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DISSERTATION

**OVINE LENTIVIRUS-INDUCED DISEASE:
EARLY PULMONARY INFLAMMATION
AND VIRAL REPLICATION**

Submitted by

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Department of Pathology

In partial fulfillment of the requirements

for the degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2001

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED
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LENTIVIRUS INDUCED DISEASE: EARLY PULMONARY INFLAMMATION AND
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REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT OF DISSERTATION

OVINE LENTIVIRUS INDUCED DISEASE: EARLY PULMONARY INFLAMMATION AND VIRAL REPLICATION

Ovine lentivirus (OvLV) is the etiologic agent of lymphocytic interstitial pneumonia (LIP). LIP is a common, naturally occurring disease among sheep and is a model of a similar disease in humans infected with human immunodeficiency virus. Since there is considerable uncertainty as to earliest sequence of events in the host-lentivirus relationship, this investigation was undertaken to better define the factors that contribute both to pulmonary inflammation and viral replication. This was accomplished by an *in vivo* investigation of lambs experimentally inoculated with OvLV and *in vitro* studies examining lymphocyte proliferation and lentivirus replication. The lambs inoculated with OvLV were examined for pulmonary lesion development through bronchoalveolar lavage cell analyses and histological analyses at either two, four, or 18 weeks post inoculation (PI). Inflammation peaked four weeks PI with accumulations of peribronchiolar T cells and numerous alveolar macrophages. Macrophages had increased expression of MHC II but were spatially separated from the lymphoid cell accumulation. The degree of lymphoid cell accumulation was predictive of the virus infection rate of alveolar macrophages. Lymphocyte proliferation was tested *in vitro* with mitogenic (concanavalin A) and antigenic (OvLV) stimulation, both with and without alveolar macrophages. Lymphocytes from OvLV inoculated lambs responded weakly to

mitogenic stimulation compared to the medium inoculated lambs and the proliferative responses from both sets of animals was suppressed when co-cultured with autologous alveolar macrophages, regardless of whether the macrophages were infected. Finally, monocyte derived macrophages infected with lentivirus *in vitro* produce more new virus when inducible nitric oxide synthase is blocked by the amino acid analogue aminoguanidine. The blockage could be reversed when L-arginine was present at greater concentrations than aminoguanidine. The addition of exogenous nitric oxide to the culture did not influence OvLV replication rates. Collectively, these data suggest that exuberant T cell mediated pulmonary inflammation increases the recruitment of macrophages to the lung and the potential for infectious spread within the host. The subsequent control of virus replication may be due in part to the innate immune mediator nitric oxide.

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A critical and necessary component of this dissertation was the use of juvenile lambs. It was the intention of the author to always proceed with utmost respect, reverence, and humanity for these animals' sacrifice. It is the hope of the author that these and all animals sacrificed in the name of science are done for the betterment of all species that share this planet.

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DEDICATION

This work is dedicated to my best friend, biggest support, climbing partner, lover, and wife, Jennifer, who has taught me that being true to yourself and still caring about the world around you are compatible goals that will lead to a meaningful life. Thank you for everything.

**OVINE LENTIVIRUS INDUCED DISEASE:
EARLY PULMONARY INFLAMMATION
AND VIRAL REPLICATION**

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Chapter One

Introduction

The investigation of ovine lentivirus (OvLV) infection and disease reported in this dissertation is a portion of a larger investigation on the early pathogenesis of OvLV induced lymphocytic interstitial pneumonia (LIP). The stated underlying hypotheses of this larger project were that lentiviral antigens, presented by MHC molecules, induce T cell proliferation, and recruitment of T cells contributes to the pulmonary infiltrative disease. As documented below, this common veterinary pathogen provides a unique opportunity to investigate certain aspects of lentivirus-associated disease that is not possible in other species. The investigation will attempt to fill in gaps in our knowledge of a complex host-virus relationship by dissecting the host immune response concurrently with the virus infection and replication. Although the main description of the lesion in this disease is LIP, this dissertation will focus on the primary target cell of OvLV: the macrophage. Since both macrophages and lymphocytes associated with this disease are intimately related, however, it will be necessary to discuss both populations. The reader is referred elsewhere for a complimentary discussion that focuses exclusively on the lymphocytes from the same experimental animals described in the first half of this dissertation.¹ Finally, it is noted that this investigation's comparison of virus-inoculated animals to sham inoculated animals provides a *de facto* opportunity to observe "normal" anatomy and immunological function in the absence of a specific pathogen.

General Hypothesis

Based on the current concepts of OvLV pathogenesis and the questions raised in the following literature review, I propose the following general hypothesis:

Ovine lentivirus infection of pulmonary macrophages initiates activation and accumulation of macrophages within the lung. This activation and accumulation is mediated by T cells, which proliferate and facilitate enhanced viral replication and induce further accumulation of naïve macrophages susceptible to infection. Macrophage infection rates are partially controlled by the non-specific activity of nitric oxide that results from their activation.

Specific Aims

First, to describe the sequential development of clinical signs and pulmonary lesions in lambs experimentally infected with ovine lentivirus (OvLV) during the first eighteen weeks post infection.

Second, to quantitate and correlate the pulmonary cellular infiltrate and virus infection rates. These measurements include lymphocyte accumulation and proliferation rates, macrophage accumulation and phenotype alterations (MHC II), and virus infection rates.

Third, to define alterations in the proliferative response of lymphocytes to non-specific (mitogen) stimulation associated with OvLV infection, with and without autologous macrophages.

Fourth, to examine the ability of nitric oxide to influence OvLV replication in cultured macrophages.

Review of the Literature

Ovine lentivirus is a model of human immunodeficiency virus (HIV)

According to the United States Center for Disease Control and Prevention, at the end of the year 2000, there were approximately 36.1 million people throughout the world infected with HIV. Additionally, approximately 21.8 million people died between the mid 1980's and the end of 2000 with AIDS related diseases. Obviously, improvements in prevention and treatment of HIV infection are major objectives of the biomedical communities throughout the world. Inherent in achieving these objectives is a clear understanding of the pathogenesis of lentivirus infections.

A number of arguments have been made that OvLV is a valuable model for biomedical research.^{2,5} Both OvLV and HIV are in the Retroviridae family and lentivirus genus.⁶ These two virus have similar viral replication schemes, overlapping tissue tropisms and disease manifestation. Lentivirus infections in both humans and animals result in a complex host-virus relationship with frequent development of chronic progressive disease culminating in death.⁷ As the first lentivirus isolated, ovine lentivirus (OvLV) is the prototype for this genus and studies of OvLV provided the first insight into the basic genomic structure and replication mechanisms that define this virus group.^{8,9} Lymphoid interstitial pneumonia (LIP) is a prominent lesion in ovine lentivirus pathogenesis¹⁰ and is analogous to that which occurs in the early stages of both human immunodeficiency virus (HIV)¹¹ and simian immunodeficiency virus (SIV) infections.¹² Finally, comprehensive literature is available which describes the anatomical and physiological characteristics of ovine lung anatomy,¹³⁻¹⁵ pulmonary lymphoid tissue,¹⁶ and bronchoalveolar lavage (BAL) cell populations.¹⁷ This knowledge base, combined with an

animal size that is similar to humans, facilitates multiple sampling of BAL cells and makes this a unique opportunity to investigate early lentiviral disease in a serial manner.

There has been considerable progress in retrovirus research on the molecular aspects of cell tropism and viral replication with human retroviruses. However, there is still uncertainty whether the immune system is capable of preventing or clearing retrovirus infections.¹⁸⁻²⁰ In particular, it is critical to understand the host-virus relationship if effective vaccines are to be developed. Understanding the host-virus relationship is particularly difficult to achieve in the early phases of disease in human lentivirus infections because the initial time and virus strain of infection are usually unknown. Experimental investigations of ovine lentivirus are particularly useful because the dose, strain, and timing of infection can be tightly controlled.

HIV is capable of infecting and replicating within two cell populations: macrophages and T lymphocytes. This dual tropism is due to differing chemokine co-receptors on the two cell populations: CCR5 on macrophages and CXCR4 on T cells.²¹⁻²⁵ These and other investigations have examined viral replication in relation to presence of viral receptor. However, they have provided only a rudimentary analysis of how the immune response to the two infected cell populations differs. Theoretical descriptions do exist of the differences in lentivirus replication and host immune responses with two distinct target cells.²⁶⁻²⁹ However, no *in vivo* studies have been performed within a single HIV infected individual analyzing both virus production from, and the immune response to, different target cells. OvLV is only capable of productively infecting macrophages^{30,31} and dendritic cells.³² Consequently, viral induced T cell depletion is not a confounding phenomenon in the analyses of the immune response to macrophage infections. Also,

analyses of viral infection and replication in conjunction with immune response are limited solely to a single population.

More than 20 years after AIDS was first defined, there still is no comprehensive data on individual patients showing the magnitude and breadth of the immune response to HIV infection and replication *in vivo*. This and other factors cited above support the conclusion that OvLV is a worthy model of HIV associated disease. In summary, these factors include the similarity of the genomic organization and protein composition of the viral agent, the pathological features of the disease process, the anatomical and physiological characterization of the organ system under study, the genetic diversity of the host animal, and, for certain experimental approaches, a body size sufficient for multiple sample collections and invasive procedures.

Ovine lentivirus associated disease as an opportunity to investigate regulation of the pulmonary immune response.

It has been said that necessity is the mother of invention; likewise disease is often the impetus to understand the body in health. The immunological regulation of the pulmonary system is unique compared to other organ systems based on its distinctive physiological function. Through the study of the respiratory system in disease, we gain an opportunity to understand more about pulmonary immunity in health. Central to this dissertation and central to pulmonary immune regulation is the pulmonary macrophage. Although all pulmonary macrophages appear to have a common progenitor cell they also appear to have a wide array of morphological and functional subpopulations.³³⁻³⁴

Macrophages in the lungs have been described in the vascular space, the interstitium, along the pleura, within bronchus associated lymphoid tissue (dendritic

cells), conducting airways and in the exchange airways: the alveoli. Additionally, ruminants are known for possessing a distinct population of pulmonary intravascular macrophages,³⁵ which are a potential target of OvLV infection.³⁶ It is likely that the various locations of pulmonary macrophages reflect various states of activation in macrophage sub-populations. Since the state of macrophage differentiation is known to be important in the OvLV life cycle,^{30,37-39} then anatomical location might also influence virus life cycle. The alveolar macrophage is the major immunological barrier of the lower respiratory system, thus it is critical that these cells function in a balanced manner to maintain homeostasis. This is readily apparent when one considers the vast quantities of potential antigen to which alveolar macrophages may be exposed (the average human male inhales approximately 10,000 liters of air per day). Assuming that the vast majority of these potential antigens are not true pathogens it is critical that alveolar macrophages are capable of distinguishing dangerous from non-dangerous antigens. Thus, it has been hypothesized that the ability of alveolar macrophages to down-regulate the pulmonary immune response is more common than up regulation.⁴⁰⁻⁴² In fact, evidence for the so called "balanced macrophage activation hypothesis" is sufficiently strong enough to warrant research towards pharmacological enhancement of this phenomenon.⁴³

Pulmonary macrophages use a variety of specific and non-specific mechanisms to provide this balanced immune response. These mechanisms can be thought of as alterations in their state of activation with enhanced functional capabilities over non-activated but otherwise similar cells. There are several well-defined changes in macrophage activation that occur when it is necessary for pathogen-derived antigen to be presented to T cells. One specific mechanism occurs when macrophages or dendritic cells increase production of MHC II molecules along with co-stimulatory molecules such

as B7 within lymphoid tissue.³⁵ This combination of up-regulating class II presented antigen along with co-stimulatory molecules is one of the key signals stimulating T cell proliferation and is likely to be important in OvLV associated disease. Other non-specific mechanisms include secretion of cytokines and chemokines such as IL-1, IFN- γ , and TNF- α ³⁵ into the microenvironment. These cytokines can modulate the functional state of other inflammatory cells (e.g. enhanced nitric oxide production) and also direct their trafficking patterns through altered production of adhesion molecules on vascular endothelial cells. The investigation described herein provided an opportunity to observe “normal” immunological regulation of the pulmonary system vis-à-vis the control animals by comparison and contrasting to the virus infected animals.

Natural disease associated with OvLV infection.

OvLV, also known as maedi-visna virus and ovine progressive pneumonia virus, infects sheep throughout most of the world. In fact, OvLV is endemic throughout most of the world's domestic sheep except for New Zealand, Australia, and Iceland.⁴⁴ OvLV is particularly common in North America with up to 50% of the flocks infected in intensive production regions.⁴⁵⁻⁴⁷ Sheep slaughtered at an early age (prior to 4 years old) usually do not have obvious clinical signs that are associated with OvLV infection,⁴⁸ and thus affected animals are not culled. However, older sheep naturally infected with OvLV generally exhibit clinical signs of disease and die after a protracted and progressive illness characterized by emaciation, dyspnea, and development of secondary bacterial infections.^{49,50} Ultimately, OvLV infection within a flock is a significant cause of decreased fertility, longer parturition to weaning intervals, and decreased weight gain by lambs.⁵¹

Survival times of sheep naturally infected with OvLV are not well established. Investigations have been performed in a range flock naturally infected with OvLV.⁵² In this study, LIP was observed in 76% of a western United States flock. Additionally, many of these animals had lymphoproliferative lesions in other organs such as the brain and mammary gland from which OvLV was subsequently isolated. Clinical signs of respiratory distress were observed in 78% of animals with severe LIP, although concurrent bronchopneumonia was present in approximately half of these animals. All sheep with moderate or severe lesions of LIP were seropositive for OvLV by ELISA and immunoblot assays, and OvLV was isolated from alveolar macrophages obtained from of these animals by BAL. Pulmonary abscesses related to *Corynebacterium pseudotuberculosis* infection were increased in seropositive (53%) over seronegative (30%) animals.

The lower respiratory tract is one of the principle sites of disease in OvLV-infected sheep. Typically, adult sheep chronically infected with OvLV have lungs that weigh 2-3 times more than normal organ weight. When removed from the animal, the lungs fail to collapse and have diffusely distributed small, gray foci (areas of lymphoproliferation) on the pleural surface.^{50:52:53} Histologically, the pulmonary lesions in affected animals without secondary bacterial infections consist of diffuse interstitial mononuclear cell infiltration within alveolar septae. Some animals also have alveolar type II cell proliferation or peribronchiolar and interstitial fibromuscular proliferation. Peribronchiolar and perivascular lymphoid follicles, both with and without germinal centers, can occur in sheep with severe interstitial pneumonia. Marked enlargement of the lymph nodes draining the lungs are due to diffuse lymph node hyperplasia and usually coincides with OvLV-induced LIP. The caudal mediastinal lymph node may reach 50g

from a normal size of 3-5g.⁵⁴ Studies of peripheral blood and lymph node lymphocytes revealed depressed levels of lymphocyte-generated interleukin-2⁵⁵ and decreased concanavalin A-induced suppresser cell activity.⁵⁶ These studies provide evidence that although large numbers of lymphocytes are present in the lungs of OvLV-infected sheep, they appear functionally inept. This further argues that the lymphocytes' proliferation and accumulation are an aberration of a normal immune response

Lymphoid Interstitial Pneumonia in Lentiviral Disease

Lymphoid interstitial pneumonia in HIV infected humans is, by definition, a pulmonary infiltrate of benign, small, polyclonal, non-cleaved lymphocytes within the alveolar, interlobular, subpleural and peribronchiolar septae while sparing the pleura, bronchi and blood vessels. These infiltrates commonly form lymphoid follicles along the lymphatic routes, both with and without germinal centers. There is no evidence of lymphoma, and interstitial fibrosis develops with chronic disease.⁵⁷⁻⁵⁹ The histopathological changes of LIP resemble the interstitial pneumonia found in autoimmune disorders such as Sjögren's syndrome and systemic lupus.⁶⁰⁻⁶² Recently, HIV associated LIP has also been linked to the development of mucosal lymphoid tissue neoplasia.⁶³ Improved technology using both invasive⁶⁴ and non-invasive^{65,66} techniques has increased the recognition of LIP in both pediatric and adult HIV cases. LIP is most commonly recognized in children who are vertically infected with HIV. Currently, approximately 12% of children have clinical signs of respiratory disease associated with the presence of LIP.^{67,68} However, one investigation observed 89% of HIV positive children without clinical signs of pulmonary disease still had a lymphocytic alveolitis based on BAL fluids with elevated CD8+ cells.⁶⁹ Pulmonary and non-pulmonary lymph

node hyperplasia is a consistent finding in HIV infected children.^{70:71} There is evidence that LIP may be a risk factor for future opportunistic diseases such as *Pneumocystis carinii* and bacterial pneumonia.^{69:72:73} Additionally, LIP has been associated with increased reservoirs of viral RNA in secondary lymphoid structures such as bronchus associated lymphoid tissue.⁷⁴ However, these risk factors are not universally acknowledged as other investigators have claimed that there are benefits associated with the development of LIP, such as increased resistance to opportunistic infections.^{12:71:75} Finally, there is empirical evidence for immune mediated disease basis as the lesions in humans rapidly regress with immunosuppressive therapy.⁵⁸

LIP is also recognized in other species with retrovirus infections including non-human primates,⁷⁶ domestic cats,⁷⁷ and wild cats.⁷⁸ LIP lesions nearly identical to those in HIV have also been described in sheep^{49:50:79} and goats⁸⁰ naturally and experimentally infected by lentiviruses. Several lines of evidence suggest that in both HIV and OvLV these lesions are due to an immune mediated mechanism. In sheep naturally infected with OvLV, there is a correlation between viral load, particularly in the lung, and the severity of LIP.⁵² The ability of different OvLV isolates to replicate in alveolar macrophages *in vitro* correlates with their capacity to induce LIP.⁸¹ Interferon from pulmonary leukocytes is spontaneously produced in OvLV infected sheep *in vivo*, and is produced *in vitro* when leukocytes are co-cultured with OvLV infected alveolar macrophages and is positively correlated with the severity of LIP lesions.⁵⁵ Respiratory function alterations in sheep with LIP are not well defined, however, increase in septa smooth muscle bundles have been shown to negatively correlate with *ex vivo* lung contractility.⁸²

The predominant lesion in simian species infected with SIV has also been described as a lymphocytic infiltration of interalveolar septa and hyperplasia of peribronchial and perivascular lymphoid tissue.⁸³ Because the end point of this study was when the monkeys developed clinical signs of disease, it was not possible to determine the timing or onset or lesion progression. However, these investigators used immunohistochemical staining against T and B lymphocytes, macrophages, and immunoglobulins which showed that peribronchiolar aggregates and interstitial infiltrates were predominantly B cells, whereas perivascular masses consisted mainly of T cells. A follow-up investigation of SIV pulmonary disease observed infiltrates of cytotoxic lymphocytes lacked correlation between severity of pulmonary lesions and virus gene expression.¹² These investigators concluded that the inverse relationship between pneumonia and immune status suggest that simian immunodeficiency virus pneumonia represents an immunopathological response to macrophage-tropic virus.

OvLV is known to be selectively tropic for cells of the monocyte-macrophage lineage and productive infection occurs as monocytes mature into functional macrophages.⁸⁰ However, many of the cells that accumulate in the lungs are lymphocytes. Several investigators have analyzed BAL fluid recovered from sheep naturally infected with OvLV during the chronic stages of disease.⁸⁴⁻⁸⁸ This is a site that is easily sampled and provides insight into the different immune responses that occur in the lung but outside of the interstitium. These investigators concluded that chronically infected sheep have an increased production of MHC Class II on alveolar macrophages (with variable results regarding the absolute numbers of macrophages).⁸⁹ Similar analyses of lung sections revealed an absolute and relative increase in the number of CD8+ cells with an absolute increase but relative decrease in the number of CD4+ cells in BAL fluid.^{90,91} This

CD4:CD8 ratio inversion is similar to that seen in the pulmonary manifestations of HIV associated disease and is consistent with an immune mediated mechanism.⁹² This information in conjunction with the unique role of the alveolar macrophage in the homeostatic mechanism of the pulmonary immune system^{41,93,94} suggest the possibility that the virus is disrupting the normal regulatory controls of pulmonary macrophages.

The accumulation of lymphocytes within the lungs of OvLV-infected sheep may be the result of one or a combination of mechanisms. These could include increased classical MHC II restricted antigen presentation along with co-stimulatory molecules inducing *in situ* proliferation, viral superantigen induced *in situ* proliferation, excessive secretion of proinflammatory cytokines, or loss of suppresser/anergy cytokines. The above evidence on the natural course of OvLV associated disease suggests that clinical signs and ultimately death of the host are directly attributable to an inflammatory response to the virus, yet *in vivo* cytopathic events have not been demonstrated. Thus, the fundamental question to be answered is: Why is there such a remarkable and persistent pulmonary inflammatory response to a virus that appears to be non-cytopathic *in vivo*?

Viral and immunological events have been quantified during OvLV infection of peripheral lymph nodes to investigate early pathogenesis.⁹⁵⁻⁹⁷ These investigators gave a subcutaneous inoculation of infectious OvLV and quantitated a “burst” of virus replication that occurred within the first week of infection via a cannulated efferent lymphatic vessel draining the site inoculation. Productively infected cells from this lymph node then diminished to low or undetectable levels by the end of the third week. Over this time period, the lymph node responded to OvLV infection in a manner typical of a primary response to viral antigens. The lymph node had a generalized cortical

enlargement consistent with both B cell and T cell expansion concurrent with increased numbers of CD8+ T cells emigrating from the lymph node. This was initially interpreted as a cytotoxic T cell response against virus infected macrophages. However, follow-up studies from the same laboratory documented that *in vivo* depletion of CD8+ cells in adult animals did not alter the course of primary experimental infection, whereas *in vivo* depletion of CD4+ cells reduced the primary infection rate.^{98,99} These results are in contrast to a similar experiment performed in a rhesus monkeys chronically infected with SIV in which eliminating CD8+ T cells resulted in a rapid increase in viremia.¹⁰⁰ However, several important differences should be noted between these experiments: 1) The sheep were depleted of lymphocytes first and then inoculated with virus whereas the monkeys were already chronically infected then depleted. 2) The experimental endpoints in the sheep study were based on serial dilutions of cells collected from a cannulated lymph node whereas the endpoints in the simian study were plasma viral RNA levels. 3) The sheep study used an exclusively macrophage tropic strain whereas the monkeys were infected with SIVmac and in the chronic stages of disease thus likely to have both macrophages and T cells infected with virus.

Cellular Tropism and Strain Variability of OvLV

OvLV is also known as maedi-visna virus and ovine progressive pneumonia virus and is considered a prototype virus of the lentivirus subgroup of Retroviridae.¹⁰¹⁻¹⁰³ Previous reports have described many of the features in OvLV which are analogous to those observed in HIV and consistent with this prototypical designation: (1) The viral genome contains several small open reading frames encoding peptides that regulate expression of structural genes during replication.¹⁰⁴⁻¹⁰⁷ (2) Diverse cytopathic phenotypes

exist that differ in replicative capacity.^{53;81;108} (3) OvLV is tropic for macrophages *in vivo* and replication in these cells is dependent upon their state of differentiation or activation.^{53;102;106} (4) After a prolonged incubation period, OvLV induces a persistent infection and slowly progressive diseases involving lymphoid tissue, lung and brain.^{50;102;105;106}

As previously mentioned, the predominant cell that is productively infected *in vivo* with OvLV are those of monocyte-macrophage lineage. Presumably, this observation is because macrophages are the only cell line displaying the virus receptor. Unfortunately, the literature on the OvLV viral receptor has been extremely sparse and the identity of the OvLV receptor is still largely speculative. Early reports suggested that the MHC II molecule might be the viral receptor.¹⁰⁹ However, this hypothesis has largely been discredited as the receptor has been tentatively mapped to either chromosome 2 or 4 while the sheep's MHC II gene is mapped to chromosome 23.¹¹⁰ Other recent experiments implicate a serine/threonine kinase complex in the binding/infection of visna virus.^{111;112} From the perspective of the virus, it has also been asserted that the LTR determines tropism of OvLV's host cell.¹¹³ Definitive evidence of these latter two assertions has yet to be provided.

Previous investigations have analyzed several OvLV field isolates that differ in their pathogenicity *in vivo* and in their growth characteristics *in vitro*.^{50;52;53;81;114} In these studies, PCR amplification of fragments from the LTR, *pol*, and *env* regions of OvLV was done. Sequencing of these fragments from four plaque-purified isolates showed that each virus is unique and that there is considerable genomic diversity. The virus strain that was used exclusively in this dissertation is 85/34, a field isolate from these studies that has been well characterized. 85/34 induces rapid syncytial formation and cell lysis in

fibroblast and alveolar macrophage cultures. Virus titers of this strain correlate directly with development of *in vitro* cytopathic effect (CPE). This virus strain corresponds to the “rapid/high” strains of HIV, which also rapidly induce CPE and quickly reach high titers. Additionally, a number of monoclonal antibodies were produced which specifically bind the capsid antigen of OvLV strain 85/34.¹¹⁵ Capsid antigen (p25) epitopes were compared by competition ELISA and all of the mAb reacted with one immunodominant region of capsid antigen.

In vivo comparative studies of various OvLV strains have also been done.^{53,116,117}

The plaque-purified strains that were tested, possess distinctly different *in vitro* cytopathic phenotypes including lytic (rapid/high phenotype) and persistent (slow/low phenotype) strains. Animals inoculated with the lytic strain and examined 24 weeks later developed significantly more severe lesions than those inoculated with the persistent strain. Additionally, BAL samples from OvLV-diseased lambs had significantly greater numbers of lymphocytes compared to BAL samples of uninfected lambs; the majority of these lymphocytes were of the CD8+ phenotype. OvLV was re-isolated from multiple tissues of both groups, but in greater frequency in lambs inoculated with the lytic isolate. Although precipitating antibodies were found in the serum of both groups, initial detection was delayed by 4.5 weeks in the persistent strain virus-inoculated animals; neutralizing antibody titers were low or undetectable in both groups. However, follow-up studies examined the abilities of serum from twin lambs infected with both lytic and persistent virus strains to neutralize *in vitro* cytopathic effect.¹¹⁸ In this investigation, these two OvLV strains were further defined as distinct neutralization serotypes based on the conclusion that antigenic recognition is in part determined by immunogenetic differences of the hosts. Finally, lentivirus-infected lambs and adult sheep with severe

LIP also had significantly elevated levels of spontaneous interferon (IFN) production from pulmonary leukocytes compared with uninfected animals or OvLV-infected sheep with mild or no lesions of LIP. Taken together, these results suggest that OvLV strains induce disease in a strain-dependent manner and that humoral immune responses against OvLV fail to inhibit lesion development.

The relationship of OvLV disease severity to host genetic differences was further investigated.¹¹⁹ Syngeneic twin lambs derived from embryo splitting were used in these studies. This protocol involved inoculation of one twin with the “rapid/high” 85/34 strain while the corresponding twin was inoculated with the “slow/low” 84/28 strain. The degree of LIP, as determined by histological analysis, was independent of the virus strain used for inoculation. This information, taken with the conclusions from studies on virus strain differences leads to the conclusion that the severity of lesions is dependent on both virus and host factors. Many of these investigations have used the Rambouillet breed of sheep, which appears to be variably susceptible to disease development when infected with 85/34 OvLV. This susceptibility to disease is not absolute. In fact, this variability in lesion development will be part of the evidence presented in this dissertation that host response to a given strain of OvLV is an important determinant in disease outcome. Specifically, the host’s immune response to virus infection may partially determine the infectious spread of the virus in both a positive manner as well a negative manner. This concept will be further explored in the concluding chapter.

Lentivirus Replication and Inducible Nitric Oxide Production

Measurement of plasma viremia are now standardized and routine in cats with FIV,¹²⁰ monkeys with SIV,¹²¹ and humans with HIV.¹²² However, to date, there are no

reports to the author's knowledge measuring plasma viremia in OvLV. In OvLV investigations, the quantity of virus in the blood has been poorly documented with only a single report measuring provirus DNA from concentrated peripheral blood mononuclear cells.¹²³ The apparent low level of plasma virions in OvLV infection is presumably attributable to the macrophage being the only cell productively infected; with the other lentiviruses, the bulk of the viral load is thought to come from T cells. As mentioned previously, the viral kinetic investigations of OvLV to date have largely relied on limiting dilution assays of virus infected cells in co-cultures¹¹⁸ or quantitation of viral antigen displaying cells by immunocytochemistry.¹²⁴

The immunological and cellular factors that affect OvLV transcription and translation have also been sparsely investigated. Interferon- τ has been associated with reduced viral replication.³⁸ Similar to other lentiviruses, transcriptional factors such as TAT, appear to up-regulate viral replication.^{77:125:126} It is likely that TAT binds to the LTR portion of OvLV provirus.¹²⁷ However, the antecedent factors leading to TAT activation have yet to be identified. Other investigations have also determined that integrase and rev (a post transcriptional regulatory protein) are essential for viral replication,¹²⁸⁻¹³⁰ but these investigations have not clearly defined the factors which turn viral replication on or off.

Pulmonary nitric oxide is produced from alveolar macrophages and is an important molecule in the physiological and immunological activities of the lung.¹³¹⁻¹³⁴ Nitric oxide also plays a variety of roles in cellular activation^{135:136} and has a controversial role in the replication of HIV.¹³⁷⁻¹³⁹ As mentioned previously, there is considerable evidence that IFN- γ is upregulated in retrovirus associated LIP. Since IFN- γ is a key up regulator of inducible nitric oxide synthase (iNOS),¹⁴⁰ it is reasonable that nitric oxide

levels are elevated with LIP. There is considerable *in vitro* evidence that nitric oxide is capable of mitigating the function of enzyme systems crucial to the replication of lentiviruses.¹⁴¹⁻¹⁴⁵ Potential targets of nitric oxide induced lentivirus alterations include reverse transcriptase, integrase, nuclear factor - κ beta, and protease. However, it is unclear whether these pathways are identical in both cell populations that are infected by HIV: macrophages and T cells.

There are many factors that influence the expression of inducible nitric oxide synthase genes (iNOS) in macrophages and the subsequent production of nitric oxide. One consideration is that inter-species regulation of nitric oxide production seems to vary.^{146,147} Additionally, within a species, macrophages may alter nitric oxide production based on anatomical location,¹⁴⁸ cellular differentiation,¹⁴⁹ or physiological demands.¹⁵⁰ Finally, there are a variety of infectious diseases in which nitric oxide production may be modified either directly by the pathogen¹⁵¹⁻¹⁵⁴ or indirectly, by the host's immune response to the infectious agent.^{155,156} In reality, it is likely a combination of pathogen and host influences which regulate nitric oxide.¹⁵⁷

Understanding the host-virus relationship

The previously referenced literature provides many examples of investigations that were successful in increasing our knowledge in retrovirus biology and host response to retrovirus infection. These include virus transmission, virus receptors, cell tropism, viral replication, host's cytokine profiles, cellular infiltration patterns, and disease outcome. However, there are still many knowledge gaps in each of these categories. This is particularly true in the early stages of natural disease since the time of infection is often unknown. The purpose of this investigation is to close these knowledge gaps in the early

stages of disease using both *in vivo* and *in vitro* investigative techniques. These techniques will be used to describe the pulmonary lesion development over the first weeks of infection; to analyze the inflammatory response in relationship to virus infection rates; to determine the immune suppressive function of alveolar macrophages to stimulated lymphocytes; and to define the relationship of nitric oxide to OvLV replication.

In conclusion, understanding a host-virus relationship is a formidable task with many controversies and uncertainties in the literature. These controversies can be found even within a single group of investigators. An example of such uncertainty is to be noted in two contemporary publications by the same group of SIV investigators who concluded that immune activation by *Mycobacterium* antigens enhances simian AIDS progression in one paper¹⁵⁸ and subsequently said immune activation reduces SIV replication in another paper.¹⁵⁹ This seemingly dual perspective may be inherent in the nature of retrovirus pathogenesis. This duality in nature may arise from the paradox that the retroviruses primarily infects cells of the immune system which can result in activation of the immune cells to produce more virus or activation of the immune cells to suppress virus infected immune cells. Currently, no single paradigm exists which completely explains this dual nature of host response to retroviruses. However, it is reasonable to believe that continued accumulation of data, both *in vivo* and *in vitro*, would ultimately provide a comprehensive picture of host-retrovirus relationship.

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Chapter Two

The Early Pulmonary Pathogenesis of Ovine Lentivirus Infection

Abstract

The earliest host responses and pulmonary lesions in both human (HIV) and ovine lentivirus (OvLV) infection are poorly understood. OvLV is an useful model for lentivirus pathogenesis studies because experimental infections can be performed under controlled conditions. Additionally, the host responses are simpler to analyze due to the exclusive macrophage cell line tropism of OvLV, thus lymphocyte infection and depletion are not confounding phenomenon. The purpose of this investigation was to describe the sequential development of clinical signs and pulmonary lesions in lambs experimentally infected with OvLV during the first eighteen weeks post infection. To accomplish this, thirty-three lambs were intratracheally inoculated with either OvLV strain 85/34 or cell culture medium. Clinical observations included monitoring for seroconversion, viral antigenemia, and radiographic signs of LIP development. Additionally, bronchoalveolar lavages (BAL) and/or necropsy tissue collections were performed at two, four, or 18 weeks. Finally, an infectious center assay was performed in a subset of animals using alveolar macrophages, pulmonary interstitial cells, and peripheral blood mononuclear cells to quantify changes in OvLV production. Relative to medium inoculated lambs, lymphocytic interstitial pneumonia was most prominent at four weeks post inoculation (PI) in virus inoculated animals. This pneumonia was characterized by lymphocytes accumulating and proliferating around small and medium sized bronchioles while alveolar

macrophages accumulated peripherally to the lymphoid aggregates. Flow cytometric analyses of the lymphocytes revealed that the preponderance of accumulating pulmonary T cells were CD8+. The signs of inflammation coincided with the emergence of seropositivity to viral antigens, the presence of OvLV antigenemia, and radiographic lesions of a diffuse interstitial pneumonia. Finally, cells from a subset of animals indicated that the peak in virus production occurred four weeks post inoculation in alveolar macrophages. Pulmonary inflammation and infectious virus production was not present at 18 weeks post inoculation although seropositivity persisted. This study is the first to comprehensively describe the early host-virus relationship in a natural target organ with exclusively macrophage tropic lentivirus.

Introduction

Ovine lentivirus (OvLV), also known as maedi-visna virus, is the etiologic agent of lymphoid interstitial pneumonia (LIP) in sheep.¹ LIP is a significant cause of morbidity and mortality in sheep^{2,3} and also occurs in human and simian immunodeficiency virus infections.^{4,6} The pulmonary histological lesions in chronic OvLV infection consist of numerous mononuclear inflammatory cells infiltrating the lung's interstitial compartment.¹ Additionally, virus infected macrophages from chronically infected animals can often be detected in large numbers concurrent with the presence of numerous CD8+ lymphocytes.^{7,8} In fact, it has been observed that the degree of inflammation observed in chronic disease parallels the number of virus positive cells.³ Thus, it would appear that the lesions observed in conjunction with chronic lentivirus infection are virus induced.

The lower respiratory tract is one of the principle sites of disease in OvLV-infected sheep. Typically, adult sheep chronically infected with OvLV have lungs that weigh 2-3 times more than normal organ weight. When removed from the animal, the lungs fail to collapse and have diffusely distributed small, gray foci (areas of lymphoproliferation) on the pleural surface.^{1,3,9} Histologically, the pulmonary lesions in affected animals without secondary bacterial infections consist of diffuse interstitial mononuclear cell infiltration within alveolar septae. Some animals also have alveolar type II cell proliferation or peribronchiolar and interstitial fibromuscular proliferation. Peribronchiolar and perivascular lymphoid follicles, both with and without germinal centers, can occur in sheep with severe interstitial pneumonia. Marked enlargement of the lymph nodes draining the lungs are due to diffuse lymph node hyperplasia and usually coincides with OvLV-induced LIP. The caudal mediastinal lymph node may reach 50g from a normal size of 3-5g.¹⁰

Although there are rare cases of immune system suppressing lentiviruses infections,¹¹ the usual outcome of both HIV and OvLV chronic infection suggests that the immune system is incapable of adequately controlling the infection. The question is then posed as to how the immune response and lesion development might be different in the early stages of infection, and specifically how the host response preceding the quiescent phase of lentivirus infection is different than in chronic disease. Previous investigations have only sparsely examined the host response during the early time points post infection. This has largely been due to the uncertainty of virus transmission in natural infections. Although definitive evidence is lacking, it has been hypothesized that OvLV transmission occurs primarily via lactogenic routes¹² whereas other methods of horizontal transmission,

such as copulation, are thought to be less common.¹³ It has been documented that some, but not all neonatal lambs inoculated with OvLV will develop pulmonary lesions at six months post infection.¹⁴ However, definitive investigations describing the cellular alterations and timing of events in pulmonary lesion development associated with lentivirus infections are lacking. Gaps in knowledge on lentivirus associated LIP include even basic features such as the key histological alterations, onset and progression of early lesions, the extent of *in situ* lymphocyte proliferation vs. in-migration, and the relationship between virus infection and inflammation. In order to address these gaps in knowledge, this investigation sought to describe the sequential development of early clinical signs and pulmonary lesions in lambs experimentally inoculated with ovine lentivirus (OvLV) over the first 18 weeks post infection.

Materials and Methods:

Experimental Animals

The experimental design was based on the previously described rapid induction of lymphoid interstitial pneumonia in lambs.¹⁴ Thirty-three colostrum-fed, neonatal, Rambouillet lambs were obtained from a flock that have repeatedly tested seronegative for antibodies to OvLV envelope glycoprotein and capsid antigen by agar gel immunodiffusion (AGID) test (Veterinary Diagnostic Technology, Inc. Wheat Ridge, CO). The Colorado State University Animal Care and Use Committee approved all procedures involving live animals. Lambs were randomly divided into two groups that were housed separately in raised pens while being fed milk replacement, creep and grass feed. Nineteen of the lambs were inoculated intratracheally with 10 ml of supernatant

from goat synovial membrane (GSM) tissue cultures containing a 5×10^6 TCID₅₀ of ovine lentivirus strain 85/34 while 14 lambs were sham inoculated with 10 ml of spent medium from uninfected GSM cell cultures (Table 2.1). The medium from both inoculums included RPMI 1640 supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 2mM L-glutamine (Gibco Life Technologies, Rockville, MD). Bi-weekly blood samples and weekly clinical data (body weight, temperature and respiratory effort) were collected. Bi-weekly thoracic radiographs were performed on two of the animals from each of the 18 weeks PI groups. These radiographs were evaluated for roentgen signs of pneumonia, with identifications masked, by a board certified veterinary radiologist (Dr. Richard D. Park, Colorado State University, Fort Collins, CO). Animals were euthanized by intravenous administration of xylazine (0.2 mg/kg) and ketamine (5 mg/kg) followed by exsanguination from the carotid artery at two, four, or 18 weeks post-inoculation. Three lambs died while under anesthesia and were not exsanguinated; an three lambs had other concurrent illnesses and were excluded from the study.

Table 2.1: Rambouillet lambs listed by experimental grouping which were used for OvLV early pathogenesis investigation.

Experimental Grouping					
96RS01	female	471	Medium	Anesthetic associated hypoxia	2
96RS04	male	207	Medium	Anesthetic overdose/exsanguinated	2
96RS05*	female	2319	Medium	Bacterial Bronchopneumonia	2
96RS31	male	256	Medium	Anesthetic overdose/exsanguinated	2
96RS32	female	140	Medium	Anesthetic overdose/exsanguinated	2
97RS19	male	14	Medium	Anesthetic overdose/exsanguinated	2
97RS21	female	2100	Medium	Anesthetic overdose/exsanguinated	2
97RS22*	female	329	Medium	Bacterial Bronchopneumonia	2
96RS03	female	207	Medium	Anesthetic overdose/exsanguinated	4
96RS18	male	175	Medium	Anesthetic overdose/exsanguinated	4

Ear ID	Sex	Ear ID	Intensity	Week ID
97RS03	male	536	None	Anesthetic overdose/exsanguinated 7
97RS20	male	593	Medium	Anesthetic overdose/exsanguinated 4
Two-week postnatal group with OV group				
96RS02	female	921	Medium	Anesthetic overdose/exsanguinated 18
96RS06	female	431	Medium	Anesthetic overdose/exsanguinated 18
96RS22	male	209	Medium	Anesthetic overdose/exsanguinated 18
96RS23	male	209	Medium	Anesthetic overdose/exsanguinated 18
Two-week postnatal group with OvLV group				
97RS16*	male	442	OvLV	Anesthetic overdose/failure to thrive 1
96RS07	male	658	OvLV	Anesthetic associated hypoxia 2
96RS13	male	433	OvLV	Anesthetic overdose/exsanguinated 2
96RS14	male	543	OvLV	Anesthetic overdose/exsanguinated 2
96RS15	NR	245	OvLV	Anesthetic associated hypoxia 2
96RS29	male	744	OvLV	Anesthetic overdose/exsanguinated 2
97RS14	female	323	OvLV	Anesthetic overdose/exsanguinated 2
97RS18	male	32	OvLV	Anesthetic overdose/exsanguinated 2
Four-week postnatal group with OvLV group				
96RS09	male	206	OvLV	Anesthetic overdose/exsanguinated 4
96RS10	female	146	OvLV	Anesthetic overdose/exsanguinated 4
96RS12	male	433	OvLV	Anesthetic overdose/exsanguinated 4
96RS17	NR	3103	OvLV	Anesthetic associated hypoxia 4
97RS13	male	571	OvLV	Anesthetic overdose/exsanguinated 4
97RS15	female	592	OvLV	Anesthetic overdose/exsanguinated 4
Two-week postnatal group with OvLV group				
96RS08	male	NR	OvLV	Anesthetic overdose/exsanguinated 15
96RS11	female	NR	OvLV	Anesthetic overdose/exsanguinated 18
96RS16	female	245	OvLV	Anesthetic overdose/exsanguinated 18
96RS24	female	167	OvLV	Anesthetic overdose/exsanguinated 19
96RS25	female	643	OvLV	Anesthetic overdose/exsanguinated 18
97RS17	female	208	OvLV	Anesthetic overdose/exsanguinated 19

* Indicates animals that were excluded from study. NR = Not Recorded.

Serology and Virus Isolation.

Bi-weekly serum samples were screened for the presence of anti-OvLV antibodies by AGID using the previously mentioned commercially available ovine progressive pneumonia test kit. Seronegative animals were confirmed negative by additional testing for anti-OvLV antibodies on a previously described western blot.¹⁵ Briefly, OvLV 85/34 proteins were separated by electrophoresis in a 12% sodium dodecyl sulfate - polyacrylamide stacking gel. Proteins were subsequently electrophoretically transferred to a nylon membrane. The membrane was blocked, probed with diluted serum samples, and detected with the isotype appropriate Vectastain® Elite ABC-Peroxidase kit and DAB substrate kit (Vector laboratories, Burlingame, CA) according to the manufacturer's instructions. Productive infection with OvLV was confirmed by virus isolation in goat synovial membrane tissue cultures using medium identical to that in which the virus was originally produced.¹⁷ Bronchoalveolar lavage cells were co-cultivated in a 25 cm² flask with naïve GSM cells similar to that from which the original virus inoculum was prepared. These flasks were examined daily for cytopathic effects (cell lysis or syncytial cell formation) over two weeks.

Cell Collection and Isolation.

Serial *in vivo* bronchoalveolar lavage (BAL) procedures were performed on a subset of animals under general anesthesia (5.0 mg/kg ketamine IV, 0.1 mg/kg diazepam IV, and 2% Isoflurane inhalation via endotracheal tube) at either two, four, or ten weeks PI a total of 22 times (Table 2.2). A five mm diameter bronchoscope was wedged in the right middle bronchus and flushed with approximately 100 ml of Hank's balanced salt solution.

Blood gases were measured while on ambient air in two medium inoculated and two infected animals at 10 weeks PI with a ABL 505 automatic blood gas analyzer (Radiometer Inc. Copenhagen, Denmark). An *ex vivo* BAL of the right lung was performed at all necropsies for a total of 33 times. *Ex vivo* lavages used 500 ml of Hank's solution that was poured directly into a cannulated right primary bronchus and immediately collected into a sterile container. Cell counts and viability from all lavages were performed by microscopic examination of cells with a trypan blue exclusion stain on a hemocytometer. Cell count means at each time point include pooled data from both *in vivo* and *ex vivo* BAL procedures. All BAL derived cells that were used in cell cultures had >95% viability.

Interstitial lung cells for the infectious center assays were collected at necropsy by mechanical disruption and enzymatic digestion of lung parenchyma. Briefly, lung parenchyma was collected from tissue that had an *ex vivo* BAL procedure performed. Additionally, the pulmonary vasculature was perfused with copious volumes of HBSS until the venous drainage ran clear. Subsequently, this tissue was minced, mixed with 1000 U of collagenase and 50 U of DNase in 3 ml of complete medium (RPMI 1640, 10% fetal calf serum and 1% Penicillin/Streptomycin), and incubated at 37°C in a shaker for 30 minutes. This mixture was then forced through a 16-gauge needle and incubated under the same conditions. The mixture was then strained through a 60 µm stainless steel sieve, washed twice in complete medium, and strained through a 20 µm nylon mesh. Cell counts and viability were determined by examination of trypan blue stained cells on a hemocytometer.

Peripheral blood mononuclear cells (PBMC) were isolated from EDTA treated blood collected from a jugular vein and separated with Ficoll-Paque® Plus (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's directions with slight modifications. The initial centrifuge step was altered to 670x g to more efficiently separate sheep PBMC from neutrophils. Cells were stained with trypan blue and viable cells were enumerated on a hemocytometer. All PBMC isolation procedures routinely had >95% viability.

Bronchoalveolar Lavage Cell Analysis and Flow Cytometry

Total cell counts were done on BAL samples using a hemocytometer and calculated based on the volume of fluid recovered (cells/ml). Differential cell counts were determined by manually counting 200 cells from cytopsin preparations. Lymphocyte subsets were quantitated by Brent Palmer by flow cytometry on fresh BAL samples. T cells were first enumerated using anti-CD2 antibodies (MUC2A at 1:66) (VMRD Inc., Pullman, WA) and single-color flow cytometry. Secondary analyses for CD4 and CD8 subsets was performed by dual staining for either SBU-CD4+ (44.38 at 1:5) or SBU-CD8+ (38.65 at 1:5), (both obtained from The University of Melbourne, Australia, Centre for Animal Biotechnology). The secondary antibodies were goat-anti-mouse IgG conjugated to either fluorochrome (FITC) or biotin (Caltag Laboratories, Burlingame, CA at 1:1000) as appropriate. Streptavidin-phycoerythrin (1:417) was added to samples containing the biotin-labeled secondary antibodies. The samples were washed three times and fluorescence was quantified using an Epics II flow cytometer (Beckman Coulter,

Fullerton, CA). Twenty thousand events were collected and the gate was set around the lymphocyte population based on the forward versus side scatter.

Infectious Center Assay

A subset of animals was analyzed by an infectious center assay. Briefly, 10^5 GSM cells were cultured in an eight well slide chamber. Once confluent, sample cells were added to the wells: 10^2 and 10^3 interstitial cells, 10^2 and 10^3 BAL cells, and 10^5 and 10^6 PBMC. Medium only was added to a negative control well and OvLV strain 85/34 at an MOI of 0.1 was added to a positive control well. The slide was cultured for 4 more days, fixed in cold acetone, and stored at -70°C until immunostaining for viral proteins. Slides were stained simultaneously by immunohistochemistry for detection of viral capsid using the same antibody described with the western blot method except that slides were lightly counterstained with hematoxylin. Results are reported as number of immunohistochemical positive foci per 1000 cells added to well.

Gross Examination and Tissue Preparation

After euthanasia, each animal received a complete post mortem examination. These examinations included, but were not limited to, examination of the lungs, pulmonary-associated lymph nodes, other lymphoid tissues, brain, and joint synovium. Gross lesions were not observed in non-pulmonary tissues so all subsequent analyses focused on the lungs and caudal mediastinal lymph node. Tissue weights were recorded.

The left lung was trimmed of all other tissue at the tracheal bifurcation prior to lung fixation. An endotracheal tube was secured in the bronchus and the lung was

completely submersed in, and infused with 10% buffered neutral formalin. The infusion formalin was contained in a 1 L flask secured 25 cm above the bifurcation of the trachea. Tissues from both the central and peripheral parenchyma of the caudal left lobe were trimmed 24 hours after the necropsy into cassettes and stored in 70% ethanol until processed for paraffin embedding.

Histopathology and Immunohistochemistry

Five micron sections were stained with hematoxylin and eosin and coverslipped. Pulmonary lesions were assessed with a semi-quantitative grading scheme (0 to 3+) and animal identification masked. The specific criteria for the grading of lesions are included in the results section.

Immunohistochemical staining of lung sections for viral antigen was performed. After routine dewaxing and rehydrating, tissues were heated by microwave in a proprietary antigen retrieval solution (Dako Corporation, Glostrup, Denmark) at a pH of 6.0. The primary antibody was monoclonal 3F (anti p25 capsid) and detection was accomplished with the Vectastain[®] Elite ABC-Peroxidase kit and DAB substrate kit according to the manufacturer's instructions. Cells were counterstained with hematoxylin and coverslipped. Lung parenchyma from an ewe chronically infected with OvLV and having severe LIP lesions was used as a positive control. Lung tissue from a seronegative sheep was used as a negative control.

Morphometric Tissue Analysis

Quantitative image analysis involved capturing digital images (TIFF files) of tissues through an Olympus Microscope fitted with a 3-CCD 1140x1520 detail Cool SNAP Camera (Roper Scientific GmbH, Bergkirchen, Germany). These images were subsequently analyzed with Metamorph software v. 4.0 (Universal Imaging Corp., West Chester, PA). Quantification of pulmonary inflammation in H&E sections was accomplished after first calibrating to absolute size with a stage micrometer. After calibration, the area (mm^2) of each lesion was measured and the circumference (mm) of the associated airway or vessel was also captured. The ratio of the H&E stained lesion area to the circumference was calculated to account for parallax views of the lesions.

Statistical Analysis

Statistical analyses were performed with the Graphpad® Instat V. 3.0 software (San Diego, CA). Quantitative mean data were log transformed to establish a Gaussian distribution. Comparison of mean data was analyzed for significance with a two-tailed, unpaired t-test with a Welch correction for means with different standard deviations. Results were considered significant if the P value was < 0.05 .

Results

General Overview

To address the specific aim of this investigation, three-day-old lambs were inoculated with OvLV or tissue culture medium and lesion development was analyzed

during 18 weeks. Lymphocytic interstitial pneumonia was most prominent at four weeks post inoculation (PI) in virus inoculated animals. This pneumonia was characterized by lymphocytes proliferating and accumulating around small and medium sized bronchioles while alveolar macrophages accumulated peripherally to the lymphoid aggregates. Flow cytometric analyses of the lymphocytes revealed that the preponderance of accumulating pulmonary T cells were CD8+. Alveolar macrophages producing OvLV antigens were extremely rare in tissue sections at four weeks PI. At 18 weeks PI, both the inflammation and the viral antigen producing cells were undetectable. Enumeration of infectious virus from alveolar macrophages was also highest at four weeks PI and undetectable by 18 weeks PI. Humoral responses to viral antigen were diagnostic for infection. During these inflammatory changes, general signs of clinical disease or specific signs of respiratory impairment were not observed.

Bronchoalveolar Lavage Cell Analysis

Sequential examination of BAL cells was used to assess temporal inflammatory changes in the lungs. The analysis of BAL fluid revealed an increase in the total number of cells recovered from the infected animals when compared with medium-inoculated animals. At two, four, 10 and 18 weeks PI, the BAL cell concentrations in infected animals were an average 4.0, 3.3, 1.4 and 1.8 fold higher than medium inoculated animals, respectively (Table 2.2). The number of alveolar macrophages, determined by manual differential counts of the BAL cells, were relatively decreased (in percentage of cells) but absolutely increased (in total cells per ml). The lymphocyte populations in the infected animals also had both an absolute and relative increase compared to medium inoculated

animals at all time points. The neutrophil populations had a slight absolute and relative increase at two, four, and 18 weeks PI. Significant differences between the lymphocyte and neutrophil total cell counts of the animal groups were not present despite the consistent changes at all time points in the mean values. This lack of significance despite the difference in mean values was due to a large degree of individual variation within the infected group.

Table 2.2. Analyses of bronchoalveolar lavage (BAL) leukocytes from lambs intratracheally inoculated with ovine lentivirus (OvLV) or tissue culture medium.

Animal Groups and Inoculums	n^a	Total Cells^b (x10⁵)	Macro- phages (% of total)^c (x10⁵)	Neutro- phils (% of total)^c (x10³)	Lympho- cytes (% of total)^c (x10³)	T cells (CD2+) (% of lymphocytes)^d (x10³)
2 week, medium	5+6	3.6	3.5 (97%)	0.1 (3%)	0.1 (3%)	0.2 (6%)
2 week, OvLV	2+7	14.7	11.1 (91%)	0.6 (5%)	0.2 (1%)	3.2 (22%)
4 week, medium	2+4	5.4	4.9 (93%)	9.7 (2%)	4.5 (1%)*	1.5 (34%)*
4 week, OvLV	3+6	18.4	15.4 (84%)	133.2 (7%)	154.3 (9%)*	84.9 (55%)*
10 week, medium	2+9	2.1	2.1 (100%)	1.2 (6%)	0.2 (2%)	0.5 (11%)
10 week, OvLV	7+10	2.3	1.9 (91%)	5.9 (2%)	0.5 (1%)	1.7 (34%)
18 week, medium	0+4	3.0	3.0 (96%)	2.3 (1%)	5.4 (3%)	1.9 (36%)
18 week, OvLV	0+6	5.4	4.4 (88%)	10.7 (2%)	83.3 (8%)	47.5 (57%)

^a Total number of animals measured at the indicated time point for each group with first number in column representing *in vivo* BAL and second number representing *ex vivo* BAL. Differences in numerical patterns were not observed between *in vivo* and *ex vivo* BAL counts, thus the data between the two groups were merged. Total animals = 33 (14 medium inoculated and 19 infected). Total BAL samples = 55.

^b Mean values of both *in vivo* and *ex vivo* BAL counts (in cells/ml or percentage as indicated).
Note: Due to rounding of group averages, the macrophages + neutrophils + lymphocyte combined values may not necessarily equal 100%.

^c Differential percentages were determined by manual counting of cytopsin prepared BAL cells.

^d Percentage of T cells out of total lymphocyte gate was determined by flow cytometry.

* Lymphocyte and T cell p values = 0.059 for indicated groups. All other p values > 0.10.

Flow Cytometry

Further analyses of the BAL lymphocyte populations were accomplished with flow cytometry to enumerate alterations in T cell subsets during the viral infection.

Examination of BAL fluid revealed a consistent increase in the percentage and total number of T cells in the infected animals compared to medium-inoculated animals (Table 2.2). The largest difference in the percentage of T cells was observed at two weeks PI with a 8-fold increase in the percentage of CD2+ cells in the infected lambs when compared to medium-inoculated animals. This observation is included in a trend in which more than half of the lymphocyte population was T cells. However, due to the large variation within the infected group, statistical significance was not achieved. Further phenotypic analysis was conducted on the T cell populations to determine the CD4+ and CD8+ cell counts (Figure 2.1). At two weeks PI, the BAL T cell sub-population mean from infected lambs were 53% CD4+ and 37% CD8+ cells. At two weeks PI, the BAL fluids from medium inoculated lambs contained insufficient lymphocytes to facilitate flow cytometry. At four weeks PI, medium inoculated animals had a mean of 73% CD4+ and 31% CD8+ BAL T cells, yielding a CD4+:CD8+ T cell mean percentage ratio of 2.4:1. In contrast, infected animals at this time point had an approximate 43:46% mean percentage ratio equaling an approximate 1:1 ratio. At 18 weeks PI, the mean percentage ratios of medium inoculated animals were 75:22% equaling a 3.4:1 ratio, while infected animals had an inverted mean percentage ratio of 42:61% equaling a 1:1.5 ratio.

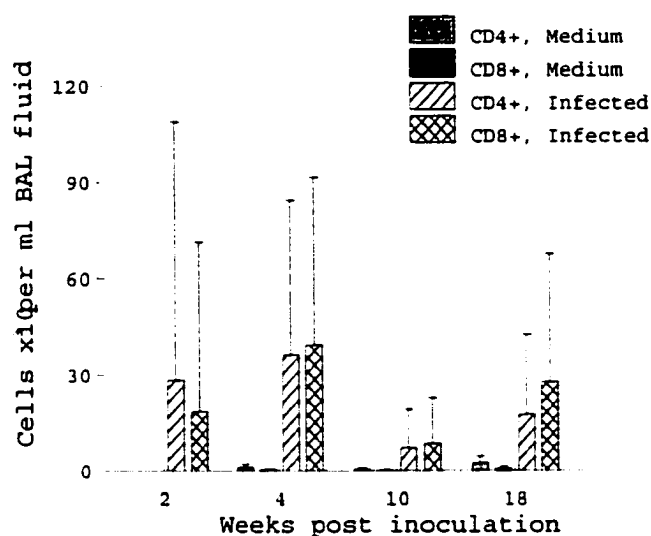


Figure 2.1. Serial changes in pulmonary inflammation in lambs inoculated with either ovine lentivirus (OvLV) or medium analyzed for bronchoalveolar lavage (BAL) fluid T lymphocyte subsets. The bars represent the mean absolute number of either CD4+ or CD8+ T cells per ml of BAL fluid (plus standard deviation) out of the total cells gated as lymphocytes.

Virus Isolation and Serological Analyses

Viral infection in inoculated lambs was confirmed through recovery of infectious virus and by determination of a rising humoral response to the viral antigens. Cytopathic effects (syncytial formations and cell lysis - Similar to Figure 2.13) were observed in BAL and GSM cell co-cultures in 56% (5 of 9) of the animals at two weeks PI. By four weeks PI, 100% (6 of 6) of the inoculated animals tested were positive by virus isolation and all infected animals (5 of 5) remained positive throughout the remainder of the study. All BAL cell samples (n=21) collected from medium inoculated animals at various time points were negative in virus isolation assay. All animals were agar gel immunodiffusion (AGID) test negative for serum antibodies to viral proteins prior to inoculation. AGID tests of serum from inoculated animals detected 16% (3 of 19) positive for OvLV specific antibodies by two weeks PI, 75% (9 of 12) by four weeks PI and 100% (6 of 6) at six weeks PI through 18 weeks PI. All of the medium inoculated animals consistently were

seronegative by AGID. Western blot assays for serum antibodies to OvLV antigen were performed on AGID negative animals to confirm they were seronegative (Figure 2.2).

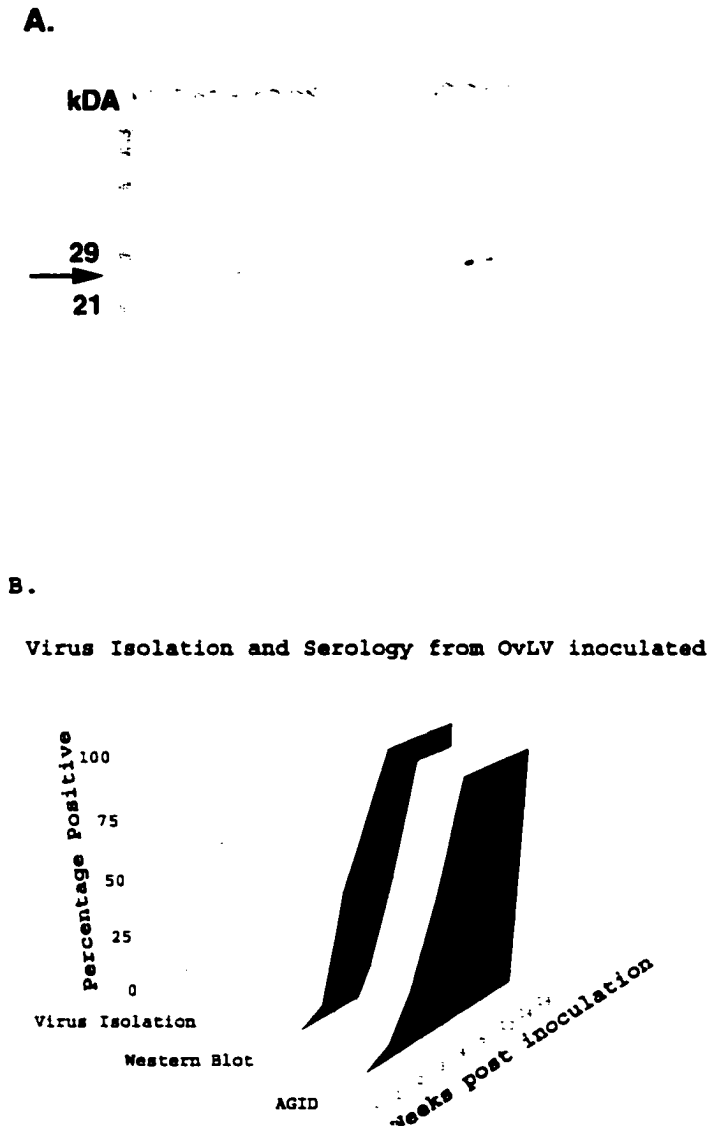


Figure 2.2. Virus isolation and serology from lambs inoculated with OvLV. A) Representative western blot in which lamb serum was screened for the presence of OvLV antigens. The arrow indicates the approximate location of p25 viral capsid. Five positive lanes are observed in this screening. B) Graphical summary of infectious status by either virus isolation, western blot and AGID for anti-OvLV antibodies. Data depicted in graph represents the median percent positive animals (out of 19 lambs total), which were tested bi-weekly. Medium inoculated animals (14 lambs total) were similarly tested and consistently observed to be negative for OvLV by all three methods.

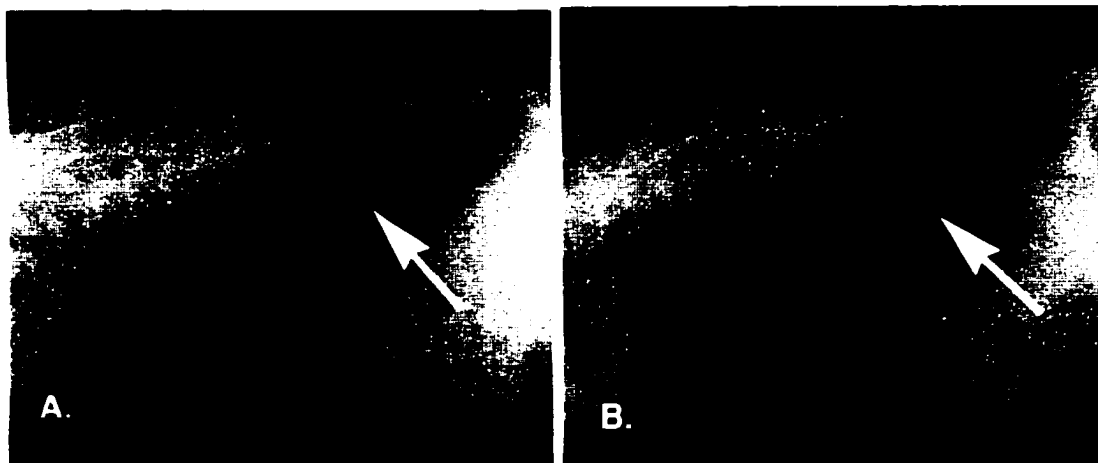
Clinical Assessment

Throughout the course of the experiment there were no observed differences in clinical parameters or respiratory function between the animal groups. These included body temperature, weight gain, and subjective assessments of respiratory effort. Arterial blood gas measurements of four animals were taken at the 10 weeks PI while under general anesthesia to minimize stress induced alterations of respiration. All animals became slightly hypoxic while anesthetized, however, significant differences in functional respiratory capacity were not observed (Table 2.3). Biweekly thoracic radiographs were taken of two medium inoculated animals and two OvLV inoculated animals over 18 weeks. The OvLV inoculated animals had a slight increase in interstitial opacities, primarily in the caudodorsal lung fields, at two and four weeks PI (Figure 2.3).

Table 2.3. Arterial blood gas values of four lambs taken at 10 weeks post inoculation while anesthetized with isoflurane, intubated, and spontaneously breathing room air with an approximate ambient barometric pressure of 642 mmHg.

Animal ID	Medium only: 96rs22	Medium only: 96rs23	OvLV: 96rs24	OvLV: 96rs25	Reference Values*
Arterial blood pH	7.42	7.31	7.46	7.45	7.32-7.50
Partial O ₂ pressure (mmHg)	43	52	48	60	>70
Partial CO ₂ pressure (mmHg)	47	64	40	38	<40
Bicarbonate concentration (mM)	29.9	31.3	28.5	26.1	21-28
Base excess (mEq/L)	4.4	2.8	4.2	2.1	-2 to +2
O ₂ saturations (%)	79.4	82.4	86.3	92.2	>90

* Reference values were adjusted for approximate barometric pressure at 5,000 altitude and taken from Veterinary Medicine: Diseases of Large Animals, by O.M. Radostits, D.C. Blood, and C.C. Gay, 1994, The Bath Press, London, UK



C. Lamb Thoracic Radiographs

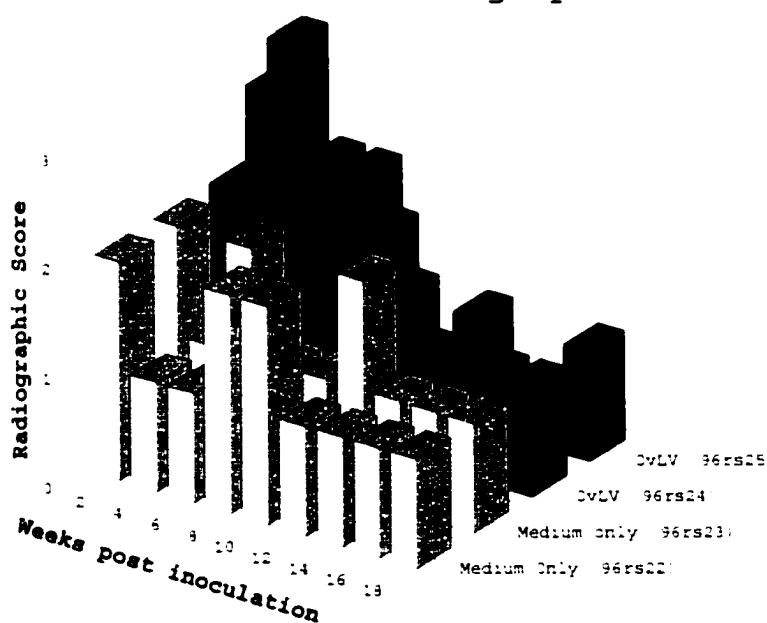


Figure 2.3: Thoracic radiographs were taken of two medium inoculated and two OvLV inoculated lambs at biweekly intervals over 18 weeks. The identity of each radiograph was masked and a qualitative score (0 = normal lung radiograph to 3 = severe interstitial opacities of caudal lung field) was assigned based by a board-certified veterinary radiologist (Dr. Richard Park, Colorado State University). A) Normal radiograph of a medium inoculated lamb at four weeks PI. The white arrow points to a well delineated primary bronchi. B) Radiograph from a lamb four weeks PI with OvLV. White arrows points to the caudal lung field with an increased interstitial pattern which obscure the primary bronchiole. This radiographic was assigned a score of three. C) The highest scores were assigned to the two OvLV inoculated lambs at either two or four weeks PI. From the sixth to the eighteenth week of the study, the radiologist was unable to distinguish the OvLV inoculated from the medium inoculated lambs.

Gross Examination

Necropsies of lambs at two and four weeks PI did not reveal gross lesions in any animals (Figure 2.4). There were slight, but insignificant, increases in the mean weights of the left lungs of infected animals (as a percentage of body weight) at two and four weeks PI. At 18-weeks PI, the lung weights were nearly identical between the two groups (both 0.58% of body weight). One medium inoculated lamb necropsied at 18 weeks PI had a single two cm solitary encapsulated abscess in the caudal right lung field while the remainder of the lung was unremarkable. There were notable gross differences in lymph node sizes between the two animal groups (Figure 2.5). The caudal mediastinal lymph node, which is the primary lymph node draining the pulmonary parenchyma, weighed (as a percentage of total body weight) significantly more (78%, $p = 0.003$) in the infected animals at four weeks PI than in medium inoculated animals. A similar observation was made in the prescapular lymph node, a lymph node not associated with direct pulmonary drainage. The infected lambs in the four weeks PI group had a mean percentage prescapular lymph node weight that was significantly heavier (93%, $p = 0.02$) than the medium inoculated animals. The mean weight differences between lymph nodes at other times were insignificant.



Figure 2.4 Formalin fixed lung from a lamb four weeks post inoculation with OvLV. The left lung from each lamb was intratracheally infused with formalin for 24 hours to achieve uniform fixation. Gross lesions consistent with lymphocytic interstitial pneumonia were difficult to visualize. However, the primary histological lesions were peribronchiolar mononuclear inflammation corresponding to conducting airways at or beyond the arrow.

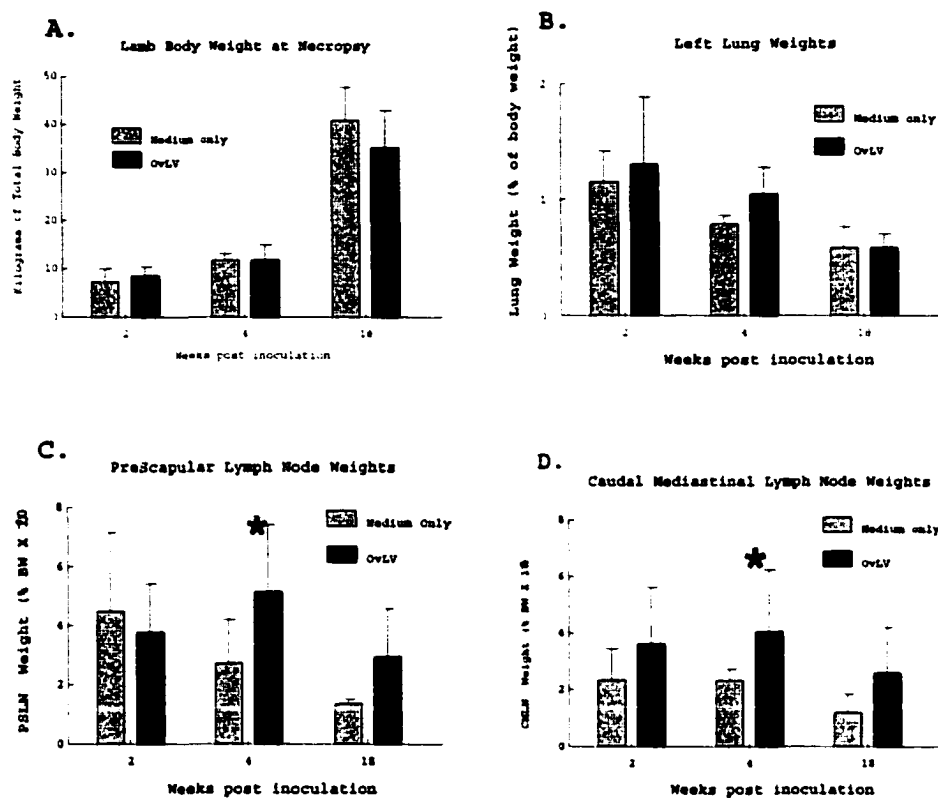


Figure 2.5 Comparison of necropsy tissue weights from lambs inoculated with OvLV or sham inoculated with conditioned medium only: A) Total body weight in kilograms. B) Left lung weight as a percentage of total body weight. C) Right prescapular lymph node weight as a percentage of total body weight (x100). D) Caudal mediastinal lymph node weight as a percentage of total body weight (x100). Both the prescapular and caudal mediastinal lymph nodes from OvLV infected lambs were significantly larger than the medium only lambs at four weeks PI. An asterisk indicates a p value < 0.05.

Pulmonary Histopathology Overview

Hematoxylin and eosin stained tissue sections were examined from the lungs of all animals with individual animal identifications masked to the observer. Interstitial pneumonias of varying severity were present at two and four weeks PI in infected animals while lesions were not observed in the medium inoculated animals (Figure 2.6). The lesions observed at two and four weeks PI in infected animals included an interstitial

infiltrate of mononuclear inflammatory cells (lymphocytes and macrophages). There was prominent mononuclear cell accumulation around medium-sized airways including scattered to dense aggregates of lymphoid cells with occasional lymphoblasts and mitotic figures in 5 of 6 of the infected animals at four weeks PI. Some of the larger lymphoid aggregates at four weeks PI appeared to partially compress the lumens of smaller airways. Alveolar macrophages accumulated circumferentially around the lymphoid aggregates. Extension of the mononuclear infiltrate from the bronchioles into the parenchyma was minimal. However, the interstitium was slightly hypercellular and congested in infected animals compared to medium inoculated animals. Additionally, at four weeks PI in OvLV infected lambs there were rare small foci of neutrophils within alveoli, although intraalveolar fibrin and edema were not observed. These neutrophils were not clearly associated with degenerative changes of pneumocytes or other pulmonary epithelial cells. There was a mild accumulation of mononuclear cells within the lamina propria of some larger airways. The peribronchiolar mononuclear cell accumulation was most prominent in infected animals at two and four weeks PI. Similarly, there was mild to moderate perivascular accumulation of mononuclear cells in OvLV inoculated lambs at two and four weeks PI. At 18 weeks PI, peribronchiolar and perivascular lymphoid accumulations in infected animals were mild to negligible. Peribronchiolar mononuclear cell accumulations in all medium inoculated animals were negligible.

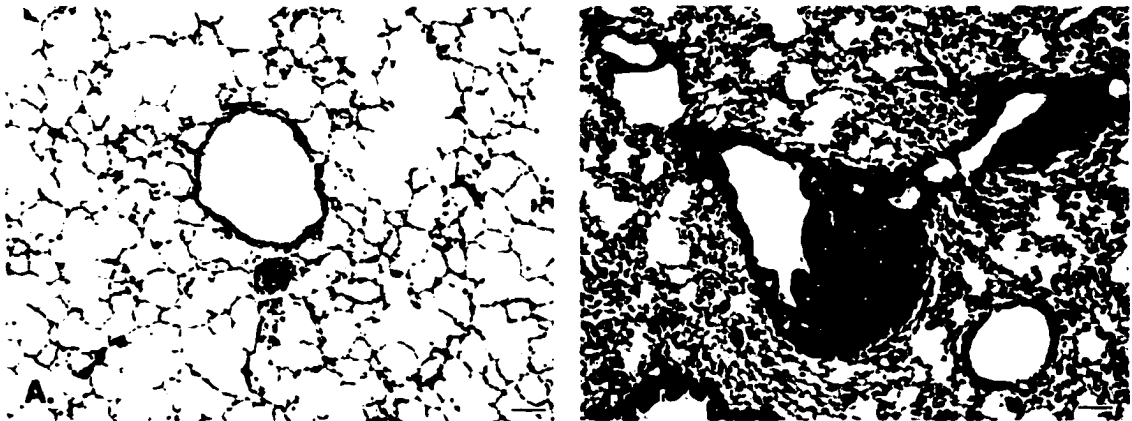


Figure 2.6 Hematoxylin and eosin stained bronchiole, arteriole, and alveolar lung tissue from (A) medium inoculated lamb and (B) OvLV inoculated lamb at four weeks post inoculation. The virus inoculated lambs had prominent peribronchiolar mononuclear cell inflammation with some extension of inflammation into adjacent parenchyma. Additionally, there was an overall increase in the cellularity of the parenchyma. Both lungs were intratracheally infused with formalin for 24 hours after necropsy to minimize atelectasis. Lungs without LIP lesions were more prone to full expansion. Bars represent 50 microns.

Criterion for Scoring and Summary of Pulmonary Lesions

All tissue identification was masked and the order of evaluation was randomized to preclude experimental group bias. A semi-quantitative scoring system was adopted to enumerate mononuclear cell accumulations in or around pulmonary structures (Table 2.4).

Table 2.4 Semi-quantitative scoring system of pulmonary mononuclear cell accumulation.

Grading	Description (per 40x field = 0.23 mm ²)
0	No mononuclear inflammatory cells observed
1	Small numbers of scattered mononuclear cells (fewer than 20 per field with no clear organization).
2	Dense aggregates of mononuclear cells (20-100 per field).
3	More than 100 cells per field and/or extension of the mononuclear aggregates around the majority of the structure's circumference or in a branching pattern to other similar structures

1. *Bronchi*: Anatomically, bronchi are the largest of the intrapulmonary air-conducting vessels and are subdivided based on the number of branches into 1°, 2°, or 3°

bronchi. Gas exchange does not occur within bronchi. Pseudostratified columnar epithelial cells line the bronchi. Subjacent to these cells is circular collagenous fibers (i.e. the lamina propria), smooth muscle, and cartilage. Submucosal glands and large blood vessels are also frequently seen associated with bronchi. The following criteria were adapted from a published description on the normal distribution of lymphoid tissue in the ovine pulmonary system¹⁸:

A. **Bronchial mucosal thickness:** This criterion described epithelial cell alterations. These alterations include either metaplastic or hyperplastic changes of the pseudostratified columnar epithelial cells. In fact no changes were observed from expected normal so criteria were not expanded and all animals received a "0" for this category.

B. **Bronchial submucosal infiltrate:** Scattered lymphoid cells were observed around the bronchi of several infected animals (Figure 2.7). These lymphoid cells were small lymphocytes with scant cytoplasm and were enumerated using criteria described in Table 2.4. Neutrophils, macrophages, or other cells were not observed in this location.

2. **Vasculature:** The vasculature of the lung predominantly follows the same anatomical branching patterns as the conducting airways. As a result, it was often difficult to determine whether loose lymphoid cells were associated with the airway or the blood vessel. Criteria were established so that if the center of a lymphoid aggregate contained a blood vessel (which was identified by flattened endothelial cells), the inflammation was deemed perivascular (Figure 2.8). If the blood vessel was on the periphery of lesions but inflammatory cells were not immediately subjacent to vessel externa then the inflammation was not considered to be perivascular.

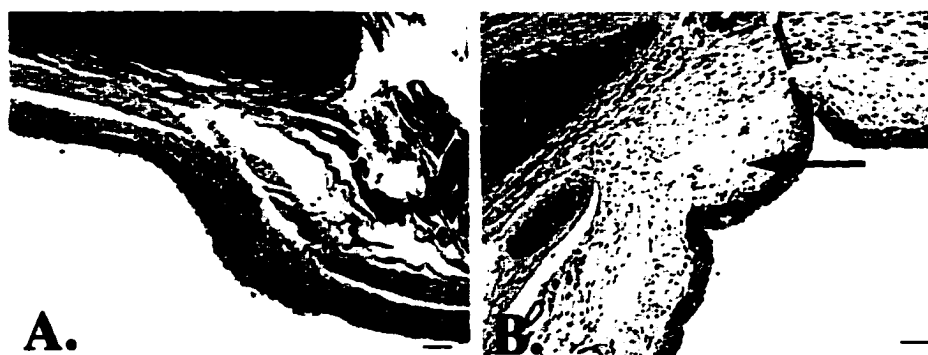


Figure 2.7. Histological alterations associated with bronchial tissue. A) Bronchus from a lamb four weeks post inoculation with medium only (96rs18). B) Bronchus from a lamb four weeks post inoculation with OvLV (96rs10). The arrowhead indicates an expanded region of lamina propria with a scattered infiltrate of mononuclear cells. The bronchus submucosa in the medium only animal is difficult to visualize. Bars indicate 50 microns.

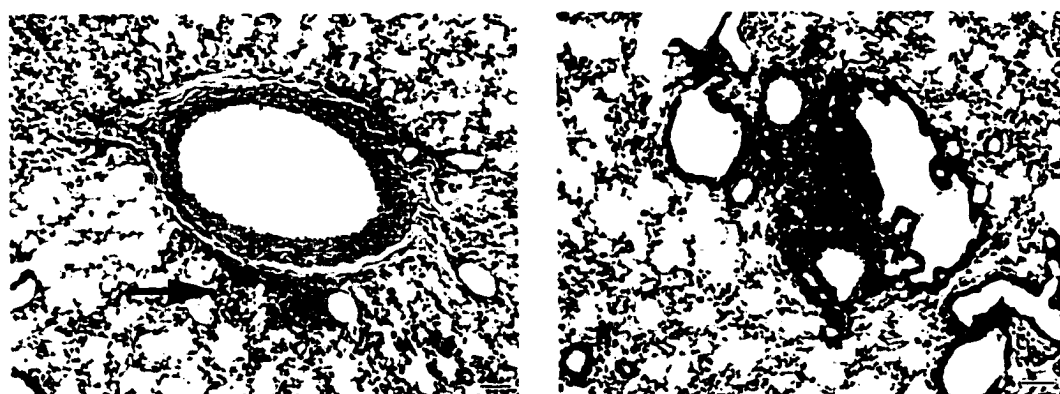


Figure 2.8. Perivascular mononuclear inflammation in lambs four weeks post inoculation with OvLV. A) A small mononuclear aggregate (arrow) is immediately subjacent to a muscular artery and is extending into pulmonary parenchyma. B) The mononuclear cell accumulation was frequently in between small bronchioles and small arterioles or venules. Bars indicate 50 microns.

A. Perivascular mononuclear cells: The degree of perivascular mononuclear cell accumulation was semi-quantitatively evaluated using the scoring system described in Table 2.4

B. Intravascular pulmonary macrophages: All lung sections came from the left

(unperfused) lung of lambs. However, blood was scarce to absent in most vessels except in the three animals which were not exsanguinated. Thus, intravascular cells were not seen and all animals received a “0” for this category.

C. Large mononuclear cells: This cell population included any cells with abundant cytoplasm and a round to reniform nucleus. It is not possible to always distinguish large lymphoblasts from small macrophages so both of these might be included in this population. The scoring system of Table 2.4 was used.

D. Small mononuclear: This cell population was distinguished by small, round dark nuclei with scant cytoplasm and primarily represents the lymphocytes. The scoring system of Table 2.4 was used.

E. Neutrophils: This cell population was identified by its prominent segmented nuclei. The scoring system of Table 2.4 was used.

3. *Bronchioles*: The bronchiole is defined as the conducting airway lined by epithelial cells which vary from columnar to cuboidal and have mild to moderate layers of connective tissue (not exceeding 6 cell layers). Submucosal glands and cartilage are not seen at the bronchiolar level. Peribronchiolar accumulations of mononuclear cells were frequently observed in lambs four weeks PI with OvLV (Figure 2.9). The scoring system of Table 2.4 was used.

A. Bronchiolar Mucosal Thickness: Alterations in the normal bronchiolar mucosa was not observed in any of the animals. Thus all animals received a “0” in this category.

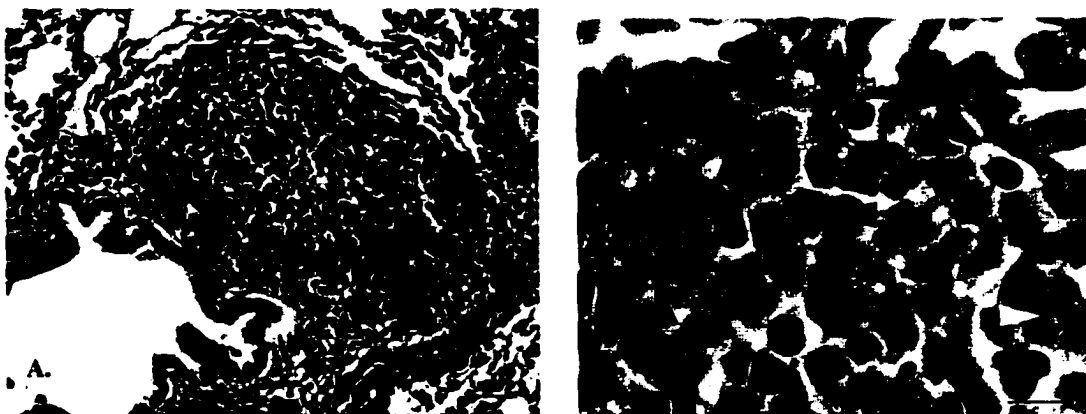


Figure 2.9 Peribronchiolar mononuclear cell accumulation in a lamb four weeks post inoculation with OvLV. A) The inflammatory cells are partially compressing both the bronchiole airway and the adjacent alveolar parenchyma. Bar in Figure A represents 20 microns. B) A higher magnification of the boxed area in Figure A shows a variety of cell types. The black arrow in the center points to a prominent mitotic figure. The black arrowhead on the left points to a large polygonal shaped cell with stippled and marginalized chromatin and abundant cytoplasm; this presumably is a macrophage lineage cell. The white arrowhead on the right points to a rare apoptotic body. Most of the other cells in this field are consistent with lymphoid cells. Bar in Figure B represents 10 microns.

B. Peribronchiolar mononuclear cell accumulation: The anatomical sub-compartment isolated in these observations was the loose connective tissue surrounding bronchioles but not primarily centered in the adjacent alveolar tissue. In addition to the cell types listed below, organizational categories of the lymphoid infiltrate were also defined. The scoring system of Table 2.4 was used.

C. Lymphoid Follicles (nodules): These structures are synonymous with the previously described dense aggregates of lymphoid cells.¹⁸ This category defines the percentage of bronchioles which have dense lymphoid aggregates and uses the same scoring system as the previous category.

D. Follicles with germinal center: These structures were extremely rare in both the OvLV and medium inoculated lambs. Follicles with germinal centers were defined as

mononuclear cell aggregates (defined above) with central area of larger cells with less stain intensity surrounded by smaller, darker cells. Again, the distinction was difficult to make and no “definitive” germinal centers were observed. However, there were rare equivocal characteristics of germinal centers and such observations were assigned a score of 1.

E. Non-organized infiltrate: This category includes lesions that did not clearly fit into other categories. Non-organized infiltrate is characterized by an apparently random scattering of cells that is of low cellular density. The scoring assignments are 0 = none observed, 1 = loose infiltrate around a few bronchioles (less than 10% of total bronchioles), 2 = intermediate or loose infiltrate around 20 to 50% of bronchioles observed, 3 = greater than 50% of bronchioles with loose infiltrate.

F. Extension of Infiltrate: This category described the degree of infiltrate extension (loose or dense) from one bronchiole to the next. 0 = none, 1 = surrounding bronchioles and starting to extend into parenchyma, but not reaching any other bronchioles, 2 = starting to bridge with other bronchioles, 3 = coalescence from one bronchiole to the next.

G. Large Mononuclear: Same as perivascular scoring system except with reference to bronchioles.

H. Small Mononuclear: Same as perivascular scoring system except with reference to bronchioles.

I. Neutrophils: Same as perivascular scoring system except with reference to bronchioles.

4. Alveolus: This is the section of lung that has previously been referred to as

“parenchyma,” or the functional part of the lung where gas exchange occurs. Based on the functional necessity and structural complexity of the tissue, a variety of categories were evaluated as documented below (Figure 2.10).

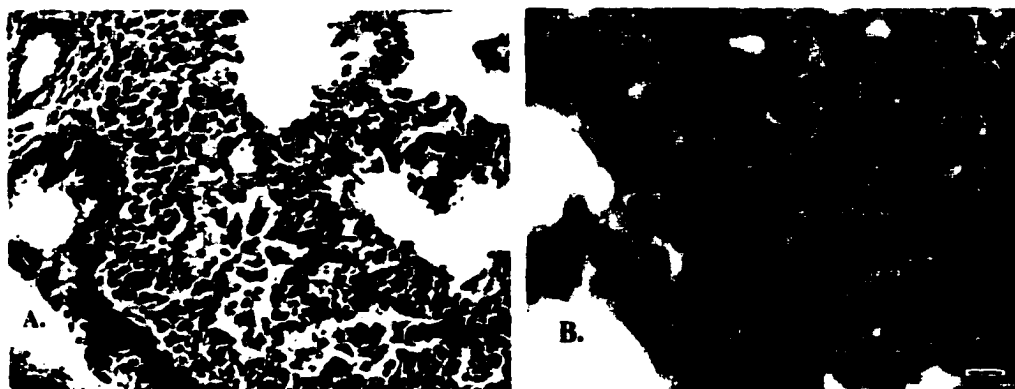


Figure 2.10 Extension of pulmonary inflammation into parenchyma in a lamb four weeks PI with OvLV. A) Similar to previous Figures, there are mononuclear cells accumulating subjacent to bronchiolar connective tissue. However, beyond this accumulation are clumps of alveolar macrophages (black arrows), some of which appear to be binucleate. The green line spans a section of alveolar wall that is markedly thickened by a variety of inflammatory cells and type II pneumocyte hyperplasia. Bar in Figure A represent 20 microns. B) A higher magnification of a thickened interstitial wall demonstrates a large number of both small and large mononuclear cells. Additionally, there are congested capillaries and the rare presence of neutrophils (black arrows). Bar in Figure B represents 10 microns.

A. Congestion with red blood cells: Pulmonary congestion was rare due to the exsanguination technique used for euthanasia. Only a few animals that died from anesthetic deaths and thus were not exsanguinated had any appreciable congestion. Pulmonary congestion was scored either 1 (minimal with a single row of red blood cells in an alveolar capillary) or 2 (more than one row of cells per capillary).

B. Alveolar Wall Thickness: A semi-quantitative score was assigned to the cellularity within the alveolar wall. Normally, only one cell is observed in a typical

histological view of an alveolar wall. An overall assessment of alveolar walls not immediately adjacent to conducting airways or blood vessels was as follows: 1 = 2 cells were frequently seen within the wall, 2 = 3-4 cells were routinely seen, 3 = more than 4 cells were routinely seen.

C. Smooth muscle: Obvious alterations in smooth muscle were not observed. Several animals with higher degree of inflammation had minimal smooth muscle hyperplasia in or around the inflammation. The animals with minimal alterations were designated 1 while all others were assigned a "0".

D. Type II Pneumocytes: Type II pneumocytes were more abundant in neonatal animals (i.e. both OvLV and medium inoculated two weeks PI groups) than in older animals. This increase of type II pneumocytes in both OvLV and medium inoculated younger groups made comparison with the older groups difficult. However, there was a small group of infected animals observed with slightly more Type II cells than their age-matched controls. These type II cells were spatially associated with LIP. These infected animals were scored 1 while the remaining animals were assigned a 0.

E. Alveolar Macrophages: The total numbers of alveolar macrophages were semi-quantitatively evaluated using the following criteria: 0 = macrophages within alveoli were rare, 1 = approximately 50% of alveoli had at least one macrophage, 2 = the majority of alveoli have at least one macrophage, 3 = there were frequently more than one macrophage per alveoli.

F. Interstitial Large Mononuclear: Same as perivascular scoring system except with reference to interstitium.

G. Interstitial Small Mononuclear: Same as perivascular scoring system except

with reference to interstitium.

H. Neutrophils: Same as perivascular scoring system except with reference to interstitium.

I. Necrosis: Interstitial wall necrosis, i.e. fibrinoid changes, was rarely observed in any animals. Several of the animals had a scant amount of fibrinoid changes in peribronchiolar interstitium that was associated with peribronchiolar neutrophils (previously scored, as shown in Figure 2.10). These animals were designated 0.5. All others were designated 0.

5. *Pleura*: The connective tissue serosal covering of the lung parenchyma was observed. Alterations of this connective tissue layer were not observed in any of the animals and thus all animals were assigned a score of 0.

Table 2.5 Semi-quantitative assessments of histological alterations in individual lambs inoculated with either tissue culture medium or OvLV at two, four, or 18 weeks post inoculation. Group medians are reported.

<u>Animal</u>	<u>Bronchi</u>	<u>Bronchi mucosal thickness</u>	<u>Bronchi submucosa infiltrate</u>	<u>Vessels</u>	<u>Intravascular mononuclear cells</u>	<u>Perivascular mononuclear cells</u>	<u>Large Mononuclear</u>	<u>Small Mononuclear</u>	<u>Neutrophils</u>	<u>Bronchioles</u>	<u>Bronchiolar Mucosal Thickness</u>	<u>Bronchiolar submucosa infiltrate</u>	<u>Non-organized infiltrate</u>	<u>Lymphoid Follicles (nodules)</u>	<u>Follicles with germinal center</u>
Two Weeks PI – Medium only:															
96RS01		0	0		0	0	0	0	0		0	0	0	0	0
96RS04		0	0		0	0	0	0	0		0	0	0	0	0
96RS31		0	0		0	0	0	0	0		0	0	0	0	0
96RS32		0	0		0	0	0	0	0		0	0.5	0.5	0	0
97RS19		0	0		0	0	0	0	0		0	0	0	0	0
97RS21		0	0		0	0	0	0	0		0	1	1	0	0
Median		0	0		0	0	0	0	0		0	0	0	0	0
Four Week PI – Medium only:															
96RS03		0	0		0	0	0	0	0		0	0	0	0	0
96RS18		0	0		0	0	0	0	0		0	0.5	0.5	0	0
97RS03		0	0		0	0	0	0	0		0	0	1	0	0
97RS20		0	0		0	0	0	0	0		0	0	1	0	0
Median		0	0		0	0	0	0	0		0	0	0.75	0	0
Eighteen Weeks PI – Medium only:															
96RS02		0	0		0	1	0	0	0		0	1	1	0.5	0
96RS06		0	0		0	1	2	1	0.5		0	1	1	0.5	0
96RS22		0	0		0	0	0	0	0		0	1	1	1	0
96RS23		0	0		0	0	0	0	0		0	1	1	0.5	0
Median		0	0		0	0.5	0	0	0		0	1	1	0.5	0

Table 2.5 Continued.

<u>Animal</u>	<u>Extension of Infiltrate</u>	<u>Large Mononuclear</u>	<u>Small Mononuclear</u>	<u>Neutrophils</u>	<u>Alveolus</u>	<u>Congestion (RBC's)</u>	<u>Alveolar Wall Thickness</u>	<u>Smooth muscle</u>	<u>Type II Pneumocytes</u>	<u>Alveolar Macrophages</u>	<u>Interstitial Large Mononuclear</u>	<u>Interstitial Small Mononuclear</u>	<u>Neutrophils</u>	<u>Necrosis</u>	<u>Pleura</u>	<u>Pleural thickening or infiltrate</u>
Two Weeks PI - Medium only:																
96RS01	0	0	0	0		2	0	0	1	0	0	0	0	0		0
96RS04	0	0	0	0		1	0	0	0	1	0	0	0	0		0
96RS31	0	0	0	0		1	0	0	0	0.5	0	0	0	0		0
96RS32	0	0.5	0.5	0		0	0	0	0	0	0	0	0	0		0
97RS19	0	0	0	0		0	0	0	0	0	0	0	0	0		0
97RS21	0	1	1	0		0	0	0	0	0	0	0	0	0		0
Median	0	0	0	0		0.5	0	0	0	0	0	0	0	0		0
Four Week PI - Medium only:																
96RS03	0	0	0	0		0.5	x	0	0	0	0	0	0	0		0
96RS18	0	0	1	0		0	0	0	0	0	0	0	0	0		0
97RS03	0	0	1	0		0	0	0	0	0	0	0	0	0		0
97RS20	0	0	1	0		0	0	0	0	0	0	0	0	0		0
Median	0	0	1	0		0	0	0	0	0	0	0	0	0		0
Eighteen Weeks PI - Medium only:																
96RS02	0	0	1	0		0	0	0	0	1	0	0	0	0		0
96RS06	0	1	1	0		0	0	0	0	0	0	0	0	0		0
96RS22	0	0	1	0		0	0	0	0	0.5	0	0	0	0		0
96RS23	0	0	1	0		0	0	0	0	0	0	0	0	0		0
Median	0	0	1	0		0	0	0	0	0.25	0	0	0	0		0

x = not evaluated due to artifactual atelectasis.

Table 2.5 Continued.

<u>Animal</u>	<u>Bronchi</u>	<u>Bronchi mucosal thickness</u>	<u>Bronchi submucosa infiltrate</u>	<u>Vessels</u>	<u>Intravascular mononuclear cells</u>	<u>Perivascular mononuclear cells</u>	<u>Large Mononuclear</u>	<u>Small Mononuclear</u>	<u>Neutrophils</u>	<u>Bronchioles</u>	<u>Bronchiolar Mucosal Thickness</u>	<u>Bronchiolar submucosa infiltrate</u>	<u>Non-organized infiltrate</u>	<u>Lymphoid Follicles (nodules)</u>	<u>Follicles with germinal center</u>
Two Weeks PI - OvLV:															
96RS07		0	1		0	1	1	1	0		0	1	1	0	0
96RS13		0	1		0	2	2	1	0		0	2	2	1	0
96RS14		0	0		0	1	1	1	1		0	1	1	0	0
96RS15		0	0		0	0.5	0.5	0.5	0		0	1	1	0	0
96RS29		0	0		0	1	1	1	0		0	1	1	0	0
97RS14		0	0		0	0	0	0	0		0	1	1	0.5	0
97RS18		0	0		0	0.5	0.5	0.5	0		0	1	1	0	0
Median		0	0		0	1	1	1	0		0	1	1	0	0
Four Weeks PI - OvLV:															
96RS09		0	0		0	0.5	0.5	0.5	0		0	1	1	0	0
96RS10		0	2		0	1	3	3	0		0	2	2	1	0.5
96RS12		0	1		0	1	2	1	0		0	2	1	2	0.5
96RS17		0	0		0	1	0	1	0		0	1	1	1	1
97RS13		0	1		0	2	2	1	0		0	2	2	0	0
97RS15		0	1		0	1	2	1	0		0	1	1	0	0
Median		0	1		0	1	2	1	0		0	1.5	1	0.5	0.25
Eighteen Weeks PI - OvLV:															
96RS08		0	0		0	0	0	0	0		0	1	1	0	0
96RS11		0	0		0	0	0	0	0		0	0	0	0	0
96RS16		0	0		0	0	0	0	0		0	0	0	0	0
96RS24		0	0		0	0	0	0	0		0	0	0	0	0
96RS25		0	0		0	0	0	0	0		0	1	0	0	1
97RS17		0	0		0	0	0	0	0		0	0	0	0	0
Median		0	0		0	0	0	0	0		0	0	0	0	0

Table 2.5 Continued.

<u>Animal</u>	<u>Extension of Infiltrate</u>	<u>Large Mononuclear</u>	<u>Small Mononuclear</u>	<u>Neutrophils</u>	<u>Alveolus</u>	<u>Congestion (RBC's)</u>	<u>Alveolar Wall Thickness</u>	<u>Smooth muscle</u>	<u>Type II Pneumocytes</u>	<u>Alveolar Macrophages</u>	<u>Interstitial Large Mononuclear</u>	<u>Interstitial Small Mononuclear</u>	<u>Neutrophils</u>	<u>Necrosis</u>	<u>Pleura</u>	<u>Pleural thickening or infiltrate</u>
Two Weeks PI - OvLV:																
96RS07	0	1	1	0		2	0	0	0	1	0	0	0	0		0
96RS13	2	2	1	0		0	1	0	0	1	2	1	1	0.5		0
96RS14	0	1	1	0		0	0	0	0	1	0	0	0	0.5		0
96RS15	0	1	1	1		0.5	0	0	0	1	1	1	1	0		0
96RS29	0	0	1	0		0	0	0	0	0	0	0	0	0		0
97RS14	0	1	1	0		0	0	0	0	0	0	0	0	0		0
97RS18	0	2	1	0.5		0	0	0	0	1	0	0	0.5	0		0
Median	0	1	1	0		0	0	0	0	1	0	0	0	0		0
Four Weeks PI - OvLV:																
96RS09	1.5	1.5	1.5	1		1	1	0	0	1	1	1	0	0		0
96RS10	2	2	1	0		1	2	0	1	2	2	1	2	1		0
96RS12	1	2	1	0		1	1	0	1	1	1	1	0	0		0
96RS17	0	2	2	0		0	0	0	0	1	0	0	0	0		0
97RS13	1	2	1	1		0	0	0	0	1	0	0	1	0		0
97RS15	0	1	2	0		0	0	0	0	2	0	0	0	0		0
Median	1	2	1.25	0		0.5	0.5	0	0	1	0.5	0.5	0	0		0
Eighteen Weeks PI - OvLV:																
96RS08	0	0	0	0		0	0	0	0	1	0	0	0	0		0
96RS11	0	0	0	0		0	0	0	0	0	0	0	0	0		0
96RS16	0	0	0	0		0	0	0	0	1	0	0	0	0		0
96RS24	0	0	0	0		0	0	0	0	0	0	0	0	0		0
96RS25	0	0	1	0		0	0	0	0	1	0	0	0	0		0
97RS17	0	0	0	0		0	0	0	0	0	0	0	0	0		0
Median	0	0	0	0		0	0	0	0	0.5	0	0	0	0		0

Histological evaluation of lymph nodes:

The only other notable alteration of tissue architecture in these animals was lymph node hyperplasia in OvLV inoculated lambs (Figure 2.11). There was hyperplasia in lymph nodes that were associated with pulmonary lymphatics (i.e. the caudal mediastinal lymph node) and peripheral lymph nodes unassociated with the pulmonary system (i.e. the prescapular lymph node). The hyperplastic patterns of these lymph nodes included an expansion of the cortex with prominent follicles and germinal centers characteristic of both a T and B cell response to antigen. Evidence of nodal hyperplasia could be seen at all time points in the OvLV infected group, although germinal centers were less prominent in OvLV infected lambs at 18 weeks PI.



Figure 2.11 Hematoxylin and eosin stained caudal mediastinal lymph nodes from experimental lambs at four weeks PI. The caudal mediastinal lymph node drains the entire pulmonary lymphatic tree and was markedly hyperplastic in OvLV inoculated animals. A) This lymph node from an OvLV infected lamb has a marked increase of the cortex. There is a prominent lymphoid follicle with a germinal center (designated by arrowheads) immediately above an afferent lymphatic, which is congested with numerous lymphocytes. The medulla is not visible in this figure. B) A lymph node from age-matched medium inoculated lamb has a poorly developed cortex and prominent medulla with dilated medullary sinuses. For both images, the bars represent 0.1 mm.

Morphometric Analyses:

The semi-quantitative observations just described were expanded using image analysis to quantitate the area of peribronchiolar and perivascular mononuclear cell accumulation (Figure 2.12). This technique measured the square area of the lymphoid aggregates in tissue section and divided by the circumference of the bronchiole or blood vessel adjacent to the lymphoid aggregate. The mean value for each group was compared by a t-test. There was a significant increase in both the peribronchiolar and perivascular lymphoid accumulation at both two and four weeks PI.

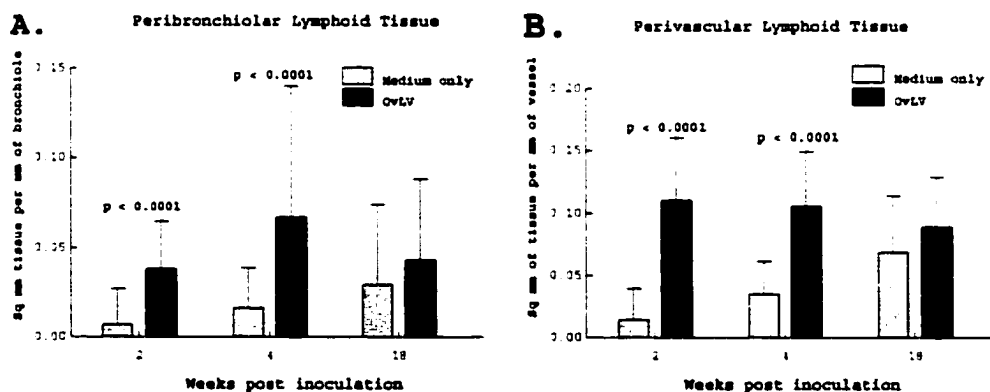
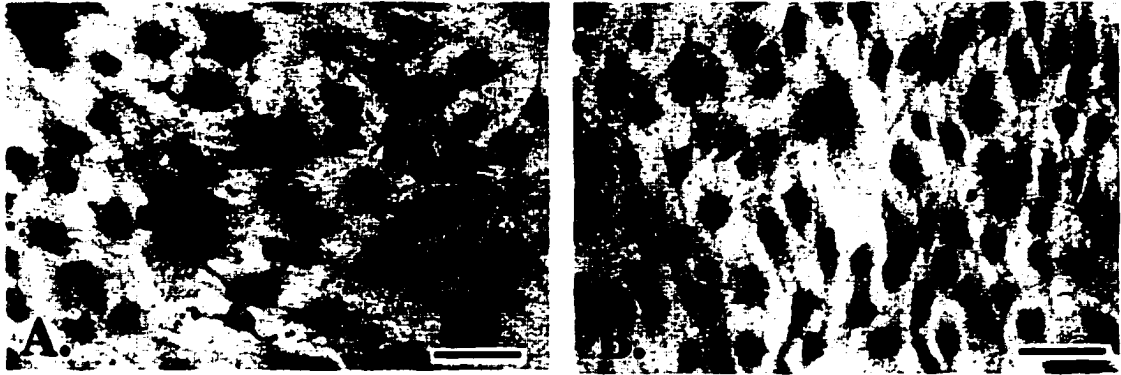


Figure 2.12 Area of (A) peribronchiolar and (B) perivascular mononuclear cell accumulation in OvLV and medium inoculated lambs at various time points. There was a significant increase in the area of mononuclear cell accumulation at two and four weeks post inoculation in both peribronchiolar and perivascular areas.

Infectious Center Assay

The production of new virus in OvLV inoculated lambs was estimated with an infectious center assay on a subset of animals. This assay co-cultures dilutions of potential virus producing cells with a population of cells that are susceptible to infection. This susceptible cell population is goat synovial membrane (GSM) cells which are subsequently stained for viral antigen and the number of foci are enumerated per test cells added to culture. Peripheral blood mononuclear cells, pulmonary interstitial cells, and alveolar macrophages were added in separate cultures. In the animals tested, the alveolar macrophages produced the most infectious virus at four weeks PI (Figure 2.13).



C. Infectious Center Assay from OvLV infected L

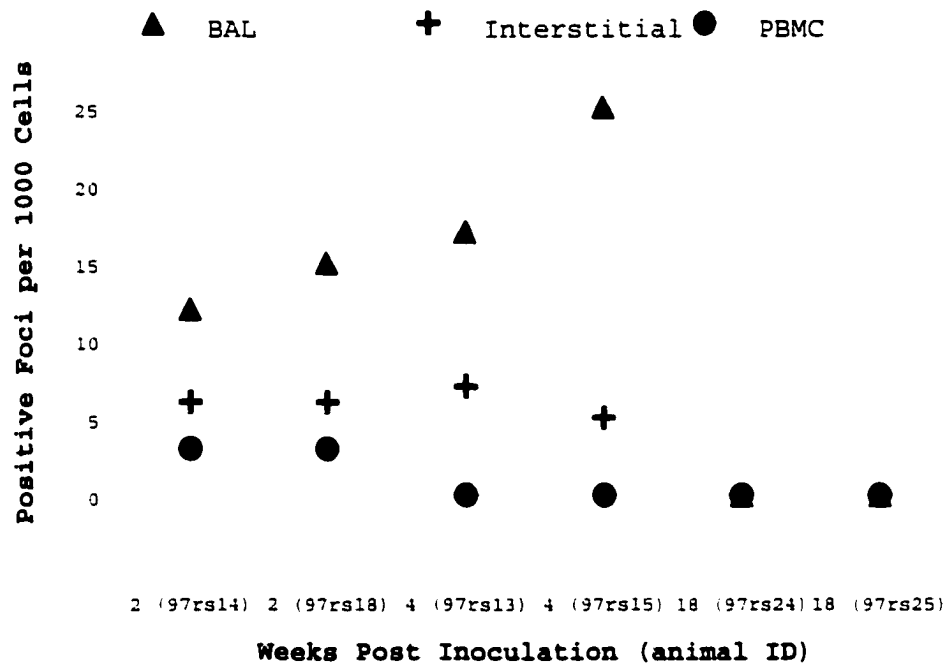


Figure 2.13 Infectious center assay for virus production from bronchoalveolar lavage cells, pulmonary interstitial cells, and peripheral blood mononuclear cells in six animals at three different times PI. A) Goat synovial membrane (GSM) culture with immunohistochemically (brown stain) positive foci for viral antigen. Note that the multinucleated cell stains positive. B) GSM co-culture from a lamb inoculated with medium only. Only the counterstain (blue) is present. Bars represent ten microns. C). Data are reported as the number of foci per 1000 cells added to the GSM culture. Co-cultures with the BAL cells, which are predominantly alveolar macrophages, produced the most positive foci at four weeks PI. Interstitial cells were second in foci numbers while PBMC produced the fewest foci. Virus was not recovered at 18 weeks PI.

Immunohistochemistry for Viral Protein

Paraffin embedded lung tissues were stained for viral antigen (capsid p25) using a peroxidase based detection method. Although multiple attempts were made using a variety of antigen unmasking procedures, the number of viral antigen positive cells in OvLV inoculated lambs was extremely low (usually 1 or 2 positive cells per entire tissue section and only at four weeks PI). The few cells which did stain positive for OvLV were macrophages located in alveoli. The low number of OvLV positive cells observed in these experimental infections occurred despite the observation of numerous antigen positive cells in chronic cases of natural OvLV infection using the same assay (Figure 2.14). The large number of sections with no antigen positive cells precluded Gaussian distribution of the data.

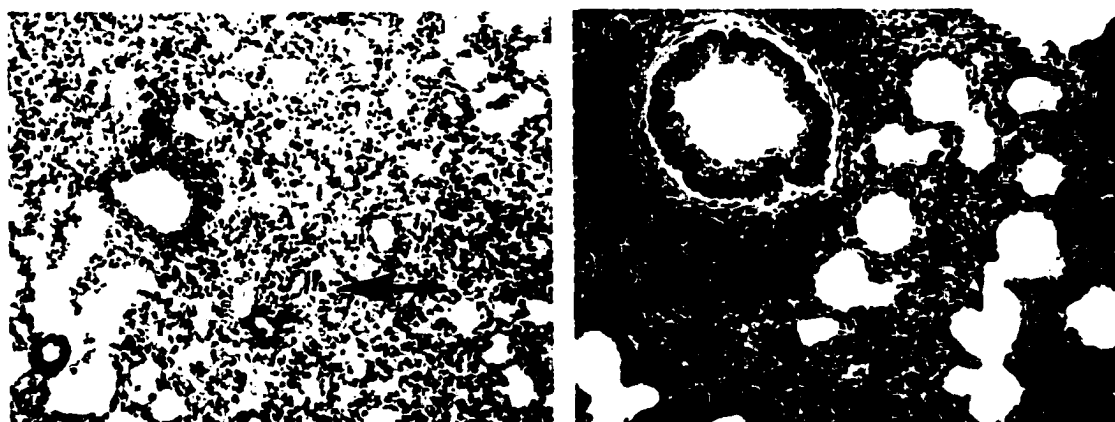


Figure 2.14 Lung sections immunohistochemically stained for viral antigen (capsid p25) using a peroxidase-based detection system and counterstaining with hematoxylin. A) Tissue from a lamb at four weeks PI with OvLV and histological lesions of LIP had rare viral antigen positive cells (arrow). B) Tissue from a ewe with a natural OvLV infection and chronic pulmonary lesions was used as a positive control for this assay. There are multiple macrophages that are immunohistochemically positive for viral antigen. Several of the cells are multinucleated. Bars represent 0.1 mm.

Discussion

The experiments described herein provide the first comprehensive description of the early host response to lentivirus infection. These studies are advantageous in sheep because they are a natural host of lentiviruses, exhibit a spectrum of changes similar to humans infected with HIV, and the pathogenesis is naturally limited to virus infection of macrophages only. The timing and incidence of lentivirus associated pulmonary lesions during the first weeks of infection were previously unknown. The combined clinical and histopathological data presented herein suggests that the majority of animals infected with OvLV will develop some degree of lymphocytic interstitial pneumonia within the first four weeks of infection and the lesions will regress by 18 weeks PI. It has been asserted that in a lentivirus infection, a peak in viral load that is followed by generation of T cells and a subsequent reduction in viral load is evidence of an adaptive immune response.¹⁹ The evidence for this assertion is particularly strong in lentiviruses that productively infect both macrophages and T cells such as the human and simian immunodeficiency viruses²⁰⁻²⁴ However, this assertion may not be completely accurate in a lentivirus system such as OvLV, which can only infect and replicate in macrophages *in vivo*, due to the macrophages' function as an antigen presenting cell.

Previous studies have described pulmonary inflammation in experimentally inoculated animals.^{15,17} However, these studies included longer incubation periods (up to six months PI) and were primarily designed to compare different OvLV strains. At six months PI, the severity of pulmonary lesions varied: approximately 25% had moderate to

severe lesions, 50% had mild lesions, and 25% had no detectable lesions.¹ The data reported in this investigation provide a clearer picture of lesion development. There were multiple lines of evidence that indicated the early pathogenesis included a peak in disease signs in all animals at four weeks PI. This evidence included histopathological alteration of lungs and lymph nodes, increases in tissue weight, pulmonary radiographic lesions, increases in BAL cell counts, and recovery of infectious virus that all peaked at four weeks PI. Additionally, four weeks PI was the primary time of seroconversion in OvLV inoculated lambs. Interestingly, the peak in lymphoid tissue hyperplasia occurred in both pulmonary and non-pulmonary lymph nodes. The prescapular lymph node is unassociated with lymphatic drainage of the pulmonary system yet had an identical response to the mediastinal lymph node. Presumably this occurs because the virus inoculum method is intratracheal, thus much of the virus is absorbed into the pulmonary capillaries and quickly dispersed throughout the body.

The development and regression of the histological features of LIP had previously been poorly documented. Normal neonatal sheep have a scarce amount of lymphoid tissue in their lungs. When sheep are infected with OvLV, lymphocytes will infiltrate, proliferate, and organize around the bronchioles and arterioles of the lungs. This process of mononuclear cell accumulation does not appear to be directly in response to virus induced cytopathic tissue changes, such as virus-induced apoptosis or necrosis. There was no evidence of alterations to the epithelial, mesenchymal, or vascular components of these medium sized air-conducting structures. In fact, the spatial relationship of these immune cells to the presumed virus infected cell is unusual compared to other viral induced inflammatory processes. In other viral diseases, such as hepatitis or dermatitis, one

frequently observe lymphocytes immediately adjacent to or infiltrating into the virus infected cell population regardless of the circulatory patterns of the tissue. OvLV associated LIP appears to differ from this lymphocyte infiltration pattern. The lymphocytes exit the pulmonary vasculature the level of bronchioles and accumulate in the peribronchiolar and perivascular connective tissue. Once emigrated from the vascular system they also proliferate *in situ* as indicated by the presence of mitotic figures. There is some lymphoid tissue organization into rudimentary follicles. Well-developed follicles with germinal centers (characteristic of B cell stimulation) were not clearly observed. Thus, it is presumed that the predominant lymphoid organization is T cell in nature.

The T cell nature of the pulmonary lesions was confirmed with flow cytometry. There was a significant increase in the number of CD2+ cells from the BAL fluid. Presumably, the primary source of these T cells was the peribronchiolar accumulation. It is worth mentioning that *ex vivo* BAL procedure should not have confounded the histological analysis of the same animal since the two procedures were done on mutually exclusive lungs. Additionally, there was a rise in the total number of alveolar macrophages in the BAL fluid. The corresponding increase in alveolar macrophages in the histological evaluation had a different pattern than the lymphocytes. Diffusely, throughout the alveoli, the number of macrophages was increased. Occasionally, there would be accumulations of macrophages around the peribronchiolar aggregates. There were, however, frequent peribronchiolar lymphoid aggregates without macrophage aggregates in close proximity. This observation raises several questions as to the functionality of the peribronchiolar lymphocytes. The focus of these new questions is now on biological necessity for armed cytotoxic lymphocytes to be in close proximity of their

target cells to function properly. This proximity is particularly important if the two principal methods of cytotoxic T cell killing of virus infected cells is to be effective: perforin-granzyme-dependent killing and Fas-Fas ligand-dependent killing.

A parallel investigation of the lymphocytes collected from these animals further analyzed the diversity and functional attributes of these cells²⁵ and is reported in part in this chapter (Figure 2.1). This investigation further determined that the BAL derived lymphocytes included an oligoclonal expansion of CD4+ T cells and a polyclonal expansion of CD8+ T cells. Furthermore, the cytokine profile of these T cells was that of a Th₁ response. This cytokine response is characterized by IFN- γ , which is a powerful activator of macrophages and IL-2, which can act in an autocrine manner to stimulate further T cell proliferation and accumulation. Collectively, the lymphocytic and macrophage alterations in the lung are consistent with a process that is viral induced, but it is still unclear whether this inflammatory response is an effective immune response. This uncertainty parallels that voiced by Eriksson *et. al.* who performed *in vivo* experiments depleting T cell subpopulations and concluded that CD8+ depletion does not alter the course of acute viremia but CD4+ depletion reduces viremia.^{26,27} Since the CD4+ population primarily affects the immune system through soluble mediators, it would follow that the absence of a soluble mediator is a potential explanation for the reduced viremia with CD4+ depletion.

There were no observations of OvLV-associated necrosis in pulmonary tissue in this investigation. The presence of rare neutrophil aggregates in the OvLV inoculated animals was matched by rare neutrophil aggregates in the medium inoculated animals. Additionally, there was not a statistical difference in the BAL neutrophil counts between

the two groups. The presence of these neutrophils could indicate a host response to a component of the tissue culture medium inoculum or could simply be an unrelated response to a bacterial pathogen that both groups of lambs contacted.

In conclusion, this investigation has defined the breadth and depth of alterations that occur in the pulmonary system of OvLV infected neonatal lambs. There is clear evidence that the inflammation is associated with virus infection, but there are also questions as to whether effective immunity is occurring. There is also evidence that viral production peaks and diminishes over the first few weeks of infection. However, it is not clear what mechanisms drive this pattern of virus production: are they host factors or are they virus factors? The following chapter will more carefully examine the relationship of the host response to virus infection rates in order to help answer these questions.

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Chapter Three

Pulmonary Inflammation and Ovine Lentivirus Infection Rate Dynamics

Abstract

The interaction of the retrovirus and host factors that affect replication during the acute stage of infection remains poorly understood. Since the macrophage is the primary target cell of ovine lentivirus (OvLV) and a central cell in regulating pulmonary inflammation, it is possible that recruitment and activation of macrophages may increase infectious dissemination of the virus within a host. Thus, a paradox is present in how an activated immune system necessary for viral replication can co-exist with an activated immune system necessary for anti-viral immunity. This study was designed to examine the alterations of OvLV infection rates in alveolar macrophages during the development of lymphocytic interstitial pneumonia. Thirty-three neonatal lambs were intratracheally inoculated with either OvLV strain 85/34 or cell culture medium. Bronchoalveolar lavages (BAL) and/or tissue collections were performed at two, four, or 18 weeks.

Immunohistochemically stained lung sections and BAL cells were enumerated for T cells, alveolar macrophages, lymphocyte proliferation, and virus infected cells.

Relative to medium inoculated lambs, pulmonary inflammation in infected lambs was most severe at four weeks post inoculation (PI) and correlated with virus infection rates of alveolar macrophages. Neither inflammation nor macrophage infections were detectable at 18 weeks PI. Humoral responses to viral proteins measured by ELISA were diagnostic for infection, but did not correlate with

macrophage infection rates. Collectively, these findings suggest that the pulmonary inflammation observed in the acute PI period is virus induced and may be part of the immune response to reduce productive infections; however, the increased number of inflammatory cells also provides additional differentiated macrophages as target cells for infectious dissemination of the virus within the host.

Introduction

Ovine lentivirus (OvLV), also known as maedi-visna virus, is the etiological agent of lymphoid interstitial pneumonia in sheep.¹ Lymphoid interstitial pneumonia is a significant cause of morbidity and mortality in sheep^{2,3} and also occurs in human and simian immunodeficiency virus infections.^{4,5} Although considerable progress has been made in retrovirus research on the molecular aspects of cell tropism and viral replication, there is still uncertainty whether the immune system is capable of preventing or clearing retrovirus infections.⁶⁻⁸ This controversy on immunity in primate retrovirus infections may be due in part to the confusion over the immune system's efficacy in dealing with two virus infected cell populations: T cells and macrophages. The experiments described herein use a natural host to investigate the early immunological and viral events that occur when retroviruses infect macrophages without lymphocyte depletion.⁹ This investigation permits a dynamic look at how the T cell response to viral antigens may inadvertently recruit naïve macrophages to the lung, providing new target cells for infectious spread of the virus within the host.

HIV is capable of infecting and replicating within two cell populations: macrophages and T lymphocytes. This dual tropism is due to differing chemokine

co-receptors on the two cell populations: CCR5 on macrophages and CXCR4 on T cells.¹⁰⁻¹⁴ These and other investigations have looked extensively at viral replication in relationship to the presence of viral receptors. However, they have provided only a rudimentary analysis of how the immune response differs to the two infected cell populations. Theoretical descriptions do exist of the differences in lentivirus replication and host immune responses with two distinct target cells.¹⁵⁻¹⁸ However, no *in vivo* studies have been performed using HIV infected individuals and analyzing both virus production from and immune response to different target cells. OvLV is only capable of productively infecting macrophages^{9,19} and dendritic cells.²⁰ Consequently, virus-induced T cell depletion is not a confounding phenomenon in the analyses of the immune response to macrophage infections. Also, analyses of viral infection and replication in conjunction with immune response are limited to a single population.

The objectives of this study were to determine the mathematical relationship of alveolar macrophage infection rates to the concurrent T cell inflammation during the early post infection period. It is unknown whether the T cell inflammation commonly observed in chronic lung lesions during lentivirus infection is effective at controlling infection of alveolar macrophages. Alternatively stated, is the lentivirus infection rate of alveolar macrophages a function of lymphocyte (T cell) accumulation and/or proliferation? As previously mentioned, lentivirus infection in many different species is followed by an acute burst in virus replication concurrent with inflammation in certain target organs. It is unknown whether the *in vivo* diminution in virus replication are primarily due to host adaptive immunity or virus

mediated such as by transcriptional activating factors. This investigation will probe this unknown through a mathematical analysis of the virus-host relationship.

Materials and Methods

Experimental Animals and Tissue Collection

The experimental design was based on the previously described rapid induction of lymphoid interstitial pneumonia in lambs.²¹ Thirty-three colostrum-fed, neonatal, Rambouillet lambs were obtained from a flock that have repeatedly tested seronegative for antibodies to OvLV envelope glycoprotein and capsid antigen by agar gel immunodiffusion (AGID) test (Veterinary Diagnostic Technology, Inc. Wheat Ridge, CO). The Colorado State University Animal Care and Use Committee approved all procedures involving live animals. Lambs were randomly divided into two groups that were housed separately in raised pens while being fed milk replacement, creep and grass feed. Nineteen of the lambs were inoculated intratracheally with 10 ml of supernatant from goat synovial membrane (GSM) tissue cultures containing a 5×10^6 TCID₅₀ of ovine lentivirus strain 85/34 while 14 lambs were sham inoculated with 10 ml of spent medium from uninfected GSM cell cultures. The medium from both inoculums included RPMI 1640 supplemented with 10% fetal bovine serum 1% penicillin-streptomycin, and 2mM L-glutamine (Gibco Life Technologies, Rockville, MD).

Serial *in vivo* bronchoalveolar lavage (BAL) procedures were performed on a subset of animals under general anesthesia (5.0 mg/kg ketamine IV, 0.1 mg/kg diazepam IV, and 2% Isoflurane inhalation) at either two, four, or ten weeks PI a

total of 22 times (Table 2.2). A five mm diameter bronchoscope was wedged in the right middle bronchus and flushed with approximately 100 ml of Hank's balanced salt solution and immediately collected for analysis. Animals were euthanized by intravenous administration of xylazine (0.2 mg/kg) and ketamine (5 mg/kg) followed by exsanguination from the carotid artery at two, four, or 18 weeks post-inoculation. An *ex vivo* BAL of the right lung was performed at all necropsies for a total of 33 times. *Ex vivo* lavages used 500 ml of Hank's solution that was poured directly into a cannulated right primary bronchus and immediately collected into a sterile container. Cell counts and viability from all lavages were performed by microscopic examination of cells with a trypan blue exclusion stain on a hemocytometer.

Enzyme linked immunoadsorbent assay (ELISA)

The serological response to OvLV antigens in infected lambs was quantified with an ELISA based on a recombinant OvLV transmembrane and capsid protein using previously described methods.²² Briefly, this assay is a solid-phase (indirect) ELISA in which recombinant OvLV proteins are bound to a 96 well plate. The test sera are subsequently incubated in the plate and the immunoglobulins binding to the antigens were quantitated with a protein-G/horse radish peroxidase based detection system via an optical density measurement. A negative/positive cut off value was calculated with known positive and negative control serum and the two-graph receiver operating characteristic method developed by Dr. Matthias Greiner, Freie Universität Berlin, Germany (<http://city.vetmed.fu-berlin.de/~mgreiner/TG-ROC/tgroc.htm>).

Immunocytochemistry

BAL cytospin preparations containing approximately 2.5×10^5 cells per slide were air dried and fixed in cold acetone. OvLV capsid p25 producing cells were detected with monoclonal antibody 3F²³ used at 1:10 dilution. BAL cells were also stained for MHC IIa (DR α epitope Figure 3.1) with monoclonal SBU 41.19, (The University of Melbourne). Detection of immunoreactivity was accomplished with the peroxidase-based Vectastain ABC Elite System (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Tissues were counterstained with Harris's hematoxylin and coverslipped.

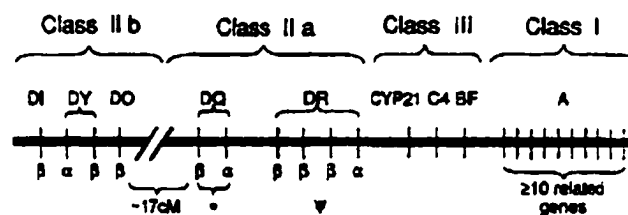


Figure 3.1: Overview of the major histocompatibility genes structure in ruminants. Antibodies against DR α encoded proteins were chosen to assess MHC IIa in BAL cells. (Figure adapted from Cell-mediated immunity in Ruminants. Goddeeris & Morrison 1994.)

Immunohistochemistry

Four sections of lung (left caudal lobe – two central and two peripheral) were stained from each animal. After routine dewaxing, all tissue sections were autoclaved in proprietary target retrieval solution at a pH of 6.0 (Dako Corporation, Carpinteria, CA) and blocked with Protein Block Solution (Biogenex, San Ramon, CA). Macrophage cells were identified using a polyclonal anti-lysozyme antibody (1:200, Dako Corp.) and T cells were identified using a pre-diluted polyclonal anti-CD3 antibody (Cell Marque - Ventanna Systems, Austin, TX). Isotype matched

irrelevant antibodies were included in the staining procedures as controls. In order to determine the relationship of mitotically active cells to virus infection rate, a subset of animals were analyzed for bromodeoxyuridine (BrDU) incorporation into pulmonary tissue. The animals were injected with BrDU diluted in saline (10 mg/kg of body weight, intravenously) one hour prior to euthanasia. Lung tissues were subsequently stained with a pre-diluted monoclonal anti-BrDU antibody (Biogenex). Tissues from non-BrDU inoculated animals and gastrointestinal tract from BrDU inoculated animals were used as controls. Detection and counterstaining was accomplished using the same method described for immunocytochemistry.

Morphometric Analyses

All morphometric procedures were performed by capturing digital images (tagged image file format) of tissues through an Olympus Microscope fitted with a three chip cooled device 1140x1520 detail Cool SNAP Camera (Roper Scientific GmbH, Bergkirchen, Germany). The 20x objective was routinely used in these analyses and in combination with the digital camera created 0.8112 mm² images. These images were subsequently analyzed with Metamorph software v. 4.0 (Universal Imaging Corp., West Chester, PA).

The infection rate of BAL cells was determined by manually counting OvLV positive cells in the cytospin preparations. Background reaction product was not observed in any medium-inoculated animals. The infection rate was defined as the number of virus antigen positive cells per total BAL cells examined at the time point. The total BAL cells on the cytospin preparations were quantitated by image analysis. The quantification was performed by capturing a low magnification image

of the cytoplasm and thresholding on the hematoxylin stained nuclei. The cell counting function was subsequently used to determine total cells per slide. The relative infection rate represents the simple fraction of OvLV antigen positive cells observed while the absolute infection rate was algebraically calculated from the relative infection rate and the total cells per ml of BAL fluid.

Total MHC IIa (DR α) production was estimated through adaptation of a previously reported image analysis based method in conjunction with immunohistochemistry.²⁴ In this technique, protein production is reported as a mathematical function of both the total number of cells with reaction product (thresholding function) and the average intensity of the reaction product of the positive cells (optical density function). The average optical density of the product was defined as $-\log[(\text{mean gray level}_{\text{specimen}} - \text{mean gray level}_{\text{background}}) / (\text{mean gray level}_{\text{foreground}} - \text{mean gray level}_{\text{background}})]$.

Quantitative assessment of immunohistochemical stained lung sections was performed using the same thresholding function to identify positive staining cells. However, after calibration with a stage micrometer, the numbers of positively stained cells were enumerated per tissue area and then adjusted per mm of bronchiole lumen to account for artifactual atelectasis or parallax view of a bronchiole (Figure 3.2).

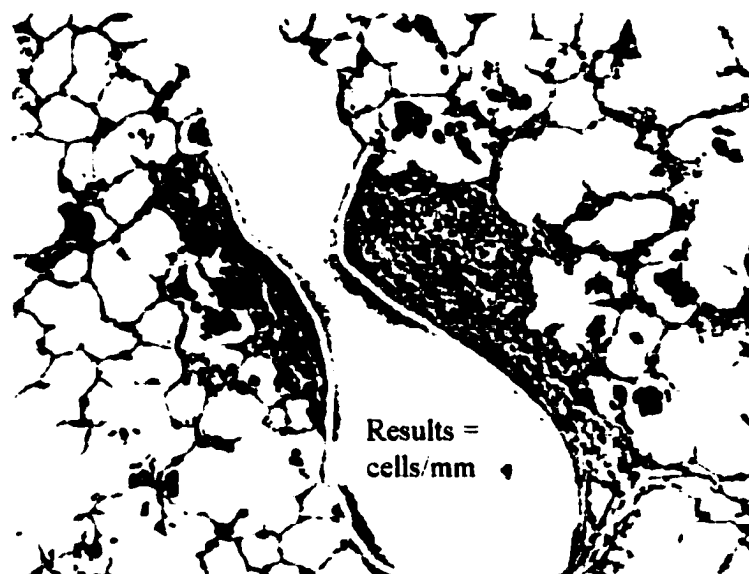


Figure 3.2 An example of image analysis technique to determine positive staining cells per lung area. In this example, differentiated alveolar macrophages are identified using lysozyme immunohistochemistry. The analysis program is gated to detect the cells within a defined color range. The number of cells per image is subsequently calculated based on the thresholded (red) area divided by the average area per cell. This number is then reported per mm of bronchiole epithelial circumference (yellow) to adjust for artifactual atelectasis or parallax view of a bronchiole lumen.

Statistical Analyses

Statistical analyses were performed with the Graphpad® Instat V. 3.0 software (San Diego, CA). Group mean comparisons were accomplished with a two-tailed, unpaired t-test with a Welch correction for means with different standard deviations. If necessary, log transformation of data was performed to achieve a Gaussian distribution. Correlation analyses were performed using a Pearson correlation coefficient of determination. A p value < 0.05 was considered significant for both tests.

Results

Quantification of Serological Response to OvLV

An enzyme linked immunoadsorbent assay (ELISA) was used to determine the relative concentration of antibodies against OvLV recombinant transmembrane and capsid proteins. This ELISA was performed on serial serum samples from lambs at biweekly intervals (Figure 3.3). This assay provided quantitative serological data that could be analyzed for correlations against the BAL viral infection rates at the same time point. 100% concordance was observed between individual ELISA and previously reported AGID results (Chapter 2); seropositive animals could be detected in the infected groups by four to six weeks PI. None of the medium inoculated animals tested positive in this assay.

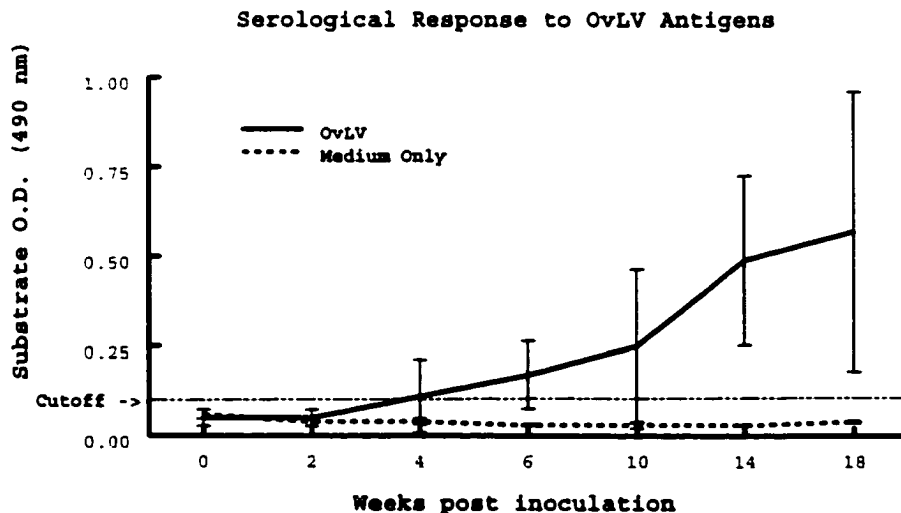


Figure 3.3. The serological responses in OvLV inoculated lambs were compared to medium inoculated lambs over 18 weeks post inoculation (n=16). Each line represents the mean ELISA substrate optical density (+/- standard deviation) which is proportional to each groups' serum antibody titer against recombinant OvLV capsid-transmembrane fusion protein. A negative/positive cut off value was calculated with known positive and negative control serum and the two-graph receiver operating characteristic method.

Immunocytochemistry for OvLV Antigen and MHC II Production

Virus infection rates within each animal were measured by immunocytochemistry of the BAL cell population to determine the percentage and total number of virus-infected cells. It was previously reported (Chapter 2, Figure 2.12) that viral antigen positive cells in paraffin embedded lung tissues from animals in this study were extremely rare (only about 1-2 cells per slide) and only observed in the infected animals at four weeks PI. This observation was in contrast with naturally infected chronic cases that were used as positive controls and which showed numerous positive macrophages associated with pulmonary lesions. OvLV infection rates were determined with immunocytochemistry on concentrated BAL cell. This assay provided data with a Gaussian distribution. OvLV capsid was detected by immunocytochemistry only in alveolar macrophages (Figure 3.4). The relative virus infection rate was highest in the animals killed at two weeks PI with approximately one OvLV antigen positive cell for every 2,857 BAL cells examined. This proportion of OvLV positive to negative cells was one positive for every 3,813 BAL cells at four weeks PI. However, because there was an average 27.7% difference in the total number of BAL cells in OvLV inoculated lambs from two to four weeks PI (data derived from Table 2.2), the absolute number of OvLV positive cells per ml of BAL fluid was highest at four weeks PI. At 18 weeks PI, the number of OvLV positive cells in BAL fluid was below the threshold for detection.

Immunocytochemistry was used to assess MHC IIa production in BAL cytospin preparations (Figure 3.5). A monoclonal antibody against DR α proteins stained alveolar macrophages with varying intensity. A relative comparison was performed using an image analysis algorithm that accounted for both the percentage

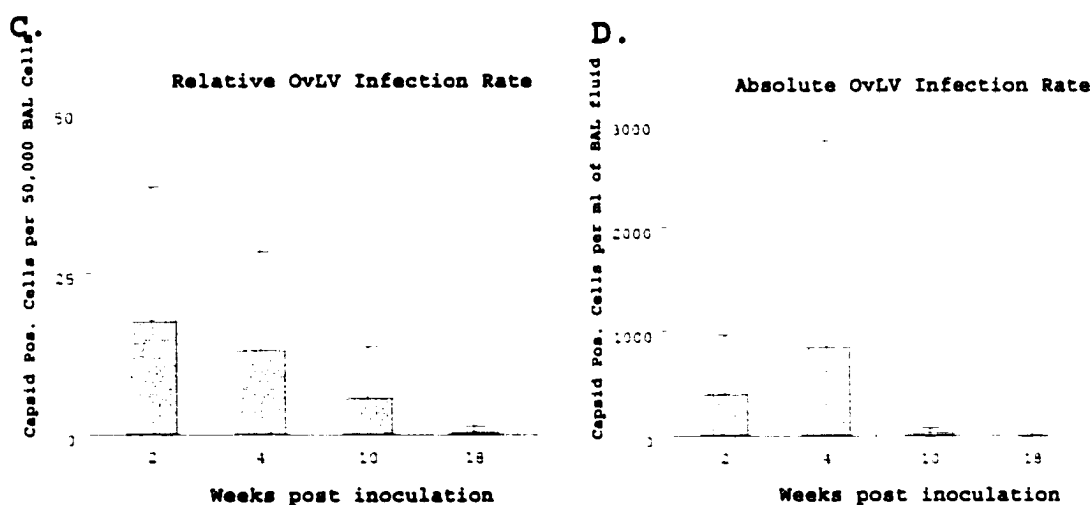
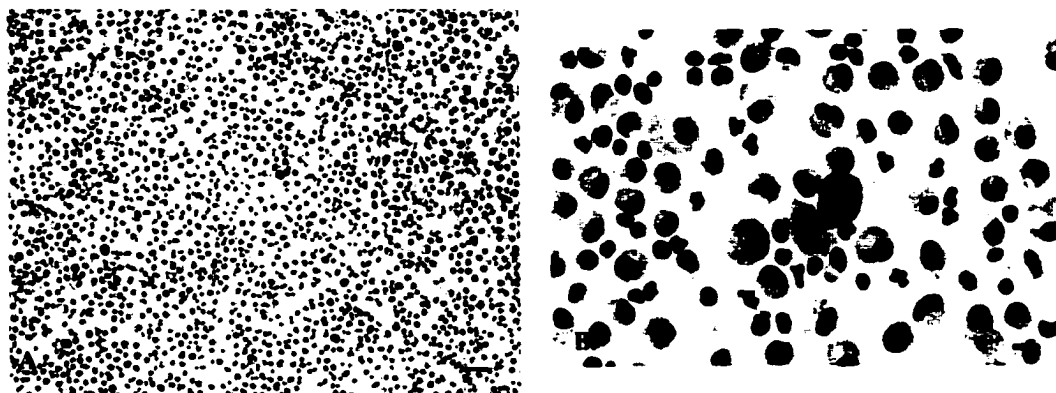


Figure 3.4. OvLV infection rates determined in infected animals by immunocytochemical staining of BAL cytopsin preparations for p25 capsid and quantitation by image analysis. Immunocytochemistry employed a peroxidase based stain and a hematoxylin counterstain. A) Representative field of a BAL cytopsin from an OvLV inoculated lamb at four weeks PI showing the scarcity of positive cells. Bar represents 20 microns. B) Higher magnification of same field in which cytoplasmic (brown) staining is observed in alveolar macrophages but not in lymphocytes (white arrow) or neutrophils (black arrow). Bar represents 10 microns. C) Bars are the relative mean (plus standard deviation) of virus positive cells per total cells in cytopsin preparation. D) Bars are the absolute mean (plus standard deviation) of virus positive cells per ml of BAL fluid.

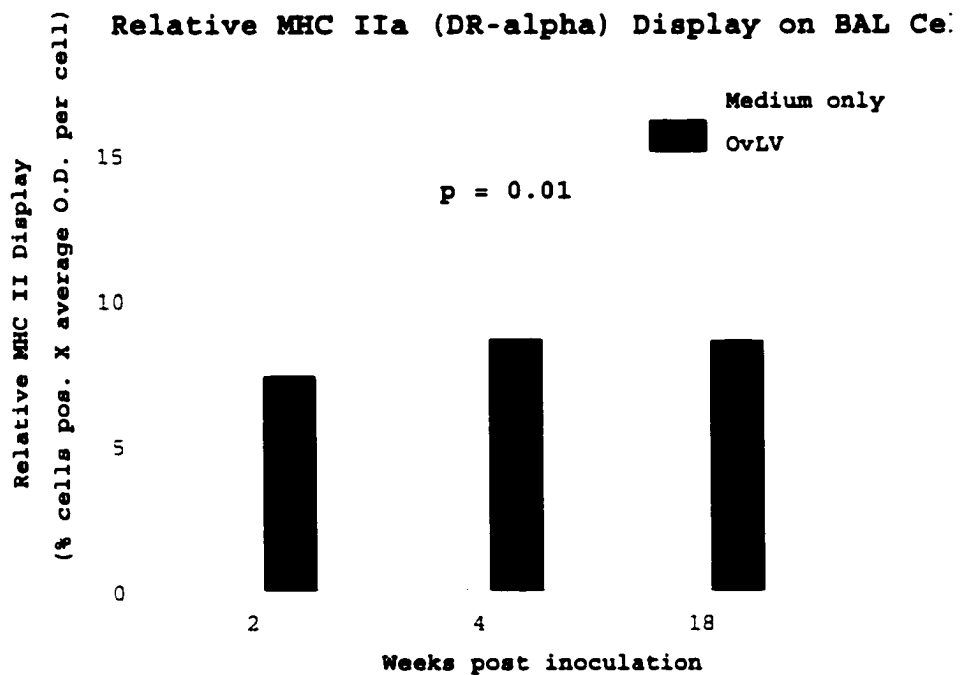
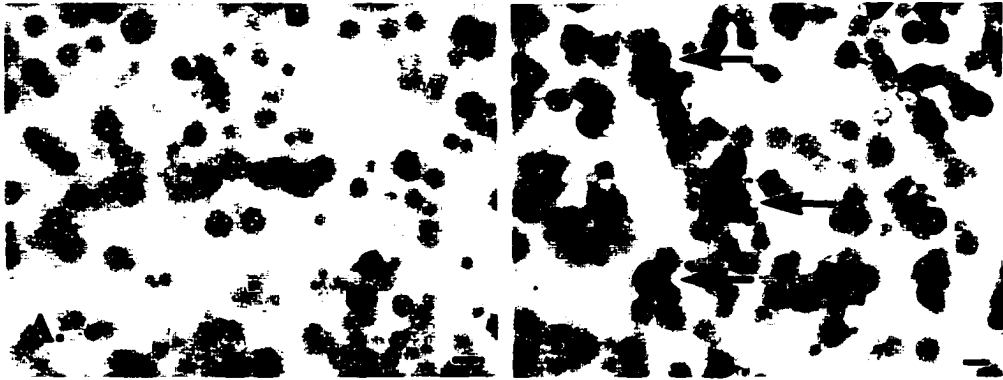


Figure 3.5 MHC II display on 34 BAL cell samples were determined with immunocytochemistry using a peroxidase based stain and a hematoxylin counterstain. A) BAL cells from lambs at four weeks PI with medium show a diffuse weak staining pattern. B) BAL cells from lambs at four weeks PI with OvLV have more positive staining cells. Additionally, the darkest staining cells are syncytial cells (arrows) consistent with OvLV infection. Both bars represent ten microns. C) Immunocytochemical mean relative presence of MHC II in BAL cells was determined with identically stained cell preparations and computer assisted image analysis. Relative levels for each animal was calculated by multiplying the percentage of positive cells by the average optical density of the positive signal. Data are reported as group means plus standard deviation.

of positive cells and the average intensity (optical density) of BAL cells. In this analysis, the mean level of MHC IIa presence was consistently higher in the OvLV inoculated lambs at all time points PI. The darkest staining cells in OvLV inoculated lambs were multinucleated (usually 2-4 nuclei per cell). These syncytial cells are consistent with OvLV induced morphological alteration, which have been previously described (Chapter 2, Figure 2.13).

Immunohistochemistry for Macrophage and T Cell Dynamics

Lysozyme is used as an immunohistochemical marker of macrophage differentiation and was used to identify pulmonary macrophages (Figure 3.6).

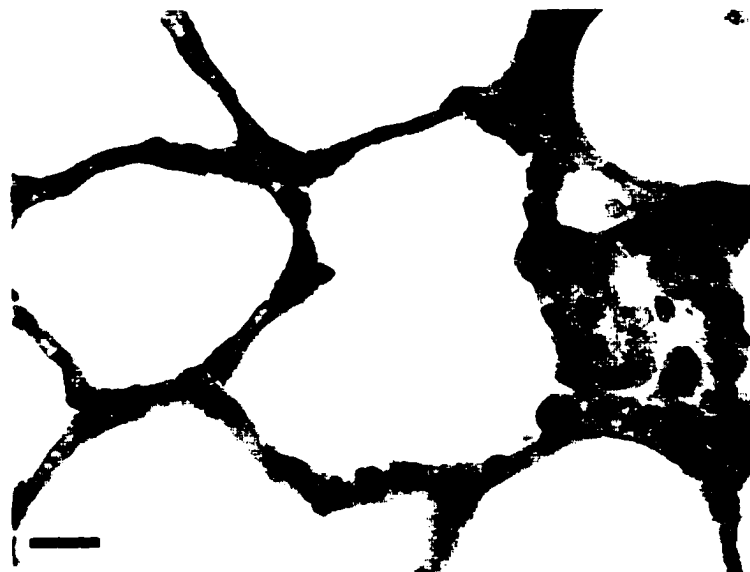


Figure 3.6 Differentiated macrophages in histological sections of pulmonary tissue were identified with anti-lysozyme immunocytochemistry using a peroxidase based stain and a hematoxylin counterstain. This image is taken from a lamb at four weeks PI with OvLV and demonstrates two brown staining macrophages: one in the alveolus and one in the alveolar septae. The primary entrance point of macrophages into the alveolus is transalveolar migration.²⁵ These macrophages do not come in direct contact with peribronchiolar lymphoid follicles. Bar represent ten microns.

Most of the pulmonary macrophages were diffusely located within alveoli. Occasionally, positive staining cells were observed in the alveolar septae. Frequently, there were accumulations of alveolar macrophages near the junction of terminal bronchioles and alveolar ducts (Figure 3.2). Circumferentially to peribronchiolar lymphoid aggregates, there was a 6.8 fold mean increase ($p = 0.002$) in the numbers of lysozyme-positive macrophages in OvLV lambs at four weeks compared to medium inoculated lambs (Figure 3.7).

The peribronchiolar lymphoid aggregates were confirmed to be predominantly T cells with anti-CD3 immunohistochemistry (Figure 3.8). The prominent peribronchiolar lymphoid aggregates in the four week PI infected group contained a 4.7 fold mean increase in the numbers of CD3+ cells compared to medium inoculated animals ($p = 0.03$). Qualitatively, there were also increased numbers of CD3+ cells in the interstitium of infected animals compared to medium-inoculated animals. The tissue macrophage and lymphocyte quantitation corroborated the observations in the BAL cell analysis with the highest counts observed in the four weeks PI infected lung tissues (Table 2.2).

Mitotically active cells were identified with anti-BrDU immunohistochemistry. This assay revealed a pattern of staining similar to that seen for the CD3+ staining cells (Figures 3.9). The predominant BrDU positive cells were morphologically consistent with the T cells and included approximately a quarter of the peribronchiolar lymphoid cells while only a rare macrophage-like cell stained positively. The peak of mitotically active cells was observed in the OvLV inoculated lambs ($p = 0.0086$) at two weeks PI with a 3.8 fold mean cell number increase in infected over medium inoculated animals.

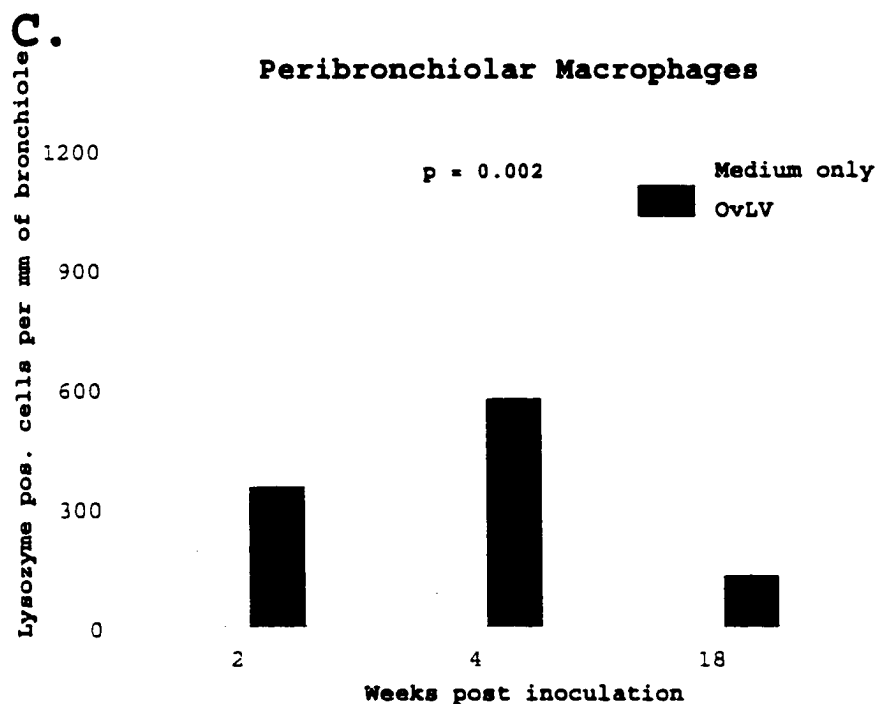
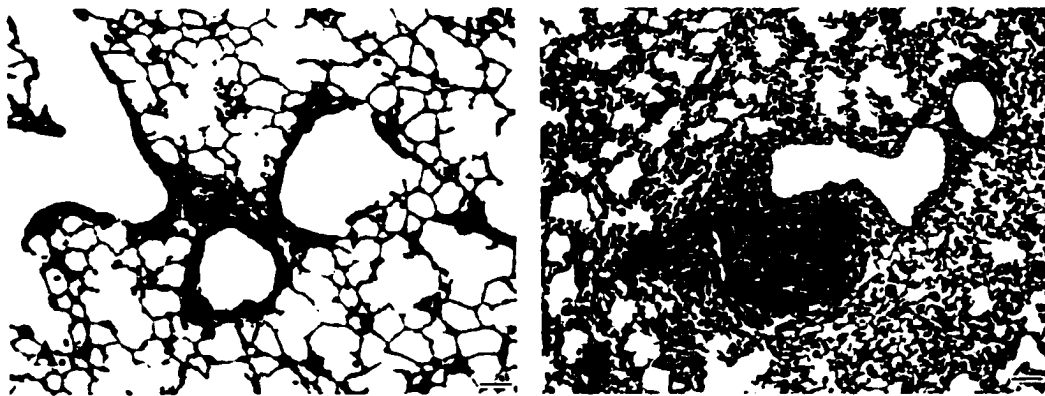


Figure 3.7. Pulmonary macrophages were detected in lung tissue from 33 lambs with anti-lysozyme immunohistochemistry using peroxidase based detection and hematoxylin counterstain. Both bars represent 50 microns. A) Tissue from a lamb at four weeks PI with medium only has scattered macrophages in alveoli near a bronchiole. Three positive staining cells are also noted in the arteriole and presumably represent intravascular macrophages. B) Tissue from a lamb at four weeks PI with OvLV. There are more alveolar macrophages than the previous image in proximity to a peribronchiolar lymphoid nodule that does not contain macrophages. C) Image analysis based quantification of the number of peribronchiolar alveolar macrophages. A significant increase was observed at two weeks PI.

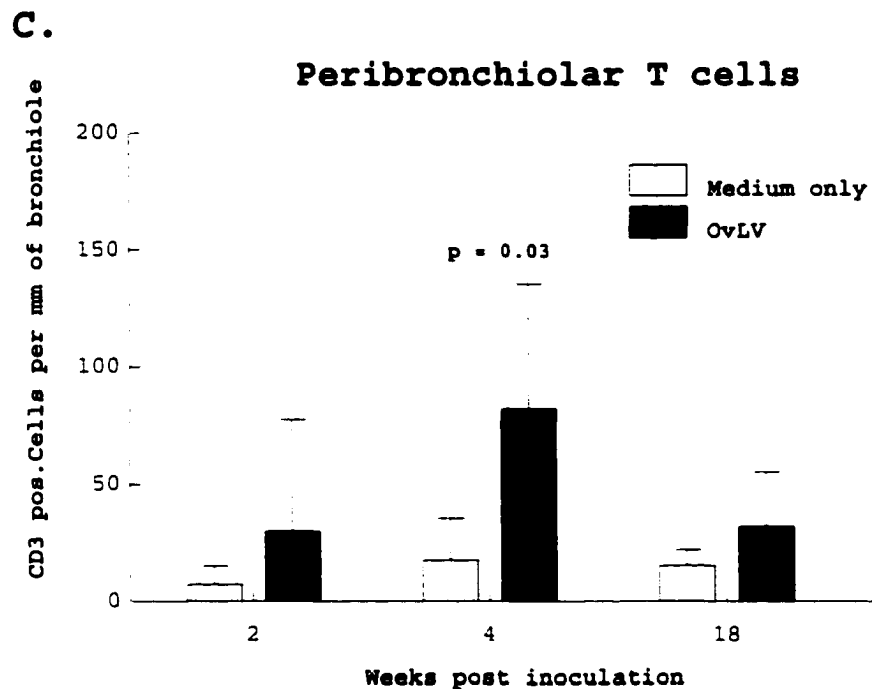
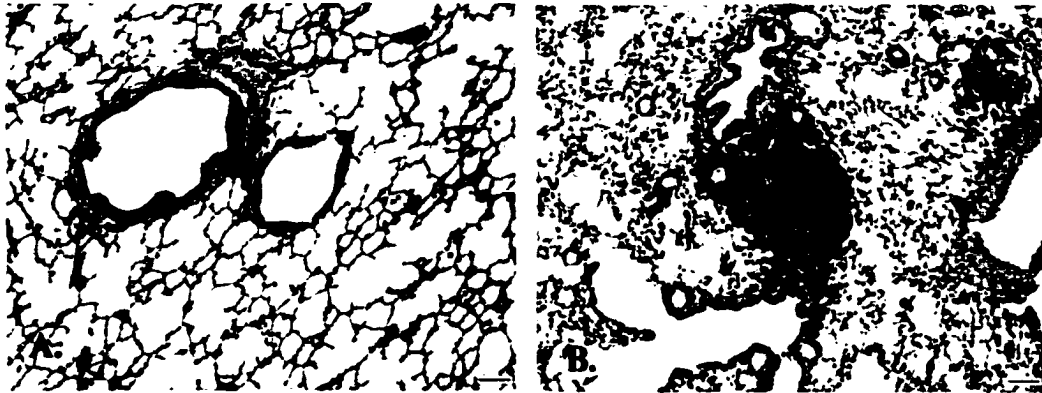
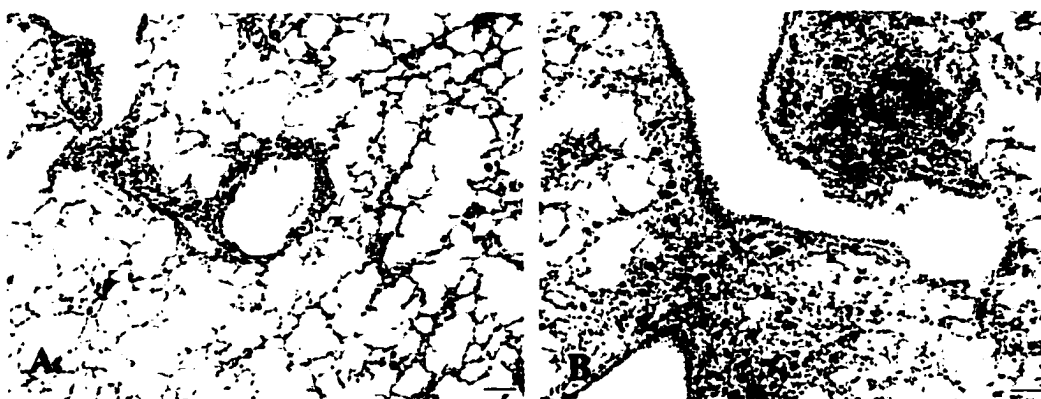


Figure 3.8. Pulmonary T cells were detected in lung tissue from 33 lambs with anti-CD3 immunohistochemistry using peroxidase based detection and hematoxylin counterstain. Both bars represent 50 microns. A) Tissue from a lamb at four weeks PI with medium only has scattered T cells within the connective tissue around a bronchiole. B) Tissue from a lamb at four weeks PI with OvLV. The majority of positive staining cells are within or close to the peribronchiolar lymphoid nodule. A slight increase in interstitial T cells is also noted. C) Image analysis based quantification of the number of peribronchiolar T cells. A significant increase was observed at four weeks PI.



Mitotically Active Cells

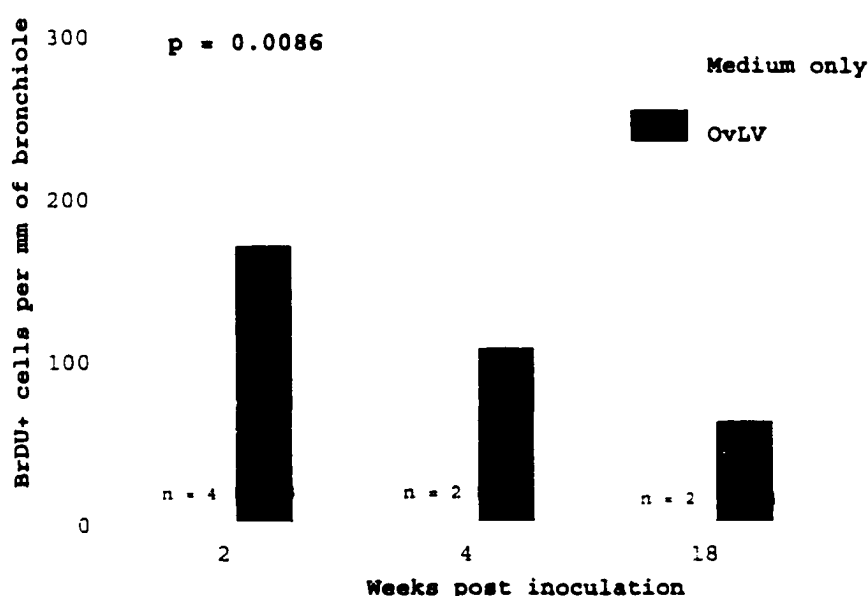


Figure 3.9. Mitotically (S phase) cells were detected in 17 lambs with anti-BrDU immunohistochemistry using peroxidase based detection and hematoxylin counterstain in a subset of experimental lambs (n per group indicated within bars). Both bars represent 50 microns. A) Tissue from a lamb at four weeks PI with medium has scattered BrDU positive cells in cells consistent with peribronchiolar lymphocytes and bronchiole epithelial cells. B) Tissue from a lamb at four weeks PI with OvLV. The majority of positive staining cells are within or close to the peribronchiolar lymphoid nodule. A slight increase in interstitial T cells is also noted C) Image analysis based quantification of the number of mitotically active cells. A significant increase was observed at four weeks PI.

Correlation Analyses

Correlation analyses were performed to detect potential functional relationships between two or more of the previously described variables. All of these analyses were performed by comparing two measured data points from the same animal at the same time PI. These analyses included serological, virological, and cellular measurements. The data are reported by the two variables compared, the coefficient of determination (r^2 or the fraction of the variance in the two variables that is shared), and the P value (Table 3.1).

The first correlation analysis tested whether humoral immunity was related to virus infection rates. The serological response to OvLV capsid and transmembrane proteins did not correlate with the absolute virus infection rate at the same time points. Additionally, to test whether the inflammatory lesions might be mediated by antibodies, such as in certain types of hypersensitivity reactions, ELISA data to total BAL cells counts were tested. There was no correlation between serological response and tissue inflammation.

Next, the virus infection rate was tested against the degree of inflammation as assessed by either BAL or tissue cell counts. The BAL total cell counts strongly correlated with the relative BAL viral infection rate at four weeks PI. Similarly, the absolute numbers of macrophages per ml of BAL fluid strongly correlated with relative infection rate at four weeks PI. Additionally, the total tissue inflammation (i.e. the combination of macrophages and T cells) had a positive correlation with both the relative and absolute virus infection rates at four weeks PI. There were no correlations between infection rates and inflammation at either two or 18 weeks PI.

BAL viral infection rates did not correlate with the number of BrDU positive cells per bronchiole basement membrane length. However, major histocompatibility IIa (DR α) presence at four weeks PI significantly correlated with the total number of virus infected cells per ml of BAL fluid.

Finally, alterations in the pulmonary macrophage to T cell ratios were examined. The predicted relationship in the normal lung is that macrophages to T cells should negatively correlate due to the proposed immune suppressive role of the pulmonary macrophage. In fact, there was a negative correlation between the macrophage and T cells tissue numbers at four weeks PI with medium. However, a positive correlation was present in the 18 weeks PI with OvLV group. No other groupings correlated.

Table 3.1 Summary of Correlation Analyses

Variable 1	Variable 2	P value	r ²
Relative Virus	BAL Total Cells	0.0010*	0.86
Relative Virus	BAL Macrophage s @ 4 weeks PI	0.0012*	0.83
Absolute Virus	Tissue Total Cells	0.0169*	0.78
Relative Virus	Tissue Total Cells	0.0201*	0.80
Absolute Virus	Tissue Macrophages	0.23	0.42
Absolute Virus	Tissue T Cells	0.27	0.29
Absolute Virus	Mitotic Rate (BrDU)	0.88	0.00
Absolute Virus	Antibody (ELISA)	0.50	0.14
BAL Total Cells	Antibody (ELISA)	0.16	0.07
Absolute Virus	MHC II @ 4 weeks PI	0.0249*	0.75
Control Group: Tissue Macrophages	Tissue T Cells @ 4 weeks PI	0.0097*	0.54 (negative)
Infected Group: Tissue Macrophages	Tissue T Cells @ 18 weeks PI	0.0020*	0.46

* Indicates a significant correlation.

r² = coefficient of determination.

Correlation coefficient (r) is positive unless indicated as negative.

Unless specified, analyses included data from all three time points PI.

Discussion

The experiments described herein provide the first description of the acute inflammatory dynamics that occur during the first weeks with lentivirus infected macrophages in a natural host and a natural target organ. This model focuses on macrophage infection since lymphocyte infection by OvLV has not been demonstrated and thus subsequent lymphocyte alterations and depletions are presumed not to occur. Inflammation can include events that are both beneficial and damaging to the host. The inflammatory dynamics examined here appear to benefit the host in the short-term but may also include potential long-term negative consequences. The short-term benefit is an immune response that correlates with a reduction in the productive infection rate. The long-term consequence is an inflammatory process that increases the number of naïve macrophages exposed to OvLV infected cells.

It has been asserted that in a lentivirus infection, a peak in viral load that is followed by generation of T cells and a subsequent reduction in viral load is evidence of an adaptive immune response.²⁶ The evidence for this assertion is particularly strong in lentiviruses that productively infect both macrophages and T cells such as the human and simian immunodeficiency viruses.²⁷⁻³¹ However, this assertion may not be completely accurate in a lentivirus system such as OvLV, which can only infect and replicate in macrophages *in vivo*. Our proposed pathogenesis begins with our observation that differentiated alveolar macrophages (identified by lysozyme positive cells) associated with OvLV infection are more activated (increased display of MHC II) than cells from uninfected lambs. The MHC II increase may be the initial signal that induces the infiltration and

accumulation of T cells. These T cells are likely to continue propagation of the inflammatory cycle by producing cytokines that recruit and activate additional pulmonary macrophages. Parallel investigations to this one indicate that the primary cytokine involved in this pathway is interferon- γ .^{12,33} Subsequent to mitotic burst of pulmonary lymphocytes, there is an increase in the accumulation and activation (increased MHC II display) of alveolar macrophages. The numbers of macrophages observed in the lesions outpace the T cell accumulation. Additionally, the macrophage and T cell accumulation correlate with the infection rate. Collectively, this series of events indicates that exuberant inflammation increases the exposure of naïve macrophages to infected macrophages.

Previous studies have described pulmonary inflammation in experimentally inoculated animals.^{4,35} However, these studies included longer incubation periods (up to six months PI) and were primarily designed to compare different OvLV strains. At six months PI, the severity of pulmonary lesions varied: approximately 25% had moderate to severe lesions, 50% had mild lesions, and 25% had no detectable lesions.¹ The dynamics of lesion development prior to the six month time period was previously unknown so the experimental design of this investigation focused on the acute time points after infection. The results reported here portends both a development and a regression phase of lesions in most animals. The development phase was predominantly characterized by increased numbers of differentiated alveolar macrophages in or around small bronchioles. The number of productively infected cells (i.e. OvLV capsid producing cells) was highest as a percentage of total alveolar macrophages at the earliest time point measured: two weeks PI. Subsequently, the infiltration and proliferation of lymphocytes around the

small airways was associated with a relative decrease in the number of OvLV antigen positive alveolar macrophages. However, because the total numbers of alveolar macrophages increased through four weeks PI, the absolute number of OvLV antigen positive alveolar macrophages peaked at four weeks PI. At 18 weeks PI, the number of OvLV antigen producing cells was extremely low and the CD8+ cells were the predominant lymphocyte subset in the BAL population. This lymphocyte subset expansion is consistent with a Th₁ immune response. However, these data, in conjunction with the previously mentioned T cell depletion experiments described by Eriksson et al^{16,17} still raise questions as to the functionality of the lymphocyte response. The four observations that 1) inflammation correlates with virus infection rate, 2) OvLV infection recruits additional macrophages to alveoli, 3) the proliferating CD8+ T cells are anatomically separated from the OvLV infected macrophages, and 4) a Th₁ cytokine response (IFN- γ based) emanates from lymphoproliferative lesions suggest that the pulmonary lymphoid lesions may be assisting infectious spread of the virus between macrophages within the host.

A number of other factors that were examined were not predictive of disease outcome and presumably not important in the pathogenesis of OvLV associated pneumonia. The serological response commonly used to indirectly diagnose OvLV infection was not predictive of lesion development. This suggests that anti-OvLV antibodies do not influence virus infection rates. The absence of any correlation between viral load and cellular proliferation (BrDU data) also suggests that mitotic activity is not an important part of viral production. This observation on macrophage proliferation is in contrast to what has been observed in synovial derived macrophages from goats infected with caprine arthritis and encephalitis

virus.^{38,39} This would indicate that virus spread within the host is predominantly a cell-to-cell infection and that mitotic activity in infected cells is not important in the determining the total number of virus infected cells within a host.

We believe that this investigation raises several new questions regarding lentivirus infections of macrophages. Most important among these is deciphering the signals that occur between infected macrophages and the responding T cells. There has been considerable work done to define the transcriptional activating factor, such as FOS and JUN, as important in regulating OvLV replication within macrophages.^{40,41} However, knowledge of the soluble and cell-to-cell signals between macrophages and lymphocytes which alter viral replication, as well as the ultimate destination of virus infected cells within a host will provide a clearer answer as to whether long term immune control of retrovirus infection is possible. Enumeration of latently infected cells, especially those serving as virus reservoir for disease recrudescence, is necessary to address this latter issue.

Since ovine lentivirus productively infects only differentiated macrophages *in vivo*, it would follow that a virion that is capable of recruiting and differentiating additional macrophages to a target organ would have an evolutionary advantage over virions less capable of such recruitment and differentiation. Questions still remain regarding the precise mechanism by which OvLV activates its host cell. Investigations are necessary to determine what signals recruit and subsequently activate macrophages. Further studies on how these signals affects replication and dissemination of the virus within the host are necessary to fully understand latency and recrudescence of lentivirus production within a host.

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Chapter Four

Lymphocyte Proliferation Associated with Ovine Lentivirus Infection

Abstract

Although there is unambiguous evidence of pulmonary lymphocyte accumulation and proliferation associated with ovine lentivirus (OvLV) infection, the mechanism inducing and augmenting this phenomena remains vague. This investigation sought to determine whether alveolar macrophages infected with OvLV have altered immune suppressive function. The experiments described in this investigation examined the responses of lymphocytes obtained from the peripheral blood to mitogenic stimulation when cultured both with and without bronchoalveolar lavage (BAL) cells. Optimal doses of the mitogen concanavalin A were established and peripheral blood mononuclear cells (PBMC) from OvLV or medium inoculated lambs were tested at various times post inoculation (PI) using ³H-thymidine incorporation. Lymphocytes from the medium inoculated lambs responded better to mitogenic stimulation at 14 weeks PI than at 2 weeks PI. The lymphocytes derived from the OvLV inoculated lambs responded poorly to mitogenic stimulation at both 14 and 2 weeks PI. Experiments were then performed in which lymphocytes co-cultured with varying concentrations of BAL cells were stimulated by mitogen. Similar to before, lymphocytes from OvLV inoculated lambs responded less to mitogen stimulation than lymphocytes from medium inoculated lambs regardless of the BAL cells' presence. It was concluded that lymphocytes from OvLV infected lambs responded poorly to mitogenic stimulation and that the immune suppressive function of BAL cells is not affected by OvLV infection.

Introduction

The previous descriptions of lesion development during the early phases of OvLV infection presented in earlier chapters state that lymphocytes are an important component of OvLV pathogenesis. However, it was also observed that there was only minimal cell-to-cell contact between the primary target cells, alveolar macrophages, and the peribronchiolar lymphoid accumulation. This observation raises questions on the effectiveness of the lymphocytes. An additional conundrum is that the pulmonary lymphocyte mitotic rate does not correlate with macrophage infection rate. Additionally, the greatest increase in lymphocytes occurs within the CD8+ T cell subset in a polyclonal pattern.¹ Finally, other investigators have observed that depletion of T cells in early OvLV infection does not increase infection rates, but rather can be associated with decreases in infection rate of macrophages.^{2,3} Thus, there remain substantial questions as to the mechanism of lymphocyte accumulation and whether these lymphocytes are part of effective immunity which controls the early infection rate in macrophages or rather these lesions are an immunopathological manifestation.

As previously mentioned, the alveolar macrophages are a major immune barrier of the lower respiratory system. A hypothesized role of alveolar macrophages is to function in a balanced manner thus maintaining pulmonary homeostasis.^{4,5} The underlying thought behind this hypothesis is that the alveolar macrophage is potentially exposed to large quantities of exogenous antigen most of which is not a true pathogen. Thus, it has been asserted that the ability of alveolar macrophages to down-regulate the pulmonary immune response is more common than up regulation. This hypothesis has been supported by *in vitro* investigations which concluded that alveolar macrophages have "lymphocytostatic" functions through a variety of mechanisms including

transforming growth factor beta (TGF- β) and nitric oxide (NO). Farmer's Lung disease (allergic pneumonitis) is an example of a disease that has been associated with a loss of immune down-regulation in alveolar macrophage.^{7,8} Lymphocytes collected from individuals with this condition have increased responsiveness to mitogen when co-cultured with autologous alveolar macrophages. Additionally, elevated levels of granulocyte macrophage-colony stimulating factor and interleukin-2 have been detected in patients with farmer's lung disease.

Pulmonary macrophages use a variety of specific and non-specific mechanisms to provide a balanced immune response. These mechanisms can be thought of as alterations in their state of activation with enhanced functional capabilities over non-activated macrophages. There are several well-defined changes in macrophage activation that occur when it is necessary for pathogen-derived antigen to be presented to T cells. One specific mechanism occurs when macrophages or dendritic cells increase expression of MHC II molecules along with co-stimulatory molecules such as B7 within lymphoid tissue.⁹ This combination of up regulating class II presented antigen along with co-stimulatory molecules is one of the key signals stimulating T cell proliferation and likely to be important in OvLV associated disease. Non-specific mechanisms which regulate the immune response include secretion of cytokines and chemokines such as IL-2, IFN- γ , and TNF- α ⁹ into the microenvironment and these mediators have been shown to be important in chronic lentivirus disease.¹⁰ These cytokines can modulate the functional state of other inflammatory cells (e.g. enhanced NO production) and also direct their trafficking patterns through altered expression of adhesion molecules on vascular endothelial cells. It has also been proposed that these mechanisms of pulmonary immune regulation may differ between species.¹¹ However, the immunoregulatory properties of

alveolar macrophages appear to be relatively selective, allowing T cell activation and cytokine secretion while inhibiting T cell proliferation within the lung.¹²

Potential disruptions of pulmonary immune homeostasis are possible in lentivirus associated lymphocytic interstitial pneumonia (LIP) since activated immune cells are more susceptible to lentivirus infection and replication.¹³⁻¹⁵ Furthermore, it has been recognized since the beginning of the AIDS epidemic that lymphocytes collected from HIV-positive individuals have altered proliferation profiles to mitogenic stimulation.^{16,17} Interestingly, lymphocytes collected from uninfected donors are capable of responding to HIV recombinant and synthetic antigens in a dose dependent manner.¹⁸ Thus, it is unclear whether these altered proliferation patterns in HIV infection are primarily due to lymphocyte or macrophage infection. The investigation conducted herein takes advantage of established patterns of lymphocyte proliferation in sheep^{19,20} and the macrophage tropism of OvLV²¹ to define alteration in lymphocyte proliferation in response to non-specific stimulation (mitogen).

Materials and Methods

Animal Sources of Cells.

Cell samples came from either rambouillet or border leicester lambs that were obtained from two flocks that have repeatedly tested seronegative for antibodies to OvLV envelope glycoprotein and capsid antigen by agar gel immunodiffusion (AGID) test (Veterinary Diagnostic Technology, Inc. Wheat Ridge, CO). The Colorado State

University Animal Care and Use Committee approved all procedures involving live animals. Cells designated as *in vivo* infected are derived from the rambouillet lambs that were intratracheally inoculated with either 10 ml of OvLV strain 85/34 (5×10^6 TCID₅₀) or spent tissue culture medium at three days post partum. Cells for negative controls are derived from lambs of the same flock intratracheally inoculated with 10 ml spent tissue culture medium. Cells were collected from these animals at various times over 18 weeks post inoculation (PI). Cells designated *in vitro* infected came from the border leicester lambs. These lambs remained seronegative for anti-lentivirus antibodies throughout the investigation.

Lymphocyte Isolation and Cultivation

Peripheral blood mononuclear cells (PBMC) were isolated from ethylenediaminetetraacetic acid (EDTA) treated blood that was collected from a jugular vein and separated with Ficoll-Paque[®] Plus (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's directions with slight modifications. The initial centrifuge step was altered to 670-x g to more efficiently separate sheep PBMC from neutrophils. Cells were stained with trypan blue and viable cells were enumerated on a hemocytometer. In a 96-well flat bottom plate, 50,000 PBMC per well were plated in complete medium, which was made with RPMI 1640, 10% heat inactivated fetal bovine serum, 1% penicillin/streptomycin, and 2mM L-glutamine (all from Gibco Life Technologies, Rockville, MD). Cultures were maintained in a in a 37°C, 5% CO₂ incubator.

Collection of Alveolar Macrophages

In vivo bronchoalveolar lavage (BAL) procedures were performed on a subset of animals under general anesthesia (5.0 mg/kg ketamine IV, 0.1 mg/kg diazepam IV, and 2% Isoflurane inhalation). A five mm diameter bronchoscope was wedged in the right middle bronchus and flushed with approximately 100 ml of Hank's balanced salt solution. The fluid was aspirated back through the bronchoscope and collected into a sterile container. Alternatively, cells were collected at necropsy, *ex vivo*, by lavaging 500 ml of salt solution into an intubated primary bronchus and retrieving into a sterile container. Cell counts and viability from all lavages were performed by microscopic examination of cells with a trypan blue exclusion stain on a hemocytometer. All BAL derived cells that were used in cell cultures had >95% viability.

Proliferation Assay

Mitogenic stimulation was done with a mannose receptor lectin, concanavalin A (Sigma Chemical Co., St. Louis, MO). A dose response curve was performed and 50 µg/ml was established as the optimal dose and used in each experiment. The immune suppressive profile of alveolar macrophages was examined by co-culturing varying dilutions of freshly isolated macrophages with PBMC. The macrophage : lymphocyte ratio ranged from 1:5 to 2:1. Stimulation of PBMC without lymphocytes was also performed. Each treatment was performed in triplicate. After treatment, cultures were placed in a 37°C, 5% CO₂ incubator for 72 hours. During the last 18 hours of this incubation, the culture was pulsed with ³H-thymidine (Amersham Pharmacia Biotech, Piscataway, NJ). Beta irradiation incorporation into cells was quantitated with the 1450 Microbeta Plus cell count harvester (Wallac Oy, Turku, Finland).

Statistical Analyses

Statistical analyses were performed with the Graphpad[®] Instat V. 3.0 software (San Diego, CA). Group mean comparisons were accomplished with either a paired or unpaired (as appropriate), two-tailed t-test with a Welch correction for means with significantly different standard deviations. A p value < 0.05 was considered significant. All graphical representations of data in the results section are reported the mean beta counts of three triplicate tests plus one standard deviation.

Results

Mitogenic Stimulation of Ovine Peripheral Blood Mononuclear Cells

PBMC were collected from lambs previously inoculated with OvLV or medium only (see Chapter 2 for animal identification) at various times post inoculation (PI). Lymphocyte proliferation in both groups of animals were tested with mitogenic stimulation. The lymphocytes collected from both OvLV and medium inoculated lambs at two weeks PI had a weak proliferative response to mitogenic stimulation (Figure 4.1). Identically stimulated lymphocytes from the medium inoculated lambs collected at 14 weeks PI had a strong proliferative response (Figure 4.2). However, the lymphocytes collected from the OvLV inoculated lambs at 14 weeks PI were lower than the medium inoculated lambs.

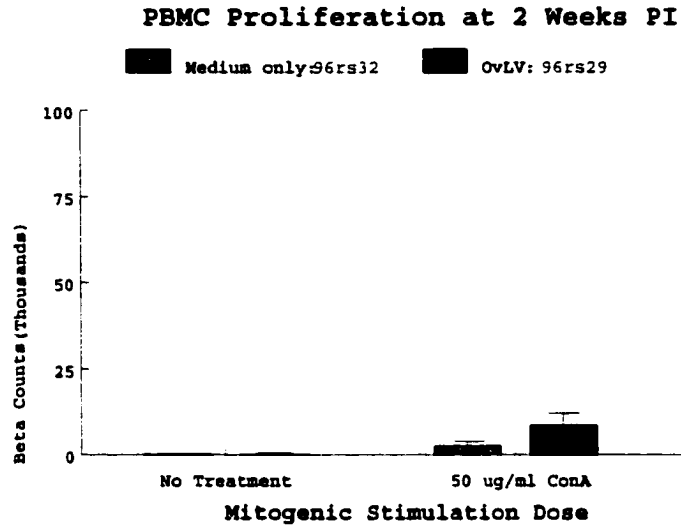


Figure 4.1 Mitogenic stimulation of PBMC from OvLV and medium inoculated lambs at two weeks PI. 50,000 PBMC were cultured with or without concanavalin A (ConA) for 72 hours and pulsed with ³H-Thymidine for 18 hours. Bars represent the mean beta counts (plus standard deviation) of three experiments. Both lambs were only slightly above background beta counts with concanavalin A stimulation.

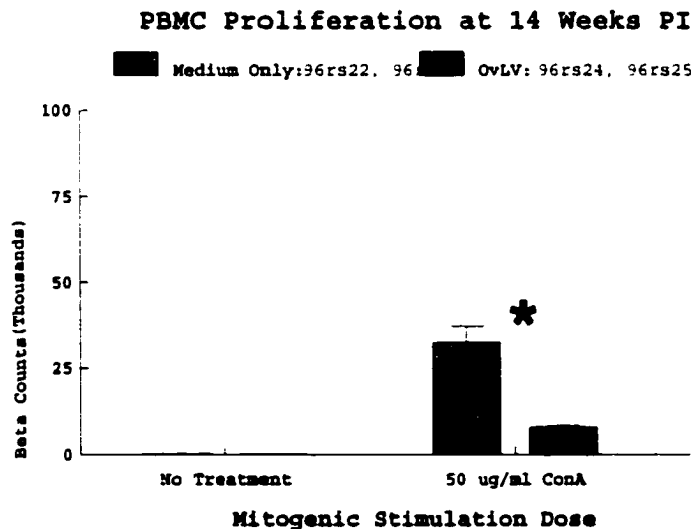


Figure 4.2 Mitogenic stimulation of PBMC from OvLV and medium inoculated lambs at 14 weeks PI. 50,000 PBMC were cultured with or without concanavalin A (ConA) for 72 hours and pulsed with ³H-Thymidine for 18 hours. Bars represent the mean beta counts (plus standard deviation) of three experiments. Both lambs were significantly above background beta counts only with concanavalin A stimulation. Lymphocytes from the medium inoculated lamb responded significantly more to the mitogen than lymphocytes from the OvLV inoculated lamb ($P < 0.001$).

Peripheral Blood Mononuclear Cell Proliferation with Autologous Alveolar Macrophages Co-Cultures

Experiments was designed to examine functional changes in infected alveolar macrophages, which may influence the proliferative capacity of lymphocytes. Normal macrophages function in a suppressive manner to mitogenic stimulation when co-cultured with autologous PBMC. Proliferation assays were designed to test for alterations of the suppressive macrophage function using either *in vivo* or *in vitro* OvLV infected BAL cells. PBMC collected from animals four weeks PI with OvLV responded significantly less to mitogen than PBMC from medium inoculated lambs (Figure 4.3). This overall reduced response occurred regardless of whether the PBMC were cultured with BAL cells. However, the PBMC from both OvLV and medium inoculated lambs increased their respective responses to mitogen when BAL cells were added up to a point (approximately one BAL cell for every two PBMC). Increasing the concentration of BAL cells beyond this point led to an overall reduction in the proliferative response.

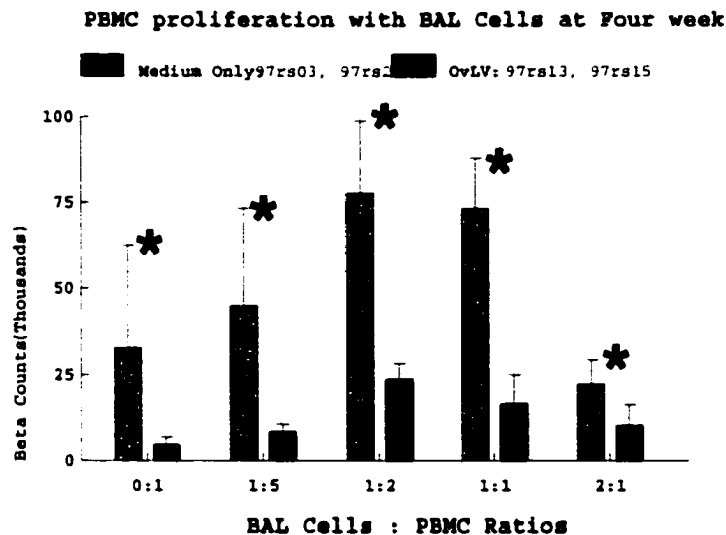


Figure 4.3 A constant number (50,000) of PBMC were co-cultured with varying numbers of autologous BAL cells from OvLV or medium inoculated lambs at four weeks PI, stimulated with concanavalin A (50 µg/ml) for 72 hours, and pulsed with ³H-Thymidine for 18 hours. Bars represent the mean beta counts (plus standard deviation) of three experiments. Similar but non-stimulated cultures from both groups consistently had beta counts less than 1,000. The beta counts of the medium only animals were significantly higher at all corresponding data points than the counts from the OvLV inoculated lambs (p values ranged from 0.0004 to 0.02). Both groups of cultures had reduced proliferation at the higher ratio of BAL cells.

A slightly different proliferation pattern was observed when the blastogenesis assay was performed with PBMC and BAL cells collected from animals ten weeks PI (Figure 4.4). There was no statistical difference between the proliferative response of PBMC collected from either the medium inoculated or infected animals at ten weeks PI and stimulated in the absence of BAL cells. When BAL cells were added at a 1:1 ratio, the proliferative response in the medium inoculated animals was slightly, but insignificantly, higher than the OvLV inoculated lambs. However, the proliferative response of the PBMC co-cultured with infected BAL cells was significantly lower compared to the culture from the medium inoculated lambs.

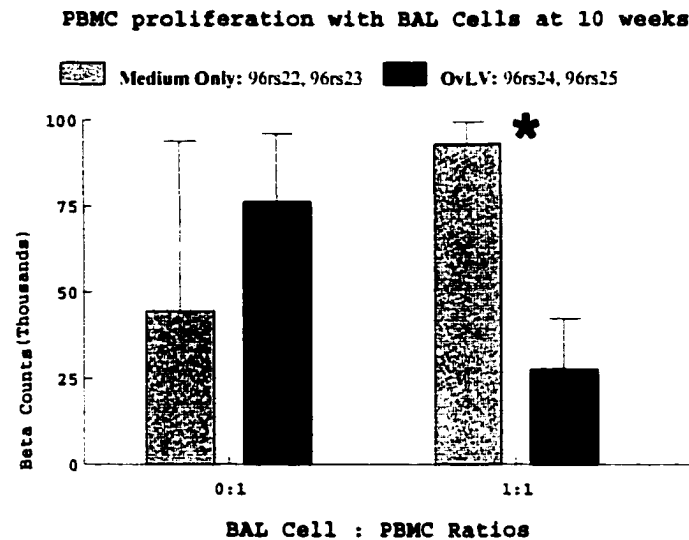


Figure 4.4. 50,000 PBMC were co-cultured with or without an equal number of autologous BAL cells from OvLV or medium inoculated lambs at 10 weeks PI, stimulated with concanavalin A (50 µg/ml) for 72 hours, and pulsed with ³H-Thymidine for 18 hours. Bars represent the mean beta counts (plus standard deviation) of three experiments. Similar but non-stimulated cultures from both groups consistently had beta counts less than 1,000. There was no significant difference in beta counts between cultures without BAL cells. The beta counts of the medium inoculated group, when co-cultured with BAL cells, was significantly ($p = 0.017$) higher than the corresponding datum point from OvLV inoculated lambs.

Finally, an experiment was designed to distinguish whether the reduced proliferative response of PBMC from OvLV infected animals was due to *in vivo* influences (i.e. the PBMC are anergic when collected) or was due to the influence of the OvLV infected alveolar macrophages. Here, BAL cells were collected from yearling rams which had repeatedly tested seronegative for anti-OvLV antibodies. The cells were adhered in 96 well plates and exposed to titrated OvLV at an MOI=3 for 24 hours. The cells were subsequently washed and the media replaced with medium containing freshly isolated PBMC from the same animal and the culture was stimulated with concanavalin A (Figure 4.5). The proliferative responses to mitogenic stimulation under these conditions were similar to previous experiments regardless of virus infection status.

Similar to before, the proliferation rate was higher with the addition of low concentration of BAL cells and lower at the higher concentrations. OvLV infectious status of BAL cells at 24 through 96 hours PI did not influence proliferation rates of PBMC.

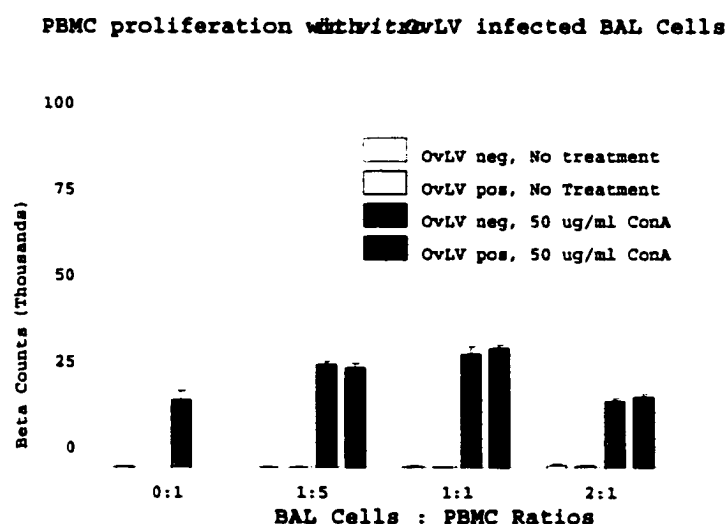


Figure 4.5 Bronchoalveolar lavage cells were collected from yearling rams seronegative for anti-OvLV antibodies. The BAL cells were split; one culture was infected *in vitro* with OvLV (MOI=2) for 24 hours (pos), while the other culture remained uninfected (neg). 50,000 autologous PBMC were subsequently added to the culture and stimulated with concanavalin A (50 µg/ml). A strong proliferative response was observed when treated with mitogen. The culture containing only OvLV infected BAL cells did not proliferate. Infection of BAL cells did not alter the level of proliferation in response to mitogen. Proliferative responses of both infected and uninfected cultures were lowest at the higher concentration of BAL cells.

Discussion

Lymphocyte proliferation assays from patients infected with HIV are commonly performed to monitor alterations in immunity associated with opportunistic infections,²² vaccinations,²³ and anti-retroviral therapy.²⁴ With these assays, clinicians commonly gauge lymphoproliferative response to mitogen as a prognostic indicator. Patients with reduced proliferative responses to mitogenic and antigenic stimulation are considered to

have a poorer prognosis.²⁵ It is uncertain in HIV whether this reduced proliferative response is primarily due to HIV infection of the lymphocyte or secondarily due to inadequate stimulation of uninfected lymphocytes. However, the data presented in this investigation suggest that lymphocytes from animals infected with exclusively macrophage tropic lentiviruses also have reduced proliferation responses.

Prior to this investigation, we considered a hypothesis that OvLV infection of macrophages resulted in a loss of normal immune suppressive macrophage function. It was hypothesized that this loss of immune suppression led to lymphocytes that were overly sensitive to non-specific environmental stimulation and exuberant lymphoproliferation, which subsequently created an environment conducive to lentivirus replication. However, the observations reported here are not consistent with this hypothesis. Normal immunosuppressive function of macrophages is maintained, regardless of OvLV infection. In fact, just the opposite, lentivirus infection leads to lymphocytes that appear to be hyporesponsive to non-specific stimulation. The reason for this seemingly anergic state of lymphocytes from lentivirus infected individuals remains uncertain.

A feasible explanation for the lymphocyte proliferation observed in the early OvLV-associated lesions is viral antigen presentation. Here we observe a dose response curve to purified viral antigen (capsid). In previous chapters, there was an observed increase in MHC II display associated with OvLV infection. The minimal amount of OvLV infected macrophage to lymphocyte cell-to-cell contact in the lung suggest that the proliferative signals may not be originating in the lungs. Alternatively, they may be originating in the lymph nodes, which were also previously described as hyperplastic associated with OvLV infection.

Not addressed in this chapter's investigation was the influence of the immune response on virus-infected cells. The data presented in this investigation in conjunction with the previous observation that proliferation rates (BrDU data) do not correlate with infection rates portends that lymphocyte accumulation, not proliferation, is the primary process occurring in response to OvLV infection of macrophages. The accumulation of lymphocytes appears capable of producing cytokine signals that recruit and activate more macrophages to the lungs. What is still unclear is how this scenario influences virus replication. The next chapter will partially explore this topic through investigation of how nitric oxide, an important product of activated alveolar macrophages, alters viral replication.

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Chapter Five

Nitric Oxide Regulation of Ovine Lentivirus Replication *in vitro*

Abstract

Ovine lentivirus (OvLV), also known as maedi-visna virus, infects and replicates primarily in macrophages. This investigation examined the role of nitric oxide in the replication of OvLV in cultured macrophages. Peripheral blood mononuclear cells were collected from OvLV-free sheep and cultured in Teflon coated flasks at a high concentration of lamb serum. The cells were subsequently infected with OvLV strain 85/34. OvLV replication was assessed under different experimental treatments by comparison of reverse transcriptase (RT) activity in culture supernatant. Cultures that were treated with exogenous nitric oxide via S-nitroso acetylpenicillamine did not have altered levels of RT activity compared to cultures treated with acetylpenicillamine. However, blockage of nitric oxide production by treatment with aminoguanidine, a molecule that selectively inhibits inducible nitric oxide synthase by competing with L-arginine, led to a significant rise in RT activity. This rise in RT activity was subsequently reversed in aminoguanidine treated cultures that were further treated with L-arginine, the normal substrate for inducible nitric oxide synthase. Finally, the number of viral antigen producing cells was also quantified after aminoguanidine treatment and found to be significantly higher than untreated cultures. Collectively, these results are consistent with nitric oxide being a negative regulator of OvLV replication in macrophages.

Introduction

Ovine lentivirus (OvLV), also known as maedi-visna virus, is the etiologic agent of lymphoid interstitial pneumonia in sheep (LIP)^{1,2}. LIP is a significant cause of morbidity and mortality in sheep^{3,4} and also occurs in human and simian immunodeficiency virus infections^{5,6}. Because *in vivo* infection and virus replication of OvLV occurs primarily in activated macrophages, and because activated macrophages are primary sources of nitric oxide, there may be a mechanistic link between lentivirus replication and nitric oxide. The purpose of this investigation was to examine the role of nitric oxide on early OvLV replication.

Pulmonary nitric oxide is produced from alveolar macrophages and is an important molecule in the physiological and immunological activities of the lung.⁷⁻¹⁰ Nitric oxide also plays a variety of roles in cellular activation¹¹⁻¹² and has a controversial role in the replication of HIV.¹³⁻¹⁵ There is considerable *in vitro* evidence that nitric oxide is capable of mitigating the functions of enzyme systems crucial to the replication of lentiviruses.¹⁶⁻²⁰ However, it is unclear whether these pathways are identical in both cell populations that are infected by HIV, macrophages and T cells, as recent experiments indicate that nitric oxide modulates HIV replication in T cells differently than in macrophages.²¹ The experiments described herein use primary cells from the natural host to investigate early OvLV replication in macrophages only without lymphocyte infection or depletion.²²⁻²⁴

There are many factors that influence the expression of inducible nitric oxide synthase (iNOS) gene in macrophages and the subsequent production of nitric oxide (Figure 5.1). One consideration is that inter-species regulation of nitric oxide production seems to vary.^{25,26} Additionally, within a species, macrophages may alter nitric oxide

production based on anatomical location,²⁷ cellular differentiation,²⁸ or physiological demands.²⁹ Finally, there are a variety of infectious diseases in which nitric oxide production may be modified either directly by the pathogen³⁰⁻³³ or indirectly from the immune response to the infectious agent.^{34,35} In reality, it is likely a combination of pathogen and host influences which regulate nitric oxide.³⁶

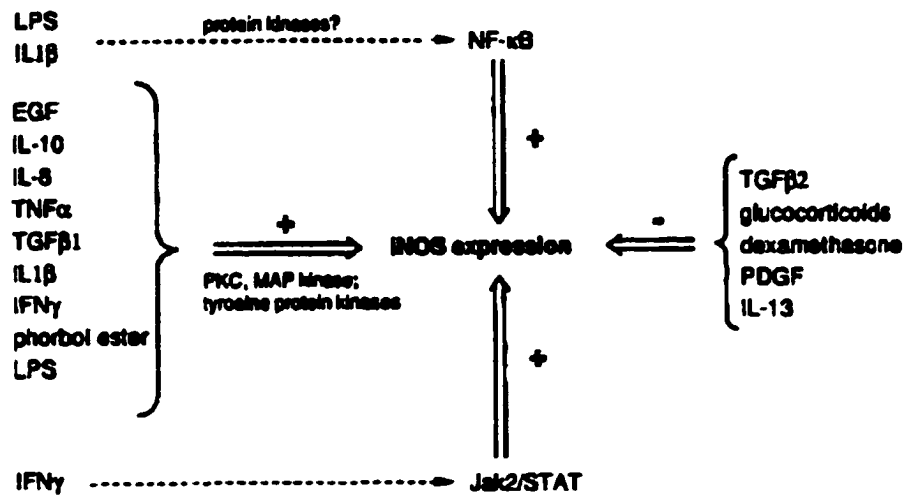


Figure 5.1 Overview of the many exogenous and endogenous factors that putatively regulate inducible nitric oxide synthase (iNOS). Many of these endogenous factors have also been linked to lentivirus infection. However, the effect of nitric oxide on the replication of lentivirus-infected cells is still controversial.
Figure adapted from Cellular and Molecular Biology of Nitric Oxide, Laskin & Laskin 1999.¹⁷

It was the objective of this investigation to clarify the role of nitric oxide in lentivirus replication. We observed treatments that inhibit nitric oxide production subsequently enhance viral replication. These observations suggest a potential mechanism in the down regulation of viral replication after the initial infection and replicative burst observed *in vivo*.

Materials & Methods

In vivo bronchoalveolar lavage (BAL) procedures were performed in yearling Border Leicester rams. These rams repeatedly tested seronegative for antibodies to OvLV envelope glycoprotein and capsid antigen by agar gel immunodiffusion (AGID) test (Veterinary Diagnostic Technology, Inc. Wheat Ridge, CO). All care and procedures involving these rams was approved and monitored by the Colorado State University Animal Care and Use Committee. BAL procedures were performed under general anesthesia (5.0 mg/kg ketamine IV, 0.1 mg/kg diazepam IV, and 2% Isoflurane inhalation). A five mm diameter bronchoscope was wedged in the right middle bronchus and flushed with approximately 100 ml of Hank's balanced salt solution. The fluid was aspirated back through the bronchoscope and collected into a sterile container. Alternatively, cells were collected at necropsy, *ex vivo*, by lavaging 500 ml of salt solution into an intubated primary bronchus and retrieving into a sterile container. Cell counts and viability from all lavages were performed by microscopic examination of cells with a trypan blue exclusion stain on a hemocytometer. All BAL derived cells that were used in cell cultures had >95% viability.

Monocyte Derived Macrophages (MDM) Cultures

MDM cultures generated from peripheral blood mononuclear cells (PBMC) were isolated from ethylenediaminetetraacetic acid (EDTA) treated blood collected from a jugular vein and separated with Ficoll-Paque[®] Plus (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's directions with slight modifications. The initial centrifuge step was altered to 670x g to more efficiently separate sheep PBMC from

neutrophils. Cells were stained with trypan blue and viable cells were enumerated on a hemocytometer. All PBMC isolation procedures routinely had >95% viability. The PBMC were cultured using conditions similar to those previously described that induce monocytes to differentiate into macrophages.^{38,39} The modified conditions included approximately 10⁶ PBMC cultured in 30 ml of macrophage differentiation medium which consisted of macrophage-SFM medium, 10% heat-inactivated lamb serum, 1% penicillin/streptomycin, and 2mM L-glutamine (all from Gibco Life Technologies, Rockville, MD). The cultures were kept in Teflon[®] coated flask and placed in a 37°C, 5% CO₂ incubator for one week. The morphology of MDM cultures were examined by centrifuging 200,000 cells onto a glass slide and immunocytochemically staining for lysozyme as marker of macrophage differentiation. The primary antibody was rabbit anti-human lysozyme (Dako Corp. Glostrup, Denmark) and detection was accomplished with the Vectastain[®] Elite ABC-Peroxidase kit and DAB substrate kit (Vector laboratories, Burlingame, CA) according to the manufacturer's instructions. Cells were lightly counterstained with hematoxylin and coverslipped.

In vitro ovine lentivirus infection

After one week, the MDM cultures were split into two flasks. One flask was infected with OvLV strain 85/34 at an MOI of 3. This OvLV strain has been previously reported to produce lesions *in vivo* and replicate to high titers *in vitro*.⁴⁰⁻⁴² After infection the cells were washed once with macrophage differentiation medium and plated into either a 48 well plate or 8 well slide chamber at 50,000 cells per well.

Culture Treatments

Aminoguanidine is an amino acid analogue that binds to the active site of iNOS⁴³ and was used to inhibit the production of nitric oxide (Figure 5.2). L-Arginine was added to the indicated cultures to compete with aminoguanidine for the iNOS active site. S-nitroso-acetylpenicillamine was used as an exogenous nitric oxide donor, while acetylpenicillamine was used as a negative control. Final culture concentrations are indicated with the data. All culture treatment chemicals were acquired from Sigma Chemical Co. (St. Louis, MO).

Stimulation of nitric oxide production in alveolar macrophages was attempted with various concentrations of lipopolysaccharide (LPS, *E. coli* O55:B5, Sigma) and an empirical dose of IFN- γ produced from a plasmid kindly provided by Dr. W.P. Cheevers using a previously described technique.⁴⁴ IFN- γ production was confirmed with the enzyme immuno assay based BovigamTM IFN- γ test according the manufacturer's instructions (CSL Veterinary, Victoria, Australia).

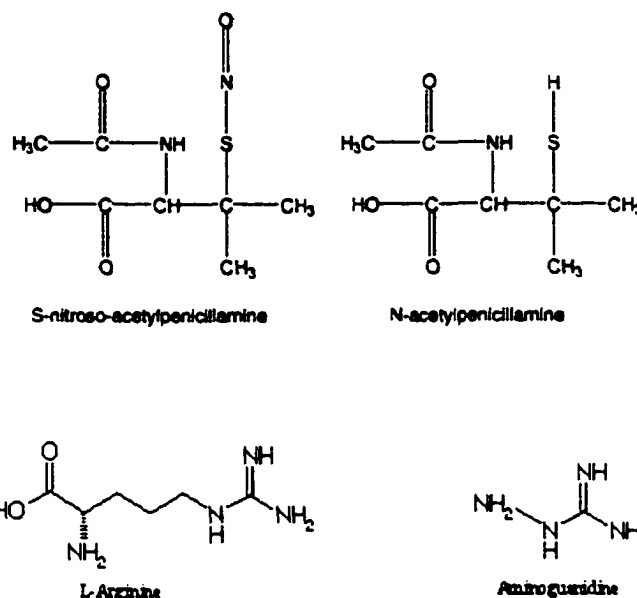


Figure 5.2 Stick figure representations of the treatment molecules used in assessing the role of nitric oxide on lentivirus replication in cultured macrophages. S-nitroso-acetylpenicillamine (SNAP) readily releases its nitrosothiol moiety under physiological aqueous conditions and has been shown to differentiate peripheral blood mononuclear cells.⁴⁵ Nitric oxide synthases metabolize L-arginine to citrulline and nitric oxide.¹⁷ Aminoguanidine was used as an L-arginine analogue that specifically blocks the inducible nitric oxide synthase enzyme.

Reverse Transcriptase Assay

OvLV relative replication rates between treated cell cultures were deduced from the reverse transcriptase activity of the cell culture supernatant. The principle of this assay is that each virion that buds off an infected cell carries reverse transcriptase; the numbers of virions produced are proportional to the enzymatic activity of reverse transcriptase. To perform this assay, supernatants were collected from identically infected cell cultures with various treatments using the Lenti-RT™ Activity Assay (Cavidi Tech, Uppsala, Sweden) according to the manufacturer's instructions with some modification. The supernatants were incubated in a microtiter plate coated with a RNA

primer along with a solution containing bromine labeled nucleotides. After incubation and subsequent washing, the bromine labeled nucleotides incorporated were quantitated using alkaline phosphatase antibodies and the appropriate substrate which was subsequently measured at 405 nm on a MR5000 microplate reader (Dynatech Laboratories, Chantilly, VA). Alterations to the procedure included testing all samples in quintuplicate and only testing non-diluted supernatants. Supernatants from uninfected MDM cultures were used as negative controls. Supernatants containing the original virus inoculum, collected from goat synovial membrane tissue cultures, were used as positive controls.

Cytotoxicity Assay

Cytotoxic effects of treatments on macrophages were assessed in parallel experiments using the MTT assay as described.⁴⁶ This assay utilizes the yellow tetrazolium salt [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide], which is metabolized by mitochondrial succinic dehydrogenase activity of viable cells to yield a purple formazan reaction product. Briefly, the culture media was replaced with phenol red-free DMEM. 25 µl of MTT solution (5 mg/ml in PBS, Gibco) was added to each cell well and incubated at 37° for 4-6 hours. Following incubation, the supernatant was aspirated and 200 µl of acidified 2-propanol (0.04N HCl in isopropyl alcohol) and 20 µl of sodium dodecyl sulfate (3% w/v in water) was added to each well to disrupt cells and solubilize the colored product. Each well was aspirated up and down multiple times with a micropipette to mix the contents of each well and the plate contents were further mixed

on a rocker for five minutes. The contents of each well were transferred to a 96 well ELISA plate and absorbance was measured on a microplate reader at 570 nm. Determinations were performed on quintuplicate wells. Mean data for treatments were compared by t-test for significant differences.

Measure of nitric oxide production

Nitric oxide concentrations of the cell culture supernatant were deduced from the accumulation of aqueous nitrate and nitrite via the Griess reaction. In the Griess reaction, nitrite readily combines with *N*-(1-Naphthyl)ethylenediamine dihydrochloride under acidic condition to form a colored azo dye which was quantitated with a microplate reader at 570 nm and compared to a standard curve made from sodium nitrite. This assay was performed using the nitrate/nitrite colorimetric assay kit (lactate dehydrogenase method) from Cayman Chemical Co. (Ann Arbor, MI) according to the manufacturers instructions.

Immunofluorescent staining

Immunofluorescent staining was used to detect iNOS and OvLV antigen in the 8 well tissue culture system. After the incubation period, the supernatants were removed, the cells were washed in saline followed by air-drying and fixation was done in cold acetone. All slides were stained at the same time by rehydrating the cells in saline, blocking with Protein Block Solution (Biogenex, San Ramon, CA), and quenching with 3% hydrogen peroxide. The primary antibodies were monoclonal 3F¹⁷ against OvLV capsid p25 and polyclonal against human iNOS²⁹ (Santa Cruz Technology inc., Santa

Cruz, CA) which were used as previously reported. Initial controls and testing of the antibody labeling procedure involved staining MDM cytopins for either OvLV capsid or iNOS (Figure 5.9). Detection was performed with an isotype appropriate secondary antibody and a peroxidase-based chromagen according to the manufacturer's instructions (both kits from Vector Labs, Burlingame, CA). Final calibration for immunofluorescent staining also included the omission of the primary antibody for determination of autofluorescence. Fluorescent labeling was accomplished using appropriate anti-isotype secondary antibodies conjugated to peroxidase and the Tyramide Signal Amplification™ Plus Fluorescence System according to the manufacturer's instructions (NEN Life Science Products, Boston, MA). Fluorescein was used to label viral antigen and cyanine 3 was used to label iNOS. Slides were coverslipped using Vectashield® mounting medium containing DAPI (Vector laboratories) and kept in the dark until viewing.

Image Analysis for co-localization

Quantitative image analysis involved capturing digital images (TIFF files) of fluorescent stained cells through an Olympus Microscope fitted with DAPI (610/675 nm), TRITC (557/590 nm), and FITC (500/525 nm) filters and a 3-CCD 1140x1520 detail Cool SNAP Camera (Roper Scientific GmbH, Bergkirchen, Germany). Five distinct images from each treatment group at each time point were captured using the 20x objective and analyzed with Metamorph software v. 4.0 (Universal Imaging Corp., West Chester, PA) using the color thresholding, overlay, and co-localization tools. All fluorescent values were normalized for the cellular density of that particular image using the number of DAPI stained nuclei. The formula for this adjustment = (fluorescence area

of image) / (total DAPI pixel area/10,000). Subsequently, the adjusted area of fluorescence corresponding to viral antigen was summed. Additionally, the area of fluorescence corresponding to iNOS production and co-localizing with viral antigen production was summed.

Statistical Analyses

All statistical analyses were performed with the Graphpad® Instat V. 3.0 software (San Diego, CA). Comparison of group mean data was analyzed for significance with a two-tailed, unpaired t test with a Welch correction for means with different standard deviations. The dose response curve of RT activity to aminoguanidine treatment was assessed by a linear regression analysis. The aminoguanidine doses were log transformed and the virus only controls were assigned to the X-axis by one log value less than the lowest treatment dose. A linear correlation was calculated with the line forced through the Y-axis at the O.D. value of the sample with no drug treatment. The index value associating viral antigen and iNOS co-localization was established with a 2x2 contingency table using the presence or absence of iNOS on one axis compared to the presence or absence of viral antigen on the other axis. Fischer's exact relative risk calculations were applied to the values. For all statistical tests, results were considered significant if the P value was < 0.05.

Results

Bronchoalveolar lavage (BAL) cell nitric oxide production and viability

BAL cells were collected from OvLV-free lambs. These cultures were stimulated to produce nitric oxide with *E. coli* derived endotoxin (LPS), recombinant interferon- γ (IFN- γ), and OvLV infection. Viability was assessed in conjunction with these treatments by the ability of the cultured cells to metabolize MTT tetrazolium salt by mitochondria enzymes into a formazan precipitate (Figure 5.3). The viability of treated cells was compared, via the concentration of tetrazolium salt, to the viability of the untreated cultures. Cultures with less than 80% viability are considered to have significant cell death. The higher doses of LPS treatment consistently reduced the cell viability below 80%. Additionally, the nitrite accumulation at these treatment doses was not consistently above 10 mM, which is the lower limit of detection for the Griess assay. These factor, combined with the lack of availability to do repeated assays led to the use of monocyte derived macrophages (MDM) for the remainder of the experiments. MDM cells consistently had excellent viability and were readily available for multiple simultaneous cell cultures.

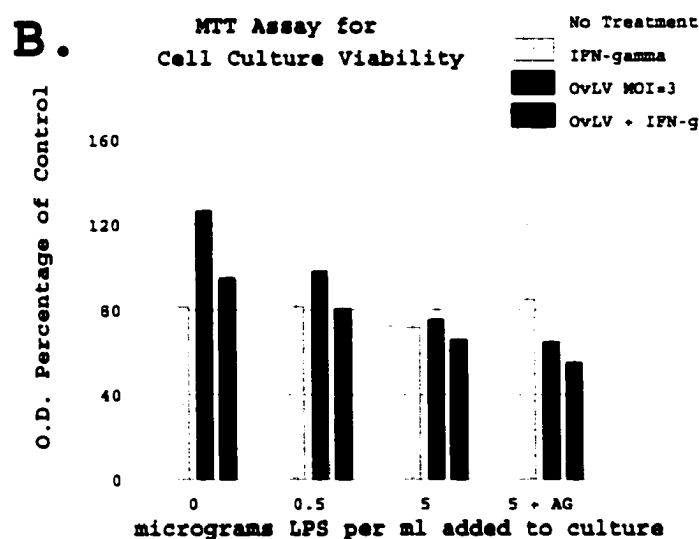


Figure 5.3 MTT tetrazolium salt assay for viability of BAL. Alveolar macrophages were exposed to various treatments in an attempt to stimulate nitric oxide production. A) Photomicrograph of non-stained cultured macrophages after five-hour incubation with MTT containing medium. The blue streaks and clumps in the cytoplasm are the metabolized formazan. B) After solubilizing the formazan, the concentrations of the salts are compared by a spectrophotometer at 570 nm. Cultures with a mean value less than 80% of the untreated cultures value are considered to have significant cell death.

Monocyte derived macrophage (MDM) cultures

After one week of culture, approximately 20% of the PBMC would differentiate from peripheral monocytes to macrophages as assessed by lysozyme immunoreactivity (Figure 5.4). The remaining 80% were washed away from the culture plates after the MDM cells were allowed to adhere. Lysozyme stain was not observed in any of the freshly isolated PBMC. The morphology of OvLV infected MDM cells was observed 48 hours post inoculation (PI). The infected MDM cells appeared to have somewhat more vacuolation to the cytoplasm; however, the lysozyme immunoreactivity appeared similar to the non-infected MDM cells. Bi-nucleated MDM cells were observed in both infected and non-infected cultures. However, there were only 1-2 bi-nucleated cells per slide in the cytopsin preparations from the non-infected MDM cells whereas there were 1-2 bi-nucleated cells per high magnification field on the cytopsin preparation from the OvLV inoculated culture. The OvLV inoculated MDM cells also had increased cytoplasmic vacuolation compared to the non-infected MDM cells.

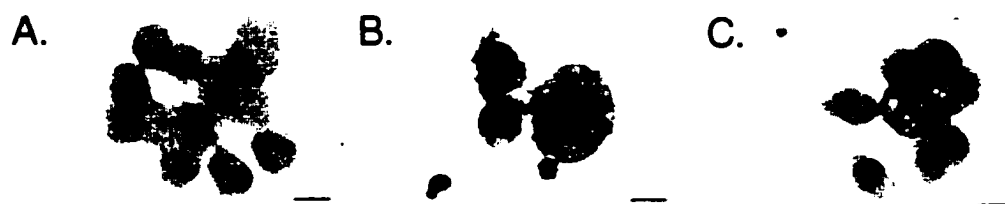


Figure 5.4 Morphology and differentiation of monocyte derived macrophages (MDM). All cells are immunocytochemically stained for lysozyme as a marker of differentiation using peroxidase based diaminobenzidine as a chromagen and hematoxylin as a counterstain. A) PBMC at the start of the culture. B) MDM cells after one week in culture with a rare bi-nucleated cell. C) MDM cells that have been infected with OvLV strain 85/34 for 3 days. All bars represent 10 microns.

Reverse Transcriptase (RT) Assay

The source of the RT activity was confirmed to be *de novo* virion from MDM cultures (Figure 5.5). In this experiment, supernatants were collected prior to virus inoculation (negative), immediately after inoculation (time 0), 24 hours after inoculation, and 48 hours after inoculation. The supernatant collected at 48 hours PI and the positive controls were the only samples that tested positive (above the baseline) of RT activity. Additionally, the virus inoculum was tested for RT activity prior to inoculation and immediately after the 30 minutes incubation period. There was a slight decrease in the RT activity of the inoculum after the incubation period. Based on this experiment, all future RT assays were performed 72 hours after virus inoculation to maximize differences between treatment and control groups.

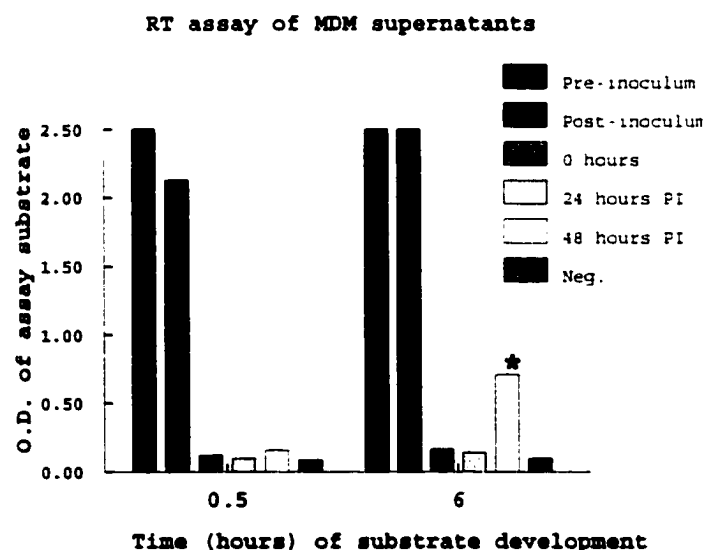


Figure 5.5 Reverse transcriptase (RT) activity of MDM cells at various times before and after inoculation. The pre-inoculum is concentrated OvLV strain 85/34 supernatant from goat synovial membrane cultures. The post-inoculum is the same supernatant after a 30-minute incubation with the MDM cells at 37° C in which there is a slight decrease in RT activity. Supernatants collected from uninfected MDM cells (negative), immediately after inoculation (time 0), and 24 hours inoculation did not have RT activity. Only supernatant collected 48 hours after virus inoculation had a significant ($p = 0.01$) increase in RT activity. Error bars are not included for bars outside the linear range of the assay.

Next, levels of RT activity were assessed when MDM cultures were treated with an exogenous source of nitric oxide (Figure 5.6). S-nitroso acetylpenicillamine (SNAP) was added to cultures at varying concentrations. Acetylpenicillamine (NAP) is a molecule without the nitric oxide donor molecule and was added as a negative control. Neither the exogenous nitric oxide donor nor the control molecule created a change in the RT levels over background. Another component of this experiment was addition of bovine hemoglobin to scavenge nitric oxide or aminoguanidine to block inducible nitric oxide synthase (iNOS). Both of these treatments, which reduce the level of aqueous nitric oxide, were associated with a significant rise in the level of RT activity.

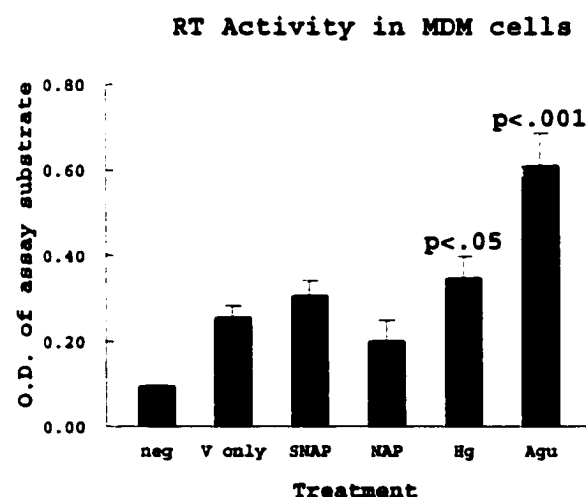


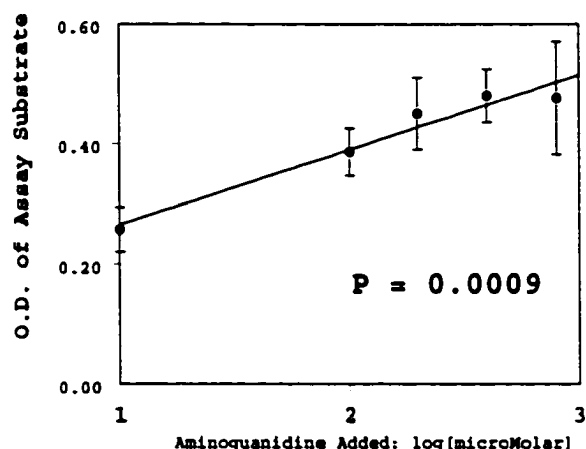
Figure 5.6 Reverse transcriptase (RT) activity of monocyte derived macrophage (MDM) cells 72 hours after *in vitro* OvLV inoculation with various concentrations of either 1 mM S-nitroso acetylpenicillamine (SNAP) or 1mM acetylpenicillamine (NAP). Cultures were also treated with 5 μ M bovine hemoglobin (Hg) or 0.2 mM aminoguanidine (Agu). The addition of exogenous nitric oxide did not change the level of RT activity above background, however chemicals that either scavenged nitric oxide or blocked its production were associated with significant rises in RT activity.

A series of experiments were subsequently designed to confirm the observation that blocking nitric oxide production was functionally related to increases in OvLV

replication. First an experiment was performed in which endogenous production of nitric oxide was blocked over a dose response curve and then RT activity assayed (Figure 5.7). Aminoguanidine was again used as an amino acid analogue of arginine, which competes with L-arginine for the functional site of iNOS. As the aminoguanidine concentration increased there was a significant increase in the RT activity. As the aminoguanidine dose rose into the toxic dose range the RT activity began to plateau. A parallel experiment, with increasing doses of exogenous nitric oxide added to the culture was performed. In this experiment, a dose response curve was not observed

In order to confirm the mechanistic connection between aminoguanidine and viral replication, L-arginine was added to a culture along with aminoguanidine (Figure 5.8). Under this culture condition, the L-arginine will compete with the aminoguanidine for the active site of iNOS. In this experiment and similar to the previous experiment, there was a significant increase in the RT activity of the aminoguanidine treated culture. This increase was partially reversed by the addition of L-arginine to an aminoguanidine containing culture. The value of the RT activity in the aminoguanidine and L-arginine combination culture was statistically at median between the virus only and virus plus aminoguanidine culture.

A. RT Activity of MDM cells with iNOS inhi:



B. RT Activity of MDM cells with addition of Nitric Oxide

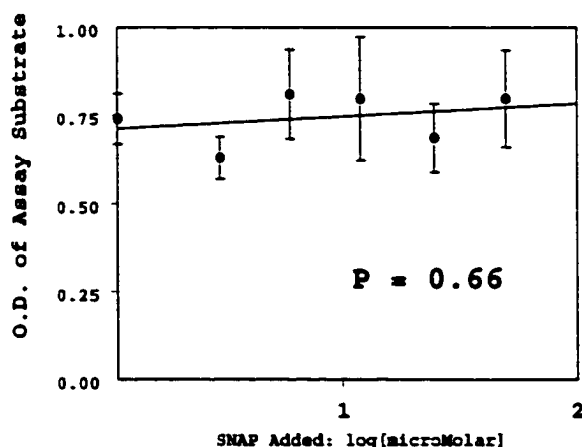


Figure 5.7 Reverse transcriptase (RT) activity of monocyte derived macrophage (MDM) cells 72 hours after OvLV inoculation with various treatments blocking or adding nitric oxide. A) Aminoguanidine concentrations were increased over a six-fold dose range in which the dose response line of RT activity was significantly different than a slope of zero. B) S-nitroso acetylpenicillamine doses were increased over an eight-fold dose range in which the dose response line of RT activity did not differ from a slope of zero. Line fitting was done with a first order polynomial equation. The zero dose for each treatment was assigned an X-value one log less than the lowest treatment dose.

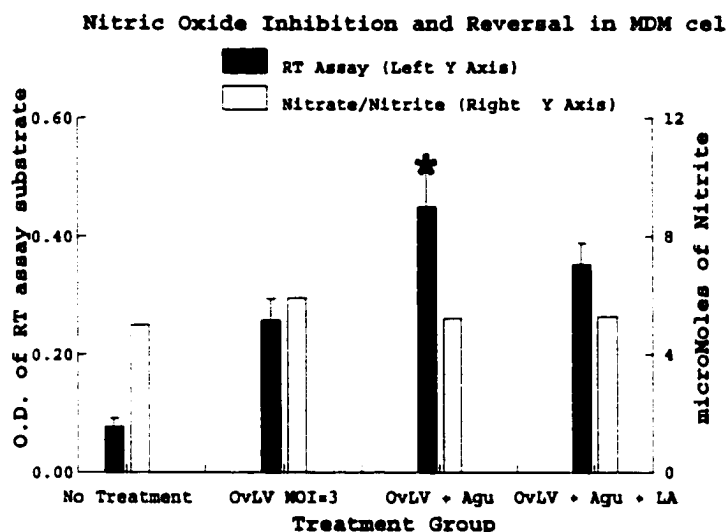


Figure 5.8 RT activity and nitrite accumulation of MDM cells 72 hours after OvLV inoculation treated with either 0.2 mM aminoguanidine (Agu) or 1.0 mM L-arginine (LA). Left Y Axis) There was a significant ($p < 0.01$) increase in the RT activity of the aminoguanidine treated culture. This increase was partially reversed by the addition of L-arginine to an aminoguanidine containing culture. The value of the RT activity in the aminoguanidine and L-arginine combination culture was statistically indistinguishable between the virus only and virus plus aminoguanidine culture. Right Y Axis) Nitric oxide was measured in the supernatant by reducing nitrate to nitrite with subsequent quantification of nitrite using the Griess reaction in which samples are compared to a standard curve of sodium nitrite. Total nitrite measured was at the lower level of detection thus results are not thought to be linear and error bars are not calculated.

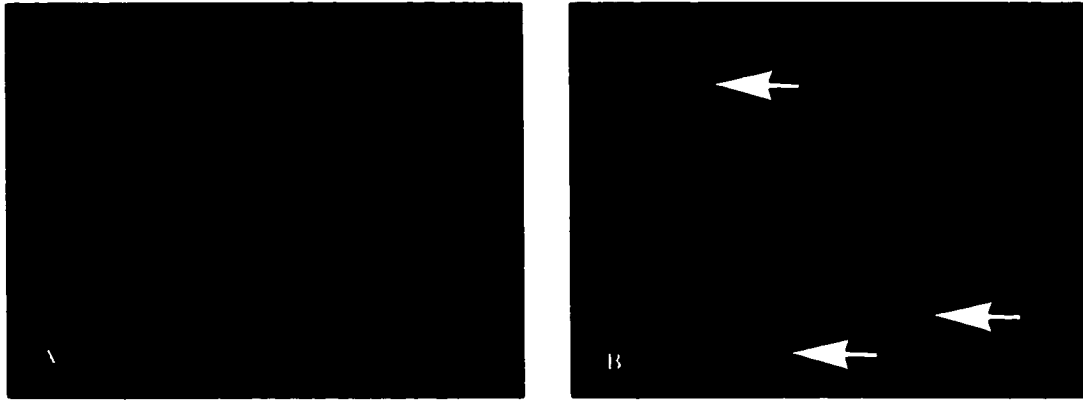
Immunofluorescent staining of iNOS and OvLV antigen

To exam the nitric oxide - OvLV replication relationship by an alternative route, the production of iNOS and OvLV antigen were measured in MDM cells by immunofluorescent co-localization technique. Antibodies against OvLV capsid and iNOS were first tested for specific staining using chromagenic detection of OvLV infected MDM cytospin preparations (Figure 5.9). Once the specific staining was confirmed, OvLV inoculated MDM cultures were examined over time for the percentage of cells producing virus antigen and how many virus antigen cells co-localized with cells

which also stained positive for iNOS (Figure 5.10). The cumulative level of viral antigen exceeded background levels only in the latter portion of the experiment (64 and 72 hours PI). Additionally, a rise above background in viral antigen associated fluorescence was only observed in the aminoguanidine treated cultures. Fluorescent co-localization was analyzed by the Fisher's exact test. This test was only applied to time points PI in which the total viral antigen fluorescence was distinguished above autofluorescence levels. At these two points in the aminoguanidine treated cultures, the presence of iNOS within a cell was not predictive of viral antigen production.



Figure 5.9 Immunocytochemical staining of monocyte derived macrophage (MDM) cells to confirm specificity of anti-OvLV and anti-iNOS antibody. A) MDM cells 72 hours post inoculation with OvLV and stained for p25 capsid. B) MDM cells from a parallel culture to panel A, but not inoculated with OvLV and stained for p25 capsid. C) Same MDM cells as panel A, but stained using an irrelevant (anti-bovine herpes virus) isotype antibody. D) Lung tissue from a mouse experimentally inoculated with *Mycobacterium tuberculosis* and stained for iNOS production. E) Same MDM cells as panel A (OvLV inoculated) stained for iNOS. F) Same as panel B (not inoculated) and stained for iNOS. All stains are peroxidase-based chromagen with hematoxylin counterstain. Note, the small fragments in the MDM panels are cytoplasmic artifacts of the vortexing necessary to dislodge the cells from the culture flask surface.



C. OvLV Antigen and iNOS expression in MDM cells

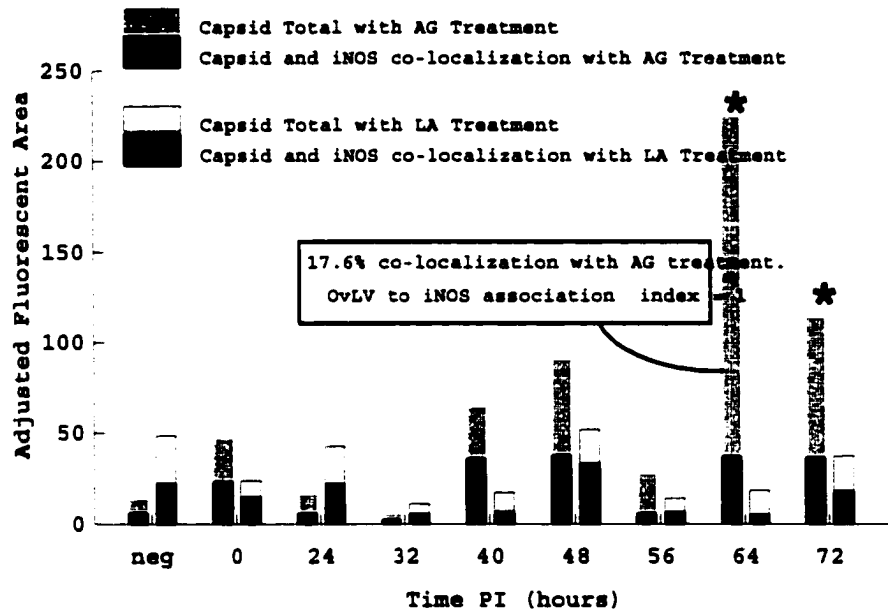


Figure 5.10 Relative co-localization of OvLV antigen (capsid) and inducible nitric oxide synthase in monocyte derived macrophages. A) L-arginine treated and B) Aminoguanidine treated immunofluorescent images of OvLV capsid (FITC - green) and iNOS (Cy3 - red) in MDM cell cultures at 72 hours PI. All nuclei stain blue (DAPI). C) Immunofluorescent co-localization of OvLV capsid antigen and iNOS in MDM cell cultures. The bar graph represents the sum fluorescent area of OvLV capsid producing cells adjusted for cellular density (see material & methods for formula). Viral antigen was significantly above background levels only at the last two timepoints measured. The bars under each graph represent the area of fluorescence at each point that co-localized with iNOS production in the same cell. OvLV capsid was significantly ($p = 0.01$) above autofluorescence levels at 64 and 72 hours PI in the aminoguanidine treated culture only. The risk assessment of iNOS and OvLV capsid co-localization in aminoguanidine treated cultures was not significant (see Figure 5.11).

	OvLV Positive Cells	OvLV Negative Cells	Totals
iNOS Positive Cell	400 (6%)	798 (12%)	1198 (18%)
iNOS Negative Cells	1514 (23%)	3853 (59%)	5367 (82%)
<i>Totals</i>	1914 (29%)	4651 (71%)	6565 (100%)

Figure 5.11 Contingency table for calculations of associative risk in co-localization. Fischer's exact calculations were used to establish a risk approximating 1. This table represents the numbers (and percentage) of cells for each of the indicated columns at 64 hours PI and treated with aminoguanidine.

Discussion

This investigation provides two lines of evidence that nitric oxide negatively regulates lentivirus replication in macrophages: First, the addition of aminoguanidine to cultured macrophages infected with OvLV significantly increases the reverse transcriptase activity of the culture supernatant. Second, in a separate experiment, the addition of aminoguanidine to a similar culture system increased the production of OvLV capsid within the cytoplasm of infected macrophages. These two alterations in the virus-infected cultures in which the production of nitric oxide is inhibited, suggest that the presence of nitric oxide may be an innate mechanism of down regulating lentiviral replication following OvLV's initial replicative burst.

Reverse transcriptase activity was used as an indicator for viral replication based on the concept that this enzyme is packaged with each virion which buds from the infected cells. Thus, the activity of RT activity in supernatant is presumed to be proportional to the number of virions in the supernatant. Since each well contained an identical number of cells, the difference in RT activity was attributed to the treatments. The manufacturer of this particular RT activity kit specifically designed this assay for quantitative analyses. Furthermore, the quantification of RT activity both before and

after virus inoculation indicated that RT activity was present only 48 hours after inoculation. This was done to ensure that RT activity measured was from *de novo* virus production and not an artifact of the original inoculation. The interpretation that the presence of aminoguanidine increases reverse transcriptase activity was further established by a significant dose response curve. The final experiment done to confirm the interpretation of the RT activity response to aminoguanidine was to compete directly with aminoguanidine for the active site of iNOS by the addition of excess L-arginine, which was able to partially reverse the affects of aminoguanidine.

The experiments involving OvLV antigen production were performed to confirm that the RT activity was truly a function of virus replication and refute the possibility that treatments were interfering with RT activity but not necessarily virus replication. This interference could include direct interference, such as L-arginine inhibiting RT activity, or indirect interference with nitric oxide reducing RT activity but not necessarily altering virus replication. The presence of virus antigen within MDM cell cytoplasm under the circumstances of this experiment is independent of reverse transcriptase activity. That is to say that although reverse transcriptase was necessary to initially create a provirus containing cell, that step was prior to any treatment and should be constant between aminoguanidine and L-arginine treated cultures. Conversely, the antigen measured in this experiment is viral capsid. The virus production was based on the number of cells containing virus capsid and not the activity of an enzyme. Thus, both the experiments involving measurement of RT activity and the experiment quantifying the number of OvLV antigen positive cells indicate that inhibition of nitric oxide synthase creates an environment which enhances OvLV replication.

It was not entirely clear why the addition of exogenous nitric oxide did not further lower the RT activity of the treated cultures. One possible explanation is that a floor effect had been reached with the endogenous production of nitric oxide and addition of exogenous nitric oxide was not going to alter that lower RT activity. Alternatively, the half-life of the exogenously added nitric oxide (estimated by the manufacturer to be only a few minutes) might have been an insufficient exposure time to reduce the RT activity. As alluded to in the introduction, the regulation of iNOS in sheep has been difficult to define due to the low levels of nitrate accumulation in sheep cell cultures. This investigation also had difficulty in establishing functional relationships based on nitrate accumulation since the quantities of nitrite in the supernatants were too close to the lower level of detection for the Griess reaction to accurately measure changes. It is possible that measuring the levels of intracellular nitric oxide might resolve this investigative obstacle.

The cell culture methods used in this investigation demonstrate the vigor of sheep MDM cells as differentiated macrophages to investigate lentivirus infection and replication. This culture's vigor along with its ease of acquisition was largely why MDM cells were chosen over other cell populations such as alveolar macrophages that appear to have shorter culture viability. Although not directly measured in this investigation, it was presumed that the cell cultures were largely depleted of lymphocytes during the washing and media change steps. The MDM cells are strongly adherent to plastic culture surfaces where as the lymphocytes are less so. Thus, intra-culture cytotoxicity from activated lymphocytes was presumed to not occur in this investigation.

In conclusion, we believe that nitric oxide is part of the innate host response to lentivirus infection that down regulates OvLV replication in macrophages. The challenge

that remains is to integrate this innate response along with the adaptive response of the host's immune system to further define host-virus relationship.

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Chapter Six

Conclusions

The key accomplishments of this investigation are as follows.

1. Neonatal lambs inoculated with ovine lentivirus (OvLV) develop a peribronchiolar lymphocytic interstitial pneumonia at four weeks post inoculation concurrent with a large influx of alveolar macrophages.
2. The alveolar macrophages have an increased level of activation characterized by increased cell surface MHC II expression. The increase in both numbers and activation status of alveolar macrophages can increase infectious dissemination of OvLV within the lung.
3. The interaction of alveolar macrophages and pulmonary lymphocytes involves only minimal cell-to-cell contact as the two cells are predominantly separated by the alveolar/interstitial septae.
4. Lymphocytes isolated from lambs recently infected with OvLV are hyporesponsive to mitogenic stimulation and only weakly responsive to antigenic stimulation. The immune suppressive functions of alveolar macrophages on lymphocyte proliferation in response to mitogenic stimulation remain intact, regardless of OvLV infection status.
5. Inhibition of inducible nitric oxide synthase by the amino acid analogue aminoguanidine is associated with increased OvLV replication in infected monocyte derived macrophages. The addition of exogenous nitric oxide to macrophage cultures does not alter OvLV replication.

This investigation focused on the early sequela of lentivirus infection in pulmonary tissue. In completing this investigation, we hopefully now have a clearer understanding of the sequence of events that occur when a macrophage exclusive lentivirus infects the unique compartment of the immune system in the lung. This investigation and others provide overwhelming evidence that inflammation observed in the early weeks of lentivirus infection is associated with the presence of replicating virus. But the interplay between the virus and the host still begs the question as to whether or not this inflammation portends an effective immune response. There was evidence provided in this investigation that inflammation was effective immunity. These include: the virus infection rate ultimately drops after pulmonary inflammation, the host's lymphocytes do respond to OvLV by producing antibodies *in vivo* and proliferating to antigen *in vitro*, and nitric oxide can act in an innate manner to down regulate lentivirus replication. Conversely, there was also evidence presented that might allow one to conclude that the pulmonary inflammation is not effective. These include: the infiltrating pulmonary lymphocytes are predominantly separated from the primary target cell by an anatomical barrier, activation of macrophages via increased class II expression is not an effective signal to indicate self-infection, and the lymphocytes present in OvLV infected individuals are hyporesponsive to non-specific stimulation. Collectively, these observations provide some evidence that immunity exists but raises more questions about the relationship of the immune system and lentiviruses.

I believe that studies on the early pathogenesis of lentivirus disease that follow this investigation should concentrate on two broad areas: 1) defining and distinguishing how the immune system differs when activated against a lentivirus-infected T cell vs. a

lentivirus-infected macrophage, and 2) deciphering the cell-to-cell and intracellular signaling pathways which can alter cell activation and subsequently alter replication of lentivirus in a cell. In the first area, I would propose that one might readily test whether exuberant inflammation is a risk factor for increased infectious dissemination of lentiviruses in a host by enumeration of latently infected cells (i.e. provirus only). If this could be done on a body-wide scale, one could measure the degree of inflammation in the early post inoculation period (say bronchoalveolar lavage at four weeks PI) and subsequently correlate that with total provirus load at a time when inflammation and virus replication has subsided. As for the second broad objective, I believe that continued investigation on both the extracellular and intracellular host signals that regulate nitric oxide production in lentivirus-infected macrophages will ultimately provide pathways for understanding lentivirus replication itself. There are, of course, many other worthy objective towards a comprehensive understanding of lentivirus pathogenesis. However, I perceive the above stated objectives as logical "next steps" in this area of work.

On a final note, I have come to believe that the primary challenge to investigators of lentivirus pathogenesis is to comprehensively and cohesively explain the lentivirus-host paradox. This paradox can be stated as "How does a lentivirus require a host with an activated immune system to replicate at the same time a host requires an activated immune system to kill a lentivirus?" Possibly, this paradox has no logical answer. An individual who observes the natural outcome of lentivirus infection, in which both the host and the virion within the host die, might conclude that there is no logical answer. However, it is the hope of this investigator that this paradox will ultimately be explained as a series of positive and negative feedback loops. With a thorough understanding of the

feedback loops, lentiviruses and their hosts might somehow learn to live in greater harmony than currently exists.