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An ongoing pulmonary cowpox virus infection suppresses an immune response to OVA peptide delivered to the lungs

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AN ONGOING PULMONARY COWPOX VIRUS INFECTION SUPPRESSES AN IMMUNE RESPONSE TO OVA PEPTIDE DELIVERED TO THE LUNGS

BY

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B.S., Biology, Kent State University, Kent Ohio, 2005

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy of Biomedical Sciences

The University of New Mexico
Albuquerque, New Mexico

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Dedication

To my wonderful mother, Margaret Hrusch, who inspired me to explore the natural world from an early age and nurtured my love for science. From digging through a muddy creek to find shell fossils, to collecting bugs and butterflies, to letting me hatch quail eggs (in the house!), you encouraged me to learn everything I could about the world around me. Thank you and I love you, Mom.

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Abstract

Cowpox virus (CPXV), a close relative of variola virus, the orthopoxvirus that causes smallpox, can suppress the immune system through a large array of immunosuppressive gene products. We developed a murine model in which DO11.10 T cells specific for an OVA peptide were transferred into BALB/c mice to assess the impact of a pulmonary CPXV infection on DO11.10 T cell proliferation in lung draining lymph nodes following intranasal OVA peptide delivery. High and low-dose CPXV infections were compared. Both doses lead to clinical illness including ruffled coat and weight loss, but the high dose is lethal and is characterized by viral dissemination to the spleen. A high-dose infection reduced DO11.10 T cell proliferation, but a low-dose infection did not. At the time that proliferation of T cells was assessed (6d post infection), 15±1% of lung dendritic cells (DCs) were infected at the high-dose, but only 5±1% of DCs at the low-dose. At both doses, infected and uninfected lung DCs had decreased expression of MHC

class II and the co-stimulatory molecules CD80 and CD86. DCs and T cells were not infected in the lymph nodes at either dose, but lymph node DCs also showed a reduction in antigen-presenting molecules. We speculated that the lung microenvironment created by infection, rather than direct infection of the DCs, suppressed DC antigen-specific T cell activation. In support, we found that alveolar lavage fluid and supernatant derived from lung homogenates from infected mice suppressed the function of uninfected lung DCs *in vitro*. Furthermore, the suppressive activity was more highly concentrated in lungs from high-dose infected mice. Cytokine analysis revealed the presence of IL-10, an immunosuppressive cytokine, in lung supernatants in mice receiving a high-dose of CPXV. We used IL-10 knockout mice in our adoptive transfer model to examine a role for IL-10 in T cell suppression in the lymph nodes. However, the knockout mice behaved similarly to BALB/c, with lack of DO11.10 T cell proliferation in the high-dose, but not the low-dose, and concluded IL-10 does not prevent T cell proliferation at the high-dose. Finally, we examined a possible virally encoded immunomodulatory protein, a soluble IFNγ receptor (IFNγR), to determine if sequestration of host-produced IFNγ contributed to the immune suppression seen in the high-dose. A mutant virus lacking the IFNγR behaved similarly to wild-type virus, and the survival and day 6 lung titers were comparable. Other virally encoded factors may play a role in suppressing DO11.10 T cell proliferation and should be examined in future experiments. These studies strongly suggest that orthopoxvirus infections create an immunosuppressive microenvironment that compromises the host pulmonary immune responses.

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I. Lung Immunology

Lung as an immune barrier organ

The mammalian pulmonary system is a complex structure that not only carries out critical oxygen and carbon dioxide exchange, but also protects itself from pathogenic microorganisms and potential noxious agents. Thus, along with oxygen and other gases, each breath draws foreign proteins and microscopic organisms deep into the lungs. Understanding the pulmonary immune response to those insults is critical for preventing and treating human disease; pulmonary infections are the primary reason for sick leave in the United States [1] and a major cause of hospitalizations [2]. Human lungs handle 10,000 liters of air daily containing fungi, bacteria, virus particles, toxins, and allergens, and must be able to physically remove or destroy these foreign objects and organisms. Many non-pathogenic organisms are simply caught in the mucus and expelled via mucociliary clearance from the lungs to be swallowed or expectorated. Others are phagocytosed and killed intracellularly. Pathogenic organisms can evade host defense mechanisms and reproduce in the lung causing pneumonia. Preventing infection requires amazing synergy between the lung parenchyma and immune system leukocytes.

During inhalation in man, air is drawn down the trachea into the two mainstem bronchi, one entering each lung, which in turn branch into smaller bronchi. The bronchi then branch into cartilage-free bronchioles, respiratory bronchioles, alveolar ducts and finally end in small air sacs, or alveoli. The trachea, mainstem bronchi and branching bronchi contain cartilage to provide rigid structure and, as are bronchioles, are lined mainly with

ciliated columnar epithelial cells, with smaller numbers of mucus-secreting goblet cells and neuroendocrine-type cells. The ciliated cells expel the mucus secreted by goblet cells, along with small inhaled particles which stick to the mucus, upward and out of the lungs in a continuous process referred to as mucociliary clearance. Mucus also serves to moisten the air and provides a direct barrier between cells in the lung and foreign particles. The alveoli are lined by 95% type I and 5% type II pneumocytes, the latter producing surfactant which coats the alveolar surfaces and helps to maintain the open structure of the alveoli during expiration. Surfactant also aids in the innate defense of the lung surfaces. The branching system that results in the alveolar surface gives the lungs a huge surface area for gas exchange, and this surface area requires constant surveillance from the immune system to prevent pathogenic organisms from entering the body. The alveoli are particularly vulnerable, because there is only a thin layer of epithelium over a thin basal membrane between the air space and blood capillaries. Along with epithelial cells, immune cells are present in the bronchioles and alveoli to protect the host from exposure to airborne pathogens.

The lungs are well-equipped to deal with foreign invaders through a variety of host defense mechanisms. The first defense mechanism is the mucus itself, providing a protective barrier over the epithelium and containing anti-microbial proteins, such a defensins, surfactants, and complement. Beneath the surfactant layer in the alveoli, alveolar macrophages (AMs) survey the alveolar space, either freely motile or loosely attached to the epithelial cells, taking up foreign particles by phagocytosis and breaking down excess proteins and immune complexes in the lungs. The epithelium also takes in

foreign particles via endocytosis and pinocytosis. Immune cells that reside within the interstitium of the conducting airways, i.e., the bronchi and bronchioles, are interstitial macrophages, dendritic cells (DCs), mast cells, neutrophils, and some lymphocytes and monocytes. Although found in the interstitial space, pulmonary DCs have a very specialized function; they sample airways similar to alveolar macrophages, except instead of moving freely over the epithelium, the cell body remains in the interstitial space, with only the dendrites reaching up between the epithelium and into the airways. Lymph vessels are in close proximity to the bronchi and bronchioles, allowing activated DCs to leave the lung and drain to the lung-associated lymph nodes (LALNs) (Figure 1). Many DCs line the airways, while lung macrophages can be found along the airways in the interstitial space (interstitial macrophages) or in the alveolar space (AMs). AMs found within upper bronchioles and bronchi are generally at the end of their life cycle and are being swept up with the mucus out of the lungs. In the alveoli, blood capillaries surrounded by endothelial cells sit directly below the pneumocytes, facilitating exchange of cells, proteins, and gases between the bloodstream and lung.

The organization of the alveoli allows gas exchange between the airways and red blood cells in the capillary bed during respiration. The epithelium also transports molecules via transcytosis from the blood into the alveolar space, including important immune mediators such as chemokines and antibodies. Leukocytes also use this capillary bed to gain access to the lung tissue, and their recruitment increases during inflammation.

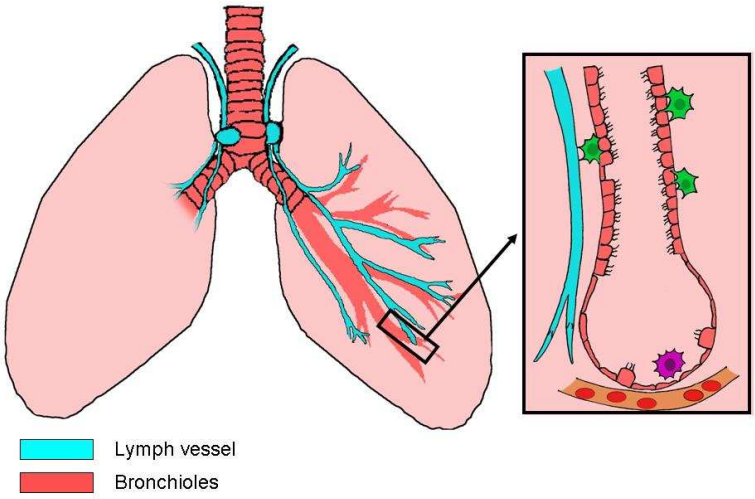


Figure 1. Basic anatomy of lungs showing the close proximity of bronchioles to lymph vessels and the major draining lymph nodes. The lymph vessels are proximal to the bronchioles which allow DCs in the epithelium to quickly enter the lymph vessels through intravasation. DCs then travel up the vessels to the lung-associated lymph nodes (LALNs). Multiple nodes are present in humans, but 2 are shown for clarity. Inset: A bronchiole, lined mainly with ciliated epithelial cells, contains many DCs (green cells) within the bronchoepithelium. These DC have dendrites which extend into the airways to sample particles entering the lung. The bronchiole ends at an alveolus lined mainly with type I pneumocytes. Phagocytic AMs (purple cell) reside in the alveolus under the mucus layer (not shown). Below the alveolus is a capillary bed where red blood cells become oxygenated. Lymph vessels terminate at the termination of the bronchioles, but fluid from the alveolar interstitual tissue can gain access to the lymphatics by being driven by pressure gradients in expiration. Monocytes and other immune cells circulating in the blood stream gain access to the lung tissue and alveolus through the capillary beds.

The lung is a very capable immune barrier, and many mechanisms exist to overcome pathogens and minimize lung damage. First, innate immune cells recognize and directly control a subset of the pathogens. Second, the innate cells activate the acquired immune response. Third, the acquired immune response targets the specific pathogen to kill the pathogen and/or infected cells. Lastly, immune memory prevents a second infection by the same organism through the life of the host. In this introduction we will examine the pulmonary immune response in a mammalian host against a model virus, the orthopoxvirus cowpox virus (CPXV). I will begin with a more general introduction on lung immunity before examining orthopoxvirus-specific immunity, with special attention to host-viral interactions and the ability of the host to respond appropriately, and survive infection, versus a poor immune response, leading to dissemination of virus and death.

Pulmonary innate immune responses to pathogens

The lungs provide a huge opportunity for pathogens to enter the human body, and many pathogens have specifically evolved to spread via aerosols, for example the virus influenza [3] and bacteria *Haemophilis influenzae* [4]. Lung pathogens can become particularly dangerous to the host if they disseminate from the lung to the blood and thereby to other organs. With only a thin barrier between the alveolar space and capillary vessels, any infectious particle must be quickly detected and destroyed after inhalation. The innate immune response to lung pathogens is carried out by two main cell types, those of hematopoetic origin and the non-hematopoetic resident lung epithelial cells, both of which create an immediate, non-specific, immune response to pathogens.

Innate immune cells of hematopoietic origin include macrophages (MΦs), dendritic cells (DCs), natural killer (NK) cells, mast cells, eosinophils, and neutrophils. The precursor hematopoietic cells reside in bone marrow, where a pluripotent stem cell can differentiate into either a common lymphoid progenitor or a common myeloid progenitor. The lymphoid progenitors differentiate into NK cells, the adaptive immune T and B cells, and a small population of specialized plasmacytoid DCs. Myeloid progenitor cells differentiate into monocytes or granulocytes, including basophils, eosinophils, and neutrophils. Mast cells also develop originally from bone marrow-derived cells but populate various organs and mucosal surfaces and replicate in situ. Monocytes travel through the blood where they can differentiate into immature dendritic cells (iDC), while other monocytes enter the lung interstitium where they take up residence and may differentiate into iDC or MΦs, depending on local cytokine feedback.

The primary innate immune cells in the alveoli, and probably the first to encounter virus entering the lungs are alveolar macrophages (AMs). AMs are important homeostatic regulators in the lung, creating a balance between immune activation, immune suppression, and tissue remodeling. As discussed above, these cells are motile within the alveolar sacs, freely moving over the epithelium, but underneath the surfactant layer, to phagocytose foreign particles and release cytokines in response to danger signals. DCs, found along the trachea, bronchi and bronchioles, are another first responder, sampling the airways with long-reaching dendrites while the cell body remains protected under the epithelium. Both of these phagocytic cells are ready to activate and begin cytokine

production in response to danger signals. These danger signals can be lipopolysaccharide (LPS) from bacteria, bacterial flagellin, bacterial CpG DNA, viral double-stranded ribonucleic acid (dsRNA), or many other conserved pathogen structures called pathogen-associated molecular patterns (PAMPs). PAMPs bind to pattern-recognition receptors (PRRs), including the toll-like receptors (TLRs) and nucleotide-binding oligomerization domain proteins (NODs). The most studied PRRs in the lung are the TLRs. Each TLR binds to a certain PAMP (Table 1), signaling through the adapter proteins MyD88 and/or TRIF, and inducing the transcription factor NFκB as well as MAP kinase signaling [5]. When NFκB activation of DCs occurs, it leads to cell activation including increased phagocytosis, increased antigen presentation, and pro-inflammatory cytokine production. DCs are required to activate naïve T cells in the lung draining lymph nodes to begin the adaptive immune response, but will be described in more detail later.

Neutrophils are an abundant leukocyte in the blood and the first cells to enter tissues after inflammatory cytokines are released from the injured area. These granular phagocytes enhance the pulmonary immune response by secreting pro-inflammatory cytokines in the lung tissue, releasing granules containing anti-microbial proteins, phagocytosing pathogens, and releasing neutrophil extracellular traps (NETs) made of DNA to catch and sequester potential pathogens [6]. NK cells are granular lymphocytes with potent cytotoxic activity that are upregulated by macrophage-produced cytokines. Virally infected cells generally display lower levels of MHC class I molecules than is typical for

Table 1. Known TLR receptors including where they are found in lung, ligand for each TLR, and the pathogen type that each recognizes [a].

TLR Receptor	Lung expression	Ligand	Pathogen type
TLR1	MΦ, Neutrophil (dimerizes with TLR2)	Triacyl lipoproteins	Bacteria
TLR2	Epithelium, DC, MΦ, T, B, Neutrophil	Peptidoglycans, Lipoteichoic acids	Fungi, Some bacteria/viruses
TLR3	Epithelium, DC	Poly I:C, dsRNA	Viruses
TLR4	Epithelium, Myeloid DC, MΦ	LPS, Glycoproteins	Bacteria
TLR5	Epithelium, Myeloid DC, MΦ	Flagellin	Bacteria
TLR6	MΦ, B, Mast (dimerizes with TLR2)	Diacyl lipoproteins	Mycoplasma
TLR7	Leukocytes	ssRNA in endosomes	Viruses
TLR8	Epithelium, Leukocytes	G-rich oligonucleotides	Viruses, Tuberculosis
TLR9	DC, B, NK	Unmethylated CpG DNA	Bacteria, Viruses
TLR10	Unknown	Unknown	Unknown
TLR11	Epithelium, DC, MΦ	Profilin	Bacteria (Toxoplasma gondii)
TLR12	Unknown	Unknown	Unknown
TLR13	Unknown	Unknown	Unknown

[a] Lung expression refers to cells that have measurable protein for each TLR (as opposed to mRNA), ligand refers to general ligands found for murine and/or human TLRs. For example, TLR9 has CpG DNA as a ligand; however, the specific CpG DNA sequences required for binding are different for different species. Pathogen type refers to the general class of organisms where each ligand is found. Limited information is available on TLR10, TLR12, and TLR13. Adapted from [7-9].

healthy cells, a change which NK cells can detect through CD94 and Ly49. Eosinophils and basophils recruited from the blood and tissue mast cells are generally considered to be involved in allergies and asthma along with parasite immunity, but also can respond more generally to pathogens entering the lungs. Eosinophils express TLR7 and are protective against respiratory syncytial virus [RSV and pneumonia virus of mice (PVM)] in a mouse model [10]. Mast cells also respond to bacteria and viruses through TLRs and respond by releasing pro-inflammatory cytokines TNFα and IL-1β [11].

Pulmonary epithelial cells should not be overlooked as important innate responders during infection. While many of the cytokines produced by AM and DC directly impact the epithelium, producing an anti-viral state in those cells, TLRs are expressed on epithelium, with ligand binding causing pro-inflammatory cytokine production (Table 2), increased transcytosis, and vascular leakage [9]. The mRNA for TLR1-11 have been found in lung epithelium, and protein for TLR2, TLR3, TLR4, TLR5, and TRL8 [8]. The high expression of TLRs suggests an important [12] role for epithelium in regulating lung response to common pathogens. For example, *Pseudomonas*, a common bacterial pulmonary pathogen, binds to TLR2 and TLR5 on lung epithelium, causing TLR upregulation and IL-8 production to promote neutrophil influx [13].

Another immune benefit provided by lung epithelium is the mucus secreted by goblet cells. Mucus contains anti-microbial proteins secreted from lung epithelium, like surfactant proteins A-D (made by type II pneumocytes), which have a primary function of lowing surface tensions in the lungs, but also bind to carbohydrate residues on

Table 2. Pro-inflammatory cytokines and chemokines produced by cells in response to TLR stimuli[a].

TLR Simulus	Cyokine	Produced By	Acts On	Function	Reference
4, 5	IL-1β	Mast, MΦ, Epithelium	MΦ, T	Activation	[11, 14]
4, 5	IL-5	Mast	Eosinophil	Granule release, IgE class switching	[15]
2, 4, 5, 9	IL-6	DC, B, Mast	T, B	Growth and differentiation	[16, 17]
4	IL-8	Epithelium	Neutrophil	Chemoattractant	[18]
2, 3, 4, 5, 9	IL-12	DC	NK, T	Activation, Th1 Differentiation	[19, 20]
2, 4	IL-18	DC, MΦ	NK, T	Induces IFNγ, Th1 response	[21, 22]
7, 9	IFNα	pDC	Many cells	MHC class I upregulation, anti-viral state	[20]
9	IFNγ	NK	Many cells	MHC upregulation, MΦ activation, Th1 response	[23]
4	TNFα	DC, Mast	Endothelium	Activation, increased vascular permeability	[5, 11]
4, 5	MIP-1α	DC, Mast, MΦ	Mono, MΦ, DC, T, NK	Chemoattractant	[24, 25]

[a]A selection of potential cytokines produced in response to TLR stimuli by immune cells. The cytokines shown are produced by the indicated cells in response to TLR stimulus alone in murine cells. Since many TLRs signal through the adaptor protein MyD88, many TLRs have a similar final outcome. For example, IL-12 is produced by DC in response to many different TLR stimuli.

pathogens, facilitating phagocytosis by immune cells. Proteins like complement and defensins secreted by leukocytes are retained in the mucus and contribute to host protection. Complement refers to over 25 small proteins produced primarily by the liver to recognize danger signals and ultimately respond by forming a membrane attack complex, which causes lysis of the target cell [26]. Complement also has other functions, including opsonization of pathogens and binding antibody-antigen complexes to facilitate clearance by phagocytes. Defensins are released by immune cells and create small pores in microbial cell membranes, leading to efflux of nutrients and eventually death of the cell (reviewed by [27, 28]).

Other epithelial responses require input from nearby immune cells, particularly AMs. Type II cells produce monocyte chemoattractant protein-1 (MCP-1) in response to macrophage-derived TNFα and IL-1 to recruit monocytes into the lung [29], and basal epithelial cells produce IL-8 in response to IL-1 alone [18]. Activated epithelium cells exhibit increased trancytosis, taking up cytokines and chemokines from the basal membrane and transporting them to the apical membrane [9]. Non-specific immunoglobulin receptor (IgR) is also upregulated on the basal membrane of epithelial cells to bring secretory IgA secreted by plasma cells in the interstitial space into the conducting airways or IgG from the blood to the alveolar space [30, 31]. There, antibodies can bind pathogens leading to opsonization by phagocytes, or inhibiting their binding to and infecting nearby cells. The pulmonary epithelium combined with the innate immune cells provides the first line of defense against pathogenic organisms

entering the lungs. If an invading pathogen is detected by these cells, a pro-inflammatory response begins, and the host's adaptive immune system is activated.

Pulmonary DCs bridge innate and adaptive immunity

DCs have a dual function in the immune system. Through TLRs and other PRRs they have innate responses to PAMPs, and through MHC class I and II, they present specific antigen to T cells leading to the adaptive, pathogen-specific immune response. The two processes are inherently linked; iDCs activated by a ligand binding a TLR upregulate antigen presentation by increasing MHC molecules on their surface along with co-stimulatory molecules CD80 and CD86, becoming activated DCs [32]. The process does not take much time as DC pick up antigen and may reach the lymph nodes by 30 minutes post-inhalation exposure [33]. Antigen plus MHC class I on the surface binds to the T cell receptor (TCR) of CD8-expressing cytotoxic T cells and activates them, while antigen plus MHC class II binds to the TCR of CD4-expressing helper T cells (Figure 2). Activated DCs also express high levels of CD1d, a lipid antigen presentation molecule that specifically binds to NK T cells. Activated DCs display potent anti-viral activity by secreting type I interferons (IFNs) and other pro-inflammatory cytokines when activated. Type I IFNs include IFNα and IFNβ and are produced mainly by plasmacytoid DCs (pDCs). IFNα causes monocytes to differentiate into DCs, enhances T cell survival, and activates NK cells. Pro-inflammatory cytokines produced by DCs include IL-1β [34], IL-6 [16], TNFα [16], IL-12 [35], and IL-23 [36]. In response to IL-12, NK cells [19] and T cells [37] make IFNγ, a critical protein for viral immunity. IFNγ is a type II interferon

that alters transcription of 30 genes, which leads to increased phagocytosis, lysosome, and iNOS activity in AMs, upregulation of class I MHC on all cells, activation of NK cells, and priming of naïve T cells for a Th1 response (reviewed by [38], [39]). Activated DCs go on to directly bind and activate cytotoxic CD8 T cells to become cytotoxic T cells and helper CD4 T cells to help B cells become activated and secrete antibody. A single DC may activate many lymphocytes due to the large surface area provided by dendrites on the activated DC and the relatively small size of naïve T cells [40]. However, this activation step does not typically occur in the lung tissue as naïve lymphocytes are generally not found in peripheral tissue. When DCs in the lung are activated, they do not remain static but migrate to a lung draining lymph node [or lung-associated lymph node (LALN)]. The lymph node houses both CD4 and CD8 naïve T cells, ready to interact with incoming DCs carrying antigen from the lung. Naïve T cells continuously cycle between the blood, lymph nodes, spleen, and other secondary lymphoid tissues so that T cells of many specificities are available to detect a matching antigen at any site in the host. The DC carrying antigen loses the chemokine receptor CCR5 that favors retention in the pheriphery, allowing the DC to enter the lymphatics and finally the lymph node through the afferent vessels. Upregulation of CCR7 occurs simultaneously with downregulation of CCR5, so not only can the DC leave the lung but it is retained in the LALN by the CCR7 interacting with ligands CCL21 and CCL19 produced by lymph node endothelium [41]. At this point the DC can bind naïve T cells to activate them while secreting pro-inflammatory cytokines to drive their differentiation. These cyokines are linked to the original TLR activated as well as signals received in the lung tissue from other immune cells or epithelium. These highly activated DCs remain in

the LALN for approximately 72 hours, activating cells and secreting cytokines before finally undergoing apoptosis [42]. This apoptotic event is thought to help regulate the immune response by not allowing over-activation. New DCs continuously cycle from the lung carrying more antigen if there is a residual bolus of antigen, as for example if an infection has not been controlled [43].

Adaptive immune response to pulmonary infections

The adaptive immune response involves cells that are pathogen-specific and able to clonally expand when activated by their target antigen and include T and B lymphocytes. Naïve T cells circulate through the lymphatics and blood until activated DCs enter a LALN bearing antigen-MHC complexes, co-stimulatory molecules CD40, CD80, and CD86, and secreting pro-inflammatory cytokines that help retain T cells in that LALN [44]. DCs acquire viral antigens in multiple ways. First, they are directly infected and present viral antigens on MHC class I molecules. Secondly, DC phagocytose infected cells, or virus particles directly, and load viral proteins in endosomes onto class II MHC molecules. A third method is cross-presentation, whereby phagocytosed material somehow enters the cytosol and is processed similar to other endogenous foreign proteins such as viral or tumor antigens and subsequently loaded onto class I MHC molecules [45]. This way even if a virus does not infect DC directly, the DC can present antigen to CD8 T cells to illicit a cytotoxic T cell response and directly infected cells can be killed.

Loose attachment of DCs and T cells occurs through co-receptors, especially ICAM-1 on DCs binding to LFA-1 on T cells, until a TCR binds MHC displaying the cognate antigen. An immunologic synapse then forms (Figure 2), creating a tight seal between DC and T cell [46]. Many MHC-TCR molecules congregate at the center of the synapse, with co-receptors forming an outside ring. The co-receptors CD80 and CD86 (also called B7.1 and B7.2, respectively) are two closely related glycoproteins [47] expressed on DCs, both of which bind CD28 on T cells. This interaction is required for a T cell to become activated and clonally expand in response to antigen. The T cell can then express the receptor CD40L, which binds CD40 on the DC leading to greater CD80 and CD86 expression on the DC surface. Activated T cells secrete IL-2, a T cell proliferation factor and survival factor. The activated T cell clonally expands and differentiates, creating an expanded clone of cells that are specific for the presented viral antigen. Activated T cells, now called effector T cells, leave the LALN though the efferent lymphatic vessel, enter the thoracic duct to the blood stream and reach the lung capillaries, where in response to chemotactic signals generated by the infected tissues and innate responders, they migrate into the tissue [48].

Effector T cells no longer require the co-stimulatory molecules CD80 and CD86 to exert their effector function. Effector CD8 T cells leave the lymph node and enter the infected tissue, able to kill an infected cell with only a MHC class I:antigen complex [44]. Furthermore, effector CD4 T cells are restimulated by B cells and macrophages in the peripheral tissues displaying MHC class II plus antigen, and respond by secreting pro-inflammatory cytokines. The major role for cytotoxic CD8 T cells during a pulmonary

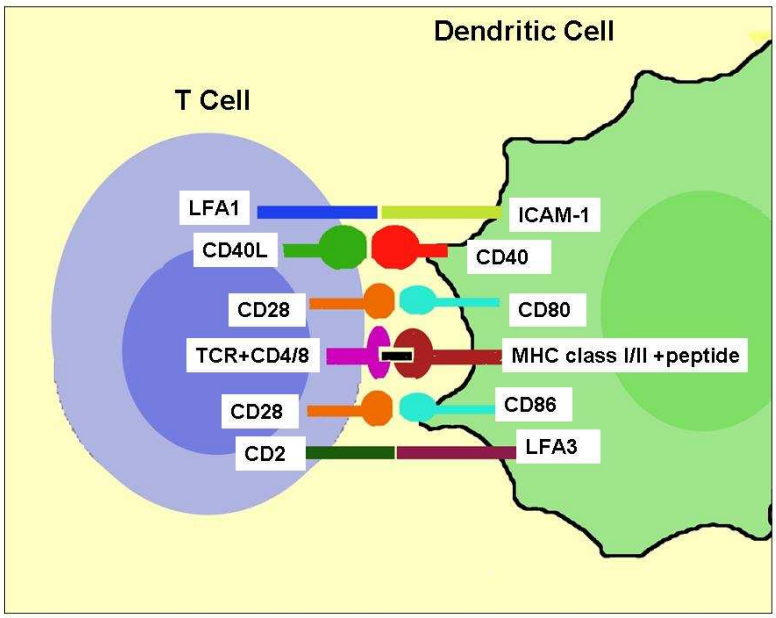


Figure 2. Basic molecules involved in DC-T cell interactions and forming the immunological synapse. In the LALN, weak interactions between LFA-1 and CD2 on T cells and ICAM-1 and LFA3, respectively, on DCs allows the TCR to sample peptide on the DC MHC molecule. When the TCR samples an MHC-carrying cognate peptide, a tight synapse forms, with co-stimulatory molecules CD80 and CD86 on the DC binding CD28 on the T cell, and CD40 on the DC binding CD40L on the T cell. These molecules group together on the cell membranes, forming a bull's-eye pattern with MHC/TCR in the middle, co-stimulatory molecules surrounding the MHC/TCR, and adhesion molecules forming the outer ring. Synapse formation leads to activation and clonal expansion of the T cell, and cytokine release from the DC.

infection is to kill any cells that are directly infected by virus, while the major role for effector CD4 T cells in that setting is to secrete cytokines, particularly IFNγ, to further activate immune cells in the lymph node and at the site of infection. A subset of activated T cells differentiate into memory T cells, which are long lived antigen-specific T cells that can reactivate quickly if cognate antigen is detected in the host .

Immature B cells reside in the lymph node, expressing membrane-bound immunoglobulins (the B cell receptor or BCR) IgD and IgM. Each immature B cell has specific antigen specificity, and activation begins with nominal antigen binding to the BCR. When the appropriate antigen is bound, the BCR is internalized and the antigen processed and presented on MHC class II. To complete activation, B cell MHC class II:antigen interacts with a TCR on an effector CD4 T cell and, similar to the T cell-DC interaction, B cell CD40L engages T cell CD40. This interaction causes B cell differentiation and clonal expansion [49]. Differentiated plasma B cells produce secreted forms of immunoglobulins, including IgG which enters the bloodstream via the efferent lymphatics and particularly when the lung is inflamed, the antibody enters the lung. The variable region of IgG can bind viral particles, preventing virus from binding host cells, while the invariable region binds Fc receptors on DCs and macrophages leading to phagocytosis. Memory B cells are long-lived B cells that continuously produce small amounts of antibody, protecting the host from future infection with the same virus. Long lived memory B cells specific for pulmonary antigen have been identified in lung tissue [50]. The host then has a steady supply of antigen-specific antibodies in the lung, and in

conjunction with memory T cells, the host is protected against subsequent infections with the same pathogen.

Pulmonary immune response to viruses

Viruses are the cause of many pulmonary infections and exploit the high number of cells making up the lung epithelium, which can become infected, allowing the virus to quickly replicate and spread. Viruses present a challenge to the immune system because they bind and enter host cells, therefore "hiding" from phagocytes. Luckily, the immune system has evolved special mechanisms to deal with intracellular pathogens, starting with specialized PRRs that recognize viral PAMPs. TLRs that recognize extracellular pathogens tend to be on the cell membrane, such as TLR4 that recognizes LPS, while TLRs that recognize intracellular pathogens like viruses are expressed in endosomal compartments of cells. DC, in particular, actively phagocytose viral particles and can become infected themselves, so that expression of endosomal TLRs is essential for host protection. For example TLR3, TLR7 and TLR9 recognize dsRNA, ssRNA and CpG DNA, respectively, common nucleic acids found in viral genomes. Vaccinia virus (VACV) DNA is detected by TLR8 in murine pDCs, which can recognize non-host poly A/T regions in DNA [51], while an unknown VACV component can activate TLR2 on murine NK cells [52]. It is unknown whether human TLRs would also recognize these viral components, as differences are evident between species. For example, human and murine TLR9s respond to different CpG DNA sequences [53], and murine TLR8 requires poly T oligodeoxynucleotides whereas human TLR8 does not [54].

Cytotoxic T cells are another essential immune cell to defend the host from viruses. These cells can bind MHC class I loaded with viral antigen to directly kill cells containing virus. Unlike MHC class II which is primarily found on professional antigen presenting cells (APCs), MHC class I is found on all nucleated cells. MHC class I, CD80 and CD86, are upregulated on human bronchoepithelial cells following exposure to rhinovirus [55]. After the lung epithelium takes in the virus, the virus begins to take over host machinery for transcription and/or translation to produce more viral proteins and eventually progeny virus. Some of these viral proteins enter proteasomes in the cytoplasm and are degraded into small peptides. These peptides enter the endoplasmic reticulum through transporters associated with antigen processing-1 and -2 (TAP1 and TAP2) proteins. TAP1 and TAP2 form a heterodimer and are associated with incompletely folded MHC class I proteins in the endoplasmic reticulum. Once peptide binds MHC class I, the complex folds in the proper conformation which allows it to dissociate from TAP1/2 and leave the endoplasmic reticulum. Finally, the MHC class I bearing antigen is exported to the cell membrane where it is visible to CD8 T cells [56]. When a CD8 T cell encounters its cognate antigen, a tight synapse forms between the T cell and the infected cell bearing MHC class I. Lytic granules contained in the T cell are released into the infected cell, leading to lysis of the infected cell thereby destroying a potential viral factory. Because this pathway is so important for viral control and clearance, many viruses circumvent it through virally encoded proteins. Viruses that are genetically unrelated, including human cytomegalovirus [57], adenovirus [58], and CPXV [59], all inhibit MHC class I molecules to avoid killing of infected cells. The host

has an important mechanism to counteract these viral immune evasion proteins. Because all nucleated cells constitutively express low amounts of MHC class I, a cell with none is considered abnormal by the immune system. Specifically, NK cells target cells without any MHC class I on the surface and kill them in a similar manner to cytotoxic T cells. In this case, NK killing receptors are inhibited by recognition of MHC class I molecules. In the absence of these molecules, natural cytotoxicity receptors (NCRs) bind the infected cell to induce target cell lysis [60].

Pulmonary DCs become directly infected by many viruses and display viral antigens on MHC class I molecules, but also phagocytose free virus particles and present the antigen on MHC class II molecules. The virus enters the cell into an early endosome of neutral pH, but as the endocytic vesicle migrates deeper into the cytoplasm, it becomes acidic and fuses with lysosomes. The vesicle contains proteases that are activated at the low pH and the virus is degraded. The vesicle, now containing viral peptides, fuses with endosomes released from the endoplasmic reticulum containing MHC class II molecules. The MHC class II molecules, unlike MHC class I, has a small protein, called class II-associated invariant chain peptide, or CLIP [61], bound to the peptide-binding grove while the protein is still in the endoplasmic reticulum. CLIP allows the MHC class II molecule to fold into a proper confirmation for endocytic loading. Viral peptides now replace CLIP in the peptide-binding groove, and the endosome moves to the plasma membrane to display the antigen-loaded MHC class II on the cell surface. DCs then present the viral antigen to activate CD4 T cells.

Some viruses, for example papillomavirus [62], do not directly infect DCs, but DCs can still present the viral antigens to CD8 T cells via a process known as cross-presentation. Even if DCs can be directly infected by a virus to display antigen on MHC class I, some DCs in the lung will remain uninfected, so having an alternate pathway to display antigen is essential for the cytotoxic immune response. For herpes simplex virus [63] and VACV [64], both direct presentation and cross-presentation are used by DCs to activate CD8 T cells. In cross-presentation, the DC phagocytoses infected apoptotic cells and debris and presents degraded viral protein on MHC class I. Although the antigen enters the cell in endosomes, some of the viral proteins are able to enter the cytoplasm and be degraded into peptides by proteasomes, transported into the endoplasmic reticulum where they are then loaded onto MHC class I molecules. Additionally, many apoptotic cells are full of active proteases which may degrade the viral proteins before the DC has even phagocytosed the debris, and viral peptide is able to escape the endosomes, or fuse with MHC class I-containing endosomes cycling from the plasma membrane [64]. Resident lymph node DCs, which are defined by surface expression of CD8α in mice, are more likely to cross-present antigens than other DCs, probably due to less free virus reaching the nodes and more draining cellular debris [45].

Viral antigen-presenting DCs activate T cells in the LALN and polarize the immune response to an anti-viral type response. This polarization, characterized by high IFNγ-, low IL-4-producing T cells, is referred to as a T_H1 response. Other types of polarization include the T_H2 response, generally associated with allergy and parasitic infection, and the Treg response, associated with suppression of the immune system by regulatory T

cells and often characterized by IL-10 secretion. Besides IFNγ, T_H1 T cells produce IL-2, a T cell proliferative factor, tumor necrosis factor-α (TNFα), which induces inflammation and supports immune cell survival, and granulocyte-macrophage colony stimulating factor (GM-CSF), causing B cell and monocyte differentiation. When CD4 T cells activate B cells in the LALN, forming a germinal center of clonally expanding, antigen-specific T and B cells, the T_H1 cytokines influence B cell class-switching to produce antibodies like high-affinity opsonizing IgG [65] and neutralizing IgA [66]. These antibodies are released from germinal center B cells in the LALN, enter efferent lymphatics and reach the lungs via the blood stream. Some IgA secreting plasma cells may reach the mucosa of the conducting airways and the IgA secreted there dimerizes and can enter the airway lumen utilizing IgR on lung epithelium to transcytose to the apical surface and bind virus particles in the bronchiolar and bronchial lumen. Serum IgG remains a monomer, and diffuses from the blood into tissues and across the epithelium into airspaces, particularly in the alveolar regions of the lung. Both antibody types inhibit infection; IgG binds virus and facilitates phagocytosis by APCs, while IgA prevents cell entry by blocking receptor sites on the virus. T_H1 cytokines act locally in the LALNs and globally by reaching the lungs and influencing the immune response there. These cytokines specifically encourage monocytes to differentiate into DCs, and AMs to produce anti-microbial reactive oxygen species and proteases. Overall the T_H1 response produces cytokines, chemokines, and antibodies effective against intracellular pathogens.

II. Pulmonary CPXV infection

Orthopoxviruses and disease

Orthopoxviruses are large double-stranded DNA viruses in the *Poxviridae* family that infect a number of mammalian hosts, including humans. *Variola major*, the causative agent of smallpox, is the most notorious family member, accounting for the deaths of millions of people before an eradication effort led by the World Health Organization was declared successful in 1980 [67]. Smallpox is not, however, the only poxvirus that can infect humans, and in recent years human infections with CPXV and monkeypox (MPV) have been increasing, presumably due to the end of the smallpox vaccination campaign and low herd immunity among human populations [68]. Because smallpox was eradicated before much technical advancement in medicine and immunology, little is known how smallpox affected the immune system and why it was so deadly. Although smallpox is known for creating large pustules on the skin, it is a respiratory pathogen and was spread by aerosols, leading to a debilitating pneumonia, viremia, and inflammation in the renal tubules [69]. Indeed the skin eruptions generally followed the extrapulmonary spread and entry into the bloodstream. MPV also leads to generalized pustules over the body, fever and malaise, but is not as lethal as smallpox. Most cases of MPV are restricted to the forested regions in western and central Africa, where the virus is endemic and infects at least primates and rodents, making control difficult [70]. A small MPV outbreak in the US was linked to pet prairie dogs who were previously housed with other exotic pets, including primates from Africa [71]. Most human CPXV infections are

caused by accidental intradermal inoculation, generally rodent or cat contact, and the infection is restricted to one or more pustules near the site of inoculation along with regional lymph swelling [72]. Importantly, fatal cases have been recorded in the past, all involving immunocompromised individuals, and concurrent eczema can exacerbate the disease [73]. In recent years, one CPXV outbreak was linked to pet rats in France [72], one case in Germany was linked to wild rats [74], and multiple cases in Eastern Europe were linked to cats having close contact with humans after being outside and presumably hunting CPXV-infected rodents [75-77]. Importantly, in a number of these cases the attending physician did not test for CPXV or any other poxvirus, and patients were given multiple antibiotics with no improvement of symptoms. CPXV is only emerging as a health concern now that a generation has grown up without the smallpox vaccine, and most cases have involved adults and children who never had the vaccine. Even the classic orthopoxvirus vaccine strain, VACV, has inadvertently infected a child whose military parent had recently been vaccinated. Luckily the child's sickness was identified readily and treatment with the recently developed anti-poxvirus compound ST-264 (now licensed as Tecovirimat) was able to reverse the infection and save the child's life [78]. Even with the eradication of smallpox, orthopoxvirus infections continue and little is known how they interact with the host immune system.

Research shows that orthopoxviruses encode many proteins that can bind to host immune proteins to inactivate them, or homologues of host proteins that alter the immune response to enhance infection rather than clear it. Some viral proteins enhance host cell survival by inhibiting apoptosis and encouraging epithelial growth to provide even more

opportunity for viral reproduction, while other proteins inhibit chemokine messages so that immune cells never reach the area of viral replication. These studies suggest that secondary infection could have played a role in the high mortality of human smallpox. The research detailed in this thesis finds a role for immune suppression by CPXV in a murine model, where infected mice are unable to respond to a second, non-viral, antigen. Besides explaining a potential virulence mechanism in smallpox, this new knowledge is important in considering treatments for current human infections of monkeypox and CPXV. That is, it helps point out the possibility of slowed immune responses to the primary infection as well as the need to be on the alert for secondary infections.

CPXV as a model orthopoxvirus

These studies utilize CPXV as a model organism to study virus-host interactions in a murine pulmonary infection. CPXV is an ideal orthopoxvirus to study in this context. It is naturally endemic in Eastern Europe, contains a full set of immunomodulatory proteins, and its natural reservoir is the rodent. Therefore a murine model is particularly relevant [76, 79, 80]. Like most orthopoxviruses, CPXV can infect other mammals as well, including humans [73], cats [76, 80], dogs [81], rats [72], and in one documented case, an elephant [74]. In humans, the disease presents with a wide range of symptoms based on age and underlying immune disorders. For example, a healthy 14-year old girl developed a single lesion at the site of infection with lymphangitis in the draining lymph node, while a 4-year old girl with atopic dermatitis developed severe generalized lesions and fever [82]. Viremia has been recorded in patients, but only in whole blood and not

serum, suggesting the virus is cell-associated [83]. The smallpox vaccine is protective for CPXV, so most of these cases were in people born after 1977, when vaccination was ended in most of Europe [68]. Because the virus currently infects humans, it is an important zoonosis to examine and understand.

Vaccinia virus is the most studied orthopoxvirus, but is lacking key immunomodulating proteins encoded by CPXV, and VACV is not naturally occurring but is a vaccine strain passed many times through humans and cell lines. For example, VACV does not encode the potent anti-apoptotic protein cytokine response modifier A (CrmA), although it does encode the less potent serine protease inhibitor-2 (SPI-2) [84]. CPXV (Brighton Red Strain) encodes four different TNF receptor homologs (CrmB, CrmC, CrmD, and CrmE), while most VACV strains encode CrmB, but truncated versions of the other Crm proteins or none at all [85]. This suggests VACV may not behave like a naturally-occurring orthopoxvirus infection. On the other extreme, ectromelia, or mousepox virus, is extremely virulent in mice and difficult to control, even in a laboratory environment. Numerous unintended outbreaks have occurred in mouse colonies even when using stringent methods to prevent contamination [86, 87]. Cowpox is virulent enough to be lethal in mice, but is not known to spontaneously spread through carefully maintained mouse colonies.

CPXV infects epithelium permissively and leukocytes non-permissively

The CPXV virion, like other orthopoxvirus in its genus, is brick-shaped with a distinctive envelope containing conspicuous surface tubules [88]. Beneath the envelope lies an outer membrane, and beneath this membrane lateral bodies encase the inner membrane and nucleosome. Lateral bodies contain densely packed viral proteins, like early viral transcription factors, that are released into infected cells. Like other orthopoxviruses, CPXV readily enters most nucleated mammalian cells, and does not seem to have a specific receptor required for entry. VACV has been found to bind glycosaminoglycans (GAGs) [89], found on most cell membranes. Macropinocytosis is triggered when the virus binds to GAGs [90]. Upon entry into the host cell, the membrane fuses with an endosomal membrane and the uncoated virus is released into the cytoplasm. Proteins, including host modulators and early viral transcription factors, contained in the virion transform the host cell into a viral factory. Replication occurs in the cytoplasm and is divided into three distinct temporal phases. First, early transcripts are produced, which lead to production of immunomodulatory proteins, newly made transcription factors, and DNA polymerase. Second, intermediate transcripts are produced, which result in translation of structural proteins and assembly proteins. Lastly, late transcripts lead to the production of more proteins involved in virion packaging and release as well as proteins that will be packaged into the virion required to begin early transcription in a freshly infected new host cell [91]. Progeny virions travel through the golgi multiple times,

gathering 1-3 membrane layers, before acquiring host plasma membrane upon budding out of the host cell to become cell-associated enveloped virus (CEV) when still attached to the host cell and extracellular enveloped virus (EEV) when released. Both CEV and EEV are highly infectious; CEV can bind directly to adjacent cells, while EEV can infect cells at distant sites. Other progeny virions [or intracellular mature virions (IMV)] are released when the host cell ruptures and contain golgi membrane, but not cell membrane, and have different surface antigens than EEV. Although EEV is the predominant infectious virion required for dissemination [92], IMV infects specific cell types in the local tissue environment, and antibodies produced by one B cell clone may not be effective against both types [93].

Cells which are not permissive for replication include most leukocytes and lymphocytes (with the exception of some CCR5+ murine cells [94]). These cells can still take up the virus [95, 96], and unwrapping and release of genetic material still takes place. In fact, early viral proteins are made in these cells, but progeny virions are not produced. In human DCs, early, but not late, VACV transcripts are produced, and intracellular replication centers never form properly, leading to poor viral DNA replication [97, 98]. However, the viral proteins produced in these cells do alter cell function. These proteins act intrinsically, binding intracellular proteins, or extrinsically, by secretion from the infected cell and binding and altering neighboring cells. Therefore, the virus infects a non-permissive cell type yet functionally changes the cell without producing new virions. Directly infected cells, whether permissive or non-permissive, secrete viral proteins which bind to soluble host immune factors or to nearby cells *in vivo*, so that even

uninfected cells are surrounded by viral proteins. These proteins change the host's normal anti-viral immune response, and will be discussed in greater detail below.

Immunomodulation by CPXV

CPXV is one of the largest poxviruses at 224Kbp, and like other orthopoxviruses, contains many genes that encode immunomodulatory proteins [99-102]. The original source of these immunomodulatory genes were likely host-cell derived and include homologues of host cytokines, chemokines, or other proteins that bind to and neutralize host proteins, including intracellular signaling proteins. CPXV is an excellent orthopoxvirus to study the role of immunomodulatory proteins in murine experimental systems, because, as discussed above, it is pathogenic in mice, and rodents are its natural reservoir [79], yet it only causes mild disease in humans [72]. The proteins produced by orthopoxviruses in the course of intracellular infection can act directly within the infected cell, or be secreted and bind host proteins important in regulating the host immune response (Figure 3). Examples of virus-encoded proteins that act within cells are cytokine response modulator A (CrmA), which inhibits IL-1β converting enzyme [103, 104], and proteins that interfere with NF-κB signaling [105-107]. Examples of CPXV proteins secreted from an infected cell that can neutralize host immune proteins are four TNFα receptor homologues [108], an IFNγ-binding protein [109], and a chemokine-binding protein [100, 110]. The importance of orthopoxvirus immunodulatory gene products in virulence has been demonstrated by infecting susceptible hosts with mutant

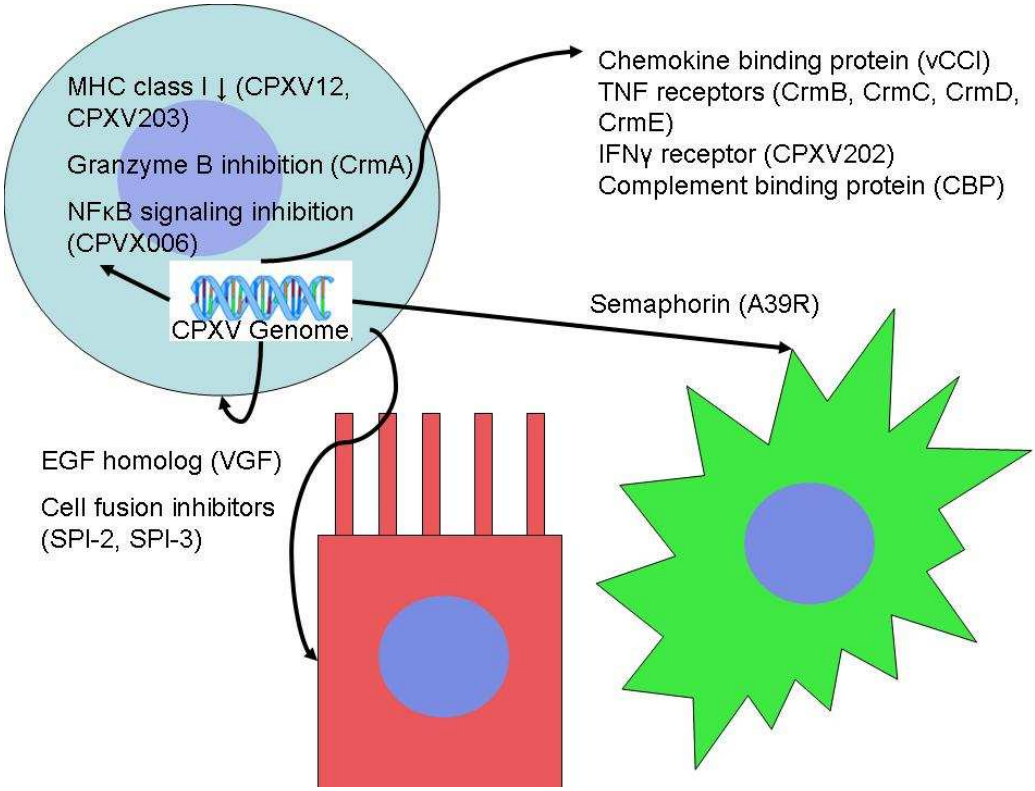


Figure 3. Examples of CPXV immunomodulatory proteins. A directly infected cell (blue cell, top left) produces viral proteins which alter the intracellular environment and secretes viral proteins to affect the extracellular environment. Some viral factors act specifically on epithelium (red cell, bottom left), such as viral growth factor (VGF) to promote cell survival and proliferation. Cell fusion inhibitors disrupt tight junctions and encourage cell-to-cell viral spread. Other viral proteins act specifically on phagocytes, for example the semophorin A39R binds plexin C1 on DCs and neutrophils, causing local actin rearrangement and inhibition of phagocytosis [111]. Many soluble factors do not bind cells (upper right), but bind important immune mediators produced by immune cells. These factors prevent DC maturation, chemotaxis to areas of inflammation, and complement-mediated cell death.

viruses created by disrupting single genes. For instance, a CPXV mutant lacking CrmA, a protein which promotes survival of infected cells by inhibiting caspases and granzyme B [112, 113], was attenuated in both an intradermal and intratracheal murine infection [114]. Vaccinia virus (VACV) lacking protein C16 (homologous to CPXV protein BR33), a protein which blocks IL-1R signaling, was attenuated in an intranasal infection as a result of allowing faster T cell recruitment to the infected lung [115]. Other than the aforementioned studies on mutant viruses, research on immune modulation by orthopoxviruses have typically used purified cells in culture systems. For example, this strategy demonstrated that VACV infects and inhibits DC function *in vitro* by decreasing MHC class II [116] and CD1d [117] on the cell surface, leading to poor antigen presentation. Furthermore, CPXV was shown to inhibit NF-κB signaling in human myeloid THP-1 cells, and deleting the CPXV gene responsible (CPXV-006) restored the ability of the THP-1 cells to produce inflammatory cytokines, as well as showing reduced virulence in a mouse model [107]. Few studies have assessed whether CPXV induces immunosuppressive effects on the host during an *in vivo* infection and addressed potential mechanisms. In an intranasal CPXV model, mortality correlated with high virus titers in the lungs and dissemination of virus to the spleen, although viremia was not observed [118, 119]. Smee *et al.* [120] found high levels of host cytokines in lung homogenates following an intranasal inoculation with CPXV, including IL-6, IL-1β, IL-2, and IFNγ, and suggested a "cytokine storm" and overstimulation of the immune response led to mortality in the mouse model. Notably, IL-10, an important suppressor of the immune system, was also elevated.

Intratracheal model of CPXV infection

Our CPXV mouse model uses the intratracheal inoculation route to generate a pulmonary infection in mice. Intratracheal injection of CPXV elicits a reproducible infectious dose leading to an extensive lung infection from the bronchus to the deep lung. A much lower infectious dose is required compared to the commonly used intranasal inoculation route of pulmonary infection; similar viral lung titers and clinical scores are achieved by $3.2x10^3$ PFU/ms I.T. or $2x10^5$ PFU/ms I.N. [114]. In the intranasal infection, many virions are trapped in the nasal turbinates, creating sinusitis and rhinitis in addition to pneumonitis. In contrast, the intratracheal model leads primarily to severe pneumonitis with consistent lesions throughout the lung tissue and with limited nasal involvement [114]. Once virus enters the lung, it can be detected by 1 day post-infection (dpi) in AMs lining the airways. By 3dpi infection it has spread to bronchiolar epithelial cells, and by 6dpi most lung cells are infected (Figure 4). Epithelial hyperplasia is apparent with edema and necrosis, and mononuclear cells are present around the bronchioles. It should be noted that in the mouse only the trachea is surrounded by cartilage and, therefore, all conducting airways are referred to as “bronchioles”.

In the current work, for the most part we shall examine two doses of CPXV, a low-dose ($5x10^3$ PFU/ms I.T.) and a high-dose ($5x10^4$ PFU/ms I.T.). Clinical signs of illness are observed at 3-4dpi in the high-dose model and the mice succumb to infection 9-12dpi (Figure 5A), whereas in the low-dose infection, clinical signs begin at 5dpi and dissipate by 14dpi, and the mice recover fully. Both doses lead to the bronchopneumonia described

above, but more virus is present in the lungs 6dpi in the high-dose infection (Figure 5B), and similar clinical symptoms of ruffled coat, weight loss, and lethargy. However, in the high-dose infection only, the virus disseminates to multiple organs, including the spleen and liver ($<1x10^3$PFUs/organ). Also with the high dose, morbidity as measured by weight loss indicates the outcome of infection. Weight loss greater than 20% at 6dpi indicates an eventual fatal outcome [118].

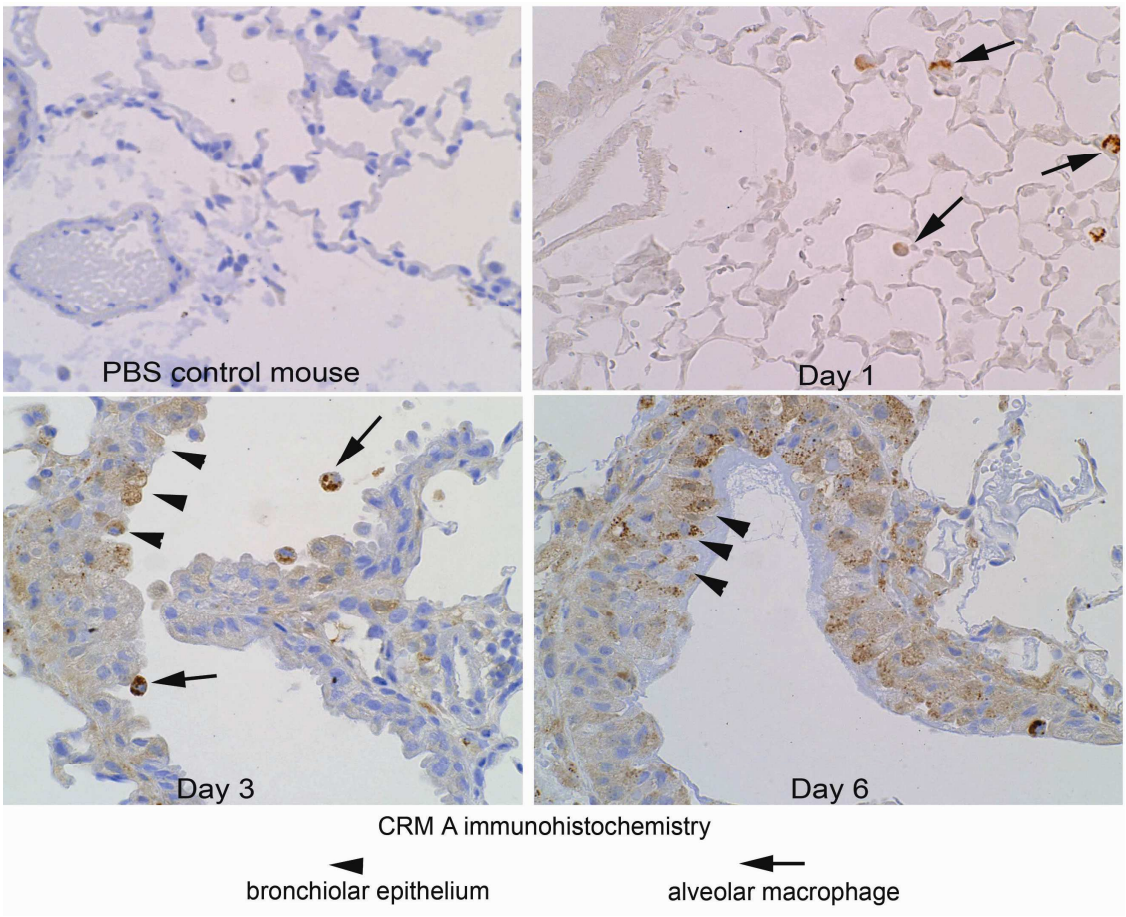


Figure 4. Lung tissue sections showing time course of cells infected after intratracheal inoculation of a high-dose of CPXV. Brown staining indicates presence of an early viral protein, CrmA, and therefore an infected cell. Upper left: A control lung section showing no viral protein and normal airways. Upper right: 1dpi shows infected AMs in the alveolar spaces, with no other cell type involved. Lower left: On 3dpi, some bronchoepithlial cells are infected along with AMs. Lower right: By 6dpi, most of the bronchoepithelium is infected, and the epithelium shows pronounced hyperplasia and inflammation. Figure kindly provided by J.A. Hutt, DVM.

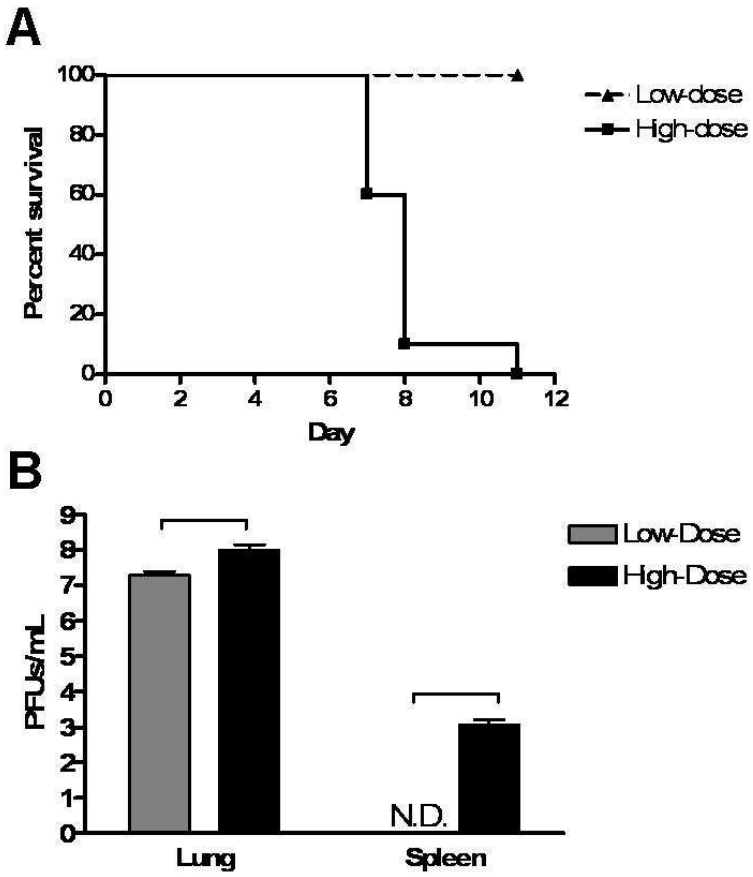


Figure 5. Mice survive a low-dose of CPXV, but a high-dose of CPXV leads to dissemination of virus and death. A) Mice survive a low-dose intratracheal CPXV infection, but not a high-dose infection. N=10 mice per group, curves are significantly different using a log-rank test with P=0.0004. B) Initial inoculum dose of virus, and lung and spleen titers 6dpi after low-dose and high-dose infection. No virus was detected in the spleens of the low-dose group. N=12 mice per group from 3 separate IT experiments, with a bar indicating a significant difference by 2-way ANOVA.

Viral dissemination from the lungs to vital organs and periphery

There are a few hypotheses, none exclusive of each other, on how orthopoxviruses spread from the lungs after a pulmonary innoculation. The virus does not disseminate in a low-dose infection, suggesting a large amount of virus entering the lung must stay within the lung or perhaps local lymphatics. Once virus is established in epithelial cells and lesions form, vascular necrosis may lead to release of virus particles directly into the blood stream, where they preferentially seed the liver and spleen. Because no detectable virus is found in blood via plaque assay in our mouse model, it is most likely very few particles disseminate this way. However, in two human infections, CPXV was detected by PCR in whole blood, but not serum, up to 4 weeks after infection [83]. Rather than free virus traveling through the blood, cell-associated virus particles may "hitch a ride" on leukocytes traveling through the blood stream. In fact, Paola D. Vermeer *et al.* found that VACV preferentially infects human airway epithelial explants via the basolateral membrane or non-confluent cell growth areas, and suggests that apical infection of lung cells may be rare [121]. Instead, breaks in the lung epithelium may allow virus to enter, or virus may travel via lymphatics to LALNs and then via efferent lymphatics enter the blood stream, either free or cell-associated. VACV also preferentially infects a canine kidney cell line (MDCK) through the basolateral membrane [122], which may explain the renal tubule damage described in smallpox patients [69], as well as how other organs become targeted during infection.

By12dpi, mice that received a sublethal intranasal dose of virus have serum antibody and are clearing the virus from all organs, while mice that received a lethal dose did not have detectable antibodies before death on 7-8dpi [118]. Antibodies play in important role in viral clearance. Mice lacking functional B cells (immunoglobulin heavy chain knockout mice) were unable to clear virus as quickly as control mice, and developed pox on the nose, tail, ears, and paw pads, symptoms not seen in BALB/c mice after pulmonary challenge, and indicative of wide-spread dissemination [123].

III. Project hypothesis and AIMs

Our laboratory previously demonstrated that peripheral human DCs infected *in vitro* with CPXV were unable to secrete anti-viral cytokines and unable to stimulate an allogeneic T cell response [124]. The laboratory also developed a murine pulmonary model of CPXV infection to determine a dose that is lethal and sublethal [123]. Because rodents are the natural reservoir for CPXV, we wondered if murine pulmonary DCs were suppressed by CPXV similar to human DCs. Pulmonary DCs would be first responders during a naturally occurring infection, and yet the effect of CPXV on these cells has not been tested. VACV has been shown to suppress primary DCs, such as Langerhans' cells in the skin [125], but is also highly immunogenic [126] and used widely as a vaccine vector [79]. We decided to assess the function and maturation level of murine pulmonary DCs after infection *in vitro*, using sort-purified cells, and *in vivo*, by infecting mice intratracheally and examining maturation of lung and LALN DCs during infection. We hypothesized that a pulmonary CPXV infection would suppress an immune response to a second immunologic challenge in a dose-dependent manner by directly infecting and suppressing dendritic cells (DCs).

Aim 1: Determine if a murine pulmonary CPXV infection can suppress the immune response to intranasally inoculated OVA peptide in a dose-dependent manner.

We analyzed whether an ongoing pulmonary CPXV infection could suppress an antigen-specific immune response in the LALN. We decided that introducing a second pathogen

to the lungs would be complicated and lead to high morbidity in a murine system and instead decided to introduce a peptide as the non-viral antigen. We selected OVA peptide to use in our murine infection model, as the peptide and OVA peptide-specific T cells are available that are well-characterized. The DO11.10 transgenic mouse strain, produced at Washington University, St. Louis, by Ken Murphy and Dennis Loh in 1989, produces OVA-peptide specific naive T cells [127]. These mice were created by introducing a murine hybridoma cell, specific for the C-terminus 323-339 peptide from chicken ovalbumin [128], into a BALB/c embryo and backcrossing the resulting mice to BALB/c mice. The resultant mice are hemizygous for the transgene and 50% of their T cells are OVA-peptide specific. The Loh laboratory also developed a monoclonal antibody, KJ1-26, to bind the transgenic TCR, leading to simple identification of transgenic T cells. We isolated T cells from the spleens of DO11.10 mice and adoptively transferred them into BALB/c mice. This ensured the expected immune response to CPXV from the normal BALB/c mouse, but with the added ability to examine DO11.10 T cells during the pulmonary infection. Importantly, DO11.10 mice share the H-2^d MHC haplotype with BALB/c mice so that donor DO11.10 T cells are treated as self in the recipient BALB/c mice. An adoptive transfer model of this type is commonly used to study hypersensitivity Th2-type responses in the lung, and much is known regarding the amount of peptide that must be given to illicit a response. Adoptive transfer of DO11.10 cells has also been used to show an OVA-specific T cell response during infection with influenza, but in that model, the virus itself encoded OVA and displayed the epitope on the viral membrane [129]. OVA-specific adoptively transferred T cells proliferated 5dpi after infection with the OVA-influenza, but not with wild-type influenza, and, as expected, the majority of

dividing cells were found in the draining lymph nodes rather than in the lung or spleen. Both viruses were cleared in a similar time frame, suggesting OVA peptide acted like another viral antigen, and did not help or hinder the immune response. In uninfected BALB/c mice, we also determined that adoptively transferred CFSE+ cells traffic to the LALN, and the labeled cells need at least 72hr post-OVA intranasal inoculation to clonally expand (Figure 6).

Aim 2: Assess whether pulmonary DC function during CPXV infection *in vitro* and *in vivo* is suppressed and, if so, whether suppression requires direct infection (cis effect) or is a result of suppressive factors in the microenvironment.

We examined the maturation of DCs post infection by analyzing the amount of MHC class II, CD80, and CD86 on the cell surface. Because lack of maturation markers only indicates potential deficiencies in DCs, we also functionally tested these DCs by using them to stimulate naïve DO11.10 T cells. In these experiments, we were able to distinguish between directly infected DCs and uninfected DC by using CPXV encoding enhanced green fluorescent protein (GFP-CPXV) on an early viral promoter. Goff *et al.* inserted GFP into the CPXV genome between the J4R and J5L regions on a VACV early/late promoter, and found it did not interfere with the kinetics of replication or LD50 in mice [130]. Because it is linked to an early promoter, even non-permissive cells are able to produce GFP after taking up virus. Using permissive Vero E6 cells, we found that GFP was produced by 2hpi and the mean fluorescent intensity of GFP increased over time (Figure 7). In DCs, this allowed us to examine cells infected *in vitro* at different

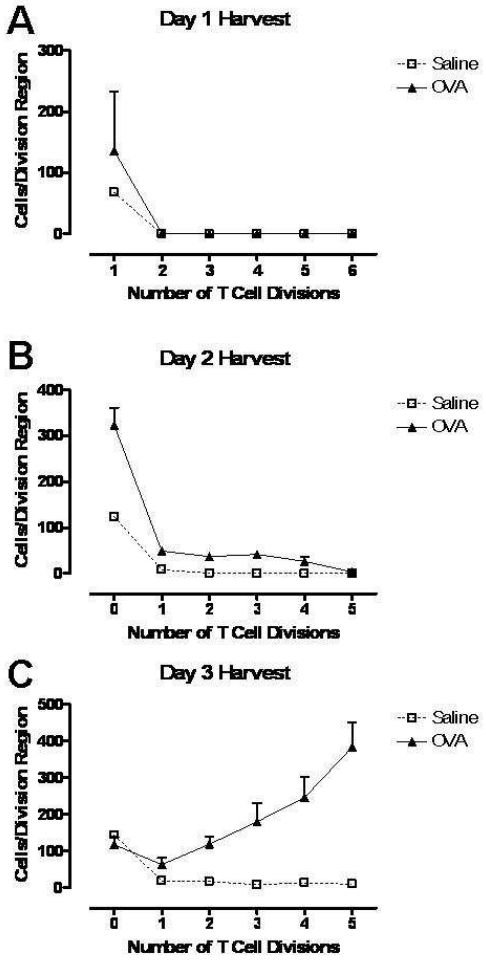


Figure 6. Intranasal OVA peptide stimulates adoptively transferred OVA-specific T cells to proliferate over 72hr. BALB/c mice were given $1x10^7$ DO11.10 T cells intravenously, which were labeled with CFSE intracellular dye, and one day later the mice were challenged intranasally with OVA peptide. Mice were sacrificed and LALN removed for analysis at 24hr (A), 48hr (B), and 72hr post intranasal inoculation. (C). The x-axis shows the number of T cell divisions a cell has undergone based on CFSE intensity (0=cell has not divided, 5=cell has been through 5 divisions, see Supplemental Figure 1 for gating strategy). The y-axis is the total number of CD4+ cells displaying the indicated divisions.

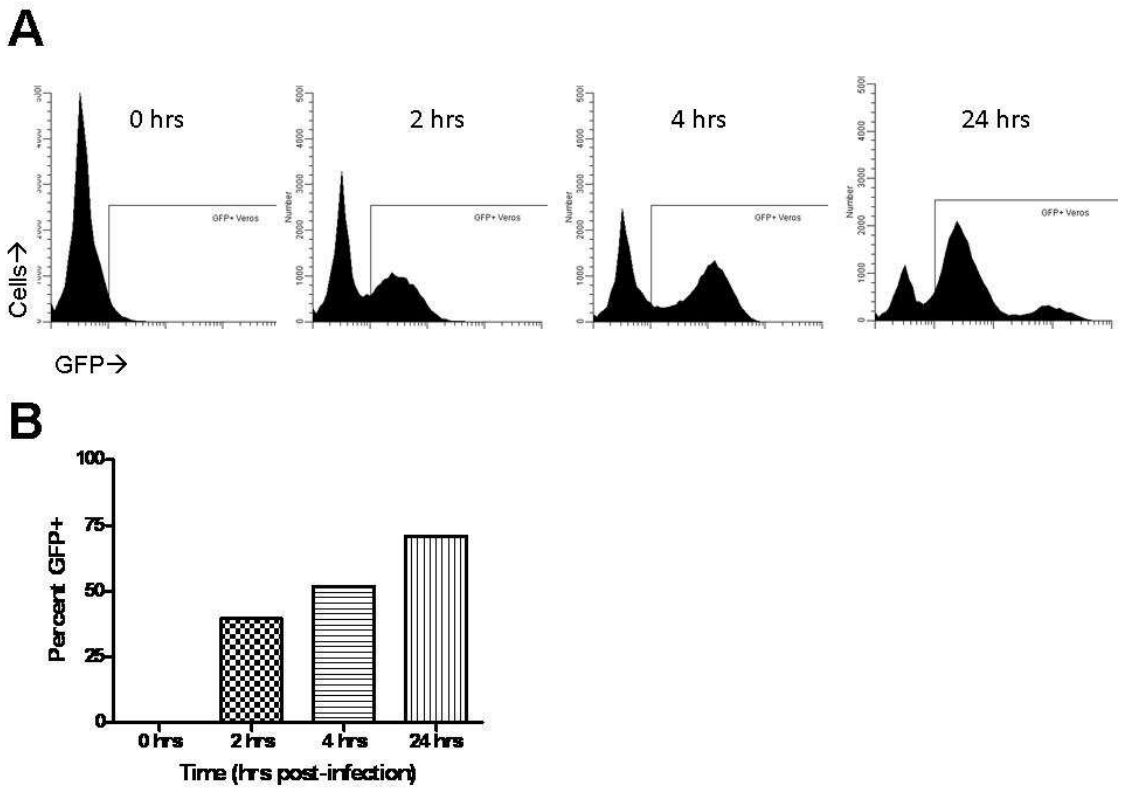


Figure 7. Time course of GFP expression in Veros following infection with GFP-CPXV. A) Histograms show GFP expression by 2hpi with GFP-CPXV. The 24hr time point shows two distinct peaks of GFP, a high-MFI peak, probably corresponding to cells originally infected which have not yet lysed, and a low-MFI peak, which most likely corresponds to cells infected by progeny virions produced by the originally infected cells. A monolayer of Vero cells were infected at MOI=5 for the time indicated. The cells were trypsinized, fixed in paraformaldehyde, and analyzed by FACS for GFP expression. N=1 experiment, with each time point run in duplicate.

multiplicities of infection (MOI) and determine how many cells were infected and how expression of GFP corresponds to typical maturation markers on the cell surface.

Aim 3: Examine potential immunosuppressive molecules that could lead to DO11.10 T cell suppression in high-dose infection. To explore host-pathogen interactions, we will examine one host factor (IL-10) and one viral factor (IFNγ receptor) that could play a role in immune suppression caused by CPXV.

The third aim focuses on potential pathways that could suppress the immune response during a CPXV infection. Because 40-50 genes are encoded by CPXV that potentially alter the immune response, there were many pathways to explore. Because we have an interest in host-pathogen interactions, we decided to look at both a host protein that could suppress the immune response, IL-10, and a viral protein, vIFNγR. We chose IL-10 because it is produced in lungs following a CPXV infection [120] and is known to suppress DC activation and antigen presentation [131, 132]. IL-10 is produced by many leukocytes, but primarily monocytes, macrophages, and T cell subsets. AMs produce IL-10 after clearing apoptotic and necrotic debris in lungs [133], and during CPXV infection the lungs exhibit lesions containing necrotic tissue [134]. When IL-10 binds the IL-10 receptor on monocytes (expressed constitutively at low levels), MHC class II and CD86 are down regulated, even in the presence of the pro-inflammatory cytokine IFNγ [135]. When cultured in the presence of IL-10, monocytes preferentially differentiate into macrophages instead of DCs [136]. DCs that are already differentiated, and then treated with IL-10, are unable to mature further in response to LPS and have a decreased ability

to stimulate antigen-specific T cells [137]. Based on these data, we hypothesized that IL-10 might suppress the immune response to OVA peptide seen during CPXV infection and that if one infected an IL-10 knockout mice with a lethal CPXV dose, they might be capable of mounting a DO.11.10 T cell proliferative response following an intranasal OVA-peptide instillation.

Finally, we examined whether a viral factor, vIFNγR, might bind IFNγ and help to explain the suppressed OVA-peptide immune response in the adoptive transfer murine model. An earlier study done in collaboration with Tim Erwin determined that IFNγ was required for effective recovery from CPXV in the mouse lung infection model. When mice were given antibodies that bind endogenous IFNγ one day prior to CPXV infection, a normally sublethal dose of virus became lethal (Figure 8). This data suggested that IFNγ made very early post-infection, possibly by NK cells, was critical for mounting an immune response to control the virus. Because CPXV, like most orthopoxviruses, encodes a soluble IFNγ receptor [138], I hypothesized that the receptor is a virulence factor, and lack of the receptor would allow a more robust immune response to occur during infection. Thus, following a lethal CPXV infection with a mutant CPXV in which the vIFNγR gene was disrupted, we asked whether DO11.10 T cells would be capable of proliferating in response to intranasal OVA-peptide.

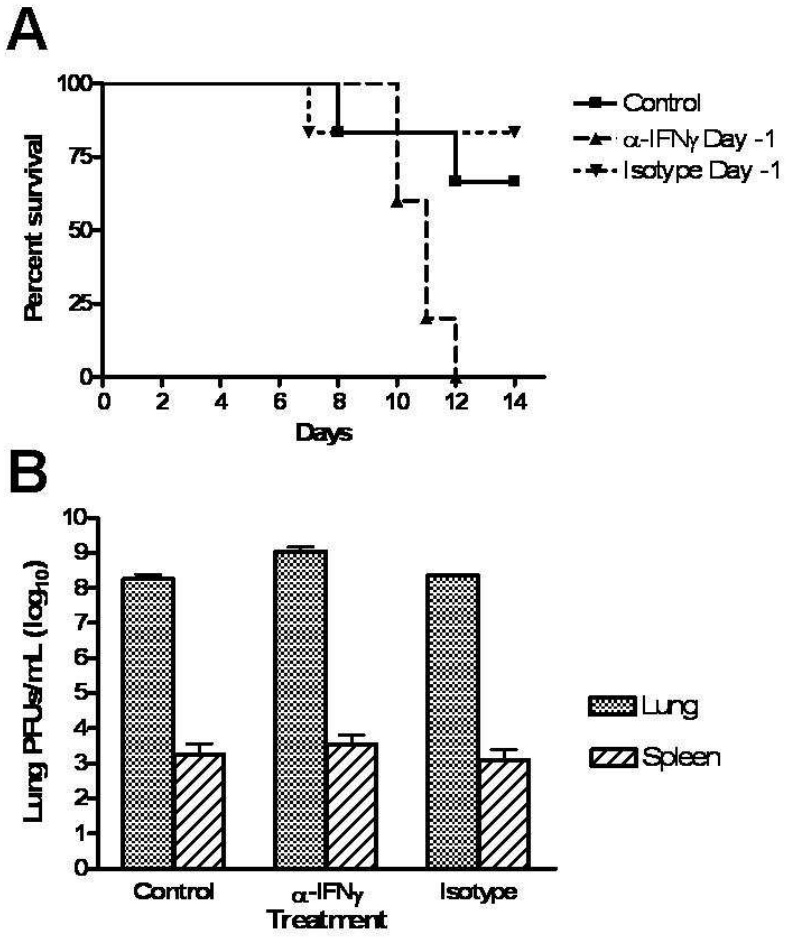


Figure 8. Survival and organ titers of mice treated with ant-IFNγ antibodies. A) Survival of CPXV-infected mice is reduced if the mice are given anti-IFNγ antibodies intraperitoneally (1mg/ms XMG1.2 antibody in a single dose) one day prior to intratracheal infection. Graph shown is one representative of 2 separate experiments with N=6 mice per group. B) Lung and spleen viral titers were similar for all three groups on 7dpi (N=3 mice per group with one experiment).

Part IV: Results

A high-dose pulmonary CPXV infection suppresses a primary immune response to OVA.

A mouse model was developed to determine if a pulmonary CPXV infection could alter the normal immune response to an irrelevant antigen delivered to the respiratory tract. BALB/c mice were infected with a low- or high-dose of CPXV ($5x10^3$ or $5x10^4$ plaque-forming units (PFUs) per mouse, respectively) via intratracheal injection (d0). Both doses led to clinical illness, including weight loss and ruffled coat, but the high-dose was eventually lethal with mice succumbing by day 9 (Figure 5A) and was characterized by viral dissemination to the liver and spleen (Figure 5B, also shown by [118]). To track a specific antigen response in low- and high-dose infected mice, $OVA_{323-339}$ peptide-specific transgenic DO11.10+/- T cells [128] labeled with CFSE were injected into the infected mice via the tail vein on d2pi. The adoptively transferred cells were stimulated to divide by giving intranasal OVA peptide d3pi, and LALNs were removed 6dpi for analysis by flow cytometry (Figure 9A). CFSE+CD4+ T cells proliferated in uninfected animals as well as in animals infected with a low-dose of CPXV, but few proliferating cells were present in the high-dose infection group (Figure 9B, C). To assure proliferation was affected, rather than recruitment of the adoptively transferred T cells, the LALNs were analyzed 2d after OVA stimulation (5dpi). The total number of CFSE-labeled T cells in the LALN was the same 2d post-OVA inoculation for all groups (Figure 9D; both

dividing and non-dividing cells enumerated), indicating that donor DO11.10+/- T cells were recruited and retained in the LALN regardless of infectious dose.

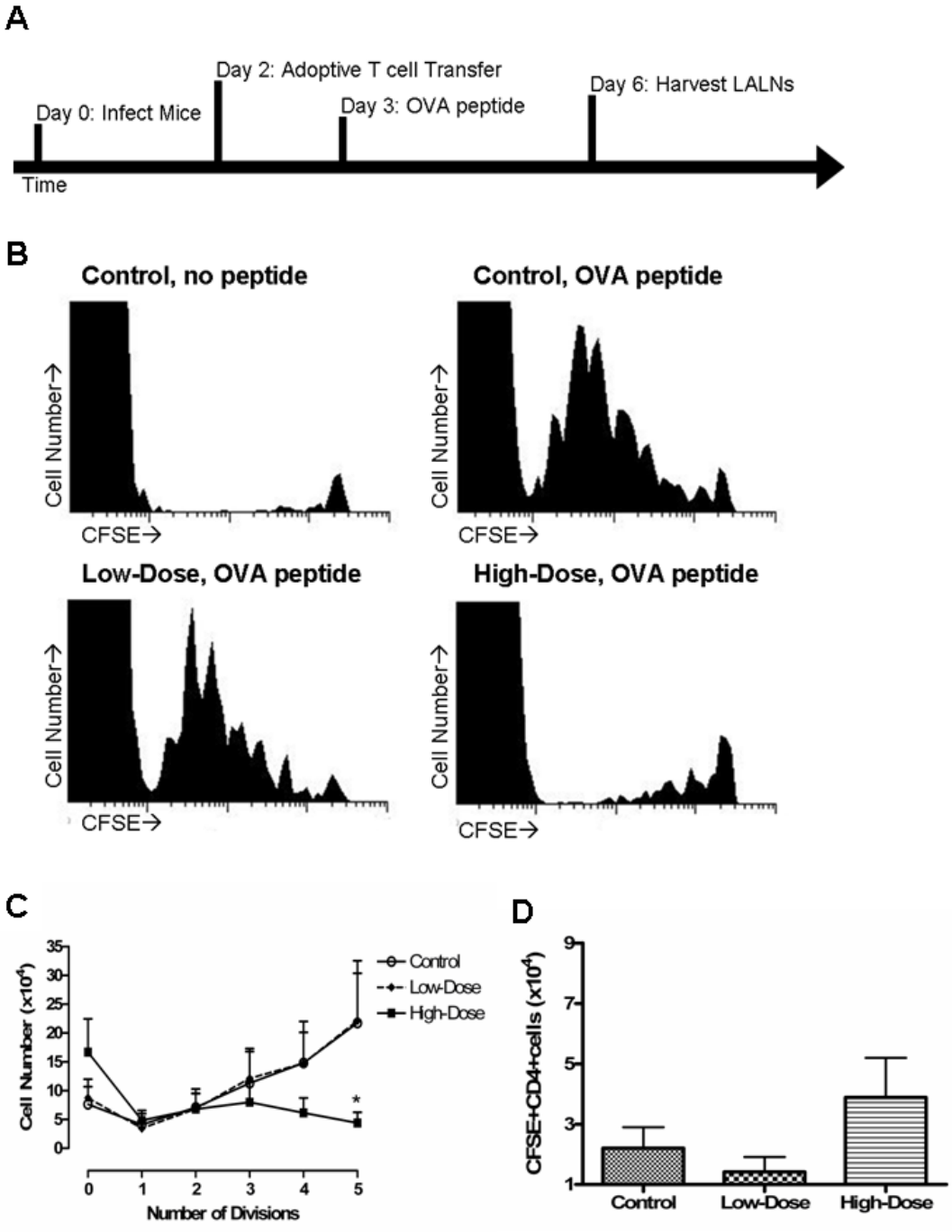

A
Day 0: Infect Mice
Day 2: Adoptive T cell Transfer
Day 3: OVA peptide
Day 6: Harvest LALNs
Time
B
Control, no peptide
Control, OVA peptide
Low-Dose, OVA peptide
High-Dose, OVA peptide
Cell Number→
CFSE→
C
Cell Number (x10⁴)
Number of Divisions
Control
Low-Dose
High-Dose
*
D
CFSE+CD4+cells (x10⁴)
Control
Low-Dose
High-Dose

Figure 9. High-dose CPXV inhibits DO11.10+/- T cell proliferation in the LALN. (A) Timeline of mouse model. Mice were infected intratracheally on day 0 with $5x10^3$ PFUs (Low-Dose), $5x10^4$ PFUs (High-Dose), or 0 PFUs (Control). On 2dpi mice from all groups were injected intravenously with 10^6 DO11.10 T cells. On 3dpi mice received intranasal OVA peptide. Mice were euthanized and the LALNs harvested for analysis 6dpi. (B) CFSE curves from CD4+ T cells in the LALN of mice receiving Control and no OVA peptide (upper left), Control and OVA peptide (upper right), low-dose infection and OVA peptide (lower left), or high-dose infection and OVA peptide (lower right). One representative mouse is shown in each panel. (C) The number of T cell divisions in each group is represented graphically. X=0 indicates that no cell division has taken place; X=5 indicates that each cell has undergone 5 divisions. (n=9 mice per group, data is average +/- SEM and asterisk (*) indicates a significant difference from control by 2-way ANOVA with $p<0.05$) (D) Harvesting 5dpi (2d post CFSE-DO11.10 inoculation) shows similar recruitment of CFSE+CD4+ T cells to LALNs for each group (n=6 per group; Data is average +/- SEM with no statistical difference between groups).

CPXV inhibits DO11.10+/- T cell proliferation in vitro.

The inability of T cells to divide in LALNs during a high-dose infection could result from direct CPXV infection of antigen presenting DCs and/or DO11.10+/- T cells, which compromised their function, or from an indirect viral effect on the immune cells caused by mediators released into the lung microenvironment created during the high-dose infection that reached the LALNs. Most cells critical for generating a primary immune response do not support a productive infection with orthopoxviruses that yields progeny virions, but the virus can nonproductively infect these cells and express early viral proteins [96]. DCs infected by VACV have reduced MHC class II expression [116], and orthopoxviruses can interfere with antigen presentation [59, 116, 117]. CPXV proteins can also inhibit NFκB signaling [105, 107, 118], an important pathway in immune cell activation.

To examine the possibility that direct CPXV infection of DCs could explain our *in vivo* results, we used an *in vitro* assay to determine whether CPXV-infected DCs were suppressed in their ability to stimulate DO11.10+/- T cell proliferation. Pulmonary DCs were infected with CPXV overnight at a multiplicity of infection (MOI) of 0.1, 1, or 10. 24hr later spleen-derived CD4+ DO11.10+/- T cells and OVA peptide were added to the infected DCs or to control uninfected DCs (MOI=0). DCs incubated with CPXV, even at a MOI=0.1, had a reduced ability to stimulate DO11.10+/- T cells compared to uninfected DCs (Figure 10A). Suppression of T cell proliferation was equivalent in cultures with DC infected at MOIs of 1 and 10. To rule out the possibility that T cells

could be directly infected by virions carried into culture from infected DCs, we infected splenic CD4+ T cells with a CPXV mutant that expressed green fluorescent protein (GFP-CPXV) under the control of a viral early/late promoter [130]. Flow cytometric analysis demonstrated that T cells were not infected (based on the absence of GFP expression) at any MOI tested, up to a MOI=10 (data not shown), consistent with previous research [96, 116]. In contrast, purified lung DCs took up virus, remained viable (data not shown) throughout the experiment, and were infected at a rate proportional to the MOI (Figure 10B). However, approximately half of the lung DCs appeared to resist viral entry or early gene transcription. In regards to the proliferation assay (Figure 10A), it appeared that infection rates of 40-50% of the DCs was associated with maximum suppression. Therefore, the numbers of uninfected DCs present in cultures showing suppression should have compensated for the loss of function of the infected DCs. This suggested that infected DCs might affect the function of uninfected DCs and/or uninfected T cells. Infected DCs expressed GFP by 4hr post-infection and the mean fluorescent intensity (MFI) of GFP increased with time (Figure 10C), indicating that GFP and possibly other early viral proteins progressively accumulated within infected cells. This result is compatible with the possibility that secreted virokines might have accumulated in the culture fluid over the first 24 hours of incubation of DCs with CPXV and could have affected uninfected DCs.

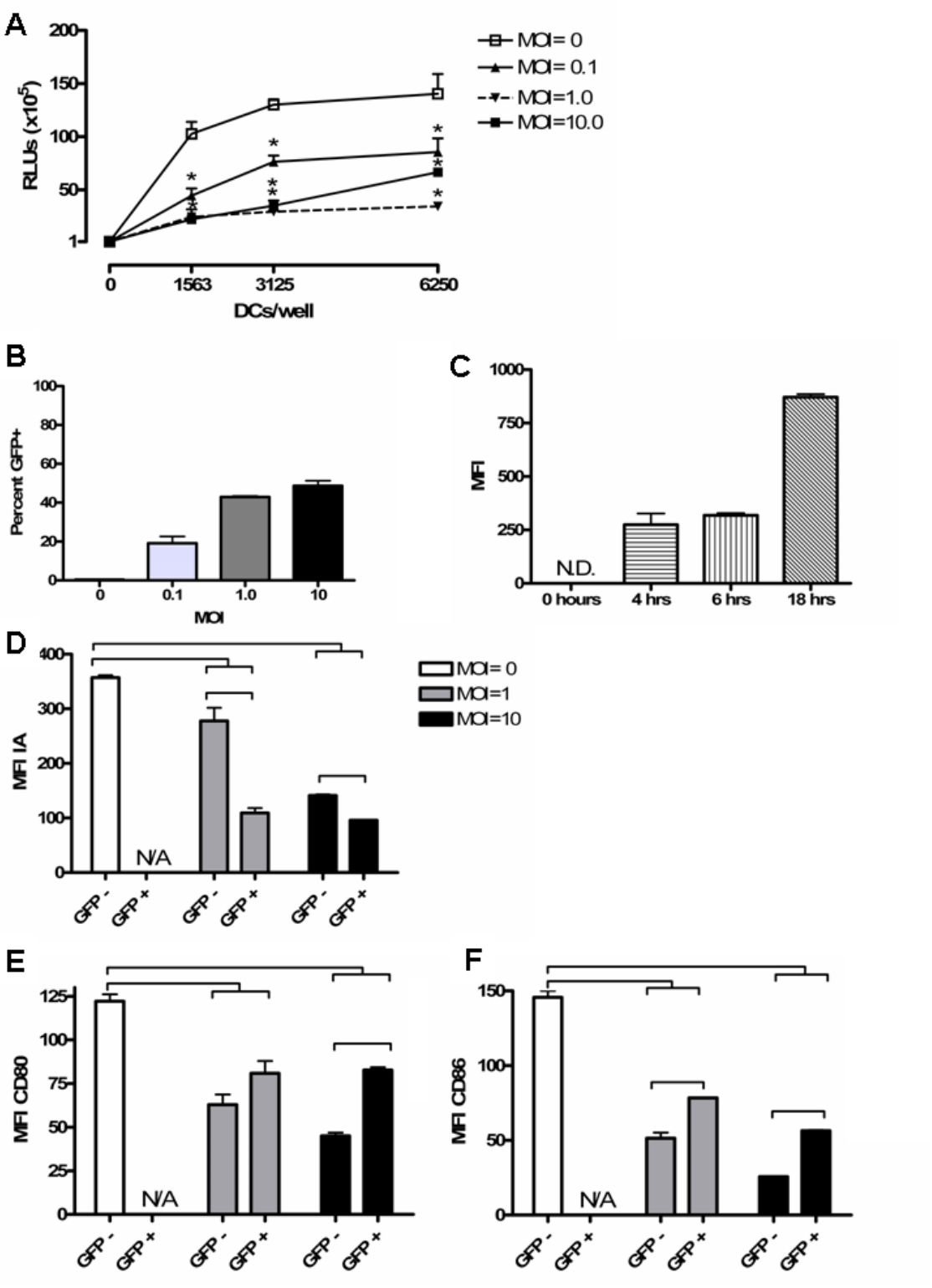


Figure 10. CPXV inhibits antigen-specific T cell proliferation in vitro. (A) Sort-purified pulmonary DCs (CD11c+Autofluorescent-) were cultured 24hr with CPXV at a

MOI of 0, 0.1, 1.0, and 10, and CD4+ DO11.10+/- T cells and OVA peptide were added for a further 72hr. Proliferation was measured by BrdU uptake. One representative experiment of three is shown. (B) DCs were incubated with GFP-CPXV to determine percent infected at each MOI as indicated by GFP expression (n=3 experiments with 3 replicates per treatment per experiment, data is average of replicate averages +/- SEM). (C) MFI of GFP at 4, 6, and 18 hr post infection using MOI=10 (n=1 experiment with 3 replicates per treatment, data is average+/-SD). Surface cell staining measured in MFI of (D) IA, (E) CD80, and (F) CD86. Shown is one representative experiment of three and the data shows the average +/- SD of replicate values. Bars indicate a significant difference at $P<0.05$ by 2-way ANOVA.

Next we examined surface expression of MHC class II (IA), CD80, and CD86 on DCs, all required for effective T cell stimulation, at 18hr post-infection with GFP-CPXV. Infected DCs (identified as GFP-positive) showed a reduction in all three surface markers. In addition, non-infected GFP-negative DCs in the infected culture wells also had significantly reduced expression of these markers (Figure 10D-F), suggesting that either infected DCs expressed surface molecules that changed the phenotype of their uninfected neighbors on contact or the culture fluids contained mediators that affected uninfected DCs. Mediators in the culture fluids might be virally encoded secreted proteins or endogenous host mediators secreted from infected DCs. These *in vitro* studies suggested that DC-stimulated antigen-specific T cell proliferation might be suppressed in the presence of CPXV by both direct infection of immune cells, particularly DCs, as well as by secreted factors.

A subset of lung DCs is infected in a pulmonary CPXV infection.

Because, in the presence of CPXV-infected lung DCs, DO11.10 T cell proliferation was inhibited, we asked whether 6d following CPXV infection, lung DCs were infected and whether the phenotype of both infected and uninfected DCs was altered. Mice were infected IT with the GFP-CPVX mutant to perform this analysis. Despite the high viral burden in the lungs 6dpi (up to $1x10^8$ infectious particles per lung, data not shown), only 5.1+/-0.9% or 15.4+/-1.4% of DCs were infected in the low- or high-dose infections, respectively (Figure 11A), while 70+/-5.6% of AMs were infected at the high dose (Figure 11B). We assessed the surface phenotype of lung DCs at 3dpi and 6dpi

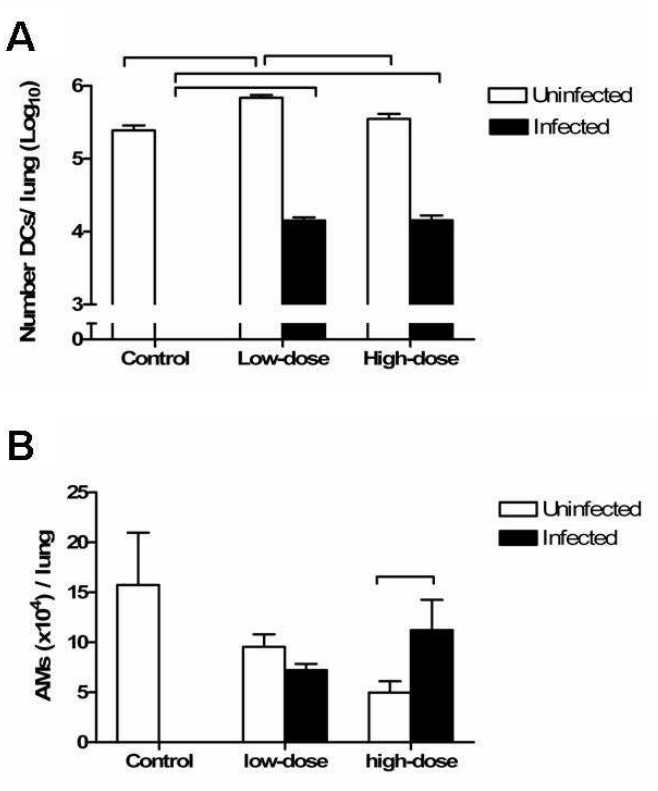


Figure 11. Few pulmonary DCs, but many AMs, are infected with GFP-CPXV in vivo. (A) Total uninfected and infected DCs recovered from lung tissue or (B) AMs recovered from BAL. Data shown is average +/-SEM from 3 experiment with n=3 mice per group per experiment. Bars indicate a significant difference at $P<0.05$ by 2-way ANOVA.

to determine if they expressed the expected maturation markers needed to present antigen. Antigen-presenting DCs must have high levels of MHC class II molecules (I-A) along with CD80 and CD86 to effectively stimulate naïve CD4 T cells in the LALN [42]. Other antigen presenting molecules we analyzed included CD40, a co-stimulatory molecule, and CD1d, which is used to present lipid antigen to γδ T cells. We also measured CCR5, which is upregulated on DCs trafficking to the LALN [42]. At 3dpi, the lethally infected mice showed a lower percentage of DCs expressing I-A, but a higher percentage of DCs expressing CD80, CD86, CD40, and CD1d (Figure 12A). At 6dpi, fewer DCs in the lungs of CPXV-infected mice expressed I-A compared to uninfected controls, with a larger difference seen at the high dose of CPXV infection (Figure 12B). In contrast to the *in vitro* CPXV DC infection in which DCs showed decreased expression of CD80 and 86, the percent of DCs expressing CD80 and CD86 was similar for both infectious doses and control mice. CD40, however, was expressed on significantly more DCs in the low-dose infection compared to control or high-dose groups. We were unable to assess the difference in marker expression between infected versus uninfected DCs, because we were unable to compensate for the high MFI of GFP in the infected DCs. However, 49.9% fewer DCs expressed I-A in high-dose versus control mice, whereas only 15.4% were infected. Thus, many uninfected DCs demonstrated reduced I-A expression, and this decrease was most likely a result of factors present in the microenvironment of the infected lung. Furthermore, these results suggested that in CPXV-infected mouse lungs, both infected and uninfected lung DCs that take up OVA peptide and traffic to the LALNs would be compromised in their ability to present antigen to OVA-specific naïve T cells there.

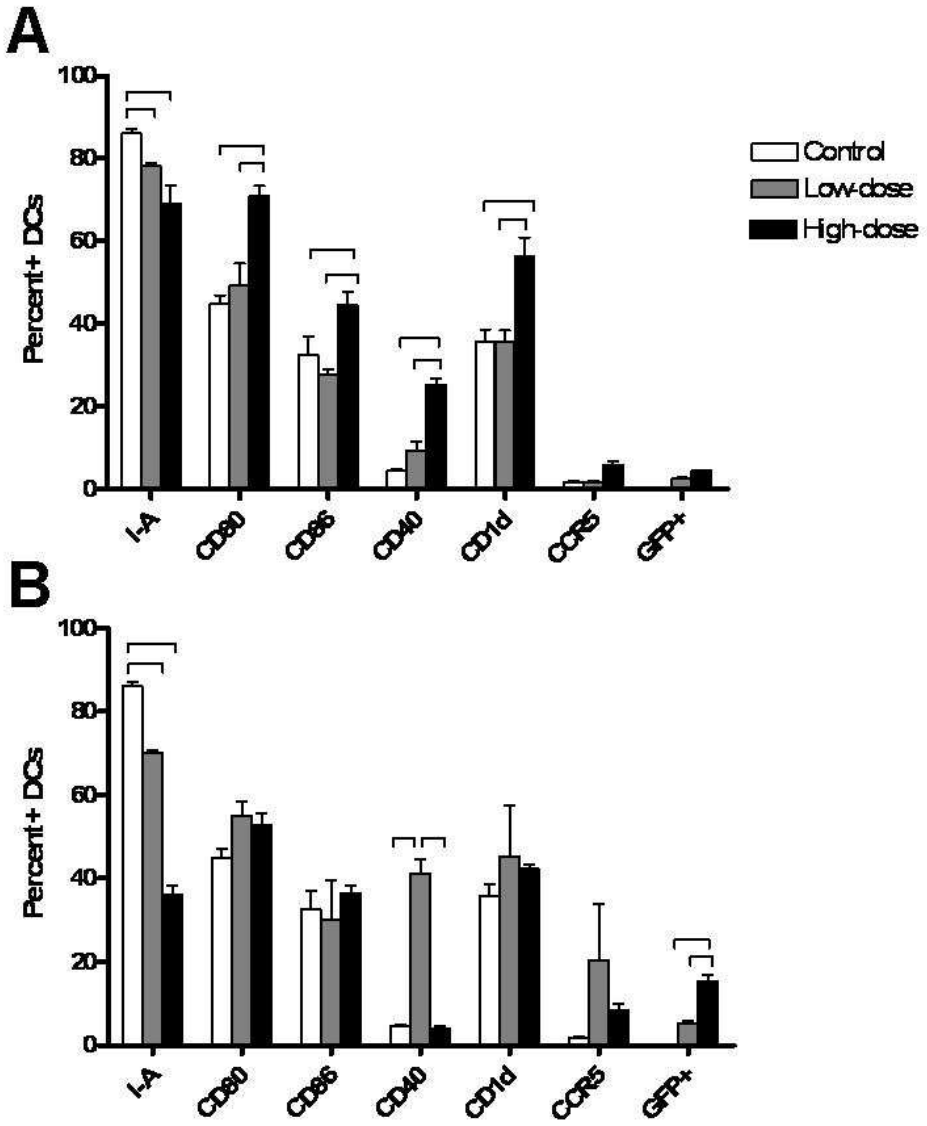


Figure 12. Surface markers on DCs and GFP expression in DCs at 3dpi (A) and 6dpi (B). Lung DCs were stained with antibodies for the indicated surface markers (n=9 mice per group). Data shown is average +/-SEM from 3 experiment with n=3 mice per group per experiment. Bars indicate a significant difference at $P<0.05$ by 2-way ANOVA.

Neither LALN DCs nor T cells are infected during a pulmonary CPXV infection.

We next determined whether DCs and T cells in the LALNs were directly infected, which could contribute to the lack of OVA peptide-induced T cell proliferation and whether immunologically relevant surface markers on DCs were reduced. Mice were infected with GFP-CPXV, and LALNs were harvested 6dpi. No GFP+CD4+ or GFP+CD8+ T cells were present in the LALNs (Supplemental Figure 2) from mice with either low- or high-dose infections. Furthermore, no GFP+ DCs (CD11c+ cells) were found in the LALNs (Figure 13A). Because IA, CD80 and 86 were lower on uninfected DCs incubated with infected DCs *in vitro* and IA was lower on uninfected DCs from infected mouse lungs 6dpi, we examined maturation markers on LALN DCs. A lower percentage of DCs from the high-dose group expressed IA and CD80 compared to control mice, and fewer DCs from the high dose group expressed co-stimulatory molecules CD80 and CD86 compared to the low-dose group (Figure 13A). The MFI of CD80 was reduced in the high-dose infection group as compared to both the low-dose and control groups, while both low- and high-dose groups had lower CD86 MFIs than control mice (Figure 13B). These data are consistent with a defective ability of LALN DCs to present antigen with greater defects in LALN DCs from mice that had received the high-dose infection, despite no evidence for direct CPXV infection. We examined further what molecules related to maturation, function and trafficking to the LALN, ie., CD25, CD1d, CD8α, CD11a, and CCR5, were expressed on DCs in the LALN of CPXV-infected mice. CD25 is expressed only on mature DC and is correlated with robust antigen presentation [139].

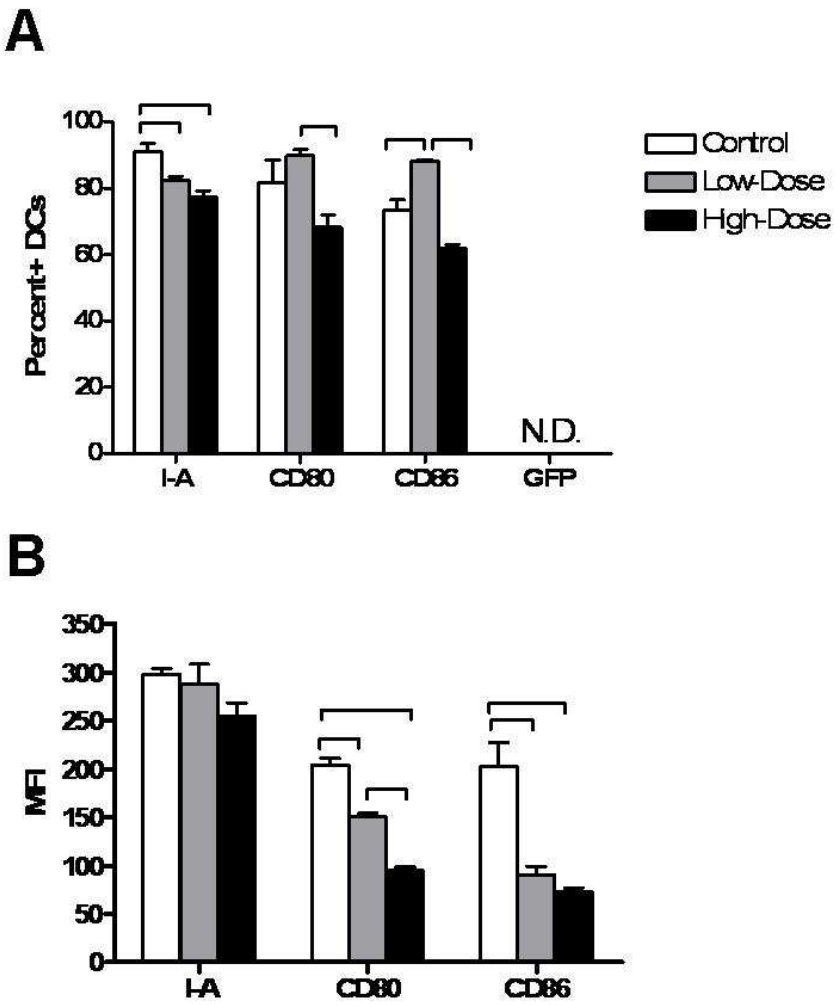


Figure 13. I-A, CD80, and CD86 are decreased on LALN DCs in infected mice. LALNs were harvested 6dpi and analyzed by immunophenotyping. (A) Percent of DCs expressing IA, CD80, CD86, and (B) MFI of IA, CD80, and CD86. LALN from 10 mice were pooled per group with a total of 3 experiments. Data shows average +/- SD and bars indicate a significant difference at $P<0.05$ by 2-way ANOVA.

As mentioned before, CD1d is required to present lipid antigens, and is known to be important in clearing VACV [117]. CD8α is a marker for resident lymph node DCs, and in one poxvirus model, CD8α-expressing DCs are not efficient as presenting viral antigen from the lung [140]. CD11a (LFA-1) is an adhesion molecule closely related to CD11c and helps initiate DC-T cell interactions [141]. CCR5 is down-regulated and CCR7 upregulated when DC are activated, leave the lung, and enter the lymph node [142]. CD1d, as shown previously [117], was expressed on fewer DCs in the high-dose infection group and showed a lower MFI compared to control mice (Figure 14). Fewer DCs expressed CD11a and CCR5 in the infected groups compared to control group, which is not surprising considering many LALN DCs in infected mice most likely recently trafficked from the lung and therefore do not express high levels of cell adhesion molecules or CCR5. Overall, the surface marker analysis from LALN DCs indicated that, despite no infected DCs in the LALN, DCs lack the required MHC and co-stimulatory molecules to effectively stimulate T cells to divide. The high-dose infection showed the lowest number of DCs expressing I-A, and the lowest MFI for both CD80 and CD86, which could account for the suppressed DO11.10 T cell proliferation in the adoptive transfer experiment. Although the low-dose shows some decrease in antigen presenting molecules, the decrease may not be severe enough to completely block presentation and T cell activation.

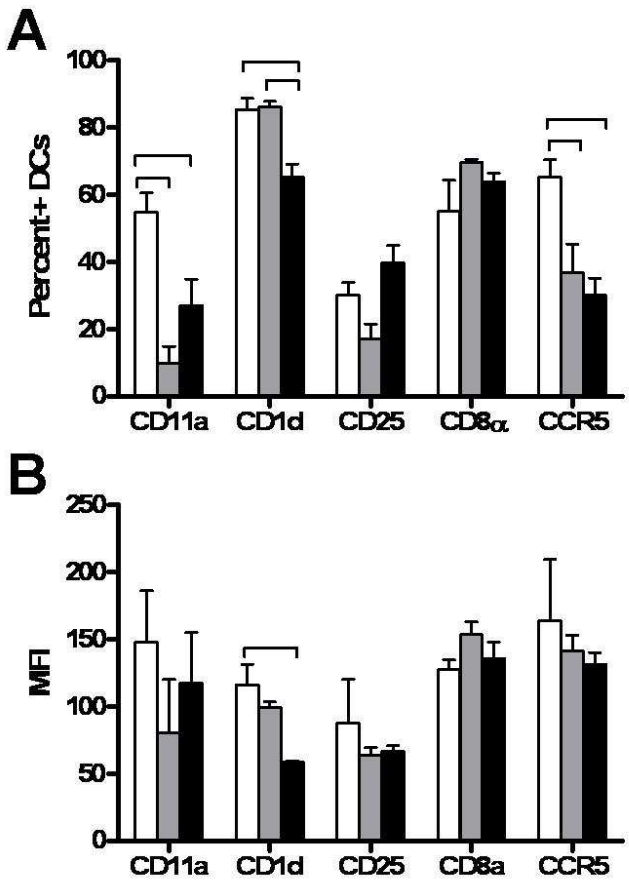


Figure 14. Percent DCs positive for (A) and MFI (B) of CD11a, CD1d, CD25, CD8*α*, and CCR5 on CD11c+ LALN cells following CPXV infection. LALN were harvested 6dpi. LALN from 10 mice per group were pooled, with 3 separate experiments averaged. Data shows average +/-SD and bars indicate a significant difference at $P<0.05$ by 2-way ANOVA.

LALN cell suspensions from infected mice can stimulate DO11.10 T cell proliferation.

Decreased LALN DC IA, CD80 and CD86 together with the absence of CPXV infection of LALN DCs or T cells suggested that the CPXV-infected lung might create an immunosuppressive microenvironment with drainage of suppressive mediators to the LALNs. It followed that the suppressive effect might be reversed if LALN DCs were removed from the inhibitory milieu. At 6dpi when suppression was present, LALNs were harvested and pooled from 10 mice per treatment group. To assess APC function, LALNs were processed to single-cell suspensions, mitotically inactivated, and incubated with uninfected DO11.10+/- T cells and OVA peptide. The low number of DCs recovered from LALNs made purification by either magnetic sorting or flow cytometry impractical, but FACS analysis revealed a similar percentage of DCs in LALN from all groups (Figure 15A). LALN cells from control, low-dose, and high-dose infection groups all supported T cell proliferation (Figure 15B), with the high-dose group being even more stimulatory than control and low-dose infected LALNs, suggesting that suppression induced by the high-dose CPXV lung infection was reversible. Similar results were obtained using lung cells enriched for DCs removed 3dpi, when OVA peptide would normally be given. At the high-dose, these cells could also support T cell proliferation *ex vivo* (Supplemental Figure 3). These data imply that infected lung tissues might continuously secrete immunosuppressive cytokines, virokines, or other immunomodulatory factors that drain to LALNs to inhibit T cell proliferation.

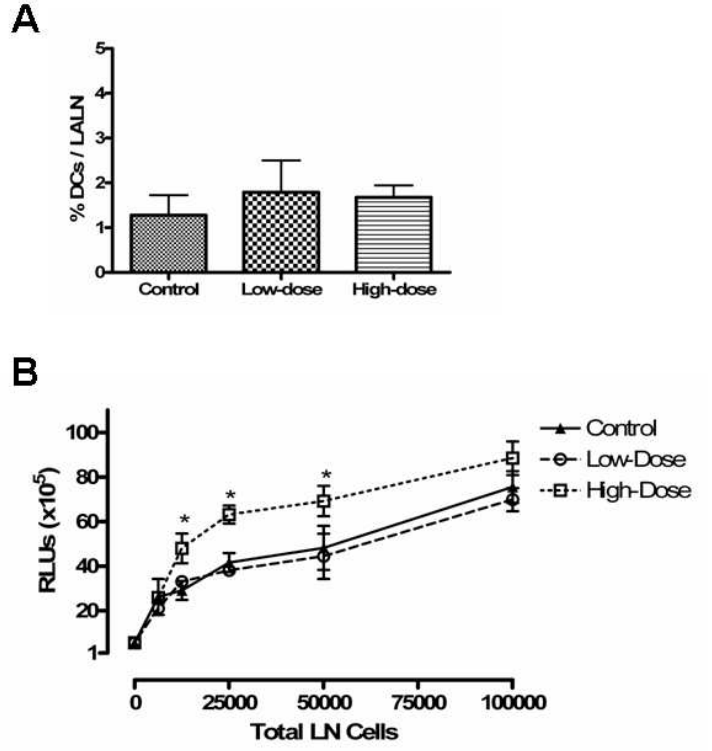


Figure 15. LALN cells from infected mice can present antigen and stimulate DO11.10+/- T cells in vitro. Pooled LALNs from 10 mice/group were harvested 6dpi and treated with mitomycin C to mitotically inactivate dividing cells. (A) The percent of DCs in the LALN was determined by CD11c+ staining and was similar for all groups. (B) LALN cells were treated with OVA and used to stimulate uninfected DO11.10 T cells. T cell proliferation was measured by BrdU uptake and expressed as relative light units (RLUs). One representative of two experiments is shown. Asterisk (*) indicates a significant difference with $p<0.05$ compared to control by 2-way ANOVA.

CPXV-infected lungs produce factors that suppress DC-mediated DO11.10 T cell proliferation.

To test the possibility that the CPXV-infected lungs generated immunosuppressive factors, we incubated DCs and T cells from uninfected mice with fluids collected from infected lungs. Two methods were used to collect infected lung fluids. First, lungs from infected mice (6dpi) were lavaged with complete medium. Second, lungs were harvested, minced in complete medium, and incubated overnight to allow release of mediators into the supernatant. DCs were incubated overnight in the collected bronchoalveolar lavage fluid (BAL) or in lung supernatant (LS) from infected or control mice. BAL was used directly as the culture medium on freshly isolated DCs, but LS from both uninfected and infected lungs was toxic to DCs after overnight incubation (data not shown). Therefore, LS was diluted with complete medium at a 1:1 ratio, i.e., 50%LS, because viability of DCs treated at this dilution was comparable to untreated DCs after 3 days in culture (data not shown). After overnight treatment with either BAL or LS, CD4+ DO11.10+/- T cells and OVA were added to the pretreated DCs and the co-incubation proceeded for 72hr after which proliferation was assessed by BrdU uptake. LS from low-dose and high-dose infected mice inhibited T cell proliferation, but the inhibition was greater with supernatants from the high-dose infected lung (Figure 16A). BAL from infected mice also suppressed T cell proliferation, but there was no significant difference between the infectious doses (Figure 16B). These data show that the inability of DO11.10+/- T cells to proliferate in the high-dose CPXV-infected animal does not require direct infection of

either the T cells or DCs, but rather may result from secreted soluble factors produced by infected lung tissue.

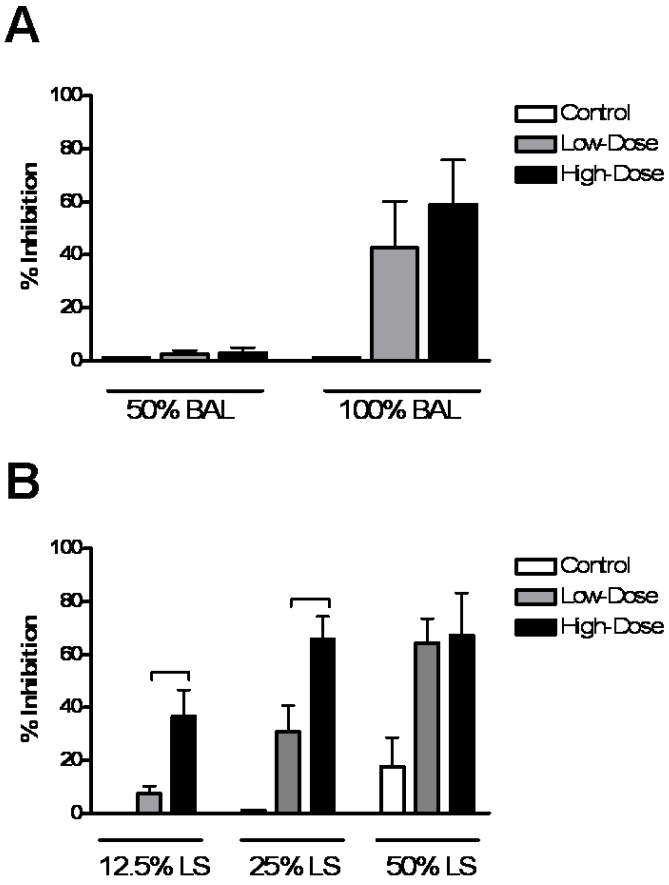


Figure 16. Mediators from infected lungs are sufficient to suppress DC-supported T cell proliferation. Pulmonary DCs were incubated overnight in (A) lung supernatant (LS) or (B) BAL from control, low-, and high-dose infected mice. DO11.10 T cells and OVA were added to the culture at 24hr, and the culture continued for an additional 72 hr. BrdU uptake was used to measure T cell proliferation. Percent inhibition was calculated using supernatant-free media as 100% proliferation. Data is shown as the average of three experiments +/-SEM, using LS and BAL collected from 3 separate experiments for all groups. Bars indicate a significant difference at $P<0.05$ by 2-way ANOVA.

LS and BAL from infected mice contain factors that bind and inhibit detection of multiple host cytokines.

The suppressive factors contained in LS and BAL from infected mice could be virally encoded proteins that suppress the immune system, but no antibodies are currently available to measure those proteins. Knowing that CPXV encodes viral IFNα/β receptor, IFNγ receptor, and vCCI chemokine receptor along with other host-binding proteins, we measured host cytokine levels in LS and BAL, and then determined interference by CPXV proteins. For this assay, we first directly measured the amount of host cytokines IL-1β, IL-6, MIP-1α, RANTES, IL-10, IL-12, IFNα, IFNβ, and IFNγ present in LS and BAL. In LS, endogenous levels of IL-10 and IL-6 were significantly higher in high-dose compared to low-dose or control groups, although the total amount of IL-10 never exceeded 64pg/ml (also pg/lung) (Figure 17, see Supplemental Figure 4 for additional cytokine data). Although a low amount, it suggested a poor pro-inflammatory response in the lungs and, instead, an immunosuppressed response. High levels of IL-1β were noted only in control mice, and the level significantly dropped during infection in a dose-dependent manner. Of the other cytokines measured, none were measured in any large amount, in either naïve or infected lungs, and any differences among groups were not significant. Next we added exogenous cytokine to each sample and measured again to determine if any endogenous proteins were present in the LS and BAL that could bind and interfere with the measurement and possibly the function of host cytokines. Thus, when the amount of cytokine added to the sample did not equal the amount measured, it was presumed that a virally encoded suppressive molecule was

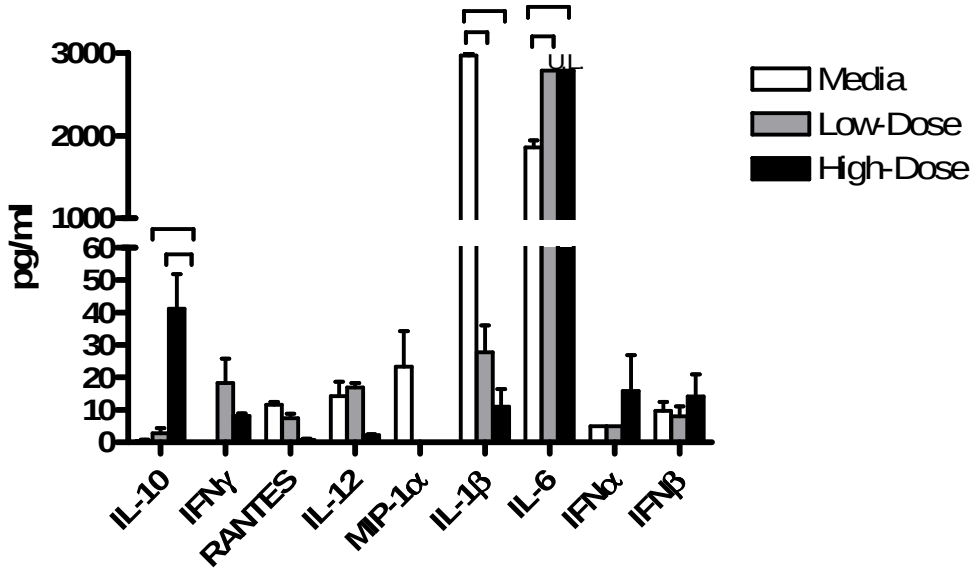


Figure 17. IL-10 is increased in LS from high-dose lungs compared to low-dose lungs. Luminex analysis was performed on LS from 3 mice per group with each sample in duplicate. Data from two separate luminex experiments (and LS from two separate I.T. infection experiments) were averaged. Bars indicate a significant difference at $P<0.05$ by 2-way ANOVA.

present. For all cytokines measured, no inhibition of cytokines was noted in LS from uninfected mice. However, when exogenous cytokines were added to LS from infected mice, the readout from the multiplex assay was reduced for several cytokines. In particular, IFNγ was inhibited in both LS (Figure 18A) and BAL (Figure 18B) samples, and even when a large amount was added (1500pg/ml), none was detected by luminex. Other cytokines inhibited include chemokines MIP-1α and RANTES (Figure 19A). The effect was specific, because certain cytokines, such as IL-1β and IL-10 (Figure 19B), were readily detected by luminex (See Table 2 for complete set of cytokines tested). Interference with host IFNγ observed in BAL suggested large amounts of CPXV proteins were present in BAL, even though little host cytokine was measured. For both LS and BAL, the inhibition was greater for the high-dose and less for the low-dose infections. The inhibition of MIP-1α was greatest, and even by adding 23,000pg/ml of MIP-1α, none could be measured in the assay in the LS and BAL samples from high-dose infected mice.

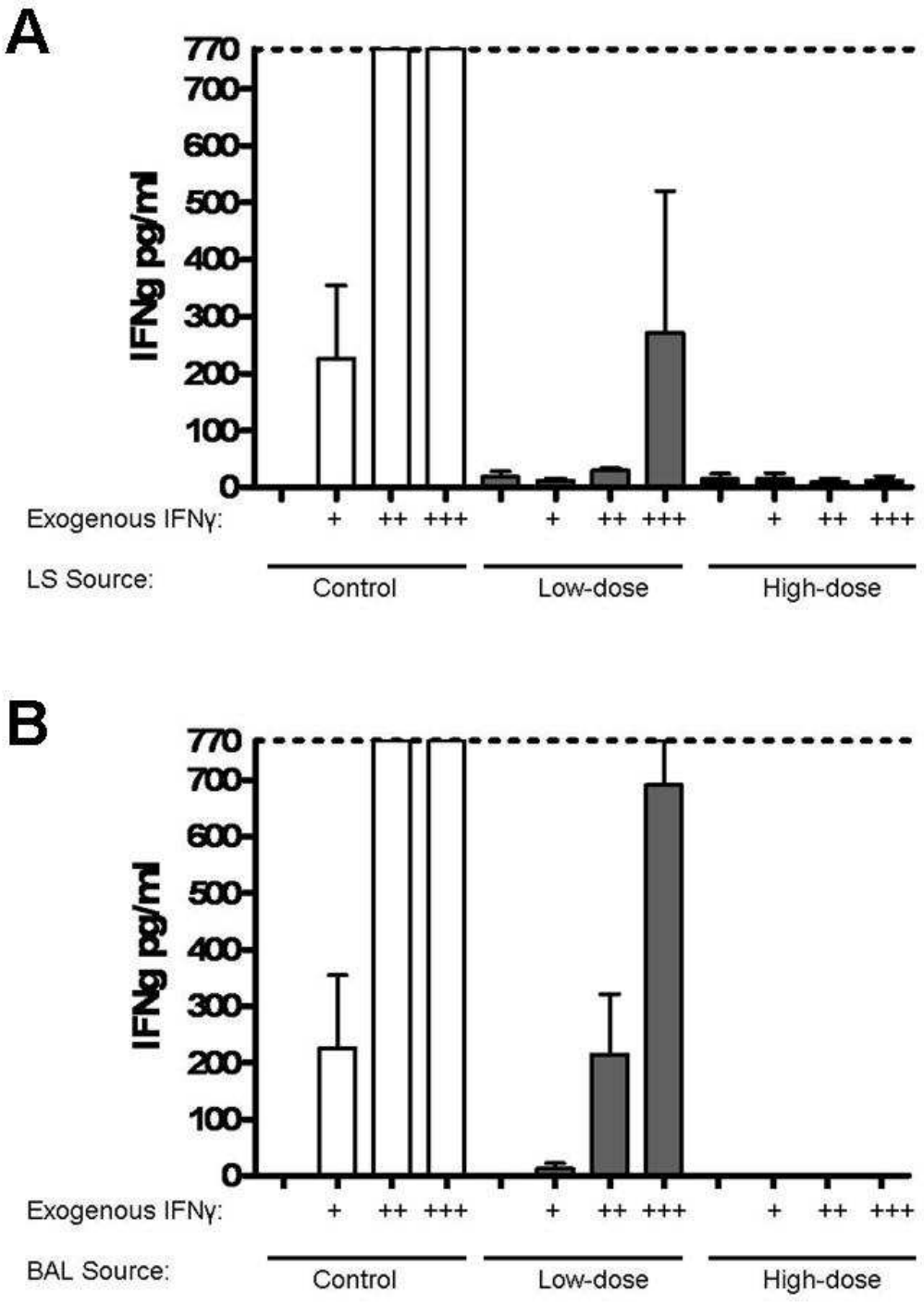


Figure 18. LS and BAL from CPXV-infected mice inhibit IFNγ cytokine measurements. Exogenous recombinant mouse IFNγ was added to LS or BAL from 3

individual mice. Increasing amounts of IFNγ were added to determine how much was inhibited. Pluses indicate the amount of interferon added to the sample; (+) indicates 150pg/ml, (++) indicates 1000pg/ml, and (+++) indicates 1500pg/ml. The upper limit of detection is shown by a dotted line. In the high-dose LS, no IFNγ was measured in the samples even with the highest amount of cytokine added exogenously.

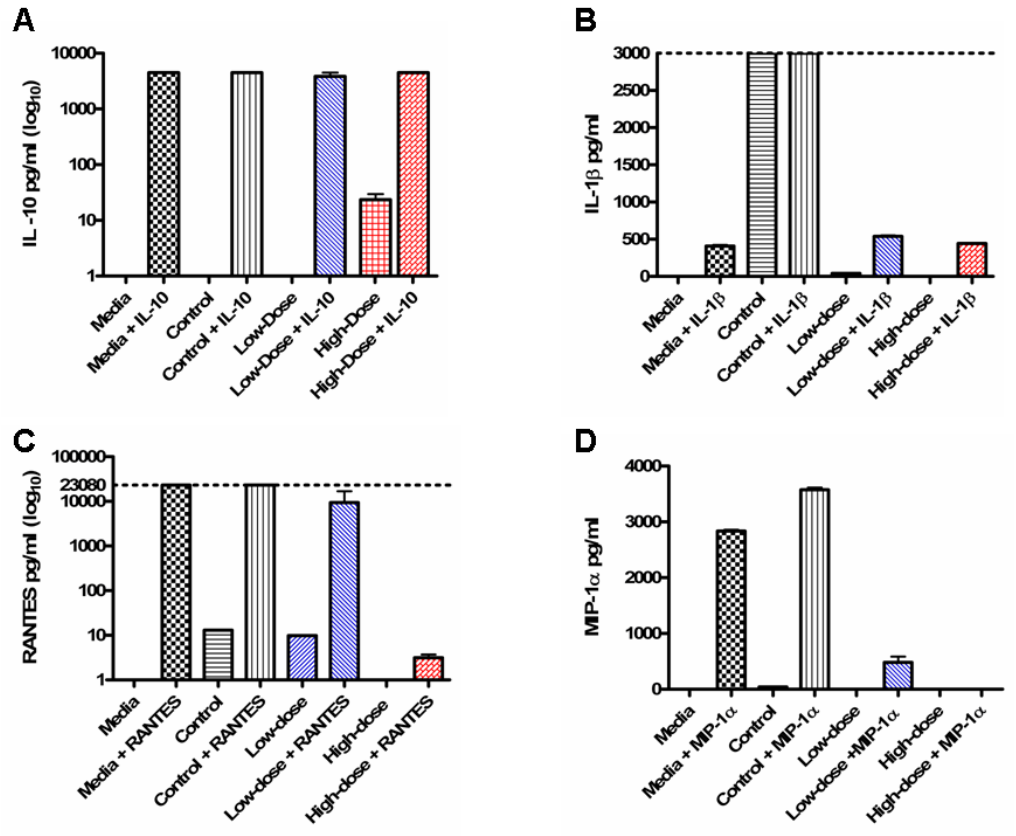


Figure 19. CPXV inhibits detection of the chemokines RANTES and MIP-1*α*, but not IL-1β or IL-10. Known amounts of exogenous cytokines were added to LS from 3 individual mouse lungs per group. For IL-10 (A) and IL-1β (B), the amount added was equal to the amount detected, indicating no inhibition of cytokine measurement by luminex. For RANTES (C) and MIP-1α (D), the detected cytokine was less than expected, indicating inhibition by CPXV.

Table 3. Complete list of cytokines tested in CPXV inhibition assay and results. A cytokine is inhibited if the amount measured was at least 25% lower than the same amount added to media. Not inhibited refers to no reduction in measured cytokine compared to media. Results for IL-12 were inconclusive because of sample variation in all groups and less than 5pg/ml measured in the media + IL-12 sample.

Inhibited	Not Inhibited	Inconclusive
IFNα	IL-1β	IL-12
IFNβ	IL-10	
IFNγ		
RANTES		
MIP-1α		

AMs are a potential source of immunosuppressive cytokine IL-10.

IL-10 has been well-characterized as an immunosuppressive cytokine and therefore worth investigating as an immunosuppressive factor during CPXV infection. It was also the only cytokine from our panel with a greater concentration in LS and BAL from the high-dose infected mice (up to 100pg/ml) than in lung fluids from the low-dose infected mice (<10pg/ml). This might explain at least partly the good LALN T cell proliferation present in the low-dose infected mice as compared with the poor proliferation seen in high-dose infected mice. The primary producers of IL-10 in the lungs are AMs, although it can also be produced by regulatory T cells, monocytes, mast cells, and tolerogenic DCs. IL-10 down-regulates the immune system by decreasing MHC class II expression, blocking the effects of the pro-inflammatory cytokines such as IFNγ and TNFα, and blocking NFκB signaling. To determine whether CPXV could directly induce IL-10 production in AMs, we isolated AMs by collecting adherent cells from lavage from naive mice and infecting them with CPXV at an MOI=1 or MOI=10. Supernatants were removed at 24hpi and analyzed for IL-10 and other cytokines. Along with AMs, we also analyzed supernatants from sort-purified lung tissue DCs also infected *in vitro* for 24h. A subset of pulmonary DCs is tolerogenic and known to produce IL-10, leading to a regulatory T cell response and anti-inflammatory cytokine production [36]. This is the desired response during allergen exposure or chronic lung inflammation. Although not a desired response during an active infection, it has been described as the primary DC response during a pulmonary infection with Mycobacterium bovis bacillus Calmette-Guerin (BCG) [35]. Post CPXV exposure, lung DCs produced no IL-10 at an MOI=0, but at an MOI=1, they produced

~7pg/ml, and at an MOI=10 they produced only ~13pg/ml (Figure 20A). In contrast, AMs infected at an MOI=1 produced ~10pg/ml IL-10 and AMs infected at an MOI=10 produced ~60pg/ml IL-10 (Figure 20B). Both cell types showed a dose response to CPXV supporting the concept that CPXV induces an anti-inflammatory response rather than a pro-inflammatory response. However, it should be noted that for both DCs and AMs, the amounts of IL-10 secreted were very small and so the actual impact of IL-10 on DO11.10 T cell proliferation cannot be determined from this assay. A number of other cytokines were analyzed from AM and DC supernatants, but no other cytokines could be detected in line with previous studies in our laboratory with human DCs in which CPXV was shown to be incapable of stimulating cytokine or chemokine secretion by monocyte derived and peripheral blood myeloid and plasmacytoid DCs [124]. In fact, infected DCs stopped producing MIP-1α and RANTES (or produced it and vCCI sequestered it). Furthermore, uninfected AMs and AMs infected at an MOI=1 produced a small amount of TNFα, while almost none was measured from those infected with an MOI=10. Taken together, these data show that CPXV-infection causes AMs to take on a more immunosuppressive role with elevated IL-10 and decreased TNFα, while the virus fails to induce DC cytokine secretion, although it is possible that the DCs do secrete cytokines, but they cannot be measured because of sequestration by CPXV encoded proteins secreted by directly infected DCs.

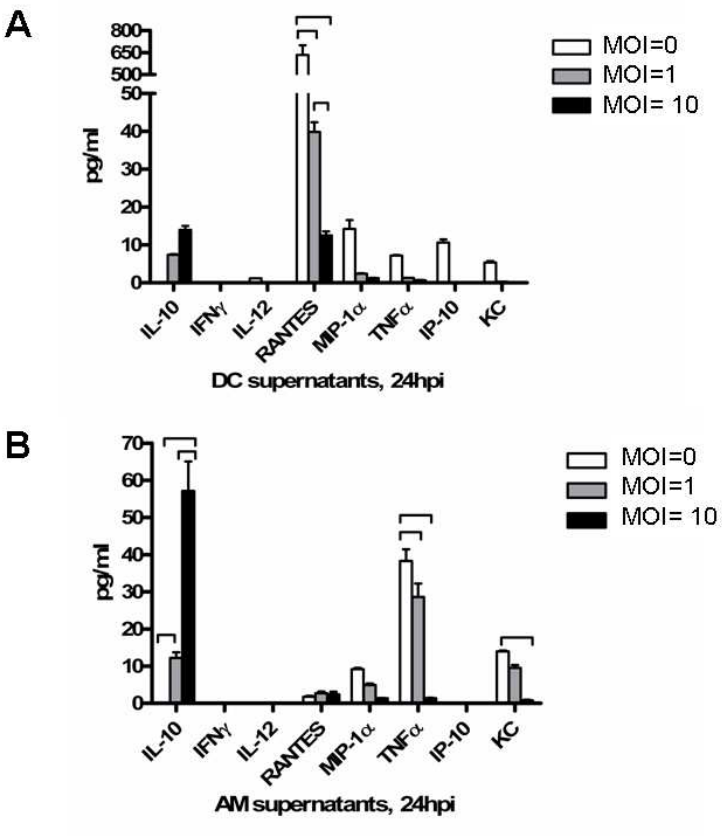


Figure 20. Cytokine response of DC (A) and AM (B) following CPXV infection in vitro. Supernatant from sort-purified pulmonary DC, or AM recovered from lavage, were examined by luminex. N=3 wells per treatment, with $1x10^5$ cells/well in 0.5 ml/well, from 1 experiment. Bars indicate a significant difference at $P<0.05$ by 2-way ANOVA.

The absence of host IL-10 in a high-dose CPXV lung infection fails to allow OVA peptide-induced T cell proliferation in LALNs.

Cytokine data suggested that IL-10 may play a very important role in CPXV infection as it is measured in LS, produced by AM infected *in vitro*, and not inhibited by CPXV proteins. IL-10 also shuts down antigen presentation and DC function, which makes it a prime candidate for inhibiting DO11.10 T cell proliferation in the adoptive transfer model. To examine this issue, we obtained IL-10 knockout mice (IL-10-/-) on a BALB/c background to further study the importance of IL-10 *in vivo* during CPXV infection. We hypothesized that mice lacking IL-10 would have a more robust immune response to CPXV and clear the virus faster, and as a side effect OVA peptide induced T cell proliferation could occur even in the high-dose infection group.

Infected IL-10-/- mice had no difference in survival compared to BALB/c mice, and the low-dose infected group survived while the high-dose group succumbed to infection. Consistent with that, only viral dissemination to the spleen was observed in the high-dose group (Figure 21A). Next we used the DO11.10 T cell adoptive transfer model to determine if IL-10 was required to prevent T cell proliferation. As before, mice were infected intratracheally with CPXV (d0), intravenously inoculated with DO11.10+/- T cells (2dpi), intranasally inoculated with OVA (3dpi), and LALN harvested (6dpi) to examine DO11.10 T cell proliferation in the LALN. The results indicated that IL-10 did not play a role in suppressing T cell proliferation, as cell proliferation was low in both infected groups (Figure 21B). One issue discovered in utilizing IL-10-/- mice was poor

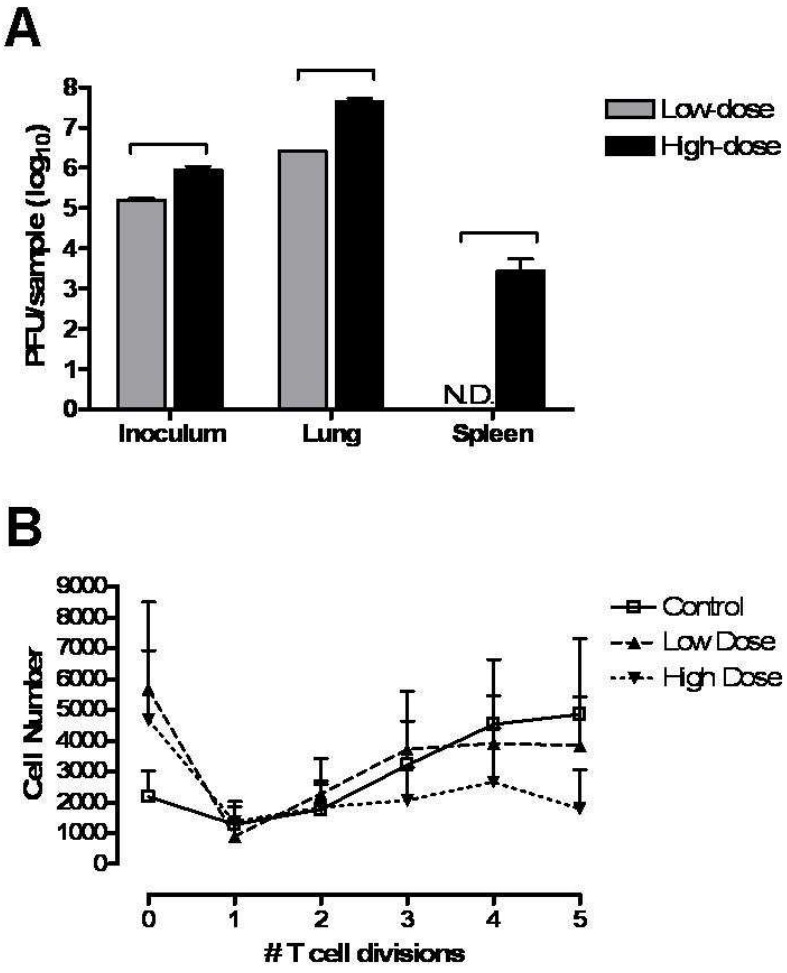


Figure 21. DO11.10 T cell proliferation is inhibited in high-dose infected IL-10-/- mice. (A) Titers of lungs and spleens 6dpi in IL10-/- mice, indicating the high-dose group had virus disseminate to the spleen. A single inoculum for each dose was tested in duplicate, and lungs and spleens tested with 3 mice per group. N.D. = not detected. Shown is the average +/-SEM. (B) The number of T cell divisions in each group is represented graphically. X=0 indicates that no cell division has taken place; X=5 indicates that each cell has undergone 5 divisions.

overall recruitment of the CFSE labeled DO11.10 T cells to the LALN, so that even uninfected mice had lower cell recruitment than would be seen in a wild type BALB/c mouse. The reduced LALN numbers likely results from early spontaneous colitis in IL-10-/- mice [143] and inflammation in the gut resulted in enhanced DO11.10 T cell recruitment to the mesenteric lymph node which competed with recruitment to the LALN (Supplemental Figure 5). However, the data is robust enough to conclude that CPXV can prevent OVA peptide induced DO11.10 T cell proliferation in LALN despite no IL-10 in the host. The high-dose infection data in the knockout mice was similar to that in the wild type BALB/c, and dissemination occurred only in the high-dose infection (Figure 21B). Previous data from our lab showed that BALB/c mice given anti-IL-10 antibodies survived infection as well as isotype-treated mice [123], and the current experiments showed that IL-10-/- handle CPXV infection similarly to BALB/c mice. Together these data indicate little to no role for IL-10 as the suppressor of DO11.10 T cell proliferation during CPXV lung infection.

A high dose pulmonary infection with CPXV lacking the IFNγR gene also results in suppression of DO11.10 T cell proliferation in LALNs.

Because very little host cytokine was measured in LS and BAL, but the inhibition assay suggested a large amount of CPXV protein is secreted into LS and BAL, it was possible that viral immunomodulatory proteins had a greater effect on immune suppression than any host factor. A key host cytokine needed to survive a pulmonary CPXV infection is IFNγ. Early IFNγ produced by NK cells upregulates antigen presentation on DCs,

phagocytosis by AMs, and induces other immune cells to activate and produce pro-inflammatory cytokines. Importantly, a preliminary study showed IFNγ was required to produce an effective immune response to survive a low-dose infection. If BALB/c mice infected at a low-dose received anti-IFNγ at -1dpi, survival was decreased (Figure 8). Anti-IFNγ had no effect if given 7dpi (data not shown), suggesting that the cytokine was needed early in the immune response to control infection. Because the virus produces IFNγR, all host IFNγ may already be sequestered by the viral proteins later in infection, but early production of IFNγ may act on immune cells before viral replication has taken place and large amounts of viral IFNγR are produced. Therefore, there is reason to suspect IFNγR is an important virulence factor, and a possible contributor to poor OVA peptide-induced T cell proliferation in the LALNs of high dose infected mice in the adoptive transfer model.

We obtained a viral mutant with the single gene encoding viral IFNγR disrupted. This virus has similar replication kinetics in Vero cells to wild-type virus (WT-CPXV). We also found that following intratracheal inoculation, similar viral titers were present in the lung 6dpi after either low-dose or high-dose infection doses compared to the same doses given to BALB/c mice. Unlike WT-CPXV (or a revertant strain in which the disrupted gene was reinserted into the viral genome), lung fluids collected mice infected with the CPXV IFNγR mutant (ΔCPXV-IFNγR) did not inhibit IFNγ in a cytokine inhibition assay, indicating the appropriate gene was knocked out (Figure 22A). In contrast, ΔCPXV-IFNγR behaved like WT-CPXV as lung fluids from mice infected with either strain inhibited MIP-1α and did not inhibit IL-10 (Figure 22B-C). In collecting the lung

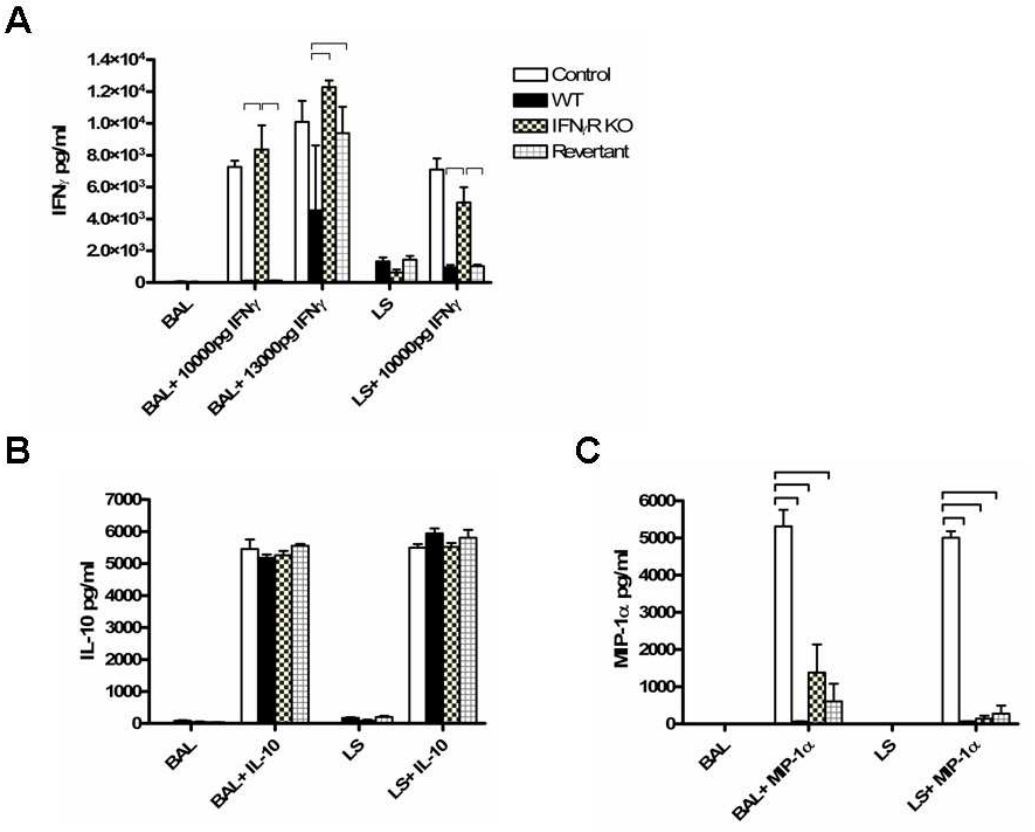


Figure 22. ΔCPXV-IFNγR virus does not inhibit IFNγ added to LS and BAL samples. (A) IFNγ measurements after adding a known amount of exogenous IFNγ to samples. (B) MIP-1a is inhibited by all three viruses. (C) IL-10 is not inhibited by any of the viruses. Samples were run in duplicate with 3 mice per group. Shown is average +/-SEM. Bars indicate $P<0.05$ by two-way ANOVA.

fluids for measuring cytokine inhibition in the Luminex assays, we added a protease inhibitor cocktail to both the LS and BAL to ensure no loss of cytokines. The protease inhibitors did prevent cytokine degradation, as endogenous IFNγ was elevated in this assay as compared to previous studies with the WT-CPXV when protease inhibitors were not added. However, the cytokines IL-6, IL-12, RANTES, and MIP-1α were measured at levels similar to the levels present in samples without protease inhibitors (Supplemental Figure 6). Interestingly, more endogenous IFNγ was measured in LS collected from the lungs of WT-CPXV and revertant infected mice, whereas only half as much was present in LS collected from ΔCPXV-IFNγR infected virus. Despite some IFNγ present, exogenous IFNγ added to LS from WT-CPXV and revertant was inhibited as expected, and the amount of exogenous IFNγ measured in LS from the mutant was similar to the amount added. Therefore, the mutant CPXV should be incapable of inhibiting IFNγ in vivo, which might allow for a proficient anti-viral cytokine response and in the adoptive transfer model enhanced DO11.10 T cell proliferation following OVA peptide intranasal innoculation.

ΔCPXV-IFNγR behaves similarly to WT-CPXV and revertant in DO11.10 T cell adoptive transfer model.

We next assessed whether the mutant virus lacking IFNγR would be attenuated in the adoptive transfer model. We hypothesized that the mutant would have a less suppressed response and allow for some T cell proliferation to occur in the high-dose infection. Mice were infected with either high-dose WT-CPXV, ΔCPXV-IFNγR, or revertant virus

(0dpi), received DO11.10 T cells intravenously (2dpi), OVA peptide intranasally (3dpi), and were harvested (6dpi) to examine the LALN for proliferating DO11.10 T cells. Control uninfected mice showed a good proliferation response, but none of the high-dose infected groups showed much proliferation (Figure 23A). All infected groups had a significantly lower amount of T cells demonstrating 5 divisions compared to controls, and no infected group was different from the other. Mice infected with ΔCPXV-IFNγR virus had fewer CFSE+CD4+ T cells recruited to the LALN compared to mice infected with the revertant virus (Figure 23B), by an unknown mechanism. In the high-dose infections with all three viruses, the viral titers in the lungs at 6dpi (figure) and the rate of death was the same, so disrupting one immunomodulatory protein could not negate the suppressive effects of other viral immunomodulatory proteins.

Despite the inability to inhibit IFNγ, the mutant virus contains many other immunomodulating proteins, which must account for the inability of the host to overcome the immunosuppression induced by the virus. In the high-dose infection, fewer T cells were recruited to the LALN after adoptive transfer in the ΔCPXV-IFNγR infected group compared to the revertant (and a similar increase in LALN cells is seen with WT-CPXV). The IFNγR encoded by CPXV seems to have minimal influence on the outcome of DO11.10 T cell proliferation in the adoptive transfer model. Other virulence genes may play a role in immune suppression, and will be discussed in detail below.

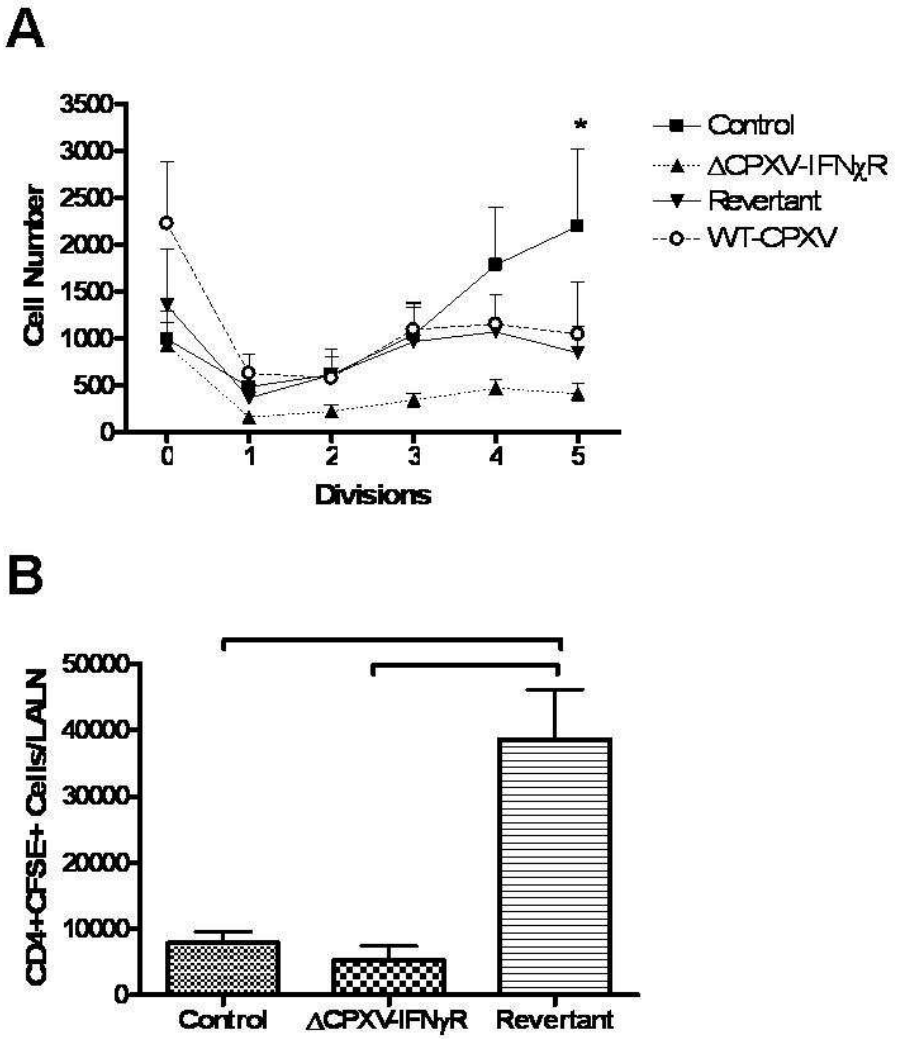


Figure 23. T cell proliferation is similar between ΔCPXV-IFNγR, WT-CPXV and revertant in an adoptive T cell transfer experiment, but fewer total CD4+CFSE+ T cells were found in the LALN in the ΔCPXV-IFNγR group. (A) Mice were infected (d0), received $1x10^6$ DO11.10 T cells (d2), intranasal OVA (d3), and LALN were harvested (d6) for analysis of T cell proliferation. Control mice had more T cells that had undergone 5 divisions than any of the virally-infected groups, and there was no difference

between infected groups. (B) Mice infected with ΔCPXV-IFNγR had significantly fewer total CD4+CFSE+ cells compared to mice infected with the revertant virus. Control N=8, A686 N=8, A687 N=9, WT N=3, combined from 3 separate adoptive transfer experiments.

V. Methods

Virus

CPXV (Brighton Red strain), ΔIFNγR-CPXV virus, and revertant virus were provided generously by David Pickup (Duke University), and GFP-CPXV was acquired from ATCC. All strains were grown in Vero E6 cells as follows: Cells were grown in complete DMEM [supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM nonessential amino acids, and 2mM HEPES buffer] in 1000cm^2 roller bottles at 37°C, 5% CO_2 until 95% confluent and then infected with CPXV at 0.1 virus particles per cell. After a 2 hr adsorption step, media containing virus was removed and replaced with complete DMEM with 2.5% FBS. After 20 hr, cytopathic effect was observed and the roller bottles were placed at -80°C overnight. After thawing, medium and cells were centrifuged at 3500g for 20min at 4°C. Pellets were resuspended in 1mM $MgCl_2$/ 10mM Tris HCl pH 9.0 and homogenized in a dounce homogenizer on ice. Resulting fluid was centrifuged at 150g for 5min to remove nuclei. Virus was purified by layering over 36% sucrose/10mM Tris HCl and centrifuging at 13,500rpm for 3 hr at 4°C. Virus pellet was resuspended in 10mM Tris HCl pH 9.0 and aliquotted (0.5ml each) into Cryovial tubes (Nalgene). Titer was determined by plaque assay using 3 randomly chosen vials with 3 replicates each, and averaging the plaque-forming units (PFUs).

Plaque assay

Vero E6 cells were plated in 48 well plates (Corning) and allowed to grow to confluency in a humidified 5% CO_2 incubator at 37°C. Organs were harvested into 1ml 1mM $MgCl_2$/10mM Tris HCl pH9.0 and frozen at -80^oC until determination of PFUs. Samples were thawed in a 37^oC water bath, homogenized using a BeadBeater (Biospec Products, Bartlesville, OK) and sonicated for 30 sec. Samples were serially diluted with complete DMEM with 2.5% FBS, and 100µl of each sample was placed on the Vero cell monolayers in duplicate. Virus was allowed to adsorb on cell monolayers in a humidified 5% CO_2 incubator at 37°C for 1.5hr. Samples were then aspirated and replaced with 500µl of complete DMEM with 2.5% FBS. Plates were incubated for 24-26 hours at which point overlay media was aspirated and the wells were stained with 0.1% (w/v) crystal violet in 5% total volume ethanol and 94.9% total volume physical formalin (10% buffered formalin) for 30min. After drying the plate overnight, plaques were counted using a light box.

Mice

Female BALB/c mice were obtained from Harlan and housed in the UNM specific pathogen free facility and used in experiments between 8-12 weeks of age. DO11.10 mice were bred in-house from breeding pairs initially obtained as a kind gift from Dennis Loh [127]. Male DO11.10+/- mice were bred to female BALB/c mice, and offspring were selected for expression of the OVA specific-TCR by immunophenotyping blood samples. Female IL-10-/- mice were obtained from Jackson Laboratories and used between 8-10

weeks of age. All animal studies were performed as described under University of New Mexico IACUC approved protocols.

Inoculations

For intratracheal inoculations, 2,2,2-tribromoethanol-anesthetized 8-12 wk old female BALB/c mice were inoculated intratracheally with 50μL of CPXV in DMEM containing 2.5% fetal bovine serum (FBS, Hyclone); control mice were inoculated with 2.5% FBS in DMEM only. Mice were intratracheally injected as described previously [114]. Two randomly selected mice were euthanized 15min after surgery, lungs removed and homogenized, and the samples tested by plaque assay to determine deposition of virus in the lungs. For intranasal inoculation of OVA peptide (Ovalbumin peptide 323-339, 95% purity, BioPeptide, San Diego, CA), isoflurane-anesthetized mice received 10ug/ms OVA peptide in 50μl of liquid placed over their nasal passages with a pipette and observed for inhalation.

Adoptive T cell transfer

DO11.10+/- mice were euthanized and spleens removed. Splenocytes were treated with red blood cell lysis buffer and run through a nylon wool column containing warm RPMI (Invitrogen) supplemented with 10% FBS to enrich for T cells. Enriched cells were incubated with 10mM CFSE (BD Biosciences) in PBS plus 0.05%BSA at 37ºC for 8 min. Cells were washed twice with RPMI medium and twice with sterile saline. CPXV-

infected and control BALB/c mice received $1x10^7$ cells in 0.5mL sterile saline via tail vein inoculation. A sample of cells was retained for FACS analysis to assure CFSE uptake.

Lung and lymph node harvest and staining

Mice were euthanized with CO_2, and lungs or LALNs harvested into Hanks' Balanced Salt Solution (HBSS). LALNs were homogenized between frosted glass slides and the cell suspensions treated with red blood cell lysis buffer (0.15M Ammonium Chloride, 1mM Potassium carbonate, 0.1mM disodium EDTA in sterile water). The remaining cells were counted and resuspended in staining buffer (2% FBS, 0.2mM EDTA in PBS). Lungs were digested with DNAse (0.03mg/ml)/collagenase (0.7mg/ml) in medium (RPMI 1640 supplemented with 10% FBS, 100U/ml penicillin/streptomycin, 2mM L-glutamine, 1mM sodium pyruvate, 1mM nonessential amino acids, and 2mM HEPES buffer) for 1hr at 37°C. Live cells were isolated by 30% Percoll (GE Healthcare) gradient centrifugation. $1x10^6$ cells were added to each staining tube and treated with purified anti-CD16/32 (Fc block, BD Biosciences) for 10min prior to adding additional antibodies. Cells were stained using monoclonal antibodies CD11c-PE, CD11c-FITC, CD80-PE, CD86-PE, IA-PE, CD3-PE, CD4-PerCP (BD Biosciences), KJ1-26-Alexa647 (BioLegend), and isotype controls on ice for 20min, washed 3 times, and resuspended in 0.5% paraformaldehyde until analysis. Samples were run on a four-color capable FACSCalibur (BD Biosciences) and analyzed with Winlist software.

Lung fluid collection

At 6dpi, mice received an intraperitoneal injection of heparin followed by a lethal injection of the 2,2,2-tribromoethanol. Lungs were lavaged via the trachea with 1ml complete medium or 1ml PBS plus protease inhibitor cocktail (1:100 dilution of Sigma #P8340). The bronchoalveolar lavage fluid (BAL) was centrifuged at 14000rpm for 30min and the supernatant collected. The lungs of the same mice were perfused by injecting 10ml sterile saline into the left ventricle of the heart, after which the lungs were removed and minced with surgical scissors into 2-5mm^2 pieces in 1ml medium or 1ml medium plus protease inhibitor cocktail (1:100 dilution). The minced lung was allowed to incubate 24h at 37ºC to allow cytokines and soluble mediators to be released from the tissue. Supernatant was then removed from the lungs and centrifuged at 14000rpm for 30min to remove cell and/or viral debris. Both BAL and lung supernatant had less than 10 pfu/ml CPXV (the limit of detection) as determined by plaque assay.

Pulmonary DC purification

BALB/c mice were injected IP with heparin and a lethal dose of 2,2,2-tribromoethanol. Lungs were lavaged 3 times with 1mL 0.5mM EDTA/PBS to remove alveolar macrophages. Lungs were perfused to remove blood cells by injecting 5-10mL sterile saline into the left ventricle with a 16g needle until lungs became white. Lungs were harvested, minced into ~2mm^2 pieces, digested with a DNAse/collagenase solution at 37°C for 1hr, and enriched for mononuclear cells on a 2-density Percoll (1.030/1.075)

gradient as previously described [144]. Pulmonary DCs (CD11c+Autoflourescent-) were isolated from the mononuclear cells on a MoFlo flow cytometer (Supplemental Figure 7 shows gating strategy) and used in experiments if purity was ≥85%.

In vitro T cell proliferation assay

Pulmonary DC were sort-purified as described above, and CD4+ T cells were MACS-sorted (Pan T cell isolation kit, Miltenyi Biotec) from spleens of DO11.10+/- mice. Cells were plated in 96-well white opaque plates. T cells were stimulated to divide by adding DCs with OVA peptide (1.0μg/ml final concentration) or Concavalin A (Invitrogen) to the culture wells. BrdU was added to the DC-T cell co-culture 48hr and BrdU uptake was measured 24hr later by chemiluminescent ELISA (Roche Applied Science). The relative light units (RLU) indicated relative cellular proliferation.

Cytokine analysis and inhibition assay

Cytokine measurements were performed by Luminex bead-based multiplex assay using cytokine measurement kits for IL-1β, IL-6, IL-10, IL-12, IFNγ, MIP-1α, RANTES (Invitrogen) or IFNα/β (Panomics). Each sample was run in duplicate using 50μL/well. Standard curves and cytokine measurements were generated on StatLIA software (StatLIA Enterprise 3.2, Brendan Technologies, Inc). For the inhibition assay, luminex standard cytokines (20-plex mixed cytokine standard, Invitrogen #LMC0006) and/or recombinant mouse IFNγ (Invitrogen #PMC4031) were added to LS or BAL in a 96-well

multiscreen filter plate (Millipore product #MAUF01010) and allowed to incubate for 30min at room temperature to allow the added cytokines to bind to any ligands in the LS or BAL. Three different dilutions of cytokines were made containing an increasing amount of cytokine. For example, recombinant IFNγ was added to samples to create a final concentration of 500pg/ml, 1000pg/ml, or 1500pg/ml to help determine approximately how much cytokine was inhibited. LS, BAL, or Medium only were treated with diluent, 500pg/ml, 1000pg/ml, or 1500pg/ml IFNγ. For assays measuring inhibition of other cytokines, the luminex standard cytokine mix was used instead of recombinant IFNγ, and the mix was diluted to produce the same final concentrations of IFNγ that are outlined above. The cytokines measured were compared to a standard curve prepared on the same plate to determine the exact amount of each cytokine added.

Statistical analysis

Statistical analysis was performed as described in figure legends using GraphPad Prism Software (GraphPad Software, Inc; San Diego, CA).

VI. Discussion

Viruses, including orthopoxviruses, have multiple strategies to evade the host immune system. These strategies may reduce the host response not only to the primary infecting virus, but also suppress the immune response to secondary infections. In the present study, we determined whether a pulmonary CPXV infection could alter the immune response to OVA peptide as a surrogate for a secondary pulmonary infection, because this typical orthopoxvirus produces a number of proteins known to interact with the host immune system [99, 101]. We found that CPXV suppressed OVA-specific T cell proliferation *in vivo* during a high-dose, but not low-dose, pulmonary infection and that direct infection of antigen presenting DCs or responder T cells was not necessary. Instead, soluble mediators, either viral-derived, host-derived, or both, likely drained from the infected lung tissue via the lymphatics to LALNs where they suppressed antigen-specific T cell proliferation.

Our *in vitro* studies confirmed and extended the work of others that demonstrated that infection of DCs with orthopoxviruses affected their *in vitro* immune function [125]. Thus, CPXV infection of DCs prior to adding them to cultures of T cells plus antigen reduced the proliferation of the responder T cells. Proliferation was compromised even when there were adequate numbers of noninfected DCs in the cultures to provide optimal proliferation. For example, as shown in Figure 10A., with 12,500 DCs per well at an MOI of 10 that produces a 40-50% DC infection rate, about 6,250 DCs should have been uninfected and the T cell proliferation might have been doubled unless there were factors

in the culture medium inhibiting all DC antigen presentation, T cell proliferation or both. Furthermore, in an overnight culture of DCs with CPXV, IA, CD80 and CD86 were reduced on both infected and uninfected DCs. These *in vitro* studies (Fig. 10D-F) correctly predicted that both uninfected and infected lung DCs in the lungs and LALNs of CPXV-infected mice would demonstrate decreased accessory molecules and that the antigen presenting capacity of LALN cells might be regained once they were removed from the microenvironment of the lung.

Early in our studies we asked whether failed proliferation of DO11.10 T cells in LALNs in the high dose infection might result from viral infection of these cells resulting in either their death or disability. Proliferating T cells can be infected with VACV [95] *in vitro*. However, T cells could not be infected by CPXV *in vitro* and using GFP-CPXV, and we found that no T cells were infected in the LALN at the peak of infection in either the high- or low-dose infections when activation of T cells should have been present. Furthermore, direct killing of the T cells was unlikely to play a role, because the number of CFSE-labeled DO11.10 T cells recovered from LALNs from control and infected groups after the intranasal inoculation of mice with OVA-peptide was equivalent. Thus, we believe that the major cause for the suppression of T cell proliferation during the high dose CPXV infection was the presence of soluble suppressor factors in the LALN. Monkeypox virus, closely related to CPXV, has been shown to suppress CD4+ and CD8+ T cell activation without directly infecting them, but the T cells had to physically contact infected PBMCs; the mechanism was not determined [145].

What were the possible inhibitors of the T cell proliferative response to OVA peptide in the LALNs? We favor the hypothesis that both CPXV-derived and host-derived factors contribute to the suppression. However, because the suppressive effect was so dependent on the viral inoculum dose, the virus-derived factors likely dominated. Among the many immunomodulatory proteins that CPXV encodes are type I and type II interferon inhibitors, four TNFαRs, a chemokine inhibitor and a semaphorin [111, 146-148]. There are also a number of genes that encode proteins of uncertain function that might also have as yet to be described immunomodulatory function [102]. The secreted CPXV type I interferon suppressor protein functions as a soluble IFNα/β receptor. It can affect uninfected DCs, by interfering with IFNα/β-induced cell maturation including upregulation of MHC class I and II and co-stimulatory molecules [147]. Another important viral protein, vaccinia complement-control protein (VCP), also found in CPXV [149], is both secreted by infected cells and expressed on the cell surface [150]. VCP acts in two ways; when cell-associated, it binds complement on the cell surface and prevents lysis of the infected cell, and when secreted, it prevents complement activation and recognition of virus particles for opsonization. Complement is recognized by DCs to aid in phagocytosis and subsequent activation [26]. Not only can the virus directly cause immune suppression by producing viral-encoded immunomodulators, but these proteins can cause the host's own cells to respond inappropriately to the infection.

As mentioned before, IL-10 produced by macrophages is an immunosuppressive cytokine, and was reported to be elevated in the serum [114] and lungs [120] of mice after an intranasal CPXV challenge. TGF-β, especially in conjunction with IL-10, creates

a regulatory anti-inflammatory environment in the lung, and some infectious agents, notably tuberculosis, promote this response [151]. In a pulmonary model of *Francisella tularensis*, macrophages produced prostaglandin E(2) (PGE-2), leading to poor IFNγ and IL-2 responses [152]. PGE-2 can be released in response to apoptotic cell uptake by macrophages [133], and has also been shown to directly inhibit T cell proliferation by blocking calcium flux [153]. Indoleamine 2, 3-dioxygenase is produced by CD11c+ lung cells, and acts to suppress T cell proliferation by depleting tryptophan [154]. However, in our model, IL-10 did not play a role in inhibiting DO11.10 T cell proliferation. Although important in lung homeostatsis, IL-10 may be overshadowed by other cytokine and virokine responses during CPXV infection. Some orthopoxviruses encode a homolog of IL-10, but CPXV does not [101], suggesting other immunomodulators play a more important role for this virus.

We examined a role for the secreted viral protein that binds IFNγ and prevents IFNγ from binding to its receptor on target cells. Recombinant VACV lacking the IFNγ viroreceptor was less virulent in nude mice [155], so there was a potential for the CPXV lacking IFNγR to be less virulent in our model. IFNγ is produced early in infection to activate immune cells in the lung, inhibits macrophage-produced immune suppressing proteins such as IL-10, and facilitates the development of a T_H1 response in LALNs [156]. Although we saw binding of murine IFNγ to the viral IFNγR, as shown in the cytokine inhibition assay, there was no difference in OVA-peptide induced T cell proliferation by DO11.10 T cells in an adoptive transfer experiment comparing the IFNγR CPXV mutant to WT-CPXV. We measured significant binding of IFNγ *in vitro* by lung supernatants

from CPXV infected mice, suggesting a significant concentration of IFNγR was present *in vivo*. However, it should be pointed out that another group showed lower binding affinity of CPXV IFNγR for mouse IFNγ than for rat IFNγ or IFNγ from other mammalian species [138]. It is possible that *in vivo*, the affinity is not high enough for presence or absence of the viroreceptor to make a difference in either immune response in LALNs or in lung viral titers.

Whether they are virus or host-derived, suppressive factors were present in the LS and BAL, suggesting either a dominant soluble factor or a combination of factors were released from infected tissues. Perhaps the BAL samples are most instructive, even though they were not as suppressive, because they contain a real-time snapshot of the soluble factors in the infected lung. BAL from infected mice presumably contained both virokines and host-derived proteins from bronchoalveolar epithelial cells and AMs. Furthermore, in the high dose infection, the majority of AMs was infected and could have been an important source of virokines, as they produced GFP after *in vivo* infection. AMs might also be an important source of suppressive host-derived proteins. AMs have a dual role of both producing pro-inflammatory cytokines in response to infection and then secreting suppressive factors to prevent lung tissue injury. In pulmonary influenza, for example, AMs were required to reduce the lung viral burden, but at the same time inhibited the function of influenza-specific cytotoxic T lymphocytes in the lung [133, 157]. Furthermore, after AMs phagocytose apoptotic cells, they respond by producing IL-10, TGF-β, and PGE-2, and can no longer mount an appropriate innate response to *Streptococcus pneumoniae* [133]. When AMs were depleted prior to pulmonary VACV

infection, mice exhibited higher viral burdens in the lungs, increased lung inflammation, and decreased survival [158]. Although AMs are necessary to control viral burden, necrosis and apoptosis of infected lung epithelium during a CPXV infection may lead AMs to express a significant immunosuppressive function.

A possible contributory mechanism to explain the suppression of DO11.10 T cell proliferation in the high-dose infected mice was that sufficient OVA peptide failed to reach the LALNs. The low number of DCs in the lungs during a high-dose infection compared to the low-dose infection suggests that pulmonary DC recruitment was poor in the high-dose infection. Indeed, CPXV encodes a viral CC chemokine inhibitor (vCCI), which binds 15 CC chemokines, including MIP-1α and RANTES [100], chemokines important in monocytes and DC recruitment. The vCCI protein is currently being explored as a potential therapeutic for asthma because of its potent ability to reduce leukocyte recruitment to the lungs of mice [110]. However, while we observed differences in DC recruitment to the lungs, there were no significant differences in percentages of DCs recovered from the LALNs from any of the groups, suggesting that recruitment to the LALNs where the OVA peptide specific T cell proliferative response occurred was not significantly affected.

Taken together, the data is most consistent with the scenario that OVA peptide-carrying DCs that reached LALNs were unable to stimulate T cells due to low levels of antigen-presenting molecules and the presence of other inhibitors in the microenvironment that directly inhibited T cell proliferation. What are the implications for the host? The

outcome of a high dose pulmonary infection is immune suppression that inhibits the ability of the host to fight both the ongoing viral infection and any secondary infectious agents. Influenza [159, 160] and HIV [161] are both well-known for dampening the host's immune response and leaving the host vulnerable to secondary infections. Unlike a high-dose CPXV infection, a low-dose infection would not result in immune suppression. CPXV was the original smallpox vaccine and the analogy of a low-dose viral infection to vaccination is appropriate. It is closely related to VACV, the vaccine virus used to eradicate smallpox, and to MVA, a severely altered VACV missing most of the immunomodulatory viral genes, which is the current vaccine strain [102]. As long as CPXV (or VACV) infects only a few cells and DC function is not severely compromised, immunity to a subsequent smallpox exposure should be expected. Furthermore, vaccination uses an intradermal route, which in general provides fewer cells for a productive infection unlike the lung where the bronchoalveolar spaces provide access to large numbers of epithelial cells, which result in an efficient highly productive infection. We and others have found that numbers of CPXV virions equivalent to what would be a lethal dose if given by a pulmonary route fail to kill the mouse if given by either a subcutaneous or intradermal route [114]. Instead of succumbing to infection by non-pulmonary routes, they survive and become resistant to a pulmonary infection. This dose effect may also explain the attenuation of mutants that have a decreased ability to proliferate *in vivo*. The may not replicate quickly enough to generate the amount of immunomodulatory proteins needed to sequester pro-inflammatory proteins produced by the host. Thus they are unable to generate enough immunomodulation to overcome the host response, resulting in the host clearing the virus and recovering.

In summary, a pulmonary CPXV infection inhibited the immune response to a non-viral antigen, and the response depended on the viral inoculum. A high-dose infection leads to an immunosuppressive microenvironment in the lungs and LALNs, and DCs or T cells did not need to be directly infected to account for the failure of OVA peptide-specific T cells to divide in response to OVA peptide. Rather, the infected lung produced immunosuppressive factors, which likely drained to LALNs and suppressed an antigen specific T cell response. We tested two potential factors, host-derived IL-10 and CPXV derived IFNγR, and found that neither are responsible for the failure of DO11.10 T cells to proliferate during a high-dose infection. This model will provide an important tool for dissecting the host pathogen interactions during a CPXV infection and can be used to examine other pathogens as well.

VII. Summary and Future Directions

In this work I established a murine model to assess if an ongoing pulmonary orthopoxvirus infection could suppress an immune response to a second, non-viral, antigen. With intranasally delivered OVA peptide as the second antigen, I found that a high-dose, but not a low-dose, CPXV infection inhibited OVA peptide-specific T cells in LALNs from dividing. This led to a number of experiments to elucidate what part of the immune response was inhibited. Previous data in the lab, as well as from outside groups, had established the potential for orthopoxviruses to inhibit DC function. As DCs are required for naïve T cell activation, we looked at DC and T cell interactions *in vitro* and found that infected DCs, provided with OVA peptide, could not stimulate OVA peptide specific DO11.10 T cells to divide. Using GFP-CPXV, I found that only a portion of DCs were infected *in vitro* as shown by their becoming GFP+, but a majority had a lower MFI of I-A, CD80, CD86. *In vivo*, a similar phenomenon occurred, where fewer DCs in the lung and lymph node expressed antigen-presenting and accessory molecules compared to controls. In the lung, few DCs were infected, and in the LALNs, no infected DCs were present. This led to a new hypothesis: viral proteins secreted from infected lung cells or host-derived anti-inflammatory molecules could drain to LALNs and suppress DC function there. In support of this hypothesis, I showed that LS and BAL could suppress uninfected DC function. Luminex analysis showed that LS from high-dose infected mice had higher IL-10 than low-dose or control mice, with few pro-inflammatory cytokines. However, infected IL-10-/- mice looked similar to BALB/c in the OVA peptide T cell adoptive transfer model. I ruled out IL-10 as a dominant suppressive factor. Finally, I

examined one viral factor that had a potential to impact the immune system, CPXV IFNγR. Using a CPXV mutant lacking this potential immunomodulatory protein, I found that OVA peptide T cell proliferation was actually lower in the high-dose group than the low-dose, and the virus behaved similarly to wild-type virus in terms of its ability to replicate in the lung and kill its host. Therefore, the dominant suppressive factor was neither host IL-10 nor CPXV IFNγR, but it (or they) was secreted from infected lungs and was apparently able to alter DC maturation in the lungs and/or drain to the LALN to affect DCs there.

A number of experiments would help elucidate the mechanism of inhibition of OVA peptide T cell proliferation following intranasal antigen deposition during a high-dose CPXV infection. As our IL-10-/- mouse and ΔIFNγR-CPXV virus experiments show, the model system lends itself to studying subtle immune defects after infection, and could be used to study other mutant viral strains. By applying other CPXV single gene mutants and mouse gene knockouts, this model can be used to investigate the impact of specific virally encoded factors and/or host proteins on the development and/or maturation of an immune response during a CPXV infection. Because factors secreted by infected cells in the lung can influence uninfected cells in the LALNs, I would focus specifically on CPXV proteins known to be secreted by infected cells. For example, CPXV encodes a type I IFN binding protein, and the VACV equivalent protein (B18) suppresses DC activation in the presence of IFNα and IFNβ [147] A mutant CPXV lacking the type I IFN binding protein might be attenuated in the adoptive transfer model because IFNα may be a more important mediator in this model than IFNγ. CPXV, depending on strain,

encodes 3-4 different TNF-binding proteins, CrmB, CrmC, CrmD [99], and CrmE [146], which suggests TNF regulation is very important for CPXV survival and replication in the host. The CPXV strain used in this work, CPXV Brighton Red, does encode all four TNF-binding Crm proteins [146]. TNF is produced mainly by MΦs and T cells and acts on immune cells to promote survival and an anti-viral state, while binding to non-immune cells to encourage apoptosis of infected cells. CrmB, CrmD, and CrmE are all secreted proteins that could bind TNFα produced *in vivo*. Ultimately, this could prevent TNFR-induced signaling in DCs, which induces NFκB and DC maturation. CPXV lacking one or more Crm proteins may be attenuated *in vivo*, and support DO11.10 T cell proliferation in the adoptive transfer model.

The *in vitro* DC-T cell co-culture is a simpler system that could help pinpoint the suppressive factor. To rule out most host-derived suppressive factors, one could collect supernatant from infected Vero cells and use that as a part of the medium (in decreasing concentrations) in the DC-T cell cultures. Using Vero cells would help to rule out a role for host proteins although one would need to test what possible Vero-derived proteins might also accumulate. To further break down which factors are responsible, Veros could be infected, and supernatant removed at 2hr, 6hr, and 24hr. If the supernatants are suppressive even at 2hr, an early gene product is likely responsible for suppression. The temporal gene transcription for orthopoxviruses is well-characterized, and promoters with corresponding proteins are well known for most orthopoxviruses [162]. If only the 24hr post-infection supernatant was suppressive, it would suggest a late gene product is responsible for the suppression. One disadvantage of this system is that the late

supernatant contains protein from all stages of replication, so is less informative if suppression is found with 24hr post-infection supernatant.

Besides finding the suppressive factor, more work is necessary to examine the infected lung microenvironment effect on DCs and T cells. The experiment showing LALNs removed from the infected host are able to stimulate DO11.10 T cells means that resident DCs can overcome the suppression. If the DCs are truly only transiently affected by the milieu, mice could be infected with a low-dose and high-dose of virus, and LALN harvested 6dpi. The LALN would then be washed and plated for 24 hrs, 48 hrs, or 72 hrs in fresh medium. At each time point, cells could be examined by FACS to determine the level of I-A, CD80, and CD86. If the cells regain expression of these surface markers, it means the DCs are able to reverse the effects of the infected lung microenvironment. If the surface markers remain the same, other explanations could still account the experimental results. For instance, removing the LALNs and creating a single-cell suspension may allow resident LALN DCs more access to the T cells and peptide than can occur *in vivo* during infection. Also, the ex-vivo LALN experiment only showed that APCs in the lymph node could present antigen to naïve T cells from an uninfected mouse. It is possible the T cells within the infected mouse are not capable of responding. To test this, I could perform an adoptive transfer experiment, where the mice are infected (d0), given CFSE-labeled DO11.10 T cells (d2), and innoculated with OVA peptide (d3) as in previous experiments to recruit the DO11.10 T cells to the LALN. However, at the harvest (d6), the LALNs would be removed, washed, and cultured with exogenous OVA peptide. If the infected lung milieu caused suppression of DO11.10 T cell proliferation in

the high-dose infection, the transferred T cells should be able to proliferate *ex-vivo* without the suppressive factors present.

In the adoptive transfer model, I did not determine if the peptide was loaded onto DC in the high-dose infected group or if the DCs that migrated to the LALN carried antigen. It would be possible to track the peptide by linking it to GFP (or similar fluorescent molecule), so that if DCs pick up the antigen, they would become GFP+. Other groups have shown that CD11c+ cells will pick up OVA peptide-GFP in the lung and traffic to the LALN [12]. In the high-dose infection in mice, it is possible that DCs taking up OVA peptide are being killed in the lung, or the DCs have OVA peptide displayed on MHC class II molecules but do not have enough MHC class II or co-stimulatory molecules to stimulate T cells once the DCs reach the LALN.

Besides discovering the fate of OVA peptide in the high-dose infection, it would be interesting to use whole OVA protein instead of OVA peptide in the adoptive transfer model, and determine if the whole protein acts similar to peptide. The peptide only shows that DCs can or cannot present peptide and stimulate naïve T cells. It has been shown that OVA peptide can directly bind MHC class II on the plasma membrane, and requiring no internalization of the peptide [128]. Giving whole protein requires the DC to process the protein first and then present. In the high-dose infection, because the peptide is not sufficient to stimulate T cells, it is likely whole protein would not be sufficient either. In the low-dose infection, OVA peptide is sufficient to stimulate, but it is unknown if the DCs have any defect in processing.

Although I did not detect CPXV infection in T cells, the microenvironment could directly impact the ability of T cells to respond to DCs carrying antigen. I could test this by incubating the T cells in LS and BAL from infected animals while stimulating the T cells to divide using anti-CD3 and anti-CD28 antibodies, which mimics activation by antigen-presentation. If the T cells incubated in LS and BAL from infected mice cannot proliferate, it could indicate T cells are directly impacted by the microenvinoment independent from DCs.

Another possibility is that DCs presented antigen, but did not secrete T_H1 inducing cytokines such as IL-12 or IL-18. In response to some cytokines, DCs become tolerogenic and down-regulate T cell responses or cause naïve T cells to differentiate into inducible Tregs [163]. Murine Tregs prevent maturation, including upregulation of MHC class II, CD80, and CD86, of immature DCs that they contact [164]. Tolerogenic DCs (tDCs) can be induced by IL-10, IL-10/TGFβ, and IL-10/IL-6 [36], and our cytokine data also showed a significant increase in IL-10 and IL-6 in the high-dose LS. More recently, human tDCs have been generated without IL-10, but TLR7/8 stimulation is needed during differentiation [165], and it is still unknown what, if any, TLRs can be stimulated by CPXV in murine cells. Although tDCs display similar MHC class II, CD80, and CD86 on the surface compared to conventional DCs, they have less CD40 and low IL-12 production, and may express programmed death ligand-1 (PD-L1) and programmed death ligand-2 (PD-L2) [165]. There is also evidence that plasticity exists and a tDC may revert back to a less suppressive type under pro-inflammatory conditions. Recently the DC

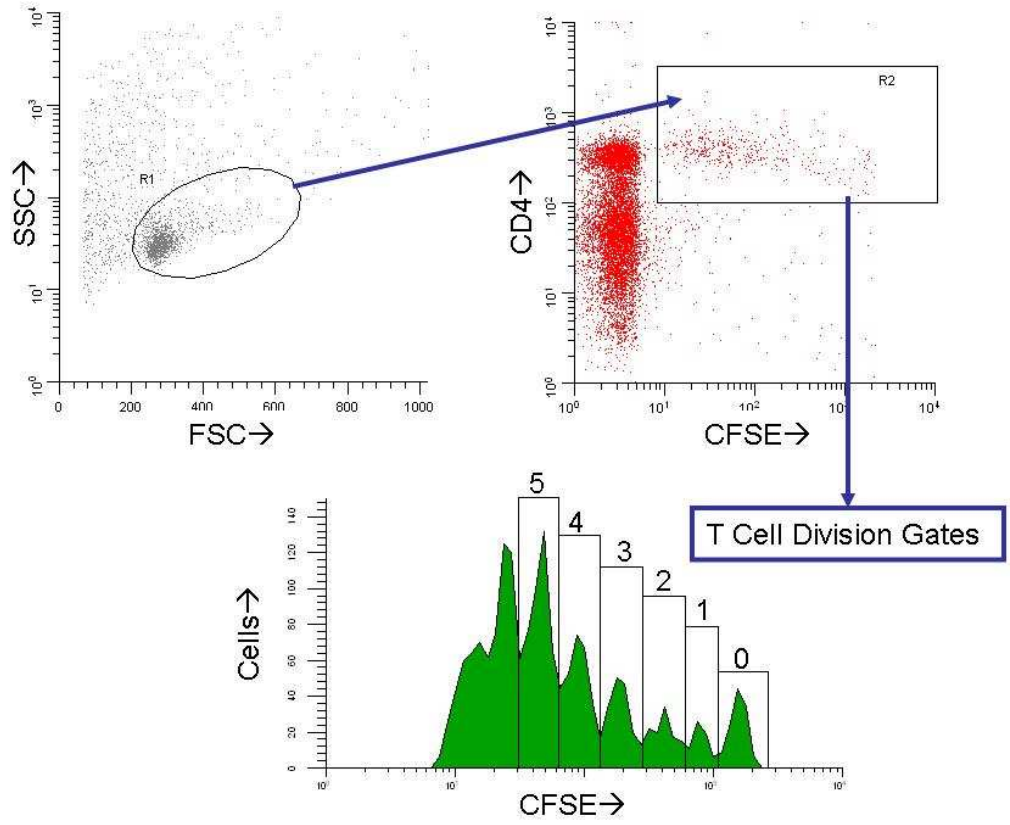
surface marker CD70 has been shown to correlate to a pro-inflammatory cytokine response and a low IL-10 producing DC [166]. CD70+ DCs are also implicated as the most important stimulator of naïve CD8 T cells during an influenza infection, and DCs lacking CD70 are poor antigen presenting cells [167]. This surface marker would be useful to measure during a CPXV infection to determine if the LALN DCs in a low-dose and high-dose infection have a tDC phenotype, and if so, how many compared to control mice.

Another mechanism by which DCs inhibit T cell function is programmed death ligand PD-L1 and PD-L2 [47]. PD-L1 and PD-L2 bind programmed death-1 (PD-1) on T cells and have varying functions depending on other stimulatory or inhibitory molecules present on the interacting DC and T cell [168]. If CD80 and CD86 expression is high on the DC, and PD-L1 is also present, DC can still activate T cells, but the T cells produce less IL-2 [168]. Hepatitis C virus has been shown to increase PD-L1 expression on human peripheral DCs, and the cells expressing high PD-L1 and low CD86 were poor stimulators of T cells in a mixed leukocyte reaction [169]. Cytomegalovirus causes high-level expression of PD-L1 in murine DCs, and these DC are tolerogenic, leading to poor antigen-specific T cell proliferation and T cell anergy [170]. In a murine chronic mycobacteria disease model, DCs in lung lesions express high PD-L1 and PD-L2 and are unable to stimulate naïve T cells, but blocking those molecules *ex vivo* restores the ability of the DCs to stimulate IFNγ production by T cells [171]. Because this pathway is exploited by numerous pathogens, these molecules could also play a role in CPXV infection. Because the LALN DCs in CPXV-infected mice have less CD80 and CD86

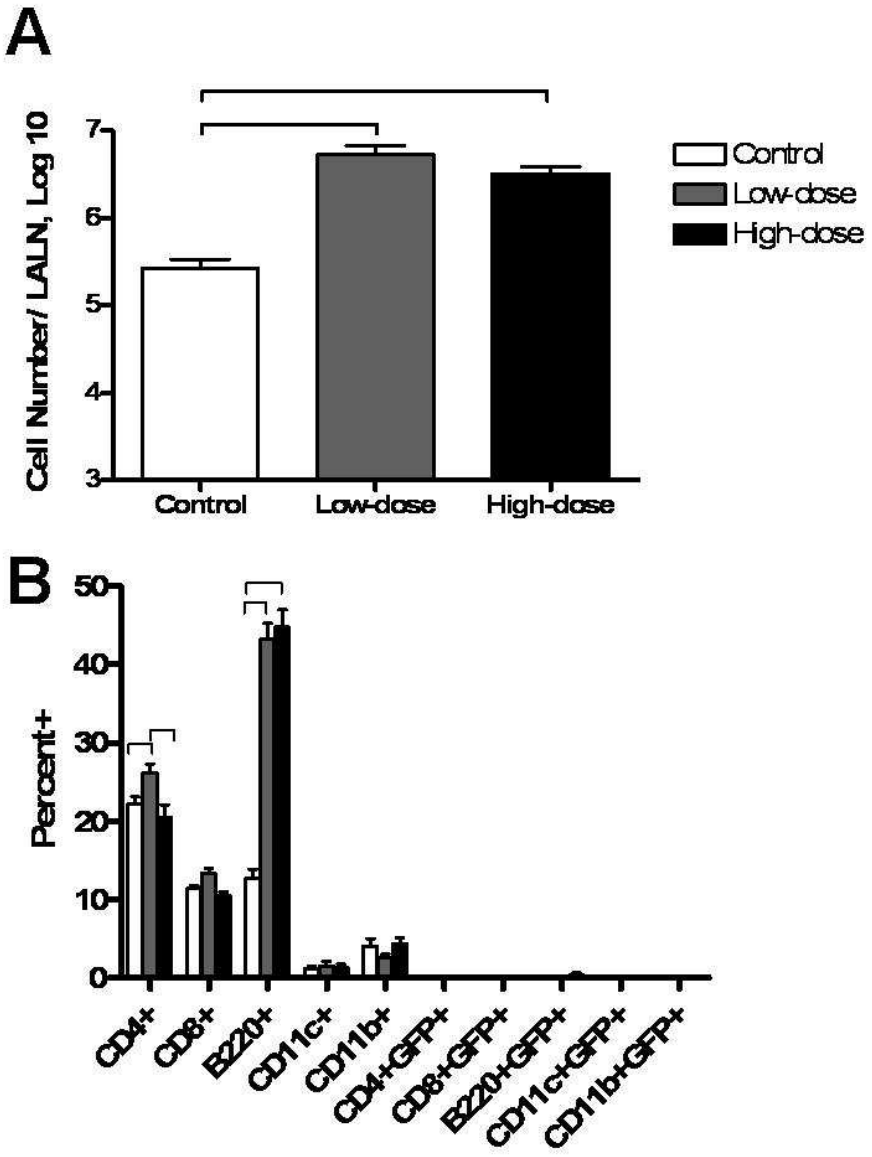
stimulatory molecules, inhibitory molecules PD-L1 and PD-L2 could provide the dominant T cell signal. In my model, I examined the co-stimulatory molecules CD40, CD80, and CD86, but did not examine potential inhibitory molecules such as PD-L1 and PD-L2, and these molecules could provide more insight into DC function during a pulmonary CPXV infection.

Overall, the mouse model I developed could be used beyond the study of orthopoxviruses to examine subtle immune suppression with a variety of infectious agents. It does not require the pathogen to be genetically modified, allowing many respiratory pathogens to be used. OVA peptide and DO11.10 mice are readily available commercially, and the intranasally instillation of OVA peptide leads to good recruitment of T cells even in uninfected mice. The assay is extremely sensitive, and could be useful in many different applications. As discussed earlier, many respiratory pathogens are thought to dysregulate the immune system and the adoptive transfer model can test if T cell clonal expansion in inhibited by an ongoing infection. Thus, the system should facilitate a broader understanding of the complexities of host-pathogen interactions.

VIII. Supplemental Figures

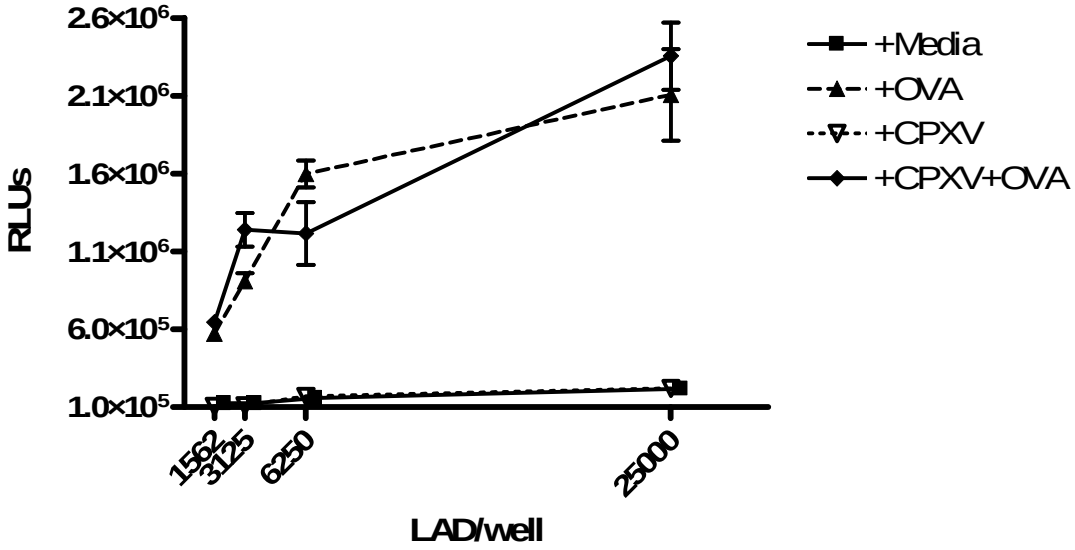


Supplemental Figure 1. Gating Strategy for T cell proliferation. Lymphocytes from LALNs were first gated out of total cells on a forward vs. side scatter plot. Next, CD4+CFSE+ cells were gated out and displayed as a histogram. T cell division gates were placed using control, uninfected mice, which received intranasal OVA peptide, as a base line for proliferation. The number of divisions for each part of the CFSE curve is indicated by a number over the gate. The percent and/or total amount of T cells within each division gate was averaged per group to create the T cell division graphs as shown in figures 6A-C, 10C, 21B, and 23A.

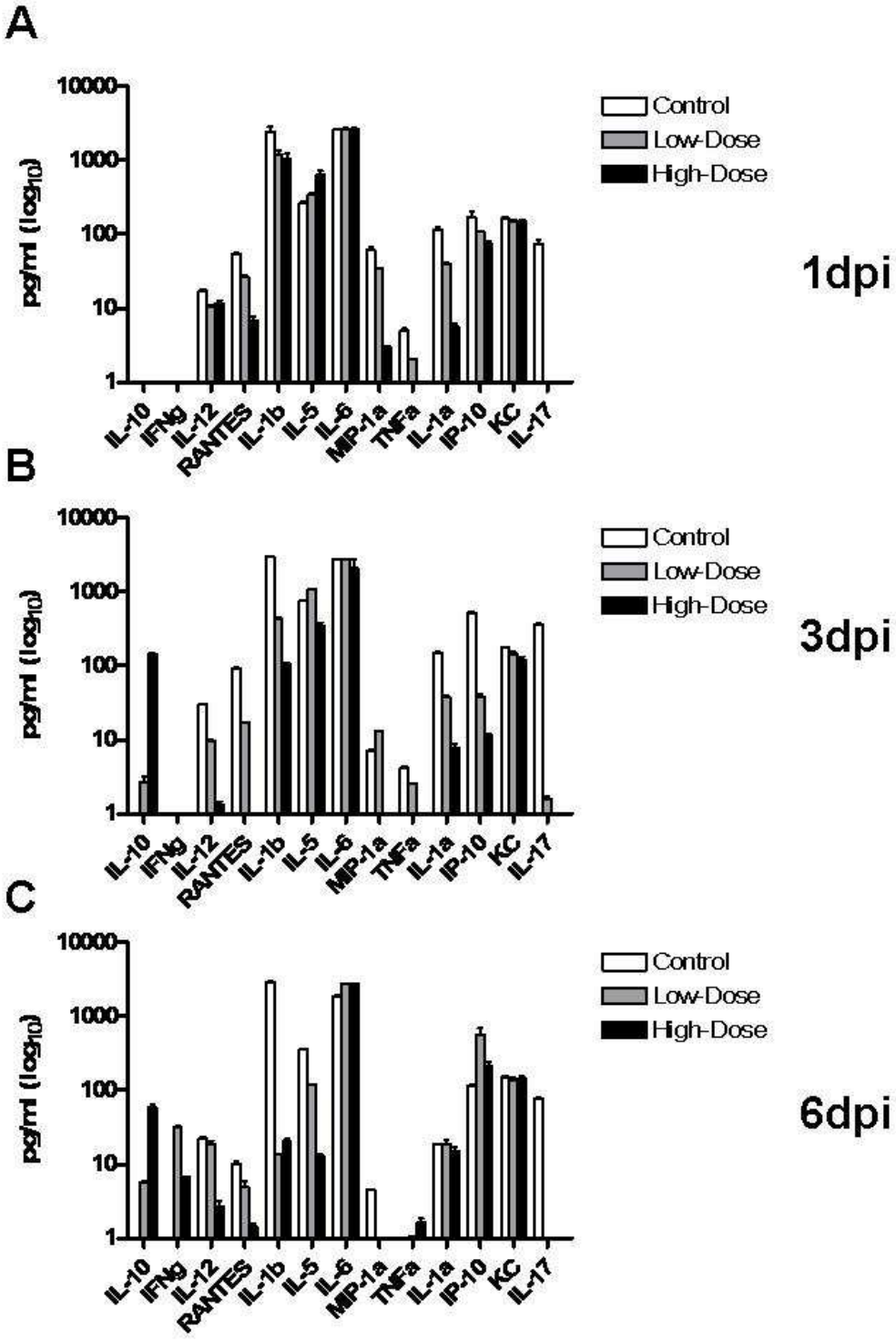


Supplemental Figure 2. Mice infected with a low-dose or high-dose of CPXV have increased LALN cells, but no infected cells. (A) Total number of LALN cells in each group 6dpi. N=10mice/group (B) Mice were infected with GFP-CPXV, and the LALN for CPXV-infected cells indicated by GFP expression. Shown is the percent of LALN

cells in each group expressing each surface marker alone, and the number of each cell type expressing GFP. GFP+ cells were found in the LALN in one experiment only, in 1.5% of B220+ cells. N≥3mice/group. Bars indicate a significant difference ($P<0.05$) by 2-way ANOVA.

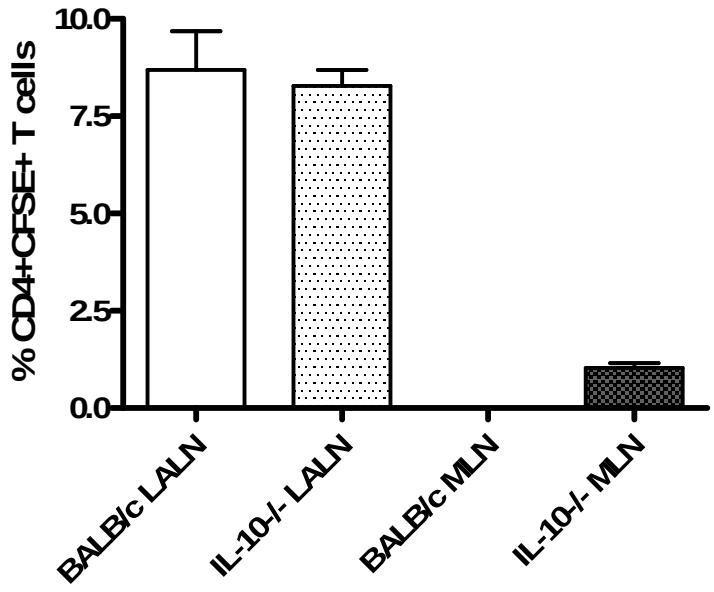


Supplemental Figure 3. A lung cell population enriched for DCs, removed 3dpi after a high-dose infection, can stimulate naïve T cells to divide. Loosely adherent DCs (LAD) were isolated from infected mouse lungs 3dpi, irradiated, and cultured with naïve DO11.10 T cells with or without OVA peptide for 72hrs. BrdU uptake was measured by chemiluminescent ELISA. Increasing RLU value correlates to increasing amounts of T cell proliferation. LAD from naïve or CPXV-infected animals stimulated T cell to divide as long as OVA peptide was added to the culture. N=1 experiment, using cells pooled from 3 infected mice or 3 uninfected mice.

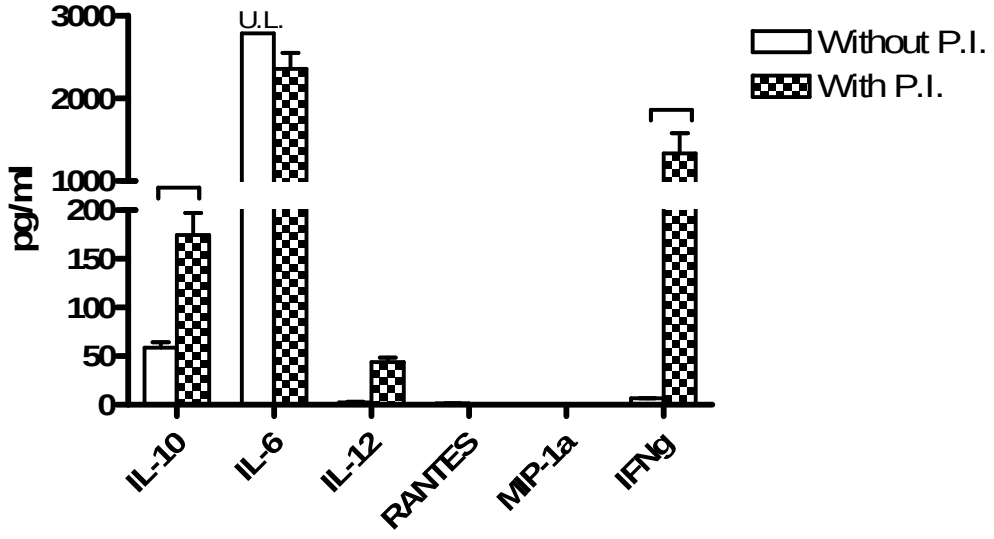


Supplemental Figure 4. Analysis of 13 cytokines over the course of a low-dose and high-dose CPXV infection. The cytokines listed were measured in LS from mice 1, 3,

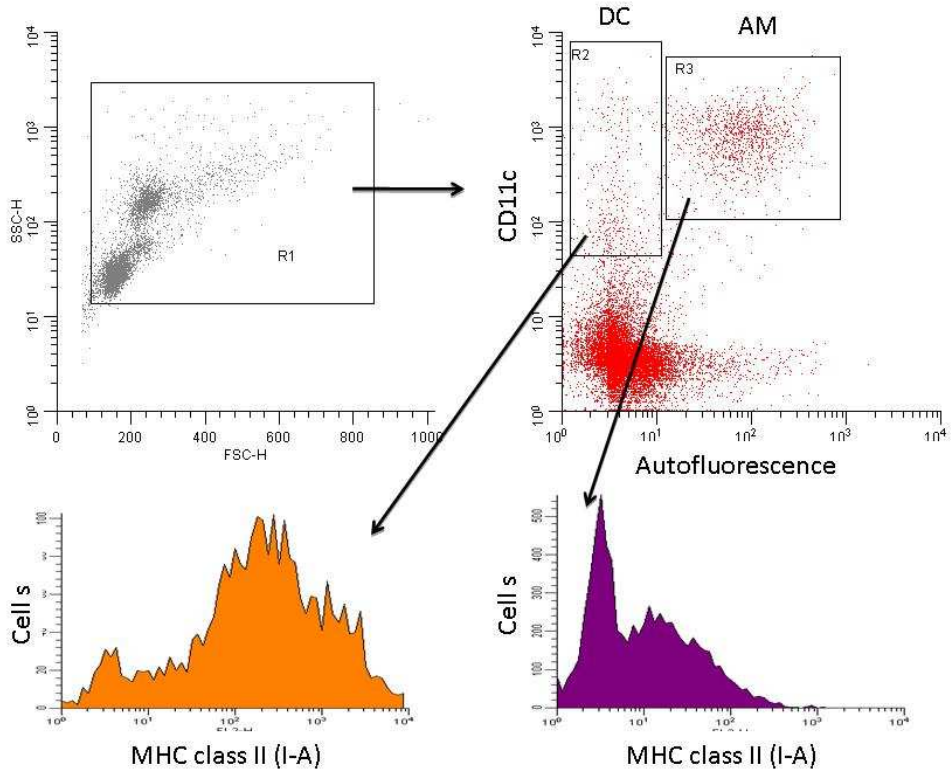
and 6dpi. Lungs were removed on the day indicated, minced, and incubated in complete RPMI for 24hr, at which time the supernatant was removed for analysis. Shown is the average of 3 mice per group per time point, with 1 experiment. No protease inhibitor was used in samples from this assay.



Supplemental Figure 5. IL10-/- mice have CD4+CFSE+ cells in the mesenteric lymph node (MLN) after a T cell adoptive transfer. Mice were given adoptively transferred CFSE-labeled DO11.10 T cells (d0) and inoculated intranasally with OVA (d1) to track DO11.10 T cells in BALB/c and IL-10-/- mice. On day 4, LALN and MLN were harvested and examined by FACS to locate CFSE+ cells. No CFSE-labeled cells were found in MLN in BALB/c mice, but a small percentage was found in all of the IL-10-/- mice tested. N=1 experiment, with 3 mice/group.



Supplemental Figure 6. Protease inhibitor affects the amount of some cytokines measured in LS from infected mice. LS from high-dose mice, treated with a protease inhibitor cocktail, is compared to a different experiment where no protease inhibitor was used. Each experiment shows the average cytokine level from 3 mice, with each sample run in duplicate. Bars indicate $P<0.05$ by 2-way ANOVA, and the upper limit of cytokine detection is noted with "U.L". A significant difference was measured for IL-10 and IFNγ.



Supplemental Figure 7. Gating strategy for pulmonary DCs. Pulmonary DCs vary in size, are CD11c+, and most express high levels of MHC class II. Therefore a wide forward/side scatter gate is used which encompasses all live, nucleated cells (R1). DCs express low to high CD11c (R2). Although AM are also CD11c+ (R3), they are autofluorescent, and express low levels of MHC class II. Gating on R2 and R3 shows the differential expression of MHC class II on each cell type. CD11c+Autofluorescent- cells are functionally able to stimulate naïve T cells, while CD11c+Autofluorescent+ cells are unable to stimulate naïve T cells [172].

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