DISSERTATION

CATIONIC LIPOSOME-DNA COMPLEX-BASED IMMUNOTHERAPEUTIC AND IMMUNIZATION STRATEGIES FOR CONTROL OF LA CROSSE VIRUS AND *LEISHMANIA MAJOR* INFECTIONS

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ABSTRACT

CATIONIC LIPOSOME-DNA COMPLEX-BASED IMMUNOTHERAPEUTIC AND IMMUNIZATION STRATEGIES FOR CONTROL OF LA CROSSE VIRUS AND *LEISHMANIA MAJOR* INFECTIONS

The morbidity and mortality affiliated with vector-borne diseases are staggering, and the associated economic and social hardships are overwhelming, especially in populations without the political or financial means for effective control or treatment. Many of these diseases continue to re-emerge in former endemic areas and/or emerge in new parts of the world, and conventional means of control are often inadequate due to the appearance of pesticide-resistant vectors, drug resistant-pathogens, and the collapse of vector control programs, among other factors. At present, safe and efficacious vaccines and therapeutics for prevention and treatment of many of these diseases are lacking.

La Crosse virus (LACV), family *Bunyaviridae,* is a mosquito-borne pathogen and is the leading cause of pediatric arboviral encephalitis in the United States. LACV is not only an important human pathogen, but it is also a model for development of vaccines and antiviral therapies for other pathogens in the family *Bunyaviridae*. Many bunyaviruses are designated as National Institute of Allergy and Infectious Diseases (NIAID) priority pathogens and there is cause for concern that they could be exploited as bioweapons. Aerosol infection animal models are essential to test the efficacy of candidate vaccines and antiviral therapies for these important pathogens. There are no

human vaccines or antiviral treatments for LACV or other members of the family. Accordingly, in Chapter 2, aerosol and intranasal inhalational challenge models of LACV infection in mice were developed and tested. Following aerosol or intranasal challenge with LACV, 100% of normally-resistant adult mice developed clinical signs of LAC encephalitis and died. LACV was detected in high titers in the nasal turbinates, brains and lungs of aerosol- or intranasally-challenged mice, as well as in the sera and livers of mice challenged intranasally. Brains of LACV-challenged mice exhibited histologic lesions of meningoencephalitis, and LACV RNA was detected and amplified from brains of challenged mice. To our knowledge, this is the first report of aerosol-transmission of LACV leading to the development of lethal encephalitis in adult mice. The experimental challenge models described herein should be useful tools in the eventual development of sorely-needed vaccines and antivirals for the prevention or treatment of bunyavirus aerosol infections.

Immunotherapy using cationic liposome-DNA complexes (CLDC) has been shown to promote antiviral, antitumor, and antibacterial immune responses in various experimental animal models. This protection relies on non-pathogen-specific activation of soluble and cellular innate immune effectors. In Chapter 3, we evaluated the ability of CLDC to protect adult mice from the development of encephalitis in a LACV aerosol challenge model. Both pre-challenge (prophylactic) and post-challenge (therapeutic) administration of CLDC significantly increased survival in LACV-challenged animals in this model system. Intraperitoneal administration of CLDC elicited reductions in viral titer in both peripheral tissues and the central nervous system (CNS) and decreased the severity of CNS lesions in treated mice compared to sham-treated control mice.

Protection was associated with increased expression of IFN-α5 and IFN-β1 in the spleen, as well as IFN-γ in the brain. Systemic depletion of natural killer (NK) cells prior to treatment was found to abrogate the full protective ability of CLDC administration, suggesting an integral role for this cell type in CLDC-induced protection. These data indicate that CLDC is an effective antiviral immunotherapy that provides both prophylactic and therapeutic protection against an otherwise lethal aerosol challenge with a viral pathogen.

Leishmania major, one causative agent of Old World cutaneous leishmaniasis, is a vector-borne protozoan parasite transmitted by the bite of infected female phlebotomine sand flies. At present, there is no vaccine approved for use in humans for the prevention of *L. major* infection. Chapter 4 describes significant protection against infection with *L. major* induced by CLDC-based immunization against the immunomodulatory salivary peptide maxadilan (MAX) from the sand fly *Lutzomyia longipalpis*. Following intraperitoneal or subcutaneous immunization with MAX, both lesion sizes and parasite burdens of infected footpads of immunized mice were significantly reduced in comparison to those of non-immunized mice. The protection elicited using a CLDC adjuvant exceeded that elicited using a human-approved aluminum hydroxide adjuvant (Alhydrogel®); additionally, notable inflammation and tissue damage present at the site of immunization when Alhydrogel® was employed as adjuvant was completely absent in mice immunized with CLDC as adjuvant. Intracellular cytokine staining of CD4+ lymphocytes identified important differences in IFN-γ and IL-4 production in the context of infection due to immunization or lack thereof. The protection described herein highlights the importance of salivary immunomodulation in the initiation of vector-borne

pathogen infections, and provides compelling evidence in support of the inclusion of salivary molecules as antigens in the formulation of subunit vaccines intended to protect against transmission of arthropod-borne pathogens.

In these studies, we utilized CLDCs as both immunotherapeutics and vaccine adjuvants to induce innate and Th1-type immune responses protective against infection with LACV and *L. major,* both of which are arthropod-borne intracellular pathogens that require the induction of pro-inflammatory Th1-biased immune responses for clearance. This body of work presents evidence that CLDC administration induces protective immune responses against aerosolized LACV challenge and parenteral challenge with *L. major*, suggesting that CLDCs are versatile and effective vehicles for the elicitation of immune protection against pathogens susceptible to pro-inflammatory and Th1-biased immune responses, and are worthy of future exploration and further application.

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CHAPTER 1

LITERATURE REVIEW

As the field of immunology has grown rapidly in breadth and complexity over the past 200+ years, so too has our knowledge of the innate immune system. The wealth of recent knowledge has revealed the significance and intricacy of innate immunity in terms of guiding and regulating ensuing adaptive immune responses. Over 2 decades ago, Charles Janeway first hypothesized that in addition to serving as a first line of defense against pathogen invasion, innate immune responses might also function to initiate and direct the development of the ensuing adaptive immune responses (1989). In the decades since, the identification and characterization of various pattern recognition receptors (PRRs) of the innate immune system (including the Toll-like receptors (TLRs), NOD-like receptors (NLRs) and intracellular receptors for nucleic acids), has enriched our understanding of how immune responses are both initiated and shaped via initial interactions with pathogens and other microorganisms. Armed with an increased comprehension and appreciation of the early immunological events that determine the quality and character of subsequent responses, investigators have increasingly focused their research efforts on utilization of the innate immune system for prevention and treatment of disease. The discovery of the naturally-occurring ligands for various PRRs and the production of alternative synthetic agonists have empowered researchers with the ability to activate the innate immune system with amazing specificity, allowing for

suppression, induction, or enhancement of distinct immune responses for control and treatment of infection or disease.

Toll-like Receptors and Innate Immunity

The innate immune system detects the presence of pathogens using a collection of PRRs that recognize and bind to various pathogen-associated molecular patterns (PAMPs), or conserved structural features shared by many different groups of microorganisms, providing immune differentiation between noninfectious structures of the host and infectious foreign materials. PRRs are expressed in a variety of cellular locations, including within intracellular compartments and upon the cellular surface, or can alternatively be secreted into the fluids of the bloodstream or host tissues (Medzhitov and Janeway, 1997). Recognition of foreign, non-self structural motifs by PRRs serves to not only initially detect both the presence and nature of the pathogen, but also to induce an immediate inflammatory immune response against that pathogen, as well as to initiate the development of an adaptive immune response suitable for control of the particular pathogen (Janeway and Medzhitov, 2002; Akira et al., 2006; Sansonetti, 2006).

Among these PRRs is a family of membrane-bound receptors termed the Toll-like receptors (TLRs), which are expressed primarily (though not exclusively) on innate immune cells such as macrophages, neutrophils, and dendritic cells (DCs). A total of 13 different TLRs have been identified in mammals (Takeda et al., 2003; Tabeta et al., 2004; Zhang et al., 2004), and each serves to recognize and bind to a particular natural ligand or set of ligands (many of which have been identified), resulting in the expression of a wide variety of genes involved in host defense including major histocompatibility complex (MHC) and co-stimulatory molecules, antimicrobial peptides, and a variety of

inflammatory chemokines and cytokines. For example, TLR3 recognizes the double-stranded RNA present in the genome of some viruses (Alexopoulou et al., 2001), TLR4 binds to the lipopolysaccharides of Gram-negative bacteria (Medzhitov et al., 1997; Poltorak et al., 1998; Qureshi et al., 1999; Hoshino et al., 1999), and TLR5 recognizes bacterial flagellins (Hayashi et al., 2001). Of particular interest to this body of work is TLR9, which is selectively expressed within the endosomal compartment of human and murine plasmacytoid DCs (pDCs) and B cells, as well as murine monocytes and myeloid DCs (Iwasaki and Medzhitov, 2004), where it surveys endocytosed material and engages unmethylated CpG oligodeoxynucleotide sequences overexpressed in bacterial DNA (in comparison to mammalian DNA, which is mostly methylated) and in various viral genomes (Krieg, 2000; Hemmi et al., 2000). Interaction between TLR9 and ligand leads to the recruitment of the adapter protein MyD88 (myeloid differentiation primary-response protein 88) to the receptor complex (Medzhitov et al., 1998; Schnare et al., 2000), leading ultimately to the activation of signaling cascades that induce the production of various pro-inflammatory cytokines (including IFN-α, IFN-β, IFN-γ, TNF-α, and IL-12), chemokines, and co-stimulatory molecules via signaling through NF-κB-mediated (Meylan et al., 2006; Akira and Takeda, 2004) and interferon regulatory factor 7 (IRF-7)-dependent pathways (Sato et al., 2000; Barnes et al., 2002). The cytokine profile induced following activation of TLR9 (primarily the production of IFNs and resultant activation of IFN-dependent antiviral mechanisms) is particularly effective for treatment or prevention of viral infections (Stetson and Medzhitov, 2006a; Kawai and Akira, 2006), and these topics will be discussed in further detail later. A direct role for TLR9 activation in host responses to viral infection has been demonstrated by the

observations that production of type I IFNs by pDCs following challenge with either herpes simplex virus- (HSV) 1 or HSV-2 is abrogated using TLR9$^{-/-}$ mice (Lund et al., 2003; Krug et al., 2004). In fact, prophylaxis with TLR9 agonists has been shown to elicit a temporal window of protection against viral challenge that can last for a number of weeks (Klinman, 2004; Amlie-Lefond et al., 2005), suggesting that TLR9-based immunotherapeutics might be useful in inducing protection against viral pathogens released in acts of bioterrorism or biological warfare.

The cytokine and chemokine milieu induced following ligation of TLRs ultimately determines the development and nature of the subsequent acquired immune response via the attraction of additional effector cells to the site of infection, resulting in a specific pattern of cytokine secretion and activation of primarily antigen-specific T-helper 1 (Th1) or T-helper 2 (Th2) cells. In fact, the induction of any acquired immune response (especially Th1-cell responses) is dependent upon the initial activation of the innate immune system via PRRs such as the TLRs (Akira et al., 2001; Medzhitov, 2001), highlighting the importance of PRRs in induction of both the innate and subsequent adaptive immune responses. The integration of innate immune responses and downstream adaptive responses is a task performed by antigen presenting cells (APC), principally DCs. Ligation of TLRs on DCs leads to maturation of immature DCs into professional APCs, stimulating the expression of co-stimulatory molecules necessary for activation of T cells and inducing the production of cytokines that serve to regulate T-cell differentiation and thus the quality of subsequent adaptive immune responses. Research utilizing TLR9 agonists as vaccine adjuvants has repeatedly demonstrated their ability to induce Th1-type immune responses (Lipford et al., 2000; Kim et al., 1999; Chu et al.,

1997; Roman et al., 1997) and cytotoxic T-lymphocyte (CTL) development (Sparwasser et al., 2000; Lipford et al., 1997). Due to the Th1-bias of the cytokine response following TLR9 activation, ensuing adaptive immune responses are primarily cell-mediated and are thus appropriate for control of intracellular pathogens such as viruses and certain protozoan parasites (e.g., *Leishmania major*) (Zimmerman et al., 1998). Additionally, it has been demonstrated that the immunostimulatory capability and adjuvant activity of TLR9 agonists can be dramatically enhanced by combining them with lipid carriers (Krieg and Davis, 2001).

Cationic Liposome-DNA Complexes

Cationic liposomes have been shown to significantly enhance the innate immune stimulatory properties of certain adsorbed molecules, particularly those with intracellular receptors, such as the non-methylated CpG motif agonists of TLR9 (Gursel et al., 2001; Krieg, 2002; Zaks et al., 2006), the TLR3 agonist polyI:C (Zaks et al., 2006), and ssRNA and dsRNA agonists of TLR7/8 (Wong et al., 1999; Hamm et al., 2007). In fact, CpG-induced production of cytokines (such as type I and II IFNs) can be increased between 10- to 100-fold when the CpG agonists are complexed with cationic liposomes (Dow et al., 1999b; Sellins et al., 2005), forming cationic liposome-DNA complexes (CLDC). This potentiation of the innate immune response is multifactorial, dependent upon the abilities of cationic liposomes to protect adsorbed nucleic acids from extracellular degradation while simultaneously facilitating endocytic uptake and localizing the complexes to the early endosomal compartment of targeted cells, the primary site of expression for nucleic acid PRRs (Akira and Takeda, 2004; Akira, 2006; Zaks et al., 2006; Takeuchi and Akira, 2007). In addition, the immunostimulatory properties of

CLDC have also been shown to be regulated in part by TLR-independent pathways and receptors, such as the cytosolic DNA sensor DAI (DNA-dependent activator of IFN-regulatory factors) (Ishii and Akira, 2006; Stetson and Medzhitov, 2006b), activation of which leads to increased production of both pro-inflammatory cytokines and type I IFNs via activation of NF-κB-mediated and IRF-3- and IRF-7-dependent pathways, respectively (Takaoka et al., 2007; Takaoka and Taniguchi, 2008). A proposed model of CLDC processing leading to stimulation of innate immune responses is provided in Figure 1.1.

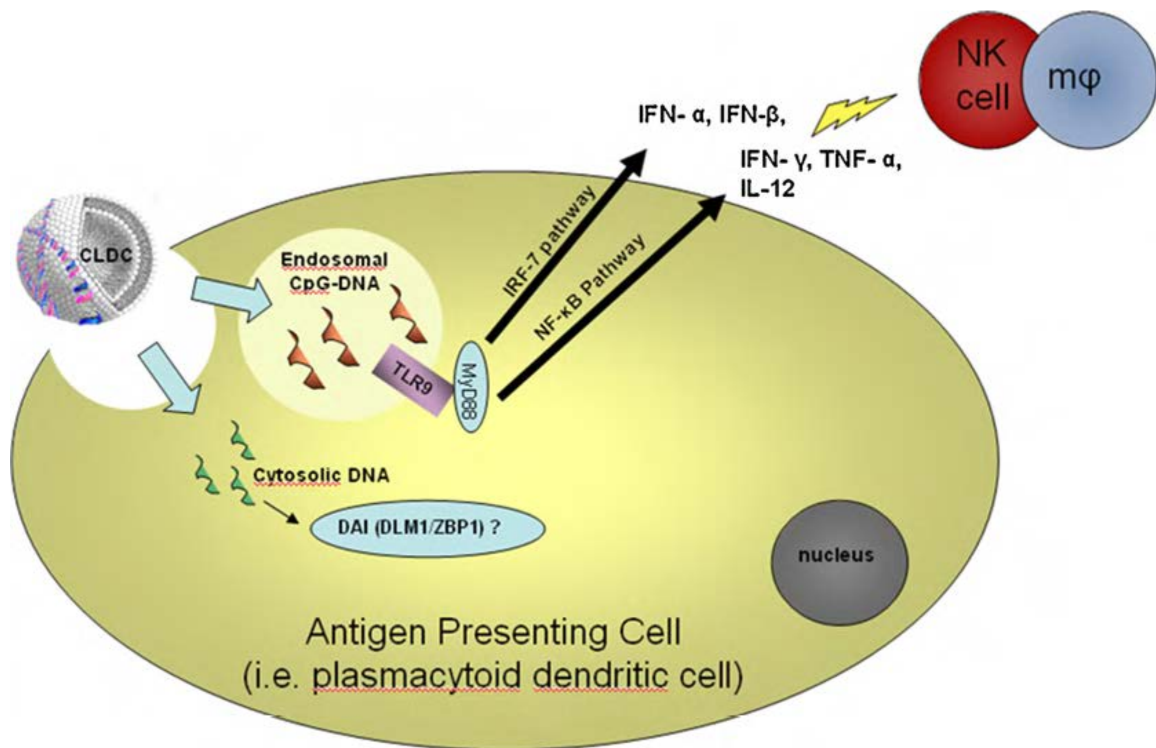

Figure 1.1. Possible routes of CLDC processing within a human host resulting in stimulation of the innate immune response. Figure is borrowed from Logue et al., 2010. Treatment with cationic liposome-DNA complexes (CLDCs) protects mice from lethal Western equine encephalitis virus (WEEV) challenge. *Antiviral Res.* **87(2):**195-203.

The first detailed descriptions of intravenous treatment with liposome-DNA complexes leading to stimulation of the innate immune system were from studies

designed to assess their efficacy in immunotherapeutic treatment of tumors (Dow et al., 1999a). The authors determined that the observed CLDC-induced antitumor effects were a result of stimulation of the innate immune system, specifically the activation of natural killer (NK) cells and resultant production of IFN-γ (Dow et al., 1999b). Subsequent research has shown that CLDCs are actively endocytosed by APCs such as pDCs and ferry molecules to endosomally-expressed TLRs such as TLR3, TLR7/8, and TLR9. Ligation of TLR9 along with cytosolic DAI leads to prompt non-specific activation of the immune system, principally in the spleen and draining lymph nodes. This response is typified by the release of pro-inflammatory cytokines, including TNF, IL-1β, IL-6, IL-12, IFN-γ, IFN-α and IFN-β (Ishii et al., 2004; Klinman et al., 2004). The ability of CLDCs to selectively activate potent innate immune responses characterized by robust production of pro-inflammatory cytokines and IFNs strongly underscores their potential clinical use as non-specific immunotherapeutics. CLDCs or liposomal CpG oligodeoxynucleotide (ODN) complexes have not only been shown to exert notable anticancer activity in various animal tumor models (Dow et al., 1999a; Dow et al., 1999b; Whitmore et al., 1999; Whitmore et al., 2001; Lanuti et al., 2000), but have also been successfully used for immunotherapeutic treatment of acute viral (Gowen et al., 2006; Gowen et al., 2008; Logue et al., 2010) and bacterial infections (Goodyear et al., 2009; Troyer et al., 2009).

In addition to its remarkable ability to activate the innate immune response and elicit production of Th1-biased cytokine responses, administration of CLDCs also stimulates the upregulation of co-stimulatory molecules on DCs and macrophages, functional maturation of APCs, and NK cell activation and infiltration (Dow et al., 1999b). Furthermore, the positive charges associated with the cationic complexes allow

for direct loading of most peptide and protein antigens, making CLDCs also quite-effective as vaccine adjuvants. When formulated together with antigens, CLDCs not only retain their ability to potently stimulate innate immune responses and activate immune cells, but serve as a platform to directly target bound antigens to DCs and other APCs. Vaccines utilizing CLDCs as adjuvants have been demonstrated to induce balanced cellular and humoral immunity, eliciting strong responses from both CD4+ and CD8+ T-cells, as well as inducing antibody responses either comparable to or exceeding those induced by Freund's complete adjuvant or aluminum hydroxide adjuvants (Chen, et al., 2008; Zaks, et al., 2006; Dow, 2008). The cross-priming and induction of CD8+ T cell responses has been attributed to the unique ability of the liposomal formulation to transport antigens into the cytosol and engage the MHC class I pathway (Walker, et al., 1992; Chikh and Schutze-Redelmeier, 2002; Zaks, et al., 2006). Additionally, some of the adjuvant effects of CLDCs have been attributed to cationic liposomes themselves (Latif and Bachhawat, 1984), as they have been shown to not only activate DCs directly (Cui, et al., 2005), but also to induce cellular necrosis (Khazanov, et al., 2006), which can lead to the activation of innate immune responses due to the liberation of endogenous immune activators, such as uric acid (Shi, et al., 2000). Due to the described potency of CLDCs as immunotherapeutics and adjuvants and their abilities to induce strong T cell responses and Th1-biased cytokine production, we elected to utilize CLDCs as both immunotherapeutics for treatment of aerosolized La Crosse virus infections and as an adjuvant in a vaccine formulation intended to protect against challenge with *Leishmania major,* both of which will be discussed in detail later (see Chapters 3 and 4, respectively).

Interferons and Interferon-induced Antiviral Effectors

Interferon (IFN) was first described in 1957 as an inhibitor of influenza virus replication (Isaacs and Lindenmann, 1957). Since then, three separate classes of IFNs have been identified, and the production of IFNs is now recognized as providing an initial line of defense against viral infections by both inducing the production of antiviral proteins and initiating an appropriate, cell-mediated adaptive immune response (Samuel, 2001). Type I IFNs (including IFN-β and various subtypes of IFN-α, among others) bind to the ubiquitously-expressed IFN-α receptor and are absolutely required for the development of potent immune responses against viral infection (Pietras et al., 2006). Type II IFN (IFN-γ), which binds to the IFNGR receptor complex, is produced by effector CD4+ Th1 cells, CD8+ T cells, and NK cells. Although a principal function of IFN-γ is the activation of macrophages, it additionally serves to mediate a wide spectrum of immune responses to a variety of pathogens (including intracellular pathogens such as viruses) via its abilities to induce the polarized development of Th1 cells, as well as to enhance both antigen processing and MHC expression (Sijts et al., 2002; Steimle et al., 1994). Type III IFNs, also known as IFN-λ or IL-28/29, have recently been discovered and are also recognized as regulators of the antiviral immune response. Presently, they are not completely understood, and are not discussed in detail here (for a review of the Type III interferon family, see Donnelly and Kotenko, 2010).

Most nucleated cell types are capable of producing IFN-α/β in response to viral infection via binding of viral structures (such as dsRNA, CpG DNA, or envelope glycoproteins) to PRRs expressed intracellularly or in endosomal compartments (Kawai and Akira, 2006). Production of type I IFNs and subsequent binding of receptors on

infected cells and nearby uninfected cells leads to activation of signaling cascades that ultimately induce the production of a variety of antiviral effector molecules, leading to the development of an antiviral state of resistance in which intracellular viral replication is inhibited (Pietras et al., 2006). Production of IFN-α/β also activates NK cells, which then act to selectively kill virus-infected cells. Additionally, IFN-α/β production serves to induce the production of MHC class I molecules in most cell types, which both increases the resistance of uninfected cells to killing by NK cells and increases the susceptibility of infected cells to killing by CD8+ cytotoxic T cells (Pietras et al., 2006).

Many of the antiviral effects of IFN production are due to activation of IFN-induced proteins, such as protein kinase R (PKR), the 2',5'-oligoadenylate synthetase (OAS) and RNase L, the RNA-specific adenosine deaminase (ADAR1), and the myxovirus-resistance (Mx) proteins. Protein kinase PKR is a constitutively-present, IFN-inducible RNA-dependent protein kinase that inhibits the translation of mRNA into protein, effectively interfering with synthesis of viral polypeptides (Clemens and Elia, 1997; Samuel, 1993). OAS is a constitutively-present, IFN-inducible PRR for viral dsRNA (Rebouillat and Hovanessian, 1999) which functions in concert with RNase L to lead to the degradation of both viral and cellular RNA (Floyd-Smith et al., 1981; Wreschner et al., 1981). ADAR1 is induced by IFN and covalently edits RNA substrates via deamination of adenosine to inosine, which both modifies the protein-coding capacity of the RNA transcript and destabilizes the dsRNA helix (Patterson et al., 1995). The Mx proteins are GTPases that serve to identify and bind to viral ribonucleoproteins, interfering with either the transport of viral nucleocapsids to the nucleus or with viral RNA transcription, depending on the cytoplasmic or nuclear location of the Mx proteins,

respectively (Staeheli et al., 1993; Haller et al., 1998; Haller and Kochs, 2002). By interfering with various cellular functions required for the production of viral genomes and other required structural elements, IFN-induced antiviral effectors provide an important line of defense against viral infections, effectively arresting early spread of the infection and allowing time for the development of a cell-mediated adaptive immune response. The central significance of the IFNs and their effects in immune defense against viral infection is underscored by the fact that the genomes of many viruses encode for mechanisms that interfere with or evade the antiviral responses induced by IFNs. For example, the NS1 protein of influenza virus sequesters dsRNA activators of PKR (Lu et al., 1995), the E3L protein of vaccinia virus binds to dsRNA activators of OAS (Rivas et al., 1998), and many viruses encode for soluble IFN receptor homologues (Alcami et al., 2000; Colamonici et al., 1995; Smith et al., 1998). Such observations highlight the central importance of IFN production in controlling initial viral replication and spread to neighboring cells, and provide sufficient rationale for the use of IFN-inducing immunotherapies such as CLDCs as approaches toward the prevention and treatment of infection with viral pathogens.

La Crosse Virus

La Crosse virus (LACV), family *Bunyaviridae*, genus *Orthobunyavirus,* California serogroup, is a mosquito-borne pathogen endemic to various regions of the Midwestern United States (Calisher, 1994; Rust et al., 1999). LACV is predominantly-transmitted by the bite of its principal vector, *Aedes triseriatus*, and is amplified and maintained in a transmission cycle involving Eastern chipmunks (*Tamias striatus grinseus*) and Eastern gray squirrels (*Sciurus carolinensis*), which serve as the primary

amplifying verterbrate hosts. Transmission from mosquitoes to vertebrate hosts occurs when an infected female mosquito takes a blood meal, transferring LACV to the vertebrate via the mosquito's saliva. While the natural rodent hosts do not become ill following infection, subsequent LACV replication leads to the development of a viremia sufficient to infect naïve mosquitoes seeking an ensuing blood meal (Borucki et al., 2002). Infection of the vector has little deleterious effect on the health of the mosquito, and LACV can also be maintained in the mosquito population via transovarial or vertical transmission (Watts et al., 1973), in which the virus replicates in the ovaries and embryos of the mosquito and overwinters in her eggs, which hatch in the spring to release LACV-infected offspring (Beaty et al., 2000).

LACV has a tripartite, negative-sense RNA genome consisting of large (L), medium (M), and small (S) RNA segments (Obijeski et al., 1976). The L segment encodes the RNA-dependent RNA polymerase, the M segment encodes a polyprotein that is post-translationally processed to form the Gn and Gc glycoproteins and a nonstructural protein NS_M, and the S segment encodes the nucleocapsid (N) protein and an additional nonstructural protein NS_S in overlapping reading frames (Elliott, 1990; Schmaljohn, 1996; Bishop, 1996). The pleomorphic virion is comprised of helical structures containing the L, M, and S RNA segments encapsidated with the nucleocapsid protein, polymerase molecules required for transcription of the negative-sense viral genome, and a host-derived lipid envelope intercalated with the Gn and Gc glycoproteins (Bishop, 1996).

Infection of naïve mosquitoes occurs following the ingestion of an infected blood meal from a viremic host. LACV first infects epithelial cells of the mosquito midgut, and

pending successful midgut escape, LACV then disseminates to the hemocoel, where it subsequently transits to and replicates in various tissues including the fat body, neural ganglia, heart, ovaries, and eventually the salivary glands at 7-16 days following virus ingestion (Beaty and Calisher, 1991; Schmaljohn, 1996). LACV replicates to high titers in the salivary glands and can subsequently be transmitted to vertebrate hosts via the mosquito's saliva. While the natural transmission cycle principally involves chipmunks and squirrels as amplifying vertebrate hosts, humans can act as tangential vertebrate hosts, and LACV infection of humans can potentially lead to serious disease, especially in children. In fact, > 90% of symptomatic LACV infections occur in patients younger than 15 years old (Rust et al., 1999; McJunkin et al., 2001).

LACV was initially isolated post-mortem in 1964 from the brain of a 4 year-old female patient from Minnesota diagnosed with meningoencephalitis who subsequently died in La Crosse, Wisconsin in 1960 (Thompson et al., 1965). LACV has since become an important public health problem in the United States, where it has remained the most common cause of pediatric arboviral encephalitis (Calisher, 1994), with an incidence in endemic areas exceeding that of bacterial meningitis (20-30 cases per 100,000 children under 15 years of age) (McJunkin et al., 2001). Annually, there are approximately 300,000 LACV infections, 70-130 cases of which have diagnoses of severe neurological involvement; the estimated case-fatality rate is approximately 0.3% (Rust et al., 1999; McJunkin et al., 2001; Jones et al., 1999). The actual number of cases of La Crosse (LAC) encephalitis is thought to be vastly underrecognized and underdiagnosed (Calisher, 1994), due in part to the close resemblance between clinical symptoms of severe LAC encephalitis and those of herpes simplex virus encephalitis. Accordingly,

LACV is now considered in the differential diagnosis of herpes simplex virus PCR-negative cases in patients with potential exposure to LACV (Sokol et al., 2001; Wurtz and Paleologos, 2000). In humans, severe LACV infection of the central nervous system (CNS) commonly presents as meningoencephalitis with inflammation largely confined to the cerebral cortex, often resulting in seizures and eventual coma (McJunkin et al., 1997; Kalfayan, 1983). Milder infections are presumably far-more common and lead to flu-like symptoms, such as vomiting, fever, stiff neck, and headache (McJunkin et al., 2001). Following recovery, many patients experience significant neurologic sequelae, including continued seizures, learning disabilities, and attention deficit hyperactivity disorder (McJunkin et al., 2001; Balfour et al., 1973). The financial burden related to these sequelae has been projected to range from $48,775 – $3,090,398 over the lifetime of each patient (Utz et al., 2003). Unfortunately, at present there is neither a licensed vaccine nor a standard antiviral therapy for prevention of LACV infection or treatment of LAC encephalitis (Hollidge et al., 2010).

In the past, most cases of LAC encephalitis have occurred in states of the upper Midwest; more recently, however, cases have been identified in North Carolina, West Virginia, Tennessee, and Missouri (Jones et al., 1999; CDC, 2010; Haddow and Odoi, 2009; Haddow et al., 2011). The recent isolation of LACV from field-collected larvae and male *Aedes albopictus* (an aggressive, day-feeding species introduced into the North American continent within the last two decades) and the connection of this vector to human cases in Tennessee collectively are a cause for concern to public health (Gerhardt et al., 2001; Erwin et al., 2002) as *Ae. albopictus* has been shown to be a competent vector of LACV (Tesh and Gubler, 1975, Hughes et al., 2006) and LAC encephalitis is

on the rise in the South, where recent cases have been reported in Louisiana, Alabama, Georgia, and Florida (Lambert et al., 2010).

The pathogenesis of LACV infection in mammalian hosts or experimental animals has not been completely determined, and our understanding of how peripheral infection spreads to the CNS to cause clinical manifestations of disease is limited to a small number of reports (Gauld et al., 1975; Amundson and Yuill, 1981; Amundson et al., 1985; Ksiazek and Yuill, 1977; Bennett et al., 2008). Peripheral injection of LACV does not typically result in the development of encephalitis in adult mice, even when administered at quite high subcutaneous doses (Johnson, 1983; Janssen et al., 1984). Prior studies from our own laboratory have shown that less than 10% of adult mice develop neurological disease when given as much as 1×10^5 infectious units of virus subcutaneously or intramuscularly (unpublished observations). As mice age, they become decreasingly susceptible to the development of LAC encephalitis, an observation similar to that made in human populations, in which infection causes neurological disease primarily in younger patients (Johnson, 1983; McJunkin et al., 2001). In models of subcutaneous inoculation of suckling mice, LACV replicates initially in striated skeletal muscle tissue near the site of infection, eventually leading to infection of the vascular endothelium and development of a viremia. The virus is then thought to bypass the blood-brain barrier and enter the CNS through infection of the vascular tissue supplying the brain (Johnson, 1983; Janssen et al., 1984; Griot et al., 1993a; Griot et al., 1993b); once in the brain, LACV infects and replicates in neurons, ultimately inducing neuronal apoptosis (Pekosz et al., 1996). Previous studies using the murine model of LACV infection have established that the neuroinvasiveness of LACV is directly related to its

capability to develop a high viremia, which in turn is correlated with previous viral replication in striated muscle tissue (Janssen et al., 1984). A more recent study involving intraperitoneal or intranasal inoculation of weanling mice concluded that after first replicating in various tissues near the site of inoculation, LACV can then infect the nasal turbinates via the bloodstream, eventually making its way into the CNS via infection and ascent of the olfactory nerves (Bennett et al., 2008). Disease presentation and organ pathologic changes in the mouse model are similar to that seen in human infections, making the murine model of LACV infection a useful tool with which to mimic the course of disease in humans, allowing for study of the transit of LACV from the periphery to the CNS and subsequent mechanisms of neuropathogenesis (Hollidge et al., 2010). An additional point of relevancy to this body of work is the observation that infection with LACV is dramatically modulated by the activity of mosquito saliva, which interferes with the induction of protective IFN responses, among other antiviral immune responses. The importance of this immunomodulation will be highlighted in the subsequent sections of this dissertation concerning potentiation of *Leishmania* infection by sand fly saliva. The salivary-antigen immunization approaches explored in Chapter 4 might be extrapolated to natural transmission models of not only LACV, but also the many other pathogens of the *Bunyaviridae* transmitted by the bites of infected arthropod vectors.

Previous studies have identified both IFN-α and IFN-β as important mediators of protection against murine LACV infection (Blakqori et al., 2007). The importance of the IFN response in protection against infection with LACV is further highlighted by *in vivo* studies that have shown that the main biological function of the LACV nonstructural

protein NSs is to usurp the antiviral type I interferon response in mammalian hosts (Blakqori et al., 2007). Additionally, production of IFN-γ is known to exert direct antiviral responses through the induction of various antiviral effectors, including PKR, OAS and RNase L, and the dsRNA-specific adenosine deaminase (dsRAD) (Boehm et al., 1997). In fact, IFN-γ is critical for antiviral responses in the CNS, in which non-cytolytic clearance of intracellular viruses is required for preservation of neuronal function (Chesler and Reiss, 2002). Also, research involving the IFN-induced MxA protein has demonstrated that it binds to and effectively sequesters LACV nucleocapsid proteins in the cytoplasm, interfering with successful amplification of the viral genome, production of infectious virions, and thus the spread of the infection to neighboring cells (Kochs et al., 2002). These combined observations further highlight the importance of the IFN response in protecting against LACV infections.

In addition to being an important human pathogen, LACV is also a well-studied model system that can be utilized for the development of antiviral therapies and vaccines for other pathogens in the family *Bunyaviridae,* many of which are designated by the National Institute of Allergy and Infectious Diseases as priority pathogens. Many members have been characterized as likely agents of bioterrorism due in part to their ability to induce serious illness in humans and the potential to be transmitted by aerosol (Sidwell and Smee, 2003). Additionally, the potential of a second wave of infection involving conventional transmission by indigenous vector species is cause for concern. Indeed, this sequela of a bioterrorist attack could have greater impact on public health than the initial aerosol event. Rift Valley fever virus (RVFV), also a member of the *Bunyaviridae,* is considered by many to be an optimal bioterrorism agent because of its

potential to emerge in new areas with devastating consequences to both human and animal populations. Considering the impact of the *Bunyaviridae* on human and veterinary health (Calisher, 1994), the expeditious development of novel, safe, and efficacious vaccines and therapeutics is clearly warranted. Aerosol infection animal models are essential to test the efficacy of candidate vaccines and antiviral therapies for these important pathogens. Accordingly, in work described herein, inhalational models of LACV infection were developed and tested (see Chapter 2); in addition, CLDC was investigated as a potential anti-LACV immunotherapeutic (see Chapter 3).

Leishmaniasis

The leishmaniases are a group of zoonotic vector-borne diseases caused by infection with obligate intracellular protozoa of the genus *Leishmania,* transmitted by the bite of infected female sand flies of the genera *Phlebotomus* and *Lutzomyia.* Leishmaniasis is presently a concern for more than 350 million humans living in more than 88 endemic countries on 6 continents throughout the world, with an estimated worldwide prevalence of more than 12 million cases and an annual increase of 1.5-2 million new cases (World Health Organization, Leishmaniasis home page: http://www.who.int/leishmaniasis/en/; Desjeux, 2004). Leishmaniasis is commonly referred to as a group of several clinical diseases, as infection with various species of *Leishmania* can lead to a wide spectrum of clinical presentations, including cutaneous, mucocutaneous, and visceral leishmaniasis, depending in part on the site of parasitic replication within macrophages of the dermis, naso-oropharyngeal mucosa, or mononuclear phagocyte system, respectively (Herwaldt, 1999). There are more than 30 different species of *Leishmania* parasites, 20 of which are pathogenic for humans

(Cupolillo et al., 2000), and disease manifestation is additionally dependent upon the particular species involved in infection. Following infection with the various species of *Leishmania* that lead to cutaneous leishmaniasis (such as *L. major, L. tropica, L. aethiopica, L. mexicana,* and *L. amazonensis,*), amastigotes replicate in macrophages resident in the dermis, resulting in a wide range of clinical manifestations from subclinical, self-healing infections to chronic disease typified by the formation of ulcerating lesions and scarring. In some cases, particularly those involving *L. braziliensis,* cutaneous infections can metastasize and disseminate to macrophages resident in the naso-oropharyngeal mucosa, resulting in mucocutaneous leishmaniasis, a disfiguring form of the disease characterized by degradation of mucosal membranes (Herwaldt, 1999). Infection with other species of *Leishmania* (such as *L. chagasi/infantum* and *L. donovani*) can lead to visceral leishmaniasis, also known as kala-azar, in which parasites spread from cutaneous sites to the bone marrow, liver and spleen, commonly leading to parasitemia and mortality (Pearson and de Queiroz Sousa, 1996; Herwaldt, 1999).

Leishmaniasis has been classified as both a zoonosis (linked to wild and/or domestic animal reservoir hosts) and an anthroponosis (linked to human reservoir hosts), with parasites cycling between intracellular forms in immune cells of the vertebrate hosts and extracellular forms in the digestive tract of vector sand flies. Transmission of *Leishmania* parasites to mammalian hosts occurs when an infected female sand fly seeking a bloodmeal regurgitates the elongate and flagellated metacyclic promastigote form of the parasite into the skin of the host, along with various biologically-active components of sand fly saliva (Rogers et al., 2002b). Regurgitated promastigotes

subsequently enter and replicate within phagocytic cells (primarily macrophages) present in the skin at the bite site. Once inside the macrophage, *Leishmania* develop into the round, non-flagellated amastigote form of the parasite and replicate within phagolysosomes, ultimately leading to lysis of the macrophage and subsequent spread to nearby macrophages (Handman and Bullen, 2002). Subsequent infection of sand flies occurs following ingestion of blood containing infected macrophages. The infected bloodmeal is transported to the posterior midgut, a starting point from which *Leishmania* parasites subsequently migrate anteriorly toward the stomodeal valve while differentiating through a number of stages of development (Bates, 2007). At the juncture of the foregut and midgut, the haptomonad promastigote forms of the parasite attach themselves to the stomodeal valve, forming a parasite plug which impairs the bloodfeeding abilities of the fly, eventually causing regurgitation of metacyclic promastigotes (the flagellated, free-swimming form of the parasite infective to vertebrates) into a subsequent bite wound, where they infect resident macrophages, completing the natural transmission cycle (Schlein et al., 1992; Rogers et al., 2002b).

Immunology of Leishmaniasis

Our present understanding of the immune response following infection with *Leishmania* parasites relies heavily on observations stemming from experimental mouse models of infection, and although the results cannot be directly applied to infection of dogs or humans, this research has led to many important conclusions concerning the cell-mediated immune response required for control of infection. Effective immunity to infection with *Leishmania* parasites is dependent upon the interconnected actions of many cells of the immune system, but none play a more important role than T cells

(Reiner and Locksley, 1995). While CD4+ CD25+ regulatory T cells play crucial roles in mediating parasite persistence and the maintenance of immunity to reinfection (Belkaid et al., 2002a) and CD8+ T cells are known to play an important protective role via the production of IFN-γ (Müller et al., 1993; Belkaid et al., 2002b), CD4+ T cells have been identified as the primary mediators of resistance or susceptibility to infection. In fact, the murine model of cutaneous leishmaniasis persists as one of the best models for understanding the immune mechanisms mediating the balance between Th1 and Th2 CD4+ cell populations.

As *Leishmania* parasites are obligate intracellular pathogens, control and clearance of infection are dependent upon the development of a CD4+ mediated Th1 cellular immune response characterized by the production of cytokines (e.g., IL-12, IFN-γ and TNF-α) that lead to the activation of infected macrophages, eliciting the production of nitric oxide (NO) by inducible nitric-oxide synthase (iNOS), which results in killing of intracellular parasites (Reiner and Locksley, 1995). The development of this protective Th1 response is dependent upon the early production of IL-12 and TNF-α by macrophages and/or DCs, which leads to the activation of NK cells and early production of IFN-γ, which further supports the outgrowth of Th1 cells and subsequent control of infection (Scharton-Kersten and Scott, 1995). In contrast, the development of a CD4+ mediated Th2 cellular immune response characterized by the secretion of Th2-biased cytokines (e.g., IL-4 and IL-13) leads to alternative activation of macrophages and polarizes the immune response towards an antibody-mediated response ineffective against intracellular organisms such as *Leishmania* parasites. The development of this non-protective Th2 response is dependent upon the early production of IL-10 and TGF-β

by macrophages and/or DCs, which promotes the outgrowth of Th2 cells and the production of IL-4, which can subsequently negate the actions of Th1 type cytokines. In the presence of a Th2-biased immune response, production of IFN-γ is inhibited and thus infected macrophages do not become activated to produce NO and instead produce arginase-1, leading to uncontrolled replication of intracellular parasites and ultimately to the development of progressive disease (Reiner and Locksley, 1995; Matthews et al., 2000). The amino acid substrate *L*-arginine is shared by both the Th1 and Th2 inducible enzyme systems; iNOS catabolizes *L*-arginine into protective NO, while arginase-1 hydrolyzes *L*-arginine to urea and *L*-ornithine, a molecule required for the production of polyamines vital for growth of *Leishmania* within macrophages (Modolell et al., 1995). As a result, macrophages can either destroy or host intracellular *Leishmania* amastigotes, depending on the relative balance of iNOS and arginase-1, which is contingent upon initial priming of either Th1 or Th2 cells. Thus, early induction of an innate immune response that leads to the outgrowth of Th1 cells is an important facet of the development of immune responses appropriate for control of *Leishmania* infections (Liese, et al., 2008). Figure 1.2 provides a visual representation of healing vs. non-healing immunological responses to infection with *L. major*.

Although the definitive host cells for *Leishmania* parasites are macrophages, DCs are the primary cell type involved in antigen presentation and instructing the development of ensuing adaptive T cell responses, and thus early events involving DCs (such as IL-12- and type I IFN-induced activation of NK cells to produce protective IFN-γ) are of paramount significance in determining the immunological events that subsequently occur (Liese et al., 2008). For example, ligation of TLR9 in the endosomal compartment

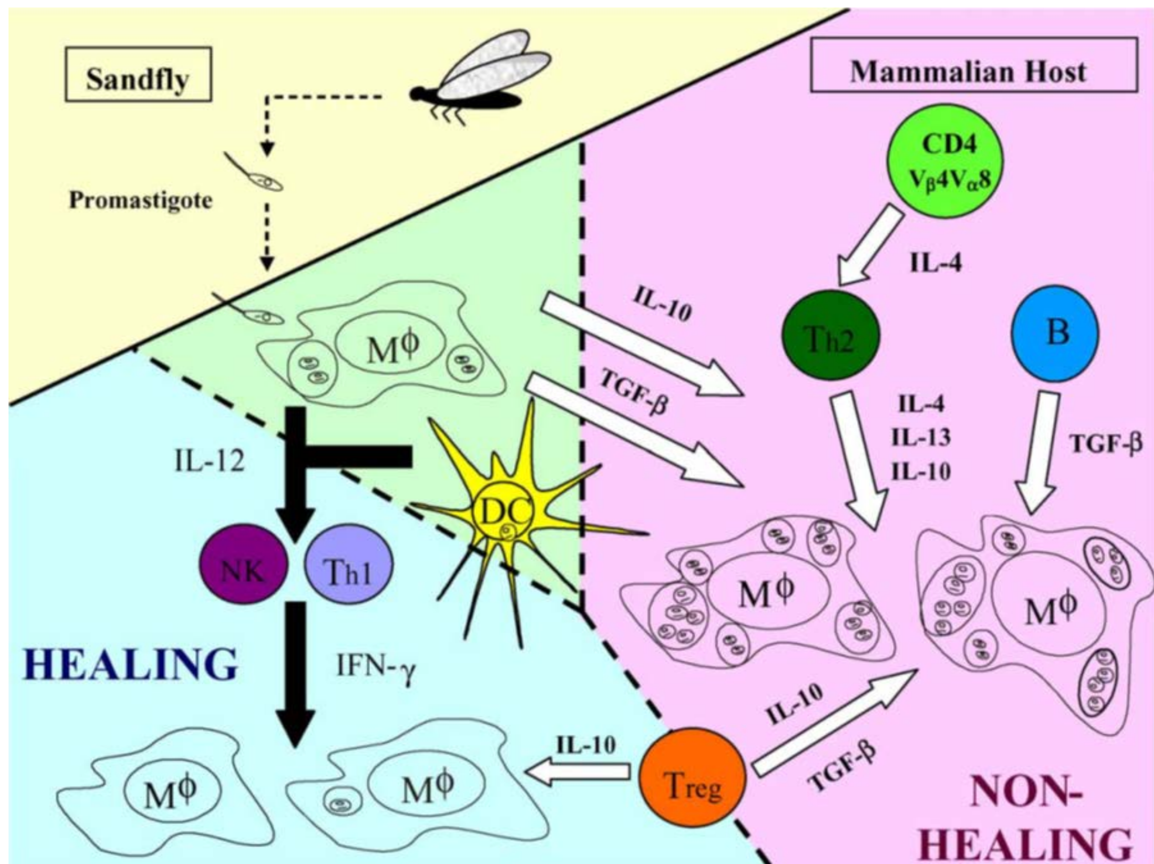

Figure 1.2. ***Leishmania major*: healing and non-healing immunological responses**. Following *L. major* infection of macrophages or DCs, IL-12 production by infected cells induces activation of NK cells, CD4+ Th1 differentiation and IFN-γ production. IFN-γ stimulates iNOS expression and NO production in the macrophage, mediating parasite killing and a healing response. Failure to produce IL-12 (or production of IL-4/IL-13) results in uncontrolled parasite replication within the infected cells facilitated by IL-10 production. IL-10 production by CD4+ CD25+ T regulatory cells both facilitates non-healing disease and maintains latent infection and long-term immunity. Figure is borrowed from Alexander and Bryson, 2005. T helper (h) 1/Th2 and *Leishmania:* paradox rather than paradigm. *Immunol. Lett.* **99(1):**17-23.

of myeloid DCs by genomic parasite DNA following parasitic degradation has been shown to play a part in protective innate and adaptive immune responses to *L. major* (Liese et al., 2007; Liese et al., 2008; Schleicher et al., 2007). The importance of this initial immune response, particularly TLR-mediated signaling pathways, is further illustrated by the observation that normally-resistant C57BL/6 mice with MyD88-

deficiency are highly susceptible to *L. major* infection and respond with an ineffective Th2-biased response (Muraille et al., 2003).

Leishmania parasites themselves employ a number of mechanisms that aid them in eluding potentially-protective actions of the host immune system. These include evasion of complement-mediated lysis prior to entering host cells (Brittingham et al., 1995), "silent" entry of macrophages that avoids the oxidative burst normally associated with phagocytosis (Mosser and Edelson, 1987), and additional cell-specific mechanisms including those aimed at macrophages (such as interfering with NO-mediated killing functions and phagosomal maturation and acidification), DCs (such as inhibition of chemotaxis and DC-maturation), and the recruitment of CD4+CD25+FoxP3+ regulatory T cells that themselves inhibit the development of protective immune responses (Brandonisio et al., 2004; Belkaid et al., 2002a; Peters and Sacks, 2006; Nylen et al., 2007). In addition, *L. major* parasites initially phagocytosed by polymorphonuclear neutrophil granulocytes are capable of utilizing this cell type as a vector for undetected entry into macrophages, effectively avoiding detection by or activation of host macrophages (van Zandbergen et al., 2004; Laskay et al., 2008). Such strategies for immune evasion further illustrate the importance of early induction of appropriate immune responses in control of *Leishmania* infections, and stress the need for interventions that fulfill this requirement.

Most mouse strains (e.g., C3H, C57BL/6, and CBA) challenged with *L. major* effectively control the infection and develop self-healing cutaneous lesions similar to those seen during human infection; in these resistant mouse strains, clearance of infection has been shown to be mediated by IFN-γ-producing CD4+ Th1 cells. In fact, a single

treatment with anti-IFN-γ monoclonal antibodies prior to challenge with *L. major* was shown to block the outgrowth of Th1 cells (Scott, 1991), rendering otherwise-resistant C3H/HeN and C57BL/6 mouse strains susceptible to infection and progressive disease development (Belosevic et al., 1989; Sadick et al., 1990). Other strains of mice (e.g., BALB/c) are exceedingly susceptible to infection with *L. major,* developing progressive cutaneous disease that eventually leads to systemic infection and death; this strain of mouse responds to infection with *L. major* by developing a non-protective Th2-biased immune response, typified by IL-4, IL-13, and IL-10 production and the absence of macrophage activation and clearance of intracellular parasites (Matthews et al., 2000). The central role of IL-4 in progression of cutaneous disease is underscored by the observation that an increase in Th1-like immune responses and clearance of *L. major* infection can be induced in BALB/c mice via treatment with anti-IL-4 monoclonal antibodies (Sadick et al., 1990; Nabors and Farrell, 1994). However, although a great deal of research has implicated IL-4 as a definitive marker of susceptibility to *L. major* infection, the actions of regulatory cytokines such as IL-10 and TGF-β have also been shown to elicit important effects that contribute to susceptible vs. resistant immunological phenotypes (Scharton-Kersten and Scott, 1995). Such observations have made it apparent that effective immunity against infection with *L. major* is highly-dependent upon the early induction of an innate immune response that leads to the outgrowth of Th1 cells appropriate for control of intracellular infections. Unfortunately, research has shown that the development of this protective immune response is perturbed not only by the parasite itself, but also by the actions of immunomodulatory salivary components co-injected during vector transmission.

Salivary modulation of *Leishmania* infection

Leishmania major, one causative agent of Old World cutaneous leishmaniasis, is transmitted by the bite of infected female sand flies of the species *Phlebotomus papatasi,* and is 1 of 10 species of *Leishmania* deemed to be of public health significance (Bates, 2007). As *L. major* is vector-transmitted, the inoculum contains not only infective metacyclic promastigotes, but also sand fly saliva containing various salivary components. The salivary molecules of blood-feeding arthropods serve multiple roles, including acting as anesthetics, inducing vasodilation, and inhibiting both coagulation of blood and the development of host inflammation and a subsequent immune response (Kamhawi, 2000; Schoeler and Wikel, 2001; Rogers et al., 2002a; Titus et al., 2006). By dilating the blood vessels of the vertebrate host, the sand fly increases blood flow to the bite site, maximizing its chances of a successful blood meal. Additionally, via suppression of the host inflammatory response, the sand fly interferes with the ability of the host to become sensitized to its saliva, again increasing the chances of successful bloodfeeding and solidifying that particular vertebrate as a source of a potential future blood meal. Due to this immunomodulation, any pathogen transmitted via sand fly bite will be confronted by a skin environment that has been substantially altered by the effects of the co-injected saliva; depending on the quality of the immune environment induced at the bite site, there is a possibility that pathogens might benefit from the changes induced by the saliva. It is now widely-accepted that arthropod saliva can serve to enhance the infectivity of pathogens transmitted to the vertebrate host during the bloodmeal (Titus and Ribeiro, 1988; Mbow et al., 1998; Belkaid et al., 1998; Jones et al., 1989; Labuda et al., 1993; Edwards et al., 1998). Many have speculated that this phenomenon may, in

part, account for the impressive ability of many arthropods to so effectively transmit such a wide variety of pathogens (including the causative agents of many diseases of worldwide health importance, such as leishmaniasis, onchocerciasis, lymphatic filariasis, Chagas disease, malaria, African trypanosomiasis, and dengue fever).

The initial observations concerning the role played by sand fly saliva in transmission of *Leishmania* parasites were a result of research involving co-injection of mice with *L. major* promastigotes and salivary gland lysates (SGL) from the New World sand fly *Lutzomyia longipalpis*. Inclusion of sand fly SGL in the inoculum led to an unexpected degree of exacerbation of infection: cutaneous lesions grew 5-10 times larger than lesions of mice injected without SGL, and the parasite loads within the lesions contained as many as 5,000-fold more parasites (Titus and Ribeiro, 1988). The dramatic exacerbation of infection caused by co-injection of sand fly SGL has subsequently been attributed to a multitude of effects on the vertebrate host immune system. Sand fly saliva has been experimentally shown to inhibit the activation of T cells (Theodos and Titus, 1993; Titus, 1998) and macrophages (Theodos and Titus, 1993), to simultaneously block the production of Th1-biasing cytokines (Mbow et al., 1998) and augment the production of Th2-biasing cytokines (Norsworthy et al., 2004; Mbow et al., 1998; Belkaid et al., 1998), and to decrease the production of molecules that serve to kill intracellular parasites, such as macrophage-produced H_2O_2 and NO (Hall and Titus, 1995; Waitumbi and Warburg, 1998; Gillespie et al., 2000; Norsworthy et al., 2004). As effective control and clearance of *L. major* infection is dependent upon the outgrowth of Th1 cells and subsequent activation of macrophages to kill intracellular parasites, it is quite apparent that sand fly saliva-mediated immunomodulation at the inoculation site leads to the

development of an immune environment in which *L. major* can thrive and successfully develop an initial focus of infection. Therefore, as an important initial step in efforts to block or neutralize the exacerbative effects of sand fly saliva on *L. major* infection, successful identification of the salivary component(s) responsible for such immunomodulation became a priority for a number of research groups.

The saliva of *Lu. longipalpis* contains a wide variety of biologically-active molecules, including anti-platelet factors, anti-clotting factors, and vasodilators, many of which are also capable of immunomodulation (Kamhawi, 2000). The salivary component of chief interest to our laboratory and these studies is a vasodilatory peptide termed maxadilan (MAX) (Lerner et al., 1991; Lerner and Shoemaker, 1992). MAX earned its name due to its impressive vasodilatory effects: at the time of its characterization, MAX was the most powerful peptide vasodilator known (maximum dilation), capable of eliciting more than 500 times the vasodilatory activity of Calcitonin Gene-Related Peptide (CGRP), the strongest vasodilatory peptide known at the time (Lerner and Shoemaker, 1992). MAX was subsequently cloned, and functional studies showed that the recombinant 63-amino acid peptide product was capable of eliciting responses qualitatively and quantitatively similar to those of the native peptide found in sand fly saliva (Lerner and Shoemaker, 1992). Studies revealed that MAX binds to and activates the mammalian type I receptor for the pituitary adenylate cyclase-activating neuropeptide (PACAP), which is expressed on both vascular and neural tissues and mediates the potent vasodilatory effects of both PACAP and MAX (Moro et al., 1996; Moro and Lerner, 1997; Eggenberger et al., 1999). Of particular relevance to this study are the observations that PACAP receptors are also found on macrophages, immature

DCs, and T cells (Arimura and Said, 1996; Torii et al., 1998). Thus, in addition to its remarkable ability to affect the vertebrate vasculature, MAX has also been shown to elicit profound immunosuppressive and anti-inflammatory effects (Bozza et al., 1998; Brodie et al., 2007; Guilpin et al., 2002), including modulation of DC phenotype and function (Wheat et al., 2008), inhibition of T cell activation (Qureshi et al., 1996), and modulation of a wide variety of macrophage functions (Brodie et al., 2007). MAX-specific effects with the potential to exacerbate and affect control of *L. major* infection include modulation of DC functions (such as delayed migration to lymph nodes, altered surface expression of co-stimulatory molecules, and modifications in cytokine secretion), decreased production of protective Th1-biasing cytokines and effector molecules that serve to kill intracellular parasites (such as macrophage-produced H_2O_2 and NO), as well as increased production of exacerbative Th2-biasing cytokines (Gillespie et al., 2000; Soares et al., 1998). In the context of *L. major* infection, the combination of MAX's multiple and varied immunomodulatory effects clearly leads to the inhibition of protective immune responses, ultimately leading to exacerbation of infection and resultant disease. In fact, some studies have suggested that successful natural transmission of *L. major* is literally dependent upon these immunomodulatory effects of salivary molecules: mice challenged with a biologically-relevant dose (10-100) of *L. major* in the absence of sand fly saliva do not become productively infected, while an identical dose of parasites co-injected with sand fly saliva leads to infection and disease (Titus and Ribeiro, 1988). These observations indicated that immunization against MAX might elicit protection against challenge with *L. major* + *Lu. longipalpis* saliva, a hypothesis that has been both tested and proven in our laboratory (Morris et al., 2001),

and that we intended to further develop and characterize with the experiments described in Chapter 4.

It has been repeatedly shown that the immunomodulatory effects of *Lu. longipalpis* SGLs can notably exacerbate infection with various *Leishmania* species of both New and Old World origin (Titus and Ribeiro, 1988; Samuelson et al., 1991; Warburg et al., 1994), and that MAX alone can substitute for the effects induced by SGLs (Morris et al., 2001). In addition, there is a convincing catalog of evidence suggesting that an anti-saliva approach towards immunization is a realistic option for prevention of leishmaniasis. Immunization with salivary proteins (such as MAX from the New World sand fly *Lu. longipalpis* or SP-15 from the Old World sand fly *P. papatasi*), whole saliva, or via pre-exposure to uninfected sand fly bites have all elicited significant protective immunity against infection with *L. major* and the subsequent development of cutaneous disease, typified by the production of anti-saliva antibodies and/or a cellular immune response characterized by high levels of IFN-γ production (Morris et al., 2001; Valenzuela et al., 2001; Kamhawi, 2000; Belkaid et al., 1998; Oliveira et al., 2008). It is hypothesized that the IFN-γ response might have the dual effects of (1) causing enhanced early destruction of parasites and (2) accelerating the development of a protective, Th1-biased anti-*L. major* response. The results from these vector-saliva-based approaches to immunizing against infection with *L. major* are impressive and convincing, and warrant further investigation and characterization of the protective immune responses induced.

Vaccines for prevention of leishmaniasis

Following infection with *L. major,* most humans elicit an immune response capable of clearing the cutaneous infection, ultimately leaving the host immune to

reinfection (Neva and Brown, 1994), making cutaneous leishmaniasis one of the few parasitic human diseases for which a protective vaccine might be developed with a reasonable and realistic expectation of success. However, there still exists no innocuous and useful human vaccine for prevention of cutaneous leishmaniasis. Proposed requirements for an effective anti-*Leishmania* vaccine include the induction of strong IL-12 and IFN-γ responses, elicitation of CD4+ Th1 responses and activation of CD8+ T cells, and the inclusion of a very potent adjuvant (Scott et al., 2004; Kedzierski et al., 2006).

A concise summary of the history of attempts to immunize against *Leishmania* infection is as follows: “leishmanization”, or inoculation with live, viable *Leishmania* parasites, remains as the only genuinely-successful immunization strategy to be used in humans, but the safety concerns involved make it an unacceptable option for widespread prevention of infection (Modabber, 1995). Vaccines utilizing killed *Leishmania* parasites have reduced the concerns of safety, but have proven to be much less efficacious, due in part to destruction of parasitic antigen epitopes and PAMPs that might otherwise elicit protective immune responses (Noazin, et al., 2009). As a result, there have been repeated attempts to produce a vaccine formulation that is as safe as killed vaccines, but as efficacious as leishmanization; however, the general lack of effective adjuvants with the ability to induce a Th1-biased immune response is a major impediment to progress. The most common adjuvants used in human immunization (the aluminum salt adjuvants) are potent inducers of antibody responses, but are not effective at eliciting antigen-specific Th1 responses (Bomford, 1980; Comoy et al., 1997).

Nevertheless, attempts to immunize against *Leishmania* parasites have yielded one particularly promising candidate, Leish-111f, which is a single recombinant polyprotein consisting of three fused molecules from *L. major* and *L. braziliensis* co-administered with either IL-12 or a human-approved detoxified lipid A derivative from the lipopolysaccharide of *Salmonella minnesota* admixed with squalene (MPL-SE). After eliciting promising protection against both *L. major* and *L. amazonensis* in mouse challenge studies (characterized by both humoral and CD4+ T cell responses), Leish-111f became the first leishmaniasis vaccine to enter human clinical trials, and has already successfully completed Phase I and II trials in healthy human subjects (Coler et al., 2007). The efficacy of the Leish-111f vaccine has been, in part, attributed to the use of the MPL-SE adjuvant, which acts through TLR4 activation and is the first T-cell adjuvant approved for use in humans (Persing et al., 2002). However, aside from this one particular success story, the development of effective anti-*Leishmania* vaccines for use in humans is still a considerable challenge. Additionally, with more than 20 different pathogenic species of *Leishmania* being transmitted by more than 30 different species of sand flies, the task of developing multiple stand-alone vaccines that target individual *Leishmania* species will be difficult. There is therefore a considerable need for innovative approaches to successful immunization against *Leishmania.*

The availability of a strong adjuvant capable of eliciting responses from CD4+ Th1 and CD8+ T cells and the production of IL-12 and IFN-γ will considerably aid in these tasks. As described previously, CLDCs possess all these qualities, and thus are a very promising option. CLDC is also a logical choice of adjuvant because it activates APCs through TLR9, a receptor known to play a part in the innate and adaptive immune

responses to *L. major* (Liese et al., 2007; Schleicher et al., 2007). Also, the development of a Th1-biased immune response following infection with *L. major* has been partially-attributed to the early production of IFN-γ by NK cells, and it has been suggested that vaccine formulations that target NK cells (such as CLDC) might effectively promote Th1 cell development (Scharton-Kersten and Scott, 1995). Furthermore, CpG ODN treatment was shown to support the development of Th1 effector cells and elicit protective immunity against infection with *L. major* in susceptible BALB/c mice (Zimmerman et al., 1998), indicating again that CLDC might be a suitable adjuvant for polarizing the immune response toward protection. An additional rationale for utilizing CLDC as an adjuvant in these experiments is based on the hypothesis that the anti-saliva Th1 immune response induced by immunization with a CLDC adjuvant might suppress the initial Th2-biased immune response induced by co-injection of MAX (Roman, et al., 1997), allowing instead for the early development of immune responses appropriate for control of intracellular pathogens such as *L. major*.

Dissertation Project

At present, safe and efficacious vaccines and therapeutics for prevention and treatment of many arthropod-borne diseases are lacking. The overall goal of this dissertation was to investigate CLDCs as potential immunotherapeutics and vaccine adjuvants for treatment or prevention of infection with arthropod-borne pathogens, whether occurring by natural or purposeful means. We utilized CLDCs due to their abilities to selectively induce both a pro-inflammatory innate immune response typified by IFN-production and a resultant Th1-type adaptive immune response, which are requirements for effective clearance of both LACV and *L. major* infections, respectively.

CLDCs were demonstrated to be novel and effective vehicles for inducing protective immunity in these two very-disparate pathogen models that both require a host immune response appropriate for intracellular pathogen clearance.

We show that (1) La Crosse virus (LACV) is transmissible via inhalation, and leads to the development of lethal LAC encephalitis in normally-resistant adult mice, (2) CLDC immunotherapy elicits prophylactic and therapeutic protection against aerosolized LACV challenge, and (3) immunization against the sand fly salivary peptide MAX using CLDC as adjuvant induces significant protection against challenge with *Leishmania major* + MAX. These findings significantly enhance our understanding of the transmission and pathogenesis of these important pathogens, and provide supportive evidence of the efficacy of CLDCs as both immunotherapeutics and vaccine adjuvants for the treatment or prevention of intracellular pathogen infection requiring induction of a Th1-biased, cell-mediated immune response for control or clearance.

CHAPTER 2

AEROSOL AND INTRANASAL CHALLENGE MODELS OF MURINE LA CROSSE VIRUS INFECTION

Introduction

The *Bunyaviridae* comprise the largest family of arthropod-borne viruses, and bunyaviruses continue to emerge throughout the world, posing significant public health risk (e.g., encephalitis, meningitis, hemorrhagic fever and respiratory distress syndromes) to humans and animals (Calisher, 1994). Many viruses in the family *Bunyaviridae* are designated as National Institute of Allergy and Infectious Diseases (NIAID) priority pathogens, including: Rift Valley fever virus (RVFV), Congo-Crimean hemorrhagic fever virus (CCHFV), La Crosse virus (LACV), Sin Nombre virus (SNV) and other hantaviruses, etc. Members of the family *Bunyaviridae* have been described as likely agents of bioterrorism because of their ability to induce serious illness in human subjects, the ease with which large volumes of infectious material can be produced, the potential for transmission via aerosol, and the current lack of prophylactic or therapeutic approaches to treatment (Sidwell and Smee, 2003). Considering the extraordinary evolutionary and epidemic potential of these viruses, their ability to emerge in new places, and their significance in human and veterinary health (Calisher, 1994), it is remarkable that licensed human vaccines or therapeutics for any of the bunyaviruses are lacking.

LACV, family *Bunyaviridae*, genus *Orthobunyavirus,* is designated as an NIAID priority B pathogen. The virus emerged as a significant human pathogen in the 1960s; the virus was isolated from the brain of a 4 year-old patient from Minnesota who was diagnosed with meningoencephalitis and subsequently died in La Crosse, Wisconsin (Thompson et al., 1965; Calisher, 1994; Rust et al., 1999). Since its emergence, LACV has remained a significant cause of encephalitis and an important public health problem in the United States (Rust et al., 1999; McJunkin et al., 2001). LACV is maintained in nature in a cycle involving *Aedes triseriatus* mosquitoes and chipmunks (*Tamias striatus grinseus*) and tree squirrels (e.g., *Sciurus carolinensis*).

Humans are tangential hosts, and pre-pubertal children are at greatest risk for severe infections. LACV infection of the central nervous system (CNS) in humans typically presents as meningoencephalitis with inflammation largely confined to the cerebral cortex, frequently resulting in seizures and coma and misdiagnosis as herpes encephalitis (McJunkin et al., 1997; Kalfayan, 1983; Sokol et al., 2001; Wurtz and Paleologos, 2000). Milder infections typically occur in older individuals and result in flu-like symptoms, such as headache, fever, and vomiting (McJunkin et al., 2001). Although the mortality rate in children is low, recovery can be associated with significant neurologic sequelae, including learning disabilities, attention deficit hyperactivity disorder and seizures (McJunkin et al., 2001; Balfour et al., 1973). Life-long costs attributed to these sequelae have been projected to range from $48,775 – $3,090,398 per patient (Utz et al., 2003). There is neither a licensed vaccine nor a standard antiviral therapy for prevention of infection or treatment of La Crosse (LAC) encephalitis (Hollidge et al., 2010).

Mouse models of LACV infection following parenteral challenge have been developed and exploited to understand the pathogenesis of the virus in vertebrate hosts. Injecting suckling mice subcutaneously (sc) with as little as one plaque-forming unit (PFU) of LACV results in viremia, neuroinvasion, and fatal encephalitis. However, by 3 weeks of age, mice are almost uniformly-resistant to sc injection, even with >5 logs of virus, and neuroinvasion does not occur (Janssen et al., 1984). In contrast, the virus is neurovirulent in both age cohorts; injecting one PFU of LACV intracranially into suckling or adult mice uniformly results in encephalitis and death. Following sc inoculation, LACV replicates initially in striated muscle tissue, leading to a viremia and infection of the brain, possibly via passage of virus through vascular endothelial cells (Johnson, 1983; Janssen et al., 1984; Griot et al., 1993a; Griot et al., 1993b). In weanling mice, intraperitoneal inoculation of LACV results in viremia and infection of multiple tissues, with the nasal turbinates yielding the greatest viral titers. Intranasal inoculation of LACV in weanling mice also results in nasal turbinate infection, and virus can directly enter the CNS via the olfactory nerves in the nasal epithelium (Bennett et al., 2008).

LACV is not only an important human pathogen, but it can also serve as a model for aerosol transmission of bunyaviruses. Development of new, safe, and efficacious vaccines and therapeutics for bunyavirus infections, whether occurring by natural or purposeful airborne events, needs to be expedited. Although LACV is naturally transmitted by the bite of an infected arthropod vector, in the studies described herein we developed inhalational challenge models using aerosol and intranasal inoculation in order to address this roadblock. We hypothesized that inhalational challenge with LACV would lead to the development of lethal LAC encephalitis in adult mice. We evaluated

the infectivity, tissue tropism, and pathogenesis of LACV when delivered in aerosol or intranasal mouse challenge models.

Materials and Methods

Mice.

5-6 week old (25g) female C3H, BALB/c, and ICR outbred mice were obtained from Harlan Laboratories (Indianapolis, IN) and National Cancer Institute (Frederick, MD). All animals were housed for a minimum of 7 days prior to manipulation or challenge in order to allow for acclimation to the research facility. All protocols and procedures involving animals were approved by Colorado State University's Animal Care and Use Committee.

Virus.

La Crosse virus (wildtype(wt) human/1960), originally isolated from a human case in 1960 from La Crosse, WI, was used in all aerosol-challenge experiments. The virus had been passed three times in suckling mouse brain followed by three passages in BHK-21 cells. Stock virus (LAC wt) was then prepared by amplification in a fourth passage in BHK-21 cells in Leibovitz's L-15 (Invitrogen, Carlsbad, CA) supplemented with 10% FCS (Colorado Serum Co., Denver, CO) and L-glutamine (Invitrogen), titrated in Vero cells in 96-well plates, and titers were determined and expressed as $\log_{10}$ tissue culture 50% infectious doses ($TCID_{50}$) per mL or per gram of tissue (Kärber, 1931). La Crosse virus (strain Lac Ori) was used in all intranasal-challenge experiments. Lac Ori virus stocks were maintained and amplified in BHK-21 cells as described above.

Aerosol challenge with LACV.

For aerosol challenge, mice were exposed to LACV in a Middlebrook Airborne Infection Apparatus (Glas-Col LLC, Terre Haute, IN) according to the manufacturer's

instructions in a BSL3 laboratory. The mice were exposed to a suspension (5mL) of LACV (2.3 x 10^8 $TCID_{50}$/mL). All aerosol challenge experiments included a nebulizing time of 20 minutes (60 ft^3/hr), followed by a 20 minute cloud decay and a 10 minute decontaminating UV exposure. Experiments typically involved the exposure of 30 to 40 mice per aerosolization. Mice were then examined twice daily for a minimum of 28 days post-exposure for the development of signs of encephalitis (including sick rodent posture, repetitive behaviors, and hind limb paralysis). All animals were humanely-sacrificed upon development of symptoms of clinical illness.

Intranasal challenge with LACV.

For intranasal challenge, mice were anesthetized with ketamine (100 mg/kg; Fort Dodge Animal health, Overland Park, KS) and xylazine (10 mg/kg; Ben Venue Laboratories, Bedford, OH). LACV was thawed just before use, diluted in whole media and delivered in a 50 µL total volume (25µL/nostril) to anesthetized mice in groups of 4-10 within a dose range of 5 x 10^1 - 5 x 10^6 $TCID_{50}$. Mice were then examined twice daily for a minimum of 28 days post-exposure for the development of signs of encephalitis, and animals were humanely-sacrificed upon development of clinical illness as described above.

Histological staining and examination of brain tissue.

Brains were harvested from 4-5 mice per challenge group at various time points following challenge (both prior to and concurrent with the development of clinical signs of encephalitis) in order to examine the histologic changes in the 2 experimental groups. The brain of each mouse was removed immediately after euthanasia, fixed in 10% neutral buffered formalin, and then coronally sectioned. Tissue sections were processed routinely, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and

eosin. Tissue pathologic changes were determined by a professional veterinary pathologist.

Determination of viral titer in tissues.

Following aerosol LACV challenge, the following tissues and organs were extracted from 3 mice per timepoint at 2, 4, 6, 8 and 10 days post-challenge: serum, brain, nasal turbinates, lung, spleen, liver and kidney. Following intranasal LACV challenge (5 x 10^4 $TCID_{50}$ per mouse), identical tissues were collected from 5 mice per timepoint at 1, 2, 3, 5, 7 and 8 days post-challenge.

Harvested tissues were placed in pre-weighed tubes containing 1 mL L-15 media plus 10% FCS and stored at -70°C until homogenized via glass dounce grinder. To determine viral titers, homogenates were briefly centrifuged (2 minutes at 14,000 rpm), diluted serially 1:10 in triplicate and added to Vero cells in a 96-well microplate format. Endpoints were determined, and titers were calculated (Kärber, 1931).

Results

Aerosol infectivity of LACV in ICR mice.

Ten 6-week old female ICR mice were exposed to 2.3 x 10^8 $TCID_{50}$ of LACV in a whole body Glas-Col aerosol exposure chamber for 20 minutes. All mice subsequently developed clinical signs of CNS infection within 14 days post-challenge (Fig. 2.1). Mice displayed a range of symptoms from sick rodent posture (lethargy or unwillingness to move, lack of grooming) to uncontrollable repetitive behavior (e.g., constant circling), to hind limb paralysis. All mice were sacrificed immediately upon observation of any CNS symptoms. The earliest time point that any mouse became sick was day 7 post-challenge (2/10 mice), while the latest development of symptoms occurred on day 14.

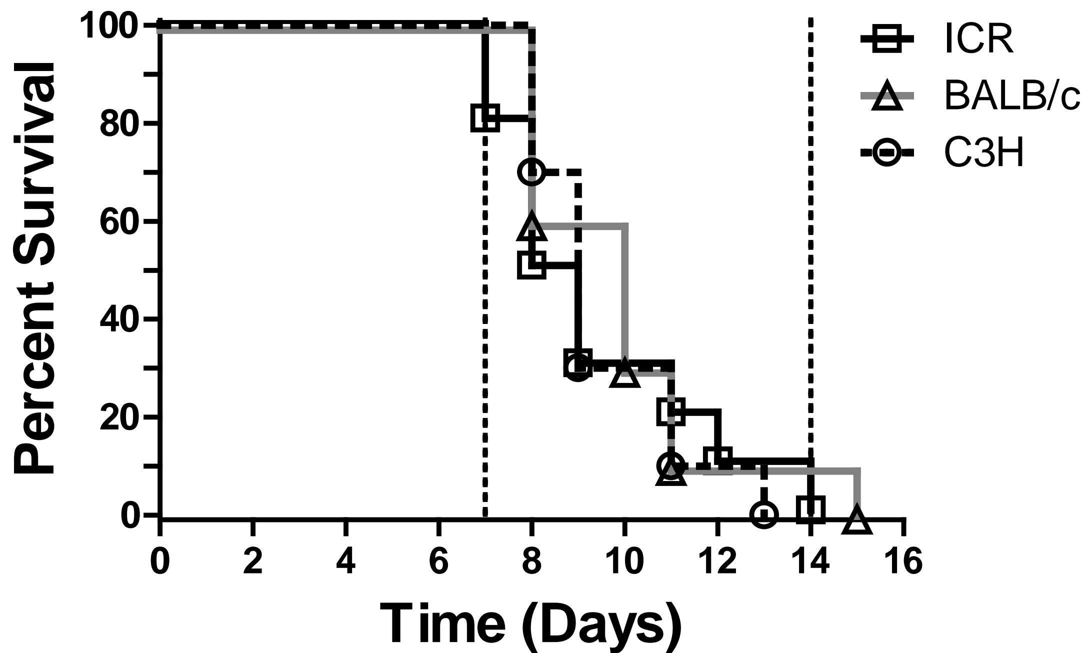

Figure 1.1. Aerosol challenge with LACV leads to 100% mortality. ICR, BALB/c, and C3H mice (*n* = 10 animals) were challenged with a suspension (5.0 mL) of LACV (2.32 x 10^8 $TCID_{50}$ /mL) via aerosol exposure and survival times were determined as described in Materials and Methods. Data shown are representative of two independent experiments.

Aerosol infectivity of LACV in BALB/c and C3H mice.

To ascertain whether similar kinetics of infection and/or levels of mortality would be observed in inbred mouse strains with differing genetic and immunologic backgrounds, ten female BALB/c mice and ten female C3H mice were challenged identically to the outbred mice. A single mouse from each group was euthanized on day 2 post-exposure, and the lungs were examined for lesions characteristic of infection (e.g., consolidation). No pathologic changes were observed in the lungs of either mouse, suggesting that early viral replication in the lungs was not prominent in mice challenged in this fashion. The remaining mice were then observed for at least 28 days for the development of signs of encephalitis. Regardless of genetic background, all aerosol-

challenged mice developed symptoms within 15 days following challenge, just as the ICR outbred mice had. The kinetics of symptom development were also comparable between the two inbred strains, as well as similar to that seen in the ICR challenge experiments (Fig. 2.1). The earliest time point for development of symptoms was day 8 post-challenge for both C3H and BALB/c strains, while the latest development of symptoms was observed on day 13 for C3H and day 15 for BALB/c mice. We chose to continue all further experiments with C3H mice because of their genetic similarity.

Histologic lesions in mice following LACV aerosol challenge.

In order to observe the pathogenesis of LACV in mice following aerosol challenge, brains were harvested at selected post-challenge time points for histological examination. Only one mouse displayed clinical signs of CNS infection at the time of sacrifice (day 10). In aerosol LACV-challenged animals, brain tissues contained histologic lesions of meningoencephalitis starting as early as day 3 post-challenge (Fig. 2.2).

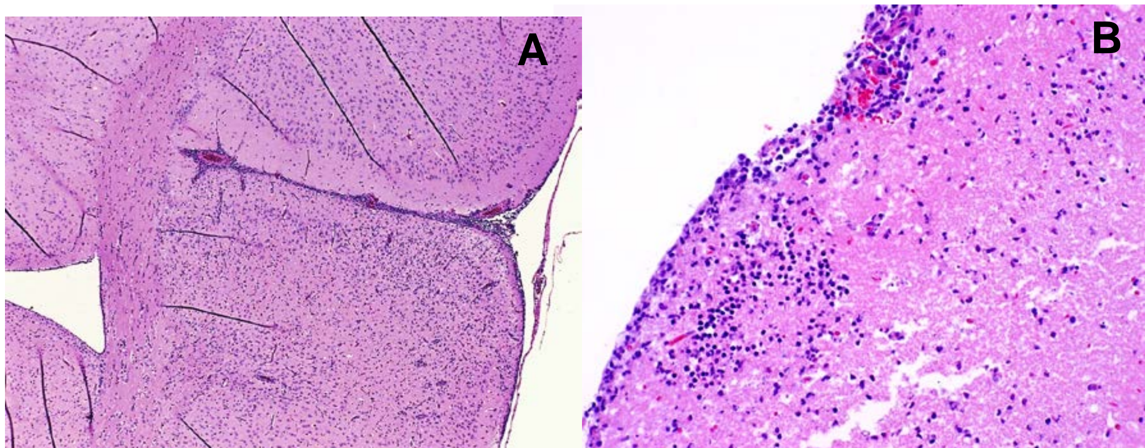

Figure 2.2. Histologic lesions in the CNS of aerosol-challenged animals. C3H mice (n = 5 animals) were challenged via aerosol with a suspension (5.0 mL) of LACV (2.32 x 10^8 $TCID_{50}$/mL) and brains were harvested at the first signs of disease. Panel A illustrates severe lymphocytic necrotizing meningoencephalitis with areas of gliosis. Panel B, at a higher magnification, shows parenchymal necrosis and inflammation. Images are representative of results obtained from three independent experiments. (A): Aerosol-challenged mouse brain, day

18 post-challenge. H&E stain, 4x objective magnification. (B): Aerosol-challenged mouse brain, day 11 post-challenge. H&E stain, 20x objective magnification.

Lesions were extensive and severe in the brains of all mice by day 10 post-challenge, regardless of symptom presentation or lack thereof. Lesions presented in a patchy, multifocal pattern, and were observed at all levels of the brain, including the cerebral cortex, hippocampus, midbrain, meninges, and brainstem. Lesions were typified by generalized meningoencephalitis with perivascular accumulations of lymphocytes and heterophils, in combination with neuronal degeneration and necrosis, rarefaction of the neuropil, and gliosis (Fig. 2.2). Lymphocytes and plasma cells were present in the meninges and in perivascular locations within the cortex. The inflammation and meningitis, although multifocal, were most pronounced in the anterior and ventral areas of the brain. In the most severely-affected brains, there were foci of liquefaction of the brain parenchyma, and the meningitis extended to the ventral brain stem area. All observed lesions were characteristically similar, but were present to variable extents; some brains presented with markedly-diminished cellular infiltrates and observable reductions in the degree of meningoencephalitis and the extent of neuronal necrosis. In addition, some areas of the brain were unaffected. Confirmation of LACV-presence was determined by amplification and detection of LACV M-segment RNA sequences via a nested reverse-transcriptase polymerase chain reaction (image not available).

Virus tissue tropisms and titer following aerosol infection.

To investigate the pathogenesis of LACV following aerosol infection, we harvested tissues from challenged mice at selected time points and titrated them for infectious virus. Tissues with detectable titers of infectious virus included the lung, nasal turbinates, and brain (Table 2.1), with the highest titers detected in the nasal turbinates

and brain. Liver, spleen and kidneys did not consistently contain infectious virus, if at all. Virus was not detected in the sera of challenged mice at any timepoint, suggesting that unlike following sc infections, the development of viremia was not a prerequisite for neuroinvasion following aerosol infection.

Table 2.1. Detection of LACV in tissues of mice following aerosol challenge[a]

Tissue	Day 2	Day 4	Day 6	Day 8	Day 10
Serum	NDV[b]	NDV	NDV	NDV	NDV
Brain	NDV	NDV	5.5	6.4	8.6
Nasal Turbinates	NDV	1.2	3.0	1.4	4.9
Liver	NDV	NDV	NDV	NDV	NDV
Lung	NDV	2.5	NDV	NDV	NDV
Kidney	NDV	NDV	NDV	NDV	NDV
Spleen	NDV	NDV	NDV	NDV	NDV

[a] **geometric mean (n = 3) $\log_{10}$ $TCID_{50}$ titer per gram or mL of tissue**
[b] **NDV – no detectable LACV at 1:10 dilution**

LACV was first detected in lungs of 2 of 3 mice on day 4 post-challenge, at which time the mean virus titer was 2.5 $\log_{10}$ $TCID_{50}$/gram of lung tissue (Fig. 2.3). LACV was not detected in the lungs of any mouse at any subsequent time point, suggesting that only transient infection of the lungs occurs following aerosol challenge or that virus titer is below the level of detection. Nasal turbinates of challenged mice were assayed in order to determine if LACV infection occurred first in these tissues, which could then lead directly to neuroinvasion and CNS infection. The nasal turbinates are located in close proximity to the brain and the rest of the CNS, and it is possible that infection of epithelial tissue could subsequently seed the bloodstream with infectious virus and/or directly infect the brain via olfactory neurons, which could discount the importance of early infection of the lungs as a prerequisite to neuroinvasion.

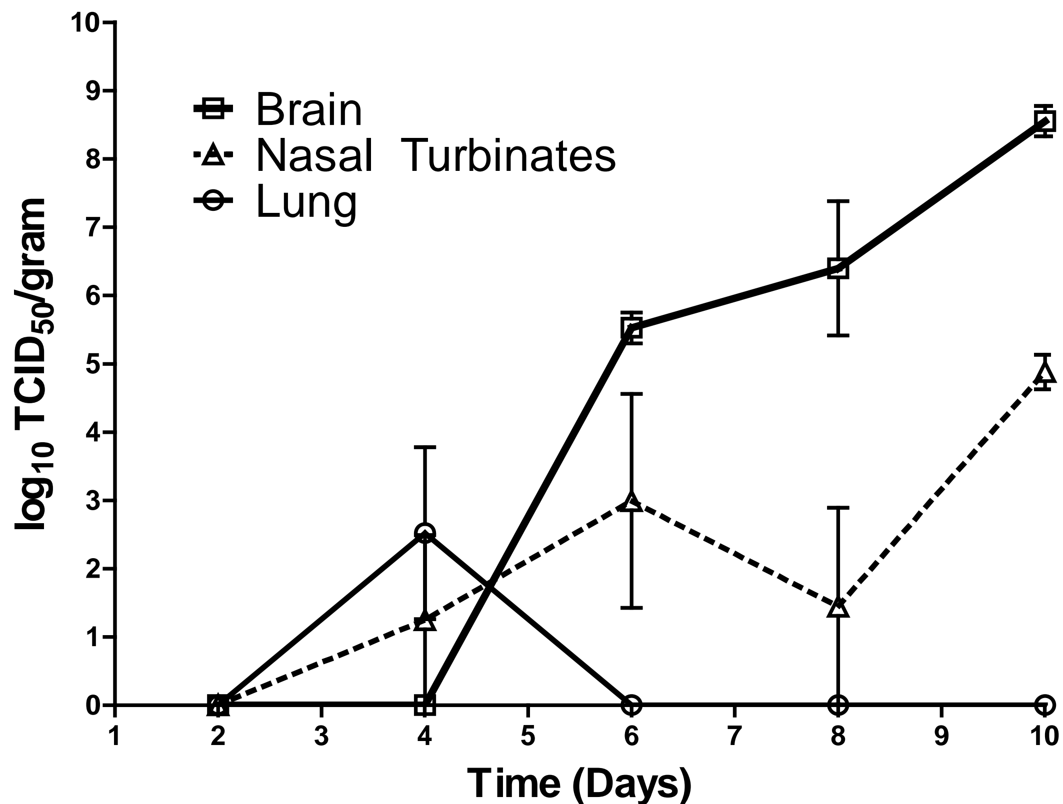

Figure 2.3. Viral titer in mouse tissues following aerosol challenge with LACV. C3H mice (n = 15 animals) were challenged with LACV via aerosol, and geometric mean viral titer (expressed as $\log_{10}$ $TCID_{50}$/gram of tissue ± SEM) were determined in the brain, nasal turbinates, and lung at the indicated time points after challenge, as described in Materials and Methods. Data are representative of two independent experiments.

Nasal turbinate infection was first detected on day 4 post-challenge (Fig. 2.3). By day 10, virus replication in this tissue had reached maximum titer (4.9 $\log_{10}$ $TCID_{50}$/gram). These results suggest that the major site of peripheral replication following aerosol challenge is the nasal turbinates.

Virus was first detected in the brains of challenged mice on day 6 post-challenge, and all brains were positive for infectious virus at all later timepoints. Brain titer increased steadily throughout the time course, reaching the greatest titer in any assayed tissue by day 10 (8.6 $\log_{10}$ $TCID_{50}$/gram) (Fig. 2.3).

Intranasal infectivity of LACV in C3H mice.

Concerns about the actual dose of virus received by individual mice and differences in lung infection rates in the aerosol model prompted exploration of intranasal inoculation as an alternative to aerosol exposure as an inhalational model of LACV infection. Intranasal challenge permits administration of a more-controlled and consistent dose of virus to each animal. Sequential dilutions of LACV were administered intranasally to C3H mice, which were then monitored for CNS disease. Symptoms were more pronounced than those seen in mice infected by aerosol, with most encephalitic mice presenting with hind limb paralysis and/or complete inability or unwillingness to move. The kinetics of disease presentation were also quite uniform in intranasally-challenged mice. Challenge with 50 µl of inoculum containing 5×10^6, 5×10^5, or 5×10^4 $TCID_{50}$ of LACV resulted in 100% mortality in all groups, and all but one mouse displayed symptoms of CNS infection by day 8 post-challenge (Fig. 2.4). This remaining mouse ultimately developed symptoms on day 14. Of those challenged with the three lower doses of virus, only one mouse (challenged with an inoculum containing 5×10^3 $TCID_{50}$) developed neurological disease and this did not occur until day 14 post-challenge. All mice challenged at 5×10^2 or 5×10^1 $TCID_{50}$ survived to day 28 without development of signs of CNS disease.

Histological lesions following intranasal infection with LACV.

To determine the pathogenesis of LACV in mice infected following intranasal challenge, brains were harvested at various time points for examination.

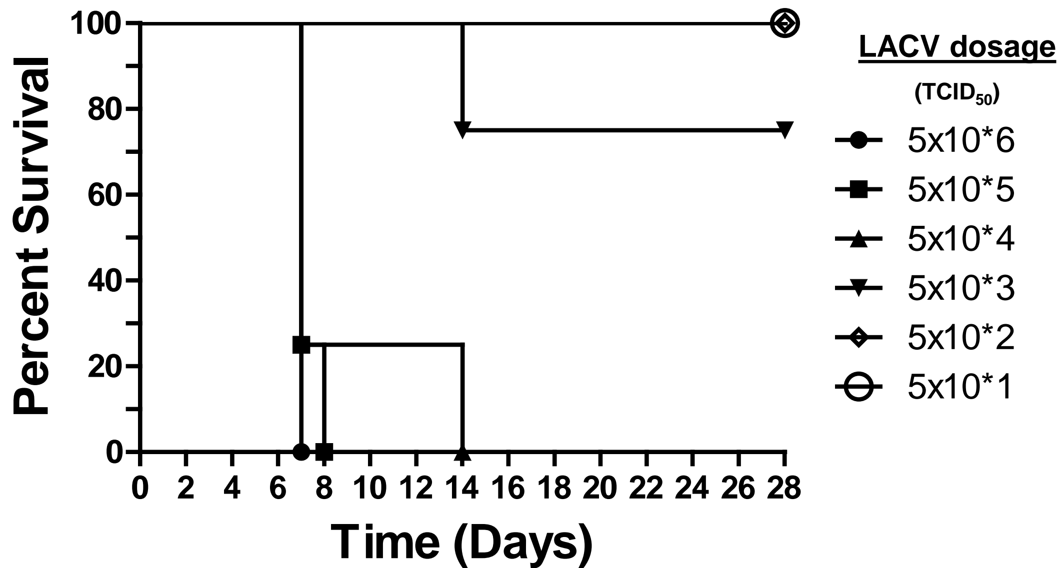

Figure 2.4. Survival of mice following intranasal challenge with LACV. C3H mice (*n* = 4 animals) were challenged intranasally with LACV (strain Lac Ori) at one of six dosages ranging from $5x10^1$ to $5x10^6$ $TCID_{50}$ and survival times were determined as described in Materials and Methods. Data shown are representative of two independent experiments.

Histological examination of the brains of mice challenged intranasally with LACV revealed inflammation of the brain parenchyma and the meninges (meningoencephalitis) in all animals euthanized following the development of clinical signs of CNS disease. The inflammation was characterized by a meningeal and perivascular infiltrate of lymphocytes and plasma cells, with variable numbers of macrophages and neutrophils, depending on the severity and acuteness/chronicity of the inflammatory process (Fig. 2.5). Necrosis was noted in association with inflammation (necrotizing meningoencephalitis) and was characterized by rarefaction of the neuropil, cellular debris, and neuronal necrosis. Very severe inflammation was present specifically in the olfactory lobes, which in some animals was so severe that there was liquefaction of the

parenchyma and replacement by foamy macrophages (i.e., gitter cells, which are microglial cells swollen with phagocytic debris from dead cells of the CNS) (Fig. 2.5B).

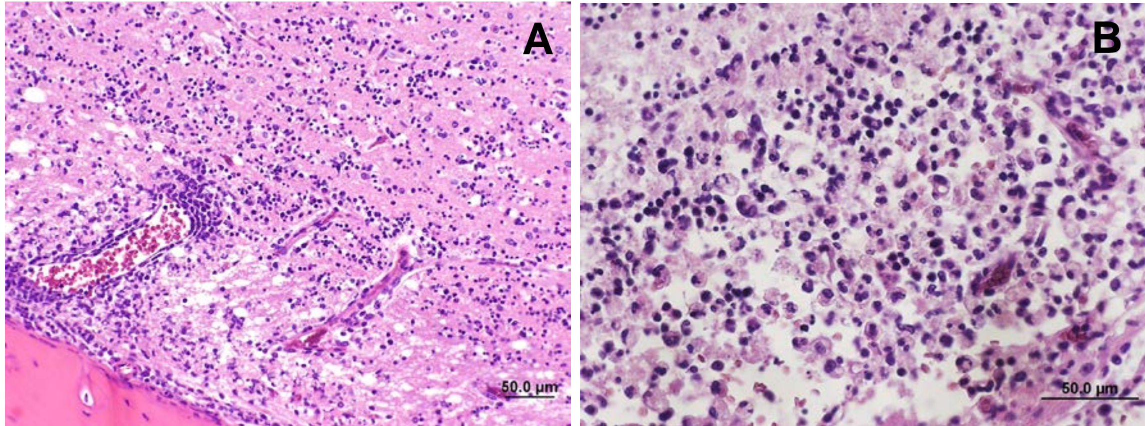

Figure 2.5. Histologic lesions in the CNS of intranasally-challenged animals. C3H mice (*n* = 5 animals) were challenged intranasally with 50 µL of inoculum containing 5 x10^4 $TCID_{50}$ of LACV and brains were harvested at the first signs of disease. Panel A illustrates severe lymphocytic necrotizing meningoencephalitis with areas of parenchymal necrosis. Panel B, at a higher magnification, shows inflammation, parenchymal necrosis, and accumulations of gitter cells. Images are representative of results obtained from three independent experiments. (A): Intranasally-challenged mouse brain, showing nasal turbinates and section 41 of the olfactory lobes; day 9 post-challenge. H&E stain, 20x objective magnification. (B): Intranasally-challenged mouse brain, showing section 41 of the olfactory lobe; day 9 post-challenge. H&E stain, 40x objective magnification.

Additionally, necrotizing inflammation was most often noted in the ventral aspect of the rostral cerebrum (the anatomical location of the olfactory tubercles), suggesting ascent of the infection along the olfactory tracts. Inflammation in the brain stem and obex was lower in magnitude and frequency, and active necrosis was absent in these caudal aspects of the brain. No significant differences in the patterns of pathologic changes were observed following intranasal vs. aerosol challenge, but the former consistently caused more-severe pathologic changes, possibly due to a higher challenge dose. LACV was confirmed to be present in the brain by amplification and detection of LACV M-segment RNA sequences via a nested reverse-transcriptase polymerase chain reaction (image not available).

Virus tissue tropisms and titer following intranasal infection.

Tissue tropisms and kinetics of infection following intranasal infection were determined. Tissues with detectable titers of infectious virus included serum, brain, nasal turbinates, liver, and lung (Table 2.2). As with aerosol infection, the greatest titers were detected in the nasal turbinates and brain.

Table 2.2. Detection of LACV in tissues of mice following intranasal challenge[a]

Tissue	Day 1	Day 2	Day 3	Day 5	Day 7	Day 8
Serum	**NDV[b]**	**1.8**	**2.7**	**NDV**	**NDV**	**NDV**
Brain	**NDV**	**2.5**	**1.9**	**5.6**	**5.5**	**6.9**
Nasal Turbinates	**NDV**	**4.3**	**4.3**	**4.7**	**4.9**	**5.4**
Liver	**NDV**	**1.4**	**1.3**	**0.5**	**NDV**	**NDV**
Lung	**NDV**	**1.5**	**2.2**	**NDV**	**NDV**	**4.3**

[a] geometric mean (n = 5) $\log_{10}$ $TCID_{50}$ titer per gram or mL of tissue
[b] NDV – no detectable LACV at 1:10 dilution

Virus was detectable in tissues as early as day 2 post-challenge (Fig. 2.6), in contrast to the aerosol-challenge model, in which infectious virus was first detected in any tissue on day 4 post-challenge.

LACV was first detected in the serum of intranasally-challenged mice on day 2 post-challenge (mean titer = 1.8 $\log_{10}$ $TCID_{50}$ /mL) (Fig. 2.6). By day 3, virus titer reached a mean titer of 2.7 $\log_{10}$ $TCID_{50}$ /mL. No virus was detected in the serum of any mouse at any subsequent time point, suggesting that viremia had been cleared or was at very low titer.

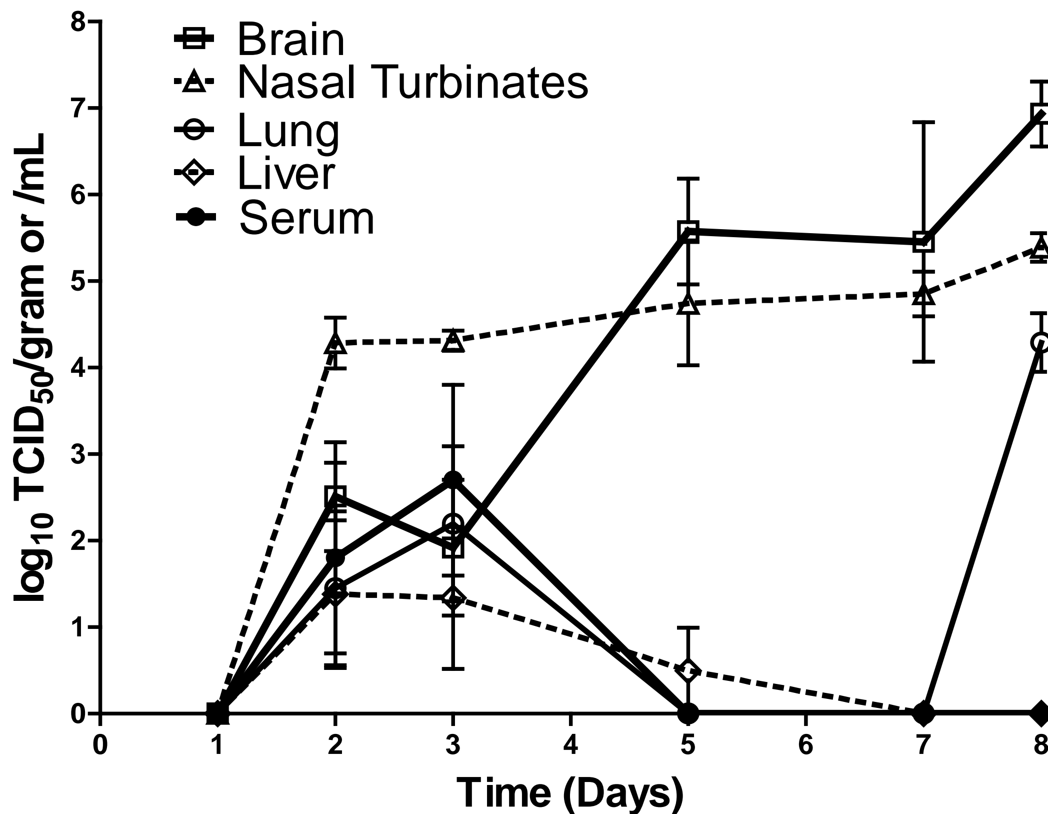

Figure 2.6. Viral titer in mouse tissues following intranasal challenge with LACV. C3H mice (*n* = 5 animals) were challenged intranasally with LACV, and geometric mean viral titers (expressed as $\log_{10}$ $TCID_{50}$/gram or /mL of tissue ± SEM) were determined in the brain, nasal turbinates, lung, liver, and serum at the indicated days post-challenge, as described in Materials and Methods. Data are representative of two independent experiments.

LACV was first detected in lung tissue on day 2 post-challenge (mean titer = 1.5 $\log_{10}$ $TCID_{50}$ /gram); by day 3, viral mean titer increased to 2.2 $\log_{10}$ $TCID_{50}$ /gram (Fig. 2.6). On days 5 and 7 post-challenge, virus was not detectable in lungs; however, virus was again detectable in the lungs on day 8 and titered at 4.3 $\log_{10}$ $TCID_{50}$/gram.

Virus was detected in the livers of intranasally-challenged mice at a maximum titer of 1.4 $\log_{10}$ $TCID_{50}$ /gram at 2 days post-challenge (Fig. 2.6). Viral load in the liver decreased steadily on days 3 and 5 post-challenge, and was not detectable at any subsequently-assayed time points.

Nasal turbinate infection was first detected on day 2 post-challenge, and mean nasal turbinate titer increased throughout the time course, reaching a maximum mean titer of 5.4 log_{10} $TCID_{50}$/gram on day 8 (Fig. 2.6). The LACV titer in the nasal turbinates on day 8 post-challenge was the highest of any of the peripheral tissues assayed from intranasally-challenged mice.

LACV was first detected in the brains of challenged mice on day 2 post-exposure; all assayed brains were positive for infectious virus at all subsequent time points. In general, mean brain titer increased throughout the time course and reached a maximum mean titer of 6.9 log_{10} $TCID_{50}$ /gram of tissue on day 8 (Fig 2.6). This was the greatest titer of LACV detected in any tissue.

Discussion

Adult mice have been demonstrated to be very resistant to LACV infection following subcutaneous or intramuscular challenge, even when challenged with high doses of virus (Johnson, 1983; Janssen et al., 1984). Prior studies in our laboratory have shown that less than 10% of adult mice develop CNS disease when injected with 1 x 10^5 $TCID_{50}$ of virus subcutaneously or intramuscularly (unpublished observations). In contrast, the current studies demonstrate that adult mice are very susceptible to aerosol or intranasal challenge with LACV. The classical model of LACV infection via peripheral routes involves initial replication in skeletal muscle tissue near the site of infection with eventual infection of the vascular endothelium and development of viremia. The virus may then bypass the blood-brain barrier and enter the CNS though infection of the vascular tissue supplying the brain (Johnson, 1983; Janssen et. al, 1984; Griot et. al, 1993a; Griot et. al, 1993b). High viremia titer is thought to condition neuroinvasiveness of LACV (Janssen et al., 1984). Aerosol or intranasal delivery of LACV might not

require a viremia to negotiate the blood-brain barrier; rather, the virus could infect tissues in the nasal epithelium, including olfactory neurons, and thus be transported directly into the CNS.

We have demonstrated that adult mice can be infected by both aerosol and intranasal challenge with LACV, leading to high rates of mortality: 100% of challenged mice succumbed to lethal LAC encephalitis within 15 days following aerosol challenge, and groups of mice challenged intranasally exhibited 100% mortality as early as day 7 post-challenge. The faster kinetics of mortality seen following intranasal challenge are likely attributable to the dose of virus administered to the respective mice and the portal of entry for the virus following intranasal and aerosol infection. Intranasal challenge would theoretically result in higher challenge doses, but might also involve a more-direct challenge of nasal epithelial tissues in close contact with the CNS, allowing for accelerated neuroinvasion and more rapid mortality. Regardless of the route of challenge, the significant titers of LACV in nasal turbinate tissues likely condition neuroinvasiveness and may overwhelm the normal immune mechanisms that protect adult mice from CNS infection. To our knowledge, this is the first report of an aerosol challenge with LACV leading reproducibly to the development of lethal encephalitis in high percentages of normally-resistant adult mice. However, because of the ability to better control the dose and route of infection, the intranasal challenge route would seem to be the better model of inhalational infection.

Following aerosol or intranasal challenge, LACV replicated in various peripheral tissues before entering the brain and causing CNS disease. In mice challenged via aerosol, LACV was first detected in the nasal turbinates and in lung tissue on day 4 post-

challenge. LACV was maintained in the nasal turbinates at high titer through day 10 of infection, but was not detected in lung tissues after day 4. LACV was first detectable in the brains of aerosol-challenge mice on day 6 post-challenge, implying that the virus must infect and replicate in peripheral tissues before neuroinvasion occurs. The virus replicated in the brain to the greatest titer found in any tissue in aerosol-infected mice. Further studies will be necessary to determine if the virus was replicating in the lungs or was a result of viremia or shedding of virus into the lower respiratory tract from nasal turbinates.

In mice challenged via intranasal inoculation, LACV was detected in the nasal turbinates and brain on day 2 post-challenge and remained there at high titers throughout the course of infection. LACV was also transiently detected in the lungs between days 2-3 post challenge. In contrast to aerosol infection, LACV was also detected in the livers and serum of intranasally-infected mice. Viremia was detectable between days 2 and 3 post-challenge, while LACV was present in the liver between days 2 and 5. The presence of a detectable viremia following intranasal challenge was a significant and notable difference between the two challenge models; viremia was not detected in the serum of any mouse infected following aerosol challenge. Viremia following intranasal infection may have been a contributing factor to subsequent neuroinvasion in the intranasal challenge model.

Intranasal infection also resulted in more rapid infection of organs and tissues. Following intranasal infection, LACV was detected concurrently in the CNS and in other tissues, in contrast to aerosol infection, in which virus was detected in peripheral tissues (lungs and nasal turbinates) days before being detected in the brain. Infectious virus was

detectable in tissues as early as day 2 following intranasal challenge, but not until day 4 following aerosol challenge. Viral titers were greater in the nasal turbinates of intranasally-challenged mice than in aerosol-challenged mice. These increased viral titers in the nasal turbinates of intranasally-challenged mice may have accelerated neuroinvasion and death.

In summary, intranasal infection leads to accelerated infection, infection of additional peripheral tissues, higher viral titers in tissues, earlier CNS infection, and earlier time to death than aerosol infection. Inoculation of high doses of LACV directly into the nasal passages may have led to rapid infection of peripheral tissues (primarily the nasal turbinates), where LACV was able to replicate to high titers sufficient to either seed the bloodstream or to ascend the olfactory neurons, allowing for quick entry to the brain and resulting in faster kinetics of mortality. It is notable that in both challenge models, the major site of peripheral replication was the nasal turbinates.

These patterns of LACV tissue tropism support the observations of Bennett et al., who showed that weanling (4 week-old) Swiss Webster mice were susceptible to lethal LACV infection by either the intranasal or intraperitoneal routes (2008). Interestingly, this research group also found the peripheral tissue with the highest titers of virus to be the nasal turbinates, and suggested that LACV could presumably enter the CNS via the olfactory neurons that bridge the nasal olfactory epithelium and the brain. The abnormal susceptibility of adult mice to intranasal and/or aerosol challenge with LACV may indeed be explained by the close association between these tissues of the upper respiratory tract and the brain/CNS. Indeed, many other arboviruses can be aerosol transmitted and some have been shown to infect the CNS directly through the olfactory tract (Kuno, 2003,

Larson et al., 1980; Ryzhikov et. al, 1995a; Ryzhikov et. al, 1995b; Vogel et. al, 1996). Our studies suggest that many other arboviruses and rodent borne viruses may also be infectious to humans when delivered via aerosol and thus are of bioterrorism concern.

Both aerosol and intranasal challenge with LACV led to the development of histologic lesions of encephalitis in the brains of challenged mice, typified by generalized meningoencephalitis with histological evidence of disease. No significant differences in the patterns of pathologic changes were observed due to intranasal vs. aerosol challenge, but the severity of lesions was consistently worse following intranasal challenge. In the aerosol model, the inflammation and meningitis, although multifocal, were most pronounced in the anterior and ventral areas of the brain. In the most severely-affected brains of aerosol-challenged mice, there were foci of liquefaction of the brain parenchyma, and the meningitis extended to the ventral brain stem area. In the intranasal model, very severe inflammation was noted specifically in the olfactory lobes, which in some animals was again so severe that there was liquefaction of the parenchyma and replacement by foamy macrophages. Additionally, in the intranasal challenge model necrotizing inflammation was most often noted in the ventral aspect of the rostral cerebrum (the anatomical location of the olfactory tubercles), suggesting ascent of the infection along the olfactory tracts in this challenge model. Accordingly, inflammation in the brain stem and obex was lower in magnitude and frequency, and active necrosis was absent in these caudal aspects of intranasally-challenged brains.

The increased severity of histologic lesions in the intranasal challenge model might again be the result of a more-direct challenge of the nasal turbinates with a higher challenge dose of virus. Alternatively, the primary route of CNS infection may actually

differ between the two challenge models. It is possible that intranasal challenge provides for a more-direct infection of the CNS via the olfactory tract (as has been demonstrated with Japanese Encephalitis virus), leading to the more-localized lesions of encephalitis found in the rostral portions of the mouse brain. Coupled with the histological observations of lesions largely confined to the anterior and ventral portions of the brains of intranasally-challenged mice, the observation that LACV consistently grew to its highest peripheral titers in the nasal turbinates strongly suggests that a direct infection of the CNS is occurring through the olfactory tract. This direct intranasal infection route may also explain the earlier and more uniform presentation of clinical encephalitis in intranasally-challenged mice.

The neurological lesions observed in the brains of mice challenged with LACV either intranasally or via aerosol were indistinguishable from those that have been observed in mice infected by vector-borne or subcutaneous routes: mainly a non-focused meningoencephalitis characterized by a large amount of cellular infiltrate (perivascular cuffing) and necrosis (Pekosz et al., 1996). Interestingly, this pattern of pathological changes is not noted in the brains of younger suckling mice challenged with LACV, in which apoptosis is thought to be responsible for most of the neural damage and the associated inflammation is mild. It has been suggested that increased cellular differentiation in the adult CNS, including increased expression levels of the anti-apoptotic regulator *bcl-2* (Pekosz et al., 1996) may account for this difference. This may explain, in part, the normally-refractory nature of adult mice to the development of LAC encephalitis. The unnatural route of infection or introduction of high titers of virus into the nasal epithelial tissues may serve to overwhelm the normal immune mechanisms that

protect adult mice from CNS infection. As with mice, older (post-pubertal) humans become decreasingly susceptible to encephalitis, which occurs almost exclusively in younger patients (Johnson, 1983; McJunkin et al., 2001). Our studies suggest that adults could experience severe neurological disease following aerosol exposure to LACV and related viruses. This is cause for concern and requires rethinking of the process for inclusion and exclusion of viruses from the NIAID priority pathogens.

In summary, inhalational (aerosol and intranasal) models of LACV infection in adult mice have been developed. Following either aerosol or intranasal challenge with LACV, 100% of normally-refractory adult mice developed clinical and histological signs of LAC encephalitis. Importantly, in the aerosol model, LACV viremia was never detected, suggesting that direct infection of the nasal olfactory epithelium can lead directly to CNS infection in absence of a viremia, which has been classically-accepted as a prerequisite to neuroinvasion. This ability of aerosol- or intranasally-delivered LACV to cause 100% mortality in normally-resistant adult mice underscores the dangerous potential of members of the *Bunyaviridae* as possible bioterrorism agents. These inhalational LACV challenge models will hopefully serve as useful tools with which to test the efficacy of sorely-needed vaccines and therapeutics to be used in the events of accidental or purposeful airborne release of members of the *Bunyaviridae.*

CHAPTER 3

IMMUNOSTIMULATORY CATIONIC LIPOSOME-DNA COMPLEXES ELICIT PROTECTION AGAINST AEROSOLIZED LA CROSSE VIRUS CHALLENGE IN A MURINE MODEL

Introduction

Viruses in the family *Bunyaviridae* designated by the National Institute of Allergy and Infectious Diseases (NIAID) as Category A, B, and C priority pathogens include Rift Valley fever virus (RVFV), Crimean-Congo hemorrhagic fever virus (CCHFV), La Crosse virus (LACV) and other related encephalitis viruses, as well as Sin Nombre virus (SNV) and other related hantaviruses (Nichol, 2001). Many recently-emerged or emerging viruses also belong to the family *Bunyaviridae*. Despite their significance in public and veterinary health (Calisher, 1994), their remarkable potential to emerge in new areas, and their significant potential for use as bioterrorism agents, vaccines and licensed antiviral treatments for many of these viruses are lacking. Furthermore, with more than 250 members, it is difficult to imagine developing protective vaccine formulations for all the many bunyaviruses that could emerge in human populations resulting from natural or purposeful events (Beaty and Calisher, 1991). Novel immunological approaches are sorely needed to prevent and control these increasingly-important pathogens.

LACV (family *Bunyaviridae,* genus *Orthobunyavirus*) first emerged as an important human pathogen in the 1960s (Thompson et al., 1965) and has since persisted as a leading cause of encephalitis and thus a considerable public health concern in the

United States (Rust et al., 1999, McJunkin et al., 2001). In endemic areas, the incidence of La Crosse (LAC) encephalitis surpasses that of bacterial meningitis (McJunkin et al., 2001), and LACV remains the leading cause of pediatric encephalitis in the United States with approximately 70-130 cases with severe neurological involvement diagnosed annually. Significantly, the actual number of pediatric infections that occur is thought to be vastly underdiagnosed (Calisher, 1994). In the past, most cases of LAC encephalitis have occurred in states of the upper Midwest; more recently, however, cases have been identified in North Carolina, West Virginia, Tennessee, and Missouri (Jones et al., 1999; CDC, 2010; Haddow and Odoi, 2009; Haddow et al., 2011). *Aedes triseriatus* (the Eastern Treehole mosquito) is the principal vector of LACV, efficiently transmitting LACV both horizontally and transovarially (Watts et al., 1973). The recent isolation of LACV from field-collected larvae and male *Aedes albopictus* (a species introduced into the North American continent within the last two decades) and the connection of this vector to human cases in Tennessee collectively are a cause for concern to public health (Gerhardt et al., 2001; Erwin et al., 2002) as *Ae. albopictus* has been shown to be a competent vector of LACV (Tesh and Gubler, 1975; Hughes et al., 2006) and LAC encephalitis is on the rise in the South, where recent cases have been reported in Louisiana, Alabama, Georgia, and Florida (Lambert et al., 2010).

Severe LACV infection of the central nervous system (CNS) in humans commonly presents with clinical signs of seizures and eventual coma, and the brains of patients display histologic lesions of meningoencephalitis consisting of cellular infiltrates largely confined to the cerebral cortex (McJunkin et al., 1997; Kalfayan, 1983). Milder infections are thought to be far more common and result in flu-like symptoms, such as

headache, fever, and vomiting (McJunkin et al., 2001). Recovery can be associated with significant neurological sequelae, including learning disabilities, attention deficit hyperactivity disorder and seizures (McJunkin et al., 2001; Balfour et al., 1973). At present there is neither a licensed vaccine nor a standard antiviral therapy for prevention of LACV infection or treatment of LAC encephalitis, providing impetus for the development of immunotherapies for treatment of this severe disease (Hollidge, 2010).

The data on the immunostimulatory and immunoenhancing properties of cationic liposome-nucleic acid complexes is well documented (Dow, 2008). When deployed in combination with certain pathogen-associated molecular patterns (PAMPs), such as non-methylated CpG motifs enriched in bacterial DNA, cationic liposomes are capable of facilitating endocytic uptake and markedly enhancing the innate immune stimulatory properties of such adsorbed molecules (Gursel et al., 2001; Krieg, 2002). It has been proposed that this potentiation of the innate immune response is due to the ability of cationic liposomes to protect adsorbed nucleic acids from extracellular degradation while simultaneously localizing the complexes to the early endosomal compartment of targeted cells, the primary site of expression for nucleic acid pattern recognition receptors (PRRs) (Akira and Takeda, 2004; Akira, 2006; Zaks et al., 2006; Takeuchi and Akira, 2007). The first detailed description of intravenous treatment with liposome-DNA complexes leading to stimulation of the innate immune system resulted from investigations of these formulations for potential immunotherapeutic treatment of tumors (Dow et al., 1999a). The authors determined that the observed CLDC-induced antitumor effects were a result of stimulation of the innate immune system, specifically the activation of natural killer (NK) cells and resultant production of IFN-γ (Dow et al., 1999b). This ability of cationic

liposome-DNA complexes (CLDC) to stimulate the innate immune system makes them uniquely appealing as non-specific immunotherapeutics and vaccine adjuvants. CLDCs or liposomal CpG oligodeoxynucleotide (ODN) complexes have not only been shown to exert notable anticancer activity in various animal tumor models (Dow et al., 1999a; Whitmore et al., 1999; Whitmore et al., 2001; Lanuti et al., 2000; Dow et al., 1999b), but have also been successfully used for immunotherapeutic treatment of acute viral (Gowen et al., 2006; Gowen et al., 2008; Logue et al., 2010) and bacterial infections (Goodyear et al., 2009; Troyer et al., 2009). CLDCs are actively endocytosed by antigen presenting cells (APCs) such as plasmacytoid dendritic cells (pDCs) and target delivery of adsorbed and protected molecules to the cellular endosomal compartment containing Toll-like receptors (TLRs) such as TLR3, TLR7/8, and TLR9. These PRRs recognize molecules common to various microorganisms and signal for the development of a non-specific immune response, resulting in the release of soluble effector molecules and the activation of innate immune cells. Unmethylated CpG ODNs efficiently bind to and activate TLR9, a signal receptor selectively expressed within the endosomal compartment of dendritic cells (DC), macrophages and B cells. Ligation of TLR9 leads to prompt non-specific activation of the immune system, principally in the spleen and draining lymph nodes. This response is typified by the release of pro-inflammatory cytokines, including TNF, IL-1β, IL-6, IL-12, IFN-γ, IFN-α and IFN-β (Ishii et al., 2004; Klinman et al., 2004). The particular cytokine milieu triggered following TLR9 ligation yields an immune response with a clear antiviral potential and Th1-type immune bias, which (if utilized as an adjuvant in the context of immunization, rather than as a stand-alone immunotherapeutic) ultimately supports the development of cell-mediated immunity

required for clearance of intracellular pathogens and control of tumor growth (Krieg, 2007). In addition, ligation of TLR9 leads to prompt cellular stimulation, characterized by upregulation of co-stimulatory molecules on DCs and macrophages, functional maturation of APCs, and NK cell activation and infiltration (Dow et al., 1999b). The immunostimulatory properties of CLDC have also been shown to be regulated in part by TLR-independent pathways and receptors, such as the cytosolic DNA sensor DAI (DNA-dependent activator of IFN-regulatory factors) (Ishii and Akira, 2006; Stetson and Medzhitov, 2006b; Takaoka et al., 2007; Takaoka and Taniguchi, 2008). Taken as a whole, the ability of CLDCs to selectively activate this type of an innate immune response suggests that they may be appropriate for use as an antiviral immunotherapy. For example, in a lethal mouse model of Punta Toro Virus (PTV) infection, CLDC administration prior to challenge was shown to increase survival, reduce viral load, and lessen the severity and presentation of disease symptoms (Gowen et al., 2006). The absence of PTV antigen in the CLDC formulation affirms that the conferred protection was due to innate, non-specific antiviral effector responses. Though the immunological mechanisms of protection were not determined in this study, it was hypothesized that the observed protection was mediated by type I interferons (IFN). Similar CLDC-induced antiviral effects were more recently reported in a murine model of lethal Western equine encephalitis virus challenge (Logue et al, 2010).

We examined the ability of CLDC immunotherapy to elicit protection against a lethal challenge with LACV, hypothesizing that CLDC treatment would protect against the development of lethal LAC encephalitis in adult mice following aerosol challenge with LACV. LACV is naturally transmitted by the bite of an infected arthropod vector,

and mouse models for parenteral LACV infection have been long established (Johnson, 1983; Janssen et al., 1984). However, many members of the family *Bunyaviridae* are designated as NIAID priority pathogens due to their potential to be used as agents of bioterrorism (Sidwell and Smee, 2003). This is due to a number of factors, including the ability of LACV and other members of the *Bunyaviridae* to induce serious illness in human subjects, the ease with which large volumes of infectious material can be produced, their potential for transmission via aerosol, and the current lack of prophylactic or therapeutic approaches to treatment (Sidwell and Smee, 2003). LACV is not only an important human pathogen, but also can serve as a model for development of therapeutics for other members of the family *Bunyaviridae*. In this regard, we recently developed a mouse model for aerosol infection with LACV (unpublished observations, see Chapter 2) and have now used this model to investigate the protective efficacy of CLDC immunotherapy for aerosol infection by bunyaviruses. CLDC immunotherapy protected mice against lethal aerosol challenge with LACV when administered pre-, co-, or post-challenge. CLDC immunotherapy, administered either prophylactically or therapeutically, provides a potential novel new approach to protect against natural or purposeful challenge with members of the *Bunyaviridae*.

In order to determine the ability of CLDC administration to reduce viral replication in challenged mice, various tissues were harvested for quantification of viral load at various time points after challenge. To verify the extent of infection and associated neuropathologic changes, brain tissues from treated and untreated mice were examined histologically for lesions of encephalitis. Innate immune responses were also evaluated via investigation of the expression of interferon genes in both the spleen and

brain. Finally, we systemically-depleted NK cells from mice prior to immunotherapeutic treatment in order to determine the importance of this cell type in CLDC-induced protection.

Materials and Methods

Mice.

5-6 week old (25g) female C3H, BALB/c, and ICR outbred mice were obtained from Harlan Laboratories (Indianapolis, IN) and National Cancer Institute (Frederick, MD). All animals were housed for a minimum of 7 days prior to respective treatments or challenge in order to allow for acclimation to the research facility. All protocols and procedures involving animals were approved by Colorado State University's Institutional Animal Care and Use Committee.

Virus.

La Crosse virus (wild type; wt), originally isolated from a human case in 1960 from La Crosse, WI, was used in all experiments. This low-passage virus stock had been passed three times in suckling mouse brain followed by three passages in BHK-21 cells maintained in Leibovitz's L-15 (Invitrogen, Carlsbad, CA) medium supplemented with 10% FCS (Colorado Serum Co., Denver, CO) and L-glutamine (Invitrogen). Stock virus (LAC wt) was then prepared by a fourth passage in BHK-21 cells and titrated in Vero cells in 96-well plates. The titer was calculated by the method of Kärber (1931).

Aerosol challenge with LACV.

For delivery of aerosolized LACV, mice were exposed to a 5 mL suspension of virus (2.32×10^8 $TCID_{50}$/mL) in a Middlebrook Airborne Infection Apparatus (Glas-Col LLC, Terre Haute, IN) under BSL3 conditions according to the manufacturer's instructions. Conditions for all aerosol challenge experiments included a nebulizing time of 20 minutes (60 ft^3/hr), followed by a 20 minute cloud decay and a 10 minute

decontaminating UV exposure (Arthun et al., 2011, submitted). Experiments typically involved the exposure of 30 to 40 mice per aerosolization. Mice were then examined twice daily for a minimum of 28 days post-exposure (maximum of 90 days post-exposure) for the development of signs of encephalitis (including sick rodent posture, repetitive behaviors, and hind limb paralysis). All animals were humanely sacrificed upon development of symptoms of clinical encephalitis.

Preparation and administration of cationic liposome-DNA complexes.

Cationic liposomes were prepared as previously described by combining equimolar amounts of DOTIM [octadecanoyloxy(ethyl-2-heptadecenyl-3-hydroxyethyl) imidazolinium chloride] and cholesterol (Templeton et al., 1997). Cationic liposome-DNA complexes (CLDC) were prepared fresh immediately prior to injection by gently mixing cationic liposomes with plasmid DNA (pMB75.6 empty vector, 3 mg/mL) in sterile Tris-buffered 5% dextrose in water at room temperature. The final concentration of plasmid DNA was 100 µg DNA/mL. For treatment of mice, 250 µL of the CLDC formulation was injected intraperitoneally (i.p.) at the appropriate timepoint. Control mice in all experiments received an i.p. sham injection of 250 µL Tris-buffered 5% dextrose in water (diluent). Additional controls included mice treated with cationic liposomes alone or plasmid-DNA alone.

Preparation and administration of CpG oligonucleotide constructs.

Mice (10-20/treatment group) were injected i.p. with either 250 µL CLDC, 50 µg Type C CpG oligonucleotide 2395 (InvivoGen, San Diego, CA) diluted in 250 µL 1xPBS, or 250 µL diluent 24 hours before aerosol challenge with LACV, and were then observed over a 4 week period for the development of clinical signs of encephalitis.

Histopathological examination of challenged mouse brains.

Brains from 4 sham-treated mice were harvested at the first signs of disease and compared to time-matched CLDC-treated mouse brains to examine the histologic changes in the 2 experimental groups. The brain of each mouse was removed immediately after euthanasia, fixed in 10% neutral buffered formalin, and then coronally sectioned. Tissue sections were processed routinely, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin. Tissues were evaluated by a professional veterinary pathologist.

Determination of viral titer in tissues.

To detect and quantify viral replication, tissues were extracted from 3 mice per treatment group at 2, 4, 6, 8 and 10 days post-challenge. Collected tissues included serum, brain, nasal turbinates, lung, spleen, liver and kidney. Once harvested, tissues were placed in pre-weighed tubes containing 1 mL L-15 medium plus 10% FCS and stored at -70°C until homogenized using a glass dounce grinder. To determine viral titers, homogenates were briefly centrifuged (2 minutes at 14,000 rpm), diluted serially 1:10 in triplicate and added to Vero cells in a 96-well microplate format to determine end point dilution titer ($TCID_{50}$) via the method of Kärber (Kärber 1931).

Sample preparation for cytokine gene expression analysis.

Tissues were extracted from 3 mice per treatment group at days 2 and 4 post-challenge. Collected tissues included brain, lung and spleen. Tissues were placed in 1 mL green bead tubes (Roche, Switzerland) containing 500 µL TRIzol Reagent (Invitrogen, Carlsbad, CA) and homogenized in a Roche Magna Lyser. Lungs and spleens were initially homogenized using two primary pulses of 30 seconds at 3500 ***g***, while brains were initially homogenized using two primary pulses of 20 seconds at 3000 ***g***. Primary tissue homogenates were collected, and 500 µL additional TRIzol Reagent

was added to each green bead tube, followed by two additional pulses as described above. Secondary tissue homogenates were collected, pooled with the primary samples, and stored at –80 °C. Total RNA was extracted following the manufacturer's protocol for TRIzol Reagent. Residual DNA was then removed via treatment with 4U of DNase I Amplification Grade (Invitrogen) in a 100 µL volume. Further RNA purification was performed using the RNeasy Mini Kit (Qiagen, Valencia, CA) RNA cleanup protocol. RNA preparations were quantified using a SmartSpec 3000 Spectrophotometer (Bio-Rad, Hercules, CA) at OD_{260}.

Cytokine gene expression analysis via quantitative reverse transcription-PCR.

5 µg total RNA was reverse transcribed to cDNA using the SuperScript III First-Strand Synthesis Kit for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The provided oligo dT was used to prime the reverse transcription reaction. Cytokine-specific DNA levels were determined using FAM dye-labeled Taq-Man probes, specific primers, and Taqman Universal PCR Master Mix (Applied Biosystems Inc., Foster City, CA). Polymerase chain reactions were prepared in a 96-well format and run on an iCycler (Bio-Rad, Hercules, CA). In order to control for differing levels of mRNA between individual samples, expression of cytokine mRNA was normalized to the expression of the hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1) housekeeping gene. Fold-changes in cytokine transcript levels between individual samples were calculated using the ΔΔCt method of relative quantification.

Natural killer cell depletion.

Systemic depletion of murine natural killer cells was accomplished by i.p. injection of 50 µg anti-asialo-GM1 antibody (Wako, Osaka, Japan). Anti-asialo-GM1 antibody was administered 24 hours prior to CLDC treatment, and again 5 days later in

order to maintain continuous depletion of the natural killer cell population. Antibody treatment resulted in 80% decreases in both lung and splenic natural killer cell counts at 24 hours post-depletion. The mice were then challenged with aerosolized LACV 24 hours after CLDC treatment. Control mice were injected i.p. with 50 µg ChromPure rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA).

Statistical analysis.

Statistical analyses were conducted using Prism 5.0 software (GraphPad, La Jolla, CA). Kaplan-Meier curves and the log-rank test were used for comparison of survival times. For survival comparison of more than two groups, the Bonferroni correction was applied. For comparisons between two groups, two-tailed t tests were performed. Data were considered statistically significant for $p < 0.05$.

Results

Intraperitoneal administration of CLDC protects mice from the development of LAC encephalitis when administered prophylactically and therapeutically.

We recently developed a murine model of aerosol-delivered LACV challenge that results in the development of LAC encephalitis in adult mice (unpublished observations, see Chapter 2). In order to examine the protective potential of prophylactic CLDC immunotherapy in this challenge model, mice were injected i.p. with CLDC at 24 hours prior to or immediately prior to aerosolized LACV challenge. To ensure that any protective CLDC effect was not merely a delay of symptom onset, mice were observed for the development of symptoms of LAC encephalitis up to 90 days post-challenge. In contrast to the cumulative survival rate of sham-treated control mice (24.5%; 13/53 mice in 3 independent experiments), the overall survival rate for mice pretreated with CLDC was 95.8% (23/24; $p = 0.0001$) for those treated 24 hours prior to challenge, and 90% (18/20; $p = 0.0001$) for those treated immediately prior to challenge (Fig. 3.1). Additional controls included mice treated with plasmid DNA alone or cationic liposomes alone. Neither of these control groups was protected to the extent of the complete CLDC formulation. In contrast to the cumulative survival rate of mice treated with CLDC 24 hours prior to challenge (95.8%), the overall survival rate was 20% for control mice treated with either plasmid DNA alone (4/5; $p < 0.0001$) or cationic liposomes alone (4/5; $p < 0.0001$), indicating that both components are required for elicitation of complete CLDC-induced protection (Fig. 3.1).

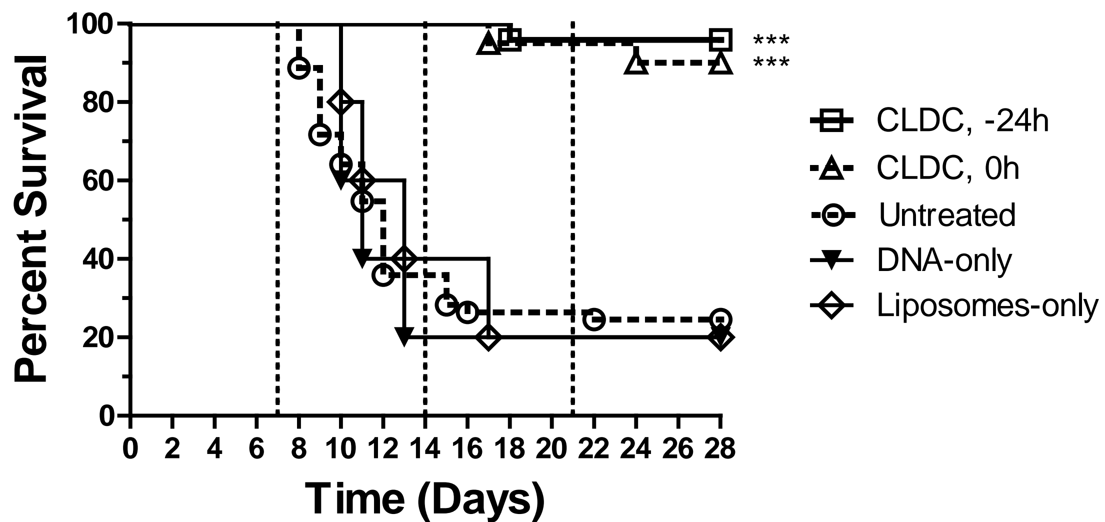

Figure 2.1. Prophylactic CLDC-induced protection against aerosolized LACV challenge. (A) C3H mice (*n* = 5-10 animals per group) were challenged via aerosol with a suspension (5.0 mL) of LACV (2.32 x 10^8 $TCID_{50}$/mL) and the effects of the timing of administration of prophylactic CLDC immunotherapy were determined. Mice were sham-injected (untreated) or were treated with CLDC 24 h prior to challenge (-24h) or immediately prior to challenge (0h), and survival times were determined as described in Materials and Methods. Groups of control mice (*n* = 5 animals) were treated with plasmid DNA alone or liposomes alone at 24 h prior to challenge. Statistical differences in survivorship were determined by Kaplan-Meier analysis, followed by a log-rank test (***, $P \leq 0.0001$). Data shown are cumulative data from three independent experiments.

CLDC-treated mice that eventually developed encephalitis survived markedly longer than the majority of those in the sham-treated control group (Fig. 3.1), demonstrating that CLDC pre-treatment both increased survival and delayed disease onset. Additional challenge experiments performed with either BALB/c or ICR outbred mice yielded similar protective results, regardless of mouse strain. Overall, prophylactic treatment with CLDC induced significant innate immune protection against an otherwise lethal aerosol challenge with LACV.

In order to examine the protective potential of therapeutic CLDC immunotherapy following aerosol infection, mice were injected i.p. with CLDC at 12, 36, or 72 hours following aerosolized LACV challenge (as above) and were observed for morbidity and

clinical outcome. Administration of CLDC within two days following aerosol infection provided significant protection against the development of clinical signs of encephalitis (Fig. 3.2).

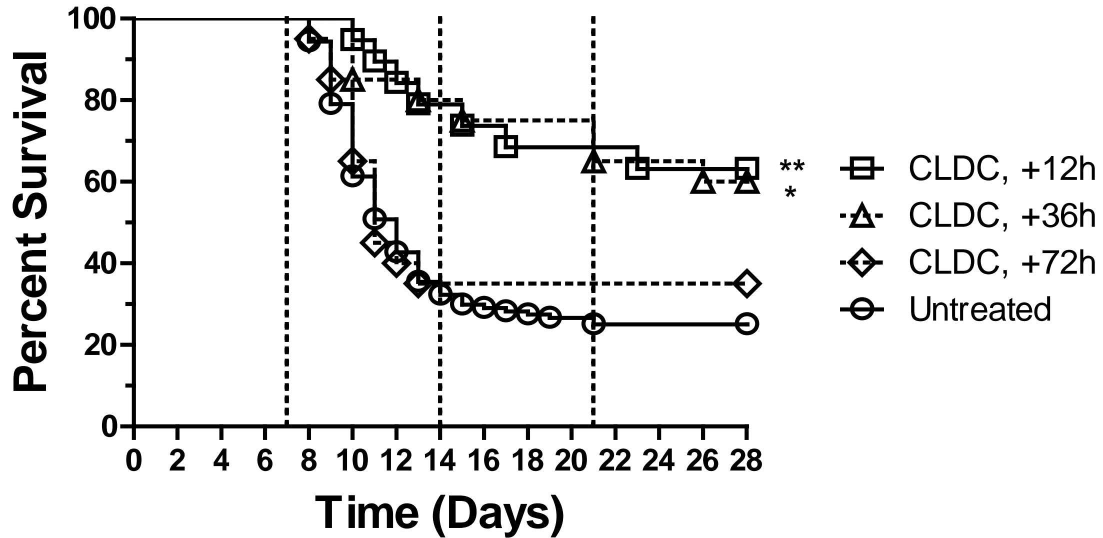

Figure 3.2. Therapeutic CLDC-induced protection against aerosolized LACV challenge is dependent on the timing of CLDC administration. (B) C3H mice (*n* = 5-10 animals per group) were challenged via aerosol with a suspension (5.0 mL) of LACV (2.32 x 10^8 $TCID_{50}$/mL) and the effects of the timing of administration of therapeutic CLDC immunotherapy were determined. Mice were sham-treated (untreated) or were treated with CLDC 12 h, 36 h, or 72 h after challenge, and survival times were determined as described in Materials and Methods. Statistical differences in survivorship were determined by Kaplan-Meier analysis, followed by a log-rank test (**, P = 0.0003; *, P = .0034). Data shown are cumulative data from three independent experiments.

Following therapeutic administration of CLDC at 12 hours post exposure, 63.2% of mice (12/19 in 3 independent experiments) survived (p = 0.0003), following treatment at 36 hours post-exposure, 60% of mice (12/20) survived (p = 0.0034), and following treatment at 72 hours post-exposure, only 35% of mice (7/20) survived (p = 0.08). The cumulative survival rate of sham-injected mice was 25% (31/124). Therapeutic CLDC immunotherapy was therefore most effective at time points closest to challenge, with protective efficacy waning closer to background levels in the 72 hour post-exposure

treatment groups. In contrast to the prophylactic treatment (Fig. 3.1), therapeutic CLDC treatment (Fig. 3.2) did not delay disease onset.

In order to investigate the mechanisms that condition the protective efficacy of CLDC treatment against aerosolized LACV challenge, we compared CLDC-induced protection to that induced by treatment with synthetic CpG-containing oligonucleotides as alternative TLR9 agonists. Mice were injected i.p. with equivalent amounts of either CLDC, Type C CpG oligonucleotide 2395, or diluent 24 hours prior to challenge with aerosolized LACV and then observed for the development of clinical signs of encephalitis. CLDC treatment yielded the highest survival rate observed (80%, $p < 0.0001$), while CpG oligonucleotide or diluent treatment yielded only 50% ($p < 0.0001$) and 10% survival rates, respectively (Fig. 3.3).

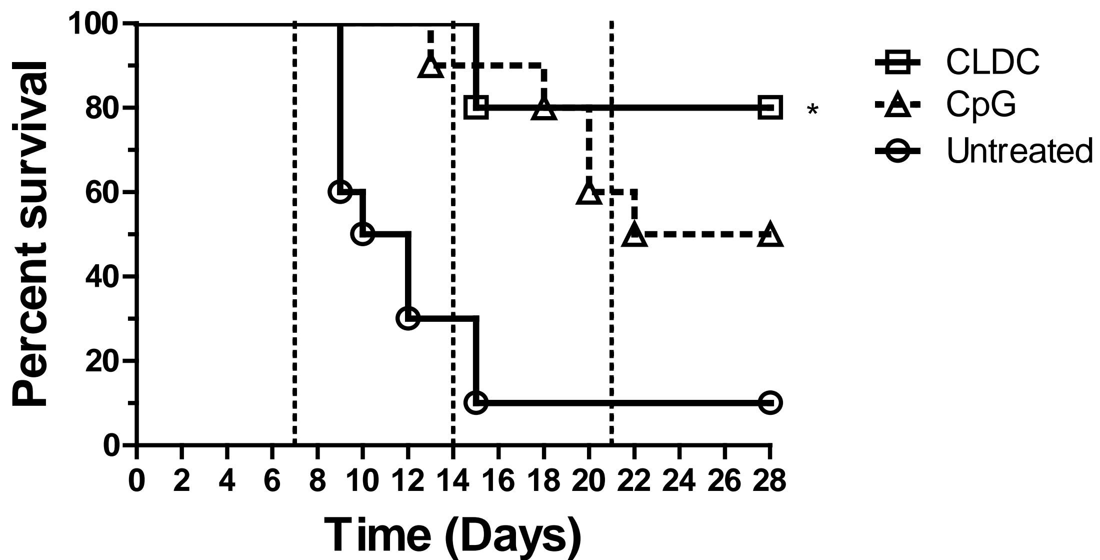

Figure 3.3. CLDC immunotherapy elicits protection against aerosolized LACV challenge exceeding that induced by CpG oligonucleotide constructs alone. C3H mice (n = 10-20 animals) were injected i.p. with either 250 µL CLDC, 50 µg CpG oligonucleotide 2395 diluted in 250 µL 1x PBS, or were sham-injected (untreated) 24 hours before aerosol challenge with LACV, and survival times were determined as described in Materials and Methods. Statistical differences in survivorship were determined by Kaplan-Meier analysis, followed by a log-rank test. Mice treated with either CLDC or CpG survived at significantly higher percentages than

untreated control mice ($p < 0.0001$). Mice treated with CLDC survived at significantly higher percentages than mice treated with CpG (*; $p = 0.0252$). Data shown are cumulative data from two independent experiments.

CLDC immunotherapy elicits protection ($p = 0.0252$) against aerosolized LACV challenge exceeding that induced by conventional immunotherapy using synthetic Type C CpG oligonucleotides alone.

CLDC reduces the severity of histological lesions in the central nervous system of challenged animals.

In order to clarify the mechanism(s) of the protection elicited by CLDC immunotherapy against lethal aerosol challenge with LACV, we next investigated whether there was a reduction in pathological changes in the brains of challenged mice due to CLDC administration. Examination of brain tissues revealed histologic lesions of meningoencephalitis in all aerosolized LACV-challenged animals, regardless of treatment or lack thereof (Fig. 3.4).

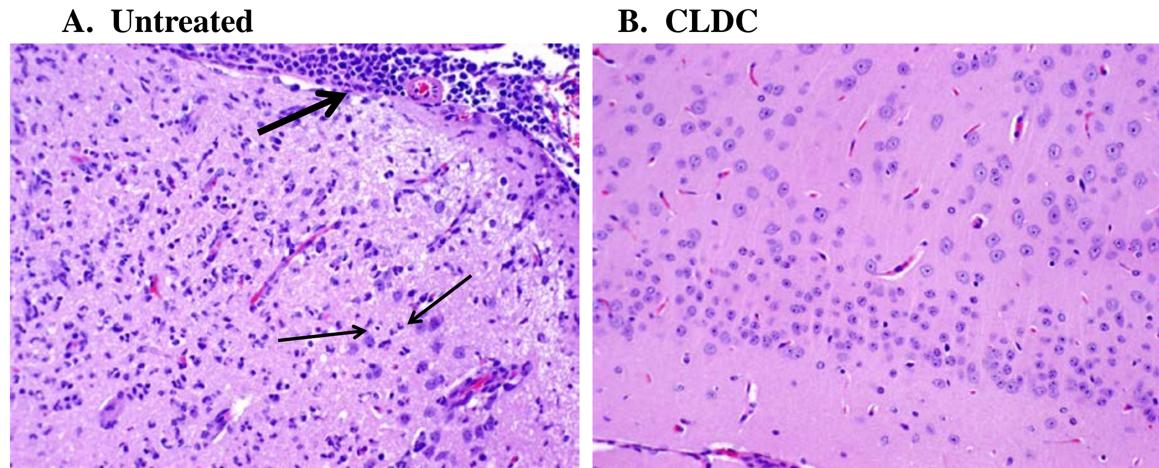

Figure 3.4. CLDC pre-treatment reduces the severity of histologic lesions in the CNS of challenged animals. C3H mice ($n = 4$ animals) were challenged via aerosol with a suspension (5.0 mL) of LACV (2.32 x 10^8 $TCID_{50}$/mL) and the effects of prophylactic CLDC administration on histologic changes in the brain were determined. Mice were sham-injected (untreated) or were treated with CLDC 24 h prior to challenge. Brains from four untreated mice were harvested at the first signs of disease and compared to time-matched CLDC-treated mouse brains. Direct

comparison of representative brain sections of untreated (A) vs. CLDC-treated (B) mice reveals significant differences in the severity of histological lesions of encephalitis, typified by meningitis (thick arrow), lymphocytic infiltration, and necrosis (thin lines). Images are representative of results obtained from three independent experiments. (A): Untreated mouse brain, day 18 post-challenge. H&E stain, 20x objective magnification. (B): CLDC-treated mouse brain, day 18 post-challenge. H&E stain, 20x objective magnification.

Lesions presented in a multifocal pattern, and were observed at all levels of the brain, including the cerebral cortex, hippocampus, midbrain, meninges, and brainstem. Lesions were typified by generalized meningoencephalitis with perivascular accumulations of lymphocytes and heterophils, in combination with neuronal degeneration and necrosis, rarefaction of the neuropil, and gliosis. Lymphocytes and plasma cells were present in the meninges and in perivascular locations within the cortex. The inflammation and meningitis, although multifocal, were most pronounced in the anterior and ventral areas of the brain. In the most severely-affected brains, there were foci of liquefaction of the brain parenchyma, and the meningitis extended to the ventral brain stem area. All observed lesions were characteristically similar, but were present to variable extents depending on CLDC treatment or lack thereof (Fig. 3.4). Lesions were categorized as extensive and severe in the brains of sham-treated mice, while those in the brains of CLDC-treated animals were consistently smaller and less severe, categorized as mild to moderate, with markedly-diminished cellular infiltrates and observable reductions in the degree of meningoencephalitis and the extent of neuronal necrosis. The presence of obvious neurological involvement in both groups demonstrated that CLDC treatment did not completely prevent invasion of the CNS by LACV, but rather lessened the neuropathology to the point that most mice did not display clinical symptoms of CNS infection, thereby increasing the chances of recovery.

Comparison of viral titer and tissue tropism in challenged animals.

The previous experiment revealed that CLDC treatment increased survival and reduced disease severity in LACV-infected mice. To investigate the pathogenesis and tissue tropisms of LACV in CLDC- and sham-treated mice that condition the differences in disease outcomes, organs were harvested at selected time points post-infection, and virus infection kinetics and titers were determined in mice in the two treatment groups (Fig. 3.5).

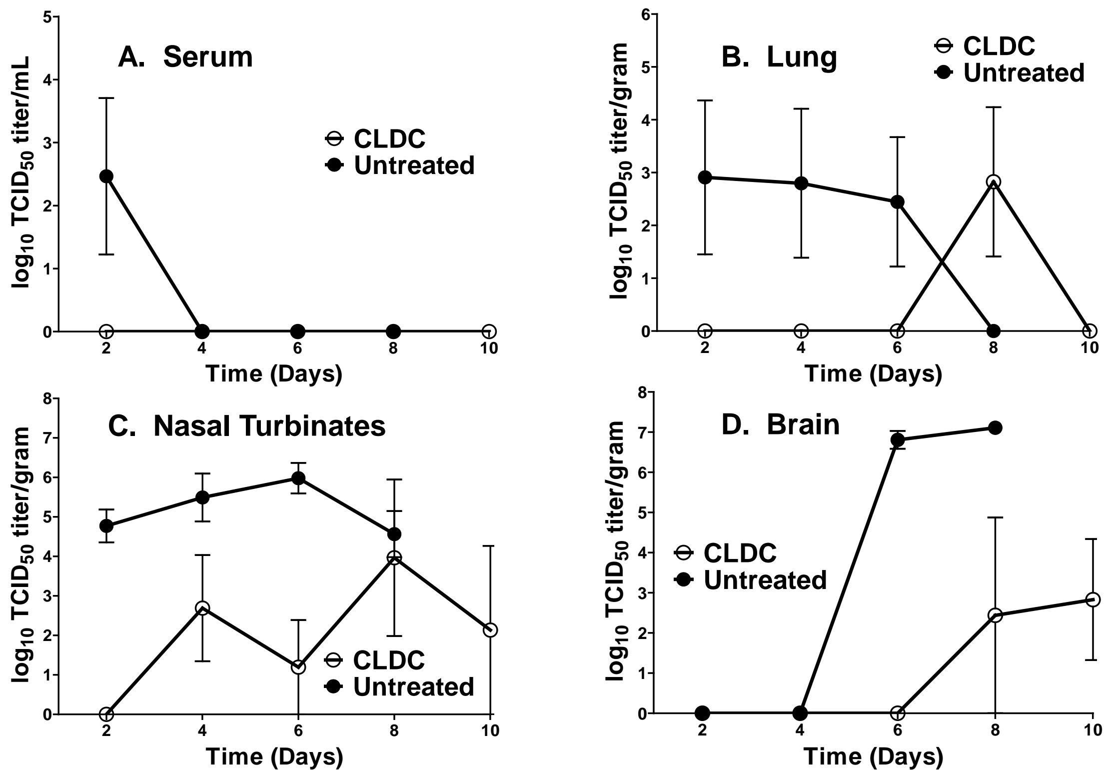

Figure 3.5. Effects of CLDC immunotherapy on viral load in mouse tissues following aerosol challenge with LACV. C3H mice (*n* = 15 animals) were sham-injected (untreated) or treated with CLDC 24 h prior to aerosol challenge with LACV. Geometric mean viral titers (expressed as $\log_{10}$ $TCID_{50}$/gram or /mL of tissue ± SEM) were determined in the serum (A), lung (B), nasal turbinates (C), and brain (D) at the indicated time points after LACV challenge, as described in Materials and Methods. Data are representative of two independent experiments.

Infectious virus was transiently found in serum and lung tissues, and once infected, persistently found in the nasal turbinates and brain (Fig. 3.5) in CLDC- and sham-treated mice. Spleen, liver, and kidneys were not consistently infected in either treatment group. Tissue tropisms were generally the same in both groups; however, viral infection of specific tissues was delayed and titers were reduced in CLDC-treated mouse tissues (Fig. 3.5A-D). In fact, all sham-treated control mice developed clinical signs of LAC encephalitis by day 8 post-challenge and were humanely sacrificed. Thus we were unable to collect tissues on day 10 for comparison to CLDC-treated mice.

LACV was transiently detected in sera of sham-treated control mice at 2 days post-challenge (2.5 $\log_{10}$ $TCID_{50}$ /mL), but viremia declined to undetectable levels by day 4 (Fig. 3.5A). LACV viremia was never detected in CLDC-treated mice at any time point post-challenge, demonstrating that a high-titered viremia is not necessary for neuroinvasion.

LACV was detected in lungs. The highest titers were detected in lungs of sham-treated control mice at day 2 post-challenge (2.9 $\log_{10}$ $TCID_{50}$ /gram). Viral titer declined in this group on days 4 and 6 post-challenge, and was undetectable by day 8 (Fig. 3.5B). Conversely, no infectious virus was detected in the lungs of CLDC-treated mice during the first 6 days post-challenge. Strangely, virus was detected in the lungs of 2 of 3 mice on day 8 post-challenge (2.8 $\log_{10}$ $TCID_{50}$ /gram), but on day 10 post-challenge virus was not detectable. The reason for this is unknown and could be attributable to CLDC treatment preventing or delaying lung infection in the majority of infected mice, or perhaps experimental error.

LACV was also detected in the nasal turbinates of infected animals (Fig. 3.5C). At every time point, the nasal turbinates of all sham-injected mice contained infectious virus. Nasal turbinate infection was detected at day 2 post-challenge, and LACV titer increased to a maximum titer on day 6 (6.0 log_{10} $TCID_{50}$ /gram) (Fig. 3.5C). Conversely, the nasal turbinates of CLDC-treated mice had no detectable LACV until day 4 post-challenge. Mean viral titers were lower in the nasal turbinates of CLDC-treated mice than in the sham-treated mice at all assayed timepoints. The maximum mean titer found in the nasal turbinates of CLDC-treated mice was 4.0 log_{10} $TCID_{50}$ /gram (day 8 post-challenge) and was notably lower than the maximum titer of 6.0 log_{10} $TCID_{50}$ /gram (day 6 post-challenge) in the sham-treated mice. Further, the nasal turbinates of all sham-injected control mice contained infectious virus, but only 1 of 3 CLDC-treated mice had detectable virus in nasal turbinates on days 6 and 10 post-challenge, and only 2 of 3 CLDC-treated mice yielded virus on days 4 and 8. These results again suggest that CLDC treatment both delayed and prevented or reduced LACV infection of tissues.

A similar trend of viral infection kinetics was observed in the brains of challenged mice (Fig. 3.5D). Virus was detected in the brains of all sham-treated control mice on day 6 post exposure, and maximum mean brain titer (7.1 log_{10} $TCID_{50}$ /gram), which was the greatest titer seen in any assayed tissue, was detected on day 8 (Fig. 3.5D). In contrast, virus was not detected in the brains of any CLDC-treated mouse until day 8 post-challenge. This titer was lower than those in the brains of sham-treated control mice at days 6 or 8 post-challenge. By day 10, the mean viral titer in the brains of CLDC-treated mice had peaked at 2.8 log_{10} $TCID_{50}$ /gram. These data suggest that CLDC administration serves to both delay infection and reduce virus replication in peripheral

tissues and the central nervous system. These results are supported by our observation that a significantly higher percentage of mice in the sham-treated group had seroconverted to LACV than in the CLDC-treated group; this suggested that CLDC immunotherapy was either preventing infection or limiting viral replication to an immunologically undetectable level in treated mice, and demonstrated that a higher proportion of sham-injected mice were indeed infected with LACV (data not shown).

CLDC elicits interferon responses in the context of LACV challenge.

The observations that CLDC treatment both delayed virus infection and restricted viral replication in challenged animals suggested induction of a protective antiviral innate immune response in CLDC-treated animals. In order to characterize the nature of this immune response and to identify potential cytokines involved, we determined whether CLDC administration would lead to increased interferon (IFN) gene expression in either peripheral tissues or the CNS of challenged animals. Levels of mRNA expression of two type I interferons (IFN-α5 and IFN-β1) and type II IFN-γ were compared between CLDC-treated and untreated animals using quantitative reverse transcriptase PCR (qRT-PCR). This approach allowed us to quantitatively determine the fold-differences in type I and II IFN mRNA expression in the spleens and brains of CLDC-treated and untreated mice (Fig. 3.6).

At both days 2 and 4 post-challenge, spleens from CLDC-treated mice (n = 3 per group) expressed significantly increased levels of both IFN-α5 and IFN-β1 mRNA transcripts (Fig. 3.6A and 3.6B). IFN-α5 expression in the CLDC-treated group was increased 36-fold over the levels in the control group on day 2 post-challenge, and on day 4 was still 14-fold higher (Fig. 3.6A).

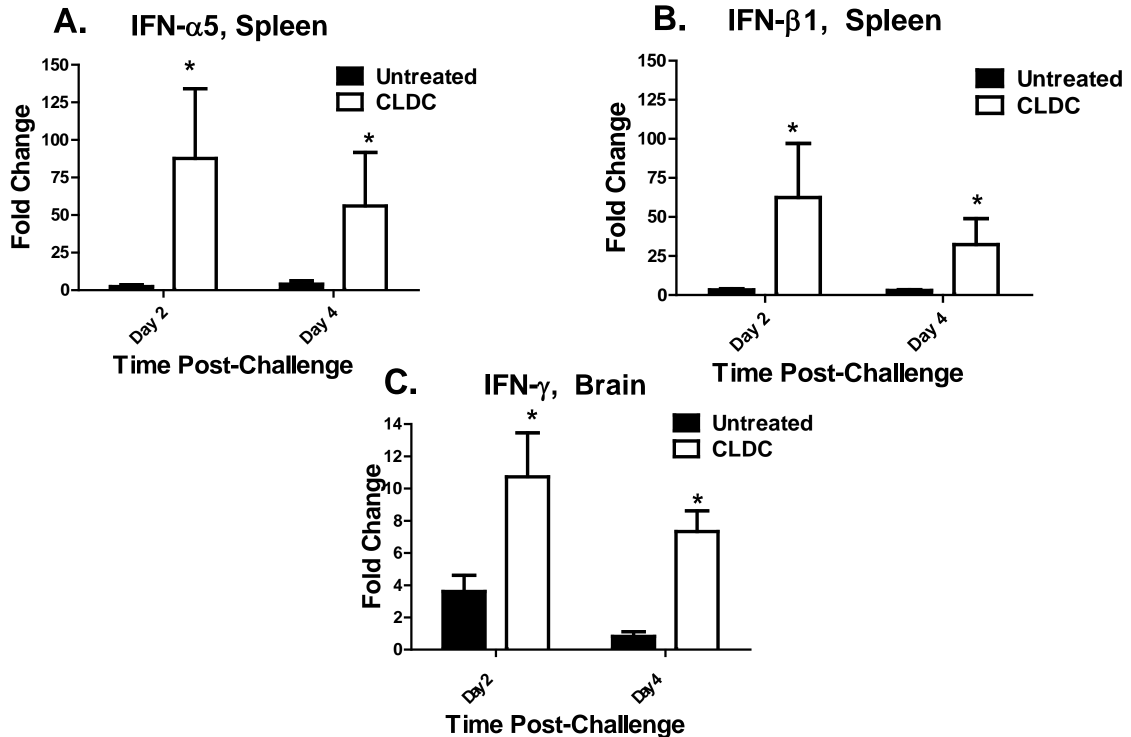

Figure 3.6. Expression of cytokine genes in the spleen and brain following CLDC administration and aerosolized LACV challenge. C3H mice (n = 15 animals) were sham-injected (untreated) or were treated with CLDC 24 h prior to aerosol challenge with LACV, and tissues were harvested at days 2 and 4 post-challenge. Cytokine gene expression in spleen and brain tissues of untreated and CLDC-treated mice (n = 3 per group) was determined via quantitative real-time PCR, as described in Materials and Methods. The mean fold change (+SEM) in mRNA expression between untreated and CLDC-treated mice is shown for IFN-α5 (A) and IFN-β1 (B) in the spleen, and for IFN-γ (C) in the brain. Statistical differences were determined using a nonparametric two-tailed Mann-Whitney test (*, $P < 0.05$). Data are representative of two independent experiments.

Mice in the CLDC-treated group expressed 19-fold more IFN-β1 mRNA than controls on day 2, and these levels were still 11-fold higher than that found in control mice on day 4 post-challenge (Fig. 3.6B). These increases in peripheral type I IFN expression were also found to be associated with elevated IFN-γ levels in the brains of CLDC-treated mice at both 2 and 4 days post-challenge (Fig. 3.6C). On day 2, IFN-γ levels were 3-fold higher in the brains of CLDC-treated mice than untreated mice, and by day 4, these differences had increased to 9-fold between the two treatments groups.

These observations provide evidence that CLDC treatment induces transcription of important antiviral cytokines that could account for the protective effects observed in treated mice.

Systemic depletion of natural killer cells abrogates CLDC-induced protection.

Previously-published studies using CLDC immunotherapy identified NK cells as the major cell type responsible for the production of IFN-γ (Dow et al., 1999a; Dow et al., 1999b; U'Ren et al., 2006; Goodyear et al., 2009; Troyer et al., 2009). In order to determine the importance of NK cells in the protection elicited by CLDC treatment in the aerosolized LACV model, anti-asialo GM1 antibodies were used to systemically deplete NK cells prior to treatment with CLDC and aerosol challenge (Fig. 3.7). This antibody preparation results in systemic NK cell depletion that lasts for at least 5 days (Habu et al., 1981; Kasai et al., 1980). Flow cytometric analysis of cells from the lungs and spleens of NK-depleted mice revealed the NK-depletion efficiency to be ~80% (data not shown).

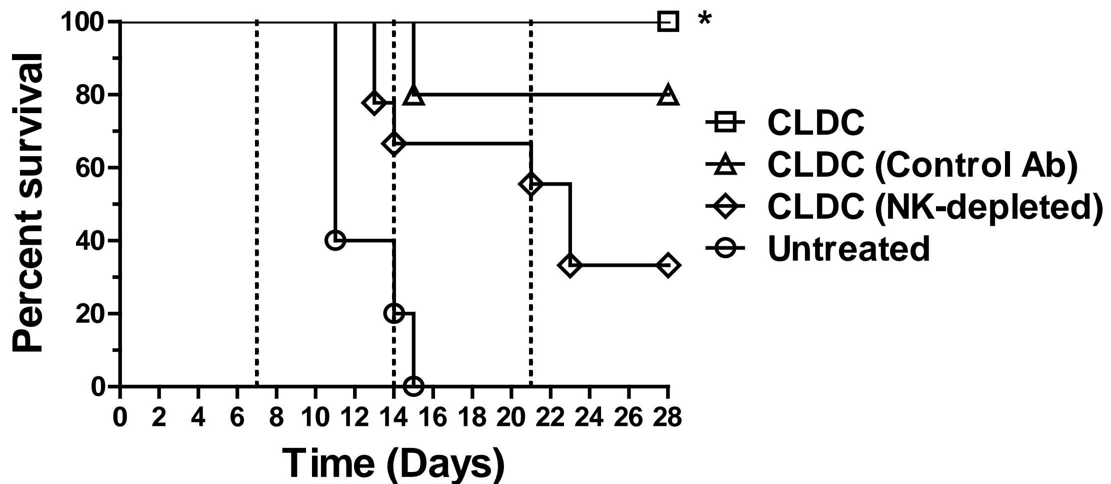

Figure 3.7. Depletion of NK cells significantly reduces survival in CLDC-pre-treated mice. C3H mice (*n* = 9-10 per group) were depleted of NK cells via i.p. injection of anti-asialo-GM1 antibody 24 hours prior to CLDC treatment and again 5 days later, as described in Materials and Methods. A control group received an irrelevant rabbit IgG control antibody. Mice were sham-injected (untreated) or were treated with CLDC and then challenged 24 hours later with

aerosolized LACV, and survival times were determined. CLDC-treated non-NK-depleted mice survived at significantly higher percentages than CLDC-treated NK-depleted mice (*;$p < 0.05$). Statistical differences were determined using Kaplan-Meier analysis followed by the log-rank test (*, $P < 0.05$). Data are representative of two independent experiments.

100% of CLDC-treated mice which had not had their NK cells depleted survived aerosol challenge, while only 33.3% of mice depleted of NK cells prior to CLDC treatment survived to 28 days post-challenge (Fig. 3.7; *; $p < 0.05$). This observation suggests that NK cells play an important role in CLDC-induced protection against aerosol challenge, and are necessary for the induction of complete protection. It is notable that the CLDC-treated, NK cell-depleted mice had a significantly higher survival rate (33.3%) than the sham-treated control mice, none of which survived ($p = 0.0084$).

Discussion

In this study, we investigated both the prophylactic and therapeutic efficacy of CLDC immunotherapy and assessed its ability to (a) prevent lethality in mice challenged by aerosol with LACV (Figs. 3.1, 3.2, and 3.3), (b) reduce the severity of CNS lesions in challenged mice (Fig. 3.4), (c) prevent, reduce, or delay infection of the sera, lungs, nasal turbinates and brain (Fig. 3.5), and (d) induce the expression of antiviral type I and II IFNs (Fig. 3.6). Adult mice are normally refractive to LACV infection when challenged peripherally, even with quite high subcutaneous doses (Johnson, 1983; Janssen et al., 1984). Previous studies in our lab have shown that less than 10% of adult mice develop neurological disease when given as much as 1 x10^5 $TCID_{50}$ of LACV subcutaneously or intramuscularly (unpublished observations). The classical model of LACV infection via peripheral routes involves initial replication in skeletal muscle tissue near the site of infection with eventual development of viremia and subsequent infection of vascular

endothelium. The virus is then thought to bypass the blood-brain barrier and enter the CNS through infection of this vascular tissue supplying the brain (Johnson, 1983; Janssen et. al, 1984; Griot et. al, 1993a; Griot et. al, 1993b). More recent studies involving intraperitoneal and intranasal inoculation of weanling mice with LACV have proposed that invasion of the CNS might alternatively occur via infection of the nasal turbinates and subsequent utilization of olfactory neurons as a means of gaining entry to the brain (Bennett et al., 2008). Our recently-developed aerosol- or intranasally-delivered LACV challenge models have also identified infection of the nasal turbinates as an important prerequisite for subsequent CNS infection (unpublished observations, see Chapter 2).

In contrast to parenteral infection, adult mice are very susceptible to LACV infection via aerosol. Aerosol challenge with LACV results in nearly 100% morbidity and mortality within two weeks of challenge. Neurological lesions are indistinguishable from those that have been observed in mice infected by vector-borne or subcutaneous routes, mainly a multifocal meningioencephalitis characterized by a large amount of cellular infiltrate (perivascular cuffing) and necrosis (Pekosz et al., 1996; unpublished observations, see Chapter 2). Interestingly, this pattern of neurological lesions is not noted in younger suckling mice, in which apoptosis is thought to be responsible for most of the neural damage and inflammation is mild. It has been suggested that increased cellular differentiation in the adult CNS, including increased expression levels of the anti-apoptotic regulator *bcl-2* (Pekosz et al. 1996) may account for this difference. This reduction in neuronal apoptosis may explain, in part, the typically-refractory nature of adult mice to the development of LAC encephalitis. Aerosol delivery of LACV may result in efficient neuroinvasion into the rostral brain via the olfactory neurons. The rapid

kinetics of infection and potentially high dosages of virus may then overwhelm the normal mechanisms that protect adult mice from a damaging inflammatory response that results in meningioencephalitis. The near 100% morbidity and mortality seen in normally-refractory adult mice when challenged via aerosol underscores the danger of these pathogens as possible bioterrorism agents.

Prior studies have successfully demonstrated the antiviral capacity and protective effects of CLDC administration against lethal subcutaneous challenge with members of the *Bunyaviridae* and *Togaviridae* (Gowen et al., 2006; Gowen et al., 2008; Logue et al., 2010). In addition, CLDC immunotherapy has been shown to significantly reduce the quantity of hepatitis B viral transcripts in a transgenic mouse model (Morrey et al., 2008). To our knowledge, however, this is the first published report of CLDC administration eliciting protection in an aerosolized virus challenge model. Prophylactic treatment with CLDC greatly increased the survival of mice challenged with aerosolized LACV (>95% compared to <25% in controls; Fig. 3.1). Therapeutic administration was found to be most protective when given at time points closest to challenge, with 60% survival seen as far out as 36 hours post-exposure. However, by 72 hours post-challenge, protective efficacy was reduced to non-significant levels ($p = 0.08$), indicating that once infection is well-established CLDC immunotherapy can no longer affect outcome (Fig. 3.2). Nonetheless, the therapeutic window for post-exposure treatment of inhalational LACV infection suggests the feasibility of CLDC treatment as a possible immunotherapeutic intervention in the event of a bioterrorist attack employing aerosolized members of the *Bunyaviridae*. The protection elicited by the complete CLDC formulation exceeded that induced by treatment with either plasmid-DNA-alone or cationic liposomes-alone (Fig.

3.1) or with synthetic CpG-containing oligonucleotides alone (Fig. 3.3). The inclusion of cationic liposomes in the complete CLDC formulation likely serves to both protect the plasmid DNA from extracellular degradation and to localize the complexes to the early endosomal compartment, allowing for maximal and targeted enhancement of the intended innate immune response.

Histological examination of the brains of CLDC-treated mice showed only mild pathological changes in the CNS compared to a severe inflammatory response in sham-treated controls (Fig. 3.4). Encephalitic lesions in CLDC-treated mice were consistently smaller and less severe, with considerably-diminished cellular infiltrates and significant reductions in the degree of meningoencephalitis and the magnitude of neuronal necrosis. Presumably, CLDC treatment reduced infection of the CNS and/or lessened the associated neuropathology to the point that most mice did not develop clinical symptoms of LAC encephalitis, thereby increasing the chances of recovery.

Pathogenesis studies (Fig. 3.5A-D) revealed that viral infection and titers in peripheral tissues and the brains of CLDC-treated animals were both delayed in initial appearance and reduced in titer in all assayed tissues. Viremia was not detected in the serum of any CLDC-treated mouse at any assayed time point. In addition, in CLDC-treated mice, lung infection was both delayed and decreased in titer following CLDC treatment, and in some cases virus infection was not detected. It is possible that CLDC-induced innate immune activation either dampens or prevents initial infection and subsequent replication of virus in the lungs (and other peripheral tissues), allowing for a delayed dissemination to other tissues (as seen in Fig. 3.5). LACV was detected in the brains of sham-treated control mice both earlier and at higher titers than in the brains of

CLDC-treated mice. Presumably, CLDC treatment reduces viral replication kinetics and titer and associated neuropathology to levels that allow mice to survive LACV infection and to avoid development of lethal LAC encephalitis.

This conclusion is further supported by our observation that sham-treated mice seroconverted to produce LACV-neutralizing antibodies at a notably-higher percentage than CLDC-treated mice (data not shown), demonstrating that a higher proportion of untreated mice were indeed infected with LACV, and suggesting again that CLDC immunotherapy either prevents infection or delays and limits viral replication in treated mice.

A particularly striking result of the pathogenesis studies was the lack of detectable viremia at any time point in all CLDC-treated mice. The classically-accepted murine model of peripheral LACV infection assumes that the generation of a high viremia is required for subsequent invasion and infection of the CNS (Janssen et al., 1984). However, despite the lack of detectable viremia in CLDC-treated mice, infectious virus was detectable in the brains of CLDC-treated mice on days 8 and 10 post-challenge (Fig. 3.5D). These results suggest that high titered viremia is not a prerequisite for neuroinvasion in our aerosol infection model. LACV could directly infect the nasal turbinate epithelium and olfactory nerves for transport into the CNS. The observation that aerosol-challenged mice that subsequently develop CNS infection did not have a detectable viremia is in contrast to the classically-accepted murine model of peripheral LACV infection, and challenges our present notions concerning the mechanism(s) of neuroinvasion by LACV and the subsequent development of LAC encephalitis in both natural and artificial transmission models. In addition, our studies also demonstrate that

adult mice are very susceptible to LACV infection and severe disease outcome when infected via aerosol and intranasally (unpublished observations, see Chapter 2). Perhaps virus infection of the olfactory neurons results in more rapid infection, different routes of infection, or greater doses of LACV being introduced into the CNS, thereby overwhelming the immune and/or molecular mechanisms that protect adult mice from lethal encephalitis when infected parenterally. Regardless, the demonstration that LACV aerosol infection results in LAC encephalitis, even in adults, underscores the potential danger of the use of LACV and/or other members of the *Bunyaviridae* as agents of bioterrorism.

Previous studies have identified both IFN-α and IFN-β as important mediators of protection against murine LACV infection (Blakqori et al., 2007). In addition, production of IFN-γ is known to exert direct antiviral responses through the induction of various antiviral effectors, including double-stranded RNA-activated protein kinase (PKR), 2'-5' oligoadenylate synthetase (2-5A synthetase), and dsRNA-specific adenosine deaminase (dsRAD) (Boehm et al., 1997). In fact, IFN-γ is critical for antiviral responses in the CNS, in which non-cytolytic clearance of intracellular viruses is required for preservation of neuronal function (Chesler and Reiss, 2002). CLDC treatment prior to aerosolized LACV challenge elicited significant fold-increases in expression of both IFN-α5 and IFN-β1 in the spleen and IFN-γ in the brain during the first 48 hours following challenge (Fig. 3.6). These observed increases in expression of important antiviral cytokines could very well account for the reductions in viral load and lessened neuropathologic changes in this treatment group, as the establishment of a type I IFN-mediated antiviral state early in or prior to infection could presumably significantly

interfere with initial viral infection, propagation and spread to neighboring tissues. Additionally, the increased expression of IFN-γ in the brains of CLDC-treated mice might serve to simultaneously control CNS infections and preserve neuronal function in those mice that did not successfully prevent LACV neuroinvasion. The importance of the IFN response in protection against infection with LACV is further highlighted by results from *in vivo* studies that have shown that LACV nonstructural protein NSs modulates the antiviral type I IFN response in mammalian hosts (Blakqori et al., 2007). The increases in IFN expression induced by CLDC administration may serve to overcome this viral defense strategy evolved to disarm important and effective antiviral innate immune responses of the mammalian host.

NK cells have been identified in previous studies utilizing CLDC immunotherapy as the major cell type responsible for the production of IFN-γ (Dow et al., 1999a; Dow et al., 1999b; U'Ren et al., 2006; Goodyear et al., 2009; Troyer et al., 2009). In our experiments, depletion of NK cells prior to treatment with CLDC and subsequent aerosol challenge significantly reduced survival percentages (Fig. 3.7), identifying an integral role played by NK cells in complete CLDC-induced protection in this challenge model. The additional observation that a small subset of NK-depleted, CLDC-treated mice were still protected from the development of LAC encephalitis suggests that other cell types or other IFN-γ-producing cells may also serve an integral function alongside NK cells in complete CLDC-induced protection. An alternative hypothesis is that as the depletion efficiency of NK cells was found not to be wholly complete (~80%, data not shown), production of IFN-γ by the remaining NK cell population in the depleted group might have accounted for the survival of some of the mice in that treatment category.

To our knowledge, this is the first published report of CLDC-induced protection in an aerosolized viral infection model, which highlights the protective potential of both prophylactic and therapeutic CLDC administration in response to a purposeful bioterrorism event using aerosolized viral pathogens. A plausible protective mechanism in this experimental system is the combination of antiviral type I and II IFN production and NK cell activation, which together lead to the development of an antiviral immunological state paired with non-cytolytic clearance of intracellular LACV in the CNS. This immunological environment has the potential to significantly interfere with initial viral propagation and subsequent spread to neighboring tissues, effectively reducing viral load and associated neuropathology to levels that allow for control of and/or recovery from LACV infection. While the exact mechanism of protection has yet to be determined, the observation that non-specific CLDC treatment can be prophylactically and therapeutically used to prevent and treat LAC encephalitis is provocative. CLDC immunotherapy may be an innovative new approach for preventing and treating aerosol and conventional infections by members of the *Bunyaviridae* and other virus families.

CHAPTER 4

CATIONIC LIPOSOME-DNA COMPLEX (CLDC)-BASED IMMUNIZATION AGAINST THE SAND FLY SALIVARY PEPTIDE MAXADILAN PROTECTS AGAINST *LEISHMANIA MAJOR* INFECTION

Introduction

The leishmaniases are a group of vector-borne diseases caused by infection with obligate intracellular protozoan parasites of the genus *Leishmania,* transmitted to vertebrate hosts by the bite of infected female phlebotomine sand flies of the genera *Phlebotomus* and *Lutzomyia.* Within vertebrates, the infective stage metacyclic promastigotes are phagocytized by macrophages, in which they differentiate into the intracellular amastigote form and replicate within phagolysosomes, leading to the development of a spectrum of diseases extending from mild cutaneous forms to lethal visceral infections (Herwaldt, 1999). More than 350 million people living in endemic regions in 88 different countries are at risk for infection, and more than 12 million people are thought to be presently infected, with an estimated 1.5-2 million new cases arising each year (World Health Organization, Leishmaniasis home page: http://www.who.int/leishmaniasis/en/; Desjeux, 2004). In addition, *Leishmania* infection of United States military personnel deployed to Afghanistan, Iraq, and Kuwait is cause for domestic concern (Centers for Disease Control and Prevention, 2004).

Infection with *Leishmania major* (*L. major*) leads to the development of Old World cutaneous leishmaniasis (Bates, 2007). Most humans infected with *L. major*

develop a protective immune response capable of clearing the cutaneous infection and ultimately rendering the host immune to reinfection (Neva and Brown, 1994), characterizing cutaneous leishmaniasis as one of the few parasitic diseases of humans for which a protective vaccine could be developed with a reasonable expectation of success. Unfortunately, there exists no innocuous and useful human vaccine for prevention of cutaneous leishmaniasis. In addition, as there are 20 different species of *Leishmania* categorized as human pathogens (Cupolillo et al., 2000), the development of multiple stand-alone vaccines that target individual species themselves is a monumental task. Novel approaches toward immunizing against infection with *Leishmania* parasites that target more general and common requirements for host infection are direly needed.

Control of infection with *L. major* is dependent upon the development of a robust CD4+ mediated type 1 (Th1) cellular immune response that supports the production of cytokines (e.g., IL-12, IFN-γ and TNF-α) that activate infected macrophages to produce nitric oxide (NO), which results in killing of intracellular parasites (Reiner and Locksley, 1995). In contrast, progressive disease is correlated with development of a CD4+ mediated type 2 (Th2) cellular immune response characterized by the production of regulatory and Th2-biased cytokines (e.g., IL-10, TGF-β, IL-4, and IL-13) ineffective for activation of macrophages, which instead support the development of an antibody-mediated response inadequate for clearance of intracellular organisms (Reiner and Locksley, 1995; Matthews et al., 2000). Therefore, proper defense against *L. major* infection is crucially-dependent upon priming of a Th1-biased adaptive immune response (Liese et al., 2008). Development of protective cell-mediated immune responses is perturbed not only by the parasite itself, but also by the actions of immunomodulatory

sand fly salivary components co-injected during natural transmission (Brittingham et al., 1995; Mosser and Edelson, 1987; Brandonisio et al., 2004; Belkaid et al., 2002a; Peters and Sacks, 2006; Nylen et al., 2007; van Zandbergen et al., 2004).

The saliva of blood-feeding arthropods contains numerous bioactive molecules that facilitate the acquisition of a blood meal, including anesthetics, vasodilators, and components that inhibit hemostasis and modulate the development of host immune responses (Kamhawi, 2000; Schoeler and Wikel, 2001; Rogers et al., 2002a; Titus et al., 2006). Depending on the quality of the immune environment induced at the bite site by salivary immunomodulation, co-injected pathogens might benefit from salivary-imposed immunological alterations. It is now widely-accepted that arthropod saliva enhances the infectivity of pathogens transmitted to vertebrate hosts during the bloodmeal (Titus and Ribeiro, 1988; Mbow et al., 1998; Belkaid et al., 1998; Jones et al., 1989; Labuda et al., 1993; Edwards et al., 1998). This phenomenon may, in part, account for the impressive ability of many arthropods to so effectively transmit such a wide variety of pathogens.

The immunomodulatory role of sand fly saliva in the transmission of *Leishmania* parasites was first demonstrated by co-injection of mice with *L. major* and salivary gland lysates (SGL) from the New World sand fly *Lutzomyia longipalpis*. Cutaneous lesions of mice challenged with both parasites and SGL grew several-fold larger than lesions of mice challenged without SGL, and the parasite loads within the lesions were increased by several thousand-fold (Titus and Ribeiro, 1988). This dramatic exacerbation of infection was attributed to the immunodulatory actions of maxadilan (MAX), a 63-amino acid vasodilatory salivary peptide of *Lu. longipalpis* (Lerner et al., 1991; Lerner and Shoemaker, 1992; Morris et al., 2001). The immunomodulatory effects of MAX are

mediated through activation of the mammalian type I receptor for the pituitary adenylate cyclase-activating neuropeptide (PACAP), expressed on vascular and neural tissues (Moro et al., 1996; Moro and Lerner, 1997; Eggenberger et al., 1999), as well as on macrophages, immature dendritic cells (DC), and T cells (Arimura and Said, 1996; Torii et al., 1998). MAX elicits potent immunosuppressive and anti-inflammatory effects capable of exacerbating infection with *Leishmania* parasites, including modulation of DC functions required for instruction of Th1 cell development, inhibition of T cell activation, decreased production of protective Th1-biasing cytokines, increased production of exacerbative Th2-biasing cytokines, and inhibition of macrophage functions required for clearance of intracellular parasites (Bozza et al., 1998; Brodie et al., 2007; Guilpin et al., 2002; Wheat et al., 2008; Qureshi et al., 1996; Gillespie et al., 2000; Soares et al., 1998). These collective results strongly suggest that MAX-mediated immunomodulation at the inoculation site leads to the development of an immune environment in which parasites thrive and successfully develop an initial focus of infection. In fact, successful natural transmission of *L. major* may literally be dependent upon salivary immunomodulation (Titus and Ribeiro, 1988). A novel approach proven to protect against infection is to alternatively direct the host immune response against co-injected immunomodulatory vector salivary components that are necessary for successful bloodfeeding and enhanced pathogen transmission (Titus et al., 2006).

The immunomodulatory effects of *Lu. longipalpis* SGLs can notably exacerbate infection with various *Leishmania* species of both New and Old World origin (Titus and Ribeiro, 1988; Samuelson et al., 1991; Warburg et al., 1994), and MAX alone can substitute for the effects induced by whole saliva (Morris et al., 2001). In addition, there

is a convincing catalog of evidence suggesting that the salivary approach towards immunization is a realistic option for prevention of leishmaniasis. Immunization with salivary proteins, whole saliva, or via pre-exposure to uninfected sand fly bites have all elicited significant protective immunity against infection with *L. major* and the subsequent development of cutaneous disease, typified by the production of anti-saliva antibodies and/or a cellular immune response characterized by high levels of IFN-γ production (Morris et al., 2001; Valenzuela et al., 2001; Kamhawi, 2000; Belkaid et al., 1998; Oliveira et al., 2008). The results from these vector-saliva-based approaches to immunizing against infection with *L. major* are impressive and convincing, and warrant further investigation and characterization of the protective immune responses induced.

Cationic liposome-DNA complexes (CLDCs) are potent activators of innate immunity whose immunostimulatory properties are mediated, in part, via activation of Toll-like receptor (TLR) 9 and the cytosolic DNA sensor DAI (DNA-dependent activator of IFN-regulatory factors) (Dow, 2008; Ishii and Akira, 2006; Stetson and Medzhitov, 2006b). In addition to their successful application as immunotherapy for acute viral (Gowen et al., 2006; Gowen et al., 2008; Logue et al., 2010) and bacterial infections (Goodyear et al., 2009; Troyer et al., 2009), the ability of CLDCs to bind and target antigens directly to antigen presenting cells (APC) make them uniquely appealing as vaccine adjuvants (Dow, 2008). CLDC-based vaccines induce balanced cellular and humoral immunity, eliciting strong responses from both CD4+ and CD8+ T cells, as well as inducing antibody responses either comparable to or exceeding those induced by Freund's complete adjuvant or aluminum hydroxide adjuvants (Chen, et al., 2008; Zaks, et al., 2006; Dow, 2008). Activation of innate immune cells by CLDCs elicits the

production of pro-inflammatory Th1 cytokines effective for promotion of cell-mediated immune responses required for clearance of intracellular pathogens (Krieg, 2007; Dow, 2008), such as *L. major*. In addition, CLDC administration leads to prompt cellular stimulation, characterized by upregulation of co-stimulatory molecules on DCs and macrophages, generalized functional maturation of APCs, and natural killer (NK) cell activation and infiltration (Dow et al. 1999b), all of which are important for effective immune clearance of *L. major*. Due to the Th1-biasing of CLDC-stimulated immune responses, we speculated that CLDC might be an effective vaccine adjuvant for generating both innate and adaptive immune responses appropriate for neutralization of MAX-potentiation and subsequent control of infection with *L. major.* We hypothesized that immunization against MAX using CLDC as adjuvant would protect mice against the development of cutaneous lesions following challenge with *L. major* + MAX.

The studies described in Chapter 4 of this dissertation show that CLDC-based MAX-immunization induces significant protection against challenge with *L. major* + MAX. Lesion sizes and associated parasite burdens in the infected footpads of MAX-immunized mice were significantly reduced in comparison to those of non-immunized mice. The degree of protection elicited using CLDC as adjuvant not only exceeded that elicited using a human-approved aluminum hydroxide adjuvant (Alhydrogel®), but was induced without causing the long-term inflammation and tissue damage at the site of immunization observed in Alhydrogel® + MAX-immunized mice. Intracellular cytokine staining of CD4+ lymphocytes of MAX-immunized challenged mice revealed an increase in the percentage ratio of IFN-γ- vs. IL-4-producing CD4+ cells, suggesting induction of a Th1-biased anti-MAX immune response protective against challenge with *L. major.*

These results indicate that CLDC is an effective adjuvant for the induction of Th1-biased immune responses required for control of intracellular pathogens, and further emphasize the importance of targeting salivary immunomodulation in thwarting establishment of infection with vector-borne pathogens.

Materials and Methods

Mice.

5-6 week old (25g) female BALB/c mice were obtained from Harlan Laboratories (Indianapolis, IN) and National Cancer Institute (Frederick, MD). All animals were housed for a minimum of 7 days prior to manipulation in order to allow for acclimation to the research facility. Mice were maintained at the Laboratory Animal Resources facility at Colorado State University, Fort Collins, CO. Animal maintenance and care complied with National Institutes of Health Guidelines (under pathogen-free conditions) for the humane use of laboratory animals and institutional policies as described in the American Association of Laboratory Animal Care and Institutional Guidelines. All protocols and procedures involving animals were approved by the Colorado State University Animal Care and Use Committee.

L. major and infection with the parasite.

Stationary phase promastigotes of *L. major* (LV39 (MRHO/Sv/59/P)) were used for all challenge experiments, and were maintained as described previously (Titus, et al., 1984). Briefly, when used in experiments, the parasites were harvested from stationary-phase cultures (Sacks, et al., 1985). The virulence of *L. major* parasites was maintained by infecting mice with parasites and re-isolating these virulent parasites from the infected mice. Prior to challenge, mice were anesthetized with ketamine (100 mg/kg; Fort Dodge Animal health, Overland Park, KS) and xylazine (10 mg/kg; Ben Venue Laboratories,

Bedford, OH). Anesthetized mice were injected subcutaneously with 10^3-10^4 *L. major* metacyclic promastigotes ± 10 ng synthetic MAX in one hind footpad.

Synthetic maxadilan.

Synthetic maxadilan was prepared by Twentyfirst Century Biochemicals, Inc. (Marlboro, MA). The 63-mer amino acid sequence used was based on the predicted sequence of mature, secreted MAX (Lanzaro et al., 1999; CDATCQFRKAIEDCRKKAHHSDVLQTSVQTTATFTSMDTSQLPGSGVFKECMKE KAKEFKAGK).

Monitoring lesion development and parasite burden in lesions.

Lesion development was followed by measuring with a Vernier digital caliper the thickness of the infected footpad compared to the thickness of uninfected footpads of control mice. Parasite numbers in infected footpads were determined using a published limiting dilution assay (Lima et al., 1997).

Preparation of CLDC adjuvant.

Cationic liposomes were prepared as previously described by combining equimolar amounts of DOTIM [octadecanoyloxy(ethyl-2-heptadecenyl-3-hydroxyethyl) imidazolinium chloride] and cholesterol (Templeton et al., 1997). Cationic liposome-DNA complexes (CLDC) were prepared fresh immediately prior to injection by gently mixing cationic liposomes with plasmid DNA (pMB75.6 empty vector, 3 mg/mL) in sterile Tris-buffered 5% dextrose in water at room temperature. The final concentration of plasmid DNA was 100 µg DNA/mL.

Immunizing against MAX.

Groups of mice (n = 5-8) were injected i.p. or s.c. at the base of the tail with 5 µg of synthetic MAX admixed with 250 µL (i.p.) or 200 µL (s.c.) CLDC adjuvant. For adjuvant comparison experiments, mice were injected s.c. at the base of the tail with 5 µg

of synthetic MAX admixed with Alhydrogel® aluminum hydroxide gel adjuvant (Brenntag Biosector, Frederikssund, Denmark) for 60 minutes on a rocking platform. Other groups of control mice (n = 5-8) were immunized with an irrelevant control antigen, lysozyme from chicken egg white (HEL; catalog no. L6876; Sigma Aldrich, St. Louis, MO), or were sham-injected with adjuvant or antigen alone. Fourteen days later, the mice were boosted in an identical fashion. Fourteen days following the boost, mice were challenged with 10^3-10^4 *L. major* metacyclic promastigotes ± 10 ng MAX.

Histopathological examination of immunization site.

Tails from 2-3 control mice, CLDC-adjuvanted MAX-immunized mice, and Alhydrogel®-adjuvanted MAX-immunized mice were harvested at 14, 42, 57, 69, and 245 days following immunization to examine the histologic changes in the 3 experimental groups. The tail of each mouse was removed immediately after euthanasia, fixed in 10% neutral buffered formalin, and then sectioned. Tissue sections were processed routinely, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin.

Anti-MAX ELISA.

Blood was collected from mice via tail bleed at 7, 14, 21, and 28 days following initial immunization, and the anti-MAX serum titer was determined by an ELISA. Briefly, ELISA plates were coated with synthetic MAX (10 µg/mL) using standard techniques (Shankar and Titus, 1995) and developed with either horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (H and L chain) (catalog no. 074-1806; Kirkegaard & Perry Laboratories, Gaithersburg, MD), HRP-labeled rat anti-mouse IgG2a (material no. 553391; Becton Dickinson Biosciences Pharmingen), or HRP-labeled rat anti-mouse

IgG1 (material no. 559626; Becton Dickinson Biosciences Pharmingen) and the TMB substrate reagent set (material no. 555214; Becton Dickinson Biosciences).

Isolation and stimulation of lymphocytes.

Following euthanasia, the popliteal and para-aortic lymph nodes were harvested from groups of experimental mice (n = 5-8) and mechanically disrupted to prepare separate single-cell suspensions in complete RPMI medium. 1 x 10^6 cells were added to each well of a 48-well plate in a volume of 500 µL complete RPMI medium. 1 µL of both Cell Stimulation Cocktail (500x; catalog no. 00-4970-93; eBioscience) and GolgiPlug™ Protein Transport Inhibitor (catalog no. 51-2301KZ (555029); BD Biosciences Pharmingen, San Diego, CA) were added to each well, and cells were then incubated for 5 h at 37ºC.

Intracellular cytokine staining and flow cytometry.

Following stimulation, cells were suspended in FACS staining buffer (PBS, 0.5% BSA, and 0.01% azide) and treated with Fc receptor block (Miltenyi Biotec). Cells were surface-labeled with PE- or APC-conjugated Abs for 20 min at 4ºC, and washed with FACS staining buffer. Cells were permeabilized via resuspension in 100 µL Cytofix/Cytoperm™ fixation and permeabilization solution (catalog no. 51-2090KZ (554722); BD Biosciences Pharmingen, San Diego, CA) for 20 min at 4ºC, and washed with 1x Perm/Wash™ buffer (catalog no. 51-2091KZ (554723); BD Biosciences Pharmingen, San Diego, CA). Intracellularly-retained cytokines (IFN-γ or IL-4) were stained with FITC-conjugated Abs for 30 min at 4ºC and washed with 1x Perm/Wash™ buffer and FACS staining buffer. Cells were analyzed by flow cytometry, conducted on a CyAn flow cytometer (DakoCytomation, Colorado, USA) using Summit Acquisition Software, Version 4.2. Live cells were gated on forward vs. side scatter characteristics

and resolved based on geometric mean fluorescence intensity (MFI) emissions generated via excitation of PE-, APC-, or FITC-conjugated Abs using a 488 nm laser.

Reagents.

The following Abs were purchased from eBioscience and used for flow cytometry: PE-conjugated anti-mouse CD4, APC-conjugated anti-mouse CD8a (Ly-2), FITC-conjugated anti-mouse IFN-γ and FITC-conjugated anti-mouse IL-4 (catalog no. 12-0041-81, 17-0081-81, 11-7311-71, and 11-7042-71, respectively). Fc receptor block was purchased from Miltenyi Biotec.

Statistical analysis.

Data for lesion progression were analyzed using ANOVA for repeated measure.

Results

L. major dose-challenge response in BALB/c mice.

In order to determine the lower limits of *L. major* challenge that would lead to detectable infection and lesion development, BALB/c mice (n = 5) were challenged subcutaneously in one hind footpad with increasing log increments of *L. major* (Fig. 4.1). Lesion development was not detected in any mouse challenged with the lowest two doses of *L. major* ($1x10^1$ and $1x10^2$ metacyclic promastigotes). In contrast, by 4-weeks post-infection mice challenged with the highest dose of parasites ($1x10^5$ metacyclic promastigotes) had developed ulcerating lesions larger than those detected in other challenge groups at any timepoint. Mice challenged with either of the two intermediate doses of parasites ($1x10^3$ and $1x10^4$ metacyclic promastigotes) gradually developed measurable, non-ulcerating lesions. Accordingly, mice were challenged with inoculums

containing $1x10^3$ - $1x10^4$ metacyclic promastigotes in all subsequent challenge experiments.

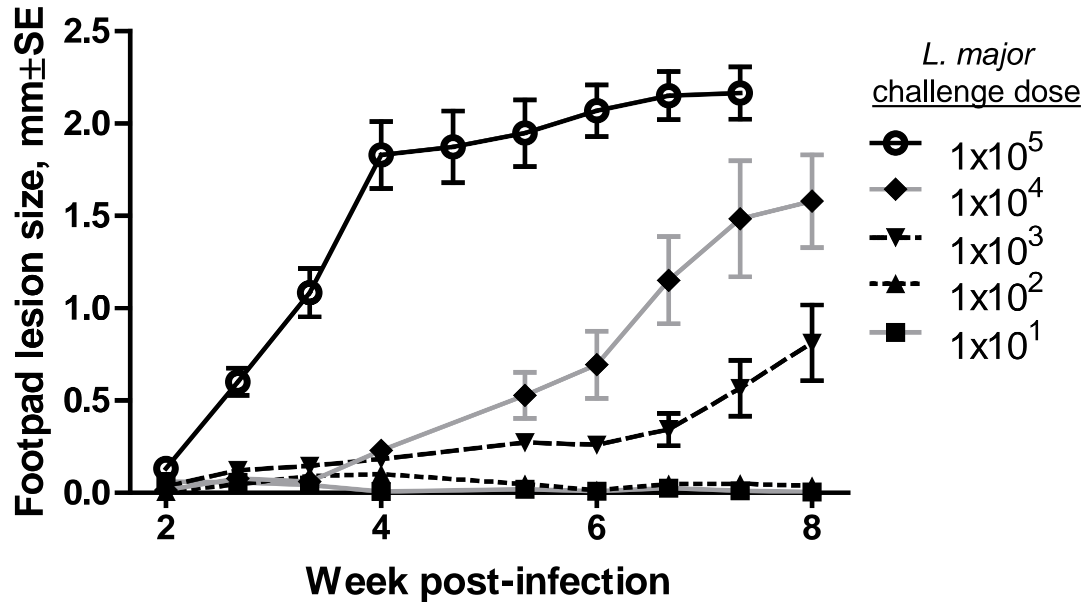

Figure 3.1. *L. major* dose-challenge response in BALB/c mice. BALB/c mice (n = 5 animals) were challenged subcutaneously in one hind foot pad with increasing log-increments of *L. major* between 1 x 10^1 and 1 x 10^5 infective-stage metacyclic promastigotes. Mean footpad lesion size (±SEM) was determined by measurement of footpad swelling using a digital caliper as described in Materials and Methods. Data shown are representative of two independent experiments.

MAX exacerbates L. major infection in BALB/c mice.

In order to investigate whether intermediate-dose *L. major* infection was exacerbated by co-injection of MAX, BALB/c mice (n = 5) were challenged subcutaneously in one hind footpad with 10^4 *L. major* metacyclic promastigotes ± 10 ng of MAX (an amount experimentally determined to be present in 2 *Lu. longipalpis* salivary glands). Mice co-injected with both *L. major* and MAX developed lesions that were significantly larger (2-3 fold; $p < 0.001$) than lesions of mice challenged with *L. major* alone (Fig. 4.2).

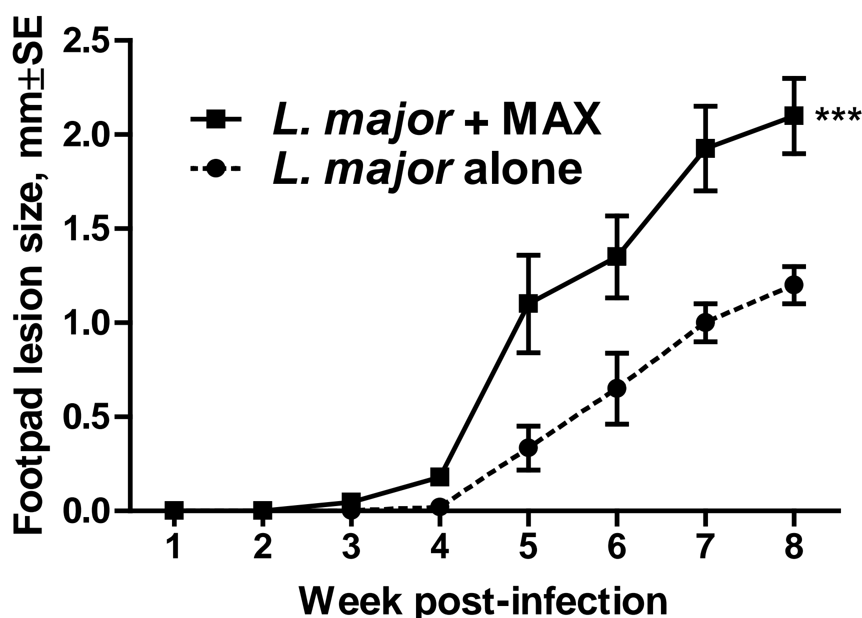

Figure 4.2. MAX exacerbates infection with *L. major*. BALB/c mice (n = 5 animals) were challenged subcutaneously in one hind foot pad with 1 x 10^4 *L. major* metacyclic promastigotes ± 10 ng synthetic MAX. Mean footpad lesion size (±SEM) was determined by measurement of footpad swelling using a digital caliper as described in Materials and Methods. Cutaneous lesions of mice challenged with *L. major* + MAX (■) were 2 to 3-fold larger than those of mice challenged with *L. major* alone (●). The asterisks denote that mice challenged with *L. major* + MAX developed lesions that were significantly larger ($p < 0.001$) than lesions that developed on mice challenged with *L. major* alone. Statistical differences in lesion progression were calculated using ANOVA for repeated measure. Data shown are representative of two independent experiments.

In addition, the parasite burden in the lesions of mice challenged with both *L. major* and MAX was increased 57-fold over that in lesions of mice challenged with *L. major* alone (Table 4.1). Thus, co-injection of synthetic MAX exacerbated infection with intermediate dose challenges of *L. major*.

Table 4.1. MAX exacerbates *L. major* infection in BALB/c mice[a]

Challenge	# of *L.major*/footpad (mean ± SEM)	Fold Increase in Parasite #
L. major	1,576,000 ± 665,750	Not applicable
L. major + MAX	90,422,000 ± 34,972,000	57

[a] At 8 weeks post-challenge, infected footpads from triplicate mice were combined and analyzed as a group for determination of the number of parasites in their footpad lesions (mean ± SEM). Results are representative of data obtained in three independent experiments.

Footpad lesion size and parasite burden are reduced in MAX-immunized BALB/c mice.

Prior studies from our laboratory concluded that MAX-immunized CBA/CaH-T6J mice were protected against challenge with *L. major* co-injected with sand fly saliva or synthetic MAX, and that protection correlated with anti-MAX antibody production and secretion of IFN-γ by CD4+ and CD8+ T cells (Morris et al., 2001); however, MAX was delivered in complete Freund's adjuvant (not suitable for use in humans), and induction of protection required multiple immunizations with large amounts of antigen (25 μg MAX per treatment). Therefore, CLDCs were utilized as an alternative adjuvant in order to expand on these previous results and investigate the protective potential of anti-MAX immunization. Mice ($n = 5$) were immunized i.p. with CLDC+MAX or treated with CLDC alone, followed by challenge with *L. major* + MAX (Fig. 4.3). Footpad lesions of MAX-immunized mice were significantly smaller ($p < 0.001$) than those of mice treated with CLDC alone at 6 weeks post-infection, and remained between 2- and 7-fold smaller through week 10 post-infection.

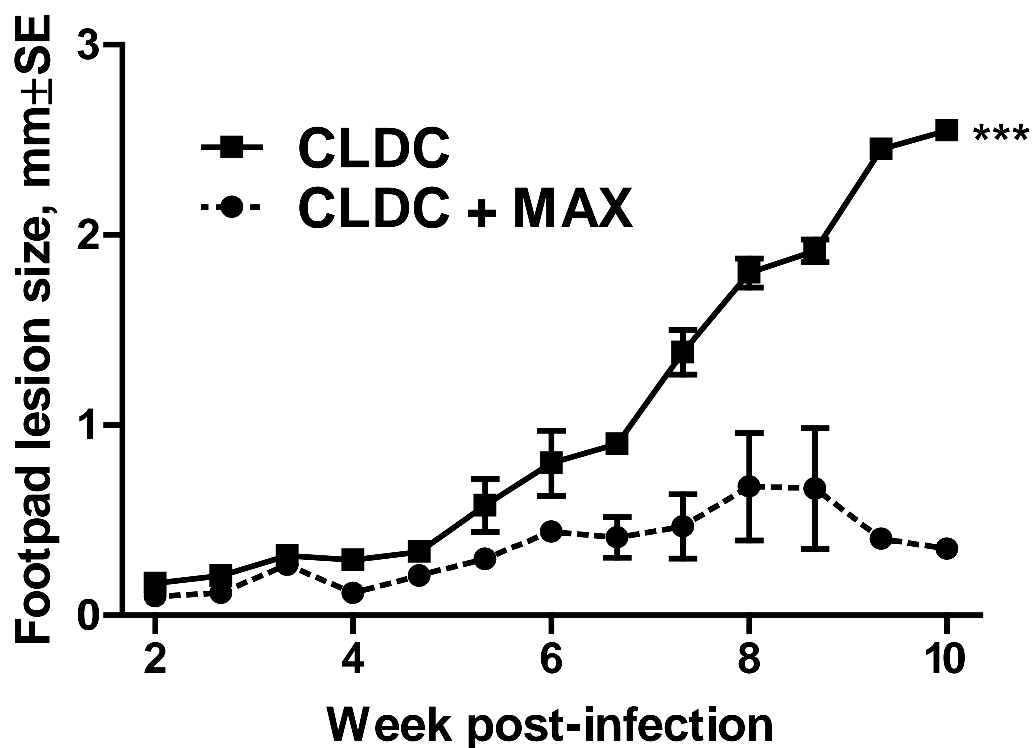

Figure 4.3. Immunization against MAX protects against infection with *L. major* + MAX. BALB/c mice (*n* = 5 animals) were immunized with CLDC + MAX or treated with CLDC alone, and were then challenged subcutaneously in one hind foot pad with 1 x 10^4 *L. major* metacyclic promastigotes + 10 ng synthetic MAX. Mean footpad lesion size (±SEM) was determined by measurement of footpad swelling using a digital caliper as described in Materials and Methods. Cutaneous lesions of MAX-immunized mice (●) were 2- to 7-fold smaller than those of mice receiving CLDC adjuvant only (■). The asterisks denote that mice treated with CLDC alone developed lesions that were significantly larger ($p < 0.001$) than lesions that developed on mice immunized with CLDC + MAX. Statistical differences in lesion progression were calculated using ANOVA for repeated measure. Data shown are representative of two independent experiments.

Visual comparison of time-matched footpads of both groups clearly revealed the significant reductions in footpad lesion size induced by MAX-immunization (Fig. 4.4). Parasite burden in lesions of MAX-immunized mice was also greatly reduced in comparison to the burden in lesions of mice treated with CLDC alone, with a 38,707-fold reduction in parasite load at day 63 post-infection (Table 4.2).

Figure 4.4. Immunization against MAX elicits reductions in footpad swelling size following challenge with *L. major* + MAX. BALB/c mice (*n* = 5 animals) were challenged subcutaneously in one hind foot pad with 1 x 10^4 *L. major* metacyclic promastigotes + 10 ng synthetic MAX, and the effects of immunization with CLDC + MAX on footpad lesion development were determined. Footpad lesion size was determined by measurement of footpad swelling using a digital caliper as described in Materials and Methods. Comparison of representative footpad lesions of mice immunized with CLDC+MAX (A) vs. mice treated with CLDC alone (B) reveals significant reductions in cutaneous lesion sizes of MAX-immunized mice (2 to 7-fold smaller than those of mice receiving CLDC adjuvant only). Images are representative of results obtained from 5 independent experiments. (A): MAX-immunized mouse footpad, day 66 post-challenge. (B): CLDC-treated mouse footpad, day 66 post-challenge.

Table 4.2. Parasite burden is reduced in MAX-immunized BALB/c mice[a]

Treatment	# of *L.major*/footpad (mean ± SEM)	Fold Increase in Parasite #
CLDC + MAX	2,608 ± 1,100	Not applicable
CLDC	100,950,000 ± 39,045,000	38,707

[a] At 9 weeks post-challenge, infected footpads from triplicate mice were combined and analyzed as a group for determination of the number of parasites in their footpad lesions (mean ± SEM). Results are representative of data obtained in three independent experiments.

These results strongly support the premise that immunization against MAX using CLDC as adjuvant protects against challenge with *L. major* + MAX, as evidenced by reductions in both footpad lesion size and parasite burden.

S.C. immunization elicits protection exceeding that induced by I.P. immunization.

In order to investigate the importance of the site of immunization and thus determine the immunization route that would induce optimal levels of protection against challenge with *L. major* + MAX, the protection elicited by i.p. immunization was compared to that elicited by s.c. immunization at the base of the tail (Fig. 4.5).

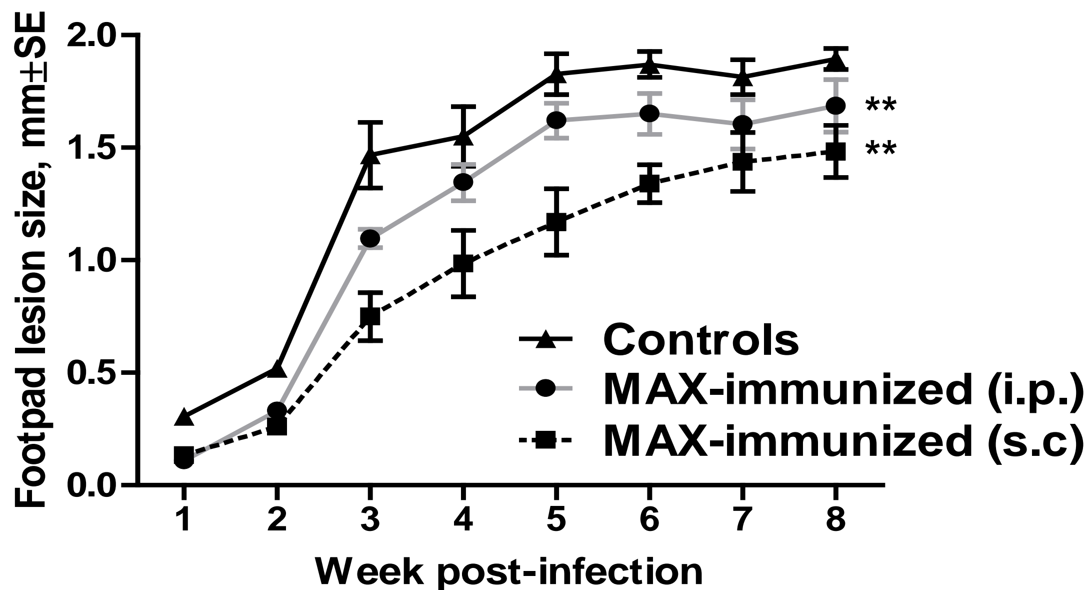

Figure 4.5. Protection elicited against infection with *L. major* + MAX by s.c. immunization exceeds that elicited by i.p. immunization. BALB/c mice (n = 5 animals) were untreated or immunized either s.c. or i.p. with CLDC + MAX, and were challenged subcutaneously in one hind foot pad with 1 x 10^4 *L. major* metacyclic promastigotes + 10 ng synthetic MAX. Mean footpad lesion size (±SEM) was determined by measurement of footpad swelling using a digital caliper as described in Materials and Methods. Cutaneous lesions of mice immunized by either route were significantly (**; p < 0.01) smaller than those of non-immunized mice. Cutaneous lesions of mice s.c.-immunized were significantly (p < 0.01) smaller than those of i.p.-immunized mice. Statistical differences in lesion progression were calculated using ANOVA for repeated measure. Data shown are representative of two independent experiments.

Footpad lesion sizes of mice immunized by either route were significantly smaller ($p < 0.01$) than those of untreated control mice, and parasite burdens in the lesions of immunized mice were also markedly reduced in comparison to those in lesions of untreated control mice (Table 4.3).

Table 4.3. S.C. MAX-immunization elicits protection exceeding that of I.P. MAX-immunization [a]

Treatment	# of *L.major*/footpad (mean ± SEM)	Fold Increase in Parasite #
MAX-immunized (s.c.)	2,847,500 ± 455,250	Not applicable
MAX-immunized (i.p.)	5,295,000 ± 933,500	1.9
Untreated Controls	37,305,000 ± 5,937,500	13.1

[a] At 5 weeks post-challenge, infected footpads from triplicate mice were combined and analyzed as a group for determination of the number of parasites in their footpad lesions (mean ± SEM). Results are representative of data obtained in two independent experiments.

These results indicate that both i.p. and s.c. MAX-immunization protects against challenge with *L. major* + MAX and elicits reductions in both footpad lesion size and parasite burden. However, footpad lesions of s.c.-immunized mice were significantly smaller ($p < 0.01$) than those of mice immunized via the i.p. route (Fig. 4.5), and the associated parasite loads were reduced 1.9-fold in s.c.-immunized mice (Table 4.3). Accordingly, the s.c. route of immunization was used in all subsequent experiments.

CLDC adjuvant elicits protection exceeding that induced by Alhydrogel®.

In order to ascertain whether the protection elicited by MAX-immunization utilizing CLDC as adjuvant was comparable to that elicited by a vaccine employing a human-approved aluminum hydroxide gel adjuvant (Alhydrogel®), the protection elicited by both formulations was compared. Mice were untreated or immunized with either CLDC+MAX or Alhydrogel®+MAX and then challenged with *L. major* + MAX as described above (Fig. 4.6).

Cutaneous lesions of mice immunized with Alhydrogel®+MAX were not significantly different than those of non-immunized mice ($p = 0.1003$), indicating that MAX-immunization utilizing Alhydrogel® as adjuvant does not elicit significant reductions in footpad lesion size following challenge with *L. major* + MAX.

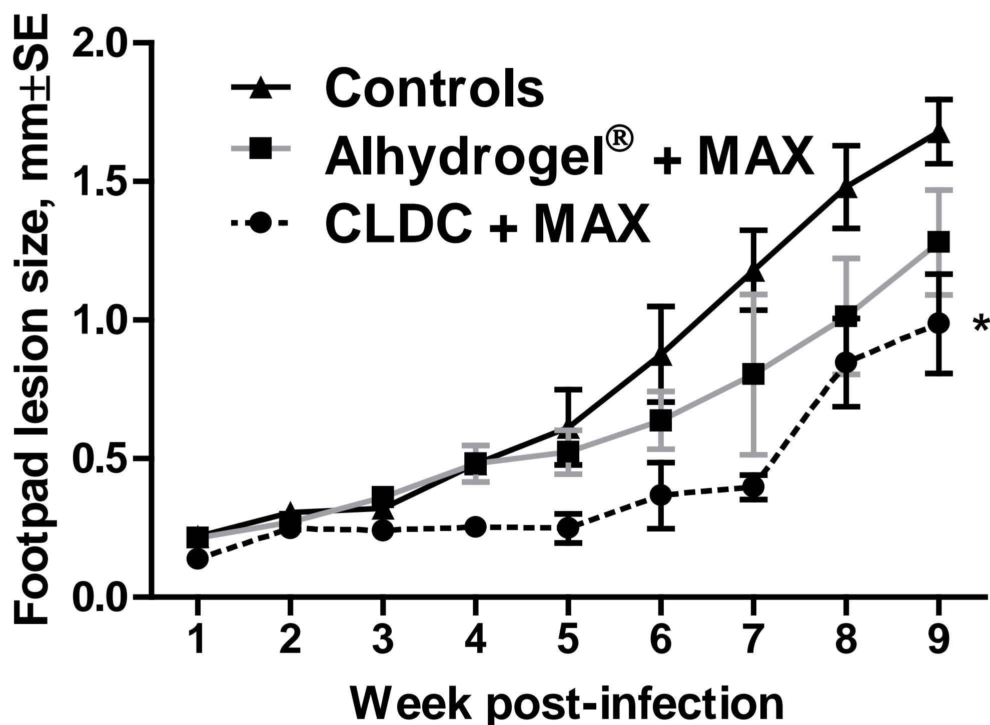

Figure 4.6. Protection elicited against infection with *L. major* + MAX by immunization with CLDC + MAX exceeds that elicited by immunization with Alhydrogel® + MAX. BALB/c mice (n = 5 animals) were untreated or immunized with either CLDC + MAX or Alhydrogel® + MAX and were challenged subcutaneously in one hind foot pad with 1 x 10^4 *L. major* metacyclic promastigotes + 10 ng synthetic MAX. Mean footpad lesion size (±SEM) was determined by measurement of footpad swelling using a digital caliper as described in Materials and Methods. Cutaneous lesions of mice immunized with CLDC adjuvant were significantly smaller than those of both non-immunized mice ($p < 0.01$) and those of mice immunized with Alhydrogel® adjuvant (*; $p < 0.05$). Lesions of mice immunized with Alhydrogel® adjuvant were not significantly different than those of non-immunized mice ($p = 0.1003$). Statistical differences in lesion progression were calculated using ANOVA for repeated measure. Data shown are representative of two independent experiments.

In contrast, cutaneous lesions of mice immunized with CLDC+MAX were significantly smaller than those of both non-immunized mice ($p < 0.01$) and mice immunized with Alhydrogel®+MAX (*; $p < 0.05$). Accordingly, the parasite burden in the lesions of mice immunized with CLDC+MAX was reduced 17-fold in comparison to non-immunized mice and 1.3-fold in comparison to mice immunized with Alhydrogel®+MAX (Table 4.4).

Table 4.4. MAX-immunization using CLDC as adjuvant elicits protection exceeding that induced by Alhydrogel®[a]

Treatment	# of *L.major*/footpad (mean ± SEM)	Fold Increase in Parasite #
CLDC + MAX	2,095,000 ± 221,250	Not applicable
Alhydrogel + MAX	2,751,500 ± 290,500	1.3
Untreated Controls	35,515,000 ± 3,750,750	17

[a] At 8 weeks post-challenge, infected footpads from triplicate mice were combined and analyzed as a group for determination of the number of parasites in their footpad lesions (mean ± SEM). Results are representative of data obtained in two independent experiments.

It was notable that although MAX-immunization with Alhydrogel® adjuvant did not significantly reduce footpad lesion size (Fig. 4.6), parasite burden in lesions was still markedly-reduced (13-fold) in comparison to non-immunized mice, suggesting that immunization with Alhydrogel®+MAX elicited some degree of protection against challenge with *L. major* + MAX. Collectively, these results indicated that MAX-immunization with CLDC as adjuvant elicits protection against challenge with *L. major* + MAX exceeding that induced by an aluminum hydroxide gel adjuvant.

MAX-immunization with Alhydrogel® adjuvant induces tissue damage at the site of immunization.

The previous experiments revealed that although MAX-immunization using either CLDC or Alhydrogel® as adjuvant elicited reductions in parasite burden following challenge with *L. major* + MAX, CLDC-adjuvanted immunization not only induced a higher degree of protection, but also elicited significant reductions in footpad lesion size. Furthermore, examination of the base-of-tail immunization site of mice immunized with Alhydrogel® + MAX indicated the presence of significant tissue damage. In order to ascertain any adjuvant-dependent differences in histopathologic changes at the site of immunization, tissue sections of mice in both immunization groups were prepared for

comparison. At 69 days post-boost, tail sections of mice immunized with CLDC+MAX appeared normal in comparison to tail sections from non-immunized mice. In contrast, tail sections of mice immunized with Alhydrogel®+MAX contained a severe inflammatory infiltrate of macrophages and lymphocytes and exhibited collateral damage to muscle tissue (Fig. 4.7).

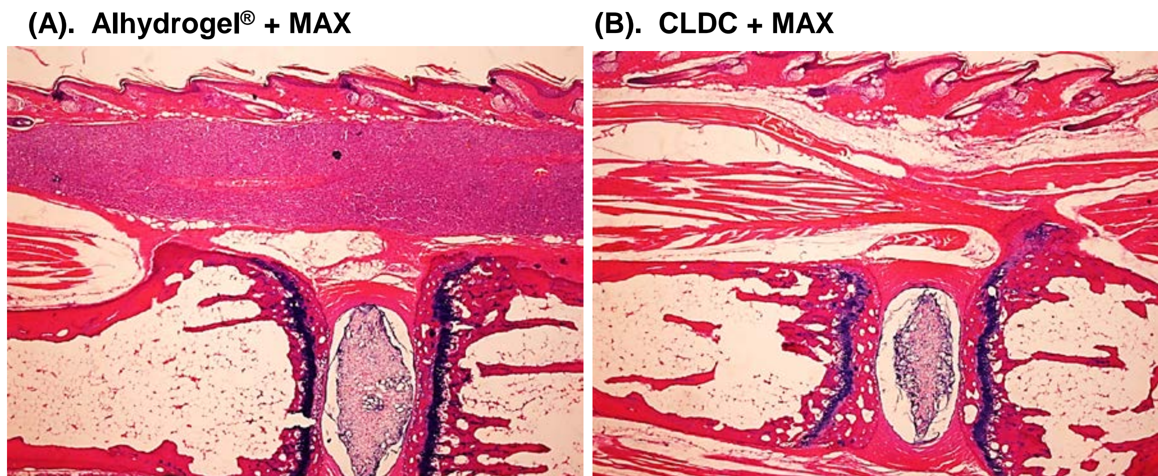

Figure 4.7. The base-of-tail immunization site of mice treated with Alhydrogel® adjuvant contains a severe inflammatory infiltrate that is absent at the immunization site of mice treated with CLDC adjuvant. BALB/c mice (*n* = 8 animals) were immunized s.c. at the base of the tail with MAX using either Alhydrogel® or CLDC as adjuvant, and were challenged subcutaneously in one hind foot pad with 1×10^4 *L. major* metacyclic promastigotes + 10 ng synthetic MAX. At various time points following immunization, tails were harvested, sectioned, and prepared for observation of histopathologic changes at the site of immunization. Comparison of representative tail sections of mice receiving Alhydrogel® adjuvant (A) vs. mice receiving CLDC adjuvant mice (B) revealed the presence of a severe inflammatory infiltrate and tissue damage at the site of immunization in group A that was absent in group B. The tail section from the CLDC-adjuvanted group appeared normal in comparison to tail sections from un-immunized control mice (not shown). Images are representative of results obtained from three independent experiments. (A): MAX-immunized mouse tail section, Alhydrogel® adjuvant, day 69 post-boost. (B): MAX-immunized mouse tail section, CLDC adjuvant, day 69 post-boost.

It was notable that this inflammatory infiltrate was present until at least 245 days post-boost, indicative of permanent tissue damage. These results indicate that MAX-immunization using a CLDC adjuvant avoids an unwanted, long-term side effect of

MAX-immunization using an aluminum hydroxide adjuvant already approved for use in humans, and warrant subsequent investigation of its approval for human usage.

Mechanism(s) of protection induced by immunization with CLDC+MAX.

Experiments were conducted to identify the immune mechanism(s) of protection against challenge with *L. major* + MAX induced by immunization with CLDC+MAX. As previous studies from our laboratory concluded that protection induced by MAX-immunization correlated with the production of anti-MAX antibodies and secretion of IFN-γ by CD4+ and CD8+ T cells (Morris et al., 2001), we assayed for the presence of anti-MAX antibodies in the sera of mice immunized with CLDC+MAX. Sera of mice immunized with CLDC+MAX did not contain detectable titers of anti-MAX IgG, IgG1, or IgG2a at any time-point following immunization, while immunization with CLDC + an irrelevant control antigen (hen egg lysozyme; HEL) elicited the production of high titers of anti-HEL IgG (Table 4.5).

Table 4.5. Mice immunized with CLDC + MAX do not produce detectable titers of anti-MAX IgG, IgG1, or IgG2a

Treatment	Antibody Isotype	Anti-MAX antibody titer, (negative control values) [a]
CLDC + MAX	IgG	0 (zero)
Positive Control	IgG	1/12,800 – 1/25,600
CLDC + MAX	IgG1	0 (zero)
Positive Control	IgG1	1/6,400 – 1/12,800
CLDC + MAX	IgG2a	0 (zero)
Positive Control	IgG2a	1/6,400 – 1/12,800
Treatment	**Antibody Isotype**	**Anti-HEL antibody titer, (negative control values)**
CLDC + HEL	IgG	1/6,400 – 1/12,800, (zero)

[a] Negative control mice were not immunized with MAX or HEL. Sera from non-immunized mice did not contain detectable anti-MAX or anti-HEL antibodies. Results are representative of two independent experiments.

These findings indicated that protection against infection with *L. major* + MAX induced by immunization with CLDC+MAX was not likely mediated by anti-MAX antibodies.

In order to ascertain whether the protection elicited by MAX-immunization was instead mediated by a CD4+ cellular immune response, the production of cytokines by CD4+ cells harvested from immunized mice following challenge with *L. major* + MAX was investigated. Lymphocytes from challenged mice were harvested from the draining lymph nodes of MAX-immunized, HEL-immunized and non-immunized mice, were stimulated *in vitro* and stained for intracellular cytokines indicative of a protective Th1- or exacerbative Th2-biased immune response (IFN-γ and IL-4, respectively) (Fig. 4.8).

Flow-cytometric analysis indicated 33% and 35% increases in the percentages of IFN-γ-producing CD4+ cells in draining lymph nodes (popliteal and para-aortic, respectively) of challenged MAX-immunized mice when compared to CD4+ cells of challenged HEL-immunized mice; in comparison to non-immunized mice, the percentages of IFN-γ-producing CD4+ cells in the popliteal and para-aortic lymph nodes of MAX-immunized mice were increased 65% and 39%, respectively (Fig. 4.8A). Conversely, the para-aortic lymph nodes of MAX-immunized mice contained 32% fewer IL-4-producing CD4+ cells than para-aortic lymph nodes of non-immunized mice and 69% fewer IL-4-producing CD4+ cells than para-aortic lymph nodes of HEL-immunized mice (Fig. 4.8B). The percentage ratio of IFN-γ- vs. IL-4-producing CD4+ cells in MAX-immunized mice was 2.53, in comparison to a ratio of 1.13 in non-immunized mice, suggesting an increased Th1-bias due to immunization with CLDC+MAX. An increased percentage ratio of IFN-γ- vs. IL-4-producing CD4+ cells is an indicator of a more Th1-biased immune response potentially capable of protecting against

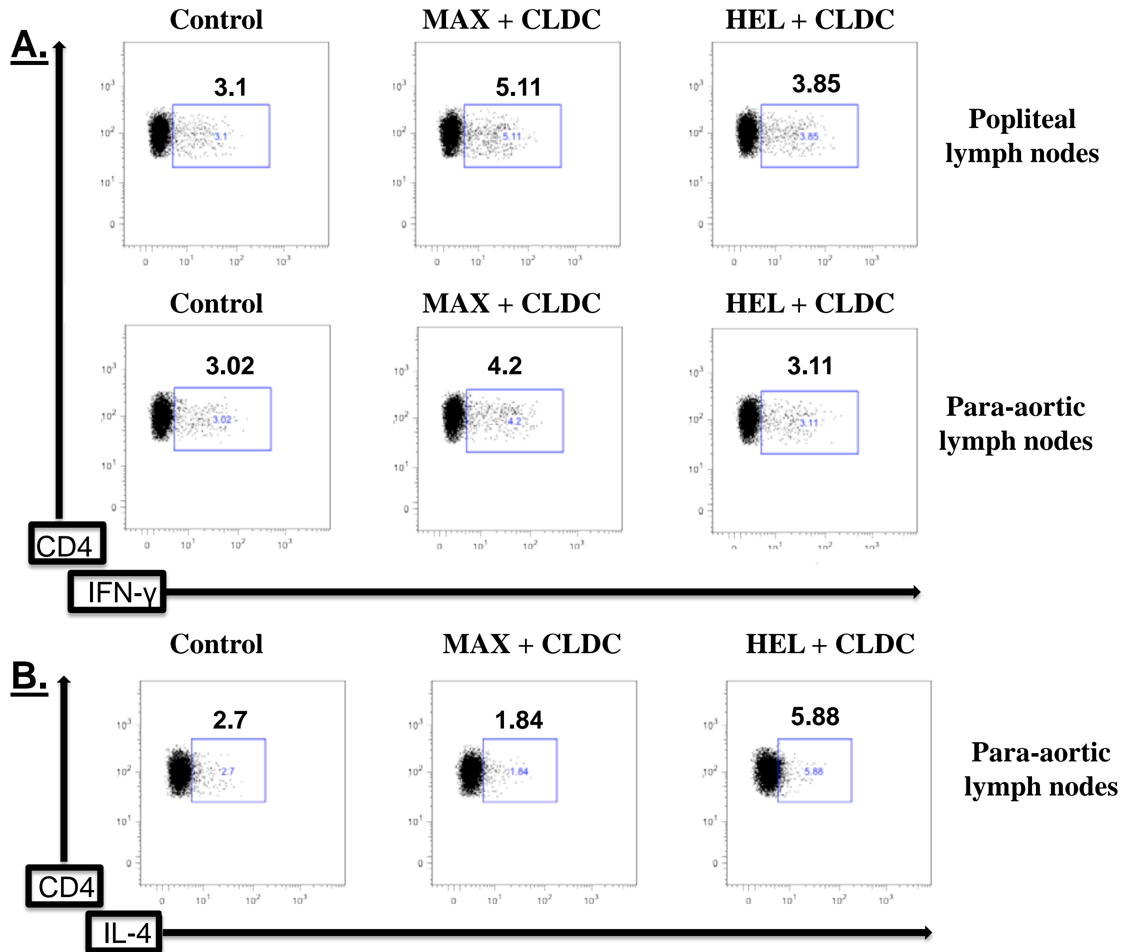

Figure 4.8. Intracellular staining of IFN-γ- and IL-4-producing CD4+ cells of MAX-immunized and non-immunized mice. Following challenge with *L. major* + MAX, popliteal and para-aortic lymph nodes were harvested from BALB/c mice (n = 5-8 animals) that were previously untreated (control), or immunized with either MAX + CLDC or HEL + CLDC. Single cell suspensions were stimulated *in vitro,* surface stained for CD4, and then stained for intracellular IFN-γ (Fig. 4.8A) and IL-4 (Fig. 4.8B), as described in Materials and Methods. Flow-cytometric analysis indicated 33% (popliteal) and 35% (para-aortic) increases in the percentages of IFN-γ-producing CD4+ cells in the draining lymph nodes of MAX-immunized mice when compared to CD4+ cells of HEL-immunized mice; in comparison to non-immunized mice, the percentages of IFN-γ-producing CD4+ cells in the popliteal and para-aortic lymph nodes of MAX-immunized mice were increased 65% and 39%, respectively (Fig. 4.8A). Conversely, the para-aortic lymph nodes of MAX-immunized mice contained 32% fewer IL-4-producing CD4+ cells than lymph nodes of non-immunized mice and 69% fewer IL-4-producing CD4+ cells than lymph nodes of HEL-immunized mice (Fig. 4.8B). The percentage ratio of IFN-γ- vs. IL-4-producing CD4+ cells in MAX-immunized mice was 2.53, in comparison to a ratio of 1.13 in non-immunized mice, suggesting an increased Th1-bias due to immunization with CLDC+MAX.

intracellular *L. major* infection; thus, the pattern of cytokine production identified in the draining lymph nodes of MAX-immunized mice challenged with *L. major* + MAX might account, in part, for the protection induced against challenge.

Discussion

In order for vertebrate hosts to effectively control infection with *L. major,* they must develop a Th1-biased cellular immune response capable of activating infected macrophages to kill intracellular parasites (Reiner and Locksley, 1995). It is of significant evolutionary interest that a salivary vasodilator (MAX) utilized by sand fly vectors to maintain blood flow and ensure a successful blood meal also modulates the immune response of the vertebrate host at the bite site, effectively interfering with the early development of protective immunity against a co-injected pathogen. Successful transmission and survival of *L. major* parasites in vertebrate hosts is strikingly dependent upon this altered immune environment at the bite site, as murine challenge with a low, biologically-relevant dose (10-100) of *L. major* in the absence of sand fly saliva does not lead to sustained infection, while an identical dose of parasites co-injected with sand fly saliva leads to progressive infection and disease (Titus and Ribeiro, 1988). These observations led to the now-proven hypothesis that immunization against sand fly salivary components elicits protection against challenge with *L. major* + sand fly saliva (Morris et al., 2001).

Recent studies have highlighted the importance of considering salivary immunomodulation during the development of vaccines intended to protect against infection with *Leishmania* parasites: vaccines which normally protect mice against needle-inoculation with *L. major* alone fail to protect against natural transmission via infected sand fly bite, in which the inoculum contains both parasites and salivary

components (Peters et al., 2009). The short-sighted goal of eliciting immune responses against vector-borne pathogens alone ignores the importance of salivary immunomodulation in allowing for initial and continued infection of vertebrate hosts. As a result, it is of paramount importance to consider the influence of salivary potentiation when formulating and developing vaccines to protect against natural, vector-borne transmission of *L. major* or any other arthropod-borne pathogen.

In this study, we investigated the ability of immunization with CLDC+MAX to protect against challenge with *L. major* + MAX, compared the elicited protection to that induced using a human-approved aluminum hydroxide gel adjuvant, and investigated the immune mechanism(s) of protection induced by CLDC + MAX immunization. Mice were challenged with *L. major* ± synthetic MAX, and the effects of MAX co-administration on infection and disease development were assessed. Co-injection of MAX elicited increases in both footpad lesion size (Fig. 4.2) and parasite burden (Table 4.1), and immunization against MAX elicited significant reductions in both lesion size (Figs. 4.3, 4.4, and 4.5) and parasite burden (Tables 4.2 and 4.3) and markedly lessened the severity of cutaneous infections, even when challenge was postponed up to 3 months following immunization. The use of a CLDC adjuvant not only induced protection exceeding that elicited by a MAX vaccine formulation employing an Alhydrogel® adjuvant (Fig. 4.6; Table 4.4), but avoided the persistent inflammation and tissue damage induced by Alhydrogel®+MAX at the site of immunization (Fig. 4.7). These observations suggest that CLDC is not only an effective adjuvant for the induction of anti-MAX immune responses protective against *L. major* infection, but also lacks a negative side effect of an adjuvant already approved for use in human populations.

Important changes in the production of Th1- and Th2-biasing cytokines were identified in the lymph nodes draining the footpad lesions of MAX-immunized mice. Increases in the percentage of IFN-γ-producing CD4+ cells were identified in MAX-immunized mice, in comparison to both non-immunized mice and those immunized with an irrelevant control antigen (Fig. 4.8A). Corresponding decreases in the percentage of IL-4-producing CD4+ cells were also identified in MAX-immunized mice (Fig. 4.8B), indicating that immunization with CLDC+MAX induced an increase in the percentage ratio of IFN-γ- vs. IL-4-producing CD4+ cells, suggesting the induction of Th1-biased immune responses in some CD4+ cells following challenge with *L. major* + MAX. An adaptive immune response to MAX characterized by an increased IFN-γ:IL-4 ratio would modify the immune environment present during initiation of *L. major* infection, making intracellular occupancy and growth of amastigotes more tenuous due to activation of macrophages and a skewing of the immune response toward clearance of intracellular parasites. Additionally, an anti-MAX immune response at the inoculation site would reprogram anti-*L. major* immunity, as priming of the developing anti-*L. major* immune response would take place in a Th1 pro-inflammatory atmosphere, generating the requisite protective immune response that interferes with early establishment of *L. major* infection. These conclusions are similar to those made in other studies investigating the effects of anti-saliva cellular immune responses on infection with *Leishmania* (Oliveira et al., 2008; Kamhawi et al., 2000; Gomes et al., 2008; Valenzuela et al., 2001; Belkaid et al., 2000), suggesting that the elicitation of cell-mediated immune responses to salivary components co-injected during natural transmission induces protective immunity against

Leishmania infection, providing supportive evidence of the efficacy of anti-salivary immunization for the prevention of arthropod-borne disease.

A particularly notable observation from this study was the complete lack of detectable titers of anti-MAX IgG in the sera of any mice immunized with CLDC+MAX (Table 4.5), suggesting that protection induced by immunization with CLDC+MAX might instead be due to cell-mediated immune responses. This is an intriguing conclusion, as anti-saliva antibodies induced by immunization with or exposure to sand fly salivary components have been suggested as important mediators of protection against subsequent challenge with *Leishmania* parasites + sand fly saliva (Morris et al., 2001; Belkaid et al., 1998; Gomes et al., 2002). The prevailing hypothesis is that anti-saliva antibodies neutralize and inhibit binding of immunomodulatory salivary components to their respective receptor(s), interfering with modulation of the host immune response. Our results suggest that even in the absence of a detectable titer of anti-MAX IgG, the cellular immune response(s) induced by immunization with CLDC+MAX are sufficient to elicit significant protection against challenge with *L. major* + MAX. This conclusion is supported by the work of Valenzuela et al. (2001), in which B-cell deficient mice (incapable of antibody production) immunized with a plasmid vaccine encoding for a salivary protein of the sand fly *P. papatasi* were protected from challenge with *L. major* + salivary gland homogenate.

One of the most intriguing observations stemming from these studies was that MAX-immunization elicited such impressive protection against challenge with *L. major* + MAX in BALB/c mice, a strain genetically biased toward a Th2-type, antibody-mediated immune response ineffective for control of infection with *L. major* (Launois et

al., 1998). Previous research has outlined an important series of immunological events leading to progressive infection that occur following challenge of BALB/c mice with *L. major*. Shortly after infection, the *L. major* antigen LACK (*Leishmania* homolog of receptors for activated C kinase) elicits early production of IL-4 by Vβ4Vα8 CD4+ T cells, leading to reduced expression of the β2 subunit of the IL-12 receptor on CD4+ T cell precursors that might otherwise develop into protective Th1 cells. Down-regulation of the IL-12 receptor interferes with the ability of BALB/c T cells to respond to IL-12, thus inhibiting the production of protective IFN-γ and NO required for the clearance of intracellular parasites (Launois et al., 1998). Furthermore, early production of IL-4 instead promotes the development of Th2 cells that support the production of anti-*L. major* antibodies demonstrated to be ineffective for control of intracellular infections. Considering the inherent programming of BALB/c mice to respond to *L. major* infection with a non-protective antibody-mediated immune response, the cell-mediated protection elicited by our CLDC+MAX vaccine in this mouse strain is quite impressive.

In addition to eliciting protection against infection with *L. major,* immunization against immunomodulatory salivary components of sand flies might have some unexpected, yet beneficial consequences worthy of consideration. The immune responses induced by immunization might not only protect against *L. major* infection, but could also interfere with the transmission of any other pathogens vectored by sand flies, even those that have yet to be identified (unknown, emerging, or re-emerging) (Travassos da Rosa et al., 1984). Furthermore, repeated exposure of immunized vertebrates to sand fly bites in endemic areas could amplify and maintain the protective anti-saliva memory

immune response through "natural" boosts, effectively extending the window of protection elicited by initial immunization.

In the experiments described herein, we elected to utilize the experimental combination of *L. major* parasites and a salivary peptide from *Lu. longipalpis,* although this is not a pathogen/vector combination found in nature: rather, *L. major* is naturally transmitted by *P. papatasi* (which does not encode MAX)*,* and *Lu. longipalpis* is the natural vector of *L. chagasi,* a causative agent of visceral leishmaniasis. However, *Lu. longipalpis* saliva modifies the host immune response and exacerbates infection with *L. major* (Titus and Ribeiro, 1988) *L. chagasi* (Warburg et al., 1994), *L. amazonensis* (Norsworthy et al., 2004) and *L. braziliensis* (Gillespie et al., 2000; Samuelson et al., 1991), suggesting that salivary immunomodulation is a natural mechanism exploited by many species of *Leishmania*. In fact, a recent study by Tavares et al. (2011) demonstrated that immunization of hamsters against *Lu. longipalpis* saliva or salivary proteins induces protection against challenge with *L. braziliensis* + *Lu. intermedia* saliva, suggesting that immunization against *Lu. longipalpis* saliva might elicit protective immune responses against various species of *Leishmania*, even those naturally-transmitted by different sand fly species. As a result, continued investigation of salivary immunomodulation is certainly justified, even if unnatural combinations of parasites and vectors are utilized. Additionally, MAX-immunization induced an immune response qualitatively appropriate for protection against infection with all *Leishmania* species, and thus it is probable that MAX-immunization would protect against challenge with any species of *Leishmania*, assuming parasites were co-injected with MAX or *Lu. longipalpis* saliva. Furthermore, the immunomodulatory activities of MAX and the murine model of

L. major infection have both been extensively characterized in our laboratory and by others, and thus the decision was made to perform mechanistic experiments using the best-defined systems available. Lastly, use of the *L. major* and *Lu. longipalpis* combination enabled us to correlate results with those of previous reports from our laboratory, allowing for interpretation of new findings within the context of an entire career's worth of historical work.

Salivary immunomodulation is not a sand fly-specific phenomenon, as the collective efforts of many research groups have identified the presence of immunosuppressive or immunomodulatory components in the saliva of most blood-feeding arthropods (Titus et al., 2006). Furthermore, vector saliva modulates infection with an impressive variety of pathogens, including various parasites (Titus and Ribeiro,1988; Mbow et al., 1998; Belkaid et al., 1998; Gillespie et al., 2000; Samuelson et al., 1991; Warburg et al., 1994; Norsworthy et al., 2004; Vaughan et al., 1999; Alger et al., 1972; Alger and Harant, 1976), viruses (Jones et al., 1989; Edwards et al., 1998; Feinsod et al., 1975; Osorio et al., 1996; Limesand et al., 2000; Edwards et al., 1998), and bacteria (Wikel and Bergman, 1997; Nazario et al., 1998; Kuthejlova et al., 2001; Zeidner et al., 2002). Thus, the implications of the protection induced by our anti-MAX vaccine are far-reaching, suggesting that immunization against salivary components should indeed be considered in other models of arthropod-borne disease, especially those of world-wide public health concern such as malaria, dengue fever, and yellow fever. The protection described herein stresses the importance of salivary immunomodulation in the initiation of vector-borne pathogen infections, and provides supportive evidence of CLDC as an effective adjuvant for the induction of immune responses required for

control of intracellular pathogens. The results of this study provide compelling evidence in support of the inclusion of vector salivary molecules as antigens in the formulation of stand-alone or subunit vaccines intended to protect against natural transmission of arthropod-borne pathogens.

CHAPTER 5

EXTENDED PROJECT DISCUSSION AND PROSPECTS FOR FUTURE RESEARCH

The morbidity and mortality affiliated with vector-borne diseases are staggering, and the associated economic and social hardships are overwhelming, especially in populations without the political or financial means for effective control or treatment. Many of these diseases continue to re-emerge in former endemic areas and/or emerge in new parts of the world, and conventional means of control are often inadequate due to the appearance of pesticide-resistant vectors, drug resistant-pathogens, and the collapse of vector control programs, among other factors. At present, safe and efficacious vaccines and therapeutics for prevention and treatment of many of these diseases (including La Crosse (LAC) encephalitis and cutaneous/mucocutaneous/visceral leishmaniasis) are lacking. The results of the studies herein suggest that cationic liposome-DNA complexes (CLDCs) are novel and effective immunotherapeutics and vaccine adjuvants for treatment and/or prevention of infection with arthropod-borne pathogens, whether occurring by natural or purposeful means. We show that (1) La Crosse virus (LACV) is transmissible via inhalation, and leads to the development of lethal LAC encephalitis in normally-resistant adult mice, (2) CLDC immunotherapy elicits prophylactic and therapeutic protection against aerosolized LACV challenge, and (3) immunization against the sand fly salivary peptide maxadilan (MAX) using CLDC as adjuvant induces significant protection against challenge with *Leishmania major* + MAX. These findings

significantly enhance our understanding of the transmission and pathogenesis of these important pathogens, and provide supportive evidence of the efficacy of CLDCs as both immunotherapeutics and vaccine adjuvants for the treatment or prevention of intracellular pathogen infection requiring induction of a Th1-biased, cell-mediated immune response for control or clearance.

Our experiments involving the development of inhalational models of LACV infection reveal that aerosol or intranasal delivery of LACV can alter the normal route of central nervous system (CNS) invasion associated with parenteral or vector-transmitted infection, bypassing the requirement of viremia for navigating through the blood-brain barrier via infection of the nasal olfactory epithelium and, presumably, ascent of olfactory neurons. These observations and those of Bennett et al. (2008) are in contrast to previous conclusions stemming from models of peripheral LACV infection, in which the neuroinvasiveness of LACV has been correlated with development of a high viremia (Janssen et al., 1984). It would be beneficial to separately titrate the olfactory nerves of LACV-challenged mice for infectious virus in order to empirically determine whether CNS invasion was indeed occurring via ascent of olfactory neurons. Future experiments involving *in situ* hybridization and/or immunohistochemical staining would allow for precise detection and localization of LACV RNA sequences and/or LACV antigens within sections of olfactory neurons, enabling definitive determination of whether neuroinvasion might be occurring via passage of LACV through the olfactory tract. In addition, comparison of viral titers in rostral vs. caudal brain sections would allow for determination of whether there was temporal dissemination of infection from the

olfactory lobes to the brainstem, which might provide additional evidence of olfactory invasion of the CNS following inhalational LACV challenge.

The unnatural route of inhalational LACV infection may overwhelm the immune mechanisms that normally protect adult mice from CNS invasion (see Chapter 2). It is interesting to speculate that this phenomenon might also occur in adult humans exposed via aerosol to LACV or related viruses, leading to development of severe neurological disease in normally-resistant age cohorts. These results not only stress the importance of the route of challenge in determining resultant pathogenesis, but also demand reconsideration of the method used for categorization of viruses as priority pathogens by the NIAID. Furthermore, as many other arthropod-borne viruses can be transmitted via aerosol and some are capable of CNS invasion through the olfactory tract (Kuno, 2003, Larson et al., 1980; Ryzhikov et. al, 1995a; Ryzhikov et. al, 1995b; Vogel et. al, 1996), our studies imply that other arthropod- or rodent-borne viruses delivered via aerosol might be infectious to humans, and would therefore be of bioterrorism concern. The inhalational LACV models developed in this study provide useful tools for testing the efficacy of vaccines and therapeutics to be used in the event of airborne release of members of the *Bunyaviridae.*

Prior studies have established the antiviral effects of CLDC administration in models of lethal subcutaneous challenge with members of the *Bunyaviridae* and *Togaviridae* (Gowen et al., 2006; Gowen et al., 2008; Logue et al., 2010); however, this study is the first report of CLDC-induced protection against aerosolized virus challenge. Prophylactic and therapeutic administration of CLDC increased the survival of LACV-challenged mice, reducing CNS infection and lessening associated neuropathology (Figs.

3.1, 3.2, and 3.4, Chapter 3). Protection was correlated with the induction of an antiviral immunological state characterized by increased expression of type I and II IFNs (Fig. 3.6, Chapter 3), and natural killer (NK) cells were identified as integral components for complete CLDC-induced protection in the aerosol LACV model (Fig. 3.7, Chapter 3). The results from Chapter 3 suggest that CLDC immunotherapy is a novel approach for prevention and treatment of aerosol or conventional infection by members of the *Bunyaviridae* and other virus families.

It is plausible that the protection described in these experiments could be improved upon by intranasal/mucosal rather than intraperitoneal administration of CLDC. As our observations suggest that CNS invasion following inhalational LACV challenge might occur via infection of the nasal epithelium/mucosa, it is not unreasonable to speculate that direct elicitation of antiviral immune responses in these tissues might drastically improve upon the protection induced by systemic administration. Mucosal CLDC immunotherapy generates nearly-complete protection against lethal inhalational murine challenge with *Burkholderia mallei*, *Burkholderia pseudomallei*, and *Francisella tularensis* (Goodyear et al., 2009; Troyer et al., 2009). It would be interesting to determine whether mucosal CLDC administration might block LACV infection of nasal turbinates and impede neuroinvasion following aerosol challenge. Additionally, it would be beneficial to determine whether CLDC-treated mice surviving LACV challenge were immune to subsequent inhalational challenge, as non-sterilizing CLDC-induced immune protection that allowed for development of subsequent anti-LACV adaptive immune responses would provide the additional benefit of "immunizing" survivors against future challenge, whether natural or artificial. Finally, experiments involving depletion of

plasmacytoid dendritic cells (pDCs) prior to CLDC treatment were inconclusive (data not shown nor discussed); it would be of interest to identify the potential role(s) of these important early producers of type I IFNs in CLDC-induced protection, as we identified a correlation between protection and increased peripheral type I IFN gene expression (Fig. 3.6, Chapter 3).

LACV is an intracellular pathogen that requires the production of Th1-biasing cytokines (e.g., IFNs, IL-12, and TNF-α) and cell-mediated immune responses for clearance. *Leishmania major* also resides intracellularly within vertebrate hosts, and requires nearly-identical pro-inflammatory and cell-mediated immune responses for control of infection. Having identified CLDCs as effective immunotherapeutics for elicitation of protective innate immune responses against aerosol challenge with LACV (see Chapter 3), we hypothesized that CLDCs might be effective adjuvants for eliciting similar Th1-biasing immune responses against a Th2-biasing immunomodulatory salivary peptide that impedes development of protective immune responses and exacerbates infection with *L. major*.

The protection against challenge with *L. major* + MAX elicited by immunization with CLDC + MAX was discussed in detail in Chapter 4. A particularly intriguing observation in these studies was that protective Th1-biased cellular immune responses were elicited in BALB/c mice, which are inherently biased toward development of Th2-type immune responses ineffective for control of *L. major* infection. In addition to their inability to down-modulate IL-4 production and respond to potentially-protective IL-12 signaling (discussed in Chapter 4), BALB/c mice do not respond with an early NK cell response following challenge with *L. major* (Scharton-Kersten and Scott, 1995), and thus

lack the production of early IFN-γ associated with effective control of initial infection. Furthermore, although MAX-immunization induced increased percentage ratios of IFN-γ- vs. IL-4-producing CD4+ cells in draining lymph nodes (see Chapter 4), continuous production of IL-4 and IL-13 by BALB/c mice following infection with *L. major* (Matthews et al., 2000) would abrogate the full protective potential of increased IFN-γ production. Therefore, the same ineffective programmed immune responses of BALB/c mice that make our protective results notable may have also exerted profound limitations on the extent of the protective efficacy of our vaccine formulation. Although MAX-immunization induced notable changes in CD4+ cell cytokine production as well as a significant delay in infection following challenge with *L. major* + MAX, immunized BALB/c mice still exhibited progressive disease development that would eventually lead to mortality due to disseminated infection. It would be beneficial to perform these immunization experiments using mouse strains resistant to progressive infection with *L. major* (e.g., C3H, C57BL/6, or CBA mice), as it is likely that the protection would be more complete, with more pronounced reductions in footpad lesion size and parasite burden due to immunization. The increased percentage ratio of IFN-γ- vs. IL-4-producing CD4+ cells identified in MAX-immunized BALB/c mice would be far-more protective if elicited in resistant mice, as production of IL-4 and IL-13 would be reduced (if not absent), and thus protection elicited by production of IFN-γ would not be abrogated. Additionally, the mechanism(s) of immune protection might be more-easily investigated using resistant mouse strains not inherently-programmed toward progressive disease development. It would also be beneficial to investigate the correlation between vaccine-induced protection and production of protective IL-12 and regulatory IL-10 and

TGF-β, as these cytokines are indicators of vaccine-induced resistant vs. susceptible immune responses to infection with *L. major* (Kedzierski et al., 2006). Finally, as the development of a Th1-biased immune response following infection with *L. major* has been partially-attributed to early production of IFN-γ by NK cells (Scharton-Kersten and Scott, 1995) and NK cells are the major cell type responsible for the production of IFN-γ following CLDC administration (Dow et al., 1999a; Dow et al., 1999b; U'Ren et al., 2006; Goodyear et al., 2009; Troyer et al., 2009), it would be of interest to systemically-deplete NK cells prior to immunization (as done in Chapter 3 prior to CLDC treatment) in order to investigate their role(s) in protection elicited by CLDC+MAX immunization. Further investigation into these matters is certainly warranted.

Critics of the salivary-immunization approach to prevention of vector-borne pathogen transmission have expressed concerns about the substantial variation of salivary molecules that are found in nature. The wide geographic distribution of *Lu. longipalpis* has led to genetic isolation of different sand fly populations, resulting in substantial sequence divergence in MAX encoded by sand flies of differing geographical locations. Genetic sequencing of MAX molecules from sibling sand fly species has revealed nucleotide variation as high as 12.8% and amino acid variation as high as 23% (Lanzaro et al., 1999), providing evidence of the evolutionary importance of evasion of host sensitization against sand fly salivary components (Milleron et al., 2004). Thus, successful immunization against a single natural variant of MAX might only elicit protection against *Leishmania* parasites transmitted by sand flies encoding that particular MAX variant. These hypotheses indicate that design of an anti-MAX vaccine intended to confer blanket immunity against the saliva of sand flies from various geographical

regions would require the inclusion of multiple immunogenic forms of MAX (Milleron et al., 2004; Valenzuela et al., 2001) or conserved peptide regions found in all natural variants. While this is likely true, these suspicions do not detract from the impressive protection induced by our vaccine formulation consisting of a single MAX variant, but rather highlight the need for continued research involving anti-salivary immunization and/or inclusion of salivary immunomodulators as components of subunit vaccines intended to protect against transmission of vector-borne pathogens.

Extensions of the work described in Chapter 4 have recently addressed the previously-discussed concerns of BALB/c susceptibility and MAX variation, as we have shown impressive protection against challenge with *L. major* + MAX in resistant C3H and C57BL/6 mice following immunization with a 15 amino-acid peptide (p11) spanning the C-terminal region of the full-length 63-mer MAX molecule. It is notable that the C-terminal domain is highly-conserved among the 4 known natural variants of MAX, and construction of deletion mutants has revealed that this domain is required for binding to the PACAP receptor (Moro et al., 1999). Immunization of mice with CLDC + p11 elicits the production of antibodies that bind to both p11 and full-length MAX, and we hypothesize that the improved protection induced by p11-immunization (in comparison to full-length MAX-immunization) is due to anti-p11 antibodies acting as MAX antagonists, inhibiting binding of MAX to the PACAP receptor. By effectively neutralizing the immunomodulatory effects of MAX, the p11 vaccine would not only elicit cellular immune responses protective against challenge with *L. major* co-injected with MAX (as described in Chapter 4 for full-length MAX), but might also interfere with the biological

activity of MAX, abrogating the Th2-bias that allows for the development of an early focus of *L. major* infection.

Although the induction of protection in the absence of detectable titers of anti-MAX IgG described in Chapter 4 is impressive, there would be substantial benefits to development of an anti-MAX or anti-MAX-peptide vaccine formulation capable of eliciting production of anti-MAX antibodies. Sand flies attempting to feed on immunized vertebrate hosts producing anti-MAX antibodies are less successful in obtaining a bloodmeal (Milleron et al., 2004). Bloodmeals are a required step in the maturation and development of sand fly eggs (Magnarelli et al., 1984), and there is a direct correlation between the size of bloodmeals taken by sand flies and the number of eggs that reach maturity (Ready, 1979). As a result, there is a strong relationship between sand fly feeding success and reproductive potential. Therefore, a MAX-vaccine (such as the p11 peptide vaccine) that induces anti-MAX antibody production would elicit protection against not only vector-transmitted *L. major,* but would also interfere with the reproductive success of sand flies, reducing the overall vector population, thereby effectively decreasing pathogen transmission. These effects could have far-reaching implications in the epidemiology of the leishmaniases in endemic areas, especially if immunization induced protection in natural vertebrate reservoirs, such as canines that serve as amplifying hosts for certain *Leishmania* species.

As we utilized the unnatural parasite/vector combination of *L. major / Lu. longipalpis* (as discussed in Chapter 4), it would be valuable to test the protective efficacy of our anti-MAX and anti-p11 vaccines against hamster challenge with *L. chagasi*-infected *Lu. longipalpis,* a parasite/vector combination found in nature. If we

were to elicit significant vaccine-induced protection in this natural transmission model, it would further legitimize anti-saliva immunization as a realistic approach to control of arthropod-borne disease and increase the likelihood of inclusion of salivary molecules as antigens in future vaccines intended to protect against transmission of arthropod-borne pathogens.

In these studies, we chose to utilize CLDCs as both immunotherapeutics and vaccine adjuvants to induce innate and Th1-type immune responses protective against infection with LACV and *L. major,* both of which are arthropod-borne intracellular pathogens that require the induction of pro-inflammatory Th1-biased immune responses for control and clearance. This body of work presents evidence that CLDC administration induces protective immune responses against a very formidable LACV aerosol challenge and a parenteral challenge with *L. major*, suggesting that CLDCs are versatile and effective vehicles for the elicitation of immune protection against pathogens susceptible to pro-inflammatory and Th1-biased immune responses, and are worthy of future exploration and further application.

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