Additionally, consideration for activation of TLR2 signaling should be taken into account in vaccine design for arenaviruses. The ideal vaccine candidate for arenaviruses would stimulate an efficient cytotoxic response and generate long

lasting memory. Knowing the role of TLR2/Mal/MyD88 signaling in generating specific CD4⁺ and CD8⁺ T cell responses, and taking into consideration the inability of LASV to induce memory markers in T cells, the role of the proinflammatory response becomes essential in developing the most effective vaccine towards LASV.

Chapter Seven

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