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DISSERTATION

ANALYSIS OF PULMONARY T LYMPHOCYTES IN OVINE LENTIVIRUS INFECTION

Submitted by
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In partial fulfillment of the requirements
For the degree of Doctor of Philosophy
Colorado State University
Fort Collins, Colorado
Summer 1999

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Committee on Graduate Work

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ABSTRACT OF DISSERTATION

ANALYSIS OF PULMONARY T LYMPHOCYTES IN OVINE LENTIVIRUS INFECTION

Ovine lentivirus- (OvLV-) induced lymphoid interstitial pneumonia (LIP) is a model for a pulmonary disease associated with human immunodeficiency virus (HIV) infection. While the mechanisms of lentivirus-associated lymphoproliferative diseases are poorly understood, T cells are thought to play an important role in the initiation and perpetuation of the lesions. To further elucidate their involvement in LIP, T cells present in the lungs of lambs experimentally-infected with OvLV were characterized. Neonatal lambs were inoculated with OvLV and infection was confirmed through the detection of anti-viral antibodies, the isolation of virus, and histological assessment of pulmonary inflammation. Increased numbers of T cells were present in bronchoalveolar lavage (BAL) fluids of OvLV-infected lambs relative to BAL fluids of sham-infected, age-matched control lambs. Moreover, the normal ratio of CD4+ to CD8+T cells was inverted in the BAL fluid of infected animals, consistent with the selective expansion of virus-specific cytotoxic T lymphocytes (CTLs). To further examine the T cells involved in LIP, a semi-quantitative RT-PCR method for analyzing ovine T cell receptor β-chain variable region (TCRBV) gene expression was developed. The PCR method used TCRBV primers that were derived from an analysis of the ovine TCRBV gene repertoire in which 34 TCRBV gene rearrangements were isolated from an ovine spleen anchored PCR library. Eighteen unique TCRBV gene segments were represented among these cDNA clones, which were clustered into 13 families based on their similarity to human TCRBV genes. Semi-quantitative RT-PCR analyses of TCRBV gene expression revealed increases in the relative

expression of TCRBV 4S1 (P=0.05) and TCRBV 7S1 (P=0.01) by T cells in the lungs of OvLV-infected versus control animals. Analysis of TCRBV complimentary determining region 3 (CDR3) nucleotide sequences, suggests that TCRBV 4S1 T cells were oligoclonally expanded, while TCRBV 7S1 populations were polyclonally expanded. Additionally, RT-PCR analysis of cytokine gene expression by these lung T cells revealed a 35-fold increase in IFN-γ expression and a 3-fold increase in IL-2 expression in infected versus control lambs, while IL-10 and IL-4 were expressed at equivalent levels in both groups. Analysis of magnetically-selected lung T cell subsets revealed TCRBV 4S1 T cells to be primarily CD4+, while TCRBV 7S1 T cells were predominantly CD8+. Further analysis of T cell subsets correlated IFN-γ expression predominantly with CD8+ T cells. Collectively, these findings suggest that the heterogeneity of virus-specific, CD8+ T cells in the lungs of OvLV-infected lambs is limited, and that *in situ* proliferation of these T cells contributes to the pathogenesis of lentivirus-associated LIP.

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Chapter 1

Literature Review

Introduction

Lymphoid interstitial pneumonia (LIP) is a lymphoproliferative disease of the lung that has been associated with pediatric human immunodeficiency virus (HIV) infection. The pathogenesis of HIV-associated LIP is not well understood, but individuals with this condition have an increased number of leukocytes in the interstitium of the lung, the majority of which are alveolar macrophages and T lymphocytes. Preliminary examination of T lymphocyte subpopulations present in the lungs of individuals with HIV-associated LIP has revealed a preferential expansion of CD8+ T cells.

Ovine lentivirus, which is closely related to HIV, also induces a similar pulmonary condition in sheep similar to that induced by HIV. Like HIV-associated LIP, OvLV-associated LIP results in perferential expansion of CD8+ T cells in the lung and, therefore, provides an excellent animal model for the study of lentivirus-induced LIP. Because of the marked increase in T cell numbers in the lungs of individuals with lentivirus-associated LIP, in conjunction with the crucial role of T cells in immune responses, the present study focuses on the influence of pulmonary T cells in the initiation and perpetuation of this lymphoproliferative disease of the lung.

Lymphoid Interstitial Pneumonia

Lymphoid interstitial pneumonia is associated with lymphoproliferation in the interstitium and bronchus-associated lymphoid tissue (BALT) of the lung. LIP presents clinically with progressive dyspnea, cough, fever, chills, and weight loss (Koss *et al.*, 1987). Lymphadenopathy and hepatomegaly also may be present, and abnormalities of serum proteins and immunoglobulins are frequent (Fishback & Koss, 1996). Chest radiographs usually reveal bilateral reticulonodular or interstitial infiltrates (Rutkin *et al.*, 1998). Pulmonary function tests, such as spirometry, reveal reduced lung volumes and diffusing capacities, which provide a sensitive indicator of disease in LIP. Until recently, diffusing capacity was considered the single most sensitive functional index of disease progression (Fishback & Koss, 1996). LIP results in a chronic and sometimes debilitating condition that can be life-threatening if no action is taken to alleviate the problem.

Microscopic examination of lung interstitium from individuals with LIP reveals diffuse, prominent, interstitial infiltrates of lymphocytes, immunoblasts, plasma cells, fibroblasts and scattered macrophages; eosinophils and neutrophils are rare (Koss, 1995; Liebow & Carrington, 1973). Lymphoid infiltrates usually are centered on the airways, but they invariably infiltrate the interstitum focally or diffusely. Another compartment of the lung involved in LIP is the bronchus-associated lymphoid tissue (BALT) which consists of organized aggregates of lymphoid tissue within the bronchial walls. Although a few lymphocytes associated with LIP overflow into the alveolar lumens, the infiltrates generally remain interstitial or in the BALT (Harty *et al.*, 1995; Sharland *et al.*, 1997a).

Lymphoid interstitial pneumonia was first described in 1966 in association with certain autoimmune diseases in adults (Rutkin *et al.*, 1998; Marquis *et al.*, 1993). The strongest correlation between autoimmune disease and LIP was observed in individuals with Sjogren's syndrome, which is a chronic inflammatory autoimmune disorder characterized by the triad of keratoconjunctivitis sicca, connective tissue disorder and pulmonary lesions (Strimlan *et al.*, 1976). Since this initial association, the prevalence of LIP has been documented in 3 out of 343 (0.9%) Sjogren's patients (Strimlan *et al.*, 1976). Futhermore, 9-25% of patients with LIP have Sjogren's Syndrome (Koss *et al.*, 1987; Koss, 1995; Nicholson *et al.*, 1995). LIP also has been observed in conjunction with other autoimmune diseases such as myasthenia gravis, autoimmune hemolytic anemia, systemic lupus erythematosus and Crohn's disease (Morris *et al.*, 1987). An additional link between autoimmune disease and LIP is the presence (in many patients with LIP), of autoantibodies reactive with double stranded DNA and of rheumatoid factor (Fishback & Koss, 1996). The well-established association between LIP and autoimmune disease suggest that LIP may result from immune dysfunction, which allows for uncontrolled expansion of pulmonary leukocytes. The occurrence of malignant lymphoma in adult patients with LIP suggests that LIP may be the benign end of a spectrum of lymphoproliferative disorders up to, and including, malignant lymphoma.

While compelling arguments can be made that LIP is an autoimmune disorder (the pulmonary equivalent of Sjogren's syndrome), LIP has been associated also with certain viral diseases. One of the first viral agents associated with LIP was Epstein Barr Virus (EBV). *In situ* hybridization has revealed EBV DNA in the lungs of 8 out of 10 HIV positive children with LIP, but not in the lungs of control patients (Andiman *et al.*,

1985). EBV has been isolated from the lungs of a large number of individuals with primary or secondary diffuse interstitial pneumonia (DIP) which clinically is identical to LIP. One study examined, 54 patients with DIP for the presence of EBV DNA and found 30% to be positive, while a control group of 32 all tested negative.

In addition to these correlations with EBV, the development of LIP has been associated with other viral infections. Individuals with chronic active hepatitis are at increased risk for the development of LIP (Morris *et al.*, 1987). In animals, LIP-like lesions, consisting of a large number of cytotoxic T cells, are observed in sheep infected with ovine lentivirus. The human immunodeficiency virus (HIV), another member of the lentivirus genus, also has been associated with LIP. The link between LIP and the HIV-1 was first noted in 1983, and it has been documented subsequently in an increasing number of reports. HIV-2, first identified in 1986 in association with AIDS in Africa, also induces LIP-type lesions (Couderc *et al.*, 1991). In fact, the association between HIV-infection and LIP is so strong, particularly in children, that the presence of LIP in HIV-positive individuals under 13 years of age (if HIV positive) was incorporated into the Center for Disease Control (CDC) case definition of AIDS in 1985.

Human Immunodeficiency Virus

The discovery of what is now considered the AIDS pandemic was first noted in Los Angeles in 1981 when an outbreak of serious pneumonia was reported (Morbidity and Mortality Weekly report, 1981). Curiously, this pneumonia was caused by *Pneumocystis carinii* an opportunistic fungus that normally does not cause disease. This lead researchers to realize that the affected patients had a new type of immunodeficiency

disease. At this time, the disease was mainly limited to the homosexual population but, as the pandemic spread, other groups such as intravenous drug users and individuals that received blood transfusions began to develop the disease. Epidemiological examination revealed that AIDS was spread by intimate contact with infected individuals, suggesting that it was caused by an infectious agent. Initially the infectious agent was named human T cell leukemia virus (HTLV III) because of its similarity to HTLV I and II, but in 1983 two independent research groups isolated a new virus and named it the human immunodeficiency virus (HIV) (Barre-Sinoussi *et al*, 1983; Gallo *et al*., 1983). Once HIV has been contracted it will eventually cause the loss of immune competence through the inexorable decline and eventual depletion of CD4+ T cells.

HIV is able to infect cells which express the CD4 receptor on their surface as well as the coreceptors CXCR4 (found on CD4+ T cells) and CCR5 (found on macrophages). The HIV gp120 envelope protein binds the CD4 receptor, and the membranes of the virus and the cell fuse, a process governed by the viral gp41 envelope protein. The virus core and its contents then are brought inside the cell. Once inside the cell, the single stranded RNA, which is complexed with reverse transcriptase, is reverse transcribed into a complementary single strand of DNA. The single-stranded retroviral DNA is copied in the cell cytoplasm into double-stranded retroviral DNA which migrates to the cell nucleus and becomes integrated into the host cell DNA by way of another unique retroviral enzyme, integrase. Once integration occurs the viral DNA is referred to as provirus. Within the host cell nucleus, proviral DNA, when activated is transcribed to produce viral RNA. Some strands of viral RNA act as messenger RNA which are

translated to produce proteins essential for the production of HIV, while other viral RNA molecules are encapsulated into virus particles.

The human immunodeficiency virus is an enveloped particle of 90-120 nm that buds from the plasma membrane of the infected cell. The viral membrane encompasses two single stranded copies of viral RNA. HIV has nine known genes and is the most complex retrovirus studied thus far. The viral genome is flanked at each end by long terminal repeat (LTR) sequences containing three regions, U5, R, and U3. The LTRs control the expression of viral genes via complex processes requiring cellular RNA polymerase II as well as auxiliary transcription factors. The LTRs contain several regulatory elements: the TATA box, which is involved in core promoter functions; upstream enhancer regions, to which cellular transcription factors, such as Sp1 and NF-κB bind to activate HIV transcription: a negative regulatory element located at the 5' end of the U3; and a trans-acting responsive sequence (TAR).

Between the LTRs, lie the structural genes gag, pol and env, as well as several regulatory regions which regulate transcription. The gag genes, or group specific antigens, encode the viral nucleocapsid, while the polymerase (pol) gene encodes protease, reverse transcriptase and integrase polypeptides. The envelope (env) gene encodes glycosylated proteins (gp 120 and gp 41) that make up the viral envelope. One of the first viral genes to be transcribed is *tat*, which encodes a regulatory protein that accelerates transcription of the HIV provirus. Another regulatory gene expressed during the early phase is called *nef*. Initially, it was thought the role of *nef* was to suppress transcription, but more recently *nef* has been shown to modify the cell to make it more amenable to the production of HIV virions. A third regulatory protein called *rev*, is a

regulator of structural gene expression. Three other ill-defined gene products are produced: *vif*, which enhances the infectivity of cell-free virus; *vpr*, a weak transcriptional activator, and; *vpu*, which is necessary for efficient virion budding.

The course of HIV infection has been divided into 4 distinct phases (Figure 1.1A). The first phase is characterized by flu-like symptoms sometimes referred to as seroconversion disease. It takes place 6-18 weeks after infection with HIV. During this period, there is a decline in CD4 + T cells with a concurrent increase in infectious virus in the plasma (Figure 1.1B). Predictably, a normal adaptive immune response to the virus ensues, as noted by the development of antibodies to HIV surface and core proteins (ie., env and p24 proteins) respectively. Additionally, HIV-specific cytotoxic T cells are expanded. The immune response seems to control the acute illness by lowering the number of virions in the blood and, thus, CD4+ T cell numbers return to normal.

This immune response, however, does not eradicate the virus. This marks the beginning of the second stage of the disease known as the asymptomatic phase. This phase lasts for 2-12 years, and it is marked by a slow and insidious decline in the number of CD4+ T cells, although the immune responses (e.g., antibodies and cytotoxic T cells reactive with the virus) remain at high levels. This is referred to as the asymptomatic phase because the virus is hard to detect in the peripheral blood, and patients are relatively free of symptoms. While virus levels in peripheral blood remain low and in some cases are undetectable during this period, viral RNA, in the form of neutralized virions, is detectable in plasma, and large numbers of virions are present on the surface of follicular dendritic cells in germinal centers and in lymphoid follicles. The follicular

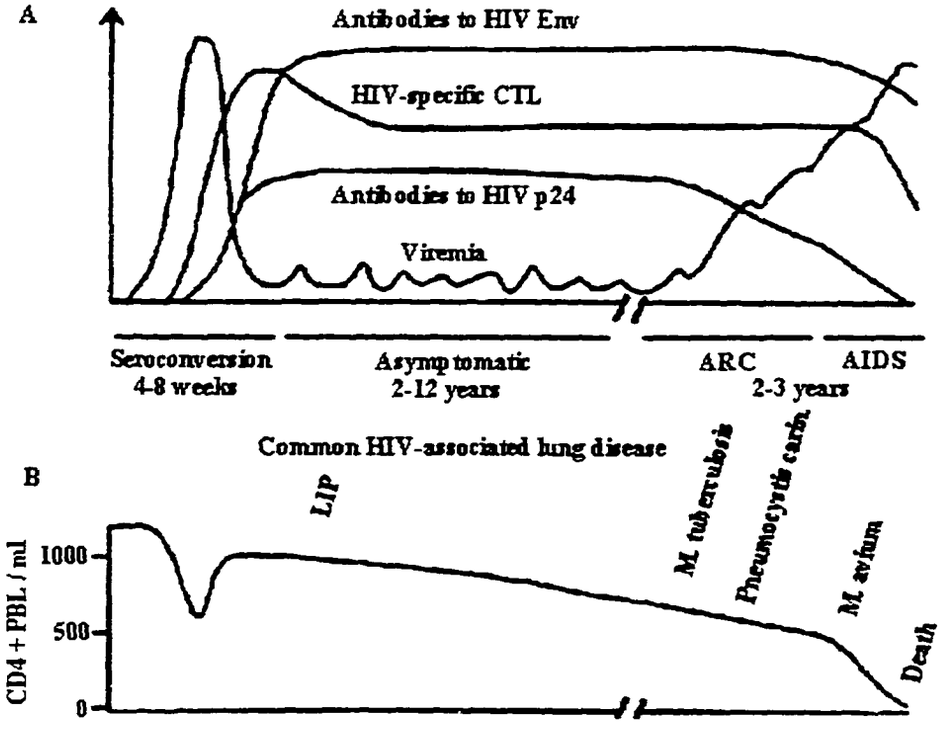

Figure 1.1. Schematic course of A) HIV infection B) HIV-associated lung disease. Adapted from HOW DOES HIV CAUSE AIDS? Science vol. 260, page 5113

dentritic cell has been implicated as an important reservoir for HIV during this phase (Burton *et al.*, 1997). Follicular dendritic cells have surface Fc and complement receptors to which virus, opsonized by antibodies or complement components, binds and persists for long periods of time. It is still not clear if virus associated with dendritic cells is infectious, but dendritic cells are found in germinal centers containing CD4+ T cells; it has been postulated that these may be the foci of infection even when the peripheral blood is negative for virus. Following the asymptomatic phase, is the third stage of disease, during which AIDS related complex (ARC) develops. This stage is characterized by an increase in virus particles in the blood and a decline in HIV-specific immune responses. At this point, opportunistic infections start to appear because CD4+ T cell numbers have dropped to a point where the individual is unable to mount an effective immune response. When CD4+ T cell numbers decline to fewer than 200/mm^3, patients are said to have progressed to the final stage of the disease known as Acquired Immune Deficiency Syndrome (AIDS). At this stage of the disease, virus production is increased and the immune system is severely impaired as a result of the loss of T helper function. Pulmonary opportunistic infections by *Mycobacterium* or *Pneumocystis carinii* commonly develop (Figure 1.1B). Opportunistic infections are the final cause of death for most individuals who progress to full-blown AIDS.

Today AIDS is not just a disease of homosexuals or intravenous drug users; it has become a worldwide problem for heterosexual couples and their children. Globally, pediatric HIV infection is a significant factor in child health. Since the pandemic began over 20 years ago, over 1.5 million children have become infected by HIV as estimated by the World Health Organization (Sharland *et al.*, 1997b). Worldwide, more than 1000

HIV-positive babies are born each day. Rates of vertical transmission by HIV-positive mothers vary worldwide, with reports ranging from 15-20% in Europe to 25-35% in Africa (European Collaborative Study, 1991; St. Louis *et al.*, 1993). An increased rate of transmission is seen in premature infants, the first-born of twins, and in women with more advanced HIV disease, marked by low CD4 T cell counts and high plasma HIV RNA concentrations (Sperling *et al.*, 1996; Duliege *et al.*, 1995). Higher rates of vertical HIV transmission have been associated with vaginal birth when compared to caesarian section delivery. Breast-feeding also has been implicated in HIV transmission; the additional risk has been estimated to be 7-22% (Dunn *et al.*, 1992). For infants and young children, AIDS is already among the 10 leading causes of death, surpassing all other infections, and it is likely to be among the five leading causes of death within the next several years. With the increase of pediatric HIV infection in recent years, there has been an increase also in the number of cases of HIV-associated LIP.

HIV-associated LIP

The incidence of LIP in children has risen in recent years because of the HIV pandemic. As stated above, the prevalence of HIV-associated LIP in children was high enough that in 1985 its presence in children under the age of 13 became part of the case definition of pediatric AIDS. A recent revision of the definition of AIDS lists LIP as a class P-2 (symptomatic infection), subclass C (LIP) disease criterion (Center for Disease Control, 1985). In HIV positive children, LIP develops in about 40% of the cases, while it is only seen in about 4% of adults (Joshi *et al.*, 1985; Joshi *et al.*, 1990; Scott, 1991). The course of LIP in children is highly variable; spontaneous resolution, episodic

worsening, and progression to frank respiratory failure are all possible outcomes (Andiman *et al.*, 1985; Rubinstein *et al.*, 1986). In one study, 95 children with vertically acquired HIV infection who were treated at three London hospitals, were evaluated to assess the respiratory morbidity caused by LIP. In this study, a diagnosis of LIP was made in 33% of the cases; these patients slowly developed progressive hypoxia with eventual respiratory failure, resulting in a mean survival time of approximately 33 months (Sharland *et al.*, 1997a). Intravenous immunoglobulin and corticosteroids have been used to treat LIP with variable success, however, administration of immune suppressive drugs to children with an immunosuppressive disease is not a desirable treatment regimen (Rubinstein *et al.*, 1988).

LIP also occurs in adults infected by HIV, but it is far less common than in children. Most adults with interstitial lung disease have nonspecific interstitial pneumonitis, rather than LIP (Schneider, 1996). The term non-specific interstitial pueumonitis was proposed for episodes of respiratory dysfunction that occur in HIV-infected patients in the absence of an opportunistic infection or neoplasm. LIP in adults usually occurs later than in children, and it is most commonly seen in individuals with AIDS-related complex, generalized lymphadenopathy, and polyclonal hypergammmaglobulinemia. As in children, there may be lymphocytosis of up to 38% in BAL, fluid with CD8+ T cells comprising 80-90% of the lymphocyte population (Wallace *et al.*, 1984). Anti-retroviral drugs such as chlorambucil and zidovudine have been used to treat LIP in adults, though the success of these therapies generally is poor.

The prognostic significance of HIV-associated LIP is somewhat confusing. Some studies have shown that patients with HIV-associated LIP have relatively good outcomes

and do not seem to suffer long-lasting or debilitating effects from the pneumonia. In addition, some researchers have found that the prevalence of opportunistic infections is decreased in this subpopulation and survival time is longer on average, suggesting that LIP may reflect an effective antiviral immune response (Itesuc *et al.*, 1993). Others, however, have proposed just the opposite; that HIV-associated LIP is actually quite detrimental and that it may predispose the individual to other opportunistic pathogens (Sharland *et al.*, 1997a; Agostini *et al.*, 1991).

The ability of BAL to predict survival of patients with HIV infection also has been examined. In one study, 115 HIV-positive individuals were evaluated by BAL. Forty two of those 115 patients did not have opportunistic infections (OI), and 11 of those 42 died (Agostini *et al.*, 1991). Analysis of BAL cells from the 11 patients who had a fatal outcome, revealed an increase in CD3+, CD8+ lymphocytes compared to the survivors. CD8+ T cell mediated lymphocytic alveolitis, a major component of LIP, therefore has been associated with a significant increase in mortality. Using univariate analysis, Augostini *et al.* (1997) associated a number of factors with more rapid mortality among individuals older than age 30. These include HIV disease status, risk category for HIV infection, number of pathogens isolated, BAL neutrophilia, and low numbers of pulmonary CD4+ T cells. The presence of alveolitis, or of increased numbers of alveolar macrophages and CD3+ BAL T cells was found to suggest a better prognosis for the patient, again correlating increased numbers of leukocytes in the lung (definition of LIP) with increased survival.

The ability of BAL cells to reflect the immunological mechanisms of interstitial disease has been demonstrated by a number of investigators. BAL has been used

routinely to investigate the immunocompetence of cells present in the lung interstitium. Most of the available information concerning pulmonary immune responses to HIV has come from the analysis of cells obtained by BAL. Analysis of the cells in BAL fluid from individuals with LIP has been important in the investigation of the pathogenesis of the disease. T cell clones derived from BAL have been shown to have cytokine profiles consistent with those derived from lung interstitium (Garlepp *et al.*, 1992). Phenotypic examination of BAL and interstitial cells from patients with LIP has revealed concordance in the CD4/CD8 T cell ratios of these two compartments (Tada *et al.*, 1992). This suggests that BAL T cell populations reflect those in the interstitium, indicating that the analysis of BAL cells may be valid for studying interstitial diseases such as LIP.

Ovine Lentivirus and OvLV-associated LIP

Lentiviruses of sheep are the etiologic agents of chronic wasting diseases, which can involve one or more organ systems including the lungs, mammary gland, and central nervous system. These conditions usually are marked by a prolonged incubation period and an insidious onset, which slowly progresses to cachexia (DeMartini *et al.*, 1993; Haase, 1986; Narayan *et al.*, 1993). The ovine lentivirus OvLV, as it is now known, was first isolated by Sigordson in 1960. OvLV has been recognized as a medical problem in sheep herds in North America, South America, Africa and Europe since the early 1900s (Adams *et al.*, 1984; Madewell *et al.*, 1987; Palsson, 1976) (Figure 1.2). The recent discovery that the causative agent of AIDS (HIV) is a lentivirus has increased the interest in OvLV, due to its potential use as a model to better understand various aspects of HIV disease.

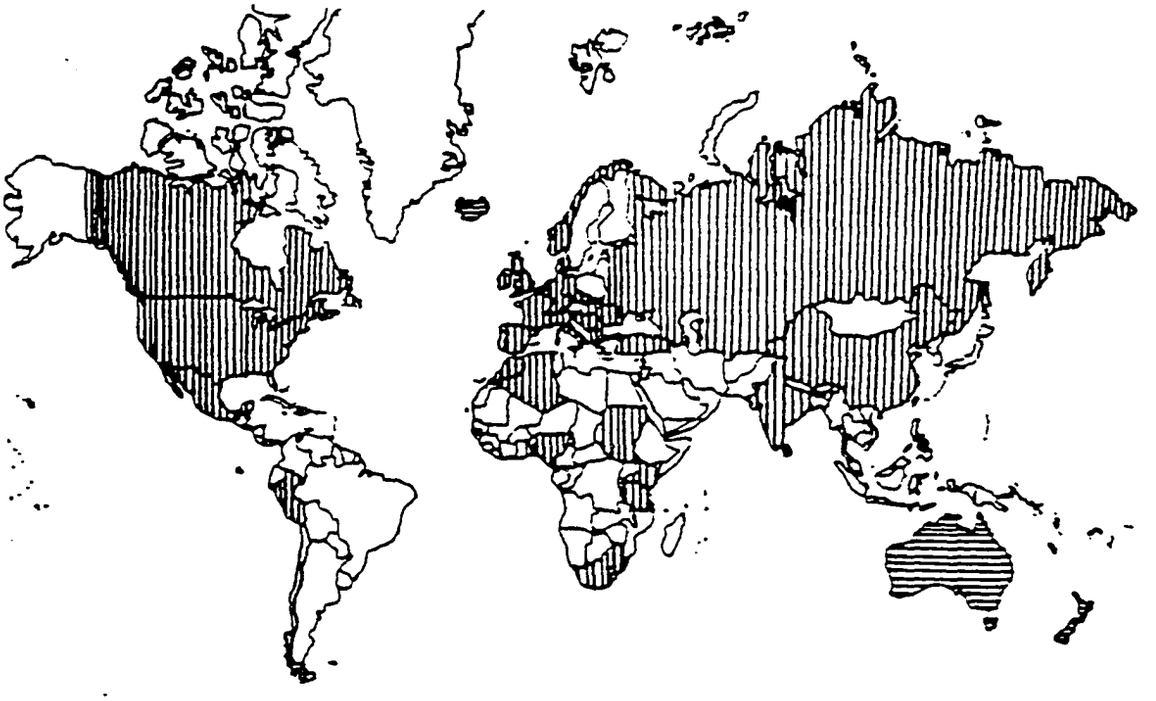

Figure 1.2. Geographic distribution of OvLV. Vertical bars denote areas where OvLV infection is present, horizontal bars denote areas where OvLV infection is not present, and areas with no bars indicate regions where OvLV infection status is unknown. Figure from Ph.D. dissertation of Scott Brodie, Department of Pathology Colorado State University

The genome of OvLV is quite similar to that of HIV. It contains *gag*, *pol* and *env* genes which have several short, multiply spliced open reading frames (ORFs) that encode proteins which have regulatory functions. All of these genes are flanked by classical lentivirus LTRs that contain U3, R and U5 regions. It has been shown that the protein encoded by the *rev* gene of maedi visna virus (MVV), a prototypic OvLV, affects viral gene expression *in vitro*. (Tiley *et al.*, 1990; Tiley *et al.*, 1991; Tiley & Cullen, 1992). and it has been established that a functional *rev* gene is essential for the establishment of a productive infection (Toohey & Haase, 1994).

The *tat* gene of OvLV encodes a 10-11 kDa protein (Davis & Clements, 1989; Gourdou *et al.*, 1989) which is a positive transactivator of viral transcription. The *tat* gene product has been shown to increase steady-state levels of MVV, LTR-directed mRNAs. The *vif* gene of MVV encodes a 29 kDa protein which is detectable in the cytosol late in the lytic cycle *in vitro* (Audoly *et al.*, 1992). Its function in MVV remains unclear, but it is hypothesized to be similar to that of HIV *vif* protein.

Interstitial pneumonia is the most commonly observed sequella of OvLV infected animals. Pulmonary inflammation induced by the virus is characterized by a progressive mononuclear cell infiltrate and lymphoid proliferation (Begara *et al.*, 1996; Lairmore *et al.*, 1988b). Clinical signs such as labored breathing and progressive weight loss are generally observed in animals two years and older (Cutlip *et al.*, 1988; De Boer *et al.*, 1979; Gates, 1981). Following the onset of clinical signs, the mean survival time is three to eight months. As with HIV infection, secondary pulmonary infection is quite common (Brodie *et al.*, 1992).

OvLV-induced chronic progressive pneumonia or LIP is denoted by a substantial increase in the size and weight of the lungs, often 2-3 times heavier than age matched uninfected lungs (Cutlip *et al.*, 1979). Because of the massive influx of immune cells and fluid, the lungs do not function properly, resulting in less oxygen exchange. The lungs of OvLV infected sheep have diffusely scattered gray foci (1-2mm) which microscopically, are revealed to contain large numbers of lymphocytes and macrophages within the alveolar septae peribronchiolarly (DeMartini *et al.*, 1993). Cuffing of pulmonary arterioles and airways is commonly seen, as is the development of lymphoid follicles, which may include germinal centers. OvLV-induced pulmonary disease resembles LIP associated with HIV infection both clinically and histologically.

Characterization of BAL cells either from naturally infected or experimentally infected sheep has revealed further similarities to the LIP associated with HIV-infection. The absolute numbers of both macrophages and lymphocytes are increased in the BAL fluid of animals infected with OvLV and presenting LIP. Typically, there is a decrease in the percentages of CD4+, CD5+ and gamma delta T cells, while there is an increase in the percentage of CD8+ T cells. Thus, an inversion of the normal CD4/CD8 T cell ratio is observed (Begara *et al.*, 1996; Lujan *et al.*, 1995). This suggests that CD8+ lymphocytes are important in the pathogenesis of lesion development in the lung and specifically in the genesis of lymphocytic alveolitis associated with OvLV infection.

One of the hallmarks of human lentivirus infection is immunosuppression which results from the decline in CD4 helper T cells. Cachexic sheep infected with OvLV also have decreased numbers of circulating CD4 lymphocytes, but not to the extent observed in HIV-infected individuals (Gorrell *et al.*, 1992). Despite this, infected, and even

cachexic sheep, do not develop opportunistic infections to the degree that HIV-positive individuals who have progressed to AIDS do. The profound immunosuppression associated with HIV infection results from the virus's ability to infect CD4+ T cells (Levy, 1993). OvLV however, does not infect T cells and therefore does not cause profound immunosuppression (Brodie *et al.*, 1995). Phytohemagglutinin (PHA) stimulation of peripheral blood mononuclear cells (PBMCs) obtained from OvLV-infected sheep produces 20-fold less virus than that obtained through the induction of macrophage differentiation, indicating that the primary target of infection is macrophages, not T cells. Further, co-cultivation of infectious PBMCs with fresh, uninfected PHA blasts does not facilitate virus replication (Gorrell *et al.*, 1992). It has been demonstrated also that efferent lymph cells, the best *in-vivo* source of purified lymphocytes, lacked virus and did not yield virus upon *in vitro* cultivation. Conversely, cultivated blood-derived macrophages were highly permissive for viral replication. Gorrell *et al.* (1992) revealed that the richest source of infected cells in fresh PBMC were CD45RA+, major histocompatibility complex class II +, nonadherent cells, a phenotype that is characteristic of dendritic cells. Thus, dendritic cells, not monocytes or CD4+ T cells are likely the predominant infected cell type in the blood of OvLV-infected animals. Dendritic cells have been shown to be important also in HIV infection by the virtue of their ability to harbor virus over long periods of time, and it has been hypothesized that they may be a crucial reservoir for virus when drug therapy has reduced viral burdens in blood (Burton *et al.*, 1997; Zoeteweij & Blauvelt, 1998).

In the lungs of OvLV-infected sheep (Brodie *et al.*, 1995) and HIV-infected humans (Salahuddin *et al.*, 1986), alveolar macrophages are the primary target cell for

virus. Alveolar macrophages have been shown to be more susceptible to infection by HIV than freshly isolated monocytes (Rich *et al.*, 1992). The importance of cell tropism in lentivirus disease is illustrated by simian immunodeficiency virus (SIV) infection of macaques. Progeny virus from cells transfected with a proviral SIV DNA clone replicates in T cells but not in macrophages. Animals infected with this virus become CD4 + T cell depleted, and develop opportunistic infection, but do not display the SIV-induced pneumonia characteristic of natural infection. Serial passage of bone marrow cells, infected with the lymphotropic SIV clone, through uninfected macaques produced a viral isolate able to infect macrophages in culture. Macaques infected with this macrophage tropic isolate were shown to develop SIV pneumonia (Sharma *et al.*, 1992).

The susceptibility of cells to virus infection is determined in part by the virus receptors and the ligands with which they interact. As previously mentioned the CD4 molecule has been shown to function as a receptor for the HIV. A putative cellular receptor for visna virus was identified by Dalziel *et al.* (1991). Using a virus overlay protein blot assay, they identified a group of polypeptides of apparent M_r 30K to 33K which interacted with visna virus and which is present on permissive but not non-permisive cells. Furthermore, they demonstrated that the interaction of visna virus with target cells could be blocked with a rat polyconal anti-ovine major histocompatibility complex class II anti serum. Futher, preincubation of visna with purified, soluble MHC class II molecules resulted in a marked decrease in virus-induced syncytum formation. Expression of MHC class II on ovine cells, however, is not in itself sufficient to render these cells susceptible to infection. For example, B lymphocytes and activated T lymphocytes express MHC class II molecules, but they do not appear to be infected by

the virus. This suggests that ovine MHC class II antigen is a necessary, but not a sufficient component of a cellular receptor for visna virus. Antibodies reactive with an unidentified 50 kDa protein of sheep choroid plexus cells block binding of MVV to these cells in culture (Crane *et al.*, 1991) suggesting that this molecule may represent another component of the cellular receptor for OvLV.

Although the immune response to OvLV is ineffective in eliminating viral infection, both humoral and cell-mediated immune responses do occur in infected sheep (Reyburn *et al.*, 1992; Kajikawa *et al.*, 1990). Studies have shown that precipitating antibodies appear in the serum of experimentally infected animals between 2 and 4 weeks post-inoculation (Begara *et al.*, 1996). The appearance of antibodies against the four major structural proteins of OvLV, p25, p16, p14 and gp 105, begin to appear in about 3 weeks, depending on the strain of virus. Sheep infected with OvLV, usually develop high titers of neutralizing antibodies within 4 to 8 months, but they are not effective in neutralizing new antigenic variants of virus which inevitably form *in-vivo* (de la Concha-Bermejillo *et al.*, 1995).

The dynamics of cell-associated viremia and antibody responses during the early stages of OvLV infection have been examined (Juste *et al.*, 1998). This study demonstrated that cell associated-viremia peaked between 2 and 6 weeks after inoculation. In OvLV-infected lambs, mean anti-p25 titers peaked 5 weeks after inoculation, then slowly declined. Antibody titers against p25, an internal OvLV structural protein, were not correlated with degree of cell-associated viremia, suggesting that these antibodies have limited antiviral activity. In contrast, a negative correlation between cell-associated viremia and antibodies against transmembrane protein (TM),

which peaked between 7-16 weeks after inoculation. was observed. A recent report indicates that TM protein of OvLV carries a neutralization epitope (Kwang *et al.*, 1995): thus the strong negative correlation between cell-associated viremia and mean anti-TM titer suggests that these antibodies may limit the spread of the virus.

A number of studies have examined cell-mediated immune (CMI) responses to OvLV. Virus specific lymphocyte proliferation has been demonstrated in response to both purified virions and to recombinant p25, the major core protein of maedi-visna virus (MVV) (Reyburn *et al.*, 1992). By depleting T cell subsets from purified PBMCs of infected sheep, Reyburn et al (1992) demonstrated that for most animals CD4+ T cells are the major lymphocyte subset responding to whole viral antigen or p25 core protein. In some infected animals, however, proliferation to visna antigen could be completely abrogated only by depletion of both T cell subsets, and in two of the animals studied CD8+ lymphocytes appeared to contribute to this *in vitro* proliferation.

Canulation of lymphatic ducts of OvLV-infected sheep has been used to monitor viral load as well as the immune response in efferent lymph after infection (Bird *et al.*, 1993). This work demonstrates that lentivirus infected cells begin to leave the lymph node around 9 to 18 days post-infection. The maximum frequency of virus infected cells leaving the nodes, however, was quite low, with only 0.001% of the total efferent cells harboring virus. As mentioned previously, there is a large number of circulating CD8+ T cells, yet the direct lentivirus-specific cytotoxic activity is quite low; only 20% of the animals tested demonstrated any virus specific cytotoxic activity.

Blacklaws *et al.* (1995) assessed CMI in lymph nodes of OvLV-infected animals and demonstrated that virus was detected in nodes by co-cultivation beginning 4 days

post-inoculation (PI), with a maximum seen between 7 and 14 days. Even at 14 days PI, the number of infected cells was quite low with the maximum frequency of infected cells being only 23 $TCID_{50}$ per 10^6 total lymph node cells. In these studies T cell proliferative responses to MVV were first detected on day 7, and they were consistently detected through day 18. Virus-specific CD8+, cytotoxic T cell precursors (CTLp) were isolated from infected nodes at 10 days PI. Unlike the lung, phenotypic studies of isolated lymph node cells demonstrated no major changes in the proportions of macrophages, CD1+ interdigitating cells, CD4+ T cells or CD8+ T cells. The persistent rise in CD8+ T cells in the lungs, as opposed the lymph node, occurs because the lung is a site of active virus replication. While CD8+ T cells are expanded in the lymph node subsequent to the impart of viral antigen, it appears that OvLV-specific CD8+ T cells rapidly leave the node.

The pulmonary immune response to MMV in infected sheep is characterized by a decrease in the percentage of CD4+, CD5+, and γδ+ T cells, with a concurrent increase in the percentage of CD8+ T cells, resulting in an inversion of the normal CD4+/CD8+ ratio (Begara *et al.*, 1996; Lujan *et al.*, 1995; Watt *et al.*, 1992b). A correlation between increases in CD8+ T cells and severity of pulmonary lesions was demonstrated by Lujan *et al.* (1995). Furthermore, cultures of pulmonary leukocytes from lambs with severe lung lesions produced significant amounts of IFN (Lairmore *et al.*, 1988a). This suggests that the release of IFN from pulmonary leukocytes, possibly CD8+ leukocytes, may serve to potentate inflammation with in the lung.

OvLV Model of LIP

Due to the many similarities between HIV and OvLV, and due to the clinical similarities between the pulmonary diseases induced by both viruses, the OvLV model can be used to study pediatric, HIV-associated LIP. HIV and OvLV both have similar genetic, morphologic and replicative strategies (Gonda *et al.*, 1985; Gonda, 1990; Davis *et al.*, 1987). The rapid induction and broad range of disease are advantages of the OvLV model. HIV-induced LIP usually takes from 5 to 81 months, with an average of 13 months, to develop (Schneider, 1996). Experimentally induced OvLV-associated LIP in neonatal lambs, however, usually develops in 2-4 weeks, thereby significantly reducing the duration of experimental assessment (Begara *et al.*, 1996). Animals experimentally infected with OvLV also exhibit a broad range of disease. Approximately, 1/3 develop severe LIP, 1/3 develop mild disease, while 1/3 show no clinical signs of disease (Watt *et al.*, 1992a). This allows the correlation of disease severity with phenotypic and functional data derived from the study of lung leukocytes.

OvLV does not infect CD4+ T cells, which allows LIP to be studied without the confounding effects of T cell depletion and immunosuppression. In children and adults, HIV- associated LIP develops during the asymptomatic phase of the disease, well before the profound loss of CD4+ T cells and the resulting immunosuppression. HIV can be separated into two variants, M-tropic and T-tropic forms. The former infects predominantly macrophage/monocytes, while the latter infects T cells (Alkhatib *et al.*, 1996; Berson *et al.*, 1996). Recently it has been demonstrated that the M-tropic variant is more readily transmitted than the T- tropic form, and that the M-tropic form predominates throughout much of the asymptomatic phase of HIV infection (Narayan *et*

al., 1988; Mosier & Sieburg, 1994). Since LIP occurs during the early stages of HIV infection, most of the virus in the lung is expected to be M-tropic infecting primarily alveolar macrophages as observed in OvLV-induced LIP.

Lastly, the OvLV model offers additional advantages over other animal models. Sheep are relatively inexpensive and easy to obtain, their temperament allows for easy handling, and their size is sufficient to permit the collection of large numbers of cells from the lungs. The size of the lamb also allows for *in-vivo* lavages to be performed, thus, enabling one animal to be repeatedly tested over time. There is no evidence that OvLV is able to infect humans; thus the virus poses no threat to researchers working with it.

T cells and their role in specific immunity

T cells appear to play an integral role in the initiation and perpetuation of LIP. A more complete understanding of the T cell response to OvLV will permit the elucidation of the mechanisms of LIP. Historically, the T cell receptor (TCR), a unique fingerprint for individual T cells, has been used to characterize T cell responses. This approach can provide useful information on the T cell response by determining if T cells are expanded clonally or oligoclonally, indicative of a conventional response to antigen (Ag). Alternatively, polyclonal expansion is consistent with superantigen (sAg) or with the non-specific recruitment of T cells by cytokines.

The TCR was first identified in the laboratory of J. P. Allison in 1983 (McIntyre & Allison, 1983). Identification and isolation of the T cell receptor was accomplished by the production of a library of monoclonal antibodies reactive with various T cell clones

and then screening the monoclonals to identify those that were clonotypic. These clonotypic antibodies reacted only with the immunizing T cell clone, suggesting that they might detect a unique receptor on the T cell surface. Clonotype specific antibodies then were used to show that each T cell expresses about 3000 T cell receptor molecules on its surface. The TCR later was found to be a heterodimer consisting of two unique polypeptide chains, now known as the α and β-chains (Babbit *et al.*, 1985; Davis, 1990). Purified TCRs from various T cell clones were combined with different antisera. Some antisera bound the heterodimer from all clones, while others only bound one. From these reactivities, it was deduced that the TCR α and β-chains have constant and variable regions like those of immunoglobulins.

The TCR is classified as a member of the immunoglobulin superfamily because of the similar domain structure as determined by amino acid sequence analysis of the TCR α and β heterodimer and immunoglobulins. The predicted amino acid sequences of T cell receptor cDNAs demonstrated that both chains have an amino terminal variable domain with homology to immunoglobulin V regions, a constant region with homology to immunoglobulin C regions, and a short hinge domain with a cysteine residue which forms the inter-chain disulfide bond (Yanagi *et al.*, 1984; Hedrick *et al.*, 1984; Chien *et al.*, 1984). Both polypeptide chains of the TCR span the cell's lipid bilayer with a hydrophobic transmembrane domain and each possesses a short cytoplasmic tail.

The V regions of both chains contain a variable (V), joining (J), and constant (C) region. In addition, the β-chain also contains a diversity (D) region. The V, J, C, and D regions are germline encoded. In the mouse, the α and β loci are located on chromosomes 14 and 6 respectively. In humans, these loci are located on chromosome

14 and 7 respectively. Presently, the position of these genes in the ovine system is unknown.

Human and mouse T cell receptor genes have been well characterized, and most of what is known today about the TCR comes for the study of these two species. Mouse germline DNA is estimated to contain about 100 Vα segments, 50 Jα segments, and one Cα segment. The β-chain loci contains 20-30 V segments and two repeats of Dβ, Jβ and Cβ segments, each repeat consisting of one Dβ, six Jβ and one Cβ genes. The germline DNA of humans contains 50 Vα genes, 70 Jα genes, and one constant region gene. The β-chain loci contains 57 Vβ genes, 13 Jβ genes, 2 Dβ genes and 2 constant region segments (Moss *et al.*, 1992). All other mammals studied to date seem to have TCR gene organization similar to that of mice and humans, although, the number of segments differs. Preliminary studies indicate that ovine (Grossberger *et al.*, 1993a) similar bovine (Tanaka *et al.*, 1990) TCRs are also composed of genes similar to those found in human and mice.

Diversity, which characterizes the T cell response, is generated by rearrangement of TCR gene segments during T cell maturation. The mechanism by which TCR germ-line DNA is rearranged into functional receptor genes is similar to the mechanisms used to rearrange immunoglobulin (Ig) genes (Yanagi *et al.*, 1984). The pre-T cell, like the pre-B cell, expresses genes encoding recombination-activating genes (RAG-1 and RAG-2). These recombinase enzymes are known to catalyze the V-J and V-D-J joining during TCR-gene rearrangement by the same deletional or inversional mechanisms that occur in the Ig genes. Additionally, the TCR genes exhibit allelic exclusion just as Ig genes. β-chain loci are present on each of two chromosomes, and the productive rearrangement of

one of those chromosomes inhibits the rearrangement of the other β-chain loci. If a nonproductive rearrangement occurs on the first chromosome, the thymocyte can undergo a second rearrangement, thus increasing the chance that a productive rearrangement will be made.

Unlike B cells, T cells do not undergo somatic mutation to create further diversity. Additionally, TCR germ-line DNA contains far fewer V genes than Ig germ-line DNA. The TCR, however, is able to generate far greater diversity than Igs through other mechanisms unique to the T cell (Chien *et al.*, 1984a). The greater diversity generated in the TCR is due in part to the increased number of J regions and in the α and β-chain loci. Also alternative joining of D gene segments in the β-chain, thus, it is possible for a Vβ gene segment to join directly with a Jβ or Dβ gene segment, generating a (VJ)β or (VDJ)β rearrangement.

In both Ig and TCR genes, nucleotides may be added or subtracted at the junctions between individual segments during rearrangement. Variation in endonuclease cleavage of the palindromic hairpin may leave additional nucleotides that are rearranged TCR genes (Lafille *et al.*, 1989b). Such P region nucleotide addition can occur in the genes encoding all the TCR and Ig genes. Additionally, there can be N region nucleotide addition catalyzed by a terminal deoxy nucleotidyl transferase (Wilson *et al.*, 1988a). This occurs in both of the TCR chains, but it only is seen in the heavy chain of the Ig. These additions produce the greatest diversity at the VDJ or VJ junctions, also known as the complimentary determining region (CDR) 3 regions.

Unlike Igs, which can directly bind antigen, the TCR recognizes processed antigen in the context of a MHC molecule. This is known as MHC restriction.

Biochemical studies by Buss *et al.* (1987) demonstrated that MHC molecules present fragments of processed antigens, and recent x-ray crystallographic studies suggest that the MHC molecules bind antigens in a cleft formed on its external surface (Bjorkman *et al.*, 1987b; Bjorkman *et al.*, 1987a). The CDRs1 and CDRs2 of the TCR are the most hypervariable regions of the germline encoded V-regions and they have been implicated in binding of MHC. The CDR3 regions are not germline encoded, they have the greatest amount of diversity, and are purported to be involved in binding of processed antigen. Therefore the induction of a T cell response requires a complex of TCR, peptide antigen and MHC molecule.

The major histocompatibility complex genes are located in a region composed of clustered genes, which regulate the immune response. The major histocompatibility complex gene products are associated with antigen presentation, intercellular recognition, and discrimination of self/non-self. There are two forms of major histocompatibility complex molecules, MHC class I and II (Chien & Davis, 1993). MHC class I molecules are expressed on the surface of all nucleated cells. MHC class I molecules are composed of a α-chain associated non-covalently with a much smaller β2-microglobulin molecule. The α-chain is organized into three external domains (α1, α2 and α3). The α1 and α2 domains interact to form a platform of eight antiparallel β strands that are bounded by two long α-helical regions. This structure forms a groove in the class I molecule which is referred to as the peptide-binding cleft. The cleft is closed at both ends and is large enough to bind a peptide of 8-10 amino acids in length. MHC class I molecules present endogenous antigens, such as those derived from intracellular pathogens and tumors antigens (Ags) to T cells that bear the CD8 co-receptor.

The glycoproteins encoded by MHC class II genes are expressed primarily on antigen presenting cells (macrophages, dendritic cells and B cells). The MHC class II molecules contain two different polypeptide chains, an α-chain and a β-chain associated by noncovalent interactions. Each chain contains two external domains. The membrane distal domain consists of the α1 and β1 domains, which form the antigen binding cleft. Unlike the MHC class I molecule the class II molecule's binding cleft is open at both ends and, therefore, is able to accommodate a larger peptide, usually 13-18 amino acids in length. MHC class II molecules present exogenous antigen to T helper cells bearing a CD4 co-receptor, thus, initiating a T helper response (Sakimama *et al.*, 1995).

The sheep MHC genes, known as the MHCOvar, have not been well characterized. The structure of the MHC class II genes have been shown to be highly conserved during evolution, thus, it is believed that the sheep MHC is similar to that of other mammals. The MHCOvar system is located on chromosome 20 and it has a similar genetic organization to that of man, with the exception of the DP subregion which does not exist in sheep (Hediger *et al.*, 1991). Southern blot analysis of ovine class II genes using probes derived from various HLA genes have detected the presence of several class II-like genes, however, further analysis is needed to provide more comprehensive information on the nature of the MHCOvar system (Chardon *et al.*, 1985).

Recognition of antigen by T cells results in the induction of a variety of effector mechanisms associated with both cell-mediated and humoral responses. The two main types of T cells, CD4 and CD8 T cells, have very different effector functions. The CD4+ T helper cells exert most of their function though the cytokines they secrete. The CD4+ T cell subpopulations can be distinguished by the cytokines they secrete (Powrie &

Coffman, 1993). The Th1 cell is defined by the secretion of IL-2, IFN-γ, GM-CSF and TNF-β. These cytokines promote the development of a classical cell-mediated immune responses; inflammation, delayed type hypersensitivity and activation of cytotoxic T cells. Th2 cells secrete IL-4, IL-5, IL-6, and IL-10. These cytokines encourage humoral immunity and, therefore, function primarily as helpers for B cell activation.

CD8+ T cells also have two potential effector populations. A CD8+ T cell can develop either into a cytotoxic T cell or a non-cytotoxic T cell. Cytotoxic T cells, or CTLs, have lytic capability and play a critical role in the recognition and elimination of altered self-cells, such as virus-infected or tumor cells. CTLs can mediate destruction of target cells by two different mechanisms (Atkinson & Bleackley, 1995). Lysis can be mediated by directional delivery of cytotoxic proteins such as perforin and granzymes which cause destruction of target cells. Alternatively, lysis of target cells may result from Fas-Fas ligand interaction (Tschopp & Nabholz, 1990; Smyth & Trapani, 1995). The interaction of the membrane bound Fas ligand on the CTL with the Fas receptor on the surface of the target cell will cause apoptosis.

HIV and the pulmonary immune response

Lymphocyte accumulation in the lung can occur as a result of a limited number of events. Lymphocyte accumulation in the interstitium of the lung can be a result from a concentration gradient-dependent chemotaxis of distant cells, immobilization of randomly migrating cells, or from the proliferation of resident and recently attracted cells (Center *et al.*, 1993). A optimal host response against pathogenic stimuli in the lung depends on rapid homing to sites of inflammation by memory lymphocytes that have

been previously primed in secondary lymphoid tissues. The first step in this process is the binding of memory cells to endothelium. This is mediated through the binding of LFA-1 integrin, expressed on the cell surface of memory T cells, to ligands expressed by the inflamed endothelium, including ICAM-1 and ICAM-2. The system VLA-4/vascular cell adhesion molecules VCAM-1 represents another complex of molecules that is involved in the lymphocyte-endothelium adhesion during inflammation (Agostini *et al.* 1993).

The expression of accessory molecules also influences several functional properties of pulmonary T cells. T-cell proliferation, cytotoxicity, and T-dependent B-cell proliferation require T-lymphocytes to bind target and accessory cells. LFA-1/ICAM and CD2/LFA-3 interactions enhance conjugate formation between the TCR and its targets. VLA-4 and CD44 also are involved in T-cell recognition and proliferation (Makgoba, *et al.* 1998). Therefore, adhesion molecules on the surface of pulmonary T-lymphocytes are involved not only in recruitment, but also in the activation of antigen-specific T cells in the lung.

Because HIV is able to infect a variety of lung cells, a strong cell-mediated-immune response usually ensues. AIDS-associated interstitial lung disease represents the prototype of a disorder that is associated with an intra-alveolar infiltration of CD45RO+ memory T cells (Agostini *et al.*, 1996). A limited number of studies have tried to elucidate the nature of the T cell response in the lungs of HIV-positive individuals. Trentin *et al.* (1996) demonstrated a bias in use of T cell receptor Vβ regions in the lungs of HIV-1-infected patients . The TCR β-chain variable region repertoire of 13 HIV positive patients at different stages of the disease were analyzed by flow cytometry and

reverse transcriptase polymerase chain reaction (RT-PCR). Using flow cytometry an overexpression of cells bearing Vβ2 and Vβ3 gene segments were found in the lungs of HIV-infected individuals compared with peripheral blood of the same subjects, as well as with the lungs and peripheral blood lymphocytes (PBL) of normal controls. TCRBV analysis by RT-PCR extended these observations and a significant bias for the expansion of TCRBV 7S1 and TCRBV 9S1 gene segments also was observed. It was also noted that the over-represented TCRBV gene segments were expressed by the CD8+ subset. The low proliferative response of T cells to various superantigens suggests that the expansion of specific Vβ–bearing subsets in the lung might result from triggering by superantigen. Superantigen involvment in HIV disease, however, remains controversial.

Although the correlates of protective immunity to HIV infection in the lung have not been clearly established, an increasing body of literature suggests that HIV-specific cellular immune responses, particularly cytotoxic T lymphocyte responses, play an important role in suppressing viral replication (Pantaleo *et al.*, 1994; Borrow *et al.*, 1994; Koup *et al.*, 1994). The importance of CD8+ T lymphocytes is supported by the finding that many HIV-infected, long-term nonprogressors have strong HIV-specific CTL activity (Klein *et al.*, 1995; Pantaleo *et al.*, 1995; Ferbas *et al.*, 1995). The CTL responses, denoted by the increased number of CD8+ T cells in the lungs of individuals with lentivirus-induced LIP, may reflect the host's immune responses to the virus. It has been suggested that CD8+ T cell activity in HIV infection might be associated with damage to pulmonary tissue and, therefore, may contribute to LIP (Autran *et al.*, 1988; Lanzavecchia, 1989).

Specific Aims:

The intent of this research was to elucidate the mechanism of lymphocyte proliferation and infiltration in the lungs of OvLV-infected lambs. Because of the marked similarity between OvLV- and HIV-induced lesions in the lungs of both species. it is likely that the mechanisms for the development of LIP in sheep and humans are similar. The hypothesis of the present study is that lymphocytes found in the lungs of individuals with lentivirus-associated LIP result from *in situ* proliferation to viral antigen. Additionally, the proliferating T cells are anticipated to promote a T helper 1 response that leads to the inflammation in the lungs.

To investigate this hypothesis, experiments were performed to phenotypically characterize T cells in the lungs of newborn lambs intratrachially inoculated with OvLV (Chapter 2). To further investigate the role of T cells in lentivirus infection, a semiquantitative RT-PCR assay was developed for the assessment of TCRBV gene expression (Chapter 4). The development of this assay required sequence data for ovine TCR β–chains variable region genes which was obtained by cloning and sequencing TCR β-chain cDNAs (Chapter 3). The semi-quantitative PCR method was used to characterize T cells in the lungs of OvLV-infected lambs (Chapter 5). Additionally, the heterogeneity of over-represented TCRBVs was examined by nucleic acid sequence analysis of the CDR3 regions. The functional attributes of T cell populations in the lungs also were explored and correlations between cytokine production, TCRBV expression, and phenotype were made.

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Chapter 2

Early pulmonary leukocyte responses during experimental ovine lentivirus infection

Abstract

Ovine lentivirus (OvLV)-induced disease in sheep most commonly presents as chronic lymphoid interstitial pneumonia (LIP), with clinical signs of progressive respiratory distress. OvLV-induced LIP results from the massive accumulation of leukocytes in the lungs of diseased lambs. The mechanisms of lymphocyte infiltration and proliferation in the lungs of OvLV-infected lambs are not well understood. The aim of this study is to demonstrate the experimental induction of OvLV-associated lung disease in neonatal lambs, and to characterize the cells recovered from the lung by bronchoalveolar lavage (BAL). In this study, 19 neonatal lambs were intratrachially inoculated with OvLV and 14 lambs were sham inoculated with culture supernatants. Infection status was assessed by the detection of virus and antiviral antibodies. Lambs inoculated with OvLV became seropositive between 2-4 weeks post-inoculation (PI). Virus was isolated from 60% of the infected lambs by 2 weeks and from 100% by 10 weeks PI. Histopathology revealed 4-week infected lambs to have the most severe pulmonary inflammation in comparison with 2- and 18-week infected lambs. Infected lambs had a 4-fold increase in the mean number of total leukocytes recovered from BAL. The mean number of lymphocytes in infected lambs rose as much as 35-fold in comparison with controls, with lambs 4 weeks PI exhibiting the most profound increase. Phenotypic analysis of T cell populations from infected lambs revealed an expansion in

both CD4+ and CD8+ T cells in comparison to controls, with an inversion of the CD4/CD8 ratio at 4 weeks PI indicative of a preferential expansion of CD8+ T cells. These data demonstrate the successful experimental induction of LIP in newborn lambs. Analysis of pulmonary leukocytes revealed an increase in CD8+ T cells, which can be temporally correlated with inflammation and, thus, with the pathogenesis of OvLV-associated LIP.

Introduction

Ovine lentivirus (OvLV), also known as maedi visna virus (MMV), is the prototypical non-primate lentivirus. It induces a chronic persistent infection denoted by the development of inflammatory lesions in the lungs, central nervous system, joints and mammary glands (Anderson *et al.*, 1985; Brodie *et al.*, 1992; DeMartini *et al.*, 1993; Narayan & Cork, 1985). The most common and debilitating clinical manifestation of OvLV-infection is progressive lymphoid interstitial pneumonia (LIP) (DeMartini *et al.*, 1993). This decease is observed in sheep flocks in the United States, Africa and Europe, and is a significant cause of morbidity and mortality (Dawson, 1980). LIP is characterized by lymphocyte proliferation and/or infiltration (Begara *et al.*, 1996) in the lungs of infected animals; resulting in lymphoid hyperplasia, hyperplasia of smooth muscle cells, alveolitis and fibrosis (Georgsson & Pálsson, 1971; Lairmore *et al.*, 1988b; Mornex *et al.*, 1994). Clinical evidence of disease includes progressive dyspnea, progressive emaciation, and development of opportunistic infections (Haase, 1986; Scott, 1991; Watt *et al.*, 1992a). To more clearly understand the mechanisms of cell accumulation in natural infection and disease, studies using experimentally infected newborn lambs are necessary.

Experimental OvLV infection of neonatal lambs has been previously used to study lentivirus-induced pulmonary disease (Lairmore *et al.*, 1986). In this study, infection was monitored serologically by agar gel immunodiffusion (AGID) and Western blot, and disease status was evaluated by histopathological scoring of lung tissue. Virus isolation and infectious center assays were employed to isolate virus and demonstrate its infectivity. Repeated monitoring of the cellular responses by examination of

bronchoalveolar lavage (BAL) cells allowed for the study of early pulmonary immune responses during the development of OvLV-associated LIP. BAL is an invaluable technique for accurately evaluating immune processes in the lung, and it has been used to track the development of a number of human pulmonary conditions, such as hypersensitivity pneumonitis, sarcodiosis, and human immunodeficiency virus (HIV)-induced lung disease (Agostini *et al.*, 1991; Agostini *et al.*, 1997; Trentin *et al.*, 1996b). It also has been demonstrated that cells recovered from BAL fluid directly mimics the leukocyte population of the interstitium in normal subjects, as well as in individuals with various types of interstitial pneumonia (Garlepp *et al.*, 1992; Tada *et al.*, 1992).

Examinations of the BAL cells have revealed major changes in the lung lymphocyte population of naturally OvLV-infected sheep (Begara *et al.*, 1996; Bird *et al.*, 1993; Lujan *et al.*, 1995). Lujan *et al.* (1995) described decreases in the percentage of CD4+, CD5+ and γδ T cells with concurrent increases of CD8+ T cells in the BAL fluid of MMV infected sheep. In many viral infections such as lymphocytic choriomeningitis virus (Fung-Leung *et al.*, 1991) and human immunodeficiency virus (HIV) (Pantaleo *et al.*, 1994), CD8+ T cell mediated immune responses are thought to be important in the clearance of viral infection. Analysis of BAL samples from OvLV-infected lambs revealed a CD8+ T cell-mediated lymphocytic alveolitis (Cordier *et al.*, 1992). The goal of the present study was to establish a productive infection in newborn lambs, and to study the permutation in lymphocyte numbers and phenotype that take place during early stages of OvLV-induced pulmonary disease. The approach allows for a more explicit examination of the kinetics of disease and the nature of the pulmonary immune response to OvLV.

Materials and Methods

Experimental Lambs. Thirty-six newborn Ramboullet lambs were obtained from a flock that had uniformly tested negative over a two year period for the presence of antibodies to OvLV envelope glycoprotein and capsid antigen, p25, by agar gel immunodiffusion (AGID). Lambs were housed in raised pens in an isolation facility at Colorado State University. Lambs were fed milk replacer, creep, and grass feed. Lambs were randomly assigned to groups and 19 intratracheally inoculated with 10 mls of goat synovial membrane tissue culture supernatant fluid containing 5×10^6 $TCID_{50}$ of OvLV. OvLV 85/34, a rapid/high strain that is lytic for macrophages and produces large nodular pulmonary lymphoid aggregates in addition to more typical LIP was used (Lairmore *et al.*, 1987). Fourteen lambs were sham inoculated with supernatant from uninfected goat synovial membrane cells.

BAL cell collection: As shown in Table 2.1, lambs were separated into groups and ethanized at 2, 4 and 18 weeks post inoculation (PI). *In vivo* lavages were performed at 2, 4, and 10 week PI or at two week intervals until the lamb was euthanized. *In vivo* lavages were performed while the lamb was under general anesthesia. Using a 5 mm bronchoscope wedged in the right middle bronchus, BAL cells were collected by flushing the lung with 100 mls of Hanks balanced salt solution (HBSS) (Sigma, St. Louis, MO.). An *ex-vivo* lavage was performed on the right lung at all necropsies. Lambs were euthanized by intravenous administration of xylazine (0.2 mg/kg) and ketamine (5 mg/kg) followed by exsanguination from the carotid artery. The lungs were removed and the right caudal lung lobe was lavaged. Lungs were perfused with 1 l of HBSS. 400 mls

of warm sterile HBSS was introduced in 50 ml aliquots through the trachea, the lung was massaged to loosen cells, and the BAL fluid was recovered by inverting the lung. BAL fluid was centrifuged at 1000 x g for 15 minutes and cell pellets were recovered, resuspended in phosphate buffered saline (PBS), and enumerated.

Serology: Virus infection was assessed by testing for a humoral response to OvLV envelope glycoprotein, p16 matrix protein, and the p25 capsid protein. Serial blood samples were collected and seroconversion was monitored using a commercial AGID assay (Veterinary Diagnostic Technology, Inc, Wheat Ridge, CO.) A reaction was considered positive when a line of identity corresponding to the positive control sera was observed. Further evidence of viral infection was obtained by Western blot analysis (Brodie *et al.*, 1992). Briefly, OvLV strain 85/34 viral proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred electrophoretically to nitrocellulose. Serum from lambs was added, incubated, and positive reactions were detected by the addition of a secondary rabbit anti-sheep antibody conjugated with horseradish peroxidase.

Virus isolation and infectious center assay: Virus isolation was performed using primary cultures of goat synovial membrane (GSM) cells. Interstitial lung cells, peripheral blood mononuclear cells, and BAL cells (5×10^6) were co-cultivated in a 25 cm^2 flask with confluent GSM cells in RPMI 1640 with 10% fetal calf serum (FCS) at 38.5^0C. GSM were observed daily for cytopathic effects, passaged at 10-day intervals, and maintained for a minimum of 30 days. A subset of lambs was analyzed by an infectious center assay.

Briefly, 10^5 GSM cells were seeded into each well of an eight well slide chamber. Once confluent, BAL cells (10^2 and 10^3), interstitial lung cells (10^2 and 10^3), and peripheral blood mononuclear cells (10^5 and 10^6) were added to individual wells. Medium was added to a negative control well and OvLV strain 85/34 at a multiplicity of infection (MOI) of 0.1 was added to a positive control well. The slide was cultured for 4 days, fixed in cold acetone, and viral capsid protein was detected by immunohistochemistry as described (Brodie *et al.*, 1995).

Histopathology: A standard method for fixation of the lung was developed to provide uniform tissues for evaluation. The left lung was trimmed of all other tissue at the tracheal bifurcation. An endotracheal tube was secured in the bronchus. The lung was then 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. Tissues from both the central and peripheral parenchyma of the caudal left lobe were trimmed into cassettes and stored in 70% ethanol until routine paraffin embedding, processing and hematoxylin and eosin staining.

Differential cell counts and single and dual color flow cytometry: Total numbers of cells recovered by BAL were determined manually using a hemocytometer or were analyzed with a Coulter counter (Beckman Coulter, Fullerton, CA.). Differential cell counts were determined from a morphological assessment of at least 200 cells after cytospin and hematoxylin and eosin staining.

Total numbers of T cells, as well as the proportion of CD4+ and CD8+ T cells, in BAL fluid were determined by flow cytometric analysis. T cell numbers were determined by single color flow cytometry using an anti-CD2 (MUC2A, VMRD Inc. Pullman WA) monoclonal antibody diluted 1:66 in phosphate buffered saline (PBS) containing 2% sheep and 2% goat serum. A secondary FITC-labeled goat-anti-mouse IgG monoclonal antibody (Caltag Laboratories, Burlingame, CA) diluted at 1:1000 in PBS with sera was used for detection of CD2 positive cells. T cell subpopulations were determined by dual color flow cytometry. First the cells were stained for CD2 as above, then for CD4 or CD8 using anti-CD4, or anti-CD8 (44.38 and 38.65 respectively, University of Melborne, Australia) monoclonal antibodies diluted 1:2 in PBS with serum. The secondary antibody was goat-anti-mouse IgG2a conjugated to biotin (Caltag Laboratories). Preparation of flow cytometry samples was performed on ice. Briefly, $1x10^6$ cells were washed twice in PBS with 2% sheep and 2% goat serum. The cells were resuspended in 50 µls of primary antibody, incubated for 20 minutes on ice and washed twice with 1 ml of PBS with serum. The fluorochrome or biotinylated conjugated secondary antibody was added in 50 µl of PBS with serum, incubated for 20 minutes and washed twice. For dual staining, the antibody incubation steps were repeated using the same methods with the second antibody. Strepavidin-PE (PharMingin, San Diego, CA.) diluted 1:417 in PBS with serum was added to samples containing the biotin-labeled secondary antibodies. Samples were washed three times and fluorescence was quantified using an Epics II flow cytometer (Beckman coulter, Fullerton, CA). Cytometry gates were adjusted (based on the forward versus side scatter) to include only the lymphocyte population, and twenty thousand events were counted.

Results

Infection status. Presence of virus in individual lambs was assessed by isolation of virus from PBMCs and BAL cells, and by the detection of anti-viral antibodies (Table 2.1)[1]. Virus isolation was the most sensitive determination of early infection, with virus recovered from 60% of tested animals at two weeks PI. By four weeks PI virus was isolated from 71% of tested animals and at ten weeks PI, virus was recovered from all inoculated lambs. AGID and Western blot detected the presence of OvLV-specific antibodies. At 2 weeks PI, only 10% of the OvLV-inoculated lambs were positive by AGID and 20% were positive by western blot. By four weeks PI, 88% of lambs tested by AGID demonstrated positive results, while 100% were positive as determined by Western blot. At 18 weeks, all OvLV-inoculated lambs were positive by AGID. Additionally, one uninfected control lamb was seropositive by Western blot at 18 weeks, although virus never was isolated from this lamb and no pulmonary lesions were found upon necropsy. All other control lambs tested negative for virus.

Histological examination. Standardized sections from the lungs of OvLV-inoculated lambs revealed varied degrees of inflammation. Inflammatory lesion scores are the summarized results from the qualitative and quantitative histological assessment performed on tissue sections. The scoring system was based on a 0 to 3 scale (0=none, 1=mild, 2=moderate, 3=severe). The primary histological region of the lung evaluated was the peribronchiolar associated lymphoid tissue. The score assigned to an individual animal was based on a cumulative assessment of the degree of this

[1] Data for individual lambs is shown in appendix 1.

Weeks PI	Infection	AGID[a] (% +)	Western (% +)	Virus Isolation[b] (% +)	Inflammation score[c]	Total cells/ml[d] X10^5	Macrophages ml[e] x10^5 (%)
2	OvLV-	0	0	0	0.08	3.58	3.52 (95.5%)
2	OvLV+	10	20	60	1.00	14.4	11.3 (89.5%)
4	OvLV-	0	0	0	0.25	5.40	4.93 (93.7%)
4	OvLV+	11	100	71	1.25	18.5	15.5 (84.2%)
10	OvLV-	0	0	0	ND	2.12	1.17 (92.5%)
10	OvLV+	100	100	60	ND	2.93	2.62 (89.6%)
18	OvLV-	0	0	0	0.37	30.4	2.97 (96.0%)
18	OvLV+	100	100	100	0.58	5.14	4.39 (87.6%)

Weeks PI	Infection	Lymphocytes/ml x10^3 (%)[e]	T cells/ml[d] x10^3 (%)[f]	CD4 + T cells/ml[d] x10^3 (%)[f]	CD8 + T cells/ml[d] x10^3 (%)[f]
2	OvLV-	6.08 (1.5%)	0.45 (6.00%)	ND[g]	ND
2	OvLV+	189 (17.3%)	85.9 (42.4%)	53.2 (53.4%)	38.5 (36.9%)
4	OvLV-	4.51 (3.2%)	2.17 (33.5%)	2.13 (72.7%)	1.40 (31.0%)
4	OvLV+	154 (10.6%)	120 (55.0%)	44.1 (43.0%)	58.3 (46.0%)
10	OvLV-	1.39 (3.4%)	0.53 (10.5%)	0.38 (61.0%)	80.0 (26.5%)
10	OvLV+	316 (6.6%)	246 (57.2%)	14.3 (46.7%)	27.6 (55.5%)
18	OvLV-	5.40 (1.3%)	3.32 (35.0%)	2.50 (78.4%)	0.56 (22.0%)
18	OvLV+	84.0 (8.5%)	63.5 (57.2%)	18.9 (41.8%)	34.5 (60.9%)

Table 2.1. Summary of immunologic, virologic, and histologic assesment of OvLV-infected and control lambs.

[a] Agar gel immunodiffusion test for antibodies to OvLV capsid and envelope structural antigens.

[b] Isolation of OvLV from PBMC, BAL cells, or lung interstitial cells by co-culture with goat synovial membrane cells.

[c] Inflammation was graded blindly, based on a cumulative assessment of the degree of inflammation in the peribronchiolar associated lymphoid tissue. Additional considerations were given to alveolar wall thickening and the number of mononuclear cells within the alveolus. Scores ranged from "0" which indicated no inflammation to a sore of "3" which indicated the most severe inflammation.

[d] Total cells/ml is based on the total number of leukocytes harvested either by in vivo or ex-vivo bronchoalveolar lavage, divided by the volume recovored.

[e] Percentage of macrophages and lymphocytes were determined by manual differential cell counts of cytospin preparations.

[f] Flow cytometry was used to determine the percentage of total T cells, CD4+ T cells and CD8+ T cells

[g] not determined

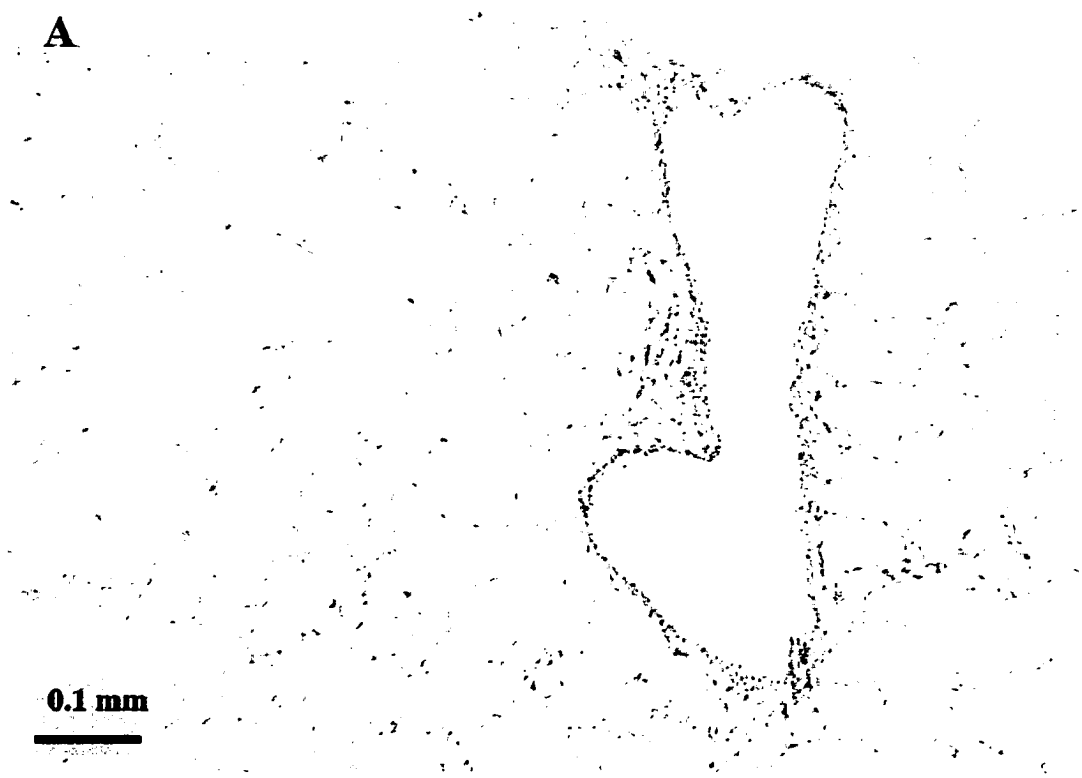
inflammation. Additional considerations were given to alveolar wall thickening due to mononuclear inflammation, and the number of mononuclear cells free within the alveolus. The highest average score of inflammation (1.25) was observed in 4-week infected lambs, with less observed in 2-week lambs and lesser still in the 18-week animals (Table 2.1). Less severe inflammation was observed also in control lambs, yet this increased over time. Inflammation in control lambs likely resulted from the constant onslaught of environmental antigens to the lung. Figure 2.1 shows a representative section of lung from a 4-week control and infected lamb.

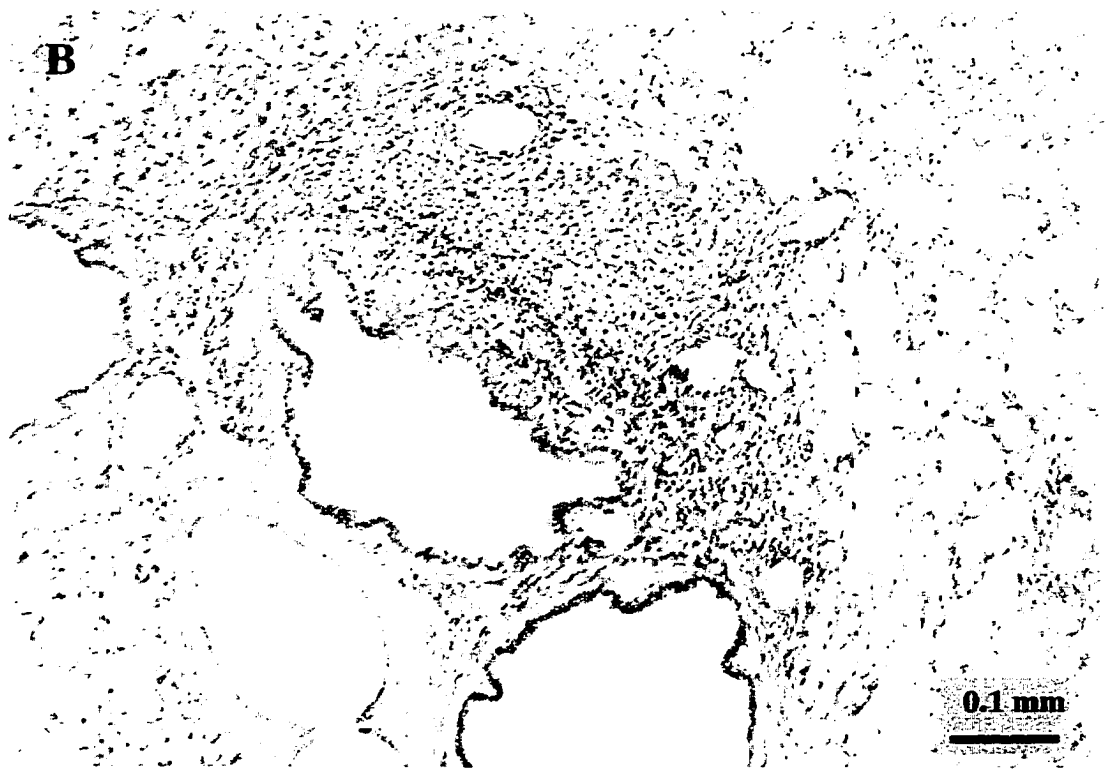
Analysis of bronchoalveolar lavage fluid. Examination of the BAL fluids from 2 and 4 week infected lambs revealed a 4-fold increase in the total number of cells recovered in comparison with controls, while at 10 and 18 weeks infected and control animals had equal numbers of total cells (Table 2.1). The mean numbers of macrophages ranged from 1-5x10^5 cells/ml in control lambs, while in infected animals means ranged from 2x10^5 to 1x10^6. Although the absolute number of macrophages was higher in infected lambs, the percentages, which ranged from 84-89%, were lower than in controls which ranged from 92-96%; 4 week infected lambs demonstrated the lowest mean values (Table 2.1).

Mean numbers of lymphocytes in the BAL of control lambs remained fairly constant ranging from 1-6x10^3 cells/ml representing from 1-3% of the total cells (Table 2.1, Figure 2.2)[2], while mean lymphocyte numbers from infected lambs ranged from 3x10^4 to 1x10^5 cells/ml and represented 6-17% of the total BAL cells. This

[2] Figures 2.2 – 2.6 are graphic representations of the data displayed in Table 2.1

Figure 2.1. Hematoxylin and eosin stained sections of lung tissue from OvLV-infected and control lambs. A) Morphology of lung tissue from an uninfected, control lamb. B) Morphology of lung lesion associated with OvLV infection.





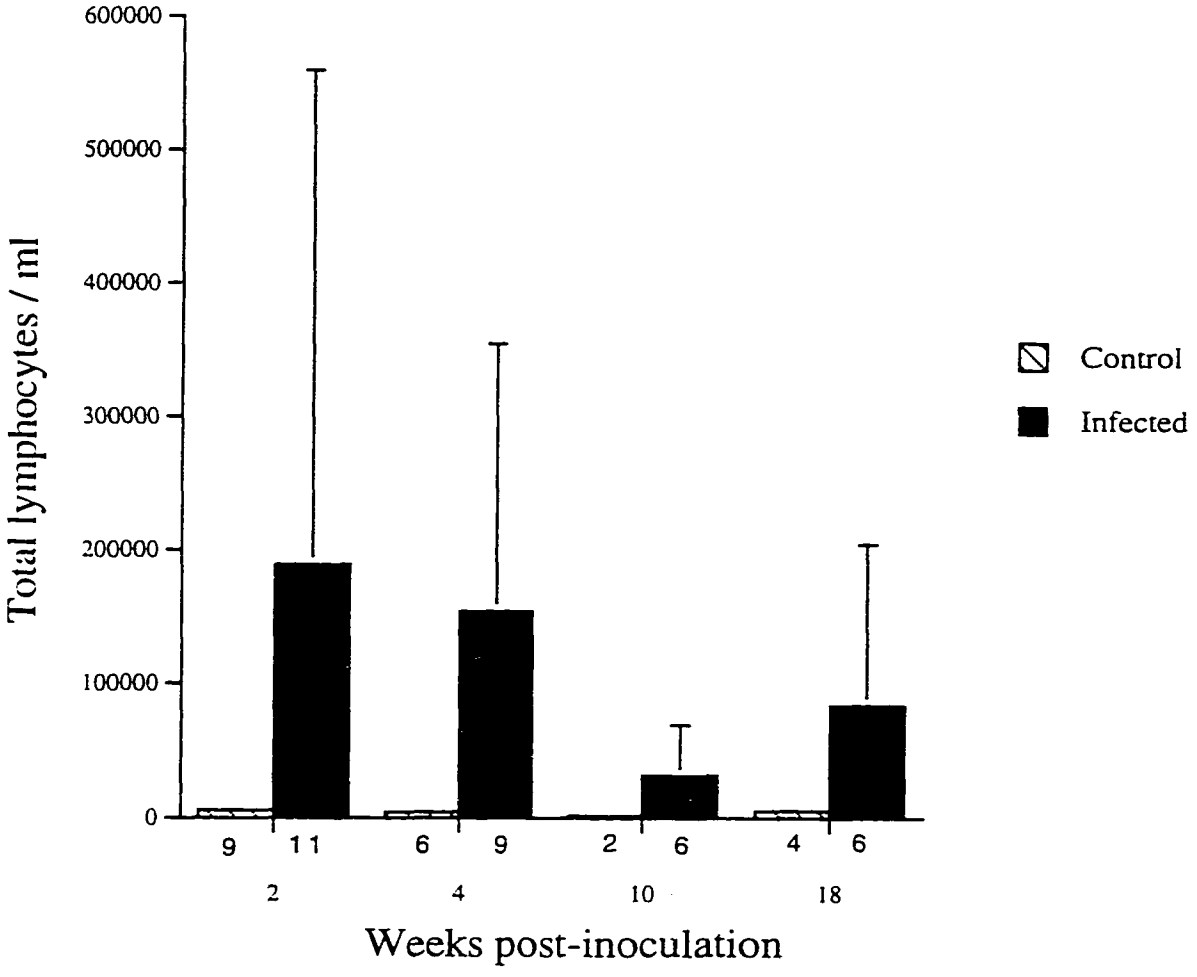


Figure 2.2. Time related changes in BAL fluid lymphocyte numbers during experimental OvLV infection. Bars represent the mean (± standard deviations) numbers of total lymphocytes obtained from *in-vivo* and *ex-vivo* lavages. The value shown below each bar indicates the number of lambs in that group.

reflects a significant increase in the mean numbers of lymphocytes at all time points. Infected animals had a 30-fold increase in the mean numbers of lymphocytes compared to controls at 2 weeks PI. This trend was magnified at 4 weeks with a 35-fold increase in the mean numbers of lymphocytes in the BAL of infected lambs. At 10 and 18 weeks, there was a 23 and 15-fold increase in mean lymphocyte numbers respectively. The total number of T cells in the BAL fluid also was significantly increased in infected lambs, with the greatest increase found at 4 weeks (Table 2.1 and Figure 2.3). As shown in Table 2.1, the percentage of T cells in control lambs ranged from 6-35% while in infected lambs the percentages were much higher, ranging from 42-57%. Phenotypic analysis of the T cell population revealed an increase in the absolute numbers of CD4+ and CD8+ T cells at all time points in contrast to the controls (Table 2.1). The percentage of CD4+ and CD8+ T cells in control lambs remained relatively constant, ranging from 62-78% and 22-31% respectively, while infected animals displayed a CD4+ T cell range of 42-51% and CD8+ T cell range of 36-60% (Table 2.1). The mean numbers of CD4+ T cells were greatest at 2 weeks in infected lambs and then steadily declined over time. The mean numbers of CD8+ T cells increased between 2 and 4 weeks, and then declined at 18 weeks (Figures 2.4 and 2.5). The mean numbers of CD4+ and CD8+ T cells in control lambs were constant thoughout the experiment, as were the CD8/CD4 ratios. As shown in Figure 2.6, CD8/CD4 ratio of infected lambs 2 weeks PI was similar to that of controls, but at later time points the ratio was inverted and favored CD8+ T cells.

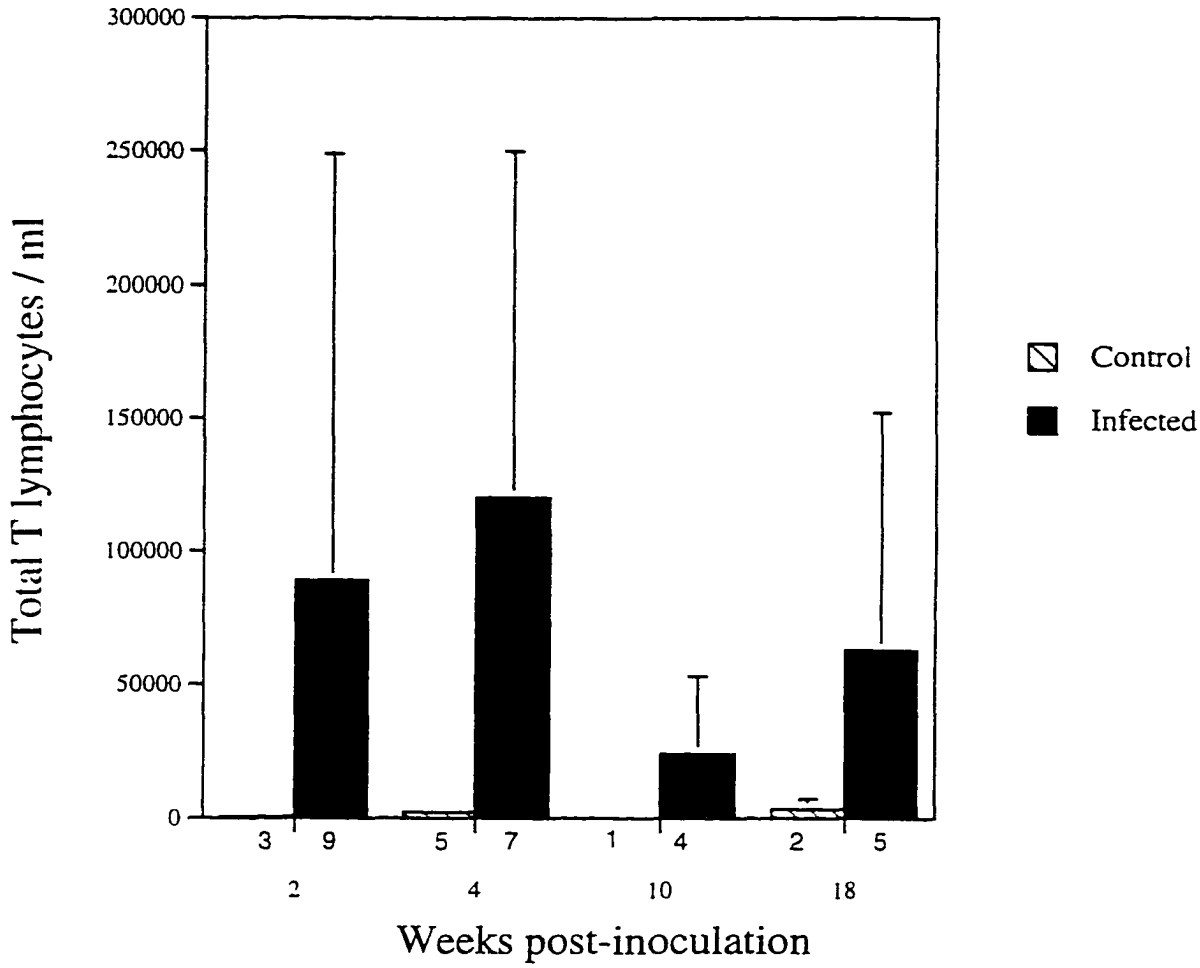


Figure 2.3. Time related changes in BAL fluid T lymphocyte numbers during experimental OvLV infection. Bars represent the mean (±standard deviations) numbers of T lymphocytes obtained from *in-vivo* and *ex-vivo* lavages. The value shown below each bar indicates the number of lambs in that group.

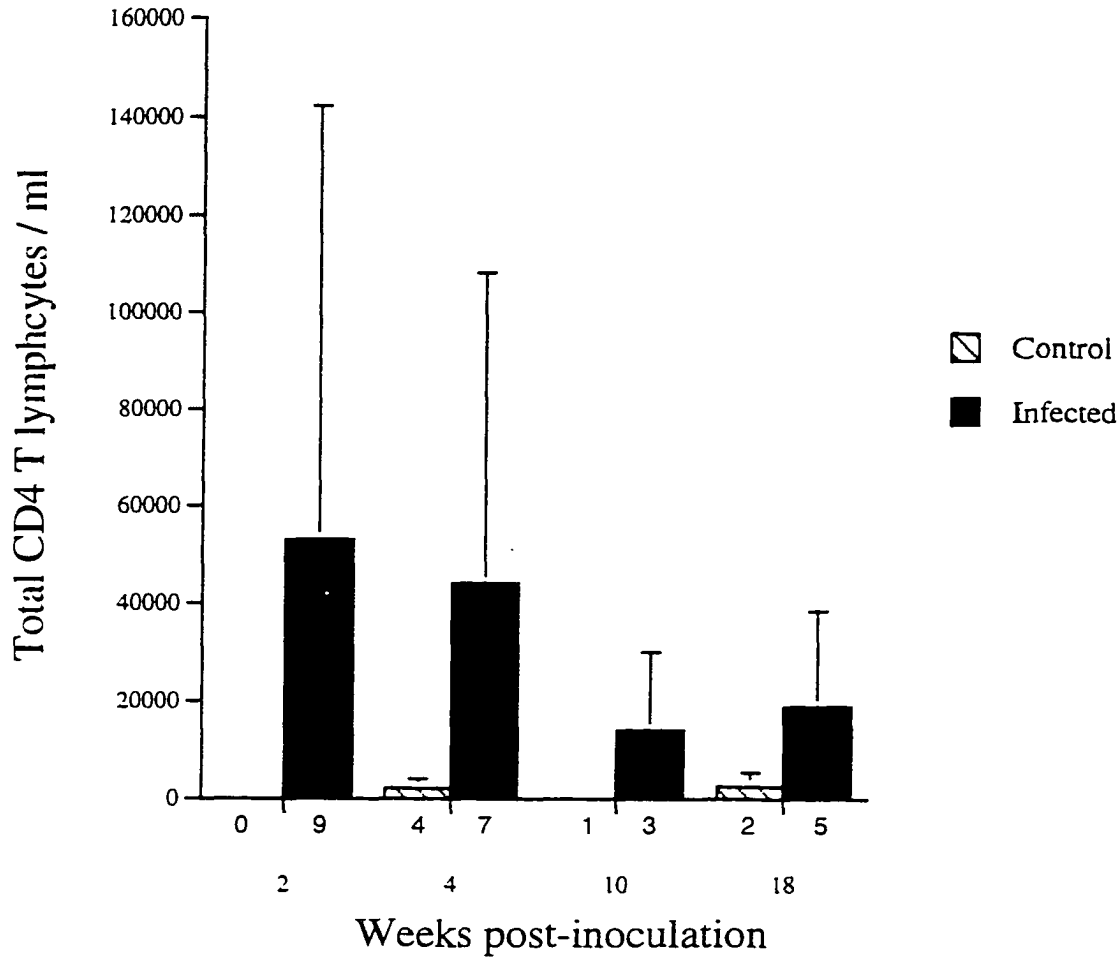


Figure 2.4. Time related changes in BAL fluid CD4+ T lymphocytes during experimental OvLV infection. Bars represent the mean (±standard deviations) numbers of CD4+ T lymphocytes obtained from *in-vivo* and *ex-vivo* lavages. The value shown below each bar indicates the number of lambs in that group.

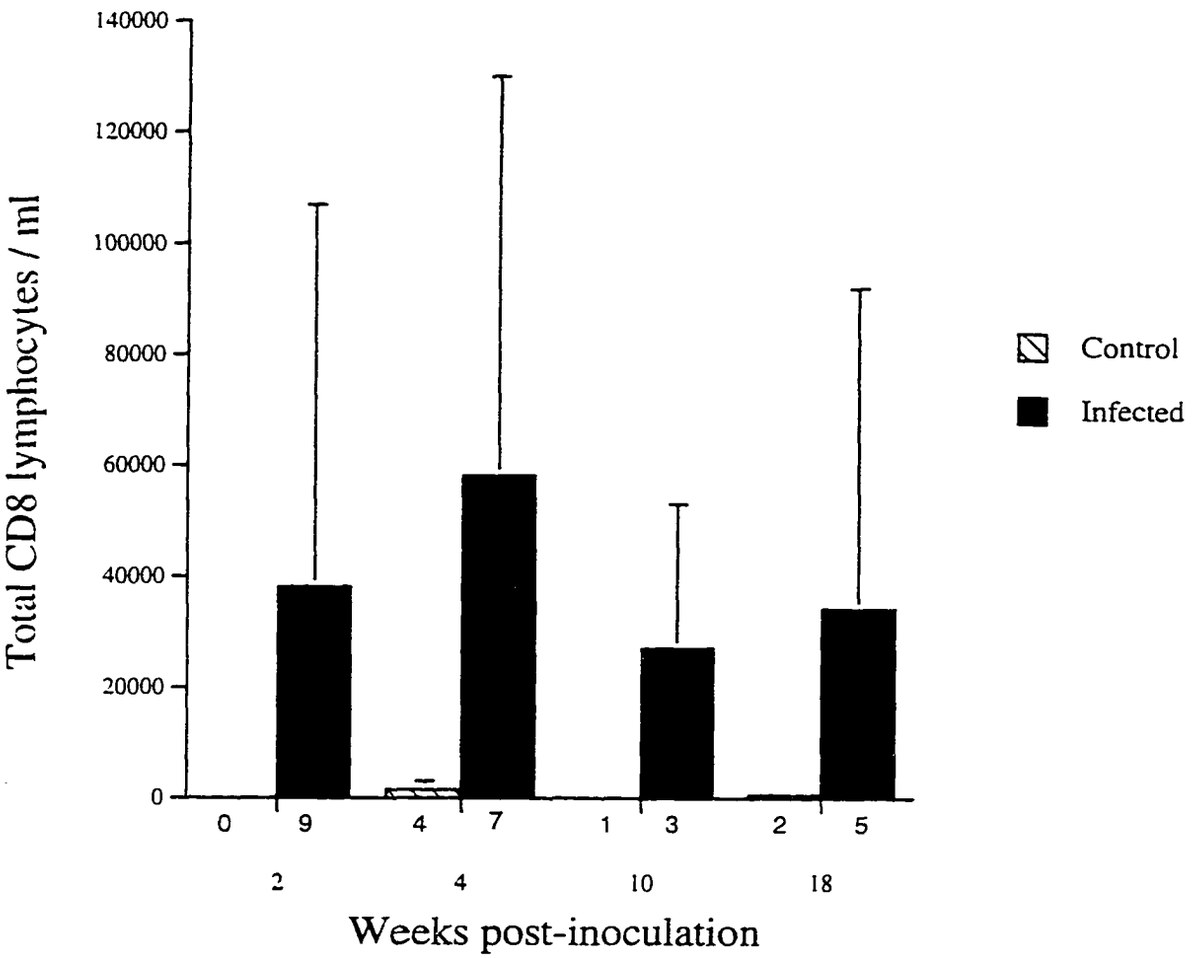


Figure 2. 5. Time related changes in BAL fluid CD8+ T lymphocyte numbers during experimental OvLV infection Bars represent the mean (±standard deviations) numbers of CD8+ T lymphocytes obtained from *in-vivo* and *ex-vivo* lavages. The value shown below each bar indicates the number of lambs in that group.

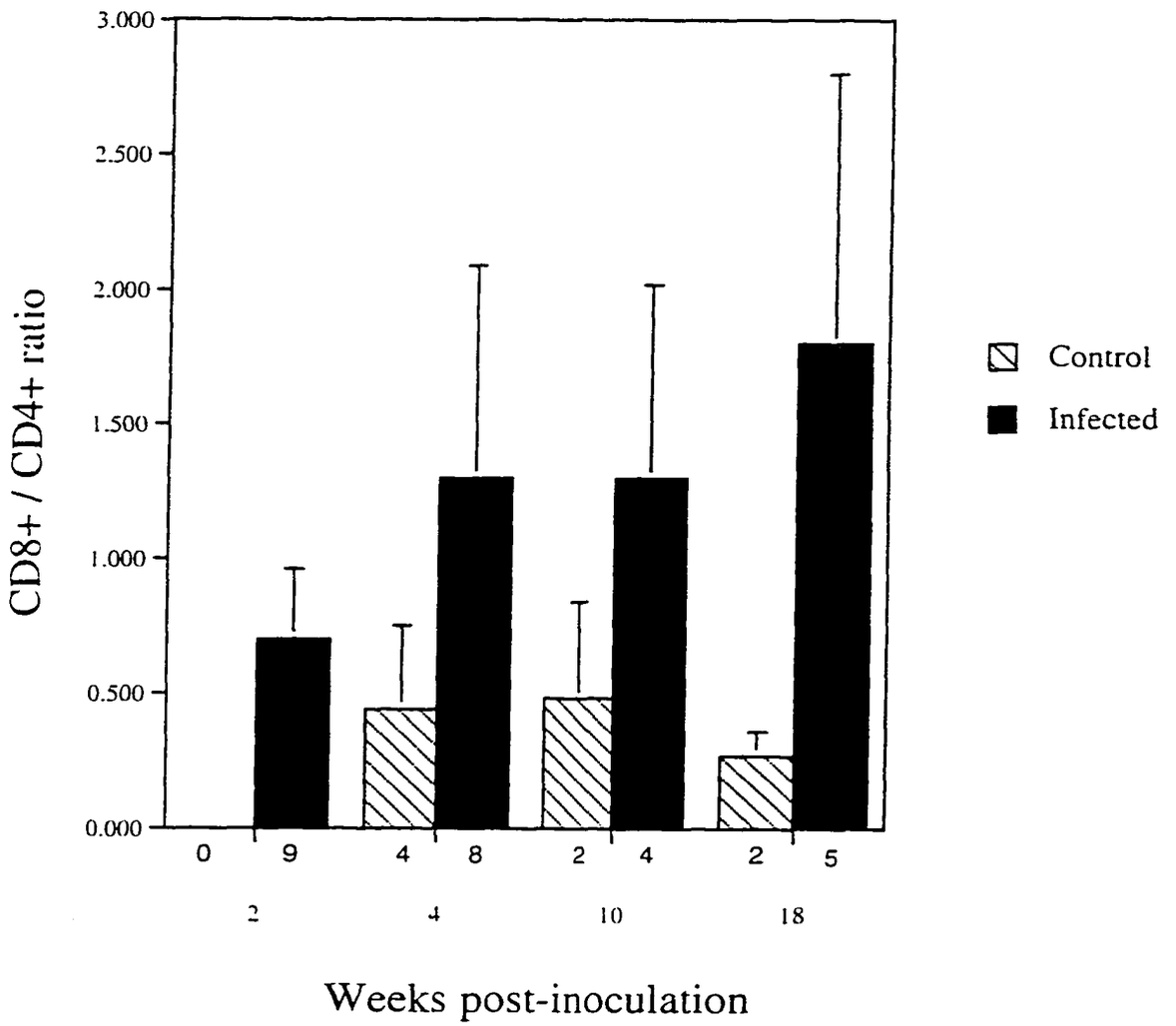


Figure 2. 6. Time related changes in the CD8/CD4 ratio in BAL fluid during experimental OvLV infection. Bars represent the mean (±standard deviations) CD8/CD4 ratios. The value shown below each bar indicates the number of lambs in that group.

Discussion

Understanding the early events in OvLV-associated pulmonary disease is crucial for elucidating the mechanisms involved in lymphocyte proliferation and infiltration into the lungs of infected lambs. To more clearly define the preliminary events in OvLV-induced disease, the use of a well-established experimental model was necessary. The present work demonstrates the successful induction of experimental OvLV-infection in lambs, as confirmed by virus isolation from PBL (data not shown) and BAL samples from infected lambs. Virus isolation was the most direct and sensitive method for determining early infection status; virus was recovered from 60 percent of the animals by 2 weeks PI. Two indirect tests of infection, Western blot and AGID (which assessed the presence of antiviral antibodies), were not as sensitive; most lambs did not test positive until 4 weeks PI. These findings are consistent with results of previous studies in OvLV and MVV experimentally infected sheep, where virus and anti-virus antibodies were detected within a few weeks to a few months after infection (Juste *et al.*, 1998; Peturrson *et al.*, 1976; Sihvonen *et al.*, 1980).

This study confirms previous findings by Watt *et al.* (1992) that demonstrated early events in OvLV-induced pulmonary disease to be marked by a significant increase in the numbers of leukocytes in the lungs. In that study, which used an adult animal model of OvLV-induced LIP, increases were observed in the total numbers of lung macrophages and lymphocytes during the first four weeks of infection. Another study by Begara et al. (1996) showed that in the first month PI, the percentages of macrophages decrease, while the percentages of lymphocytes increases. This trend is characteristic of the development of an immune response to specific antigen as seen in other viral

infections. Further analysis of pulmonary leukocytes has demonstrated a heavy bias toward T cells at four weeks PI. Because T cells are critical in directing the immune response, their significance in LIP needs to be clarified.

Phenotypic examination of the T cell population in infected lambs revealed an expansion in the absolute numbers of both CD4+ and CD8+ T cells, however, the percentage of CD4+ T cells decline while CD8+ T cells increase. At two weeks PI, infected lambs had the greatest number of CD4+ T cell and these numbers declined over time. OvLV infection, unlike HIV infection, does not lead to profound immunosuppression because the CD4+ T cell numbers do not drop below critical levels (de la Concha-Bermejillo *et al.*, 1995). CD8+ T cells, however, are highest at 4 weeks and decline over time. This suggests that CD4+ T cells arrive at the site of infection first and direct the proliferation of virus specific CD8+ T cells. This leads to an inversion of the normal CD4/CD8 ratio to favor CD8+ T cell at 4 weeks. The inversion of the normal CD4/CD8 ratio became even more pronounced at 10 and 18 weeks PI as a result of the continued decline in CD4+ T cells which seemed to migrate out of the lung before CD8+ T cells. The CD8+ T cell response mediate the clearance of virus infected cells, which results in the decreased inflammation observed in the lungs of OvLV-infected lambs 18-weeks PI. These results are similar, if not identical, to those observed in the MVV model (Begara *et al.*, 1996) and in lambs naturally infected with OvLV (Cordier *et al.*, 1992), and they are the hallmark of anti-viral immune responses. It has been noted by Lujan et al. (1995) that the numbers of CD8+ T cells in BAL fluid are potentially valuable indicators of the intensity of lung lesions caused by MVV . The present results confirm that finding, because CD8+ T cell numbers were highest at four weeks PI and histology

scoring based on inflammation and lung lesions (data not shown) also were highest at that time point.

The validity of experimentally infected lambs as a model for natural OvLV infection is uncertain. Although the cell populations found in the lungs of experimentally infected lambs mirror those of naturally infected animals, the kinetics of disease are quite different. In naturally infected sheep pulmonary disease usually takes many months to develop and a chronic condition is induced (Dawson, 1987). However, in our experimental infection, a more acute form of LIP is induced. This most likely reflects properties of the virus used in experimental inoculation or the inoculation itself. The virus used, 85/34 is a rapid/high strain which was chosen because of its ability to quickly induce severe pulmonary disease (DeMartini *et al.*, 1993). Naturally infected lambs may not become infected with such a virulent strain and, therefore, the disease induced may not be as severe. Also, experimental infection may introduce significantly more virus into the lung compared to that acquired during natural infection. Additionally, in the course of natural infection, the virus which is present in respiratory droplets shed by other infected animals, may not be inhaled as deeply, thus the infection which ensues is more superficial and the immune response is able to control it quickly.

Phenotypic changes of T cells in the BAL fluid of OvLV-infected lambs are similar to changes occurring in the lungs of HIV-infected individuals. In patients infected with HIV, the percentage and absolute number of pulmonary CD8+ T cells are significantly increased over control subjects (Agostini *et al.*, 1996; Itesuc *et al.*, 1993; Pantaleo *et al.*, 1994). Additionally, HIV positive patients without full blown AIDS show a decrease in the percentage of CD4+ T cells, but not in absolute numbers, again

parallelling OvLV infection (Agostini *et al.*, 1988). This is important because pediatric HIV-associated LIP develops before the onset of immunodeficiency and the decline in CD4+ T cells.

The standard deviation of the mean was high in total leukocyte, lymphocyte, T lymphocyte and T cell subpopulations. This reflects of a number of factors inherent to OvLV-infection, the bronchoalveolar lavage technique, and the derivation of the absolute numbers, which were based on total leukocytes/ml harvested. As previously noted in studies of naturally- and experimentally-infected lambs, the large variation between lambs is not unusual (DeMartini *et al.*, 1993). It has been documented that OvLV induces severe disease in about 1/3 of the infected sheep and no disease in another 1/3, with the rest falling in-between (Watt *et al.*, 1992a). This variability allows us to correlate our findings with disease severity. For example, two of the lambs had virtually no inflammation, four had mild inflammation and one had moderate inflammation two weeks PI (Appendix 1). This type of variability was also observed in lambs at four weeks PI. Additional variation was caused by the nature of the BAL procedure. Lavages were performed by adding HBSS while manually massaging the lung. This procedure, while done as precisely as possible, could not be performed in exactly the same manner each time, thus, resulting in differential cell recoveries. The advantage of the lavage procedure was the ability to obtain large numbers of cells, which allowed for a number of studies to be performed.

Although many of the characteristics of naturally OvLV infection and LIP are present in the experimental model not all the aspects are identical. The kinetics of experimental induced LIP in lambs is quite different from that seen in naturally infected

animals. Naturally infected lambs tend to develop a more chronic LIP which takes months to develop while experimentally induced LIP occurs within a few weeks and causes a more acute pulmonary condition. Therefore experimentally induced LIP is an accelerated version of naturally acquired disease.

These data confirm that OvLV-induced LIP is characterized by a significant increase in lung leukocytes, most specifically T lymphocytes. T cells regulate the accumulation and function of virtually all leukocytes; thus, their importance in the development of OvLV-induced LIP is paramount. Furthermore, the activation of OvLV-specific T cells in the lungs is likely the proximal event in the initiation and perpetuation of LIP, indicating the need for subsequent examination of the attributes of these cells in the lungs of infected lambs. This study revealed that lesion development and T cell numbers are greatest in 4-week infected lambs, and it is also at this point that CD8+ T cells first predominate. Analysis of T cell receptor gene expression by T cells in the BAL of lambs 4-weeks PI will help to more fully elucidate the nature of the T cell expansion in the lungs of OvLV-infected sheep.

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Chapter 3

Analysis of sheep T-cell receptor β-chain heterogeneity

Abstract

The lack of sequence data for ovine T cell receptor (TCR) genes has impeded the development of molecular techniques for analysis of T cell immune responses in sheep. Thus, the present work provides the nucleotide sequences of 34 TCR β-chain cDNA clones, isolated from an ovine spleen anchored-PCR library. These cDNA clones represent 18 unique TCR β-chain variable region (TCRBV) gene segments, which have been clustered into 13 families, each containing five or fewer members. From this analysis we have estimated the size of the ovine TCRBV repertoire to be 23, with an upperbound limit of 37. Based on comparisons of nucleotide and deduced amino acid sequences, ovine β-chain sequences were found to be similar to those of other mammals in terms of overall organization of nucleotide sequence, and in the conservation of certain amino acid residues known to be critical to β-chain structure and function. These findings provide information that will permit the development of methods anticipated to be useful in evaluating the role that specific T cell populations play in naturally occurring and experimentally induced diseases of sheep.

Introduction

The αβ T cell receptor (TCR) is responsible for mediating antigen recognition of T lymphocytes through the formation of a ternary complex with a major histocompatibiliy complex (MHC) molecule and an antigenic peptide (Davis & Bjorkman, 1988; Wilson *et al.*, 1988a; Blackman *et al.*, 1988). In order for T cells to recognize the vast diversity of antigens which might be encountered, the TCR repertoire must be equally if not more diverse. It has been theorized that there are greater that 10^{10} different TCR sepcificities (Roth *et al.*, 1989). Consequently, each TCR is as unique as a human fingerprint and, therefore, can be used as a marker to identify individual T cells.

Diversity in the TCR is located in the variable regions of the α and β-chains, which defines the specificity of the T cell receptor. These α- and β-chain polypeptides arise from the random recombination of multiple, non-contiguous germline genes which encode discrete segments of the TCR polypeptides (Chien *et al.*, 1984a; Hayday *et al.*, 1985). The variable region of the TCR contains two germ line encoded hypervariable regions, also termed complementary determining regions (CDRs) one and two. These regions are thought to play a role in MHC binding. Additionally, there is a CDR3 region that is not germline encoded. Rather, it is formed during the association of discrete variable (V), diversity (D) and joining (J) regions of the β-chain or the V and J regions of the α-chain. CDRs3 are the most diverse of the hypervariable regions and they are thought to be responsible for antigen binding (Jorgensen *et al.*, 1992).

Two methods have been employed to analyze the role of specific T cells in diseases of both humans and mice. The first technique uses monoclonal antibodies that are specific for the variable region of the TCR. This type of analysis has been used to

correlate the presence of specific T cells with many human diseases such as rheumatoid arthritis (Striebich *et al.*, 1998), systemic lupus (Sutmuller *et al.*, 1998) and AIDS (Trentin & Semenzato, 1996a). In the ovine system, however, these antibodies are not currently available and their development is tedious and time consuming. Alternatively, specific TCR usage in disease has been analyzed using reverse transcriptase-polymerase chain reaction (RT-PCR). Development of a RT-PCR method for TCR β-chain variable region gene (TCRBV) analysis is much less time consuming and tedious than the previous method mentioned. This type of analysis, however, requires information on the TCRBV nucleotide sequence. To date there is nucleotide sequence data for only a single ovine α-chain cDNA (Hein *et al.*, 1991) and for 2 truncated β-chain cDNAs (Grossberger *et al.*, 1993a). Thus, more complete information in the ovine system is needed. Here we present the results of a comprehensive analysis of ovine TCR β-chain heterogeneity which is anticipated to facilitate the development of RT-PCR method. This method will permit the analysis of ovine TCRBV gene expression by T cells which mediate pathogenesis in ovine disease.

Materials and Methods

Molecular cloning and sequencing of ovine TCR β-chain cDNAs. Sequence analysis of ovine TCRBV gene has been performed as described by Halsey *et al.* (1999). Briefly, total RNA was purified from the spleen of a single Ramboullet ewe (Chomczynski & Sacchi, 1987). First strand cDNA was synthesized, G tailed and PCR amplified (Loh *et al.*, 1989; Rosenberg *et al.*, 1992). The amplification products were separated and fragments of the appropriate size were gel purified. Gel-purified fragments were sequentially digested, ligated into pBluescript SK^- (Stratagene, La Jolla, CA) and electroporated into *E. coli* XL1 Blue (Stratagene). Positive recombinants were selected, grown and plasmid was isolated. The nucleotide sequences were determined using dideoxy chain termination. Analyses of nucleotide and deduced amino acid sequences were conducted using FASTA (Pearson, 1994) and BLAST (Gish & States, 1993) alogorithms to search the GenEMBL and PIR/SwissProt databases, respectively. Pairwise and multiple sequence alignments were performed using the algorithms of Myers and Miller (1988) and Higgins and Sharp (1989), respectively.

Results

Molecular cloning and nucleotide sequence analysis of ovine TCR β-chain cDNAs. Thirty-six plasmids containing TCR β-chain rearrangements were isolated, and the nucleotide sequences of these cDNAs were determined (Figure. 3.1). Open reading frame analysis revealed all of the rearrangements to encode functional polypeptides. Of 36 clones isolated, 34 were unique rearrangements and 22 individual TCRBV sequences were identified. Four of the TCRBV pairs displayed minimal sequence variation and were considered to represent allelic polymorphisms (Concannon *et al.*, 1986; Plaza *et al.*, 1991; Posnett, 1990). Thus, 18 total β-chain variable regions were identified.

Ovine TCR β-chain V-region polypeptide sequences. V-region amino acid sequences, predicted from the nucleotide sequences in Figure 3.1, are shown in Figure 3.2. These β-chain polypeptide sequences are similar to those of other mammals in terms of overall organization (Davis & Bjorkman, 1988; Tanaka *et al.*, 1990; Wilson *et al.*, 1988a; Isono *et al.*, 1994; Schrenzel *et al.*, 1994) and in the conservation of certain amino acid residues known to be critical to β-chain structure and function (Chothia C *et al.*, 1988; Novotny *et al.*, 1986; Garcia *et al.*, 1996).

Ovine TCRBV nomenclature. The TCRBV nomenclature proposed in Figure 3.2 is based on amino acid and nucleotide identity between ovine and human TCRBV elements (Figure 3.3), and on the nomenclature established by Arden *et al* (1995).

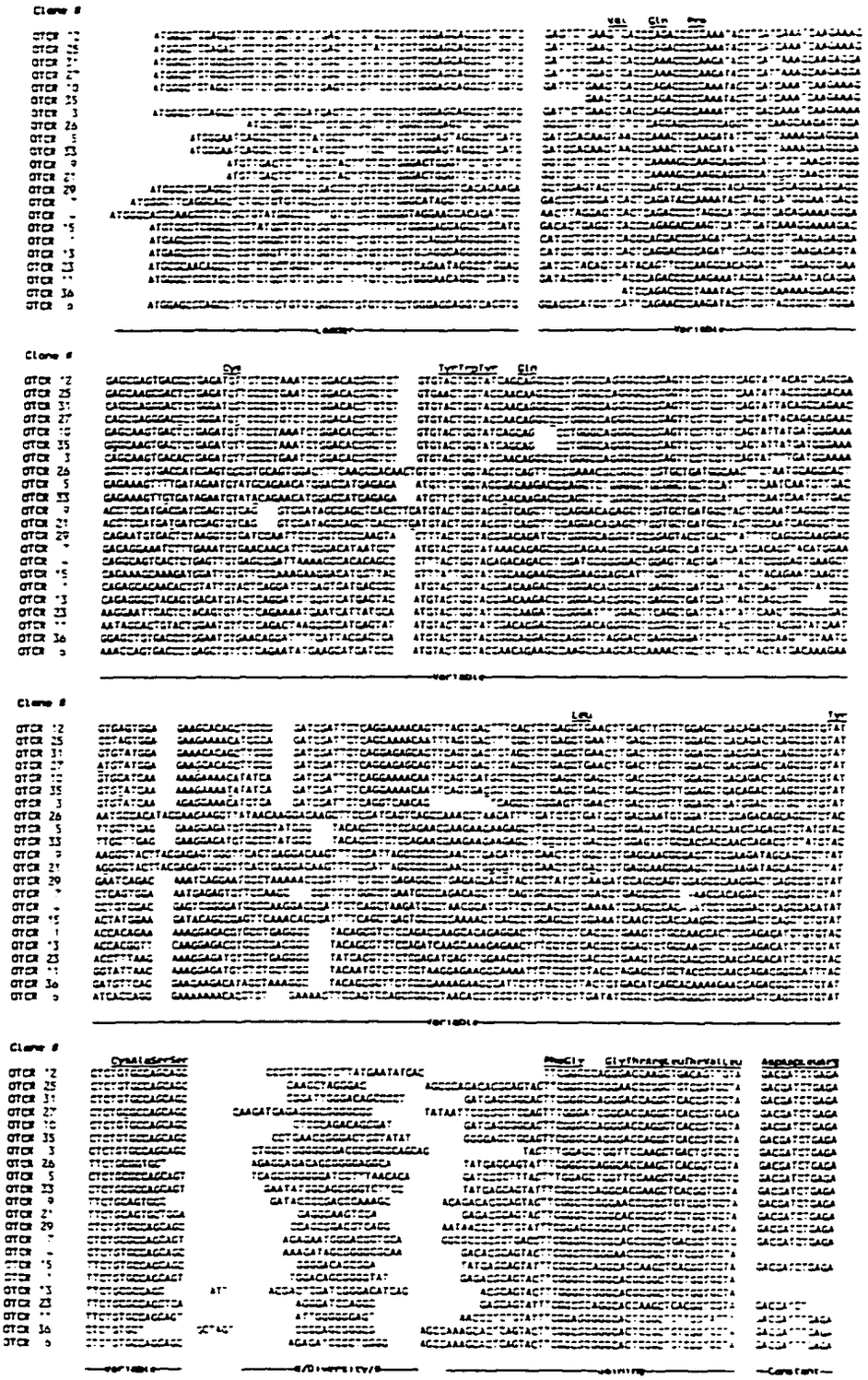

Figure 3.1. Nucleotide sequences of sheep TCR β-chain cDNAs. Consensus codons are indicated above the alignment. Underlined nucleotides denote substitutions in presumptive polymorphic variants.

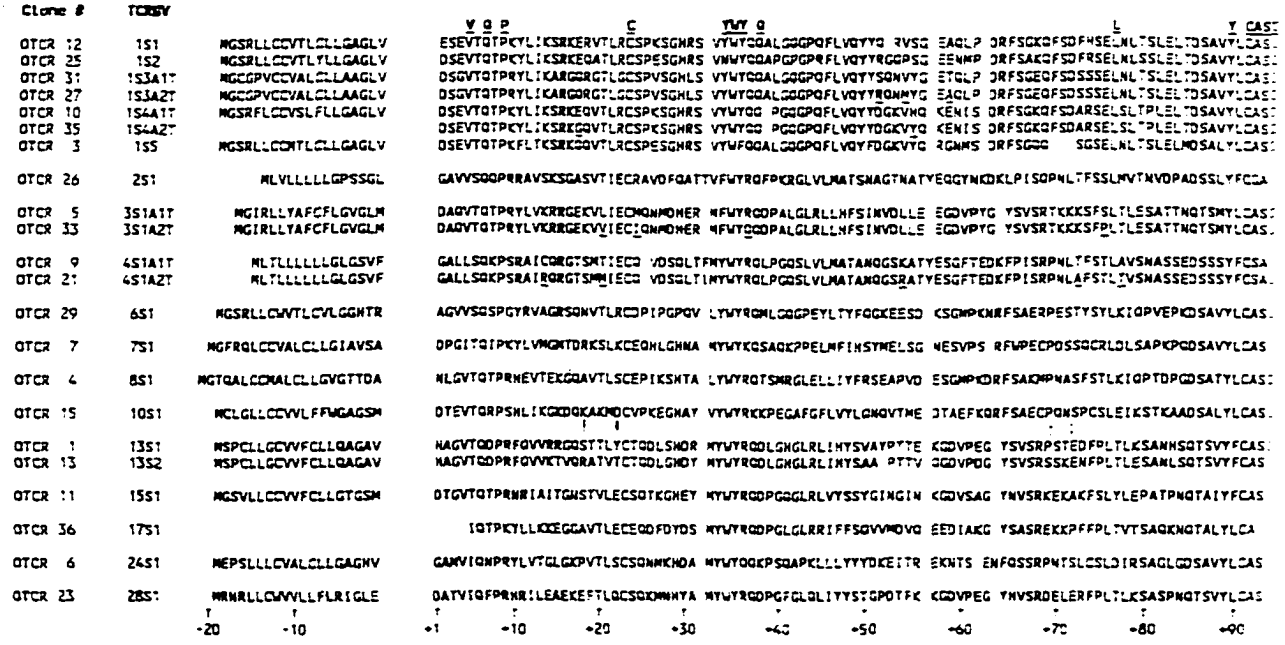

Figure 3.2. Predicted amino acid sequences of sheep TCRBV polypeptides. Consensus residues are indicated above the alignment. Underlined residues denote amino acid substitution in presumptive polymorphic variants. Numbering is based on the OTCR23 amino acid sequence.

	1S1	1S2	1S3	1S4	1S5	2S1	3S1	4S1	6S1	7S1	8S1	10S1	13S1	13S2	15S1	17S1	24S1	28S1
1S1		88	86	88	82	54	56	47	58	59	57	58	58	61	60	62	57	56
1S2	82		82	86	83	54	58	53	60	59	59	56	59	62	56	61	59	55
1S3	83	70		81	81	50	55	49	60	62	58	56	60	59	56	57	64	51
1S4	82	80	71		88	55	56	52	61	61	60	58	60	56	61	63	63	51
1S5	75	71	70	78		52	56	50	59	59	59	60	56	59	59	58	63	50
2S1	22	21	25	22	25		53	66	54	48	59	53	54	55	54	59	53	52
3S1	33	33	33	30	29	30		52	52	60	56	55	66	67	65	63	51	61
4S1	24	26	28	26	24	57	26		50	51	56	57	53	57	55	51	52	49
6S1	41	40	42	45	46	30	26	30		56	65	55	52	55	56	53	56	51
7S1	37	35	37	35	30	18	27	21	35		57	58	55	58	59	56	60	52
8S1	36	39	42	38	38	35	33	30	52	34		59	59	56	56	59	57	52
10S1	39	40	39	35	40	22	26	20	34	31	36		58	55	56	51	58	53
13S1	33	29	34	28	31	29	54	31	32	29	33	25		87	65	58	54	62
13S2	31	33	37	27	34	30	50	27	27	28	34	26	79		65	58	52	65
15S1	29	29	33	35	29	27	52	28	32	27	33	28	51	50		60	53	64
17S1	33	33	31	34	34	30	53	28	28	25	33	23	44	45	42		55	55
24S1	39	38	41	41	38	25	37	28	38	38	40	33	37	31	33	33		55
28S1	30	27	28	29	25	23	46	28	29	25	30	25	57	53	53	41	35	

Table 3.1. Nucleotide and amino acid identities of sheep TCRBV sequences. Numbers above the diagonal indicate the percentage nucleotide identity between TCRBV genes shown on the x-axis and y-axis, whereas numbers below the diagonal indicate the percentage amino acid identities. Sequences, exclusive of the leader segments, are compared.

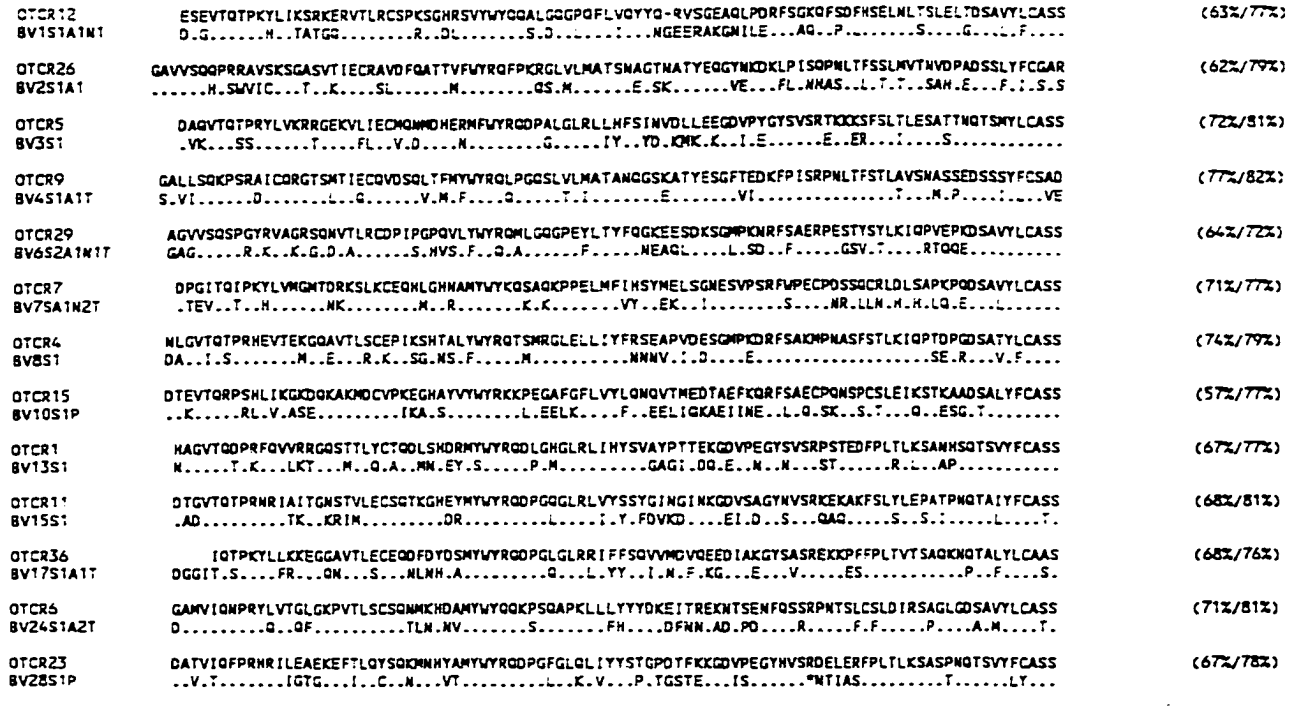

Figure 3.3. Alignments of sheep TCRBV amino acid sequences with homologous human sequences. Percentage amino acid/nucleotide identity for each pair is indicated. The asterisk in the sequence of human BV28S1P denotes a stop codon present in this pseudogene.

Amino acid and nucleotide sequence identity between ovine and human V-regions ranged from 57 –77% and from 72- 82% respectively, identities not unlike those which result from the comparison of human TCRBV sequences with those of other species (Tanaka *et al.*, 1990; Wilson *et al.*, 1988a; Isono *et al.*, 1994; Schrenzel *et al.*, 1994). Family membership indicated in Figure 3.2 was based on a determination of nucleotide sequence identity among 18 of the unique ovine TCRBV sequences (Table 3.1). Five sequences (OTCR12, 25,31,10 and 3), all of which were homologues of human TCRBV1, displayed >75% nucleotide identity to each other and, therefore, were designated TCRBV1S1-BV1S5, respectively. Similarly, two homologues of human TCRBV13, OTCR1 and 13, displayed 87% nucleotide identity with each other and, thus, were designated TCRBV13S1 and TCRBV13S2, respectively. In total, 13 distinct TCRBV families were defined by the present analysis: 2 multimembered families and 11 additional families, each containing a single member.

Estimated size of the ovine TCRBV repertoire. Thirty-four individual rearrangements were characterized, 18 of which were found to be unique TCRBV genes. Six of these TCRBV genes were present in multiple rearrangements in our analysis: TCRBV1S3 (OTCR27, 31 and 32), TCRBV1S4 (OTCR10 and 35), TCRBV1S5 (OTCR3 and 34), TCRBV2S1 (OTCR26, 28, and 30), TCRBV3S1 (OTCR5, 8, 14, 16, 18, 24 and 33) and TCRBV4S1 (OTCR9, 19, 20, and 21). The remaining 12 TCRBV genes were observed only in single rearrangements. Based on this distribution, we have estimated the size of the ovine TCRBV repertoire using statistical methods employed by others (Barth *et al.*, 1985a; Kimura *et al.*, 1987; Concannon *et al.*, 1986; Behlke *et al.*, 1985). The maximum

likelihood estimate (MLE) of the number of distinct ovine TCRBV genes, according to this analysis, is 23. The upper bound, calculated at a 95% confidence interval, is 37.

Discussion

Until now there has been little information on the ovine T cell receptor β-chain repertoire. In this study we have cloned, sequenced and analyzed 36 TCR β-chains isolated from splenic RNA of an adult Ramboullet sheep. This analysis has identified 34 unique TCR β-chains rearrangements, which contain 18 independent TCRBV regions. The ovine β-chain rearrangements have been named and grouped into 13 families based on sequence similarity to human β-chains. Based on these findings, the most probable number of ovine TCRBV genes was calculated to be 23, with an upper bound limit of 37. Studies in humans and in mice have proven the MLEs to be a more accurate assessment of the actual number of TCRBV genes than the upper bound value, that has been shown to overestimate the prevalence of these germline genes. Recent characterizations of the bovine (Tanaka *et al.*, 1990) and caprine β-chain V-region repertoires suggest that additional ovine V-regions will be identified. Bovine homologues of 8 ovine V-regions families, and of 1 V-region family that has not yet been identified in sheep, have been described. Caprine homologues of all 13 ovine V-region families have been characterized, and 5 additional V-region families, one of which corresponds to the unique bovine sequence mentioned above, have been defined. Given the modest evolutionary distance between cattle, sheep, and goats (Kohne *et al.*, 1970) and the documented similarities in the immune system of ruminants, ovine counterparts of these 3 additional families probably exist. Therefore, based on the most probable number of ovine β-chains and information on the repertoire of closely related species, our sequences likely represent the majority of variable regions in the ovine β-chain repertoire.

In this study we have presented data that indicates the overall organization of TCRBV gene segments in the ovine is very similar to that in other species. Based on a comparison of translated mouse and human TCRBV gene segments sequences, it has been noted that the ovine β-chain contains three hypervariable regions, two located in the V region and one in the V-D-J junction (Wilson *et al.*, 1988a). The existence of these unique hypervariable regions in the T cell receptor chains is significant because it suggests that the ovine TCR is required to interact with not only foreign antigen, but also polymorphic MHC restricting elements as do all other mammals studied to date. Additionally, the second hypervariable region, or CDR2 is germline encoded, thus, it defines a unique sequence present in all β-chain rearrangements possessing that V region. Therefore, it should now be possible to exploit this hypervariable region to make a panel of DNA primers, each specific for a given β-chain variable region gene, which will allow the development of assays to determine the involvement of specific T cells in ovine disease.

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Chapter 4

Differential utilization of T cell receptor β-chain variable region genes by ovine peripheral blood T lymphocytes

Abstract

A semi-quantitative RT-PCR method for analyzing ovine T cell receptor β-chain variable region (TCRBV) gene expression is described. Using this method, we have found significant variability in TCRBV expression by peripheral blood T lymphocytes of Suffolk and Ramboullet sheep. Within each animal, the relative levels of expression for individual TCRBV elements is highly variable (intra-individual variation). While these patterns of expression generally are preserved within individual animals of a given breed, the levels of expression for certain TCRBVs is highly variable within the group (inter-individual variation). Further, certain TCRBV transcripts, while of low variability among animals within a breed, are expressed at significantly different levels between the two breeds (inter-breed variation). Additionally, TCRBV expression by T lymphocytes in organized lymphoid tissues was analyzed and found not to be significantly different from that observed in peripheral blood. In total, the results of this analysis parallel those obtained from the study of other species, where the influence of MHC and other genetic loci, thymic selection, and environmental factors have been found to shape the peripheral TCRBV repertoire. These findings provide a baseline against which TCRBV expression

of protective and/or pathogenic T cell populations can be compared during the evaluation of their roles in natural and experimental diseases of sheep.

Introduction

The αβ T cell receptor (TCR) is a heterodimeric transmembrane glycoprotein which mediates major histocompatability complex (MHC)-restricted antigen recognition by T lymphocytes (Bjorkman & Davis, 1988; Ishiguro & Hein, 1994; Marrack & Kappler, 1987). Both α and β-chains consist of constant and variable domains. The variable domain of the α-chain is composed of variable (V), and joining (J) elements. The β-chain contains those elements as well as a diversity (D) region. The fine specificity of T lymphocytes is determined by the variable sequences in the amino terminal portion of the chains. The TCR variable domains have three hypervariable regions that are equivalent to the complimentary determining regions (CDRs) in immunoglobulin (Ig) light and heavy chains. Unlike the Ig however, somatic mutation does not take place in TCR genes, thus, magnifying the importance of germ-line-encoded gene segments which play a significant role in the generation of TCR diversity, and which enable T cells to recognize the vast variety of antigens encountered.

An individual's T cell repertoire is influenced by both genetic and environmental factors (Akolar P.N. *et al.*, 1993; Wong *et al.*, 1993; Woodland *et al.*, 1991). The germline genes carried by an individual are the fundamental building blocks from which the T cell repertoire is shaped. During the development of lymphoid precursor cells into mature immunocompetent T cells, diversity is generated by random somatic recombination of germ-line-encoded V-D-J regions (Chien *et al.*, 1984b; Toyonaga & Mak, 1987; Wilson *et al.*, 1988b). Further diversity is created by N-region nucleotide addition (Wilson *et al.*, 1988b) between the V-D and D-J regions, and in some T cells further variation is mediated by P-nucleotide addition (Lafille *et al.*, 1989a). It has been

demonstrated in mice that differences in germline genes also influence the T cell repertoire (Behlke *et al.*, 1986; Kotzin *et al.*, 1985).

Major histocompatibility complex (MHC) molecules, know as HLA in humans and H-2 in mice, play a prominent role in educating immature thymocytes in the thymus of both species. T cells expressing certain TCR variable region genes are preferentially retained during positive selection because of proper affinities for one of the self-MHC molecules expressed on thymic epithelium (Bill & Palmer, 1989b; Blackman *et al.*, 1989; Kisielow *et al.*, 1988). The majority of immature double positive T cells are not able to meet this requirement and die. However, some of TCRs interact with MHC molecules, but bind too tightly and, therefore, are potentially harmful for self; these cells undergo a process called negative selection and are deleted by apoptosis (Bill *et al.*, 1989a; Blackman *et al.*, 1990; Fowlkes *et al.*, 1988; Kappler *et al.*, 1987). Although the sheep MHC, known as OLA, does not have homologous counterparts to the human DP genes, the rest of the class I and II genes are quite similar. We therefore expect to find the same intricate network of selection by the ovine MHC on T lymphocytes (Puri *et al.*, 1987).

Superantigens are molecules produced by viruses and bacteria that can influence the T cell repertoire by their potent T cell stimulatory properties. There are two major types of superantigens, soluble exotoxins, such as those secreted by *Staphylococcus*, and the virally encoded superantigens, such as those encoded by mouse mammary tumor viruses (MMTV) (Choi *et al.*, 1989; Herman *et al.*, 1991; Kotzin *et al.*, 1993; Marrack & Kappler, 1990; Marrack *et al.*, 1991; Pullen *et al.*, 1992; Woodland *et al.*, 1991). MMTV are infectious retroviruses that frequently integrate stably into the mouse genome. In the mouse, deletion of T cells bearing specific Vβ elements can occur. This

can be linked to the presence of endogenous retroviruses, like MMTV, which encode the prototypic endogenously expressed superantigen Mls-1, which binds the TCR in a non-conventional manner (Pullen *et al.*, 1990; Okada & Weissman, 1989; Barth *et al.*, 1985b). Numerous endogenous retroviral sequences have been discovered in the ovine genome, but it is not yet known if any of these sequences encode superantigens (Bai *et al.*, 1996).

Analysis of TCR β-chain variable region (TCRBV) gene expression is useful for examination of the T cell repertoire (Choi *et al.*, 1989; Loh *et al.*, 1989; Dunn *et al.*, 1993; Wong *et al.*, 1993). Reverse transcription-polymerase chain reaction (RT-PCR) has been used extensively to study TCR repertoires in both mice and humans, however, reagents for employing this technique were not previously available for use in the ovine system. We have developed a method for detection of TCRBV in sheep, which allows us to analyze ovine T cell repertoires. The technique utilizes a panel of 16 unique primers whose sequences were derived from the complementary determining region two (CDR2) region of the TCR β-chain (Halsey *et al.*, 1999). These primers are used in conjunction with another primer derived from the β-chain constant region (Grossberger *et al.*, 1993b). A α-chain constant region primer set has also been incorporated into the system as an internal control (Jein *et al.*, 1991). The α-chain primer pair acts as a standard, allowing the assay to be semi-quantitative. We have applied this approach to define the TCR repertoire in peripheral blood from two breeds of sheep, and immune organs of one breed. This study provides information on the TCR repertoire of healthy sheep, which will facilitate further studies on the influence of genetic and environmental disease in ovine system.

Materials and Methods

RNA Isolation. Whole blood from five adult Ramboullet and Suffolk sheep, born and raised at Colorado State University's Rigden farms, was collected into heparized tubes. Blood samples were centrifuged for 15 minutes at 1000 x g, and the buffy coats were collected. Buffy coats were diluted 1:1 with Ca++ and Mg++ free Hanks balanced salt solution (HBSS) and mononuclear cells (PBMCs) were isolated by density gradient centrifugation (1600 x g for 40min) on Histopaque (Sigma) with a density of 1.071 g/l. PBMC were washed in HBSS and resuspended in Ultraspec (Biotecx) at a concentration of 1 x 10^7 cells/ml. Spleens, lymph nodes and thymuses were harvested from two, 4-week old Ramboullet lambs, euthanized with ketamine (5 mg/kg) and xylazine (0.2 mg/kg). Ten mgs of each tissue were homogenized in 1 ml of Ultraspec. Total RNA was isolated from all of the Ultraspec cellular lysates according to the manufacturers specifications.

cDNA Synthesis. First strand cDNA was synthesized using 5 µgs of total RNA, 1 mm dNTPs, 10 U of AMV reverse transcriptase (Promega, Madison WI.) and 13 uM oligo-dT in a volume of 25µl. The primer and template were denatured for 10 minutes at 70° C before the dNTPs, buffer, and enzyme were added. The reverse transcription (RT) reaction was conducted at 42° C for 1 hour, and terminated by boiling for 5 minutes.

TCRBV polymerase chain reaction. Amplification was conducted using a panel of 16 unique TCR β-chain variable region (TCRBV) primers in conjunction with a TCR β-chain constant region (TCRBC) primer (Table 4.1). PCR reactions (50µl) contained: 10

mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 200 μm dNTPs, and 2.5 U of Taq polymerase (Perkin Elmer Cetus, Emeryville, CA.). TCRBV and TCRBC primers were included at 1 μm. A pair of α-chain constant region (TCRAC) primers was incorporated into the reaction, each at 0.025μm. These primers provided an internal standard for assessing amplification efficiencies and also facilitated normalization of TCRBV amplification among the individual samples. All reactions were conducted in HotStart 50 reaction tubes (Molecular Bio-Products, San Diego, CA.). After an initial denaturation at 94° C for 5 minutes, 31 cycles of PCR were conducted with the following cycling profile: 94° C for 1 min, 60° C for 1 min, and 72° C for 1 min. This was followed by a final incubation at 72° C for 10 minutes. PCR products were resolved by electrophoresis in 2% agarose gels. Hae III fragments of ϕX 174 DNA (Gibco BRL) were included as molecular size markers, and all bands were visualized by ethidium bromide staining.

Quantification. Agarose gels were visualized and digitized using Eagle Eye II gel scanner (Statagene, LaJolla, CA.). Digital output was analyzed using NIH Image analysis software (Shareware, NIH, Bethesda, MD.) allowing the intensities of both the TCRBV and TCRAC PCR products to be converted to pixel values. The pixel value of each TCRBV product was divided by that of the TCRAC product to normalize the TCRBV expression among the different amplification reactions. Relative expression was determined by dividing the normalized value for each TCRBV by the sum of the normalized values for all 16 TCRBV gene segments analyzed and multiplying by 100.

Table 4.1. Oligonucleotide primers used in the Vβ RT-PCR method and the expected product sizes. All β-chain primer sequences were deduced from the CDR2 region of ovine TCRBV genes (Halsey *et al.* 1999). The β-chain constant region primer was deduced from published data (Grossberger *et al.*, 1993), as were the α-chain primers (Jein *et al.*, 1991).

Vβ	Variable Region Primer Sequences	Product Size (bp)
1.1	5'-CAG TATTACAGTCAGCGA-3'	428
1.2	5'-TTACCGCGGACAACCTAG-3'	428
1.3	5'-TACAGCCAGAACGTGTAT-3'	428
1.4	5'-TATGATGGGAAAGTGCAT-3'	422
2.1	5'-CGTCAGTTCCCGAAACGC-3'	473
3.1	5'-ATGTTGACTTGCTTCTTG-3'	430
4.1	5'-TTCCAGGACAGAGCTTGG-3'	469
6.1	5'-GGAGGAATCAGACAAATC-3'	424
7.1	5'-CAGAAGCCGCCAGAGCTC-3'	461
8.1	5'-TGCGGGGACTGGAGTTAC-3'	460
10.1	5'-AAGGAGCATTTGGGTTTT-3'	454
13.1	5'-CCTATCCCACCACAGAAA-3'	421
13.2	5'-CCGCTCCCACCACGGTTC-3'	422
17.1	5'-AACAGGATTTTGATTACG-3'	505
24.1	5'-GCCAAGCACCAAAACTGC-3'	460
28.1	5'-AAGACTCGGGATTTGGAC-3'	456
Constant Region Primer Sequences		
Cβ	5'-TCCAGATACTGTCTGGGC-3'	
CαF	5'-ATCAAGGACCCCAACCCC-3'	361
CαR	5'-GTGACCGTGTTCCGCATC-3'	

Results

Validation of the RT-PCR method. A PCR-based method was developed to analyze the relative expression of ovine TCRBV genes. Initially, the ability of each of the primers to amplify the corresponding variable region genes was assessed using plasmid templates. These plasmids contained the β-chain cDNAs from which the TCRBV sequences originally were derived (Halsey, *et al.* 1999). Amplification of these plasmids with the appropriate TCRBV primers, in conjunction with a TCRBC primer, produced single bands of the expected sizes in all cases (Figure 4.1A). Importantly, the intensities of the TCRBV products were comparable among the sixteen different reactions, indicating that the amplification efficiencies for each of these individual TCRBV primers were similar. This panel of primers subsequently was used to amplify cDNA produced from peripheral blood T lymphocyte mRNA (Figure 4.1B). In these reactions, a TCRAC internal control fragment also was amplified. Thirty-one cycles of amplification were performed for these reactions, conditions determined in previous optimization experiments to produce TCRBV and TCRAC fragment intensities which were proportional, in a linear manner, to the quantities of mRNA in the original sample (data not shown). This linearity allowed quantification of the relative expression of individual TCRBV genes by T lymphocyte populations as described in Materials and Methods.

This method proved to be highly reproducible (Figure 4.2). Three separate analyses were conducted with a single preparation of PBMC RNA, each beginning

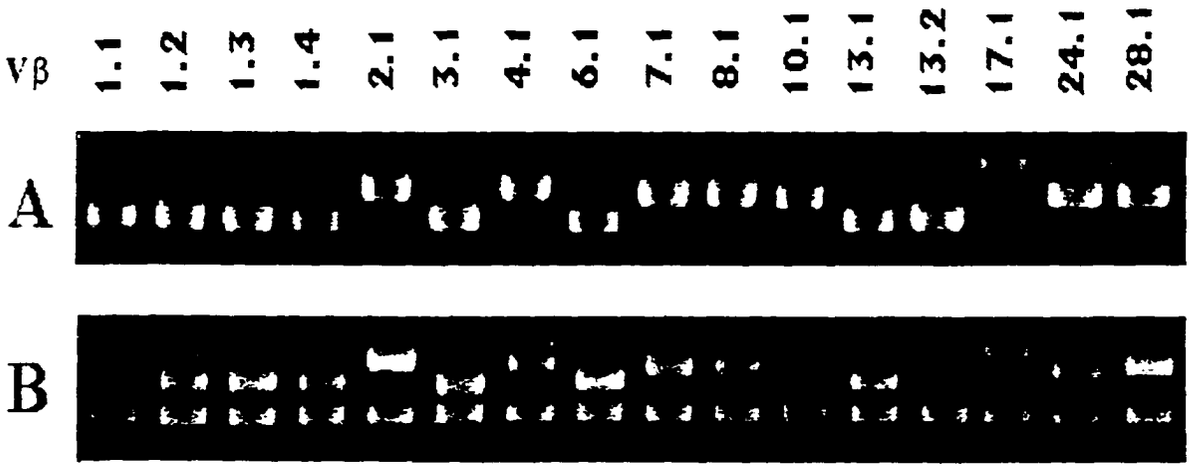

Figure 4.1. A) Representative gel of the PCR products produced by amplifying 200 nanograms of plasmid templates containing β-chain rearrangements that utilized each of the respective variable regions. **B)** Representative gel showing RT-PCR analysis of ovine peripheral blood T cell receptor β-chain variable region expression. TCR α-chain constant region primer pair (lower band) was duplexed with the β-chain primer (upper band) and products were separated on ethidium bromide-stained gels.

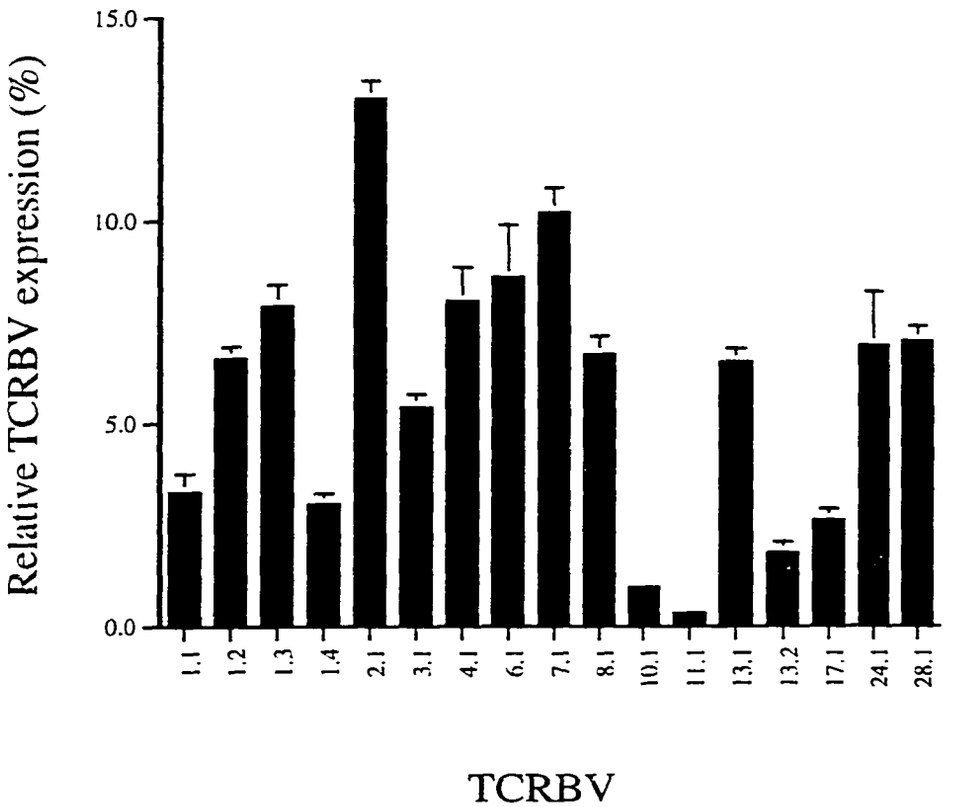

Figure 4.2. Reproducibility of the RT-PCR method. Three separate aliquots of a single RNA preparation were reverse transcribed, PCR-amplified and quantified. Bars represent the mean expression levels (± standard error) for each TCRBV.

with the reverse transcription reaction and proceeding through the PCR amplification and the quantification. Virtually identical levels of expression were obtained for each of the TCRBV genes in these three replicate assays. Specifically, the standard errors of the means for the all 16 TCRBVs analyzed were less than 15%.

Intra-individual variation in TCRBV gene expression. The relative levels of expression of individual TCRBV elements by PBMCs, within individual animals, was highly variable (see for example the pattern shown in Figure 4.2). Very low levels of expression were observed for certain TCRBV genes (e.g., TCRBV10S10), the expression of others was intermediate, while some were expressed at very high levels (e.g., TCRBV2S1). These observations indicate that the frequency of TCRBV gene expression by mature, ovine peripheral blood T lymphocytes does not reflect the stochastic utilization of germline TCRBV genes, but rather is configured by selection processes that likely occur in the thymus as has been demonstrated in other mammals.

Inter-individual variation in TCRBV gene expression. The pattern of TCRBV expression was fairly consistent in all the animals (Table 4.2, Figure 4.3). Those TCRBVs, which were expressed at the highest levels, such as TCRBVs 2S1,13S1, 28S1, 6S1, 3S1, and 1S3, were expressed at high levels in all the animals tested, irrespective of breed. Similarly, TCRBVs expressed at the lowest levels (TCRBVs 1S1, 13S2, and 10S1) were consistently low in all the animals. The rank ordering of the mean TCRBV expression levels shown in Table 4.2 illustrates these patterns.

Table 4.2. TCRBV gene expression by peripheral blood T lymphocytes of Suffolk and Ramboullet sheep. Shown are the means, standard deviations, ranges, and coefficients of variation for 5 animals from each breed. Coefficients of variation values of less than 20 indicate minimal inter-individual variation (shaded numbers). Coefficients of variation of greater than 40 indicate high inter-individual variation (boxed numbers). Below is a ranking of TCRBV usage for both breeds.

	Suffolk			Ramboullet		
Vβ	mean±stdev	range	coeff. of var.	mean±stdev	range	coeff. of var
1.1	0.68±0.42	(0.4-1.0)	**63.94**	1.25±1.15	(0.15-3.2)	**92.33**
1.2	3.86±1.19	(2.2-5.1)	30.73	5.88±0.80	(4.5-6.7)	**14.92**
1.3	6.44±1.41	(5.5-8.9)	21.90	8.23±1.25	(6.7-9.7)	**15.28**
1.4	5.59±0.67	(4.6-6.3)	**12.00**	4.37±1.31	(2.6-5.5)	25.84
2.1	17.2±6.09	(10.5-21.8)	35.32	12.7±3.35	(8.5-17.4)	26.28
3.1	7.80±1.88	(5.2-10.5)	24.22	8.55±2.49	(5.8-11.8)	29.20
4.1	6.08±3.39	(2.7-10.5)	**55.76**	4.61±0.64	(3.9-5.5)	**13.95**
6.1	7.99±1.89	(5.2-10.0)	23.26	9.55±1.32	(7.6-11.3)	**13.85**
7.1	5.69±2.73	(1.7-8.5)	**48.07**	7.33±3.37	(2.8-12.3)	**45.99**
8.1	5.19±1.76	(3.1-7.6)	34.02	6.50±1.27	(4.7-7.9)	**19.62**
10.1	2.10±1.10	(1.3-4.0)	**52.83**	2.09±2.59	(0.4-6.7)	**123.6**
13.1	11.3±2.88	(8.7-15.3)	25.89	7.88±1.78	(6.3-10.7)	22.70
13.2	0.98±0.34	(0.6-1.5)	34.94	1.53±0.77	(1.2-2.3)	**50.22**
17.1	4.65±1.45	(2.6-6.3)	31.31	3.74±0.94	(2.1-4.4)	25.33
24.1	5.78±1.25	(4.6-7.6)	21.74	6.41±0.82	(5.4-7.3)	**12.88**
28.1	8.28±1.54	(6.2-10.0)	**18.70**	9.64±0.80	(8.5-10.4)	**8.382**

More ◆——— **TCRBV Usage** ———◆ **Less**

Ramboullet 2.1, 28.1, 6.1, 3.1, 1.3, 13.1, 7.1, 8.1, 24.1, 1.2, 4.1, 1.4, 17.1, 10.1, 13.2, 1.1
Suffolk 2.1, 13.1, 28.1, 6.1, 3.1, 1.3, 4.1, 24.1, 7.1, 1.4, 8.1, 17.1, 1.2, 10.1, 13.2, 1.1

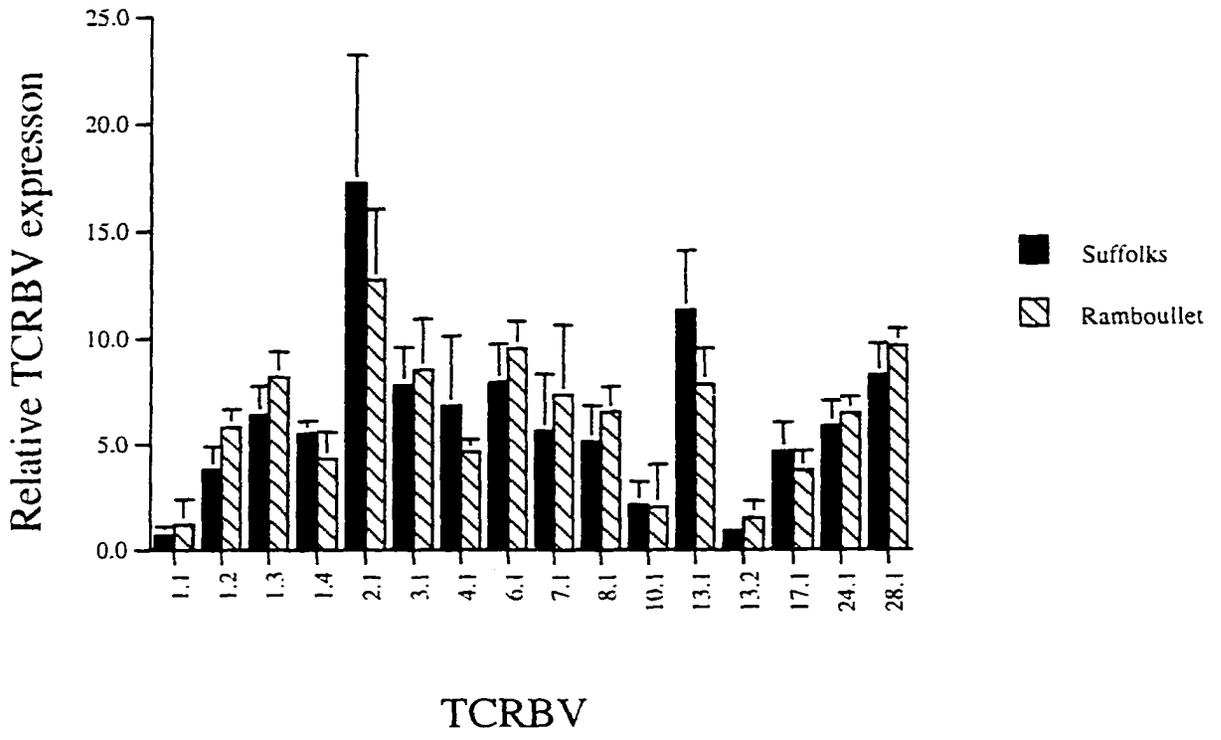

Figure 4.3. Comparision of the TCRBV gene expression in peripheral blood of Suffolk and Ramboullet sheep. Bars represent the mean (±standard deviation) of TCRBV gene expression of 5 animals.

Within this framework of relative expression established through the rank ordering of the means, considerable variability in the expression of particular TCRBVs among animals is observed. Coefficients of variation greater than 40 are indicative of widely ranging expression levels. TCRBVs 1S1, 7S1, 10S1, and 13S2 were found to vary extensively among individual sheep of both breeds. Additionally, considerable inter-individual variation in the expression of TCRBV4S1 was observed among the Suffolk sheep. This variability is reflected also in the ranges of relative expression. Expression of TCRBV4S1 and TCRBV7S1 was highly variable in the Suffolk sheep, as was that of TCRBV7S1 in the Ramboullet sheep. In each of these cases, the differences between animals with the highest and lowest expression levels were 4 to 5-fold. Even more dramatically, the ranges of expression for TCRBV1S1 and TCRBV10S1 in Ramboullet sheep varied by 21- and 16-fold, respectively. Thus, while the patterns of expression are generally conserved among all these animals, the expression of certain TCRBVs is highly variable among individuals.

While the relative expression of certain TCRBVs is highly variable among different animals, others display minimal inter-individual variation. The expression levels of TCRBVs 1S2, 1S3, 4S1, 6S1, 8S1, 24S1, and 28S1 in the Ramboullet sheep are only slightly variable as indicated by coefficients of variation of less than 20. Suffolk sheep display a similar lack of variation in TCRBVs 1S4 and 28S1, yet overall inter-individual variation is more pronounced in this breed compared to the Ramboullets.

Inter-breed variation in TCRBV expression. The overall patterns of TCRBV expression are well conserved among the two breeds (Table 4.2). TCRBVs 2S1,13S1, 28S1, 6S1, 3S1, and 1S3 are expressed at the highest levels in both breeds, while TCRBVs 1S1, 13S2, and 10S1 are expressed at the lowest levels. Nevertheless, a 2-tailed t-test revealed statistically significant ($p<0.01$) differences between the two breeds in the mean expression levels of three TCRBVs; 13S1, 13S2, and 1S2. The importance of these differences is suggested by the fact that two of these three TCRBVs, 13S1 and 1S2, occupy very different positions in the rank ordered means of the two breeds. TCRBV13S1, while expressed at the second highest level in Suffolks, is only the sixth most prevalent TCRBV in the Ramboullet repertoire. TCRBV1S2, while thirteenth in the rank ordered means of the Suffolks, is tenth in the Ramboullets. Two other TCRBVs, 4S1 and 8S1, also varied in their positions in the rank ordered means of the two breeds. TCRBV4S1 was seventh most utilized TCRBV in Suffolks but was the eleventh most prevalent in Ramboullets. In contrast, TCRBV8S1 was eighth in the Ramboullets, but eleventh in the Suffolks. These variations in the relative prevalence of particular TCRBVs among the two breeds likely reflect the importance of these TCRBVs in the repertoires of the two breeds and the effects of genetic differences in shaping the peripheral TCRBV repertoire.

TCRBV expression in peripheral lymphoid organs. TCRBV expression in spleen, lymph node, thymus and peripheral blood obtained from each of two one-month old Ramboullet lambs was analyzed. Intra-individual variation in the expression of TCRBV genes by peripheral blood T cells, as seen in Figure 4.2 and Table 4.2, was observed in these

animals as well (Figure 4.4). Expression of TCRBV2S1 and 13S1 were high, while TCRBVs 10S1, 13S2 and 17S1 were low, with the remaining TCRBVs expressed at intermediate levels. Further variation was observed when TCRBV expression in organized lymphoid tissues was compared to that in peripheral blood. Most notably, the expression of TCRBV13S1 in the lymph node of animal 1 was increased over that of peripheral blood by 70%. In contrast, the expression of TCRBV7S1 in the lymphoid organs of animal two was markedly reduced in comparison to peripheral blood. Ultimately no clear consensus patterns of TCRBV expression among organs of these two animals was observed. Thus, the relative expression of TCRBVs is varied in different anatomical sites, likely reflecting the differential migration of T cells throughout the body, as well as the exposure of individual animals to varied environmental antigens acquired through different routes.

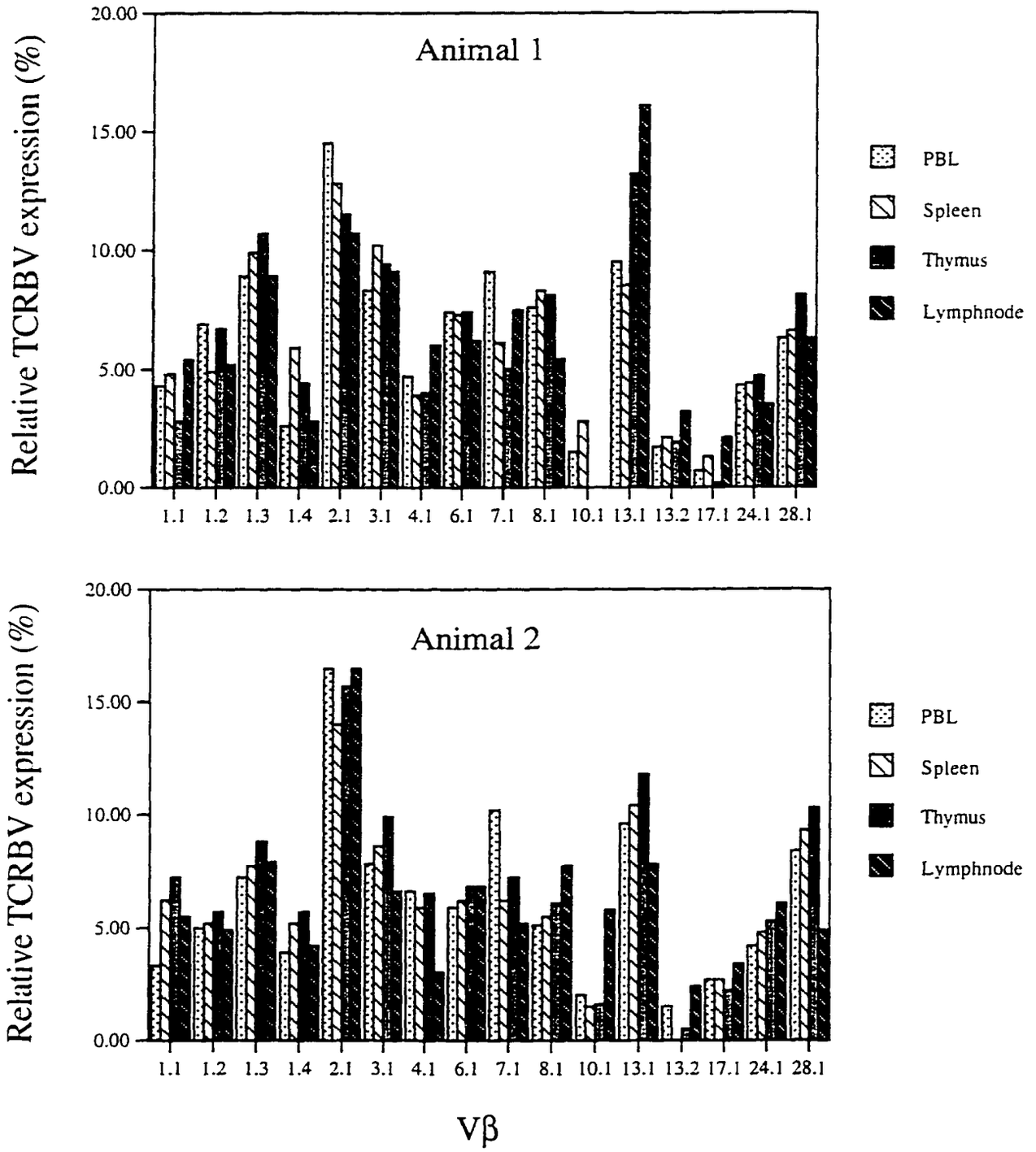

Figure 4.4. Comparision of TCRBV gene expression in peripheral lymphoid organs (spleen, thymus, caudal mediastinal lymph node, and peripheral blood) of two 1-month old Ramboullet lambs.

Discussion

We have developed a reliable and reproducible method for comparing the relative expression of 16 ovine TCRBV genes. This study focuses on TCRBV gene expression by T lymphocytes in immune organs and peripheral blood of normal sheep from two breeds. The PCR method allows for the analysis of relative TCRBV gene expression within an animal, as well as between animals. The procedure uses primers specific for the α-chain constant region as an internal control in each PCR reaction, to provide a baseline of expression between samples, allowing for a quantitative analysis of TCRBV expression.

The PCR amplification was carried out in the range of linearity, insuring that there was not a plateau in the production of product. The efficiency of amplification was also examined, demonstrating that there was comparable amplification for all the TCRBV primers. By quantifying both the α and β-primer pair products and dividing one by the other we were able to control for the standard PCR discrepancies in amplification efficiency which are observed from tube to tube. It should be noted that the values of percent relative expression of TCRBV family members do not account for the presence of TCRBV family members for which primers were not available. Accordingly, the results do not provide absolute quantitative information about specific TCRBV gene expression in a given sample, but do allow for comparison of relative levels of gene expression.

Criticisms of mRNA based techniques for the evaluation of cell surface protein expression have been raised. It has been suggested that RNA levels do not correlate well with cell surface protein expression because small populations of activated cells may

contribute significant amounts of RNA to total RNA assayed, thus, skewing the results. However, it has been demonstrated in humans and in mice that the numbers of activated cells in a normal individual is low (<1%) and that the level of expression of mRNA for TCRBV is not necessarily increased upon activation (Paillard *et al.*, 1988). Correspondingly, previous groups have found no differences between RNA and protein levels in TCRBV studies in mice using RNAse protection assays (Okada & Weissman, 1989; Singer & Balderas, 1990). In addition, many groups have reported similar TCRBV levels using Vβ monoclonal antibodies in conjunction with RT-PCR in both mice and humans (Wong *et al.*, 1993). The use of this technique, therefore, valid is for the study of an ordinarily static population of T cells such as those found in peripheral blood.

Individual animals exhibited distinct diversity with respect to TCRBV gene expression, which ranged from 25% to less than 1%. Heterogeneity in TCRBV gene expression has been well documented in humans and mice (Akolar P.N. *et al.*, 1993; Dunn *et al.*, 1993; Okada & Weissman, 1989; Singer & Balderas, 1990; Uhrberg & Wernet, 1996). TCRBV diversity in an individual is directly related to selection in the thymus and genetic traits. Most certainly genetic loci of the variable region of the β-chain influence the frequency of its recombination with the other component of the β-chain. Exogenous and endogenous antigens also play a crucial role in molding the TCR repertoire.

Several types of antigen have been identified which stimulate T cells bearing particular variable-regions β-chain elements irrespective of the other variable components of the TCR. These have been termed superantigens. Exogenous superantigens, such as the toxins produced by *Staphylococcus aureus*, stimulate T cells

bearing particular Vβ elements (Choi *et al.*, 1989; Marrack & Kappler, 1990). Interestingly, it has been demonstrated that T cells bearing human Vβ 13.2 respond to *S. aureus* toxin whereas cells bearing Vβ 13.1 do not (Choi *et al.*, 1990). Interestingly, our study has consistently shown TCRBV 13S1 to be expressed at high levels while TCRBV 13S2 is always low levels, thus, it is possible that some superantigen may be influencing the expression of these two closely related family members. Choi *et al.* (1990) demonstrated that introduction of eight amino acid residues at positions 67-77 of human Vβ 13.2 into human Vβ 13.1 were sufficient to confer the toxin reactivity to the later, suggesting that the site for toxin-binding of TCR Vβ lies in this region. Ovine TCRBV 13S1 and TCRBV 13S2 also differ at residues 69, 71 and 73 and these substitution may allow for TCRBV 13S2 to be partially deleted while TCRBV 13S1 is not.

Endogenous superantigens found in mice and known to be encoded by murine retroviruses can also influence T cell repertoires (Herman *et al.*, 1991; Marrack *et al.*, 1991). Retroviruses can encode superantigens, which can cause the deletion of T cells bearing specific Vβ elements by interaction with MHC and the variable portion of the β-chain in a non-conventional manner (Pullen *et al.*, 1990). The sheep genome has been shown to contain many endogenous retrovirus denoted by the discovery of numerous endogenous sequences which exhibit high homology with ovine pulmonary carcinoma virus (OPC), a retrovirus known to cause nasal carcinoma in sheep (Bai *et al.*, 1996). These endogenous retoviruses may influence the expression of TCRBV genes in individual animals, resulting in the variability of the TCRBV gene expression seen in sheep. Although, we have seen no deletion of specific TCRBV elements in sheep, the

entire repertoire has not yet been assayed. Thus, it is possible that deletion may be occurring in some TCRBV for which primers are not currently available.

The pattern of TCRBV expression varied between individuals of the same breed, however, there were overall commonalties. For example TCRBV 2S1 and 13S1 was always expressed at a highest levels while TCRBV 10S1 and 13S2 were always lowest (Figure 4.3). Although the sheep are from an out-bred population, the common pattern of TCRBV expression is not surprising because these individuals are of similar lineage and have been raised in the same environment and, therefore, exposed to similar antigens. The Suffolk breed exhibited more inter-individual diversity in TCRBV gene expression than the Ramboullet breed. This lack of variation in TCRBV gene expression among Ramboullet was most likely due to herd genetics rather than the breed as a whole. The breeding regime at the farm from which all the samples were taken, allows more rams to impregnate ewes in the Suffolk sheep population than the Ramboullet. It is well documented in humans that siblings have well conserved TCRBV gene expression, therefore, this herd can be seen as one large family connected by a common father and the MHC genes he has passed on to his progeny (Akolar P.N. *et al.*, 1993). These data imply that in both herds genetic factors strongly influence TCRBV repertoires, while environmental factors may be less important.

Differences in TCRBV gene expression between breeds were also identified. Two families, TCRVB 1 and TCRBV 13, contained members that were expressed at significantly different levels (P=0.01) in the two breeds. This difference in gene expression between breeds is analogous to differences see in different strains of mice. Because the animals were raised in the same environment these differences are most

likely of genetic origin. It is interesting that both families found to be expressed at different levels between breeds are multi-membered, therefore it is possible that differences between the breeds are a result of compensation by one family member for another.

Analysis of the TCRBV gene expression from the thymus and peripheral immune organs revealed similar levels of expression (Okada & Weissman, 1989). Most of the TCRBV transcripts in thymus RNA samples are from thymocytes that will never reach the periphery, whereas the peripheral TCRBV transcript are from T cells that have passed through the thymic selective processes. Because the relative transcript levels were the same in the thymus and peripheral immune organs, this suggests that thymic selection in ovine as in other species does not act solely upon the TCRBV gene segment, but more likely elements of both the α and β-chains.

In summary, these studies describe for the first time a reproducible and reliable RT-PCR method for the assessment of TCRBV gene expression in heterogeneous ovine T cell populations. In addition, the characterization of the ovine TCRBV repertoire in this study provides information pertaining to the peripheral T cell repertoire in healthy sheep. Furthermore, and most importantly, the method and the information on TCRBV gene expression of healthy individuals will facilitate the study of T cell-mediated protection and/or pathogenesis in ovine disease models.

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Chapter 5

Preferential expansion of T cells with a predominant Vβ usage in the lungs of ovine lentivirus-infected lambs

Abstract

Ovine lentivirus- (OvLV-) induced pulmonary disease in neonatal lambs is a model for pediatric human immunodeficiency virus- (HIV-) associated lymphoid interstitial pneumonia (LIP). While the mechanisms of lentivirus-associated lymphoproliferative disease are poorly understood, T cells are thought to play an important role in the initiation and perpetuation of the lesions. To further elucidate their involvement in LIP, T cells from the lungs of lambs experimentally infected with OvLV were characterized. Reverse transcriptase-polymerase chain reaction (RT-PCR) analyses revealed statistically significant increases in the relative expression of the TCR β-chain variable genes (TCRBV) 4S1 (P=0.05) and TCRBV 7S1(P=0.01) by T cells in the lungs of 4-week infected lambs, versus age matched controls. Analysis of TCRBV complimentary determining region 3 (CDR3) nucleotide sequences, suggest that TCRBV 4S1 T cells were oligoclonally expanded, while TCRBV 7S1 populations were polyclonally expanded. RT-PCR analysis of cytokine gene expression by these lung T cells revealed a 35-fold increase in IFN-γ expression and a 3-fold increase in IL-2 expression in infected versus control lambs, while IL-10 and IL-4 were expressed at equivalent levels in both groups. Analysis of magnetically-selected lung T cell subsets

revealed TCRBV 4S1 T cells to be primarily CD4+, while TCRBV 7S1 T cells were predominantly CD8+. Furthermore, analysis of T cell subsets correlated IFN-γ expression predominantly with CD8+ T cells. Collectively, these findings suggest that the heterogeneity of virus-specific T cells in the lungs of OvLV-infected lambs is limited, and that *in situ* proliferation of T cells in a T helper 1 (Th1) directed manner contributes to the pathogenesis of lentivirus-associated LIP.

Introduction

Lymphoid interstitial pneumonia (LIP) is a lymphoproliferative lung disorder associated with human immunodeficiency virus (HIV) infection. Although, LIP has been documented in HIV positive adults (Travis *et al.*, 1992), it is more commonly seen in HIV positive children (Joshi *et al.*, 1985; Joshi *et al.*, 1990; Scott, 1991). The prevalence rate of LIP has been reported to be 40% in HIV positive children with or without full-blown AIDS (Sharland *et al.*, 1997). In recent years there has been a rise in the number of cases of HIV-associated LIP, due to the global spread of HIV infection. LIP is distinguished clinically from opportunistic infections by a more indolent presentation, with less severe, more gradually appearing symptoms (Rutkin *et al.*, 1998). Typical physical findings include dyspnea, decreased lung function, and increased occurrence of bacterial infections of the lower respiratory tract (Fishback & Koss, 1996; Sharland *et al.*, 1997).

LIP is thought to be a result of aberrant immune regulation, leading to uncontrolled inflammation. HIV-associated LIP is a progressive disease characterized by an increase in lymphocytes and macrophages in the lungs, with the most profound expansion seen in the CD8+ T lymphocyte population (Wallace *et al.*, 1984). Trentin *et at.* (1996) have shown a significant over-expression of T cells bearing TCR β-chain variable region Vβ 2, Vβ 3, Vβ 7, and Vβ 9 in the lungs of HIV infected patients, and it was noted that T cells bearing these Vβs virtually all belonged to the CD8+ T cell subset. Additionally, high levels of proinflammatory cytokines such as, TNF, IL-1, IL-6, IFN-γ and IL-2 have been detected in the lungs of HIV-infected individuals (Agostini *et al.*, 1996). Inflammation associated with LIP rarely leads to complete pulmonary failure, but

it has been documented recently that LIP or CD8+ T cell mediated lymphocytic alveolitis in individuals without opportunistic infection is correlated with a high hazard of death (Agostini *et al.*, 1991).

A pulmonary lymphoproliferative condition, clinically and histologically similar to HIV-associated LIP, is observed also in the lungs of sheep infected with ovine lentivirus (OvLV). OvLV-infection is a progressive disease, marked by lymphoid interstitial pneumonia (Mornex *et al.*, 1994; Watt *et al.*, 1992) and CD8+ T cell mediated lymphocytic alveolitis (Begara *et al.*, 1996; Cordier *et al.*, 1992; Lujan *et al.*, 1995). OvLV-infection of lambs quickly induces LIP, which normally takes months to years to develop in HIV-positive children (Schneider, 1996). In our experimental model, lambs develop marked histological lesions 4-weeks post-inoculation (PI), which temporally are correlated with a significant increase in T lymphocytes, and an inversion of the normal CD4/CD8 ratio. For these reasons, OvLV-infected lambs are a particularly useful paradigm for the study of LIP pathogenesis in pediatric HIV infection.

In the study of viral pathogenesis, the applicability of an animal model system for a human disease has to be established (Narayan *et al.*, 1988). As previously mentioned, both HIV and OvLV induce similar histological lesions in the lungs of their respective hosts. HIV also shares extensive genomic homology (Gonda *et al.*, 1985; Gonda, 1990) and has similar structural and replicative mechanisms as OvLV (Davis *et al.*, 1987). HIV (Clarke *et al.*, 1990; Plata *et al.*, 1990) and OvLV (Brodie *et al.*, 1995) are both tropic for alveolar macrophages, which are the primary cell types infected in the lungs of HIV+ patients. OvLV, unlike HIV, does not infect CD4+ T cells. This allows for the study of LIP without the confounding effects of CD4+ T cell depletion and immunosuppression.

Importantly, HIV-associated LIP in children usually develops long before the onset CD4+ T cell depletion.

To evaluate the mechanisms of lymphocyte proliferation and infiltration in the lungs of OvLV-infected lambs, we have analyzed T cell receptor (TCR) β-chain variable region (TCRBV) gene expression of BAL T cells from lambs experimentally infected with OvLV. We have concentrated on the lambs at 4-weeks PI because previous work has revealed this to be the earliest time point at which there are significant increases in T cells, specifically CD8+ T cells, and the greatest amount of lesion development. We are interested in looking at early events, because information on the T cells involved in the initiation of disease is crucial for understanding the pathogenesis of LIP.

Analysis of the TCRBV gene expression allows for the identification of T cell populations that are expanded in the lungs of infected animals. Nucleotide sequence analysis of the CDR3 regions of overexpressed TCRBV transcripts provides information on the clonal origins of the expanded populations. Additionally, correlation of TCRBV expression with cytokine secretion and phenotype aids in elucidating the function of the T cells involved in disease. A better understanding of the mechanisms of lymphocyte proliferation and infiltration in the lungs during lentivirus-infection will further extend our knowledge of LIP and will aid in the development of immunotherapies to control and prevent this condition.

Materials and Methods

Experimental Animals. Newborn Ramboullet lambs were obtained from of flock that had repeatedly tested negative for presence of anti-OvLV antibodies to viral envelope glycoprotein and capsid antigen p25 by agar gel immunodiffusion assay. Lambs were housed in raised pens in a isolation facility at Colorado State University. Lambs were fed milk replacer, creep, and grass feed. Lambs were randomized and either intratracheally inoculated with 10 mls of culture supernatant fluid containing 5 x 10^6 $TCID_{50}$ of OvLV strain 85/34, or sham inoculated with goat synovial membrane tissue culture supernatant without virus. Lambs were euthanized by intravenous administration of xylazine (0.2 mg/kg) and ketamine (5 mg/kg) followed by exsanguination from the carotid artery at 4-weeks post inoculation (PI).

Sample Collection. After lethal injection and exsanguination, lungs were removed and the right caudal lung lobe was lavaged. Briefly, 400mls of warm sterile hanks balanced salt solution (HBSS) (Sigma) was introduced in 50ml aliquots through the trachea, the lung was massaged to loosen cells, and the BAL fluid was recovered by inverting the lung. BAL fluid was centrifuged at 1000 x g for 15 minutes, the pellet was recovered, resuspended in phosphate buffered saline (PBS), and cells were enumerated. Total BAL cells were pelleted, resuspended in Ultraspec solution (Biotex) at 1 x 10^7 cells/ml, and frozen at –80° C. An additional ten million cells were used for the isolation of T cell subsets. Cells were washed with PBS containing 2% goat and 2% sheep serum, and incubated with either anti-CD4 mAb (SBUT4) or anti-CD8 mAb (SBUT8) (1:2 dilution of neat supernatants) for 30 minutes on ice. After three washes, cells were then incubated

with magnetic beads (4×10^7) coupled to rat anti-mouse IgG2a (Dynal) for 30 min on ice. Cells bound by specific antibodies coupled and, thus, bound to the beads were magnetically selected, washed 3 times and resupended in 500 µls of Ultraspec RNA isolation solution.

cDNA Synthesis. First strand cDNA was synthesized using 5 µgs of total RNA, 1 mm dNTPs, 10 U of AMV reverse transcriptase (Promega, Madison WI.) and 13um oligo-dT in a volume of 25µl. The primer and template were denatured for 10 minutes at 70° C before the dNTPs, buffer, and enzyme were added. The reverse transcription (RT) reaction was conducted at 42° C for 1 hour, and terminated by boiling for 5 minutes.

TCRBV polymerase chain reaction. Amplification was conducted using a panel of 16 unique TCR β-chain variable region (TCRBV) primers in conjunction with a TCR β-chain constant region (TCRBC) primer (Table 4.1). PCR reactions (50µl) contained: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 200 µm dNTPs, and 2.5 U of Taq polymerase (Perkin Elmer Cetus, Emeryville, CA.). TCRBV and TCRBC primers were included at 1 µm. A pair of α-chain constant region (TCRAC) primers was incorporated into the reaction, each at 0.025 µm. These primers provided an internal standard for assessing amplification efficiencies and also facilitated normalization of TCRBV amplification among the individual samples. All reactions were conducted in HotStart 50 reaction tubes (Molecular Bio-Products, San Diego, CA.). After an initial denaturation at 94° C for 5 minutes, 31 cycles of PCR were conducted with the following cycling profile: 94° C for 1 min, 60° C for 1 min, and 72° C for 1 min. This was followed by a final

incubation at 72° C for 10 minutes. PCR products were resolved by electrophoresis in 2% agarose gels. Hae III fragments of ϕX 174 DNA (Gibco BRL) were included as molecular size markers, and all bands were visualized by ethidium bromide staining.

Cytokine PCR. Primer pairs for IFN-γ, IL-2, IL-4 and IL-10 were deduced from the corresponding ovine genes sequences (Genbank). 2 μls of cDNA (the same cDNA as above) was PCR amplified separately with each cytokine primer pair and a primer pair specific for the housekeeping gene G3PDH (Table 5.1). 50 μl PCR reactions were performed as for TCR PCR. Cytokine primers were used at 1 μm and G3PDH primers at 0.1 μm. The reactions were performed as described above, except each cytokine was amplified under optimal annealing temperatures and cycle numbers. IFN-γ and IL-10 were amplified at an annealing temperature of 61°C for 30 cycles, IL-2 was amplified at 59°C for 34 cycles and IL-4 amplification was performed at 61°C for 34 cycles. PCR products were visualized by ethidium bromide staining on a 2% agarose gel along with Φ-X 174 DNA/Hae III fragments (Gibco BRL) marker.

Quantification. Agarose gels were visualized and digitized using Eagle Eye II gel scanner (Statagene, LaJolla, CA.). Digital output was analyzed using NIH Image analysis software (Shareware, NIH Bethesda MD.) allowing the intensities of both the TCRBV and TCRAC PCR products to be converted to pixel values. The pixel value of each TCRBV product was divided by that of the TCRAC product to normalize the

Table 5.1. Oligonucleotide primers used in the cytokine RT-PCR method. Primers were designed based on ovine gene sequences obtained from GENBANK.

Cytokine	Primer Sequences
IFN-g +	GCTCTACGGGGAACACAATG
IFN-g -	TGCCAAGTTGGACCCTGAGA
IL-2 +	GCTCTACGGGGAACACAAATG
IL-2 -	AGTCATTGTTGAGTAGATGCTTT
IL-4 +	CTATTAATGGGTCTCACCTCCCA
IL-4 -	CTTGCCAGGCTGCTGAGATTC
IL-10 +	TGTCTGACAGCAGCTGTACCC
IL-10 -	CACTCATGGCTTTGTAGACAC
G3PDH +	TGAAGGTCGGAGTCAACGGATTTGGT
G3PDH -	CATGTGGGCCATGAGGTCCACCAC

TCRBV expression among the different amplification reactions. Relative expression was determined by dividing the normalized value for each TCRBV by the sum of the normalized values for all 16 TCRBV gene segments analyzed and multiplying by 100.

Plasmid cloning and nucleotide sequence analysis of TCRBVs. Individual TCRBV genes, amplified by PCR, were electrophoresed in 2% agarose gels to permit their separation from the TCRAC internal standard bands. Bands representing the TCRBVs were excised and eluted from the gel. Gel-purified fragments were ligated into pBluescript SK$^-$ (Stratagene, La Jolla, CA), that had been cut with Eco RV, and T-tailed by incubation with dTTP and Taq polymerase as described (Marshuch *et al.* 1990) Ligation products were introduced by electroporation into *E. coli* XL-1 Blue (Stratagene) and colonies containing recombinant plasmids were identified by using the blue/white screen. Plasmid recombinants were confirmed by PCR amplification using a TCRBC primer and specific TCRBV primers. Recombinant plasmids were prepared from individual colonies and the nucleotide sequences of the respective TCRBV rearrangements were determined by dideoxy chain termination method using the TCRBV primers specific for the cDNA.

Results

Analysis of TCRBV usage. We analyzed TCRBV gene expression by BAL T lymphocytes from OvLV-infected and control lambs to detect T cell populations expanded in the lungs of infected lambs. Figure 5.1 shows that the mean levels of expression for most TCRBV was similar in the lungs of control and infected lambs. Infected lambs, however, demonstrated elevated expression of certain TCRBV genes, specifically TCRBV 4S1 and TCRBV 7S1. The mean expression of TCRBV 4S1 was significantly increased ($p = 0.05$) in the lungs of infected lambs ($8.01 \pm 5.6\%$), compared to that found in control lambs ($2.2 \pm 0.57\%$). Similarly, mean expression of TCRBV 7S1 in the lungs of infected lambs ($12.5 \pm 2.7\%$) was elevated over the mean expression in control lambs ($4.8 \pm 3.5\%$) ($P=0.01$). A significant ($P=0.01$) decrease in expression of TCRBV 13S2 was noted in the lungs of OvLV-infected lambs. The mean expression levels were $0.48 \pm 0.6\%$ and $3 \pm 1.06\%$ in infected and control lambs respectively. Although, TCRBV 10S1 and TCRBV 17S1 were expressed at a 2-fold greater levels in the lungs of control versus infected lambs, the differences were not statistically significant. The mean TCRBV expression levels for both control and infected lambs derived from two independent experiments, which were conducted one year apart. The increases in TCRBV 4S1 and 7S1 and the decline in TCRBV 13S2 were observed in each of the individual experiments. Therefore, these alterations were not experimental artifacts, but rather genuine alterations in specific lung T cell populations in response to OvLV-infection.

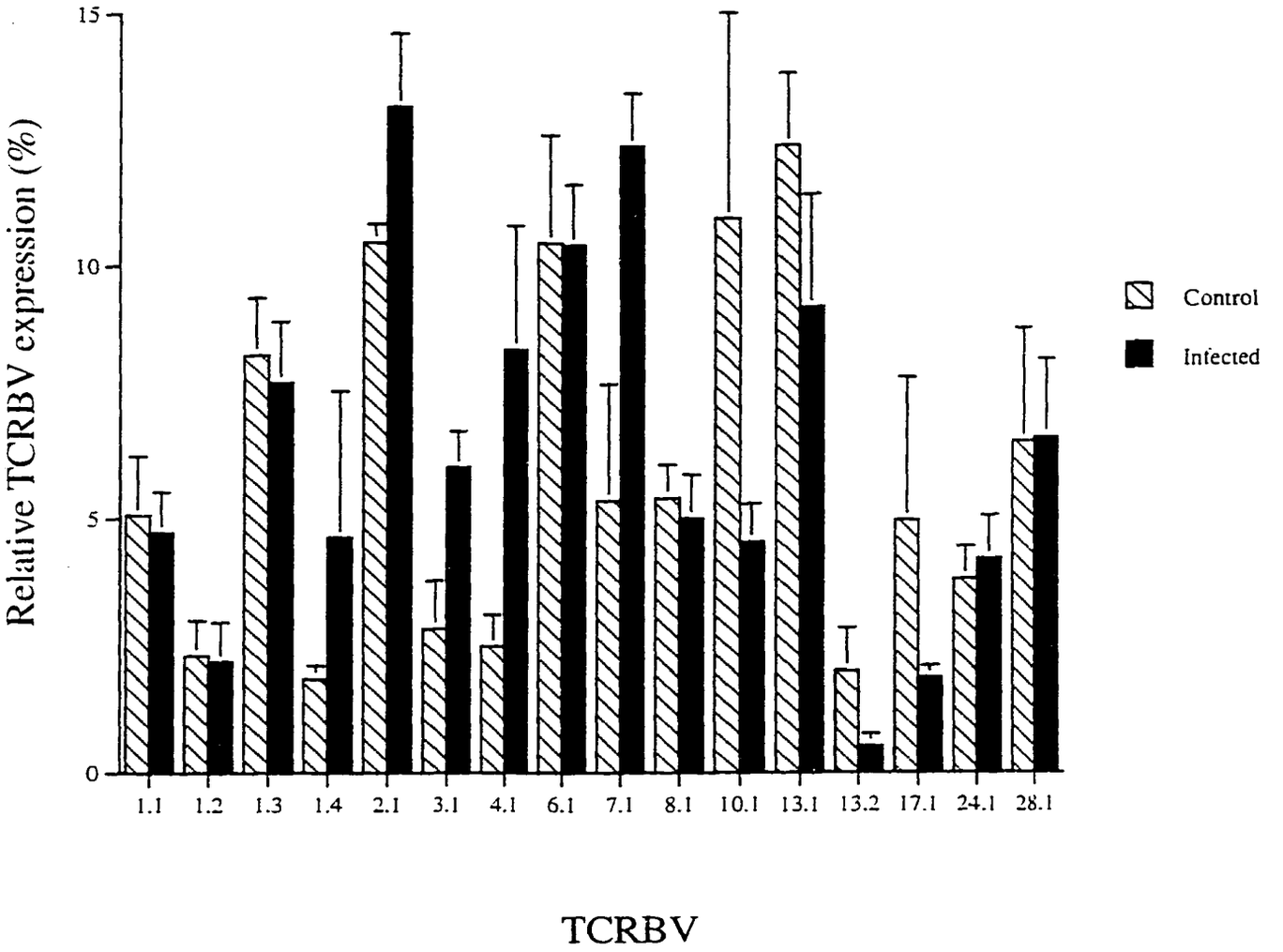

Figure 5.1. Comparision of TCRBV expression by total BAL T cells from lambs 4-weeks PI. Bars represent the means (±standard deviations) of 6 infected and 4 control lambs.

Analysis of VB juctional diversity. To assess the clonality of the T cell populations expanded in the lungs of infected animals, TCRBV 4S1 and TCRBV 7S1 PCR products from 4 infected lambs were cloned into plasmid vectors and the nucleotide sequences of individual cDNAs were determined. CDR3 region amino acid sequences deduced from this analysis are shown in Figure 5.2. A significant bias in the use of specific TCRBV 4S1 rearrangements was observed in three out of four lambs (Figure 5.2A). In lamb 1, 5 of 6 rearrangements were identical, consistent with the clonal expansion of a particular T cell in the lung of this infected lamb. Animal 2 exhibited a similar bias, with 4 identical rearrangements out of 6, again suggestive of clonal expansion in the lung. Additionally one rearrangement in lamb 1 contained 4 residues seen YGEL which were also noted in animal 4. In animal 3, TCRBV 4S1 expression was not as heavily biased by a single rearrangement, with only 2 of 5 clones exhibiting identical sequences. Notably, one of the rearrangements found in animal 3 was identical at the amino acid level to the predominant rearrangement in animal 2. While amino acid sequence identity in CDR3 particularly implicates these sequences in the response to OvLV, comparison of the nucleotide sequences revealed identity as well, suggesting that the presence of the sequence in animal 3 may be the result of PCR contamination. CDR3 sequences of TCRBV 4S1 from control lambs revealed no sequence similarity (data not shown).

Analysis of TCRBV 7S1 CDR3 sequences revealed little similarity, either within an animal or among animals, indicating a more polyclonal expansion of these T cells (Figure 5.2B). In animal 1, 2 of 6 rearrangements were identical and two more showed sequence similarities, denoted by the common use of residues, R, D, and N. Two of the four rearrangements in Animal 3 were identical. Animals 2 and 4 had completely

Figure 5.2. Predicted amino acid sequences of TCR β-chain CDR3 regions from BAL T lymphocytes. A) TCRBV 4S1 rearrangements from four infected lambs. B) TCRBV 7S1 rearrangements from 4 infected lambs. Nomenclature for ovine TCR β-chain variable and joining regions is based on nucleotide and amino acid sequence identity with homologous human β-chain elements as described by Halsey, et al. (Immunogenetics. 1999). J regions designated by W, X, Y, and Z are not homologous to any of the 13 known human Jβ elements.

A.

Animal 1			
YFCSA	GNRGI	DPLYFGAGSKLTVL	(Jβ X)
YFCSA	GNRGI	DPLYFGAGSKLTVL	(Jβ X)
YFCSA	GNRGI	DPLYFGAGSKLTVL	(Jβ X)
YFCSA	GNRGI	DPLYFGAGSKLTVL	(Jβ X)
YFCSA	GNRGI	DPLYFGAGSKLTVL	(Jβ X)
YFCS	GLQRSYGEL	HFGPGTRLTVL	(Jβ 1.1)
Animal 2			
YFCSA	AGAGGNPL	YFGPGTRLLVL	(Jβ 2.5)
YFCSA	GDLDM Q	TQYFGPGTRLLVL	(Jβ 2.5)
YFCSA	GESSREQ	TQYFGPGTRLLVL	(Jβ 2.5)
YFCSA	GESSREQ	TQYFGPGTRLLVL	(Jβ 2.5)
YFCSA	GESSREQ	TQYFGPGTRLLVL	(Jβ 2.5)
YFCSA	GESSREQ	TQYFGPGTRLLVL	(Jβ 2.5)
Animal 3			
YFCSA	GESSREQ	TQYFGPGTRLLVL	(Jβ 2.5)
YFCSA	GESSREQ	TQYFGPGTRLLVL	(Jβ 2.5)
YFCSA	GDSDQRH	TQYFGPGTRLLVL	(Jβ 2.5)
YFCSA	ESRQRG	NERLYFGNGTKLSVL	(Jβ Z)
YFCSA	Y	EQYFGPSTKLTVV	(Jβ 2.7)
Animal 4			
YFCSA	GRYGEL	HFGPGTRLTVL	(Jβ 1.1)
YFCSA	QAYNEY	HFGPGTRLTVL	(Jβ 1.1)
YFCSA	GSGPQ	ETQYFGPGTRLLVL	(Jβ 2.5)
YFCSA	GGGGMG Q	TQYFGPGTRLLVL	(Jβ 2.5)
YFCSA	GG QP	NNPLYFGGGTRLLVL	(Jβ Y)
YFCSA	SG QPNTAQ	LYFGAGSKLTVL	(Jβ X)
Vβ	**N/Dβ/N**		**Jβ**

B.

Animal 1

CASS	RDIRGNAAQ	LYFGAGSKLTVL	(Jβ X)
CASS	RDIRGNAAQ	LYFGAGSKLTVL	(Jβ X)
CASS	RQ R NT	DPLYFGAGSKLTVL	(Jβ X)
CASS	RD SNSP	LQFGIGTRLTVT	(Jβ 1.6)
CASS	SERLGGKDD	QYFGPGTKLTVV	(Jβ 2.7)
CAS	NWDSATA	GAALTFGAGSRLTVV	(Jβ 2.6)

Animal 2

CAS	NWDSGKA	GAALTFGAGSRLTVV	(Jβ 2.6)
CASS	GDGWQRAD	PLYFGAGSKLTVL	(Jβ X)
CAS	QRA	YNSRLQFGIGTRLTVT	(Jβ 1.6)
CASS	SLGG	NNPLYFGGGTRLLVL	(Jβ Y)
CASS	DSGAYDERH	FGPGTRLLVL	(Jβ 2.5)

Animal 3

CASS	NSTNTEVF	FGKGTRLTVV	(Jβ W)
CASS	NSTNTEVF	FGKGTRLTVV	(Jβ W)
CASS	RDQSSH	EQYFGPGTKLTVV	(Jβ 2.7)
CASS	RAPDSG	EQYFGPGTKLTVV	(Jβ 2.7)

Animal 4

CASS	WTADEYH	FGPGTKLTVV	(Jβ 2.7)
CASK	RTGKG	PLYFGGGTRLLVL	(Jβ Y)
CASS	ETATT	NERLYFGNGTKLSVL	(Jβ Z)
CASS	QPTGHLAAL	GPGTKLTVV	(Jβ 2.7)
Vβ	N/Dβ/N		Jβ

heterogeneous CDR3 sequences, with virtually no amino acid sequence homology between rearrangements. Of note, however, is the CDR3 sequence similarity and shared Jβ utilization of one sequence in Animal 2 with one sequence in Animal 1. As described above for TCRBV 4S1, the TCRBV 7S1 sequences from two control lambs showed no conservation of CDR3 sequence.

RT-PCR analysis of cytokine gene expression. To assess the function of T cells in the lungs of infected lambs, expression T cell cytokine genes by BAL cells was analyzed. Figure 5.3 is a representative agarose gel of IFN-γ, IL-2, IL-4, and IL-10 PCR products, as well as that of the housekeeping gene G3PDH. Annealing temperatures and cycle numbers for each set of cytokine primers were optimized for specificity and linearity (data not shown). Figure 5.4 shows the levels of expression for IL-2, IFN-γ, IL-10 and IL-4 from total BAL cells from the lungs of control and infected lambs. IFN-γ was expressed at high levels in all infected animals, with a mean expression value of 2.5±0.92, while control animals expressed virtually no IFN-γ (0.07±0.08). This reflects a 35-fold increase in IFN-γ mRNA expression in the lungs of infected lambs relative to control (P=0.007). IL-2 expression was elevated three fold in infected animals, with a mean expression level of 1.08±0.52, compared to a level of (0.38 +0.22) in control lambs (P=0.04). IL-10 was expressed at equivalent levels in both groups. Infected lambs had a mean expression of 0.67±0.25, while the value for controls was 0.55+0.37 (P=0.5). IL-4 expression was virtually undetectable in both control and infected lambs. Examination of enriched T cell subsets populations revealed IFN-γ gene expression to be twice as high in

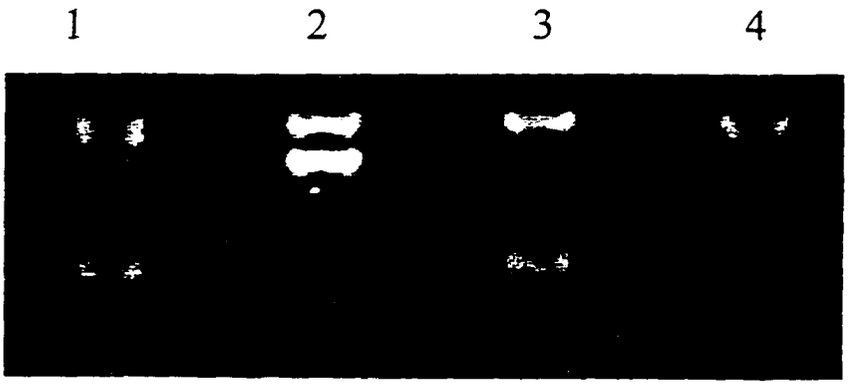

Figure 5.3. Representative gel showing RT-PCR analysis of ovine cytokine gene expression duplexed with G3PDH. Lane 1 is IL-2, G3PDH, lane 2 is IFN-γ, G3PDH, lane 3 is IL-10, G3PDH, and lane 4 is IL-4, G3PDH. The upper band in all lanes is the G3PDH product while the lower band is the cytokine

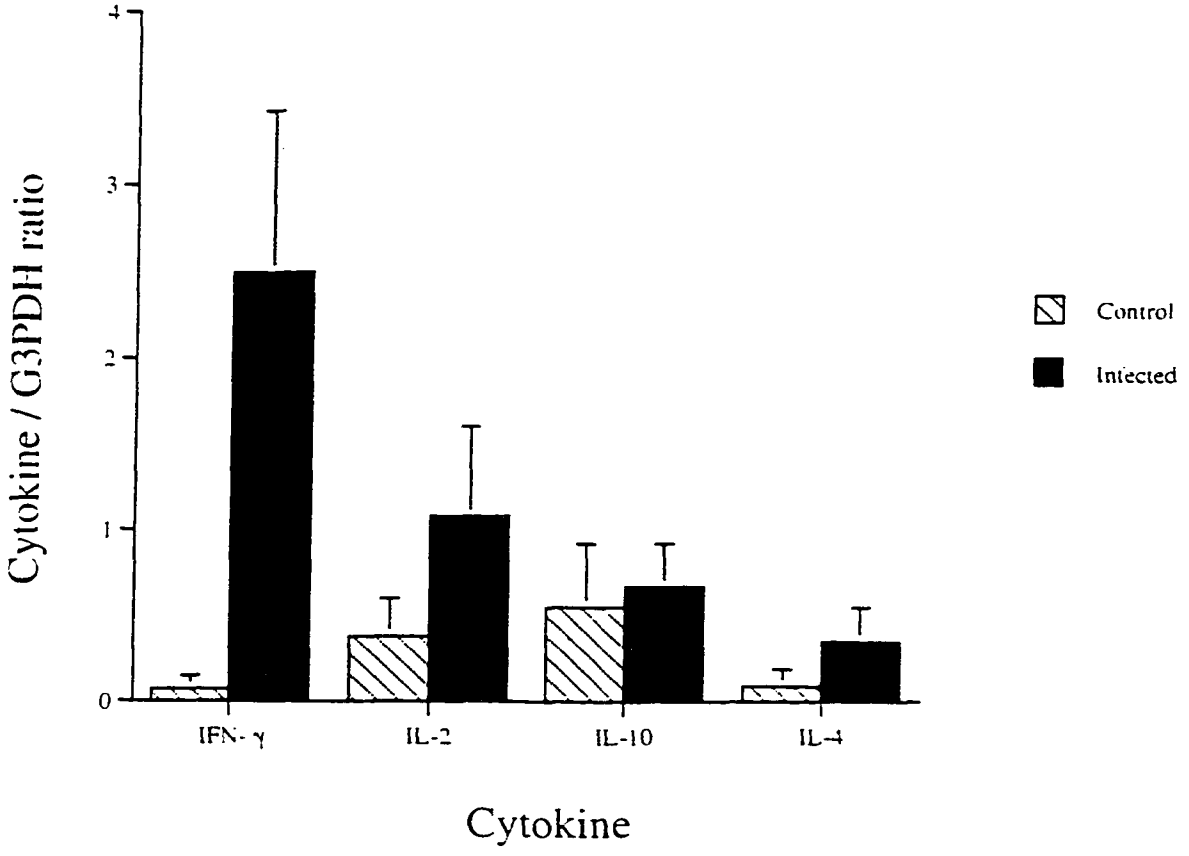

Figure 5.4. Relative cytokine mRNA levels detected by RT-PCR in total BAL cells from lambs 4 weeks PI. Bars represent the means (±standard deviations) of 4 control and 6 OvLV-infected lambs.

CD8+ T cells as in CD4+ T cells in infected lambs (Fig. 5.5), while IL-2 was expressed at similar levels in both subsets (Fig 5.6).

Phenotypic analysis of T cells expressing TCRBV 4S1 and TCRBV 7S1. The magnetically selected CD4+ and CD8+ T cell subsets described above were analyzed also for TCRBV expression. Small numbers of cells were available for each of the samples. Magnetic bead selection, in conjunction with the amplification afforded by PCR, however, made it possible to correlate TCRBV gene expression with phenotypically distinct T cell populations. The enrichment of T cell subsets by magnetic beads is indicated by the differential expression of TCRBV 4S1 and 7S1 in the selected populations versus the unselected populations. If the selections had failed to enrich the target populations, TCRBV gene expression would be the same in CD4+, and CD8+ enriched populations as in the total T cell population. Figure 5.7 demonstrates that TCRBV 4S1 expression was higher in CD4+ T cells. The mean expression of TCRBV 4S1 by CD4+ cells was 0.86 ± 0.42 where as that of CD8+ T cells was 0.35 ± 0.13. Total T cells were intermediate with a value of 0.47 ± 0.07. A similar analysis of TCRBV 7S1 expression shows it, in contrast to TCRBV 4S1, to be higher in CD8+ T cells (Figure 5.8). TCRBV 7S1 expression was lowest in CD4+ T cells (0.24 ± 0.05), highest in CD8+ T cells (0.69 ± 0.04) and intermediate in total T cells (0.27 ± 0.02). Thus, among those T cells expanded in the lungs of OvLV-infected lambs TCRBV 4S1 T cells were predominantly CD4+, while TCRBV 7S1 cells were chiefly CD8+.

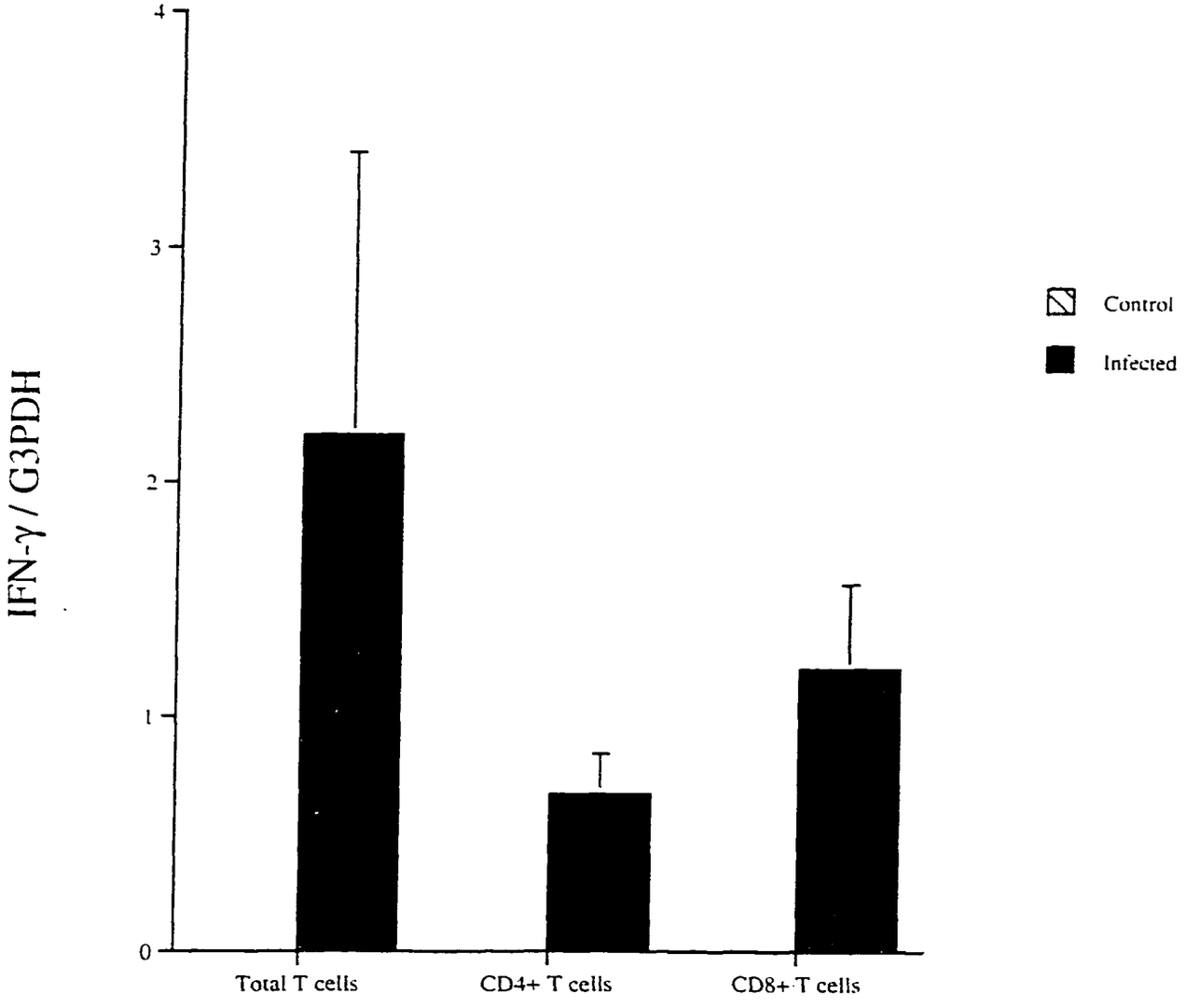

Figure 5.5. Relative IFN-γ mRNA levels detected by RT-PCR in total BAL cells and magnetically selected CD4+ and CD8+ BAL T cells from lambs 4-weeks PI. Bars represent the means (±standard deviations) of 3 OvLV-infected and 2 control lambs.

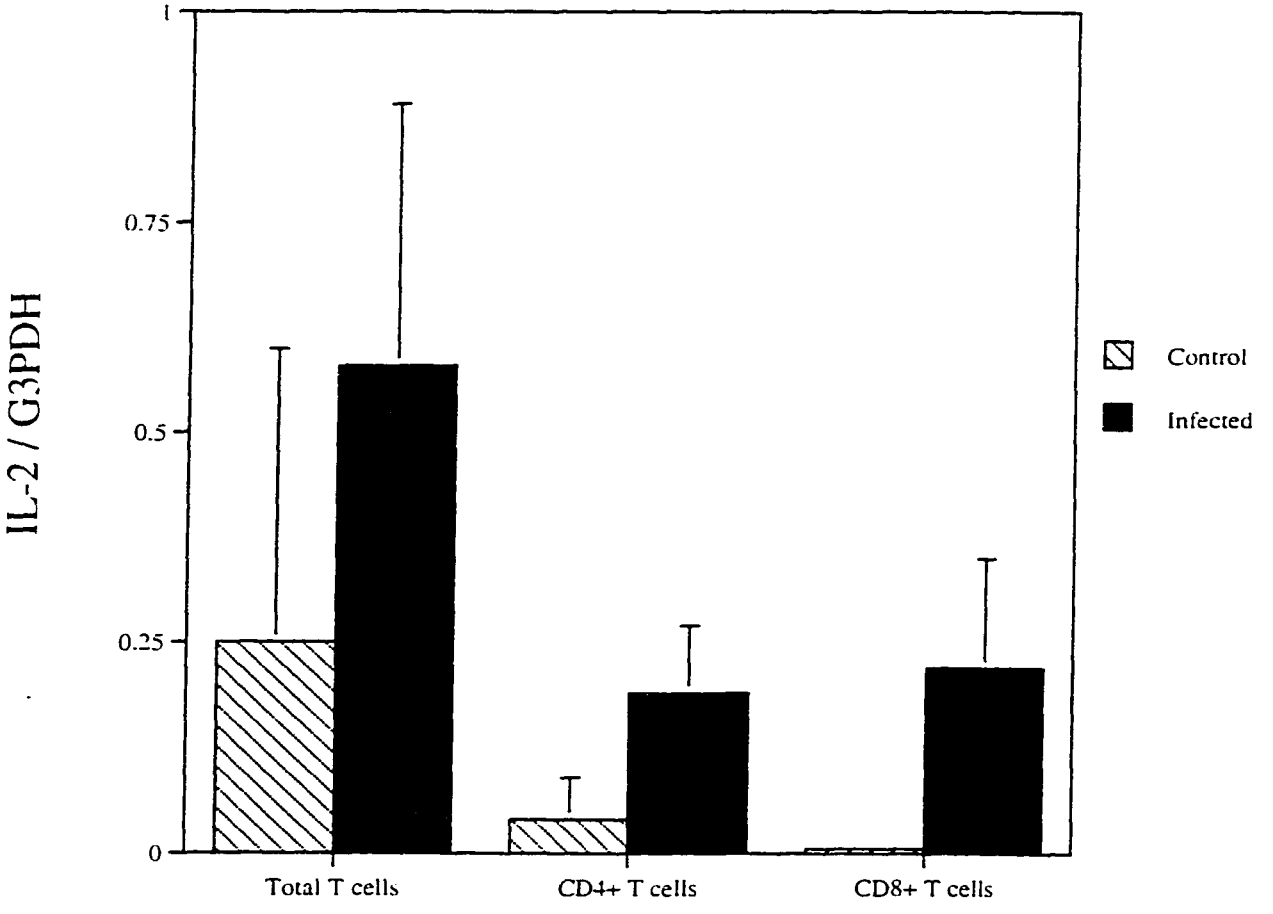

Figure 5.6. Relative IL-2 mRNA levels detected by RT-PCR in total BAL cells and selected CD4+ and CD8+ BAL T cells from lambs 4-weeks PI. Bars represent the means (±standard deviations) of 3 OvLV-infected and 2 control lambs

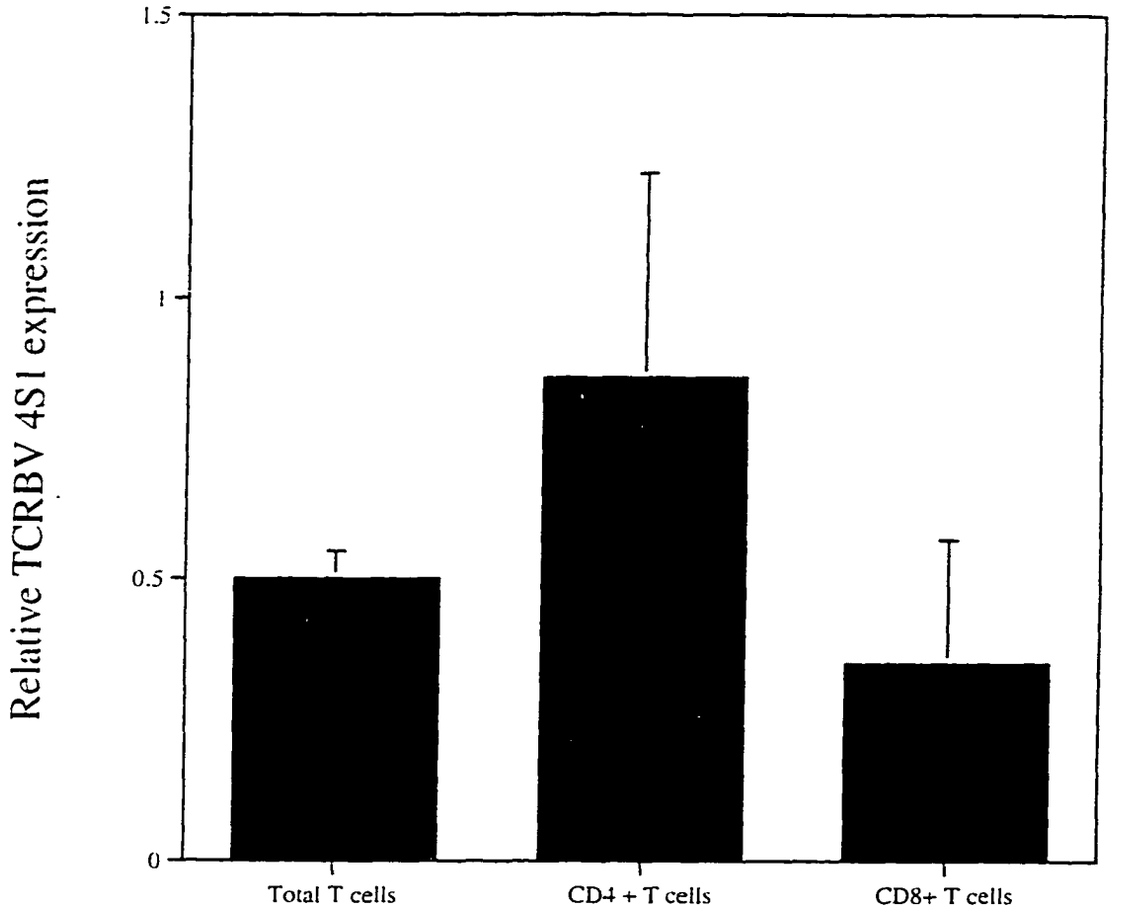

Figure 5.7. Relative TCRBV 4S1 expression detected by RT-PCR in total BAL T cells and magnetically selected CD4+ and CD8+ BAL T cells from lambs 4-weeks PI. Bars represent the means (±standard deviations) of 3 OvLV-infected lambs.

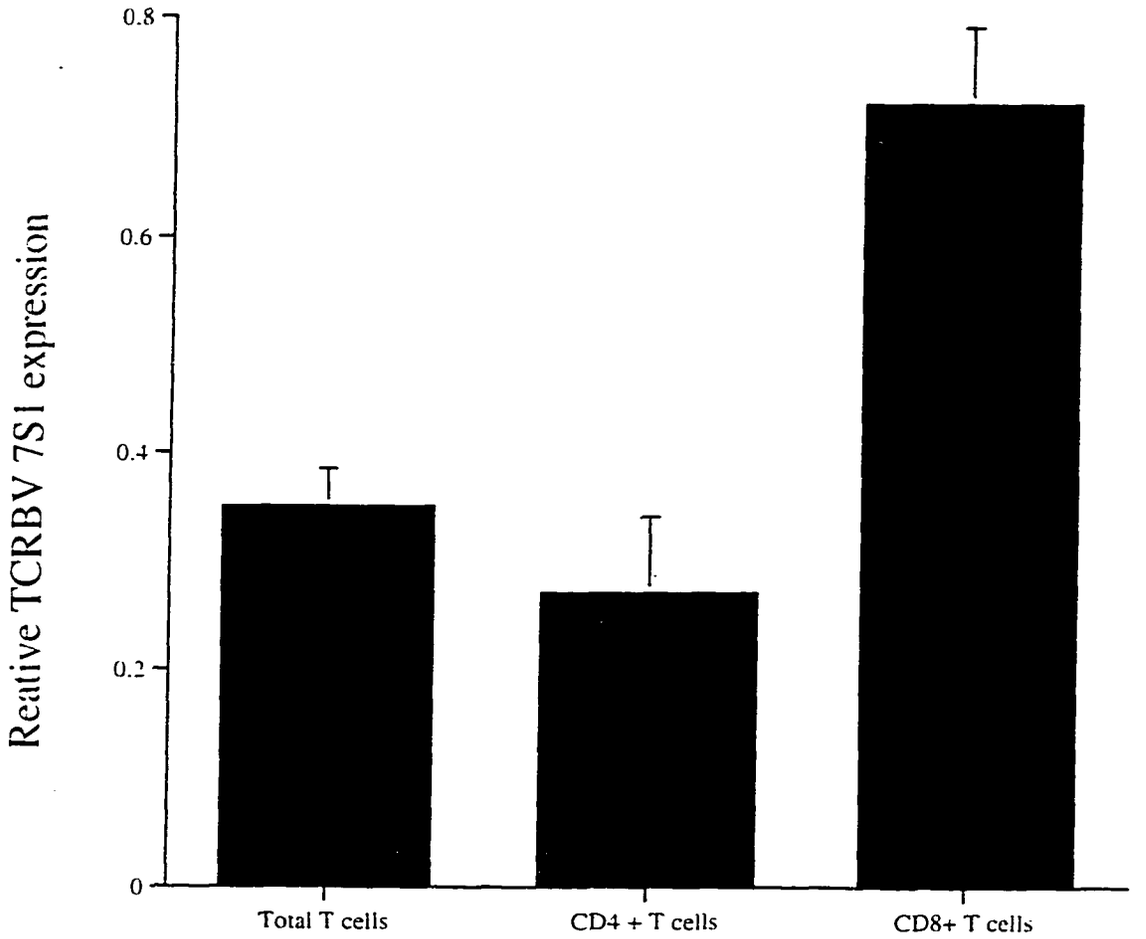

Figure 5.8. Relative Vβ 7.1 mRNA levels detected by RT-PCR in total BAL T cells and magnetically selected CD4+ and CD8+ BAL T cells from lambs 4-weeks PI. Bar represent the means (±standard deviations) of 3 OvLV-infected lambs.

Discussion

We have analyzed TCRBV gene expression by BAL T cells to determine their role in promoting leukocyte accumulation in the lungs of OvLV-infected lambs. Although, LIP results primarily from an accumulation of leukocytes in the interstitium of the lung, we have analyzed T cells recovered by BAL. It has been demonstrated that T cells isolated from the BAL fluid of healthy individuals, which is a more accessible compartment of the lung, reflect both phenotypically and functionally, T cells present in the interstitium (Garlepp *et al.*, 1992). Furthermore, T cells derived from the BAL and interstitial tissue of individuals with various types of interstitial pneumonia exhibit identical CD4/CD8 T cell ratios (Tada *et al.*, 1992). These observations indicate that analysis of BAL T cells is valid for the study of OvLV-induced LIP and other interstitial diseases.

Two TCRBV genes, TCRBV 4S1 and TCRBV 7S1 were found to be significantly overexpressed in the BAL of OvLV-infected lambs. Similar findings have been reported in HIV; T cells in the lungs of certain AIDS patients exhibit overexpression of TCRBV 2S1, 3S1, 7S1, and 9S1 (Trentin & Semenzato, 1996). The skewing of the T cell repertoire in the lung during HIV and OvLV infections likely reflects the proliferation of certain T cell populations in response to virus. This is consistent with the fact that, during both of these lentiviral infections, HIV- (Clarke *et al.*, 1990; Plata *et al.*, 1990) and OvLV-infected macrophages (Brodie *et al.*, 1995), with moderate to high viral burdens, are present in the lung.

To further define the nature of the pulmonary T cell expansion we sequenced the CDR3 regions of TCRBV 4S1 and 7S1 rearrangements from 4 infected lambs. The

CDR3 region of the β-chain is thought to bind antigenic peptide presented by MHC and, therefore, it is important in determining the nature of the T cell response. Moreover, since CDRs3 are neo-antigens unique to individual T cell progenitors, CDR3 sequence analysis provides insight into the clonal origins of the responding T cells. Sequence analysis revealed the TCRBV 4S1 CDR3 regions to be well conserved, suggesting oligoclonal expansion of T cells bearing this β-chain. Clonal expansion of CD4 + T cells has been observed in HIV infection (Roglic *et al.*, 1997; Gea-Banacloche, *et al.*, 1998). Additionally, clonal expansion of Vβ 19 T cells in peripheral blood has been noted early in HIV infection (Pantaleo *et al.*, 1994). Interestingly, patients who experience the most dramatic expansion, progressed to AIDS within 12 months, suggesting expansion of this type may reflect a limited T cell receptor repertoire, which facilitates virus escape from the immune response by limiting recognition of virus variants.

In contrast to TCRBV 4S1, TCRBV 7S1 CDR3 sequences were not conserved among infected animals. This could reflect a viral superantigen (sAg)-induced proliferation, which would cause the expansion of T cells bearing certain Vβs without regard for their CDR3 sequence and, by extension, their inherent peptide specificity. This is unlikely, however, because only CD8+ T cells exhibit greater TCRBV 7S1 expression. sAg would bind to all Vβ 7.1 bearing T cells regardless of coreceptor expression, thus, causing equal expansion of both CD4+ T cells and CD8+ T cells. The observed expansion is not likely to result either from cytokine mediated recruitment, which would cause the upregulation of ICAMs and VCAMs on the lung endothelium. Were this responsible for the influx of T cells into the lung, it would show no preference for T cells bearing particular Vβs, but would result in a lung T cell population whose

TCRBV gene expression would parallel that seen in peripheral blood. This was not observed, however, indicating that the expansion of TCRBV 4S1 and 7S1 in the lungs reflects the expansion of OvLV-specific T cells. These T cells likely were initially activated in regional lymph nodes. Post-activation, these T cells would upregulates adhesion molecules, home to the site of OvLV-infection in the lung where they would proliferate. The relative expression of TCRBVs, therefore, would be skewed to favor TCRBVs associated with virus specific T cells (in this case TCRBV 4S1 and 7S1). In humans, lymphocytosis associated with HIV-infection and mediated by CD8+ T cells appears to result from a proliferative response to virus (Itesuc *et al.*, 1993). Furthermore, in SIV infected primates, CD8-mediated lymphocytosis contributes to the development of LIP, but ultimately reduces disease severity and promotes the clearance of macrophage-tropic virus (Mankowski *et al.*, 1998).

Cytokine analysis of total BAL cells revealed that IFN-γ and IL-2 are expressed in significantly higher quantities in infected lambs that in controls. A T h 1 response, denoted by the production of IFN-γ and IL-2 previously has been noted in the lungs of OvLV-infected sheep (Lairmore *et al.*, 1988a) and in HIV-infected individuals (Agostini *et al.*, 1996; Meltzer *et al.*, 1993). Increased IFN-γ in the lungs of infected lambs could account for increased numbers of alveolar macrophages in infected lambs. IFN-γ could recruit alveolar macrophages, which subsequently become infected with virus, and present more viral peptide to T cells, inducing more IFN-γ production and establishing a cycle of macrophage infiltration and inflammation similar to that seen in the lungs of both OvLV and HIV-infected individuals. Analysis of T cell subpopulations revealed that IFN-γ was produced primarily by CD8+ T cells. Interestingly, some CD8+ T cells in

peripheral blood that produce IFN-γ have been shown to be non-cytotoxic and to secrete soluble factors that suppress HIV replication (Stranfford *et al.* 1999; Breen *et al.* 1997). In the lung, however , factors that suppress HIV replication may be unable to overcome the influence of IFN-γ on macrophage activation and recruitment. This, in part, may explain the chronic nature of the disease. In the absence of a high level of direct killing of infected macrophages by cytotoxic CD8+ T cells, macrophages remain in the lung longer and continue to produce more virus, furthering the infection. Increased IL-2 production also augments this scenario by its ability to stimulate the expansion of T cells in the lungs, thereby, facilitating the lymphoproliferative aspect of the disease (Agostini *et al.*, 1995). IL-10, produced by T cells and macrophages, also is known to control inflammation in the lung. IL-10, however, is not found at higher levels in the lungs of infected lambs, thus, it is unable to exert anti-inflammatory pressure to reduce the number of immune cells in this compartment.

The oligoclonal nature of CD4+, Vβ 4.1+ T cells is consistent with an immune response directed against a limited number of viral antigens. The polyclonal nature of CD8+, Vβ 7.1+ T cell expansion is more reminiscent of a response to many viral epitopes, presented either by many different MHC class I molecules, or one which is promiscuous in its binding of viral peptides. Regardless of the initial impetus for T cell expansion, Th 1 cytokines are produced in response to OvLV-infection and these cytokines participate in the further recruitment and expansion of immune cells in the lungs of infected animals (Figure 5.9). This leads to chronic lymphocytic alveolitis, which is perpetrated by the recruitment of new alveolar macrophage to the site by IFN-γ. These data suggest that LIP could be treated by administration of anti IFN-γ monoclonal

antibodies, or perhaps IL-10, both of which would act to counter the inflammatory Th 1 response.

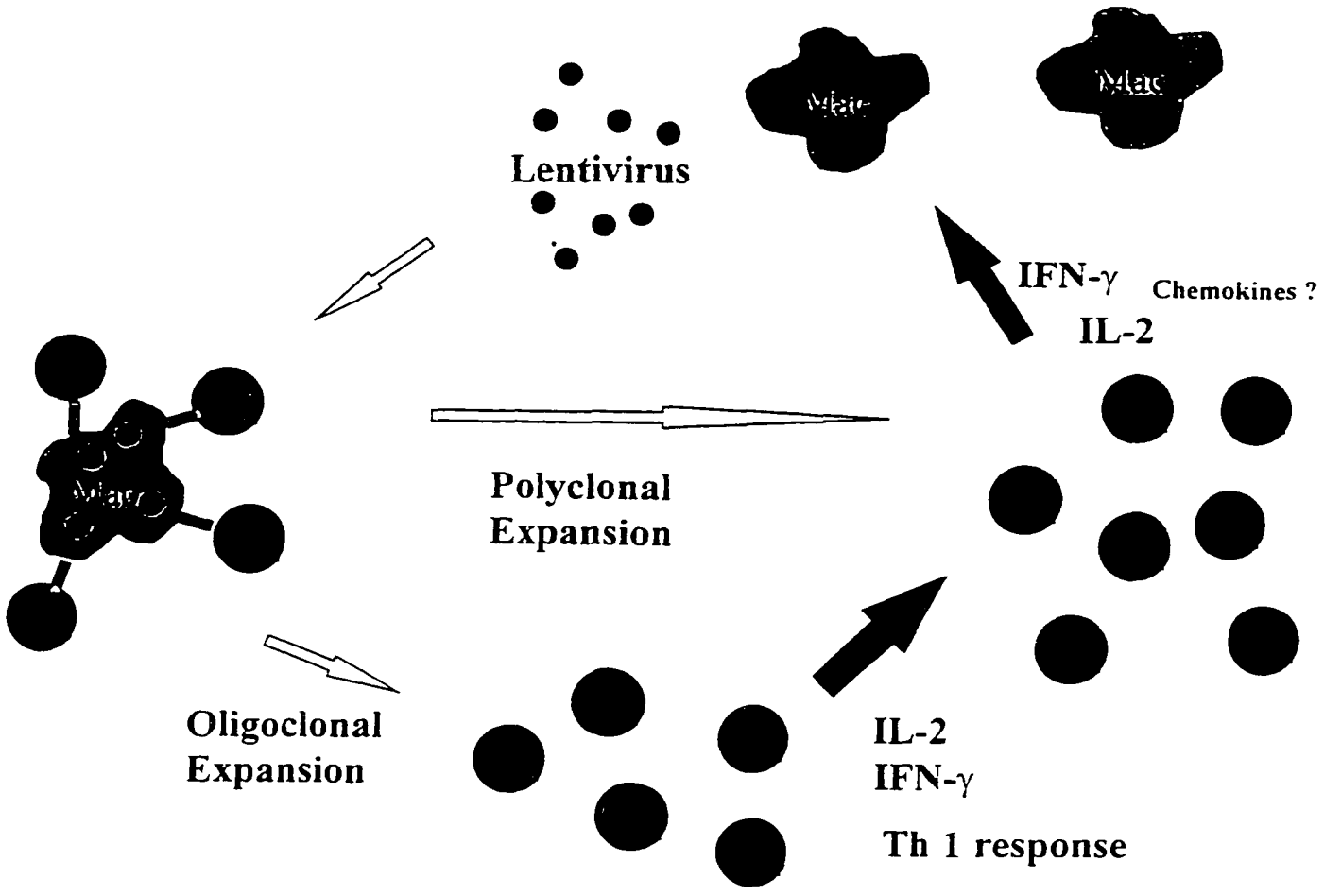

Figure 5.9. Proposed mechanisum of OvLV-induced LIP.

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Appendix 1

Summary of data for all individual animal used in this study

Lamb ID	Inoculum	Lavage	Weeks PI	%lymphocytes	Lymphocytes/ml	% T cells	Total T cells/ml	CD4+ T cells/ml	CD4 %	CD8+ T cells/ml	CD8%
96RS02	Medium	*in-vivo*	2	1 00%	6 30E+02		ND	ND	ND	ND	ND
96RS03	Medium	*in-vivo*	2	3 00%	7 20E+03	8 00%	5 76E+02	ND	ND	ND	ND
96RS04	Medium	*in-vivo*	2	8 00%	7 20E+03	5 00%	4 03E+02	ND	ND	ND	ND
96RS01	Medium	*ex-vivo*	2	2 00%	9 80E+03		ND	ND	ND	ND	ND
96RS04	Medium	*ex-vivo*	2	0 00%	0 00E+00	2 00%		ND	ND	ND	ND
96RS31	Medium	*ex-vivo*	2	5 00%	3 90E+03	9 00%	3 71E+02	ND	ND	ND	ND
96RS32	Medium	*ex-vivo*	2	1 00%	5 90E+03		ND	ND	ND	ND	ND
97RS19	Medium	*ex-vivo*	2	3 00%	5 70E+03		ND	ND	ND	ND	ND
97RS21	Medium	*ex-vivo*	2	8 00%	1 44E+04		ND	ND	ND	ND	ND
Mean				**3.44%**	**6.08E+03**	**6.00%**	**4.50E+02**	ND	ND	ND	ND
Stdev				**2.96%**	**4.43E+03**	**3.16%**	**1.10E+02**				
96RS09	OvLV	*in-vivo*	2	10 00%	6 40E+03	8 00%	5 12E+02	2 76E+02	54 90%	2 37E+03	37%
96RS10	OvLV	*in-vivo*	2	11 00%	6 82E+03	64 00%	3 96E+03	1 92E+03	48 70%	1 82E+03	46%
96RS11	OvLV	*in-vivo*	2	6 00%	7 80E+03	50 00%	3 90E+03	1 52E+03	39 40%	3 59E+03	46 10%
96RS12	OvLV	*in-vivo*	2	1 00%	1 40E+03						
96RS07	OvLV	*ex-vivo*	2	10 00%	4 90E+05	39 00%	1 91E+05	1 11E+05	58%	1 86E+05	38%
96RS13	OvLV	*ex-vivo*	2	21 00%	1 20E+06	41 00%	4 92E+05	2 66E+05	54%	1 23E+05	25 50%
96RS14	OvLV	*ex-vivo*	2	4 00%	8 40E+04	53 00%	4 45E+04	2 40E+04	54 40%	1 87E+04	42 20%
96RS15	OvLV	*ex-vivo*	2	5 00%	6 50E+04	41 00%	2 67E+04	1 63E+04	61 80%	6 93E+03	26 80%
96RS29	OvLV	*ex-vivo*	2	3 00%	1 59E+04	44 00%	7 00E+03	2 73E+03	39 60%	2 52E+03	36 90%
97RS14	OvLV	*ex-vivo*	2	2 00%	8 00E+03	42 00%	3 36E+03	2 35E+03	70%	1 14E+03	34 40%
97RS18	OvLV	*ex-vivo*	2	0 00%	0 00E+00	ND	ND	ND	ND	ND	ND
Mean				**6.64%**	**1.89E+05**	**42.44%**	**8.59E+04**	**5.32E+04**	**53.42%**	**3.85E+04**	**36.99%**
Stdev				**6.07%**	**3.70E+05**	**15.16%**	**1.64E+05**	**8.92E+04**	**9.87%**	**6.79E+04**	**7.37%**
96RS02	Medium	*in-vivo*	4	4 00%	3 60E+03	3 70%	1 33E+02	ND	ND	ND	
96RS06	Medium	*in-vivo*	4	1 00%	7 70E+02	30 00%	2 61E+02	1 64E+02	63%	6 26E+01	24 10%
96RS03	Medium	*ex-vivo*	4	1 00%	8 30E+03	64 00%	5 31E+03	4 46E+03	84%	7 97E+02	15%
96RS18	Medium	*ex-vivo*	4	1 00%	7 70E+03	46 00%	3 54E+03	2 87E+03	81%	9 91E+02	28%
97RS03	Medium	*ex-vivo*	4	1 00%	6 70E+03	24 00%	1 61E+03	1 01E+03	63%	3 82E+03	57%
97RS20	Medium	*ex-vivo*	4	0 00%	0 00E+00	ND	ND	ND	ND	ND	ND
Mean				**1.33%**	**4.51E+03**	**33.54%**	**2.17E+03**	**2.13E+03**	**72.75%**	**1.42E+03**	**31.03%**
Stdev				**1.37%**	**3.59E+03**	**22.79%**	**2.23E+03**	**1.92E+03**	**11.32%**	**1.65E+03**	**18.15%**
96RS08	OvLV	*in-vivo*	4	1 00%	1 70E+03	47 00%	7 99E+02	4 00E+02	50 90%	3 09E+02	38 70%
96RS11	OvLV	*in-vivo*	4	28 00%	1 18E+05	63 00%	7 41E+04	2 15E+04	29 70%	3 78E+04	51 60%
96RS17	OvLV	*in-vivo*	4	1 00%	1 10E+03	ND	ND	ND	ND	ND	ND
96RS09	OvLV	*ex-vivo*	4	7 00%	5 20E+05	58 00%	3 02E+05	1 36E+05	45 60%	1 51E+05	50 50%
96RS10	OvLV	*ex-vivo*	4	18 00%	4 50E+05	64 00%	2 88E+05	1 50E+05	52 70%	1 67E+05	37 60%
96RS12	OvLV	*ex-vivo*	4	10 00%	2 40E+05	63 00%	1 51E+05	3 48E+04	23 20%	1 03E+05	68 70%
96RS17	OvLV	*ex-vivo*	4	2 00%	3 80E+04	41 00%	1 47E+04	6 77E+03	46%	5 00E+03	34%
97RS13	OvLV	*ex-vivo*	4	10 00%	1 50E+04	49 00%	7 35E+03	3 38E+03	46%	3 31E+03	45%
97RS15	OvLV	*ex-vivo*	4	0 00%	0 00E+00	55 00%			50%		42%
Mean				**8.56%**	**1.54E+05**	**55.00%**	**1.20E+05**	**4.41E+04**	**43.01%**	**5.83E+04**	**46.01%**
Stdev				**9.38%**	**2.04E+05**	**8.57%**	**1.31E+05**	**6.44E+04**	**10.69%**	**7.23E+04**	**11.03%**

Lamb ID	Inoculum	Lavage	Weeks PI	%lymphocytes[a]	Lymphocytes/ml	% T cells[b]	Total T cells/ml	CD4+ T cells/ml	CD4 %[c]	CD8+ T cells/ml	CD8%[d]
96RS02	Medium	ex-vivo	10	1 00%	2 40E+03	ND	ND	ND	ND	ND	ND
96RS06	Medium	ex-vivo	10	2 00%	3 80E+02	14 00%	5 30E+01	3 80E+01	72 00%	8 00E+00	16 00%
96RS22	Medium	ex-vivo	10 ND		ND		ND	ND	ND	ND	
96RS23	Medium	ex-vivo	10 ND		ND	7 00%	ND	ND	50 00%	ND	37 00%
Mean			**1.50%**		**1.39E+03**	**10.50%**	**5.30E+01**		**61.00%**	**8.00E+00**	**26.50%**
Stdev			**0.71%**		**1.43E+03**	**4.95%**			**15.56%**		**14.85%**
96RS08	OvLV	ex-vivo	10	2 00%	8 74E+03	59 00%	5 16E+03	3 30E+02	64 00%	1 53E+02	35 00%
96RS11	OvLV	ex-vivo	10	25 00%	9 19E+05	67 00%	6 16E+05	3 17E+04	52 00%	3 05E+04	50 00%
96RS16	OvLV	ex-vivo	10	15 00%	1 64E+05	ND	ND				
96RS24	OvLV	ex-vivo	10	0 00%	0 00E+00	49 00%	0 00E+00		41 00%		68 00%
96RS25	OvLV	ex-vivo	10	60 00%	6 70E+05	54 00%	3 62E+05	1 08E+04	30 00%	5 22E+04	69 00%
97RS17	OvLV	ex-vivo	10	2 00%	1 34E+05	ND	ND	ND	ND	ND	ND
Mean			**17.33%**		**3.16E+05**	**57.25%**	**2.46E+05**	**1.43E+04**	**46.75%**	**2.76E+04**	**55.50%**
Stdev			**23.04%**		**3.84E+05**	**7.68%**	**2.99E+05**	**1.60E+04**	**14.59%**	**2.61E+04**	**16.22%**
96RS02	Medium	ex-vivo	18	10 00%	9 40E+03	65 00%	6 11E+03	4 58E+03	75 80%	9 78E+02	16%
96RS06	Medium	ex-vivo	18	2 00%	1 00E+04	5 00%	5 20E+02	4 21E+02	81%	1 45E+02	28%
96RS22	Medium	ex-vivo	18	1 00%	2 20E+03	ND	ND	ND	ND	ND	ND
96RS23	Medium	ex-vivo	18	0 00%	0 00E+00	ND	ND	ND	ND	ND	ND
Mean			**3.25%**		**5.40E+03**	**35.00%**	**3.32E+03**	**2.50E+03**	**78.40%**	**5.61E+02**	**22.00%**
Stdev			**4.67%**		**5.05E+03**	**42.43%**	**3.95E+03**	**2.94E+03**	**3.68%**	**5.89E+02**	**8.49%**
96RS08	OvLV	ex-vivo	18	13 00%	1 01E+05	51 00%	5.17E+04	2 90E+04	56 10%	3 21E+04	62 90%
96RS11	OvLV	ex-vivo	18	5 00%	1 30E+04	70 00%	9 10E+03	5 10E+03	56 60%	2 91E+03	32 80%
96RS16	OvLV	ex-vivo	18	25 00%	3 20E+05	69 00%	2 21E+05	4 86E+04	22 40%	1 44E+05	65 10%
96RS24	OvLV	ex-vivo	18	8 00%	5 28E+04	55 00%	2 90E+04	8 71E+03	30%	2 09E+04	72%
96RS25	OvLV	ex-vivo	18	7 00%	1 66E+04	41 00%	6 89E+03	3 03E+03	44 10%	4 96E+03	72 10%
97RS17	OvLV	ex-vivo	18	6 00%	2 70E+02	ND	ND	ND	ND	ND	ND
Mean			**10.67%**		**8.40E+04**	**57.20%**	**6.35E+04**	**1.89E+04**	**41.84%**	**3.45E+04**	**60.98%**
Stdev			**7.55%**		**1.21E+05**	**12.34%**	**8.98E+04**	**1.95E+04**	**15.37%**	**5.86E+04**	**16.28%**

Lamb ID	Inoculum	Lavage	Weeks PI	AGID	Western	Virus isolation	Inflamation	Total cell/ml	% Macrophage	Macrophages/ml
96RS02	Medium	*in-vivo*	2	-	-	-	ND	6.30E+04	99.00%	6.24E+04
96RS03	Medium	*in-vivo*	2	-	-	-	ND	2.40E+05	97.00%	2.33E+05
96RS04	Medium	*in-vivo*	2	-	-	-	ND	9.00E+04	92.00%	8.28E+04
96RS01	Medium	*ex-vivo*	2	-	-	-	0	4.90E+05	98.00%	4.80E+05
96RS04	Medium	*ex-vivo*	2	-	-	-	0.5	1.30E+06	100.00%	1.30E+06
96RS31	Medium	*ex-vivo*	2	-	-	-	0	7.80E+04	95.00%	7.41E+04
96RS32	Medium	*ex-vivo*	2	-	-	-	0	5.90E+05	99.00%	5.84E+05
97RS19	Medium	*ex-vivo*	2	-	-	-	0	1.90E+05	97.00%	1.84E+05
97RS21	Medium	*ex-vivo*	2	-	-	-	0	1.80E+05	92.00%	1.66E+05
Mean							**0.083**	**3.58E+05**	**96.56%**	**3.52E+05**
Stdev							**0.2041241**	**3.98E+05**	**2.96%**	**4.00E+05**
96RS09	OvLV	*in-vivo*	2	-	-	-	ND	6.40E+04	90.00%	6.21E+04
96RS10	OvLV	*in-vivo*	2	-	-	+	ND	6.20E+04	87.00%	5.39E+04
96RS11	OvLV	*in-vivo*	2	-	-	-	ND	1.30E+05	97.00%	1.26E+05
96RS12	OvLV	*in-vivo*	2	-	-	+		1.40E+05	93.00%	1.30E+05
96RS07	OvLV	*ex-vivo*	2	-	+	-	1	4.90E+06	90.00%	4.41E+06
96RS13	OvLV	*ex-vivo*	2	+	+	-	2	5.80E+06	60.00%	3.40E+06
96RS14	OvLV	*ex-vivo*	2	-	-	+	0.5	2.10E+06	92.00%	1.90E+06
96RS15	OvLV	*ex-vivo*	2	-	-	-	1	1.30E+06	81.00%	1.05E+06
96RS29	OvLV	*ex-vivo*	2	-	-	+	0.5	5.30E+05	97.00%	5.10E+05
97RS14	OvLV	*ex-vivo*	2	-	-	+	1	4.00E+05	98.00%	3.90E+05
97RS18	OvLV	*ex-vivo*	2	-	-	+	1	3.60E+05	100.00%	3.60E+05
Mean							**1**	**1.44E+06**	**89.55%**	**1.13E+06**
Stdev							**0.5**	**2.04E+06**	**11.24%**	**1.49E+06**
96RS02	Medium	*in-vivo*	4	-	-	-	ND	9.00E+04	93.00%	8.37E+04
96RS06	Medium	*in-vivo*	4	-	-	-	ND	7.70E+04	99.00%	7.62E+04
96RS03	Medium	*ex-vivo*	4	-	-	-	0.5	8.30E+05	98.00%	8.10E+05
96RS18	Medium	*ex-vivo*	4	-	-	-	0	7.70E+05	75.00%	5.70E+05
97RS03	Medium	*ex-vivo*	4	-	-	-	0	6.70E+05	99.00%	6.60E+05
97RS20	Medium	*ex-vivo*	4	-	-	-	0.5	8.00E+05	95.00%	7.60E+05
Mean							**0.25**	**5.40E+05**	**93.17%**	**4.93E+05**
Stdev							**0.2886751**	**3.57E+05**	**9.22%**	**3.31E+05**
96RS08	OvLV	*in-vivo*	4	-	+	-	ND	1.70E+05	94.00%	1.60E+05
96RS11	OvLV	*in-vivo*	4	+	+	+	ND	4.20E+05	51.00%	2.14E+05
96RS17	OvLV	*in-vivo*	4	+	+	ND	ND	1.10E+05	91.00%	1.00E+05
96RS09	OvLV	*ex-vivo*	4	+	+	+	2	7.50E+06	84.00%	6.30E+06
96RS10	OvLV	*ex-vivo*	4	+	+	+	1.5	2.50E+06	67.00%	1.67E+06
96RS12	OvLV	*ex-vivo*	4	+	+	+	1.5	2.40E+06	87.00%	2.08E+06
96RS17	OvLV	*ex-vivo*	4	+	+	ND	1	1.90E+06	97.00%	1.84E+06
97RS13	OvLV	*ex-vivo*	4	+	ND	+	0.5	1.50E+05	90.00%	1.35E+05
97RS15	OvLV	*ex-vivo*	4	+	+	-	1	1.50E+06	97.00%	1.45E+06
Mean							**1.25**	**1.85E+06**	**84.22%**	**1.55E+06**
Stdev							**0.5244044**	**2.33E+06**	**15.45%**	**1.96E+06**

Lamb ID	Inoculum	Lavage	Weeks PI	AGID[a]	Western	Virus isolation[b]	Inflamation[c]	Total cell/ml[d]	% Macrophage[e]	Macrophages/ml
96RS02	Medium	*in-vivo*	10	-	-	-	ND	2 40E+05	90 00%	2 16E+05
96RS06	Medium	*in-vivo*	10	-	-	-	ND	1 90E+04	95 00%	1 81E+04
96RS22	Medium	*in-vivo*	10	-	-	-	ND	4 40E+05	ND	
96RS23	Medium	*in-vivo*	10	-	-	-	ND	1 50E+05	ND	
Mean								**2.12E+05**	**92.50%**	**1.17E+05**
Stdev								**1.77E+05**	**3.54%**	**1.40E+05**
96RS08	OvLV	*in-vivo*	10	+	+	+	ND	4 60E+04	85 00%	4 37E+04
96RS11	OvLV	*in-vivo*	10	+	+	+	ND	4 90E+05	75 00%	3 68E+05
96RS16	OvLV	*in-vivo*	10	+	+	+	ND	1 40E+05	78 00%	1 09E+05
96RS24	OvLV	*in-vivo*	10	+	+	-	ND	2 70E+05	100 00%	2 70E+05
96RS25	OvLV	*in-vivo*	10	+	+	-	ND	1 20E+05	93 00%	1 12E+05
97RS17	OvLV	*in-vivo*	10	+	+	ND	ND	6 90E+05	97 00%	6 69E+05
Mean								**2.93E+05**	**89.67%**	**2.62E+05**
Stdev								**2.50E+05**	**10.50%**	**2.33E+05**
96RS02	Medium	*ex-vivo*	18	-	-	-	0 5	9 40E+04	87 00%	8 18E+04
96RS06	Medium	*ex-vivo*	18	-	-	-	0 5	5 00E+05	98 00%	4 90E+05
96RS22	Medium	*ex-vivo*	18	-	-	-	0	2 20E+05	99 00%	2 17E+05
96RS23	Medium	*ex-vivo*	18	-	-	-	0 5	4 00E+05	100 00%	4 00E+05
Mean							**0.375**	**3.04E+05**	**96.00%**	**297195**
Stdev							**0.25**	**1.81E+05**	**6.06%**	**1.83E+05**
96RS08	OvLV	*ex-vivo*	18	+	+	+	0 5	7 80E+05	87 00%	6 78E+05
96RS11	OvLV	*ex-vivo*	18	+	+	+	0 5	2 60E+05	94 00%	2 40E+05
96RS16	OvLV	*ex-vivo*	18	+	+	+	0 5	1 30E+06	70 00%	9 10E+05
96RS24	OvLV	*ex-vivo*	18	+	+	+	0 5	8 60E+05	89 00%	5 80E+05
96RS25	OvLV	*ex-vivo*	18	+	+	+	1	2 40E+05	92 00%	2 20E+05
97RS17	OvLV	*ex-vivo*	18	+	+	ND	0 5	4 50E+03	94 00%	4230
Mean							**0.58**	**5.41E+05**	**87.67%**	**4.39E+05**
Stdev							**0.2041241**	**4.70E+05**	**9.09%**	**3.39E+05**