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DISSERTATION

VACCINATION AGAINST BOVINE MYCOBACTERIAL DISEASES AND CHARACTERIZATION OF BOVINE γδ T CELLS

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 2001

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY BRIDGET J. VESOSKY ENTITLED VACCINATION AGAINST BOVINE MYCOBACTERIAL DISEASES AND CHARACTERIZATION OF BOVINE γδ T CELLS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

VACCINATION AGAINST BOVINE MYCOBACTERIAL DISEASES AND CHARACTERIZATION OF BOVINE γδ T CELLS

Mycobacterium bovis and *Mycobacterium avium* subspecies *paratuberculosis* are the causative agents of bovine tuberculosis and Johne's disease, respectively, both of which continue to seriously impact the agriculture industry. The development of novel vaccines for bovine mycobacterial diseases would likely reduce the incidence of bovine tuberculosis and Johne's disease. The *M. bovis* BCG vaccine has been variably efficacious against experimental *M. bovis* challenge, but due to interference with diagnostic tests for bovine tuberculosis it has not been used in the field. As with bovine tuberculosis, there are currently no widely used vaccines for Johne's disease. Subunit vaccines containing the proteins found in the culture media of growing mycobacteria, along with various adjuvants and cytokines, have been shown to be protective in small animal models of both human and bovine tuberculosis. Chapters 2 and 3 describe the development and testing of subunit vaccines for both bovine tuberculosis and Johne's disease in cattle, the natural host.

The subunit vaccine for bovine tuberculosis consisted of culture filtrate proteins (CFP) from several virulent strains of *M. bovis*, along with recombinant interleukin 2 (IL-2) and monophosphoryl lipid A (MPL). Animals were vaccinated subcutaneously three times and challenged intratracheally with a virulent strain of *M. bovis*. Immunological assessments were made throughout the course of the experiment and the protective efficacy of the vaccine was assessed 17 weeks following challenge at necropsy. Unlike

the BCG vaccine, the CFP vaccine did not interfere with the tuberculin skin test but it failed to induce strong post-vaccination cellular immune responses and induced strong post vaccination and post challenge humoral responses. In addition, the CFP vaccine significantly reduced the severity of lung lesions found in the cattle. However, the CFP vaccine significantly increased the extrathoracic spread of *M. bovis*, likely an effect of the significant TH2 response that developed. Thus, although the performance of the CFP vaccine for bovine tuberculosis did not justify its use as a prophylactic vaccine, valuable information regarding the bovine immune response to CFP vaccines was obtained.

Similar to the subunit vaccine for bovine tuberculosis, the subunit vaccine for Johne's disease was composed of CFP from a clinical isolate of *M. a. paratuberculosis*, recombinant bovine IL-2, synthetic oligodeoxynucleotides (CpGs), and dimethyl dioctadecyl ammoniumbromide (DDA). Animals were vaccinated subcutaneously on two occasions and challenged orally with a clinical isolate of *M. a. paratuberculosis* on three consecutive days. Post-vaccination and post-challenge immunological assessments were made throughout the study. Preliminary evidence suggests that the CFP/IL-2/CpG/DDA vaccine induced significantly higher percentages of αβ and γδ T cells with activated phenotypes than the adjuvants alone. Similar to the results of the *M. bovis* vaccine trial however, the CFP/IL-2/CpG/DDA vaccine failed to induce significant post-vaccination cellular immune responses to *M. a. paratuberculosis* CFP. The protective efficacy of the vaccine and the biological relevance of the activated T cell populations will be conclusively determined at the conclusion of the study.

In addition to the bovine vaccine trials, this study examined phenotypic and functional characteristics of bovine γδ T cells. γδ T cells make up as much as 75% of the

circulating T cell populations in cattle. More importantly, γδ T cells have been shown to be relevant in mycobacterial infections. This study identified and characterized the expression of activation markers on two peripheral blood populations of bovine γδ T cells. Interestingly, CD62L, CD44, and CD45R were identified as useful markers of activation on both populations of γδ T cells. This study demonstrated the propensity of bovine γδ T cells to expand in response to stimulation with various mycobacterial antigens. In addition, bovine γδ T cells were identified as potent producers of IFN-γ following stimulation with a component of the mycobacterial cell wall. The immunostimulatory component of the mycobacterial cell wall was identified as mycolylarabinogalactan peptidoglycan (mAGP). Given that the bovine tuberculosis and Johne's disease subunit vaccines described in this study failed to induce significant amounts of IFN-γ following vaccination, mAGP may be a useful adjuvant component of future CFP based vaccines for bovine mycobacterial diseases.

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CHAPTER 1

Literature Review

History of Tuberculosis

Mycobacterial diseases have plagued human kind for much of history. The Old Testament makes reference to consumption (one of the many names given to tuberculosis) in Deuteronomy 28:22 (161). Ancient Egyptian mummies with skeletal lesions indicative of mycobacterial spinal diseases (73), and the discovery of acid fast bacilli in human skeletal remains found in Germany, also reveal the presence of a mycobacterial organism as early as 5000 - 3700 BC (246). Robert Koch discovered the tubercle bacillus, the etiologic agent of this ancient disease, in 1882 (47). In Koch's time over 14% of all deaths were attributed to tuberculosis (47). Today, over 100 years after its discovery, the World Health Organization reports that over one third of the world's population is infected with the tubercle bacillus, and over 2 million people die from tuberculosis every year (2) (which is about 3.65% of all deaths). Although there has been a drop in the percentage of global deaths from tuberculosis since Koch's time (14% to 3.65%), there has been a recent resurgence in tuberculosis due to the AIDS epidemic, the emergence of multi-drug resistant strains, extensive global travel, and the lack of a consistently efficacious vaccine (2).

Genus *Mycobacterium*

The genus *Mycobacterium* belongs to the family *Mycobacteriaceae* and is the only member of this family (1). The pathogenic mycobacteria, including *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium avium* subspecies *paratuberculosis*, and others are considered slow growers, taking as long as several weeks to form visible colonies on solid media. Mycobacteria are also characterized by their acid fast staining,

owing to the presence of waxy substances in the cell wall, and by DNA with a high GC content (1).

The unique cell wall structure of mycobacteria defines and greatly contributes to the pathogenicity of this genus. In addition to the chemotype IV peptidoglycan, mycobacteria contain mycolic acids (long chain fatty acids), arabinogalactan, and lipoarabinomannan (LAM). The mycolic acids found in the cell wall of mycobacteria contribute to the hydrophobicity of the wall, making it an effective permeability barrier for many antibiotics. Several studies have demonstrated the immunomodulatory effects of LAM, including functions such as inhibition of macrophage activation (262) and the induction of many macrophage cytokines (25).

Mycobacterial Diseases

Mycobacterial species are the etiologic agents of many diseases and various disease manifestations. *M. tuberculosis* can cause "classical" pulmonary tuberculosis in man, non-human primates and a few other mammals such as elephants. Other less common manifestations of *M. tuberculosis* infection are acute progressive tuberculosis (Galloping consumption), cervical adenopathy (Scrofula), abdominal tuberculosis, cutaneous tuberculosis, genitourinary tuberculosis, tuberculosis of the pericardium, and vertebral tuberculosis (Pott's disease) (91). *M. bovis* also causes tuberculosis in man. In fact before the pasteurization of milk it was quite frequently the cause of human tuberculosis. *M. bovis* has a much broader host range than does *M. tuberculosis*, capable of infecting all warm blooded animals and some cold blooded animals as well. *M. bovis* can also cause Scrofula and abdominal tuberculosis, although it is not nearly as common today as it once was. *Mycobacterium leprae* is the causative agent of both lepromatous

and tuberculoid leprosy, which primarily affects humans. *M. avium* is ubiquitous in the environment and along with the other members of the *Mycobacterium avium intracellulare* complex (MAC) it is a common pathogen of AIDS patients (108). *M. avium* subspecies *paratuberculosis* has been identified as the causative agent of Johne's disease (ruminants) and has also been implicated in Crohn's disease (humans) (120).

Diagnosis of Mycobacterial Diseases

The medical diagnosis of tuberculosis probably had its beginnings between 460 and 370 BC when Hippocrates gave an accurate description of the clinical manifestations of tuberculosis (56). An etiologic diagnosis, one that did not depend on only the clinical symptoms of the disease, could not take place however until the causative agent was identified in 1882 by Robert Koch. A definitive diagnosis of tuberculosis today can be determined by acid fast staining of tissue or sputum and chest X-rays but the most common diagnostic test is the tuberculin skin test. Credit for this discovery also goes to Robert Koch, who in 1890 announced in error that he had discovered the cure for tuberculosis (33). In fact what he had discovered was a valuable diagnostic tool based on the induction of a delayed type hypersensitivity response after the injection of a glycerine extract of a culture of the tubercle bacilli, now called Old Tuberculin.

Old Tuberculin is essentially a crude form of the substance currently used for diagnosis. The tuberculin used today is termed purified protein derivative of *M. tuberculosis* (PPD). Although the preparation of PPD varies depending on the source, the general method is to grow virulent *M. tuberculosis* in a protein free liquid broth until it reaches stationary phase and then sterilize the culture by autoclaving. The cultures are

then filtered to remove any bacilli and particulate material, the filtrate is then concentrated, and the proteins precipitated using ammonium sulfate or trichloroacetic acid (185).

The tuberculin test provides a useful assessment of whether an individual has been exposed to mycobacteria. The antigens contained within PPD have not been well characterized, owing to the denaturing of the proteins during the sterilization procedure. It is known however, that there are antigenic epitopes present that are shared with mycobacterial species other than *M. tuberculosis*. This is an important point because if an individual has been vaccinated with *M. bovis* BCG or has been exposed to environmental mycobacteria they may have a positive reaction to PPD derived from *M. tuberculosis*. In addition to the lack of specificity of this test, it was recognized soon after the introduction of PPD as a diagnostic tool, that individuals with active tuberculosis may have false negative results (43).

A positive PPD reaction is marked by the perivascular infiltration of inflammatory cells into the lower part of the dermis (191). Antigen specific T cells that recognize antigen, which has been processed and presented by macrophages, mediate this reaction. Tumor necrosis factor-α (TNF-α) and chemokines such as macrophage chemoattractant protein (MCP-1) and interleukin-8 (IL-8) all are likely contributors to the cell influx at the site of injection (34).

The 100 years of research since Koch described the "Koch Phenomenon" (the response seen in tuberculous patients following the injection of tuberculin) have not resulted in a complete understanding of the delayed type hypersensitivity (DTH) reaction that occurs in individuals that have been exposed to a mycobacterial species. At the

center of the confusion is the question " does DTH equal protection?" Given that individuals with a positive response to tuberculin were only moderately ill and those with serious and rapidly progressing disease had a negative tuberculin response, Von Pirquet concluded in the early 1900's that tuberculin reactivity correlated with protection (43). Whether DTH and protection are separate entities or whether they are different manifestations of the same process has been in debate since Von Pirquet's observations. George Mackaness proposed in the 1960's that protective immunity to intracellular organisms was due to an antigen specific activation of macrophages and that the DTH response was an exaggerated manifestation of this mechanism (182, 183). Evidence in the early 1980's suggested that two different T cell populations mediated the protective response and the DTH response (222). Furthermore, one important observation that argues that DTH and acquired specific resistance are separate mechanisms is that IFN-γ is essential for acquired specific resistance to *M. tuberculosis* but not for the DTH response to PPD (82). One of the hypotheses that has been proposed is that DTH and protection are indeed separate mechanisms and that chemokines mediate the DTH response and cytokines mediate acquired specific resistance (223).

Treatment of Mycobacterial Infections

A Historical Perspective

Many of the early treatments for tuberculosis were non-medical and usually quite ineffective. Laying on of hands by Kings, blood letting, cupping, consumption of cod-liver oil and blood (both human and animal), blistering, inhalation of a variety of gaseous mixtures and the pumping of hot gas into the rectum were among the common early "treatments" for tuberculosis (100). The early 1900's brought the collapse theory,

which was the delivery of an artificial pneumothorax in an attempt to allow the diseased lung to rest and heal. Unfortunately, very few of these treatments offered any genuine hope to the sufferers of tuberculosis.

Some of the great accomplishments of modern medicine began with the discovery of compounds that could destroy organisms without causing damage to the patient. Paul Ehrlich was among the first to discover such compounds. In the early 1900's Ehrlich demonstrated the somewhat beneficial effects of Salvarsan, an arsenical compound, against syphilis and in the 1930's the sulphonomides were discovered (124). Unfortunately none of these compounds were useful in the destruction of the acid-fast bacillus. A laboratory at Rutgers University, headed by Selman Waksman, discovered the antibacterial properties of a soil bacterium (*Actinomyces*) against a wide range of pathogens. In 1944 William Feldman and Horton Hinshaw, of the Mayo Clinic, demonstrated that streptomycin was effective against *M. tuberculosis* in guinea pigs (100). In that same year the first human clinical trial began, consisting of one woman with terminal stage pulmonary and disseminated tuberculosis, and the results were very encouraging, as the young women recovered and was doing well several years later (233). The discovery of an antibiotic that was effective against the tubercle bacilli gave many the hope of surviving this dreaded disease.

Current Treatment

The treatment of tuberculosis has come a long way from the ineffective methods of quacks and well-intentioned physicians to today's WHO recommended short course treatment. The treatment currently used is a two month regimen of rifampicin, isoniazid, pyrazinamide, and either streptomycin or ethambutol followed by four months

of rifampicin and isoniazid (325). This short course regimen is designed to curtail drug resistance, cure the patient, and decrease the transmission of tuberculosis to others (325). If followed, and the patient is infected with a drug susceptible strain of *M. tuberculosis*, the above regimen is 98% effective (325). This is indeed an impressive figure but unfortunately poor compliance with drug regimens and the emerging drug resistant strains have greatly contributed to the threat posed from tuberculosis even in this antibiotic era.

The emergence of drug resistant strains demands the development of new drugs to combat tuberculosis. These strains are infecting individuals all over the world and 13% of the new cases in the United States are caused by *M. tuberculosis* strains that are resistant to at least one of the five front-line drugs (200). A recent study on the impact of multi-drug resistant tuberculosis in six countries reported that over 20% of the new cases of tuberculosis were caused by drug resistant strains of *M. tuberculosis* (105).

One of the most important events in the history of tuberculosis came in 1998 with the publishing of the genome sequence of *M. tuberculosis* H37Rv (74). Although the genome is the key to discovering countless new drug targets much time and energy will still need to be put into identifying those targets and developing drugs that will attack those targets.

One promising new drug candidate for the treatment of tuberculosis is PA-824, a nitroimidazopyran (275). This compound has low minimal inhibitory concentrations (MIC) against numerous different strains of *M. tuberculosis*, including several drug resistant strains. Additionally, PA-824 may also be effective against "latent" tuberculosis. In vivo experiments demonstrate that this compound is as equally effective

as isoniazid in reducing bacterial load after an intravenous infection in mice or an aerosol infection in guinea pigs.

Prevention of Tuberculosis

History of the BCG Vaccine

History tells us that the eradication of diseases caused by microorganisms is possible but many factors are involved. The management of tuberculosis involves diagnosis, treatment, and prevention, all of which are essential for the eradication of this disease. In addition to more efficient and effective methods of diagnosis and the development of novel drugs to combat tuberculosis, efficacious vaccines are desperately needed.

Scientists recognized the need for a vaccine against tuberculosis but many of the early attempts failed. Among the vaccines tried were killed tubercle bacilli, use of an avirulent strain of mycobacteria isolated from a turtle, and one attempt to attenuate a virulent organism using leeches (248). In 1900 Albert Calmette and Camille Guerin started working on a vaccine for tuberculosis at the Pasteur Institute. In 1919, after 230 passages, 13 years, and one World War, these two scientists had derived an avirulent mycobacteria from a virulent organism (123). This organism, originally isolated from a cow with a *M. bovis* infection, was designated bacille Calmette Guerin (BCG). Between 1921 and 1928 over 114,000 French infants were given BCG by the oral route, no serious side effects were attributed to the vaccination, and it resulted in decreased mortality in the recipients (248).

The reputation of the BCG vaccine has varied throughout its history. The first incident to tarnish the reputation of BCG was in 1929 when 72 children died when they

were mistakenly given a virulent mycobacterial strain rather than BCG (180). This variable efficacy of the BCG vaccine has resulted in numerous questions regarding its continued use as a vaccine for tuberculosis. The protective efficacy of BCG has been assessed in numerous clinical trials. There was a positive protective efficacy in several of these trials, ranging from as high as 80% in a trial conducted in Haiti to as low as 2% in trials in Alabama and Georgia (287). There were three trials, conducted in Chingleput, Georgia, and Illinois, that had a negative protective efficacy (145). This variable efficacy of the BCG vaccine has resulted in numerous questions regarding its continued use as a vaccine for tuberculosis.

The reasons for the variable efficacy of BCG have puzzled researchers for years, resulting in the generation of numerous hypotheses to explain this phenomenon. Among these hypotheses are that pre-exposure to environmental antigens alters the immune system in such a way that protective immunity from BCG never develops. Other hypotheses are that the immunological potencies of the different vaccines varied, there were differences in the genetic susceptibility of the populations in the individual studies, and the areas in which the studies were conducted had strains of *M. tuberculosis* with varied pathogenicity. It is important to mention that although some of the aforementioned hypotheses are difficult to demonstrate experimentally they are not mutually exclusive and the poor performance of BCG in some circumstances and its inconsistency is most likely due to many different factors.

The variable efficacy of the BCG vaccine, the reemergence of tuberculosis concomitant with the HIV epidemic, the emergence of multi-drug resistant strains of *M. tuberculosis*, and the overwhelming number of deaths and people infected with *M.*

tuberculosis have heightened the quest for new and improved vaccines for tuberculosis. Vaccine development is an exciting area of research and tuberculosis researchers are exploring several design strategies looking for a tuberculosis vaccine that would be effective at curbing the global tuberculosis epidemic. Some of the strategies currently being explored are the use of subunit vaccines, DNA vaccines, and improving upon the existing BCG vaccine.

Subunit Vaccines

Researchers have known for quite some time that live mycobacteria can confer protection to a virulent mycobacterial challenge, whereas dead mycobacteria cannot (75). Although killed mycobacterial vaccines can generate some resistance to a virulent challenge, they do not generate protective T cells that confer resistance upon adoptive transfer (220). The conclusion from these observations has been that products of an actively metabolizing organism may also confer protection. Culture filtrate proteins (CFP), or proteins excreted into the culture medium by growing mycobacteria, were shown by several laboratories to induce or elicit a cell mediated immune response (11, 12, 76, 136, 212, 224). Given that these culture filtrate proteins could induce the production of interferon gamma (IFN-γ) (11, 224), a key protective cytokine in mycobacterial infections, by splenocytes harvested from mice infected with *M. tuberculosis*, the next obvious step was to investigate the protective potential of these proteins.

Early vaccination experiments involving the vaccination of mice with *M. tuberculosis* CFP with several different adjuvants were very encouraging, as some vaccines gave equal or nearly equal protection to a challenge with *M. tuberculosis* as

BCG (10, 242). The longevity of the protection induced by the CFP vaccine was investigated in one of the early CFP vaccine trials and found to be not as long lasting as that provided by the BCG vaccine (242). Therefore, results of early CFP based subunit vaccine experiments were encouraging however enhancement of the long-term protective response would be necessary.

One method of enhancing the protective response of the CFP based vaccines was to include recombinant cytokines. Two of the cytokines incorporated into subunit vaccines were interleukin 12 (IL-12) and interleukin 2 (IL-2). IL-12 was chosen because it was previously shown to enhance the secretion of IFN-γ by $CD4^+$ T cells (84, 282). IL-2 was incorporated to enhance the proliferation of specific T cells (252). The effects of the addition of IL-2 and IL-12, individually and combined, into subunit vaccines for tuberculosis has been determined in both the murine model and the guinea pig model (21). In the murine model CFP, monophosphoryl Lipid A, IL-2, and IL-12 together conferred protection after an aerosol challenge with *M. tuberculosis* that was only slightly less than that generated by BCG (21). Interestingly, in the guinea pig model, this same vaccine did not significantly reduce the number of bacteria in the lungs. The lesions in the guinea pigs that received the CFP, MPL, and only IL-2 were however much smaller and looked very similar to the lesions in the BCG vaccinated animals. Moreover, the animals that received the CFP, MPL, and IL-2 vaccine lived much longer than the control animals (21).

Rather than vaccinating with crude CFP some investigators have used either purified or recombinant antigens that are found in the CFP. A recent publication by Peter Andersen's group describes the successful use of ESAT-6 as the antigen in a subunit

vaccine against tuberculosis in the murine model (45). ESAT-6 is a mycobacterial antigen that is found in the low molecular weight fraction of CFP and is recognized by *M. tuberculosis* infected humans (297) and *M. bovis* infected cows (236). This study showed that the use of a recombinant ESAT-6 antigen along with MPL and dimethyl dioctadecylammonium bromide (DDA) as a vaccine against an aerosol challenge with *M. tuberculosis* conferred a greater reduction of the lung bacterial load than did BCG (45).

Other antigens have also been included in subunit vaccines, but with limited success. There is some evidence that heat shock protein (hsp) 60 and hsp70, confer protection to animals challenged with *M. tuberculosis* (142, 179, 227). Others have demonstrated that vaccinating guinea pigs with a combination of recombinant hsp60, hsp70, IL-2, and MPL before challenging with a low dose aerosol of *M. tuberculosis* not only conferred no protection but also caused severe damage to the lungs (295). Although the perfect combination of adjuvants, cytokines, and antigens has not been discovered, continued research to develop more effective subunit vaccines for tuberculosis and the testing of such vaccines in larger and natural models of tuberculosis remains a worthwhile endeavor.

DNA Vaccines

A relatively new approach to vaccination is the use of DNA vaccines. DNA vaccine technology has expanded following the primary observation that the injection of protein coding DNA resulted in the production of the encoded proteins in vivo (318) and was able to generate both humoral and cell mediated immunity (296). In 1993 a group demonstrated that the injection of DNA that coded for a viral protein protected mice from influenza and stimulated both antibody and cytotoxic responses (296). DNA vaccination

is currently the focus of many research efforts because it affords the immunological advantages of live attenuated vaccines while eliminating some of the safety concerns. Additionally, DNA vaccines are cost effective and relatively easy to produce (130).

The basic components of a DNA vaccine are the gene that encodes for the protein of interest cloned into a bacterial plasmid, a bacterial origin of replication, an antibiotic resistance gene, a strong mammalian promoter and Poly A tail, and CpG motifs to act as an adjuvant (130). CpG motifs are simply synthetic oligonucleotides that are produced to resemble motifs that are common to bacterial DNA. These CpG motifs were first shown to induce IFN-γ production by NK cells (321) and have since been shown to induce many other useful immunological responses. In order to take advantage of the immunomodulatory effects of cytokines, several researchers have included cytokines into DNA vaccines (158).

To date, several animal studies have been conducted using DNA vaccines to protect against virulent mycobacterial challenges. Some have been successful and some have not. The mycobacterial antigen 85 (Ag85) complex, or components of the complex, has been used in several small animal DNA vaccine trials. The members of the Ag85 complex (Ag85A, Ag85B, and Ag85C) dominate the culture filtrate of mycobacterial cultures (316) and induce strong IFN-γ production in individuals infected with *M. tuberculosis* (171). In mouse studies DNA vaccines using the Ag85 complex, or individual members of the complex, conferred significant protection against challenge with a virulent strain of *M. tuberculosis* (22, 148, 158). Vaccinating guinea pigs with Ag85A DNA conferred no significant reduction of bacterial numbers in the lungs, as compared to control animals (21). Although there was no reduction of bacterial load, the

animals receiving the Ag85A DNA vaccine had much less extensive lung involvement and lived longer than the control animals (21).

Other mycobacterial antigens have been used in DNA vaccines for tuberculosis with variable results. DNA encoding MPT64 and ESAT-6 conferred some protection against an aerosol challenge with *M. tuberculosis* H37Rv but less than that conferred by the BCG vaccine (158). DNA encoding hsp60 has been shown to be protective in mice against a *M. tuberculosis* challenge by some investigators (179) and non-protective and even damaging in guinea pigs by other investigators (295). A DNA vaccine that encoded ESAT-6, MPT64, MPT63, and KatG was also tested in the murine model of tuberculosis and provided significant protection to a *M. tuberculosis* Erdman aerogenic challenge (205).

Recently, several investigators have tried to enhance the efficacy of DNA vaccines for tuberculosis by including additional components in the vaccine. The incorporation of a tissue plasminogen activator signal sequence, designed to increase the secretion of the protein, increased the protection associated with several tuberculosis DNA vaccines (22, 324). When combined with MPT64 and ESAT-6 DNA, the gene for ubiquitin (which targets proteins for degradation by the proteosome and subsequent presentation into Class I MHC) confers significant protection to *M. tuberculosis* challenged mice (95). The co-delivery of an Ag85B or MPT64 DNA vaccine along with a DNA vaccine coding for granulocyte-macrophage colony-stimulating factor (GM-CSF) did not increase the protective efficacy over the Ag85B or MPT64 DNA vaccine alone (157).

Recombinant BCG Vaccines

Another area of vaccine research has focused on recombinant BCG vaccines. Several BCG strains have been created that secrete cytokines such as IL-2, IFN-γ, and granulocyte-macrophage colony-stimulating factor (GM-CSF). These recombinant BCG strains have been shown to stimulate antigen specific proliferation and cytokine secretion (216) (209). The preliminary evidence for the usefulness of cytokine expressing BCG vaccines is encouraging but the protective efficacy of these vaccines remains to be determined.

In addition to the creation of BCG strains that secrete cytokines, researchers have begun to make BCG strains that resemble other organisms. One example is adding pore-forming cytolysins from *Listeria monocytogenes* in an attempt to decrease the restriction of BCG antigens to particular intracellular compartments (139). Another example would be to add the RD1 and RD2 gene clusters to BCG. *M. bovis* BCG does not contain RD1 and RD2, which are found in virulent mycobacteria (186). These genetic regions were deleted during the attenuation of *M. bovis* BCG. Many researchers feel that adding back some of these antigens (coded for in the deleted gene clusters) to BCG, while avoiding the restoration of virulence, may provide a useful vaccine against *M. tuberculosis* (138). Again, there is much work to be done in this field and protection studies should provide the necessary evidence regarding the efficacy of these organisms as vaccines.

Host Response to Mycobacteria

While it is essential that mycobacteriologists continue to search for new vaccines and drugs to combat tuberculosis, it is also essential to obtain a better

understanding of the host response to mycobacteria. Several different cell populations and cell products are involved in the host response to mycobacterial infections. These cell populations and cell products work together in complex ways to combat antigenic challenges. Unfortunately, we probably understand very little of what is actually involved in an immune response to a pathogenic organism but technology and research are helping to develop a more complete picture of how immunity operates.

Macrophages

Mycobacteria primarily reside within the macrophage, which are essential components of the innate and acquired immune response to mycobacteria. It is essential to gain a clear understanding of how mycobacteria enter the host macrophage, how mycobacteria avoid normal macrophage effector functions and finally, how the macrophages are able to control and eliminate the tubercle bacilli.

Mycobacterial Entry into Macrophages

Several mechanisms for ingestion and uptake of mycobacteria by macrophages have been proposed and demonstrated. Two of the complement receptors, CR1 and CR3 have been shown to be important components in the conventional phagocytosis of *M. tuberculosis* (255). Moreover, Ag85C has been shown to contribute to the CR3 mediated uptake (140). Given that *M. tuberculosis* expresses mannose glycans and that macrophages express mannose receptors on their surface, it seems plausible that mannose receptors would be a likely mechanism for mycobacterial uptake. Mannose receptors do in fact mediate the uptake of virulent but not avirulent strains of *M. tuberculosis* (254). Virulent strains of *M. tuberculosis* contain mannose capped forms of LAM, which are responsible for the binding and subsequent uptake by macrophage

mannose receptors (256), whereas avirulent strains do not contain these mannose caps (66).

The mechanisms of mycobacterial uptake are quite complex and the different ways in which the bacilli are taken into the macrophage can affect the fate of the organism once inside. Oxygen radicals (O_2-) are some of the non-specific anti-microbial substances produced by macrophages. The mannose receptor mediated phagocytic pathway does not induce the production of oxygen radicals, which could be an important survival mechanism for mycobacteria (18). Moreover, cholesterol associated with the plasma membrane of the macrophage is essential to entry and for the association of a protein (TACO) that protects mycobacteria from degradation within the lysosome (119).

Macrophage Effector Functions and Avoidance by Mycobacteria

Once inside the macrophage, mycobacteria reside within the phagosome (135). This vesicle will eventually acidify and fuse with lysosomes, which contain toxic enzymes, to form the phagolysosome. This environment is usually detrimental to organisms that are phagocytosed yet mycobacteria have adapted to persist within this hostile environment. Experiments comparing the electron micrographs of macrophages infected with virulent *M. tuberculosis* to those of macrophages infected with avirulent *M. tuberculosis* identified a potential avoidance mechanism. Virulent organisms were able to free themselves of the phagosome by 24 hours after infection whereas the avirulent organisms were still surrounded by the phagosomal membrane (211). Moreover, *M. tuberculosis* has been shown to escape from fused phagolysosomes in order to escape the fusion with secondary lysosomes (193). In addition to escaping from these hazardous vesicles, *M. tuberculosis* also has been shown to prevent the maturation of the

phagosome into the phagolysosome (70, 71), thereby residing in a retarded vesicle and avoiding degradation by lysosomal products. Furthermore, it was demonstrated that mycobacteria are able to inhibit this fusion of the phagosome and the lysosome by recruiting the TACO protein (106).

Another mechanism that macrophages use to destroy ingested microorganisms is the acidification of vesicles. *M. tuberculosis* and *M. avium* prevent the acidification of the vesicles in which they live (87). The mechanism for the prevention of acidification was determined to be the lack of the association of the proton- ATPase with the vacuole containing the organism (277). Although it has been experimentally demonstrated that both *M. tuberculosis* and *M. avium* prevent the acidification of the phagosome, recent evidence suggests that *M. avium*, but not *M. tuberculosis*, is resistant to the toxic effects of an acidified vesicle (122).

Macrophages are key antigen presenting cells. Macrophages process antigens and present them in the context of major histocompatibility complex (MHC) class I and MHC class II. Exogenous antigens are typically loaded into MHC class II (245) and presented to $CD4^+$ T cells, whereas endogenous antigens are typically loaded into MHC class I (154) and presented to $CD8^+$ T cells (79, 160). This mechanism allows specific antigen recognition by T cells, which in turn stimulates their effector functions. An important effector function of T cells is the secretion of IFN-γ (274). The effects of IFN-γ on infected macrophages have been very well studied. Stimulation of murine peritoneal macrophages with recombinant IFN-γ inhibits the growth of *M. tuberculosis* (243). The mechanism by which IFN-γ causes growth inhibition was demonstrated to be the induction of reactive nitrogen intermediates (RNI) in macrophages, which is toxic to the

pathogen (96, 109). TNF-α is also likely involved in this RNI mediated killing, as IFN-γ induces the expression of TNF-α receptors on the surface of macrophages and blocking TNF-α inhibits production of RNI (273).

In addition to the generation of RNI, reactive oxygen intermediates (ROI) have also been implicated as anti-mycobacterial agents. The evidence for the role of ROI in the host response to *M. tuberculosis* is controversial. There is some experimental evidence that demonstrates that ROI do not have a significant effect on the killing of *M. tuberculosis* (65). Other experiments, using mice without the gene that encodes phagocyte oxidase, suggest that ROI may have a role in the host response to *M. tuberculosis* (4, 85).

Mycobacterial components are able to directly affect the macrophage response. LAM is a major component of the mycobacterial cell wall and is also known to modulate the host immune response in the favor of the organism. LAM is able to affect the host immune response by causing the macrophage to be unresponsive to IFN-γ (261). Additionally, LAM is capable of scavenging oxygen free radicals, inhibiting protein kinase C, and preventing the transcriptional activation of genes that are typically upregulated or induced by IFN-γ (64).

Although mycobacteria have seemingly ingenious mechanisms to avoid the host immune response, macrophages are often effective at destroying mycobacteria. Mycobacterial immunologists are endlessly searching for ways that this intricate battle between the mycobacteria and the macrophage can be turned in favor of the host. Understanding the circumstances that lead both to an effective host response and to an ineffective host response is essential for the global elimination of this disease.

CD1

CD1 is a non-polymorphic MHC Class I molecule (59, 288). Humans have five distinct CD1 proteins (CD1a, CD1b, CD1c, CD1d, and CD1e) (5, 323) whereas mice have only two (CD1d1 and CD1d2) (44). The crystal structure of CD1 revealed the ability of this molecule to bind hydrophobic peptides (62) and it has also been shown to present lipid molecules (28, 263). The fact that a molecule could present non-peptides to T cells for recognition challenged one of the fundamental dogmas of immunology.

The discovery of a cellular mechanism for the presentation of non-peptides impacted mycobacteria research greatly. Owing to the very complex and lipid nature of the mycobacterial cell wall much research has been done to identify the mycobacterial antigens presented by CD1. In 1994 Beckman et al demonstrated that CD1b molecules could present non-peptide components of mycobacteria to double negative NK T cells (lacking the CD4 and CD8 molecules) (28). The human CD1b molecule was later shown to present lipoarabinomannan (LAM) to double negative NK T cells (263). Other mycobacterial antigens presented by CD1b are free mycolic acids, phosphatidylinositol mannosides, and glycosylated mycolates (199). The presentation of these lipid antigens by CD1b is dependent upon a nine amino acid targeting sequence found in the cytoplasmic tail of the CD1b molecule (152).

It is indeed exciting that CD1 molecules are capable of presenting non-protein mycobacterial components but the relevant question is, what is the immunological consequence? In other words, is the presentation of these molecules by CD1 crucial or even beneficial to the host? The fact that *M. tuberculosis* infection causes the down regulation of CD1 molecules implies some benefit to the pathogen in the lack of CD1

(272). Moreover, patients with tuberculoid leprosy express CD1 within granulomas whereas patients with lepromatous leprosy do not (264). The tuberculoid form of leprosy is indicative of a strong immune response and the lepromatous form is indicative of a weak immune response thus, this observation implies that CD1 induction corresponds to an effective immune response to *M. leprae*. Interestingly, evidence in the mouse model demonstrates that CD1d does not contribute significantly to the immune response to *M. tuberculosis* (29) (88). This does not however, indicate that other CD1 molecules are unimportant in the host response to *M. tuberculosis*.

NK T Cells

NK T cells are components of the innate immune response. Phenotypically, they have invariant αβ TCRs, NK co-receptors, and they can be either $CD4^+$ or double negative cells (30). Likely a consequence of their activated phenotype, NK T cells are capable of secreting large amounts of cytokines very quickly (30). Interestingly, these cells recognize the non-polymorphic CD1 molecules however, the antigen that is presented to NK T cells has yet to be conclusively identified (302) but there is some evidence that they recognize non-peptide antigens derived from mycobacteria (28).

The role of NK T cells in mycobacterial diseases has not been well characterized but as potent producers of IFN-γ it is likely that they are important during the innate response. Although NK T cells readily produce IL-4 there is evidence that upon in vivo stimulation with *M. bovis* BCG they can produce IFN-γ (103, 104). There is also evidence to suggest that NK T cells are involved in the granulomatous reaction, as the development of such lesions is not apparent in mice that lack NK T cells (14). A

more definitive role for NK T cells in mycobacterial infections will likely be uncovered as they are investigated further.

γδ T Cells

While searching for the genes that encoded the α and β chains of T cell receptors (TCR) Tonegawa discovered the genes that encoded the γ chain (247). This was the beginning of an exciting episode in immunology. Although a lot more is known about γδ T cells today than upon their discovery in 1984, they remain something of an enigma. The precise mechanism of antigen recognition, the characteristics that define the antigens that γδ T cells recognize, their specific effector functions, and their role in infectious diseases are among the properties of γδ T cells that remain unanswered.

The γ TCR genes were discovered in 1984 (247) and this was followed by the identification of the γδ T cell in 1986 (46). γδ and αβ T cell lineages are considered distinct, likely separating before the rearrangement and cell surface expression of the TCR (131). There are two subsets of γδ T cells. One set appears early in ontogeny and expresses very little if any junctional diversity and is therefore called invariant. Moreover, there is some evidence that these invariant γδ T cells require no classical thymic selection (9). Invariant, early subsets have also been shown to have distinct tissue tropisms. The Vγ5 subset migrates to the epidermis (17), while the Vγ6 subset migrates to the mucosal epithelia of the uterus, vagina, and tongue (150). The second subset of γδ T cells appear later in ontogeny and are highly diversified, particularly in the junctional regions. One example of this second subset is Vγ4, which migrates to the blood and lymphoid organs (35). The evidence suggests that negative selection takes place in these

variant subsets of γδ T cells but positive selection is less clear because the ligand mediating positive selection in γδ T cells has not yet been identified (9).

Antigen Specificity and Recognition

The enigmatic property of γδ T cells is largely due to the apparent vast diversity of antigens recognized and the various ways in which recognition occurs. The fact that γδ T cells possess a TCR that resembles that of αβ T cells lead to the assumption that antigen specificity and recognition would also resemble αβ T cells. γδ T cells do not fit the dogmatic mold of either $CD4^+$ αβ T cells, which recognize small peptides in the context of MHC Class II molecules or $CD8^+$ αβ T cells, which recognize small peptides in the context of MHC Class I molecules.

γδ T cell clones have been identified that recognize MHC Class I molecules (241), MHC Class II molecules (189), and CD1 molecules (238). Moreover, several experiments have led to the identification of antigens that are recognized by γδ T cell clones independently of antigen processing and presentation (253, 259, 309). γδ T cell clone LBK5 was shown to recognize the classic Class II MHC molecule IE^k (253). What is most interesting about this recognition is that the peptide that IE^k presents is irrelevant to recognition (253). Another γδ T cell clone, G8, recognizes the non-classical Class I molecule called T10 (309). Again this recognition is direct, that is requiring no processing and presentation, and the presence or absence of peptide is inconsequential (309). Finally, clone IgI4.4 recognizes a herpes glycoprotein (gI) without antigen processing and presentation (259).

In addition to the confusion over the antigen processing and presentation requirements of γδ T cells there is also a debate as to whether γδ T cells recognize protein

or non-protein antigens. The evidence that supports the recognition of protein antigens as well as non-protein antigens is quite convincing. Although this is not the expected finding, it may indicate a large repertoire of antigens capable of eliciting a γδ T cell response.

Heat shock proteins, especially when present in large quantities, are generally an indication of damaged or stressed cells. Several investigators have reported the recognition of mycobacterial heat shock proteins by γδ T cells (38, 40, 117, 133, 249). γδ T cell lines generated from PPD positive individuals responded, in the presence of antigen presenting cells (APCs), to both PPD and a recombinant form of the 65kDa mycobacterial heat shock protein (hsp) (133). Another experiment with hsp65 demonstrated that not only did γδ T cell lines respond to the heat shock protein but they also responded to cells that had been pulsed with small fragments of hsp65 (40). This indicates a need for APCs but is also reminiscent of antigen recognition by αβ T cells. The aforementioned evidence that γδ T cells recognize heat shock proteins has resulted in the hypothesis that one of the roles of γδ T cells is to specifically recognize damaged or defective self.

Concurrent with the experiments that demonstrated recognition of heat shock proteins and peptides were numerous experiments demonstrating recognition of protease resistant antigens by γδ T cells (81, 114, 203, 232, 283, 284). An elegant experiment conducted by Pfeffer et al in 1990 was the first demonstration that γδ T cells recognized non-traditional antigens. This experiment was exciting because it demonstrated the proliferation of γδ T cells in response to protease resistant mycobacterial lysate, not using γδ T cell lines but γδ T cells isolated from healthy human volunteers (232). In an attempt

to define the "protease resistant" ligand for human γδ T cells Tanaka et al tested numerous non-peptide compounds. Their work demonstrated that human γδ T cell clones and γδ T cells from healthy donors were stimulated by monoalkyl phosphates (284). Additionally, prenyl phosphates stimulated γδ T cells independently of any antigen processing and presentation (203). Interestingly, the non-protein mycobacterial products that stimulate γδ T cells are produced by pathogenic strains of mycobacteria in much greater quantities than in the vaccine BCG strain (81). Thus, these non-protein mycobacterial products are potentially immunologically important antigens.

Effector Functions

Equally as diverse as the antigens recognized by γδ T cells are the effector functions of γδ T cells. Human γδ T cells are capable of specific lysis of *M. tuberculosis* pulsed target cells and the secretion of IL-2 (208) and IFN-γ following stimulation with *M. tuberculosis*. The production of IFN-γ is dependent upon the secretion of IL-1 and IL-12 by infected macrophages (265). Much of the work on γδ T cells has involved mycobacteria and thus the cytokines studied tend to be the Th1 type cytokines. However, there is evidence that they are capable of secreting TH2 type cytokines (IL-4 and IL-5) as well (228). In addition to lysis and secretion of cytokines, γδ T cells also express mRNA for numerous chemokines such as MIP-1α, MIP-1β, RANTES, and lymphotactin (36). Some investigators suggest that the functions of γδ T cells place them in a more innate immunological role (37) while other studies suggest that γδ T cells function as cells of the acquired response, capable of specific recognition and possibly immunological memory responses (141). The diversity of the antigen specificities and the functions of γδ T cells are fascinating.

γδ and Infectious Diseases

It did not take long after their discovery for γδ T cells to be implicated in various immunological roles during infectious disease processes. In 1989 they were shown to significantly increase in numbers in the draining lymph nodes of mice following a *M. tuberculosis* challenge (153) and to accumulate in lepromatous granulomas (197). Murine γδ T cells specific for mycobacterial antigens accumulate in the draining lymph nodes early during a *M. bovis* BCG infection and then decrease as αβ T cells expand (149). This observation, among others, lead to the hypothesis that the role of γδ T cells in mycobacterial infections was a "first line" of defense. This observation and hypothesis was challenged by other experiments that showed a concurrent increase in αβ and γδ T cells after infection with BCG (126). Moreover, this experiment demonstrated the lack of γδ T cell accumulation during the anamnestic response to BCG. There is also some evidence that γδ T cells play a protective role in mycobacterial infections. Mice that are depleted of CD4 and CD8 T cells are still able to stabilize the growth of BCG while SCID mice are unable to do so, indicating a potential role for γδ T cells in protection (151). They have also been shown to be protective in other infectious disease models, such as murine listeriosis (198).

Many of the aforementioned papers lead to the controversy over the potentially protective role of γδ T cells in *M. tuberculosis* infection. An accurate analysis of this question was accomplished by comparing the response to an aerosol challenge of *M. tuberculosis* in normal mice versus γδ-KO mice by D'Souza et al. The major finding of this experiment was that although γδ T cells did not appear to play a role in reducing the bacterial load in mice they did affect the granulomatous response (89). The

granulomas in the γδ-KO animals were much more pyogenic when compared to the predominantly lymphocytic granulomas of the control mice. The hypothesis that came out of these experimental observations was that, perhaps through the secretion of chemokines such as MCP-1, γδ T cells control the cellular influx into the granuloma (89).

Bovine γδ T Cells

Characterization

Although γδ T cells have been discussed previously, it is important to discuss what is known about bovine γδ T cells and how they differ from human and murine γδ T cells. Probably the most obvious unique feature of bovine γδ T cells is their number. Bovine γδ T cells are very prominent in the circulation, especially during the first few months of life (20). Bovine γδ T cells can make up as many as 75% of the peripheral blood mononuclear cells (137). There are at least two possible explanations for the large number of γδ T cells found in ruminant animals. Firstly, γδ T cells are often associated with epithelial surfaces and ruminant animals have a large amount of epithelial surfaces in the gut. Secondly, ruminants could simply depend more heavily on γδ T cells for immune functions than do species with fewer γδ T cells (137).

At least two populations of bovine γδ T cells have been identified. The major distinguishing feature between the two populations is the differential expression of the WC1 antigen. Recently, it was also demonstrated that these two populations could also be distinguished by their propensity to populate different areas of the body (181). The more information that is uncovered about these two distinct populations of bovine γδ T cells the more likely it becomes that they are more than just phenotypically separate, but are in fact functionally distinct as well.

Perhaps the best characterized of the two populations is the WC1$^+$ γδ T cell. The distinguishing feature of these cells is the WC1 surface molecule (206). WC1 is a glycoprotein with a molecular mass of 220 kDa (184). Although the WC1 gene family exists in humans and mice, it is much more simplistic, having perhaps one or two genes, compared to the eight to ten in cattle and it is not expressed on human or murine γδ T cells (314). No specific function has yet been identified for WC1 but sequence homology reveals that it is most like CD5 and CD6 (315) and it has been shown to augment proliferation (132). In addition to the expression of WC1, this population of bovine γδ T cells can also be defined by the lack of CD2, CD4, and CD8 expression (181). The WC1$^+$ γδ T cells are the dominant population of γδ T cells in the circulation, marginal zones of the spleen, skin, and lamina propria (181).

The WC1$^-$ γδ T cells are not as well defined as the WC1$^+$ γδ T cells. WC1$^-$ γδ T cells express the γδ TCR and CD3 but unlike WC1$^+$ γδ T cells, they also express CD2 and have variable expression of CD8 (181). WC1$^-$ γδ T cells can be found in the circulation but are not present in the same high proportions as the WC1$^+$ γδ T cells. WC1$^-$ γδ T cells are found in the intestinal tract and rather than populating the marginal zones of the spleen the WC1$^-$ γδ T cells populate the red pulp (181). Although no progress has been made to define the MHC restriction of bovine γδ T cells it is possible, given the variable CD8 co-expression on WC1$^-$ γδ T cells, that these cells are MHC Class I restricted.

Function

There is evidence to not only suggest that bovine γδ T cells have different functions than human or murine γδ T cells but that WC1$^+$ and WC1$^-$ bovine γδ T cells are

functionally distinct as well. As stated previously, human and murine γδ T cells differ from bovine γδ T cells in that they do not express WC1 genes (314). Moreover, there is recent evidence to suggest that bovine γδ T cells do not express CD28 as do at least a portion of human γδ T cells (143). This is an interesting observation because it not only points out an important difference between human and bovine γδ T cells but it also reveals that CD28 is not a co-stimulatory molecule for bovine $WC1^+$ or $WC1^-$ γδ T cells. Observational and experimental evidence suggests that $WC1^+$ and $WC1^-$ γδ T cells have different functional capabilities. Firstly, $WC1^+$ and $WC1^-$ γδ T cells populate different portions of the body. Phenotypically, it has been demonstrated that $WC1^+$ γδ T cells express larger quantities of CD62L than do the $WC1^-$ γδ T cells. Moreover this observation has been correlated to the fact that $WC1^+$ γδ T cells accumulate at artificial sites of inflammation, whereas $WC1^-$ γδ T cells do not (317). Another important functional difference between $WC1^+$ and $WC1^-$ γδ T cells is the ability for type I IFNs to stimulate the growth of $WC1^-$ γδ T cells while suppressing the expansion of $WC1^+$ γδ T cells (292).

$CD4^+$ T Cells

$CD4^+$ T cells are essential cells in the host immune response to mycobacterial infections. $CD4^+$ T cells are protective, they produce IFN-γ, and are mediators of immunological memory (13, 39, 127, 207, 219, 221, 230). The discovery of the crucial role of $CD4^+$ T cells in the immune response to mycobacterial infections was the result of several very elegant experiments.

$CD4^+$ T Cells Are Essential and Protective

It has been known for quite some time that the cell mediated component of the

immune response, not the humoral component, is involved in protection against mycobacterial infections. The conclusive demonstrations that $CD4^+$ T cells were important in this immune response began to emerge in the late 1980's. Isolated $CD4^+$ T cells from mice infected with *M. tuberculosis* could provide protection to T cell deficient mice that were challenged with *M. tuberculosis* following adoptive transfer (221). Moreover, mice that were depleted of $CD4^+$ T cells in vivo and subsequently challenged with either *M. tuberculosis* or *M. bovis* BCG developed exacerbated bacterial loads in the spleens (207, 230).

To investigate these observation further, mice that lacked Class II MHC molecules, and were therefore devoid of $CD4^+$ T cells, were challenged with *M. bovis* BCG (168). These animals died due to the BCG infection and were also unable to produce measurable amounts of IFN-γ (168). More recently, Class II deficient mice were challenged with *M. tuberculosis* and they were also found to be much more susceptible to infection than the control mice (285). Moreover, a M. *tuberculosis* challenge of CD4-KO mice demonstrated that the lack of $CD4^+$ T cells results in decreased survival and higher bacterial loads in the lung, liver, and spleen (61). In addition to the evidence for an essential role of $CD4^+$ T cells in the mouse model, it is likely that $CD4^+$ T cells play a crucial role in human tuberculosis, as individuals that are HIV positive are much more susceptible to mycobacterial infections (24, 267).

$CD4^+$ T Cells Produce IFN-γ

The aforementioned experiments have conclusively demonstrated that $CD4^+$ T cells are important in the host immune response to *M. tuberculosis*. Additional experiments have demonstrated the effector function of $CD4^+$ T cells that seems to confer

protection is the production of IFN-γ. IFN-γ is the key protective cytokine in the host response to *M. tuberculosis* due to its role in activating macrophages (82, 110). $CD4^+$ T cell clones, obtained from PPD positive individuals, are capable of producing IFN-γ in response to mycobacterial antigens (39). Additionally, $CD4^+$ T cells harvested from mice that were infected with *M. tuberculosis* produced IFN-γ in response to both purified culture filtrate protein fractions (224) and live *M. tuberculosis* (225). As mentioned previously the lack of functional $CD4^+$ T cells not only increases the susceptibility to mycobacterial infections but also ablates the early production of IFN-γ (61, 168).

$CD4^+$ T Cells and Immunological Memory

Immunological memory can be defined as a response, following a resolved primary infection or exposure, that is accelerated and confers at least partial protection upon a secondary challenge. It is this response that one hopes to achieve through vaccination.

Immunological memory to mycobacterial infections was demonstrated by infecting mice with *M. tuberculosis*, treating the infection with isoniazid and then challenging the mice a second time (169). This experimental design allowed for the identification of the cell type responsible for immunological memory to *M. tuberculosis*. $CD4^+$ T cells, isolated from mice that were infected with *M. tuberculosis* and treated with isoniazid for 30 days, were able to confer protection to T cell deficient mice following adoptive transfer (219). This $CD4^+$ T cell population was then further characterized by flow cytometry. Again, mice were infected with *M. tuberculosis* and treated with isoniazid for 30 days before the secondary challenge. These studies demonstrated that the $CD4^+$ T cell populations that emerged, concurrent with immunological memory, were

phenotypically $CD44^{hi}/CD45RB^{lo}$ and $CD44^{hi}/CD45RB^{int}$ (127). This study did not however demonstrate the functional ability of these cell populations to confer protection to T cell deficient *M. tuberculosis* infected mice. A recent study by Andersen et al demonstrated by adoptive transfer experiments that although the $CD44^{hi}/CD45RB^{lo}$ $CD4^+$ T cells from memory mice could confer some protection a much more significant amount of protection was mediated by the $CD44^{lo}/CD45RB^{hi}$ $CD4^+$ T cells from memory mice (13).

$CD8^+$ T Cells

The evidence that $CD8^+$ T cells had a potential protective role in the host response to *M. tuberculosis* infection began to accumulate in the late 1980's. $CD8^+$ T cells could transfer protection to T cell deficient and *M. tuberculosis* infected animals (221) and mice depleted of $CD8^+$ T cells had more bacteria in the spleens following an intravenous challenge with *M. tuberculosis* than did control mice (207). Although both of the aforementioned experiments used antibodies for selection or depletion, the evidence that $CD8^+$ T cells were involved in protection was convincing. Additionally, $CD8^+$ T cells were also shown to mediate specific cytolysis of *M. tuberculosis* infected macrophages (93).

The development of mice lacking functional class I MHC molecules allowed a more accurate assessment of the role of $CD8^+$ T cells. β_2M-KO mice had increased numbers of bacteria in their lungs, developed granulomas with caseous necrosis, and died much earlier than the control animals following an intravenous injection with *M. tuberculosis* (112). The conclusion from these observations was that indeed $CD8^+$ T cells played a significant role in the immune response to *M. tuberculosis*. Unfortunately this

experiment did not rule out the effect of other cell populations that are β_2M dependent, such as populations of T cells restricted by non-polymorphic MHC molecules such as CD1. CD8-KO mice, when infected with a low dose aerosol of *M. tuberculosis*, show no significant difference in bacterial load up to 50 days post infection as compared to the control mice (88). Recent experiments with CD8 KO mice and in vivo depletion of $CD8^+$ T cells suggest a role for $CD8^+$ T cells during the chronic stage of infection (293, 300).

Cytokines

One of the essential effector functions of immune cells is the secretion of cytokines and chemokines. There are many cytokines and chemokines that have demonstrated and proposed roles in the host response to *M. tuberculosis*. Three cytokines, IFN-γ, TNF-α, and IL-12 are absolutely essential to the control of a *M. tuberculosis* infection (27, 82, 83, 110, 111).

IL-12 is a macrophage-derived cytokine that induces the production of IFN-γ by T lymphocytes (166). Exogenous IL-12, given concomitantly with an intravenous *M. tuberculosis* infection, was demonstrated to increase the survival of mice, decrease the bacterial load, and delay the lung pathology (113). Moreover, IL-12 augments the IFN-γ production by $CD4^+$ T cells (84). To further investigate the role of IL-12 in the host response to *M. tuberculosis*, IL-12 $p40^{-/-}$ mice were infected intravenously with *M. tuberculosis* (83). These mice were unable to control the growth of *M. tuberculosis* in the lung, liver, or spleen and were not able to produce detectable IFN-γ (83). These experiments taken together demonstrate the essential role of IL-12, presumably due to its role in stimulating IFN-γ production.

IFN-γ is a T cell cytokine that plays a pivotal role in the immune response to mycobacterial diseases (82, 96, 109, 110, 243). Numerous in vitro and in vivo experiments demonstrate a role for IFN-γ in mycobacterial infections. IFN-γ has been shown to inhibit the growth of *M. tuberculosis* in murine peritoneal macrophages (243). IFN-γ induces many of the antimicrobial effects of macrophages, including the induction of RNI (96, 109). IFN-γ KO mice were used to confirm the essential in vivo role for IFN-γ. Subjecting IFN-γ KO mice to either an intravenous or aerosol challenge with *M. tuberculosis* resulted in decreased survival, increased pathology, increased bacterial loads, and failure to produce RNI (82, 110). Other cytokines, such as IL-18 also influence the production of IFN-γ (218) and are therefore also important in the immune response to mycobacteria (278).

TNF-α is produced mainly by macrophages and it plays a role in the activation of macrophage anti-microbial functions and in the inflammatory process (60, 98, 107, 164). TNF-α has been shown to be an important component in granuloma formation, as the administration of TNF blocking antibody causes both the regression of established granulomas and the disruption of developing granulomas (162). Intravenous infection of mice lacking the TNF-α receptor and in mice depleted of TNF-α showed a higher *M. tuberculosis* load in all organs tested and the lack of production of RNI (111). Furthermore, the aerogenic infection of $TNF^{-/-}$ mice with *M. tuberculosis* clearly demonstrates the necessity for TNF-α in the lung (27). These mice were unable to control the infection, which resulted in the death of all animals by day 35, and they had poorly formed, necrotic granulomas (27). In addition to the essential role of TNF-α early

after *M. tuberculosis* infection, it has also been shown to be important during the chronic stage of *M. tuberculosis* infection (294).

Bovine Tuberculosis

M. bovis is the etiologic agent of tuberculosis in many species of animals, including cattle and a number of wild animals. *M. bovis*, once a serious health concern for humans, is now more of an economic threat to the global cattle and deer industries. The identification of *M. bovis* within cattle or deer herds results in significant financial loss, due not only to a decrease in revenue from milk and meat sales but also from export and movement restrictions placed upon the infected herds. Several countries have implemented bovine tuberculosis eradication programs with mixed success. Some countries, including the United States, have failed to totally eradicate the disease but have been able to dramatically reduce the prevalence with test and slaughter approaches. The bovine tuberculosis eradication programs in other countries, such as New Zealand, Ireland, and England have been somewhat less successful due to the existence of wild animal reservoirs.

History of Bovine Tuberculosis in the United States

The bovine tuberculosis eradication effort began in 1917 when the prevalence of the disease was 5% (187). The basic principal of the eradication program was to use the tuberculin skin test to test all animals and slaughter all reactors. This method was quite successful, as the prevalence dropped to 0.02% by 1983. Unfortunately, not much progress has been made since. The consistent prevalence of *M. bovis*, albeit low, is due in part to the inadequate sensitivity of the diagnostic test, increased herd size, increase Mexican cattle importation, and to an increase in the number of farmed cervid herds

(187). In 1999 there were 6 infected cattle herds; 3 in Texas, 2 in Michigan, and 1 in North Dakota. Some of the Texas herds have been infected since 1985 but the herds are far too large to depopulate. In addition to the infected cattle herds in Michigan, there are also reports of infected farmed deer herds, wild deer, coyotes, raccoons, and a black bear (49). Identifying this disease in the wildlife is causing concern because of the risk of establishing a stable wildlife reservoir, which would make it even more difficult to eradicate the disease and likely increase the risk of transmission to both farmed herds of cattle and deer as well as humans.

Problems with Wild Animal Reservoirs in England and New Zealand

Countries without persistent wildlife reservoirs and with test and slaughter eradication programs have been fairly successful at controlling bovine tuberculosis. The existence of wildlife reservoirs that have the potential to spread *M. bovis* into the farmed cattle or deer herds makes disease control much more difficult. England and New Zealand are prime examples of countries struggling with wild animal reservoirs of bovine tuberculosis.

The primary reservoir for *M. bovis* in New Zealand is *Trichosurus vulpecula*, the brushtail possum. While the prevalence of *M. bovis* in New Zealand cattle is less than 1% , with nearly 3% of the herds on movement restriction, the prevalence of *M. bovis* in possums is estimated to be 1-5% (23). This means that there are between 700,000 and 3.5 million *M. bovis* infected possums in New Zealand. Furthermore, these animals excrete and harbor large numbers of bacteria (23). The control of possum populations by poison has dramatically reduced the prevalence of *M. bovis* in cattle (55). Additionally,

the control of possums by poisoning is considered to be more cost effective than vaccinating cattle (159).

The major barrier to the eradication of *M. bovis* from England is thought to be the existence of *M. bovis* in badgers. The control of this reservoir is much more complicated than with the possums of New Zealand because the badger is a protected species. The British government has just sponsored a large bovine tuberculosis experiment in badgers, designed to definitively demonstrate that badgers are capable of transmitting *M. bovis* to cattle (188).

In addition to the culling of wildlife reservoirs of *M. bovis*, vaccination of the wildlife has also been proposed. Experimental vaccine trials involving both possums and badgers have been conducted (6, 7, 51, 276) The vaccination of possums with BCG affords significant protection against a virulent *M. bovis* challenge (6, 7, 51). There have not been as many detailed protection studies performed in the badger, owing to its status in England, but the experiments that have been conducted also suggest a protective effect of BCG (276). Although the results of experimentally vaccinating wildlife with BCG have been relatively encouraging, logistical factors such as delivery remain the primary obstacles.

Bovine Immune Response to *M. bovis*

Not nearly as much is known regarding the bovine immune response to *M. bovis* as is known about the human and murine response to *M. tuberculosis.* The similarities of most mammalian immune systems and the similarities between *M. bovis* and *M. tuberculosis* have led to the assumption that many responses in one host to one organism are similar in another host to another organism. There are however, in vitro, ex

vivo, and in vivo analyses of the bovine immune response to *M. bovis* but because of the lack of reagents and the size of the animal the information is not as detailed as the mouse system.

Monocytes from *M. bovis* infected cattle, in the presence of T cells, have been shown to inhibit the growth of live *M. bovis* (175). Additionally, virulent *M. bovis* induces the transcription of TNF-α, IL-1β, and IL-6 mRNA by bovine alveolar macrophages (8). Peripheral blood mononuclear cells, isolated from *M. bovis* infected cattle, have been shown to proliferate and produce IFN-γ in response to several *M. bovis* derived proteins (129, 236, 240). T cell proliferation in response to *M. bovis* protein ESAT-6 is particularly high and evident from 5 –20 weeks post *M. bovis* infection (240). Both $CD4^+$ and $CD8^+$ T cells, isolated by magnetic sorting from *M. bovis* infected cows, displayed strong proliferation and production of IFN-γ in response to live *M. bovis*, and *M. bovis* derived antigens (176).

Role of Bovine γδ T Cells in Bovine Tuberculosis

Bovine γδ T cells have not been studied in bovine mycobacterial infections as thoroughly as murine and human γδ T cells in *M. tuberculosis* infections but some important experiments have been conducted. $WC1^+$ γδ T cells are among the first to arrive in response to an intradermal injection of bovine PPD in *M. bovis* sensitized and infected animals (99). Peripheral blood changes of $WC1^+$ γδ T cells following infection with *M. bovis* suggest an accumulation of this cell type in early *M. bovis* lesions (237). Furthermore, the SCID-bo model of bovine tuberculosis (SCID mouse reconstituted with bovine lymphoid cells) suggests a similar role in lesion development for bovine γδ T cells as for murine γδ T cells (266).

Prevention of Bovine Tuberculosis

The eradication of bovine tuberculosis will consist of many different components, including the use of sensitive diagnostic tests, the control of wild animal reservoirs, and most importantly the development of an efficacious vaccine. The *M. bovis* BCG vaccine has been used in several large animal vaccine trials and is currently the "gold standard". Although the efficacy of the BCG vaccine has varied somewhat the main drawback of its use is the fact that it compromises the tuberculin skin test. The tuberculin skin test is used in many countries as the primary diagnostic test for bovine tuberculosis. Vaccinating animals with BCG would make it difficult to distinguish between vaccinated and infected animals using the tuberculin skin test.

M. bovis BCG has been used in many bovine tuberculosis vaccine trials but until recently the results were difficult to assess, due to the high doses of both the vaccine strain and the challenge strain (134, 320). High doses of BCG have been shown to be less effective at inducing the protective immune responses, as compared to lower doses (125). Recent cattle vaccine trials conducted in New Zealand have demonstrated protection using doses that more closely mimic natural infection. In cattle, the protective effect of BCG can be demonstrated for either subcutaneous or aerosol delivery, although the intratracheal route affords the most protection against a virulent *M. bovis* challenge (53, 54). Despite the fact that BCG delivered by the intratracheal route is capable of preventing the formation of lung lesions, reducing the number of lymph node lesions, and decreasing the number of culture positive cows following a virulent *M. bovis* challenge, it does not prevent infection (54).

Similar to the vaccines that are being developed to combat *M. tuberculosis* infection, subunit and DNA vaccines are being developed for cattle trials as well. The goal is to develop a vaccine that confers protection while keeping the integrity of the diagnostic test intact. The murine model was used to assess the efficacy of a variety of subunit vaccines against an aerosol challenge with *M. bovis* (42). A subunit vaccine consisting of *M. bovis* CFP and DDA afforded more protection than that delivered by the BCG vaccine (42). The encouraging results of an effective subunit vaccine for *M. bovis* infections, in a small animal model, provide the basis for a similar vaccine to be used in a large animal vaccine trial. DNA vaccines have not been used extensively in cattle but the recent evidence that DNA vaccines can confer protection against *M. tuberculosis* in mice and guinea pigs warrants the investigation of the application of such technology in cattle vaccines. A DNA vaccine encoding the *M. bovis* antigen, MPB83, protected mice against an intravenous challenge with virulent *M. bovis*, which was moderately more protective than the BCG vaccine in this experiment (63). Moreover, to investigate the potential of this vaccine for use in cattle, three calves were vaccinated and their immune response to bovine PPD was measured. The animals that were vaccinated with the MPB83-DNA vaccine, as compared with animals that received only the vector, had stronger lymphocyte stimulation indices in response to bovine PPD and MPB83 (63). This preliminary data justifies further investigation of the protective effects of this vaccine in cattle.

Johne's Disease

Johne's disease is a chronic enteritis of ruminants that is caused by *M. avium* subspecies *paratuberculosis* (*M. a. paratuberculosis*) and was described well over one

hundred years ago. Animals are thought to become infected early after birth, either by the ingestion of contaminated milk or feces. There is also some evidence that *M. a. paratuberculosis* can be transmitted to fetuses prior to birth (281). The chronic stage of the disease can last for several years after the initial infection before clinical signs become apparent, the most defining being severe diarrhea. The disease is fatal if treatment is not provided. Because the disease is difficult to detect before the emergence of clinical signs, and because of the expense, treatment is usually not administered. If the animal is valuable and treatment is desired the regimen is isoniazid at 20 mg/kg for the life of the animal (269).

Johne's disease is prevalent on every continent and causes tremendous global economic loss annually. In the United States a recent study revealed that 21.6% of the nations dairy herds are infected with *M. a. paratuberculosis* (310). In New York State 61% of the 33 herds tested were positive (217) and in Missouri 74% of the dairy herds tested were positive (289). Due to the loss of milk production in infected cows and the need to replace culled animals, Johne's disease is responsible for between $200 and $250 million in annual loss, in the United States alone (226). Other reports suggest estimated total economic losses in the United States as high as $1.5 billion annually (270).

Immune Response to *M. a. paratuberculosis*

Despite the tremendous impact of this disease on the global economy, very little information is available regarding the immune response to *M. a. paratuberculosis* infection. It is imperative that the host cellular immune response to *M. a. paratuberculosis* be studied, in order that new preventative and control methods can be

established. The unique tropism of *M. a. paratuberculosis*, to the gut, suggests that the immune response may differ from that seen with other mycobacteria species.

Clinically, there appear to be at least two forms of the disease, similar to that seen in the clinical manifestations of leprosy. Histologically, the lepromatous form of the disease is marked by a primarily macrophage granuloma with numerous bacilli and the tuberculoid form of the disease is defined by a granuloma dominated by lymphocytes and few bacilli (58). Interestingly, sheep that were defined histologically as tuberculoid had a much higher stimulation index in response to Johnin-PPD, than did those that were classified as lepromatous (58). In a follow up experiment it was also shown that animals that had been classified as tuberculoid also produced higher levels of IFN-γ and IL-2 in response to Johnin-PPD (57). Furthermore, the numbers of $CD4^+$ and $CD8^+$ T cells were decreased in the lesions of sheep that were histologically lepromatous (178). These two distinct manifestations of Johne's disease are thought to represent early (tuberculoid) and terminal (lepromatous) stages of the disease (72).

The in vitro response of $CD4^+$, $CD8^+$, γδ, and B cell populations, from *M. a. paratuberculosis* infected cows, have been studied very little. $CD4^+$, $CD8^+$, and γδ T cells from *M. a. paratuberculosis* infected cows have all been shown to proliferate in response to *M. avium* PPD (PPD-A) and $CD4^+$ and $CD8^+$ T cells, but not γδ T cells, also produce IFN-γ in response to PPD-A stimulation (26). γδ T cells have also been demonstrated to have an immunomodulatory role, apparently lysing $CD4^+$ *M. a. paratuberculosis* specific T cells (67). B cells from clinically symptomatic animals are found, in some cases, in high numbers in the periphery with lower than normal proliferative responses (304). In accordance with the aforementioned stages of Johne's

disease, animals with sub-clinical infections produce more IFN-γ than those with clinical disease (271, 280).

Vaccine Efficacy against *M. a. paratuberculosis* Infection

Infection with *M. a paratuberculosis* usually goes undetected and untreated until the clinical symptoms begin to appear. Unfortunately, infected animals have been shown to excrete organisms 18 months before the onset of clinical symptoms (69). Furthermore, animals showing clinical signs can secrete up to $5x10^{12}$ organisms per day in their feces (68). Therefore, prevention of transmission is essential for the control of this disease.

Interestingly, very little effort has focused on vaccine development for Johne's disease. The majority of the vaccination attempts for Johne's disease have used either killed or attenuated mycobacteria and have been assessed by observing the number of natural cases of Johne's disease in vaccinated versus non-vaccinated animals. A vaccine trial conducted in the Netherlands between 1984 and 1989, using a heat-killed vaccine, reported a 10% decrease in the number of animals destroyed due to Johne's (301). This trial did not however, have non-vaccinated controls to analyze at the same time period as the vaccinated animals, making it difficult to attribute the 10% decrease to an effect of the vaccine. A two-year vaccine study with goats in Madrid, using a killed commercial vaccine, demonstrated a 20% reduction in the number of animals euthanized due to Johne's disease (86). This vaccine trial was conducted in adult animals, presumably already infected with *M. a. paratuberculosis*. Finally, an attenuated vaccine study conducted in lambs revealed a marginal decrease in bacterial numbers within tissue

samples but the total length of the study was less than eight months in duration, thus giving no indication of long term protection (156).

Summary

Mycobacterial diseases have plagued human and animal kind for thousands of years. Although successful methods of treatment and prevention of mycobacterial diseases have been developed, significant numbers of humans and animals continue to suffer from various diseases caused by the organisms that make up the family *Mycobacteriaceae*. Two important components of the effort to control mycobacterial diseases are the development of efficacious vaccines and a more comprehensive understanding of the host immune response to mycobacteria. The subsequent chapters describe the development and testing of novel vaccines for bovine tuberculosis and Johne's disease and the characterization of an important cellular component of the bovine immune system.

CHAPTER 2

Vaccination of Cattle with *Mycobacterium bovis* Culture Filtrate Proteins and Interleukin-2 for Protection against Bovine Tuberculosis

Abstract

Despite eradication efforts, bovine tuberculosis remains a significant threat to the agriculture industry of many countries. The *M. bovis* BCG vaccine has been shown to be variably efficacious against a virulent *M. bovis* challenge in cattle. Notwithstanding this, the *M. bovis* BCG vaccine has not been used as a control measure for bovine tuberculosis because it interferes with diagnostic tests for bovine tuberculosis. Vaccines that effectively protect cattle against infection with *M. bovis* or limit the severity of the disease, while not compromising diagnostic tests, are needed. This study describes the development and evaluation of a subunit vaccine for bovine tuberculosis. Cattle were vaccinated with varying doses of *M. bovis* culture filtrate proteins (CFP), recombinant interleukin 2 (IL-2), and 200 μg of monophosphoryl lipid A (MPL). This vaccine formulation did not interfere with the tuberculin skin test and significantly reduced the severity of lung lesions found in cattle following a virulent challenge with *M. bovis*. Despite these favorable responses, the CFP/IL-2/MPL subunit vaccine failed to induce significant post-vaccination cellular immune responses and led to increased extrathoracic dissemination of *M. bovis*. Future subunit vaccines for bovine tuberculosis must demonstrate the ability to limit the severity of disease and prevent dissemination.

Introduction

Bovine tuberculosis, caused by *Mycobacterium bovis*, is a devastating disease affecting the world's agriculture industries. Bovine tuberculosis is responsible not only for financial losses due to a decrease in meat and milk production, but also financial losses due to movement restrictions. It is estimated that the prevalence of *M. bovis* in New Zealand cattle is about 0.2% and about 3% of the herds are on movement restriction

(23). Although the prevalence of *M. bovis* in New Zealand's cattle seems low, the country has been unable to totally eradicate the disease, due in part to the estimated 3.5 million infected possums, which are thought to transmit the disease to cattle (23). England is also struggling with the eradication of *M. bovis*, but the situation there is more complicated as the major wildlife reservoir (the badger) is a protected species (146). The efforts to control *M. bovis* infections in the United States have been more successful because of the absence of a prevalent wild animal reservoir. There are however, pockets of the country that continue to struggle. The border of Texas and Mexico has been an area with persistently infected cattle herds (234) and Michigan is working to control *M. bovis* infections in the white tail deer populations (257).

Currently the only available vaccine for bovine tuberculosis is *M. bovis* BCG. The BCG vaccine has been variably efficacious in cattle (31, 53) but it is not used as a preventative measure because it compromises the diagnostic tests for bovine tuberculosis (115). There is therefore a great need for an effective vaccine for bovine tuberculosis that does not compromise the tuberculin skin test. Much of the recent mycobacterial vaccine research has focused on vaccines that are composed of the culture filtrate proteins derived from various mycobacterial species (10, 21, 42). The mycobacterial proteins secreted into the culture medium during growth were identified as immunostimulatory (11, 224) and have thus been incorporated into vaccines with various combinations of adjuvants and cytokines, with variable effectiveness (10, 21, 42, 45, 177, 227, 242). One of the more effective combinations of CFP, adjuvants, and cytokines in the mouse model of tuberculosis was CFP from *M. tuberculosis*, monophosphoryl lipid A, and murine IL-2 (21). This combination provided some protection to mice that were given an aerosol

challenge with *M. tuberculosis* and dramatically reduced the lung pathology of guinea pigs that were given an aerosol challenge with *M. tuberculosis* (21). In addition to the protective potential of this combination, there was no DTH response induced by PPD following vaccination (21). *M. bovis* culture filtrate based vaccines have also been demonstrated as protective and non-DTH inducing in the murine model of bovine tuberculosis (42).

In this study the effect of different combinations of *M. bovis* CFP and bovine IL-2, in combination with the adjuvant MPL, on the bovine immune system were studied and the protective efficacy was assessed. In contrast to the immune responses induced by the BCG vaccine the CFP/IL-2 vaccines induced high levels of CFP specific antibodies and low levels of IFN-γ and IL-2 production following whole blood stimulation with bovine PPD. The high dose CFP/IL-2 vaccine, in combination with MPL, did not induce a DTH response to bovine PPD, whereas the BCG vaccine did. Additionally, the CFP/IL-2 based vaccine reduced the severity of lung lesions seen in cattle following intratracheal challenge with *M. bovis*, as compared with the control animals. However, the animals that were vaccinated with the CFP/IL-2 based vaccine did have a significant increase in the number of lesions found outside of the thoracic cavity.

Materials and Methods

Animals

Seventy-two Friesian cross calves were obtained from tuberculosis-free accredited herds. All animals were from an area in New Zealand where both farmed and feral animals were free of tuberculosis. All animals at the onset of this study were approximately 6 months old and were negative in response to bovine PPD by the whole

blood IFN-γ assay (CSL Ltd., Australia). All animals were grazed on pasture inside an isolation unit for the duration of this study.

Bacterial strains

M. bovis BCG Pasteur 1172P2 was used to vaccinate animals in group 10 of this study. This strain has been used in previous bovine vaccination studies (53, 54). *M. bovis* WAg201 was used as the challenge organism in this study. This organism was originally isolated from a tuberculous possum and has been used successfully to infect cattle in several previous studies (52-54). Both *M. bovis* BCG and *M. bovis* WAg201 were grown to mid-log phase in Tween albumin broth (Dubos broth base [Difco Laboratories, Detroit, Mich.]) supplemented with 0.006% (vol/vol) alkalinized oleic acid, 0.5% (wt/vol) albumin fraction V, and 0.25% (wt/vol) glucose. All dilutions prior to inoculation of cattle were made in Tween albumin broth. The CFU of the inoculum was determined by plating 10-fold serial dilutions on Middlebrook 7H11 (Difco) supplemented with 0.5% (wt/vol) albumin, 0.2% (wt/vol) glucose, and 1% (wt/vol) sodium pyruvate.

Vaccine Preparation

Production of *M. bovis* CFP

The CFP used in these studies was prepared as previously described (42). The CFP was prepared from cultures of three different field isolates of *M. bovis* (862422, 896146, and 903053). All three isolates were grown to mid-log phase in sodium pyruvate alanine salts (SPAS) medium (0.03% [wt/vol] Bacto Casitone [Difco], 0.005% ferric ammonium citrate, 0.4% [wt/vol] K_2HPO_4, 0.2% [wt/vol] citric acid, 0.1% [wt/vol] L-Alanine, 0.12% [wt/vol] $MgCl_2 \cdot 6H_2O$, 0.06% [wt/vol] K_2SO_4, 0.2% [wt/vol] NH_4Cl,

0.18% [vol/vol] 10N NaOH, 0.5% [wt/vol] sodium pyruvate). The bacteria were grown in roller bottles with gentle rolling. The supernatants were harvested by filtering out the bacteria with a 0.22 μm filter unit. The culture filtrate was then concentrated by ultrafiltration using an ultrafiltration stirred cell (Amicon, Danvers, Mass.) with a PM10 membrane (molecular weight cut off of 10 kDa)(Millipore, Bedford, Mass.). Following concentration the CFP was dialyzed against PBS for a minimum of 48 hours with three buffer changes. The CFP was then filter sterilized with a 0.22 μm filter unit and the protein concentration was determined using bicinchoninic acid assay (BCA)(Pierce, Rockford, IL). The final vaccine formulation was prepared by first pooling equal quantities of CFP from each strain and then lyophilizing the mixture. Analysis of the CFP by two-dimensional gel electrophoresis revealed a complex mixture of proteins.

Recombinant Bovine IL-2

The recombinant bovine IL-2 was prepared in *Pichia pastoris* as described previously (306) and the specific activity of the protein was 2,500 U/μg. Briefly, RNA was isolated from bovine lymphocytes that had been stimulated with concanavalin A. RT-PCR was then used to prepare the cDNA that encoded the full length mature IL-2 protein. The cDNA was then cloned into the expression plasmid pPIC9 (Invitrogen Corp., Carlsbad, Calif.). Recombinant bovine IL-2 was produced by using this construct to transform cultures of *P. pastoris*, by methods described previously (307). Recombinant IL-2 was purified using immobilized metal-chelating affinity chromatography with Talon (Clontech Laboratories Inc., Palo Alto, Calif.). The biological activity of the purified recombinant IL-2 was determined by an IL-2 bioassay, as described previously (53). Endotoxin levels were measured in the IL-2 using the

Limulus amebocyte lysate assay (E. Toxate; Sigma Chemical Co., St. Louis, Mo.) and found to be <0.1 endotoxin unit/μg of protein.

Adjuvant

The adjuvant used in this study was monophosphoryl lipid A (MPL) (Corixa/Ribi ImmunoChem Research Inc., Hamilton, Mont.). MPL is a non-toxic derivative of *Salmonella minnesota* R595 lipopolysaccharide. MPL was resuspended to 1 mg/ml in sterile water containing 2 μl of triethylamine per ml. Animals receiving adjuvant only or one of the CFP based vaccines received 200 μg of MPL per dose.

Vaccination with CFP and DTH Responses

Twelve animals were randomly placed into 3 groups (n=4). One group received one subcutaneous dose of 10^6 BCG, one group received three subcutaneous doses of 1 mg CFP/250 μg IL-2/200 μg MPL two weeks apart, and one group received no vaccine. All twelve animals were skin tested with bovine PPD (Ministry of Agriculture and Forestry, Central Animal Health Laboratory, Upper Hutt, New Zealand) thirteen weeks after the initial vaccination. Each animal was injected intradermally with 100 μl of a 1 mg/ml solution of bovine PPD. The thickness of the skin was measured in mm just prior to injection and then the injection site was measured again 72 hours later. The DTH responses were reported as the increase in skin thickness between the pre-injection measurement and the 72-hour measurement.

Vaccination Protocol and Assessment of Immune Responses

Animals were randomly placed into 10 groups (n=4). The vaccines that the animals in each group received are described in Table 2.1. Group 1 received only

Table 2.1. Vaccine Formulations

Vaccine Group	Dose of CFP	Dose of IL-2 (μg)	Dose of BCG (CFU)
1	-	-	-
2	-	50	-
3	-	250	-
4	200 μg	-	-
5	200 μg	50	-
6	200 μg	250	-
7	1 mg	-	-
8	1mg	50	-
9	1 mg	250	-
10	-	-	10^6

Animals in vaccine groups 1-9 were vaccinated three times at 2 week intervals. Animals in vaccine group 10 were vaccinated two times at 6 week intervals. The vaccines for groups 2-9 were prepared by combining the given amount of CFP and IL-2 with 200 μg of MPL per dose.

MPL, groups 2-9 received some combination of high, low, or nil doses of CFP and high, low, or nil doses of bovine IL-2 and Group 10 received 10^6 colony forming units of BCG. At 0, 2, 4, 7, and 11 weeks after the initial vaccination blood samples were taken from each animal for antibody ELISA, IL-2 and IFN-γ assays.

Challenge and Necropsy

Concurrent with the dose response study described above, an additional 20 animals were randomly placed into groups 1, 9, and 10 to increase the group size in order to accurately assess the protective efficacy of the CFP vaccines. Seven animals were assigned to both groups 1 and 9 and six animals into group 10. These animals were vaccinated as the other animals in their groups. In addition to the animals in groups 1, 9, and 10, the four animals from group 5 were challenged intratracheally with $5x10^3$ CFU of virulent *M. bovis* at 13 weeks after the initial vaccination as described previously (53). Table 2.2 summarizes the groups, the vaccines received, and the challenge dose. Briefly, animals were anesthetized and an endotracheal tube with a fine cannula was inserted per os into each trachea. A 1.5 ml inoculum, containing $5x10^3$ CFU of *M. bovis* was injected and flushed with 2 ml of saline. Blood samples were collected from all animals at 0, 2, 5, 10, and 17 weeks post challenge. 17 weeks post challenge all animals were killed by captive bolt and severance of the carotid artery. Procedures for identifying macroscopic lesions and processing for histopathologic testing have been described previously (53). Samples for bacterial culture were taken from the left and right bronchial lymph nodes and from the anterior and posterior mediastinal lymph nodes. Samples for bacterial culture were also taken from any other tuberculous lymph node or organ. Samples

Table 2.2. Vaccine and Challenge Doses

Vaccine group	No. of animals	Dose of CFP	Dose of IL-2 (μg)	Dose of BCG (CFU)	Challenge dose (CFU)
1	11	-	-	-	$5x10^3$
5	4	200 μg	50	-	$5x10^3$
9	11	1 mg	250	-	$5x10^3$
10	10	-	-	10^6	$5x10^3$

Animals in vaccine groups 5 and 9 were vaccinated three times at 2 week intervals. Animals in vaccine group 10 were vaccinated twice at 6 week intervals. Animals in all four vaccine groups were challenged with virulent *M. bovis* 13 weeks after the initial vaccination.

collected for bacterial culture were homogenized using a Tenbroeck grinder (Wheaton, Millville, N.J.) and processed for the isolation of mycobacteria, as previously described (53).

Antibody ELISA

M. bovis AN5 culture filtrate was diluted in carbonate buffer (pH 9.6) to 3 μg/ml. 100 μl of this suspension was added per well to 96 well ELISA plates and incubated at 4°C overnight. The ELISA was carried out as previously described (319). All serum used in this assay was stored at –20°C until the assay was performed. Results are expressed as an absorbance index, calculated by expressing the value found for the sample as a fraction of the binding of a strong reference sample multiplied by 100.

IFN-γ and IL-2 Assays

Within 4 hours of collection, 1.5 ml aliquots of heprinized whole blood were added to two separate wells. 100 μl of either PBS or a 0.3 mg/ml solution of bovine PPD was added to the wells and incubated for 24 hours at 37°C. The plasma supernatants were stored at -20°C until the samples were processed. The supernatants were analyzed for IFN-γ using a sandwich ELISA kit (CSL Ltd.) as previously described (244) and for IL-2 using a bioassay as previously described (102).

RT-PCR of Lesions

Sections of tuberculous lymph nodes and lungs were placed into test tubes with approxiately 5 ml of Ultraspec (Cinna/Biotecx, Friendswood, Texas) and snap frozen in liquid nitrogen. Lung lesion samples were removed from three animals in group 10, six animals in group 1, and six animals in group 9 (one sample from each animal). Lymph node lesion samples were removed from three animals in group 10,

seven animals in group 1, and seven animals in group 9 (some animals had lesions in multiple lymph nodes and samples were taken from each). These samples were then stored at -80°C until RNA could be isolated. Each sample was homogenized and total RNA was extracted. 1 μg of RNA from each sample was reverse transcribed using murine Maloney-leukemia virus reverse transcriptase (Life Technologies, Grand Island, NY.). The cDNA was diluted and 10 μl aliquots were amplified using Taq polymerase (Promega, Madison, WI.) and bovine cytokine specific primers. The primer and probe sequences used in this study are given in Table 2.3. The PCR reactions were then loaded onto a 1% agarose gel and run at 150 volts for 20 minutes. The gels were then denatured, neutralized, and blotted overnight onto nitrocellulose. Fluorescein labeled probes that were specific for bovine cytokine and housekeeping genes were then hybridized and detected with a chemiluminescent detection system. The blots were then analyzed digitally and pixel values were assigned to each sample by intensity and size. Mean pixel values and standard error of the means were determined for each group and each gene amplified.

Histological Analysis

Tissue samples collected from tuberculous lungs and lymph nodes were fixed in buffered formalin, embedded in paraffin, and cut in 6 μm sections. Each sample was stained with haematoxylin and eosin, and Ziehl-Neelsen stains. Each section was examined several times without knowledge of experimental groups and graded with the following scale; - = absent, ± = minimal, + = small, ++ = moderate, +++ = abundant. The scale was used to assess the amount of mineralization, and the numbers of giant cells, neutrophils, and lymphocytes, in the lung lesions. In the lymph node sections the number

Table 2.3. Bovine Cytokine Primers and Probes

IL-12 p40 sense: 5' AGG AAG ATG GAA TTT GGT CCA antisense: 5' TCA ATA AGC AGG CTC TCC TCA probe: 5' AAG CAG CAG AGG CTC CTC TGA	**TNF-α** sense: 5' TCC GGG ATG TGG AGC TGG CGG antisense: 5' TTG AAC CAG AGG GCT GTT GAT probe: 5' AGA GGG AAG AGT CCC CAG GTG
IL-2 sense: 5' CAC TAA CTC TTG CAC TCG TTG antisense: 5' CTC TGG GGT TCA GGT TTT TGC probe: 5' ACG TGC CCA AGG TTA ACG CTA	**IL-10** sense: 5' CCC TGT CTG ACA GCA GCT GTA antisense: 5' TGT GGC ATC ACC TCT TCC AGG probe: 5' GAC CCA GTC TCT GCT GGA TGA
IFN-γ sense: 5' CCA GAT GTA GCT AAG GGT GGG antisense: 5' GCT CTC CGG CCT CGA AAG AGA probe: 5' ATT CCG GTG GAT GAT CTG CAG	**IL-1α** sense: 5' ATG AGC CAC TTC GTG AGG ACC antisense: 5' TGA TGA TTT CTT CTT CTG TAT probe: 5' GCC AGT GGG AAG ATT CTG AAG
IL-4 sense: 5' GGG TCT CAC CTA CCA GCT GAT antisense: 5' GCT CAA TTC CAA CCC TGC AGA probe: 5' GAA TTC ATG CAT GGA GCT GCT	**IL-1β** sense: 5' GAG GCT GAT GAC CCT AAA CAG antisense: 5' GGG CTA GCC AGC ACC AGG GAT probe: 5' GCC ATG GAG AAG CTG AGG AAC

All of the sense and antisense primers and the probes were designed using the specific mRNA sequence in GenBank.

of eosinophils was assessed, rather than the number of lymphocytes. The relative amount of necrosis was described as the percent of the section that was necrotic.

Statistical Analyses

Analysis of variance (ANOVA) on $\log_{10}$ –transformed data was performed on all of the antibody, IFN-γ, and IL-2 responses, as well as the bacterial counts. The raw data for both the lung lesion scores and the intradermal tuberculin test responses were evaluated by ANOVA. The Fishers's exact test was used to evaluate the proportions of cattle from different vaccine groups with lung and lymph node lesions. The χ^2 test was used to evaluate the comparison of the percentage of culture positive thoracic lymph nodes. Finally, ANOVA was used to analyze the comparison of the cytokine expression profiles of lung and lymph node lesions between different vaccine groups.

Results

Vaccination with CFP does not induce DTH responses in cattle

New vaccines for bovine tuberculosis must not interfere with the diagnostic test for bovine tuberculosis, the tuberculin test. In order to assess the effect of the CFP vaccine on the tuberculin test, four animals that had been vaccinated with CFP/IL-2/MPL, four animals that had been vaccinated with BCG, and four non-vaccinated animals were skin tested with bovine PPD 13 weeks following vaccination. The animals that had been vaccinated with BCG all developed significant DTH responses to the bovine PPD by 72 hours after injection (Table 2.4). In contrast, none of the animals from the CFP vaccinated group, or the animals from the non-vaccinated group, developed any significant response to the bovine PPD (Table 2.4).

Table 2.4. Skin Test Responses to bovine PPD

Vaccine formulation	Increase in thickness (mm)
BCG (10^6)	4.6 ± 0.6 *
Non-vaccinated	0.0 ± 0.0
CFP/IL-2/MPL (1 mg/250 μg/200 μg)	0.1 ± 0.1

Animals in the BCG group were vaccinated once, animals in the CFP group were vaccinated three times at two week intervals, and the animals in the non-vaccinated group were not vaccinated. The animals were skin tested with intradermal injections of 100 μg of bovine PPD, 13 weeks after the initial vaccination.

*=significantly greater than the non-vaccinated group ($p<0.05$).

Effect of Different Doses of CFP and IL-2 on Immune Responses

To determine the immunological effects of CFP/IL-2 based vaccines, ten groups (n=4) were vaccinated with different combinations of CFP and IL-2 or with control vaccines. Table 2.1 summarizes the vaccine formulations given to each group of four animals in this study.

All animals were tested for serum antibodies to *M. bovis* CFP at 0, 2, 4, 7, and 11 weeks following the first vaccination. The highest antibody responses were seen at 7 weeks. Animals from groups 5, 6, 8, and 9 produced significant antibody response to *M. bovis* CFP (Table 2.5). Animals receiving BCG, IL-2 alone, CFP alone, or no vaccine had low antibody responses to CFP (Table 2.5).

All animals were tested for IFN-γ production in response to bovine PPD at 0, 2, 4, 7, and 11 weeks following the first vaccination. Table 2.5 shows the peak IFN-γ responses, which were observed at the 4 week timepoint. Group 10 (BCG) had the highest IFN-γ response. The CFP groups produced very little IFN-γ. The only CFP vaccinated group whose IFN-γ production was significantly greater than the control group (group 1) was group 5 (low CFP/low IL-2) (Table 2.5).

All animals were also tested for IL-2 production in response to bovine PPD at 0, 2, 4, 7, and 11 weeks following the first vaccination. The peak IL-2 responses were observed at the 4 week timepoint. As with IFN-γ production, the BCG group (Group 10) produced the highest IL-2 response. Again the only CFP group that produced IL-2 levels above that of the control group (Group 1) was group 5 (low CFP/low IL-2) (Table 2.5).

Table 2.5. Post Vaccination Immune Responses

Group	Vaccine formulation		Ratio of mean response at 4 wks to response prevaccination		Mean antibody response 7 weeks postvaccination
	M. bovis CFP	IL-2 (μg)	IL-2[a]	IFN-γ[b]	Ab[c]
1	-	-	1.1 ± 0.2	1.3 ± 0.3	6.9 ± 1.6
2	-	50	0.9 ± 0.3	0.9 ± 0.3	6.9 ± 2.3
3	-	250	0.8 ± 0.2	1.0 ± 0.4	5.5 ± 1.4
4	200 μg	-	1.6 ± 0.4	1.7 ± 0.7	6.9 ± 1.4
5	200 μg	50	5.3 ± 0.3*	7.4 ± 3.2*	29.0 ± 6.2*
6	200 μg	250	2.0 ± 0.6	2.0 ± 0.7	22.5 ± 5.5*
7	1 mg	-	3.1 ± 1.0	5.2 ± 2.9	9.4 ± 1.3
8	1 mg	50	2.2 ± 1.2	2.5 ± 0.9	19.3 ± 1.9*
9	1 mg	250	2.1 ± 0.4	1.9 ± 0.4	17.8 ± 4.5*
10	-	-	21.9 ± 13.6†	29.8 ± 21.2†	9.0 ± 0.9

Peripheral blood cultures from each animal were stimulated with bovine PPD and supernatants were analyzed for IFN-γ[b] and IL-2[a] production. Serum was also analyzed for specific antibodies to *M. bovis* CFP[c]. IL-2 responses are expressed as stimulation indices; data are presented as the ratio of the mean responses at 4 weeks to the response prevaccination ± SE. IFN-γ responses are expressed in OD indices; data are presented as the ratio of the mean response at 4 weeks to the response prevaccination ± SE. Maximum antibody responses were measured 7 weeks following the first vaccination; data are expressed as absorbance indices ± SE.

*= significantly > than control group ($p < 0.05$), †= significantly > than all groups ($p < 0.05$).

Protective Efficacy of The CFP/IL-2 Vaccine

The protective efficacy of the high dose CFP/ high dose IL-2 vaccine and the low dose CFP/ low dose IL-2 vaccine was assessed in a vaccination/challenge experiment. The low dose CFP/low dose IL-2 group was included in the challenge experiment because this was the only CFP vaccine group to demonstrate significant cellular responses post vaccination. Table 2.2 summarizes the vaccine formulations, challenge dose, and number of animals included for each group.

The animals were challenged intratracheally with $5x10^3$ cfu of *M. bovis* 13 weeks after the administration of the first vaccine. All animals were slaughtered approximately 17 weeks post-challenge. Each animal was evaluated for the presence of tuberculous lung lesions and the lesions found were scored based on the number and size of the lesion, and whether or not the lesion was consolidated (Table 2.6). In addition, the number of acid fast bacteria in the lungs of the animal were determined (Table 2.6). The BCG vaccine significantly reduced the number of animals with tuberculous lung lesions whereas neither CFP group in this study significantly reduced the number of animals with lung lesions (Table 2.6). Both the BCG group (group 10) and the high dose CFP/high dose IL-2 group (group 9) had significantly lower lung lesion scores than the non-vaccinated group (group 1) (Table 2.6). There were no significant differences in the lung bacterial counts between the four groups in this challenge study (Table 2.6).

In addition to the lung evaluations, the lymph nodes, both inside and outside of the thoracic cavity, were also evaluated. The number of cattle with lesioned lymph nodes is summarized, for each of the four groups, in Table 2.7. Nearly all of the

Table 2.6. Lung Lesions

Vaccine Group	No. of cattle with lung lesions/total no.[a]	Lung lesion score[b]	Lung bacterial count[c]
1	11/11	3.8 ± 0.12	2.53 ± 0.14
10	5/10*	1.00 ± 0.39*	1.96 ± 0.32
9	8/11	2.09 ± 0.48*	2.63 ± 0.20
5	4/4	2.75 ± 0.63	2.62 ± 0.32

Following slaughter, the lungs of all animals were carefully examined. The presence of macroscopic lung lesions was noted[a]. Mean lung lesions scores for each vaccine group are given ± SE[b]. Lung lesions were scored in the following manner: 4=>100 small lesions or a consolidated lesion >60 mm in diameter, 3=25-99 small lesions or a consolidated lesion 20-60 mm in diameter, 2=10-24 small lesions, 1=1-9 small lesions, and 0=no lesions.

*=significantly different from the control group (group 1) ($p < 0.05$).

Geometric means for the bacterial counts ($\log_{10}$ CFU per gram of tissue) ± SE are also given[c].

Table 2.7. Lymph Node Lesions

Vaccine Group	No. of cattle with lesioned lymph nodes/total no.[a]		No. of lesioned lymph nodes/ animal[b]	Lymph node bacterial counts[c]	% of thoracic lymph nodes *M. bovis* culture positive
	All Sites	Outside			
1	11/11	1/11	3.18 ± 0.38	2.42 ± 0.14	84
10	7/10	0/10	1.20 ± 0.33*	1.86 ± 0.18‡	53*
9	11/11	5/11¶	3.36 ± 0.28	2.53 ± 0.12	73
5	4/4	0/4	3.5 ± 0.29	2.41 ± 0.14	81

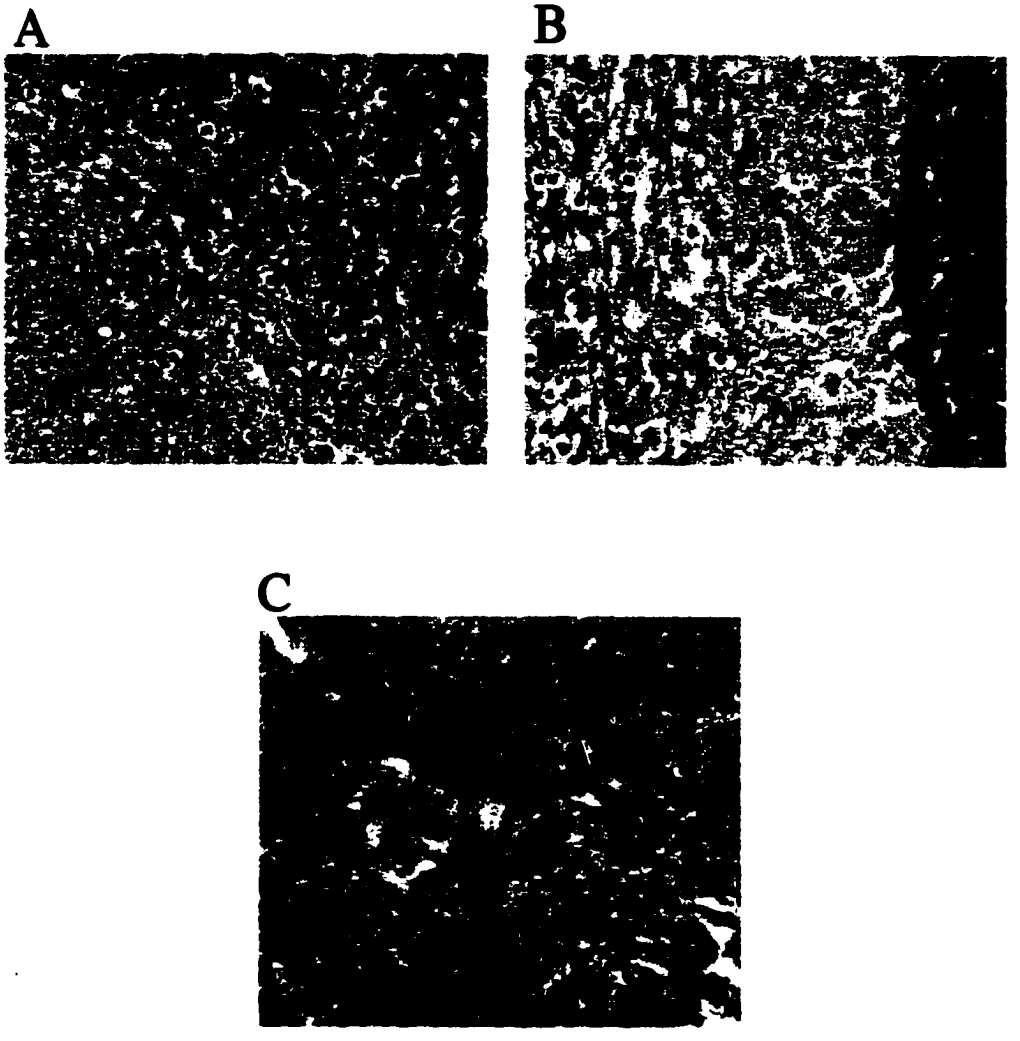
Following slaughter, the lymph nodes (thoracic, head, and mesenteric) were carefully examined. In addition to the number of cattle per group with lesioned lymph nodes[a], the mean number of total lesioned lymph nodes per animal[b], ± SE, was noted for each group. ¶=significantly greater than the control and BCG-vaccinated groups ($p< 0.05$), *=significantly less than the control group (group 1) ($p< 0.05$). Geometric means for bacterial counts ($\log_{10}$ CFU per gram of tissue) ± SE were also determined[c]. ‡=significantly less than the group 9 ($p< 0.05$).

animals in this study had lymph node lesions. The BCG group (Group 10) had three animals without lymph node lesions, but this was not statistically significant (Table 2.7). A significant number of animals in group 9 had lesions outside of the thoracic cavity (Table 2.7). Only one other animal, from group 1, had extrathoracic lesions (Table 2.7). Additionally, the mean number of lesioned lymph nodes per animals, the mean bacterial counts in the lymph nodes, and the number of thoracic lymph nodes that were culture positive were noted for each group (Table 2.7). The animals in the BCG group (Group 10) had fewer numbers of lesioned lymph nodes, lower bacterial counts, and fewer thoracic lymph nodes that were *M. bovis* culture positive (Table 2.7). Neither CFP group varied significantly from the control group (Group 1) in the number of lesioned lymph nodes per animal, the lymph node bacterial counts, nor the number of thoracic lymph nodes that were culture positive for *M. bovis* (Table 2.7).

Histological Analysis of Lung and Lymph Node Lesions

Sections of lung and lymph node tissue from each group were examined without prior knowledge of groups. The lung sections from each group were assessed for relative amounts of necrosis and mineralization and relative numbers of giant cells, neutrophils, and lymphocytes. The location of the lymphocytes in the lung section was also noted. Typically the lesions consisted of a central necrotic focus, that was usually mineralized, surrounded by mantles of epithelioid macrophages and lymphocytes. Figure 2.1A-C shows a representative section from the BCG group (A), the high dose CFP/high dose IL-2 group (B), and the non-vaccinated group (C). Table 2.8A-D summarizes the findings for each lung section reviewed. All three of the vaccine groups tended to have

Figure 2.1. Lung Lesion Histology



Representative photographs of lung sections stained with hemotoxylin and eosin (magnification 400x) from Group 10 (A), Group 9 (B), and Group 1 (C). The arrows in A and B indicate central areas within the granulomatous lesions that have mineralized. The N in A-C indicate areas of necrosis within the granulomatous lesions.

Table 2.8. Lung Histological Analysis

A

Group	Animal#	Necrosis	Mineralization	Giant Cells	Neutrophils	Lymphocytes
Adjuvant	2	80%	+++	-	++	++(per)
Adjuvant	6	75%	+++	±	+	+++(per)
Adjuvant	8	60%	+++	+	±	+++ (per/clus)
Adjuvant	9	70%	+++	+	±	++(clus)
Adjuvant	10	85%	+++	+	+	++(per)
Adjuvant	12	90%	+++	±	±	±
Adjuvant	41	75%	+++	±	±	++(per)
Adjuvant	43	25%	++	+++	±	++ (sheets/clus)
Adjuvant	62	50%	+++	-	+	++(per)
Adjuvant	72	80%	+++	±	+	++(per)
Adjuvant	85	25%	+	±	±	+++(clus)

B

Group	Animal#	Necrosis	Mineralization	Giant Cells	Neutrophils	Lymphocytes
BCG	4	50%	+++	+	±	+++ (per/clus)
BCG	30	10%	+	+++	+	++(clus)
BCG	67	15%	±	-	++	++(per/clus)
BCG	69	<1%	-	-	+++	++(per)

Section of lung tissue were stained with hematoxylin and eosin and scored based on relative amounts of necrosis and mineralization and relative numbers of giant cells, neutrophils, and lymphocyes, without prior knowledge of groups. Lesions were scored with the following scale: -=absent, ±minimal, +=small, ++=moderate, +++=abundant. Necrosis is described as the relative percent of the section that was affected.

Table 2.8. Lung Histological Analysis (cont.)

C

Group	Animal#	Necrosis	Mineralization	Giant Cells	Neutrophils	Lymphocytes
High CFP/IL-2	17	30%	++	+	±	++(perivas)
High CFP/IL-2	19	30%	++	++	+	++(sheets)
High CFP/IL-2	24	40%	+++	++	±	++(per)
High CFP/IL-2	33	20%	+++	±	±	++(per)
High CFP/IL-2	40	75%	-	±	++	++(per)
High CFP/IL-2	56	35%	+++	+	+	++(per)
High CFP/IL-2	58	60%	+++	±	+++	++(per)
High CFP/IL-2	82	20%	++	++	±	++(clus)

D

Group	Animal#	Necrosis	Mineralization	Giant Cells	Neutrophils	Lymphocytes
Low CFP/IL-2	5	50%	+++	+	±	++(per)
Low CFP/IL-2	22	10%	++	±	±	++(perivas)
Low CFP/IL-2	23	50%	+++	±	++	+++ (perivas)
Low CFP/IL-2	29	20%	+++	+	±	++(per)

Section of lung tissue were stained with hematoxylin and eosin and scored based on relative amounts of necrosis and mineralization and relative numbers of giant cells, neutrophils, and lymphocyes, without prior knowledge of groups. Lesions were scored with the following scale: -=absent, ±minimal, +=small, ++=moderate, +++=abundant. Necrosis is described as the relative percent of the section that was affected.

less necrosis than the non-vaccinated group, but the BCG group had the least amount of necrosis (Table 2.8A-D). The BCG group also appeared to have less mineralization than the control group (Table 2.8A & 2.8B). The lymph node sections from each group were assessed for relative amounts of necrosis and mineralization and relative numbers of giant cells, neutrophils, and eosinophils. Table 2.9A-2.9D summarizes the histological findings for each section examined. The lymph node lesions examined were variable, even within the groups. The BCG groups seemed to have less necrosis and mineralization than the other groups but again this was variable (Table 2.9A-2.9D). There were no major differences between the control group and the CFP vaccinated groups (Table 2.9A, 2.9C, and 2.9D).

RT-PCR Analysis of Lung and Lymph Node Lesions

At the conclusion of this study, lymph node and lung lesions were removed from animals in groups 1, 9, and 10. Routine RT-PCR followed by southern blot analysis was performed on lung and lymph node lesions from several animals in each of the three groups. Bovine actin was chosen as the housekeeping gene and each sample was analyzed for the relative amount of actin, in order to demonstrate that similar amounts of RNA were reverse transcribed for each sample. Figures 2.2A-2.2H and 2.3A-2.3H represent the relative mRNA expression for each group and each cytokine investigated, in comparison to the relative expression of actin mRNA, in both the lung (Figure 2.2) and lymph node (Figure 2.3) lesions. Overall, the lesion cytokine expression profiles were similar in all three groups examined, for both the lung and lymph nodes. There were however, a few significant differences. The animals in group 9 (high CFP/high IL-2) had higher levels of IFN-γ in the lung lesions, as compared to the animals

Table 2.9. Lymph Node Histological Analysis

A

Group	Animal#	Necrosis	Mineralization	Giant Cells	Neutrophils	Eosinophils
Adjuvant	6	80%	+++	+	±	-
Adjuvant	41	90%	+++	±	±	-
Adjuvant	12	90%	+++	++	±	-
Adjuvant	43	20%	++	+++	±	-
Adjuvant	8	60%	++	+	+	-
Adjuvant	9	90%	+++	±	+	-
Adjuvant	72	80%	+++	+	+	-
Adjuvant	10	75%	++	++	±	-
Adjuvant	72	50%	++	±	++	-
Adjuvant	62	15%	+	-	±	+
Adjuvant	6	10%	+	±	±	-
Adjuvant	2	2%	±	±	±	-
Adjuvant	43	2%	-	-	±	+++

B

Group	Animal#	Necrosis	Mineralization	Giant Cells	Neutrophils	Eosinophils
BCG	4	80%	+++	±	+	+
BCG	69	50%	++	±	±	-
BCG	67	75%	+++	±	++	-
BCG	26	40%	+	+	++	-
BCG	57	50%	++	±	++	-
BCG	30	25%	+++	++	±	-
BCG	71	30%	+	±	+	-
BCG	30	5%	±	+	±	-
BCG	32	1%	±	-	±	++
BCG	64	1%	-	-	±	++

Section of lymph node tissue were stained with hematoxylin and eosin and scored based on relative amounts of necrosis and mineralization and relative numbers of giant cells, neutrophils, and eosinophils, without prior knowledge of groups. Lesions were scored with the following scale: -=absent, ±minimal, +=small, ++=moderate, +++=abundant. Necrosis is described as the relative percent of section.

Table 2.9. Lymph Node Histological Analysis (cont.)

C

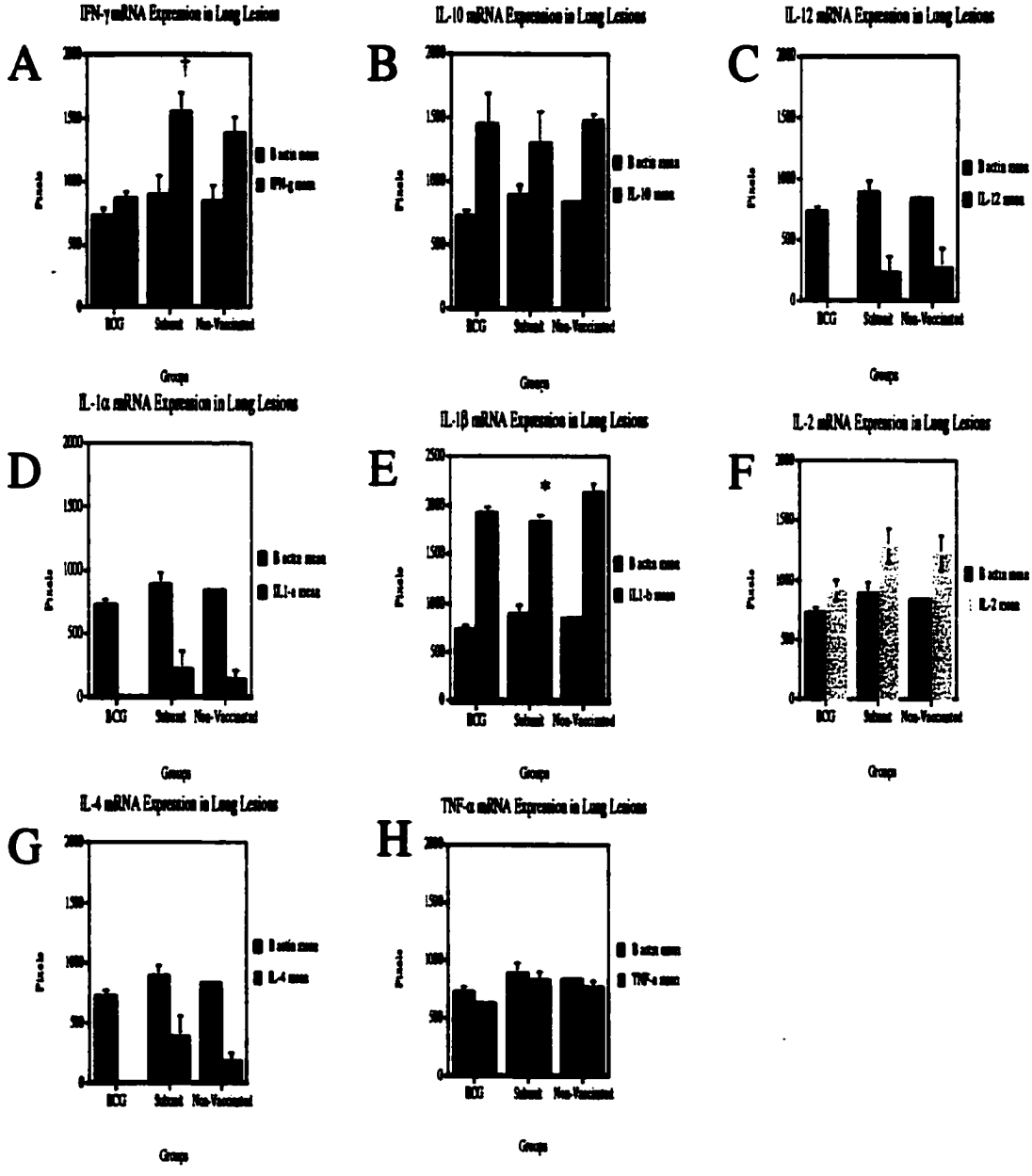
Group	Animal#	Necrosis	Mineralization	Giant Cells	Neutrophils	Eosinophils
High CFP/IL-2	19	60%	+++	±	±	-
High CFP/IL-2	24	80%	+++	+	±	-
High CFP/IL-2	68	50%	++	±	+	-
High CFP/IL-2	82	50%	++	±	±	-
High CFP/IL-2	17	90%	+++	++	±	-
High CFP/IL-2	33	30%	++	±	+	-
High CFP/IL-2	40	40%	++	+++	±	-
High CFP/IL-2	58	60%	++	±	±	-
High CFP/IL-2	85	70%	+++	±	±	-
High CFP/IL-2	21	30%	++	±	++	-

D

Group	Animal#	Necrosis	Mineralization	Giant Cells	Neutrophils	Eosinophils
Low CFP/IL-2	5	95%	+++	-	+	-
Low CFP/IL-2	22	95%	+++	+	+++	-
Low CFP/IL-2	23	50%	++	±	±	-
Low CFP/IL-2	29	10%	±	-	±	-

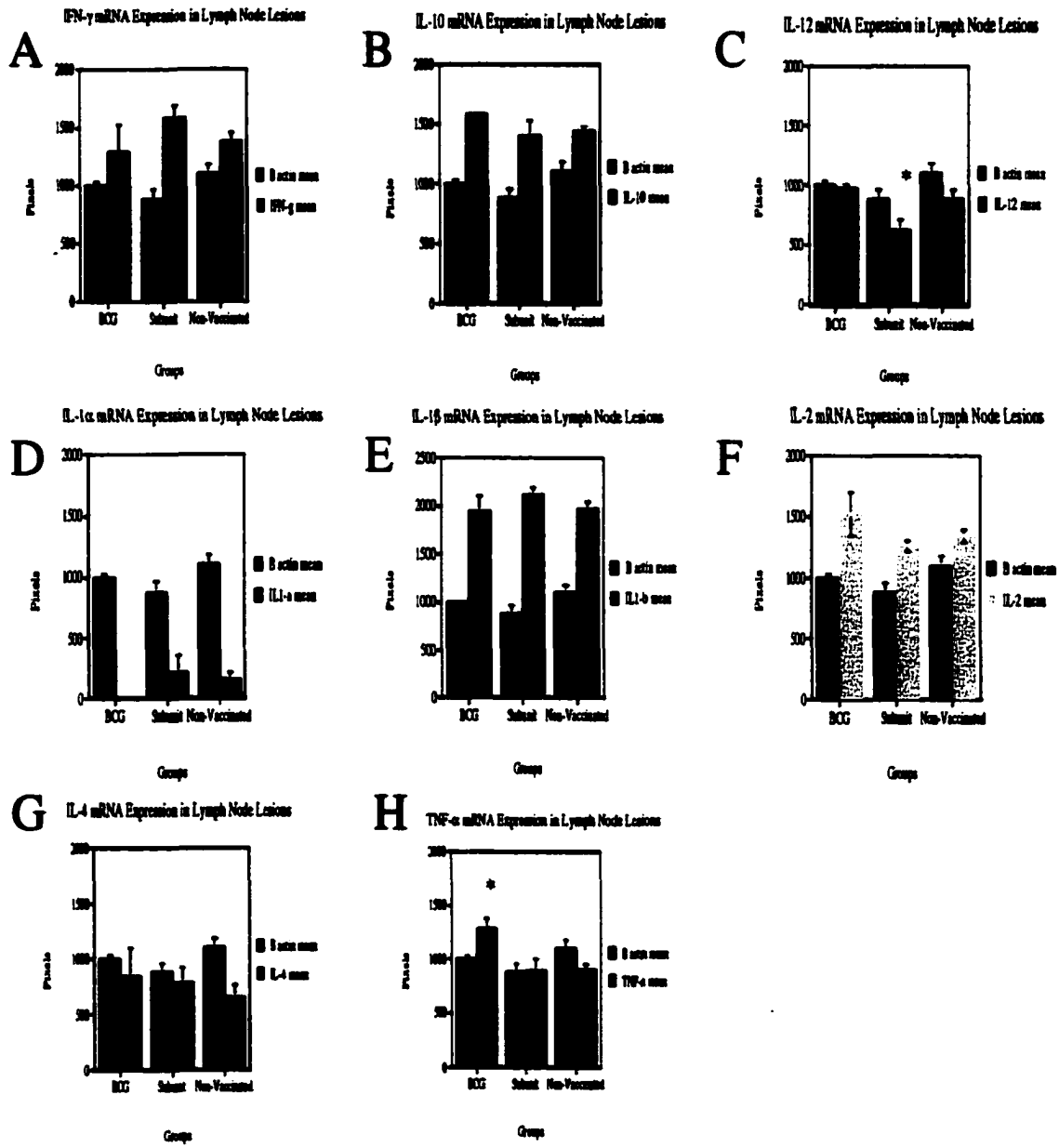
Section of lymph node tissue were stained with hematoxylin and eosin and scored based on relative amounts of necrosis and mineralization and relative numbers of giant cells, neutrophils, and eosinophils, without prior knowledge of groups. Lesions were scored with the following scale: -=absent, ±minimal, +=small, ++=moderate, +++=abundant. Necrosis is described as the relative percent of section.

Figure 2.2. Lung Lesion RT-PCR



mRNA of lung lesions from groups 1, 9, and 10 was used for RT-PCR and Southern Blot analysis of bovine cytokines. Data are expressed as mean pixel values ± SE. †= significantly different than BCG group ($p < 0.05$), *= significantly different than non-vaccinated group ($p < 0.05$).

Figure 2.3. Lymph Node Lesion RT-PCR



mRNA of lymph node lesions from groups 1, 9, and 10 was used for RT-PCR and Southern Blot analysis of bovine cytokines. Data are expressed as mean pixel values ± SE. †= significantly different than BCG group ($p < 0.05$), *= significantly different than non-vaccinated group ($p < 0.05$).

in group 10 (BCG) (Figure 2.2A). Additionally, the animals in group 9 also had lower levels of IL-1β expression in the lung lesion as compared to the animals in group 1 (Figure 2.2E). Analysis of the lymph node lesions also revealed significant differences in cytokine expression profiles. The animals in group 9 had lower levels of IL-12 expression than the animals in group 1 (Figure 2.3C). Additionally, the animals in group 10 had higher levels of TNF-α expression than the animals in group 1 (Figure 2.3H).

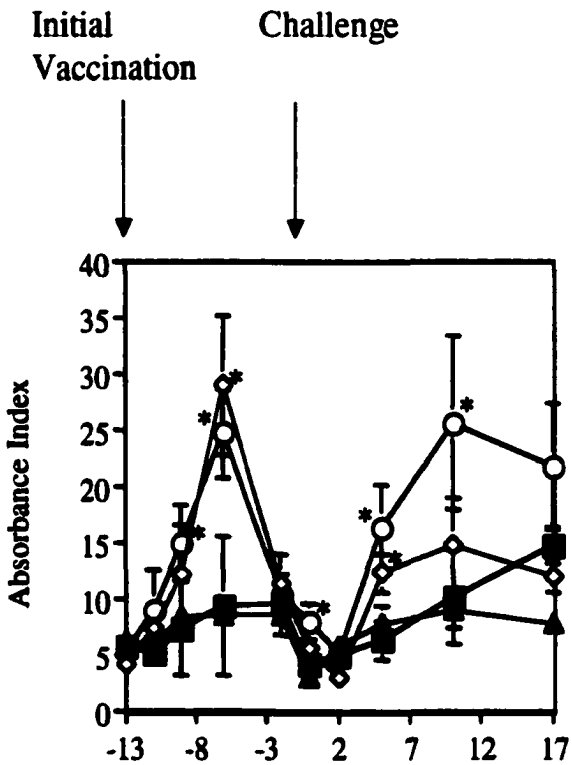
Antibody Responses Following Vaccination and Challenge

Antibody responses to *M. bovis* CFP were measured for each animal before and after challenge. Figure 2.4 shows the mean antibody responses to CFP for groups 1, 5, 9, and 10 for the duration of this study. Peak responses for both of the CFP vaccinated groups were observed at 7 weeks post-vaccination or 6 weeks pre-challenge (Figure 2.4). Very strong anamnestic responses were observed for both the CFP vaccinated groups 5 and 10 weeks post-challenge (Figure 2.4). Antibody responses in the BCG and non-vaccinated groups were very low throughout the study, increased levels were only seen in the non-vaccinated group 17 weeks post-challenge (Figure 2.4).

Cellular Immune Responses Following Vaccination and Challenge

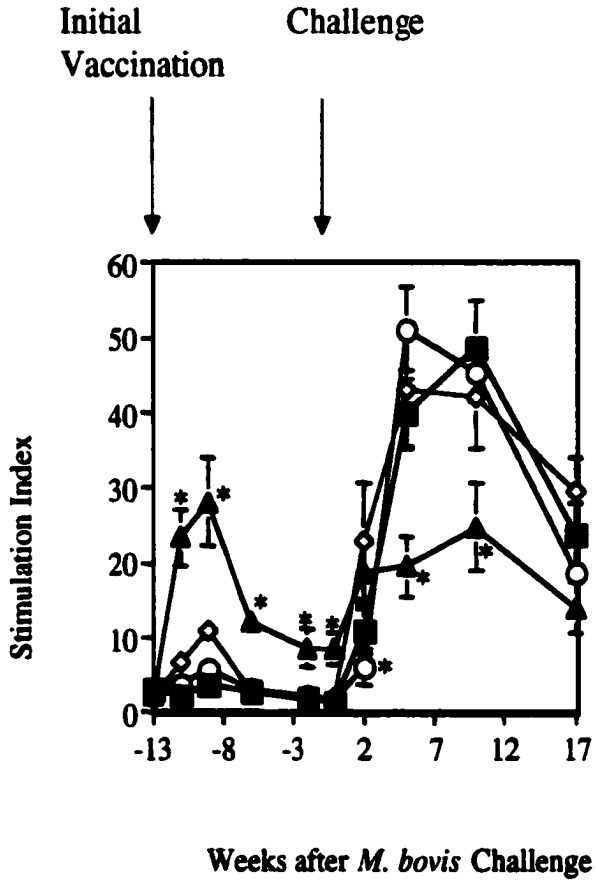
Animals that were vaccinated with BCG had high IL-2 (Figure 2.5) and IFN-γ (Figure 2.6) responses following vaccination, with peak responses recorded 9 weeks pre-challenge (Figures 2.5 & 2.6). These responses went back down by 2 weeks pre-challenge and increased slightly following challenge (Figures 2.5 & 2.6). In contrast, the non-vaccinated and CFP vaccinated animals had relatively low IL-2 (Figure 2.5) and IFN-γ (Figure 2.6) responses after vaccination. Both the IL-2 (Figure 2.5) and the IFN-γ

Figure 2.4. CFP Specific Antibody Responses



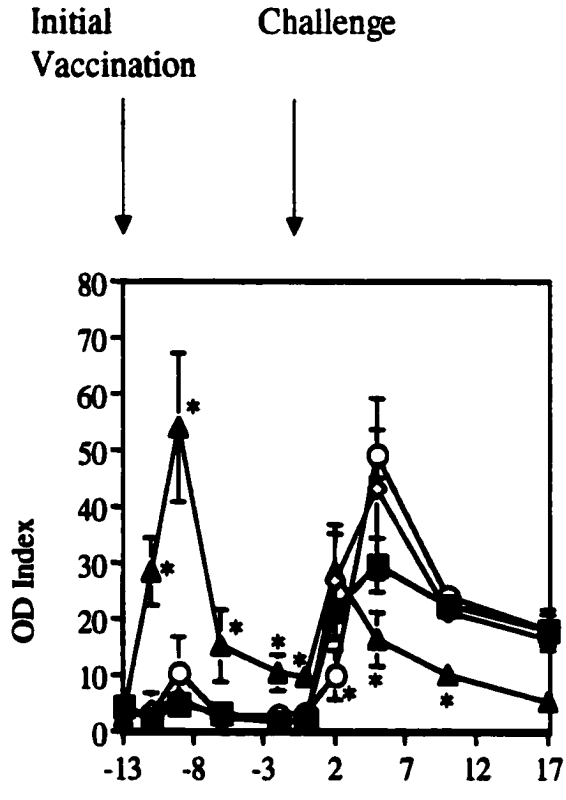
Mean antibody responses of animals vaccinated with either high -dose CFP/IL-2 (0), low-dose CFP/IL-2 (◊), BCG (▲), or adjuvant alone (■) and challenged with virulent *M. bovis* at 13 weeks after the initial vaccination (n=11 for the high-dose CFP/IL-2 and adjuvant groups, n=10 for the BCG group and n=4 for the low-dose CFP/IL-2 group) are shown. Data are expressed as absorbance indices ± SE. * = means were significantly greater than the means of the BCG and adjuvant control groups ($p < 0.05$).

Figure 2.5. Interleukin-2 Production



IL-2 released from bovine PPD stimulated peripheral blood cultures of animals vaccinated with either high -dose CFP/IL-2 (0), low-dose CFP/IL-2 (◊), BCG (▲), or adjuvant alone (■) and challenged with virulent *M. bovis* at 13 weeks after the initial vaccination (n=11 for the high-dose CFP/IL-2 and adjuvant groups, n=10 for the BCG group and n=4 for the low-dose CFP/IL-2 group) are shown. Data are expressed as mean stimulation indices ± SE. * = means were significantly different than the means of the adjuvant control group ($p < 0.05$).

Figure 2.6. IFN-γ Responses



IFN-γ released from bovine PPD stimulated peripheral blood cultures of animals vaccinated with either high -dose CFP/IL-2 (0), low-dose CFP/IL-2 (◊), BCG (▲), or adjuvant alone (■) and challenged with virulent *M. bovis* at 13 weeks after the initial vaccination (n=11 for the high-dose CFP/IL-2 and adjuvant groups, n=10 for the BCG group and n=4 for the low-dose CFP/IL-2 group) are shown. Data are expressed as mean OD indices ± SE. * = means were significantly different than the means of the other groups ($p < 0.05$).

(Figure 2.6) responses rapidly increased for the non-vaccinated and CFP vaccinated groups following challenge, with peak responses observed at 5 and 10 weeks post challenge.

Discussion

This study demonstrated that although a high dose CFP/IL-2/MPL based bovine tuberculosis vaccine significantly reduced the severity of lung lesions present following a virulent *M. bovis* challenge, the level of protection was not as high as seen with the BCG vaccine. Additionally, while the BCG vaccine induced positive skin test reactions to bovine PPD the high dose CFP/IL-2/MPL vaccine did not. Despite these encouraging findings, vaccination with the high dose CFP/IL-2/MPL vaccine also led to extrathoracic dissemination following challenge.

Although the high dose CFP/IL-2/MPL vaccine failed to induce a measurable cellular immune response following vaccination, the vaccine was effective at reducing the lung lesion severity following challenge. This result was somewhat unexpected, given the importance of a strong cellular response in controlling mycobacterial infections. The CFP/IL-2/MPL vaccine did stimulate B cells, indicated by the strong specific antibody response to *M. bovis* CFP, which could have contributed to the reduction in lung lesion severity observed. Although the cellular immune response has unequivocally been demonstrated as essential for protection against mycobacterial diseases (61, 97, 173, 174, 221) there is some evidence that B cells contribute to protection (286, 303). Alternatively, although the high dose CFP/IL-2/MPL vaccine did not induce a detectable PPD specific cellular response following vaccination, it is possible that it did induce a

response that was sufficient for the generation of a population of memory cells that were able to confer some post-challenge protection.

An unfortunate consequence of the high dose CFP/IL-2/MPL vaccine was the extrathoracic spread of the *M. bovis*. Only one animal from the non-vaccinated group had tuberculous lesions outside of the thoracic cavity, whereas five of the animals in the high dose CFP/IL-2/MPL group had tuberculous lesions in lymph nodes outside of the thoracic cavity. No animals in any of the other groups had lesions outside the thoracic cavity. It is possible that the increased CFP specific antibodies present in the animals that received the high dose CFP/IL-2/MPL vaccine contributed to the dissemination of *M. bovis*. The binding of antibodies directed against CFP that are also found on the outside of the bacillus could result in the uptake of the bacilli by monocytes through opsonization, and thus leading to the transport of *M. bovis* to extrathoracic lymph nodes. Although animals in the low dose CFP/IL-2/MPL group had high levels of CFP specific antibodies, the levels were lower than those found in the high dose CFP/IL-2/MPL group and none of the animals in the low CFP/IL-2/MPL group had extrathoracic lesions. Moreover, the increased dissemination could be a consequence of the induction of a TH2 response, as is seen in leprosy (194, 210). In this study the high dose CFP/IL-2/MPL group produced the highest IgG1 to IgG2 ratios of all other groups pre and post-challenge (data not shown), indicating that the vaccine induced a TH2 type antibody response. The disseminated form of leprosy is marked by, among other things, elevated levels of IgG1(147).

Both antibody and cellular immune responses were evaluated in animals that received various combinations of *M. bovis* CFP, IL-2 and MPL. This experiment

demonstrated that significant humoral responses could be generated in animals receiving any combination of both CFP and IL-2. Cellular responses to bovine PPD were only generated in animals that were vaccinated with the low dose CFP/IL-2/MPL vaccine, and for this reason this group was included in the challenge section of this study. The post challenge data, specifically the post mortem exam and culture of tuberculous organs, did not indicate that the low dose CFP/IL-2/MPL vaccine induced any protection in the animals to which it was given. The post vaccination cellular responses seen in the animals vaccinated with the low dose CFP/IL-2/MPL vaccine were much less than those observed in the animals vaccinated with BCG, which likely explains the poor protective efficacy of this vaccine. Moreover, the post-challenge cellular immune responses of both the high and low dose CFP/IL-2/MPL vaccinated groups are indicative of an active *M. bovis* infection as compared to the response in the BCG vaccinated group which are indicative of a more controlled infection (53, 54, 305).

The major disadvantage of using the BCG vaccine for the prevention of mycobacterial diseases, including human and bovine tuberculosis, is the inability to distinguish between vaccinated and infected individuals using the skin test. The animals in this study that were vaccinated with the high dose CFP/IL-2/MPL vaccine did not have a positive DTH response to bovine PPD following vaccination, whereas BCG vaccinated animals did. This is consistent with similar vaccine trials in other species (19, 21, 42). This was a very important finding because it demonstrated that a CFP based vaccine could provide some protection against a virulent *M. bovis* challenge while not compromising the diagnostic test. It will be interesting to investigate whether future CFP

vaccines for bovine tuberculosis can induce stronger post-vaccination cellular immune responses while leaving the animals skin test negative.

In accordance with the reduction of macroscopic lung lesion severity generated by the high dose CFP/IL-2/MPL vaccine, there was also less necrosis present upon microscopic examination than in the non-vaccinated animals. The necrosis, however, was much more severe than that in the BCG vaccinated animals, which is inconsistent with similar studies in guinea pigs (21). The mineralization of a lesion typically indicates that the lesion is older and more severe than a lesion without mineralization. The BCG vaccinated animals also had much less mineralization as compared to the other groups, again indicating that the BCG vaccine was more protective than the high dose CFP/IL-2/MPL vaccine.

Although the RT-PCR analysis of the lung and lymph node lesions from groups 1, 9, and 10 did reveal some significant differences in the cytokine expression profiles between these three groups, the results are rather difficult to interpret. We have demonstrated that within a bovine granulomatous lesion, formed in response to an intratracheal challenge of *M. bovis*, numerous cytokines are being highly expressed, regardless of the vaccine administered. This is likely a reflection of the fact that only macroscopic lesions were examined. None of the vaccines tested prevented infection and animals from all groups had macroscopic lesions. This experiment does not take in to consideration that some groups had less lesions present than others. Perhaps it would have been a more effective exercise to compare the cytokine expression profiles of several lesions from each animal.

Although the overall performance of the CFP/IL-2/MPL vaccines used in this study was not sufficient to warrant its prophylactic use against *M. bovis* infections in cattle, some valuable information regarding the bovine immune response to such subunit vaccines was acquired. It is apparent, from this cattle trial and others, that a substantial post-vaccination cellular immune response correlates with the ability of the vaccine to afford the animal protection against a virulent *M. bovis* challenge. Thus pre-challenge evaluations of novel bovine tuberculosis vaccines could save valuable time and money. It will be necessary in future vaccine development to design a vaccine that not only induces substantial pre-challenge cellular responses but also prevents the extrathoracic spread of disease. This may be accomplished by incorporating stronger TH1 inducing adjuvants and cytokines into the CFP based vaccines. It is interesting that the outcome of this large animal vaccine trial was significantly different than the outcome of similar trials in smaller animals, which is important to consider for the development of human vaccines.

Acknowledgments

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CHAPTER 3

Vaccination of Cattle with *Mycobacterium avium* subspecies *paratuberculosis* Culture Filtrate Proteins and Interleukin-2 for Protection against Johne's Disease

Abstract

Johne's disease is a serious threat to the agriculture industry of many countries. Currently, the most effective control measure for Johne's disease is the removal of infected animals from the herd. Unfortunately, by the time the animal is showing clinical signs of disease it has likely transmitted the disease to young animals in the herd. The available vaccines for Johne's disease are crude and relatively ineffective. Moreover, the chronic nature of the disease makes the evaluation of new vaccines very difficult. This study assessed the immune responses of cattle vaccinated with a *M. a. paratuberculosis* culture filtrate protein vaccine before and after a virulent oral challenge with *M. a. paratuberculosis*. Preliminary evidence suggests that the CFP vaccine for Johne's disease induced significantly larger populations of activated T cells in vaccinated animals, as compared to the non-vaccinated control animals. In addition, this study demonstrated that monitoring activation marker expression on both αβ and γδ T cells may be useful in assessing the protective efficacy of vaccines.

Introduction

Johne's disease is a chronic enteritis of ruminants that is caused by *Mycobacterium avium* subspecies *paratuberculosis*. Johne's disease is prevalent on every continent and financially it is devastating to the world's agricultural industry (69). The prevalence of Johne's disease in the United States is staggering, with some areas reporting that 74% of the dairy herds are positive for *M. a. paratuberculosis* (289). Annually this disease cost the US agriculture industry between $200 million and 1.5 billion dollars (69, 226).

M. a. paratuberculosis is thought to infect animals shortly after birth, and in fact experimental infections have demonstrated that it is difficult to establish infection in older animals (170, 279). Once animals are infected with *M. a. paratuberculosis* it can be 3-5 years before they show clinical signs of the disease (170). Johne's disease has been extremely difficult to control, in part due to the chronic nature of the disease. It is very difficult to identify infected animals before the onset of clinical symptoms. Unfortunately, animals that are in the subclinical stage of the disease have been shown to shed bacteria for as long as 18 months before the onset of clinical disease, which allows for younger animals to be infected (68).

Detection of infected animals before the onset of shedding and the prevention of infection of young animals would be instrumental in the control of this devastating disease. Diagnosis of bovine mycobacterial diseases has classically relied on measuring DTH responses 72 hours after the injection of PPD. Unfortunately, the use of *M. a. paratuberculosis* PPD (Johnin) has not been effective (94, 311, 313). Currently, the most common diagnostic tool for Johne's disease is the IDEXX *Mycobacterium paratuberculosis* Antibody Test Kit produced by IDEXX Laboratories, Inc. The sensitivity of the IDEXX kit is reported to be only in excess of 50% (77, 204). In addition to the poor sensitivity of diagnostic tests for Johne's disease, vaccines that have been developed for the prevention of Johne's disease have only been marginally effective. Many of these experimental vaccines for Johne's disease have used heat killed mycobacteria (86, 167, 301, 312), which may explain the poor performance.

In recent years, much of the research on experimental vaccines for mycobacterial diseases has focused on using culture filtrate protein based vaccines, rather than heat

killed mycobacterial vaccines (10, 21, 42, 45, 227, 308). A CFP based vaccine has never been evaluated in the bovine model of Johne's disease. There have been several combinations of adjuvants and cytokines used in CFP vaccines, with variable results (10, 21, 42, 177, 227, 308). Dimethyl dioctadecylammonium bromide (DDA) has been used successfully as the adjuvant in CFP based vaccines in both murine models of human and bovine tuberculosis (10, 42). DDA generates strong cell mediated immune responses (128, 177). CpGs, oligonucleotides designed to mimic immunostimulatory microbial DNA motifs, have been shown to be effective stimulators of murine TH1 immune responses when given orally with proteins (192) and have enhanced the protective efficacy of the BCG vaccine in the murine model of tuberculosis (116). The incorporation of IL-2 into mycobacterial CFP vaccines has also favorably augmented the immune response (21, 308).

In this study we sought, not only to evaluate the protective efficacy of a *M. a. paratuberculosis* CFP/DDA/CpG/IL-2 vaccine after an oral challenge with *M. a. paratuberculosis*, but to also investigate cellular immune responses following vaccination and challenge. Very little information is available regarding the changes in activation and memory marker expression on bovine T cells following vaccination with CFP vaccines and challenge with *M. a. paratuberculosis*. This information will be useful in the assessment of both the immunological effects of vaccination and challenge as well as the role of sub-populations of T cells ($CD4^+$, $CD8^+$, $WC1^+$, and $WC1^-$) in Johne's disease. This study also investigated the effectiveness of using various CFP fractions as skin test antigens for the diagnosis of *M. a. paratuberculosis* infection.

Materials and Methods

Animals

Ten Holstein steers were purchased from a local dairy known to have low incidence of Johne's disease. At the onset of this study all animals were between 10 and 12 weeks of age and had been de-horned, vaccinated, and weaned. The animals were fed grain and hay once daily and were allowed to graze in an isolation unit following challenge. All animals were skin test negative in response to Johnin and to CFP derived from *M. a. paratuberculosis* strain 97R-1722.

Bacterial growth conditions

A field isolate of *M. a. paratuberculosis* strain 97R-1722 (a generous gift from Dr. Robert H. Whitlock) was used to produce the culture filtrate proteins and was used as the challenge strain. Initially the strain was grown in 7H9 broth enriched with 0.025% (vol/vol) tyloxapol (Sigma) and 2mg/liter of mycobactin J. When the culture was well established it was grown in Sauton's broth, also supplemented with 0.025% tyloxapol and 2mg/liter of mycobactin J. When the culture reached mid-log phase (typically after approximately 4 weeks), 0.05% Tween 80 was added and the culture was incubated with agitation overnight. Low passaged seed lots were frozen and stored at -20°C. The number of colony forming units/ml was determined by plating 10-fold dilutions on Middlebrook 7H11 (Difco Laboratories) supplemented with mycobactin J.

Preparation of Culture Filtrate Proteins

The CFP used to vaccinate cattle in this study was prepared from *M. a. paratuberculosis* strain 97R-1722. Strain 97R-1722 was grown in Sauton's medium, supplemented with 0.025% (vol/vol) tyloxapol and 2mg/liter mycobactin J, with gentle

rolling until it reached mid-log phase. Bacterial cells were pelleted by centrifugation at 3000 rpm for 30 minutes. The culture supernatant was then filtered through a 0.22 μm-pore-size filter. The CFP was concentrated by ultrafiltration using an ultrafiltration stirred cell (Amicon, Danvers, Mass.) with a PM10 membrane (Millipore, Bedford, Mass.), which allows the concentration of proteins greater than 10,000 Daltons in size. The flow through was concentrated using a PM3 membrane (Millipore, Bedford, Mass.), which allows the concentration of proteins greater than 3,000 Daltons in size. The concentrated CFP was dialyzed against a sodium phosphate buffer for 48 hours with at least 3 buffer changes. The protein concentration of the CFP was measured using the BCA assay (Pierce, Rockford, Ill.). The CFP was then filtered through a 0.22 μm-pore-size filter and then lyophilized.

Adjuvants and vaccine preparation

In addition to the CFP from strain 97R-1722, dimethyl dioctadecyl ammoniumbromide (DDA), CpGs, and recombinant bovine IL-2 (a kind gift from Dr. Neil Wedlock) were also included as adjuvants. The CFP (greater than 10,000 Da) was resuspended in PBS to 1 mg/ml. DDA was resuspended in PBS to 1 mg/ml and then heated to 80°C for 10 minutes. CpGs were resuspended to 1 mg/ml in sterile water. The IL-2 was resuspended in sterile water to 500 μg/ml. The vaccine was composed of 500 μg CFP (greater than 10 kDa fraction), 400 μg DDA, 150 μg CpG, and 20 μg IL-2.

Vaccination and challenge

The animals were randomly divided into two groups (n=5). The first group received adjuvants alone and the second group received CFP and adjuvants. All animals were vaccinated twice subcutaneously, at three-week intervals (the first vaccine

did not include IL-2 but the second vaccine did). Animals were rested for 4 weeks and then challenged with 10^7 bacteria on three consecutive days, for a total challenge of $3x10^7$ bacteria. The challenge dose was mixed with 25 ml of milk and given orally with a 60 ml syringe. Approximately 18 weeks following challenge the animals received a vaccine boost. Vaccinated animals received 500 μg of CFP and 400 μg of DDA, whilst the non-vaccinated group received DDA alone. Table 3.1 summarizes the vaccine formulations that each animal received.

Feces collection and isolation of mycobacteria

Approximately 3 grams of feces were collected from all animals periodically after challenge. The feces were processed and analyzed by the Diagnostic Laboratory at Colorado State University Veterinary Hospital for the presence of mycobacteria.

Skin Testing

All animals were skin tested prior to the onset of this study, following vaccination, and periodically after challenge. The animals were skin tested with saline, purified protein derivative from *M. avium* strain D-4 (USDA, Ames, IA.), purified protein derivative from *M. bovis* AN-5 (USDA), Johnin (USDA), CFP (greater than 10 kDa), CFP (3-10 kDa), and a pool of 3-10 kDa and greater than10 kDa CFP. Prior to skin testing the cervical area was shaved with a surgical blade and the area was wiped with alcohol. 100 μl of a 1mg/ml solution of each antigen was subcutaneously injected into a separate site in the cervical area. The injection sites and normal skin were measured 72 hours later with calipers.

Table 3.1. Vaccine Formulations

Animal #	Vaccine #1	Vaccine #2	Vaccine #3
5507	DDA/CpG	DDA/CpG/IL-2	DDA
5724	DDA/CpG	DDA/CpG/IL-2	DDA
5819	DDA/CpG	DDA/CpG/IL-2	-
5867	DDA/CpG	DDA/CpG/IL-2	DDA
5898	DDA/CpG	DDA/CpG/IL-2	DDA
6140	DDA/CpG/CFP	DDA/CpG/IL-2/CFP	DDA/CFP
6265	DDA/CpG/CFP	DDA/CpG/IL-2/CFP	DDA/CFP
6271	DDA/CpG/CFP	DDA/CpG/IL-2/CFP	DDA/CFP
6280	DDA/CpG/CFP	DDA/CpG/IL-2/CFP	DDA/CFP
6294	DDA/CpG/CFP	DDA/CpG/IL-2/CFP	DDA/CFP

Animals were randomly divided into two groups. The first group received three vaccines that contained adjuvant formulation. The second group also received three vaccines that contained CFP in addition to the adjuvant formulation given to the first group of animals.

Flow cytometric evaluation

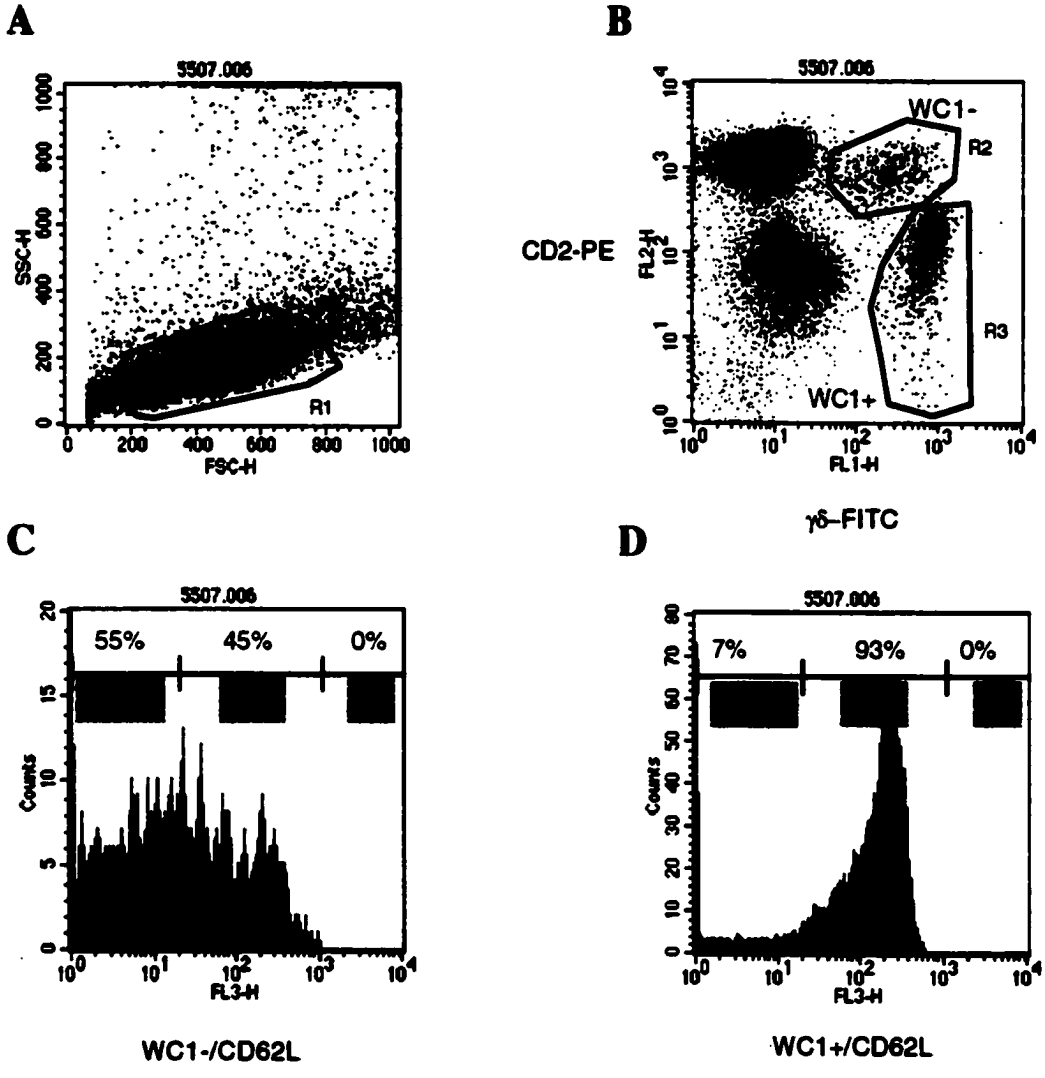
50 ml of whole blood was collected from each animal prior to the onset of this study, following vaccination, and periodically after challenge. The whole blood was collected into CPT sodium heparin Vacutainer tubes (VWR, Denver CO). The CPT tubes were centrifuged at 3000 rpm for 30 minutes and the peripheral blood mononuclear cells (PBMCs) were removed. The PBMCs were then washed twice in deficient RPMI supplemented with 0.1% sodium azide. The cells were adjusted to $1x10^7$ cells/ml and 200 µl were plated into each of 19 wells of a 96 well round bottom plate. $WC1^+$ γδ T cells, $WC1^-$ γδ T cells, $CD4^+$ T cells, and $CD8^+$ T cells from each animal were analyzed for the expression of CD62L, CD44, CD45R, and CD25 on the cell surface. Cells were stained with either mouse anti-bovine CD4 (VMRD, Pullman WA), mouse anti-bovine CD8-FITC (Serotec, Raleigh NC), or mouse anti-bovine CD2-PE (VMRD) and mouse anti-bovine δ chain-FITC (VMRD). The cells that were stained with mouse anti-bovine CD4 were then stained with anti mouse IgG-PE (Sigma, St. Louis MO). For two and three color flow cytometry cells were also stained with mouse anti-bovine CD62L (Serotec), mouse anti-bovine CD25 (VMRD), mouse anti-bovine CD44 (VMRD), or mouse anti-bovine CD45R (Serotec). These cells were then stained with anti-mouse IgG1-PerCP (Becton Dickenson, San Diego CA). Both the anti-bovine δ chain and the anti-bovine CD2 antibodies were conjugated to either FITC or PE by Chromoprobe. Cells were also stained with the appropriate isotype controls. Samples were run on the FACS Calibur (Becton Dickenson) flow cytometer and analyzed using CELLQuest software (Becton Dickenson).

An example of an analysis file is shown in Figures 3.1A-D. The lymphocytes were gated based on their forward scatter and side scatter profile (Figure 3.1A) and designated region 1 (R1). The T cell subset of interest was then gated based on fluorescence. In Figure 3.1B the cells in gate R2 are $CD2^+$ and $\gamma\delta^+$ and are therefore $WC1^-$ $\gamma\delta$ T cells and the cells in gate R3 are $CD2^-$ and $\gamma\delta^+$ and therefore $WC1^+$ $\gamma\delta$ T cells. The expression of the cell surface molecule (CD62L in Figures 3.1C and 3.1D) of interest on the T cell subset of interest was then analyzed in a histogram format. Figure 3.1C shows the expression of CD62L on $WC1^-$ $\gamma\delta$ T cells and Figure 3.1D shows the expression of CD62L on $WC1^+$ $\gamma\delta$ T cells. The histograms were divided into three regions based on levels of fluorescence intensity (Figures 3.1C and 3.1D). The more molecules of CD62L on the surface the higher the fluorescence intensity. The first region was designated "neg/lo" because the cells that fell into this region had little or no CD62L on their surfaces (Figures 3.1C and 3.1D). The second region was designated "mid" because the cells that fell into this region had a moderate amount of CD62L on their surfaces. Finally, the third region was designated "hi" because the cells that fell into this region had numerous CD62L molecules on their surfaces. Note that in this instance there were no $WC1^-$ $\gamma\delta$ T cells (Figure 3.1C) or $WC1^+$ $\gamma\delta$ T cells (Figure 3.1D) that fell into the "hi" region of CD62L expression. The percentage of the T cell subsets of interest that fell into each of the regions were then calculated and used to monitor cell populations over the course of this study.

IFN-γ ELISA

At the onset of this study, following vaccination, and periodically after challenge, peripheral blood mononuclear cells from each animal were isolated as

Figure 3.1. Description of Flow Cytometric Analysis



The flow cytometric analysis was carried out by first gating on lymphocytes (A) followed by the T cell population of interest (B) in this case $WC1^+$ and $WC1^-$ γδ T cells. Histogram plots can then be used to examine an additional cell marker only on the populations previously gated (C and D). In C the histogram shows CD62L expression on $WC1^-$ cells and D shows CD62L expression on $WC1^+$ cells.

described for flow cytometry. The PBMCs were resuspended in RPMI 1640 (supplemented with 10% heat inactivated fetal calf serum, 0.12% β-Mercaptoethanol, 5% HEPES, 5% L-glutamine (200 mM), and 50 mg ampicillin) to $1x10^6$ cells/ml. 200 µl of the cell suspension from each animal was added to 9 wells of a 96 well plate. In triplicate, either ConA (1 µg/ml), *M. a. paratuberculosis* > 10kDa CFP (10 µg/ml), or PBS was added to the wells. The plates were incubated at 37°C with 5% CO_2 for 48 hours. The plates were then removed and frozen at -80°C until the ELISAs were run. Bovine IFN-γ EASIA kit was used to qualitatively measure the IFN-γ in the supernatants (Biosource, Belgium). The kit protocol was followed. Briefly, 100 µl of the tissue culture supernatant was added to the anti-IFN-γ coated wells. Positive and negative controls were supplied with the kit and added to anti-IFN-γ coated wells. 50 µl of incubation buffer was added to each well and the plates were incubated at room temperature on a horizontal shaker (700 rpm) for one hour. The plates were washed 3 times with 1x PBS/Tween. 100 µl of the working conjugate was added to each well and incubated at room temperature on a horizontal shaker (700 rpm) for one hour. The plates were washed 3 times with 1x PBS/Tween. 100 µl of the Chromogen (TMB) was added to each well and the plates were incubated at room temperature on a horizontal shaker (700 rpm) for 15 minutes. 200 µl of the Stop Solution was added to each well and the plate was read at 450 nm against a 630 nm reference filter. Positive samples had OD values that were greater than the average OD reading of the negative control plus 0.15.

Statistical Analysis

The statistical analysis of the data from the flow cytometry was done using the Student's *t* Test.

Results

Fecal Cultures

It has been demonstrated that animals infected with *M. a paratuberculosis* shed large numbers of organisms in their feces, especially during the period immediately before clinical signs develop and during the clinical stages of the disease (68). Little information is available regarding the amount of shedding that occurs immediately after infection. Monitoring the feces of the challenged animals for the presence of mycobacteria was therefore a useful way to monitor the progression of the disease and to determine at what point the infected animals began to shed the organism. Results from samples collected at day 1, week 1, week 2, week 6, week 15, and week 30 show that only one animal at one time point tested positive (Table 3.2).

Skin tests

Currently there is no widely used skin test reagent for the detection of Johne's disease. The commercial tests that are available are time consuming and not very reliable (77, 94, 204, 311, 313). The discovery of a specific and sensitive skin test reagent for the diagnosis of Johne's disease would be very useful. The skin test reagents used in this study included *M. a. paratuberculosis* PPD and PPDs derived from both *M. avium* and *M. bovis* as controls. Additionally, three different fractions of CFP (greater than 10 kDa fraction, 3-10 kDa fraction, and a pool of both the greater than 10 kDa and 3-10 kDa fractions) were tested. At each time-point every animal received a saline injection as a negative control. At the onset of this study, 2 weeks post-vaccination, 6 weeks post-challenge, 15 weeks post-challenge, and 29 weeks post-challenge each animal received an intradermal injection of each skin test reagent. 72 hours after the

Table 3.2. Mycobacterial Colonies Isolated from Feces

	# of mycobacterial colonies isolated					
Animal #	Day 1	Week 1	Week 2	Week 6	Week 15	Week 30
5507	0	0	0	0	0	0
5724	0	0	0	0	0	0
5819	0	RIP	RIP	RIP	RIP	RIP
5867	0	0	0	0	0	0
5898	0	0	0	0	0	0
6140	0	0	0	0	0	0
6265	0	0	0	0	0	0
6271	0	0	0	2	0	0
6280	0	0	0	0	0	0
6294	0	0	0	0	0	0

At various timepoints during this study 3 grams of feces was collected and processed for the isolation of acid fast bacilli. The animals were challenged between week 2 and week 6. RIP = dead.

administration of the skin test reagents the skin thickness was measured using calipers. The results of the skin test readings are shown in Tables 3.3A-3.3E. To be considered positive, an increase in skin thickness of at least 2 mm over that of both the normal skin and the saline injection site was necessary. Animal 5724 was negative for all skin test reagents at the onset of this study (Table 3.3A) but was strongly positive in response to Johnin and slightly positive to two of the three CFP fractions 2 weeks after vaccination (Table 3.3B). As this animal received only the adjuvant vaccine, it is likely that it was exposed to *M. a. paratuberculosis* prior to the onset of our study. All but one animal were slightly positive to one or more of the skin test reagents at at least one timepoint post-challenge (Table 3.3C-E). There did not appear to be any pattern to the positive responses.

Flow Cytometric Analysis

Changes in Peripheral Blood T cells

$WC1^+$ γδ T cells, $WC1^-$ γδ T cells, $CD4^+$ T cells, and $CD8^+$ T cells were monitored over the course of this study. The percentage of total peripheral blood lymphocytes was determined for each T cell population of interest by flow cytometry. The $WC1^+$ γδ T cells made up between 18 and 30% of the total peripheral blood lymphocytes at the onset of this study (Figure 3.2A). The adjuvant group had a significantly higher percentage of $WC1^+$ γδ T Cells than the CFP group at the onset of this study (Figure 3.2A). This is likely a reflection of the age of the animals. As the animals increased in age the percentage of $WC1^+$ γδ T cells decreased in both groups of animals (Figure 3.2A). The percentage of $WC1^-$ γδ T cells remained relatively constant throughout the course of this study, remaining well below 10% of the peripheral blood

Table 3.3. Skin Test Responses

A

Baseline	Normal	Saline	Johnin	PPD A	PPD B	CFP 3-10 kDa	CFP > 10 kDa	CFP Pool
				Skin Thickness in mm				
Animal #								
5507	5	3	3	3	3	3	3	5
5724	4	4	3	4	4	4	4	5
5819	4	3	3	3	3	3	3	4
5867	4	3	5	3	3	4	3	4
5898	5	4	4	5	3	4	5	5
6140	5	3	5	4	4	5	4	5
6265	5	4	4	5	5	5	4	5
6271	4	3	3	3	3	3	4	4
6280	5	3	3	4	4	3	4	4
6294	5	4	4	4	4	4	4	5

Animals were skin tested with several antigens at several timepoints throughout this study. Data are represented as mm of skin thickness at the injection site 72 hours post-injection. Positives are indicated in red and indicate at least a 2mm increase in skin thickness over both normal and saline controls.

Table 3.3. Skin Test Responses (cont.)

B

2 Weeks Post Vaccination	Normal	Saline	Johnin	PPD A	PPD B	CFP 3-10 kDa	CFP > 10 kDa	CFP Pool
Animal #	Skin Thickness in mm							
5507	4	3	3	3	3	4	4	5
5724	4	3	19	4	5	3	6	8
5819	4	3	3	3	3	3	3	3
5867	4	3	3	4	4	4	4	4
5898	5	3	3	3	4	4	3	5
6140	4	3	4	3	3	3	4	4
6265	4	3	4	3	3	4	4	4
6271	4	3	3	3	3	3	4	4
6280	4	3	4	4	4	4	5	5
6294	4	3	3	3	3	4	4	4

Animals were skin tested with several antigens at several timepoints throughout this study. Data are represented as mm of skin thickness at the injection site 72 hours post-injection. Positives are indicated in red and indicate at least a 2mm increase in skin thickness over both normal and saline controls.

Table 3.3. Skin Test Responses (cont.)

C

6 Weeks Post Challenge	Normal	Saline	Johnin	PPD A	PPD B	CFP 3-10 kDa	CFP > 10 kDa	CFP Pool
Animal #	Skin Thickness in mm							
5507	5	4	4	5	4	5	5	6
5724	5	4	9	5	4	5	5	6
5819	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP
5867	5	5	6	4	5	5	5	6
5898	5	5	7	7	6	6	6	8
6140	5	4	4	5	4	9	5	7
6265	6	4	6	6	5	6	8	7
6271	4	6	4	6	5	4	6	6
6280	6	7	6	6	6	6	7	7
6294	5	4	4	4	4	5	4	6

Animals were skin tested with several antigens at several timepoints throughout this study. Data are represented as mm of skin thickness at the injection site 72 hours post-injection. Positives are indicated in red and indicate at least a 2mm increase in skin thickness over both normal and saline controls.

Table 3.3. Skin Test Responses (cont.)

D

15 Weeks Post Challenge	Normal	Saline	Johnin	PPD A	PPD B	CFP 3-10 kDa	CFP > 10 kDa	CFP Pool
Animal #	Skin Thickness in mm							
5507	3	3	3	3	3	4	4	5
5724	3	4	6	5	4	5	4	5
5819	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP
5867	3	3	4	3	4	4	4	5
5898	3	3	5	5	4	5	5	5
6140	3	4	3	4	3	4	4	4
6265	4	4	5	6	5	6	5	5
6271	3	2	3	3	4	5	4	5
6280	3	3	3	4	5	5	5	6
6294	3	3	3	4	4	4	4	4

Animals were skin tested with several antigens at several timepoints throughout this study. Data are represented as mm of skin thickness at the injection site 72 hours post-injection. Positives are indicated in red and indicate at least a 2mm increase in skin thickness over both normal and saline controls.

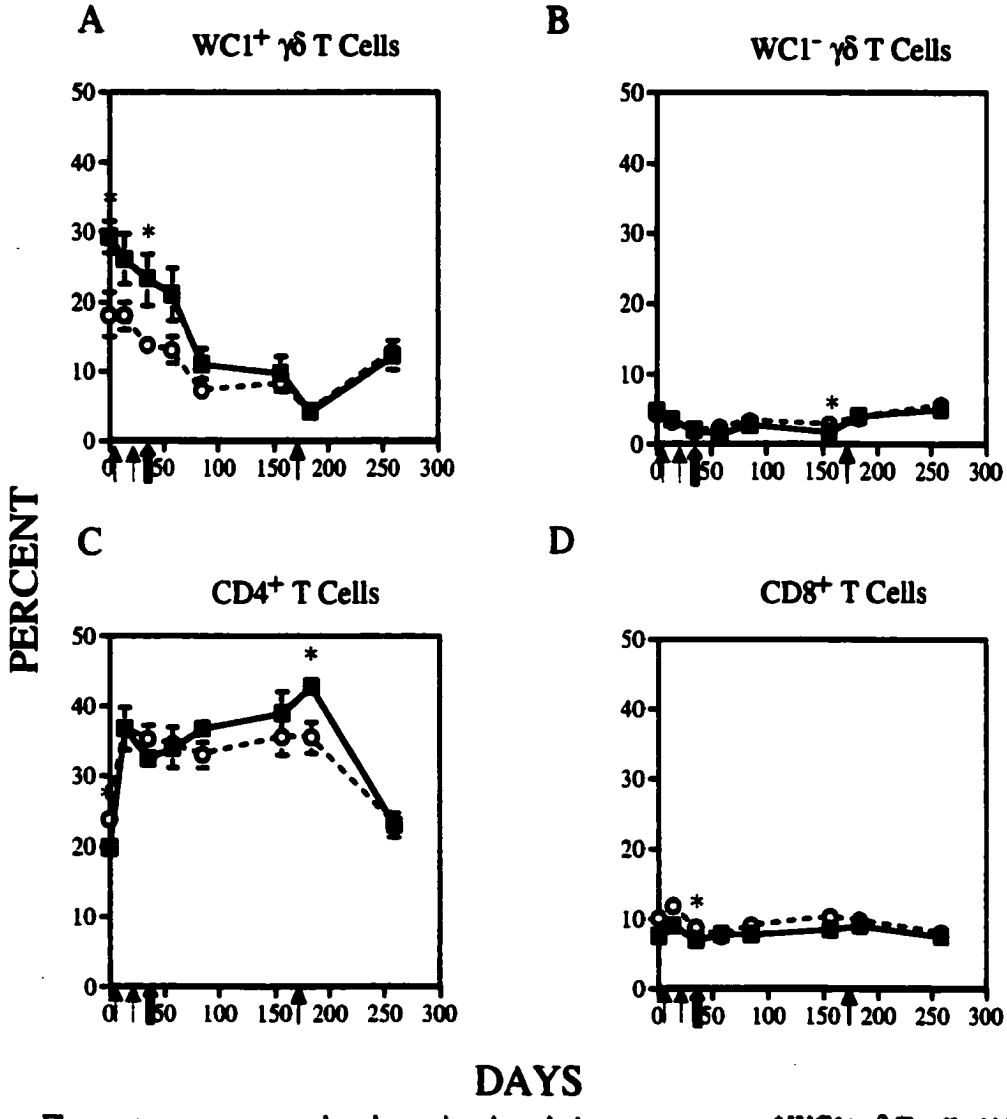
Table 3.3. Skin Test Responses (cont.)

E

29 Weeks Post Challenge	Normal	Saline	Johnin	PPD A	PPD B	CFP 3-10 kDa	CFP > 10 kDa	CFP Pool
Animal #	Skin Thickness in mm							
5507	4	4	4	3	4	4	5	5
5724	4	4	6	6	5	5	5	6
5819	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP
5867	4	4	4	4	5	5	4	6
5898	4	4	5	5	4	5	5	6
6140	4	3	3	4	4	5	5	5
6265	4	4	5	4	5	5	5	6
6271	4	3	4	5	4	4	4	4
6280	4	4	4	5	4	5	6	7
6294	5	4	4	4	4	5	6	6

Animals were skin tested with several antigens at several timepoints throughout this study. Data are represented as mm of skin thickness at the injection site 72 hours post-injection. Positives are indicated in red and indicate at least a 2mm increase in skin thickness over both normal and saline controls.

Figure 3.2. Percentages of T Cells



Flow cytometry was used to determine the relative percentages of WC1+ γδ T cells (A), WC1- γδ T cells (B), CD4+ T cells (C), and CD8+ T cells (D) at various timepoints throughout this study. Data are represented as mean percentages ± SE. Closed squares represent the adjuvant group and the open circles represent the CFP group. ↑= 1st vaccination, ↑=2nd vaccination, ↑ = challenge, and ↑=3rd vaccination. *=significantly different than adjuvant group ($p < 0.05$).

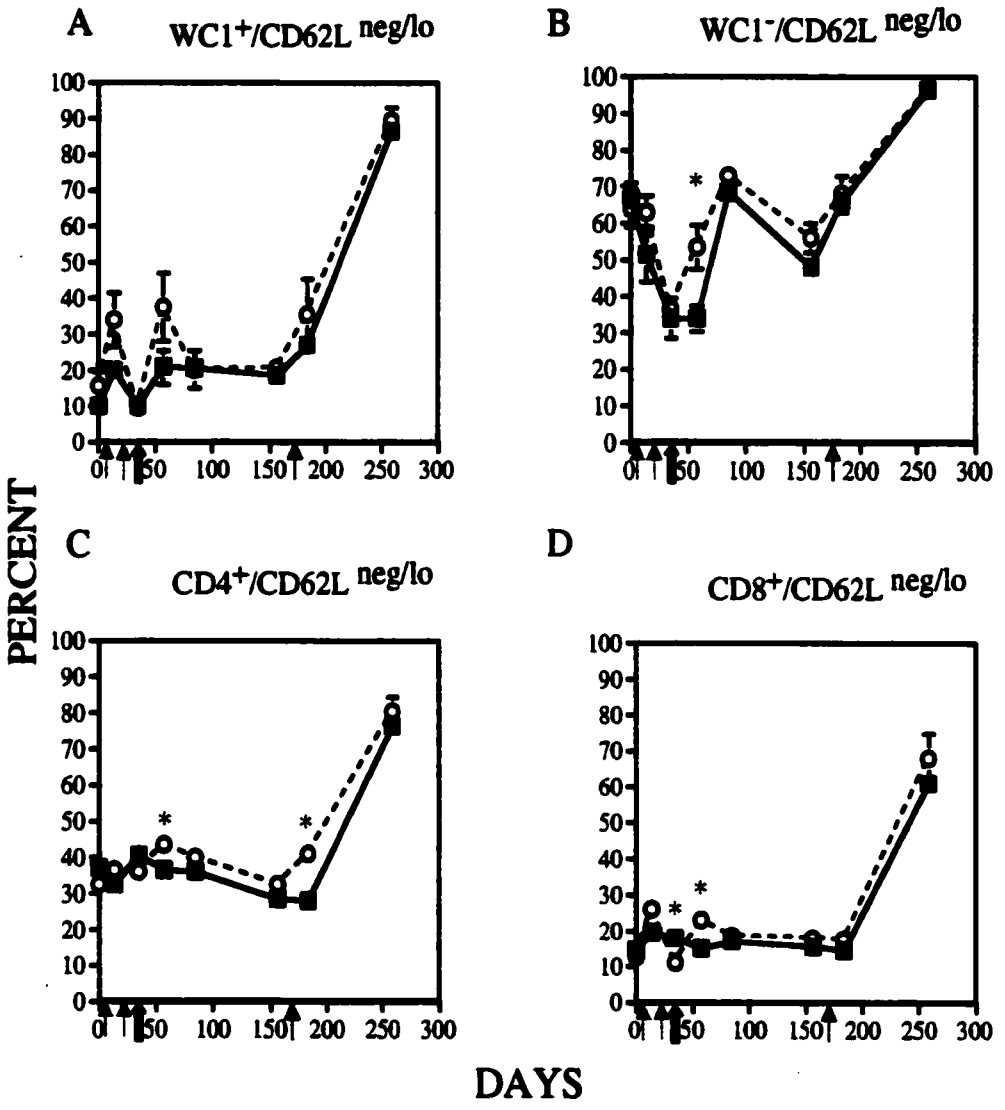
lymphocytes (Figure 3.2B). The percentage of $CD4^+$ T cells varied between 20 and 40% throughout the course of the experiment (Figure 3.2C). The changes observed in the percentage of $CD4^+$ T cells were consistent between both groups of animals, with the exception of one timepoint (Figure 3.2C). One week following vaccine #3 the animals in the adjuvant group had a significantly higher percentage of $CD4^+$ T cells than did the animals in the CFP group (Figure 3.2C). The final timepoint shows a drop of about 10% in the percentage of $CD4^+$ T cells in both groups of animals (Figure 3.2C). This is likely due to an unknown immunological challenge of the animals, as the animals were not kept in a pathogen free environment but were grazed on pasture. Similar to the $WC1^-$ γδ T cells, the percentage of $CD8^+$ T cells remained relatively constant throughout the course of the study, for both groups of animals (Figure 3.2D).

Expression of CD62L

CD62L is a cell surface molecule that allows lymphocytes to bind high endothelial venules of peripheral lymph nodes (163, 268). CD62L is expressed on naïve lymphocytes and therefore allows naïve cells to enter lymph nodes where antigen presentation can take place. Additionally, CD62L has been shown to be important for migration into sites of inflammation (15, 92, 235). Following activation the expression of CD62L is lost, allowing the lymphocytes to remain at the area of inflammation or infection. For this reason CD62L is a convenient marker of activation. In this study, lymphocytes that have little or no CD62L on their surfaces ($CD62L^{neg/lo}$) are considered activated.

The expression of CD62L on the $WC1^+$ γδ T cells was relatively similar between both groups of animals throughout the course of this study (Figure 3.3A). About

Figure 3.3. CD62L Expression



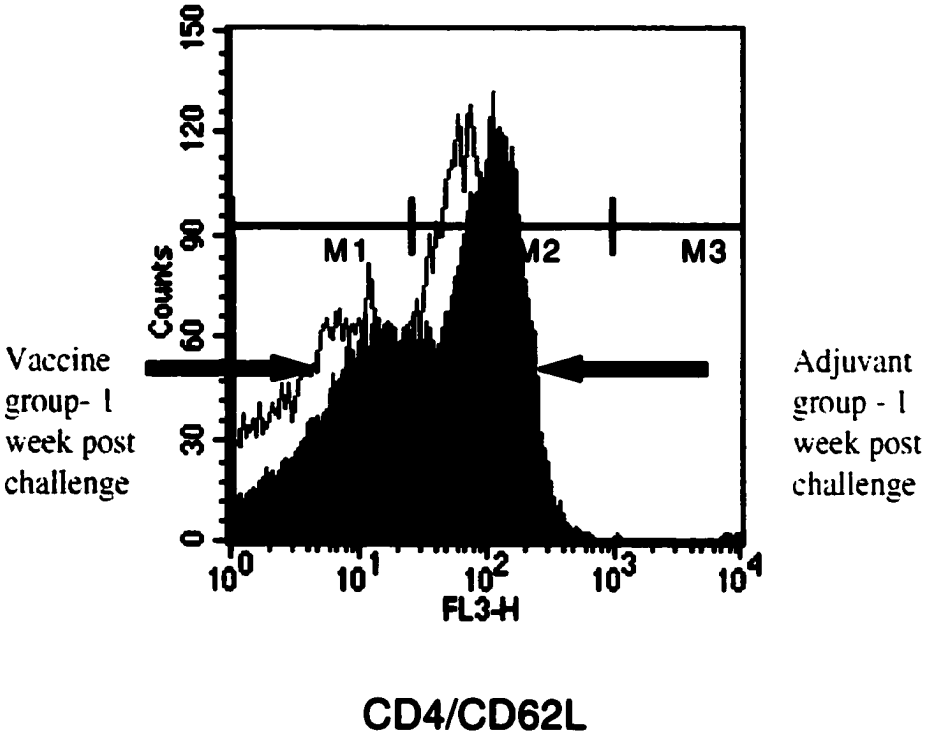
Flow cytometry was used to determine the relative percentages of CD62L $^{neg/lo}$ expression on WC1$^+$ γδ T cells (A), WC1$^-$ γδ T cells (B), CD4$^+$ T cells (C), and CD8$^+$ T cells (D) at various timepoints throughout this study. Data are represented as mean percentages ± SE. Closed squares represent the adjuvant group and the open circles represent the CFP group. ↑= 1st vaccination, ↑=2nd vaccination, ↑= challenge, and ↑=3rd vaccination. *=significantly different than adjuvant group ($p < 0.05$).

20% of the $WC1^+$ γδ T cells were of the activated phenotype throughout this study (Figure 3.3A). The $WC1^-$ γδ T cells had a higher percentage of $CD62L^{neg/lo}$ cells than the $WC1^+$ γδ T cells (Figure 3.3B). One week following challenge the CFP vaccinated group had a significantly higher percentage of $WC1^-/CD62L^{neg/lo}$ γδ T cells than the adjuvant vaccinated group (Figure 3.3B). This "activated" compartment remained elevated for the duration of this study (Figure 3.3B). The expression of CD62L on $CD4^+$ T cells was relatively constant throughout the course of this study, typically between 30 and 40% of the $CD4^+$ T cells were of the $CD62L^{neg/lo}$ phenotype (Figure 3.3C). As with the $WC1^-$ γδ T cells, the CFP vaccinated group had a significantly larger percentage of $CD4^+$ T cells that were $CD62L^{neg/lo}$, one week following challenge (Figure 3.3C). This increase in the percentage of $CD4^+/CD62L^{neg/lo}$ T cells in the CFP vaccinated group, as compared to the adjuvant vaccinated group, following challenge is also shown in Figure 3.4. The expression of CD62L on the surface of $CD8^+$ T cells was very consistent throughout this study (Figure 3.3D). As described with both the $WC1^-$ γδ T cells and with the $CD4^+$ T cells, the CFP vaccinated group had a significantly larger percentage of $CD62L^{neg/lo}$ T cells, when compared to the adjuvant vaccinated group, following challenge with *M. a. paratuberculosis* (Figure 3.3D). At the final timepoint there was a significant increase in the $CD62L^{neg/lo}$ population of all the T cell populations studied, in both groups of animals. This increase in the activated populations was likely the effect of an unrelated challenge.

Expression of CD25

CD25 is the alpha chain of the IL-2 receptor and is therefore a marker of activation. The expression of CD25 on the T cell subsets studied in this experiment was low throughout the duration of the study. The expression of CD25 on the surface of

Figure 3.4. CD62L Expression on CD4+ T Cells



Flow cytometry was used to analyze differences in CD62L expression on CD4+ T cells between the CFP vaccine group (gray) and the adjuvant group (pink).

WC1$^+$ γδ T cells, WC1$^-$ γδ T cells, and CD8$^+$ T cells was less than 5% at all timepoints studied (Figures 3.5A, B, and D). The expression of CD25 on CD4$^+$ T cells was slightly higher and more variable (Figure 3.5C).

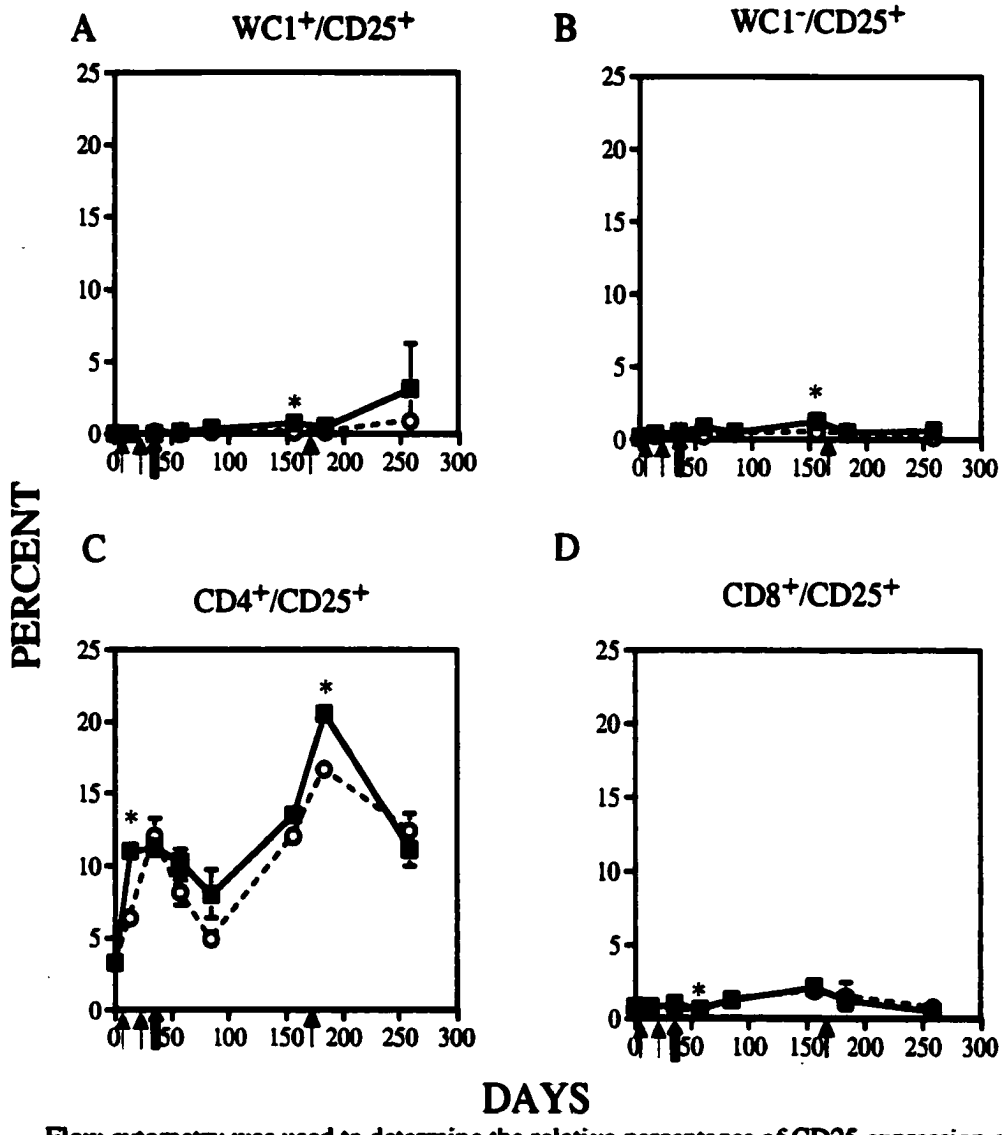
Expression of CD44

CD44 is a cell surface molecule that is classically a marker of activation and along with other cell surface molecules, a marker of T cell memory (50, 101, 251). The ligand for CD44 is hyaluronic acid and the receptor-ligand interaction allows for the adhesion of cells to stromal cells and facilitates the exiting of cells from the blood vessels into the lymph nodes or areas of inflammation (16). The percentages of WC1$^+$ γδ T cells, WC1$^-$ γδ T cells, CD4$^+$ T cells, and CD8$^+$ T cells, that were CD44$^{neg/lo}$ were investigated in this study. There were no significant differences, between the adjuvant vaccinated group and the CFP vaccinated group, in the percentages of CD44$^{neg/lo}$ populations for any of the T cell subsets studied, at any timepoint (Figures 3.6A-3.6D).

Expression of CD45R

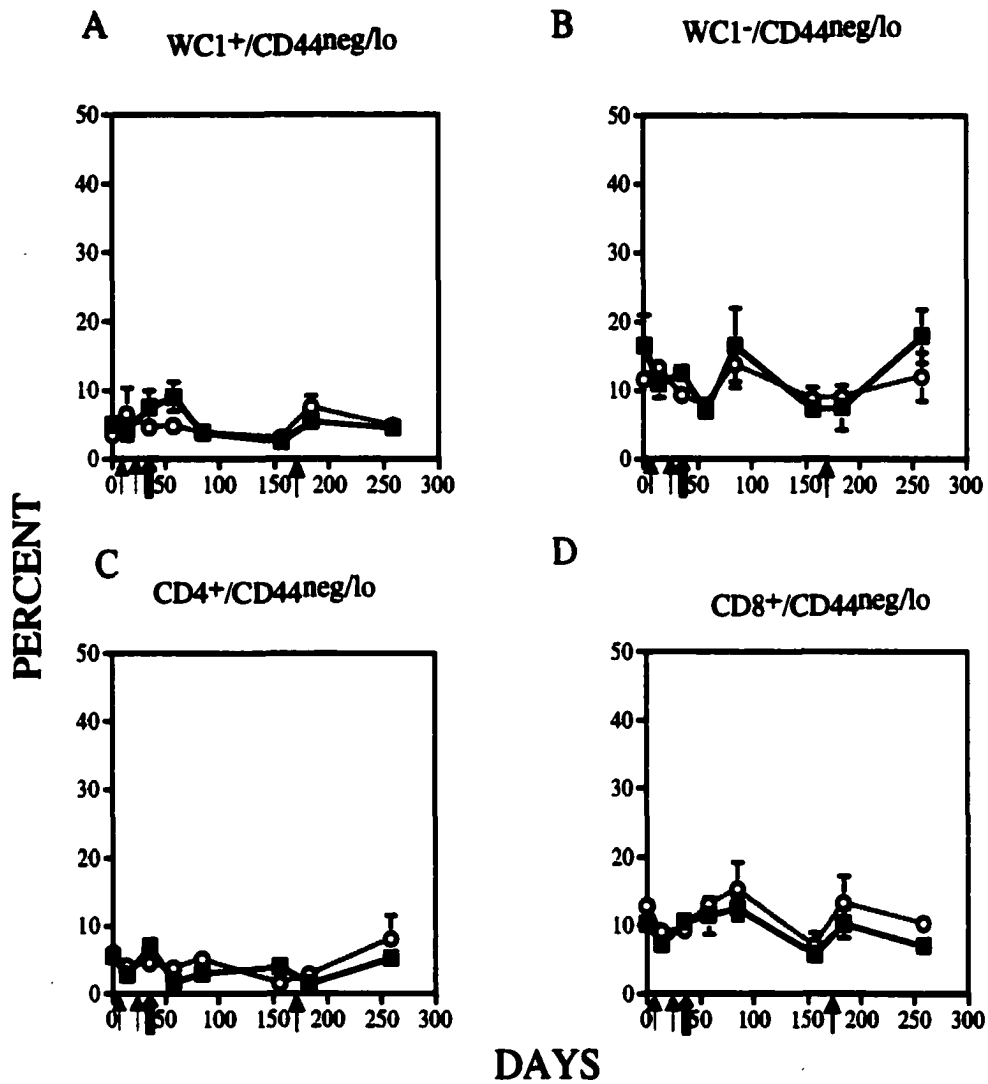
CD45 is a phosphotyrosine phosphatase that is important for cell signaling. There are many isoforms of CD45 that are the result of alternative splicing. In mice, humans, and cattle, differential expression of the CD45 isoforms distinguish naïve (CD45R$^{mid/hi}$) and memory (CD45R$^{neg/lo}$) cells (144, 172, 195). The antibody used in this study specifically recognizes bovine CD45R, which is most similar to human CD45RB. The expression of CD45R on WC1$^+$ γδ T cells was fairly dynamic throughout the course of this study (Figure 3.7A). Following the second vaccination and at two timepoints following challenge, the CFP vaccinated group had a significantly larger population of CD45R$^{neg/lo}$ WC1$^+$ γδ T cells than did the adjuvant vaccinated group (Figure 3.7A). The

Figure 3.5. CD25 Expression



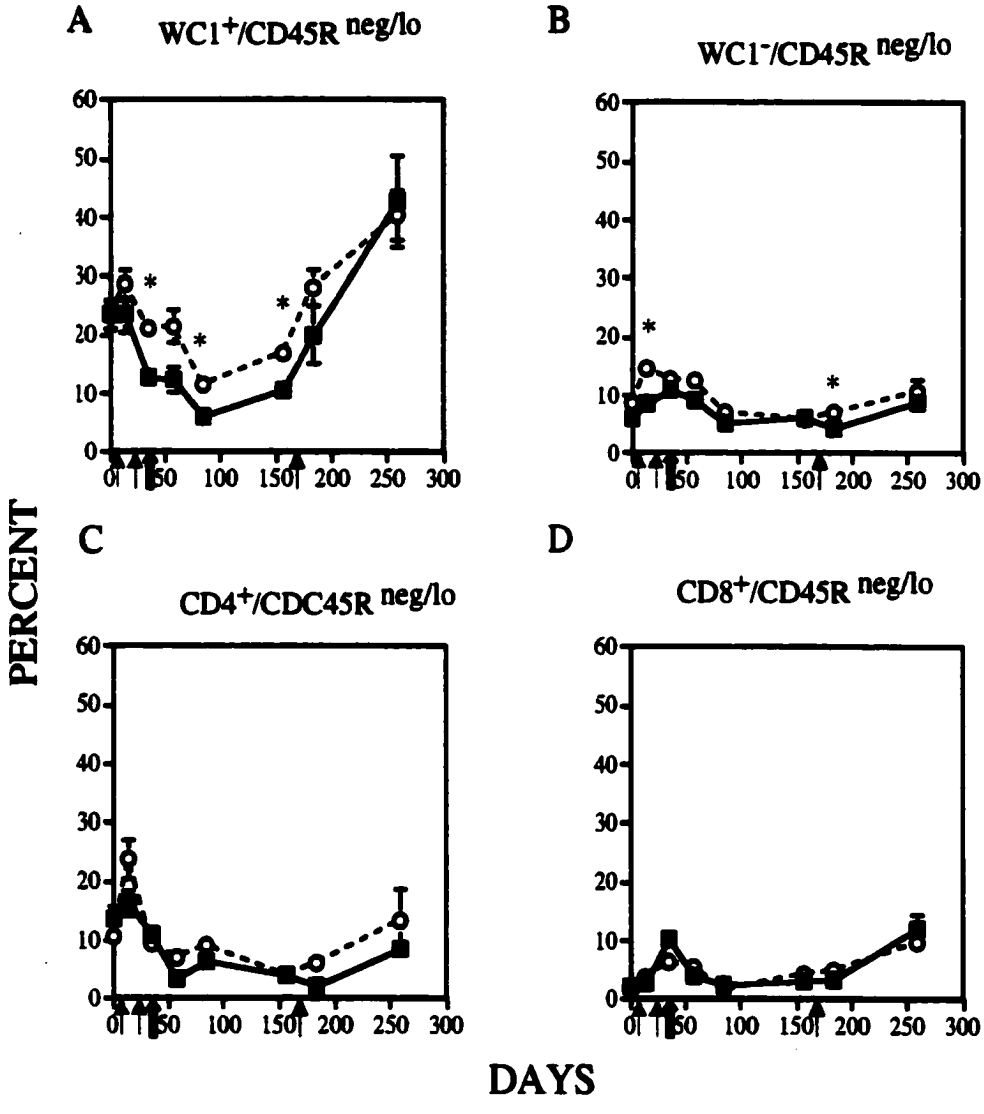
Flow cytometry was used to determine the relative percentages of CD25 expression on $WC1^+$ γδ T cells (A), $WC1^-$ γδ T cells (B), $CD4^+$ T cells (C), and $CD8^+$ T cells (D) at various timepoints throughout this study. Data are represented as mean percentages ± SE. Closed squares represent the adjuvant group and the open circles represent the CFP group. ↑= 1st vaccination, ↑=2nd vaccination, ↑= challenge, and ↑=3rd vaccination. *=significantly different than adjuvant group ($p < 0.05$).

Figure 3.6. CD44 Expression



Flow cytometry was used to determine the relative percentages of CD44 expression on WC1+ γδ T cells (A), WC1- γδ T cells (B), CD4+ T cells (C), and CD8+ T cells (D) at various timepoints throughout this study. Data are represented as mean percentages ± SE. Closed squares represent the adjuvant group and the open circles represent the CFP group. ↑= 1st vaccination, ↑=2nd vaccination, ↑= challenge, and ↑=3rd vaccination. *=significantly different than adjuvant group ($p < 0.05$).

Figure 3.7. CD45R Expression



Flow cytometry was used to determine the relative percentages of CD45R $^{neg/lo}$ expression on WC1$^+$ γδ T cells (A), WC1$^-$ γδ T cells (B), CD4$^+$ T cells (C), and CD8$^+$ T cells (D) at various timepoints throughout this study. Data are represented as mean percentages ± SE. Closed squares represent the adjuvant group and the open circles represent the CFP group. ↑= 1st vaccination, ↑=2nd vaccination, ↑= challenge, and ↑=3rd vaccination.
*=significantly different than adjuvant group ($p < 0.05$).

expression of CD45R was relatively constant on the $WC1^{-}$ γδ T cells (Figure 3.7B). After the administration of vaccine #1 and vaccine #3, the $WC1^{-}$ γδ T cells of the CFP vaccinated group had significantly larger populations of $CD45R^{neg/lo}$ cells (Figure 3.7B). This increase in the percentage of $CD45R^{neg/lo}$ $WC1^{-}$ γδ T cells in the CFP vaccinated group, as compared to the adjuvant vaccinated group, following the administration of the first vaccine is also shown in Figure 3.8. There were no significant differences in the levels of CD45R expression, between the CFP vaccinated group and the adjuvant vaccinated group, on $CD4^{+}$ T cells or $CD8^{+}$ T cells at any timepoint (Figures 3.7C-3.7D).

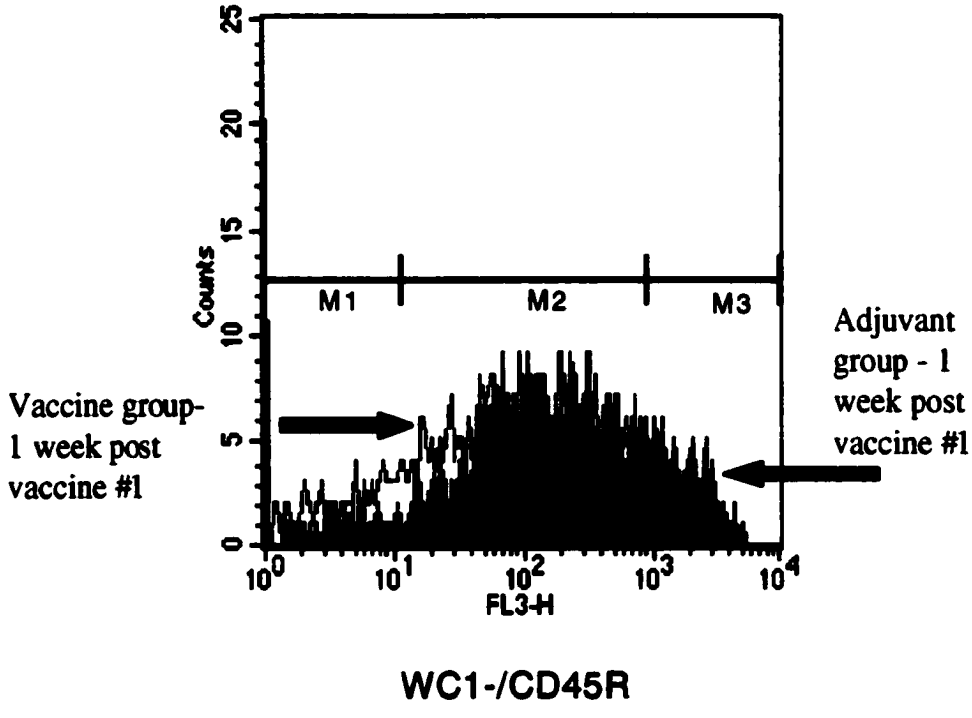
In Vitro Production of IFN-γ by CFP Stimulated PBMCs

The in vitro production of IFN-γ to *M. a. paratuberculosis* > 10kDa CFP was measured at the onset of this study, following vaccination, and following challenge. No detectable IFN-γ was produced by any of the animals following vaccination (Data not shown). One week following challenge, seven of the nine animals produced significant amounts of IFN-γ to *M. a. paratuberculosis* CFP (Figure 3.9).

Discussion

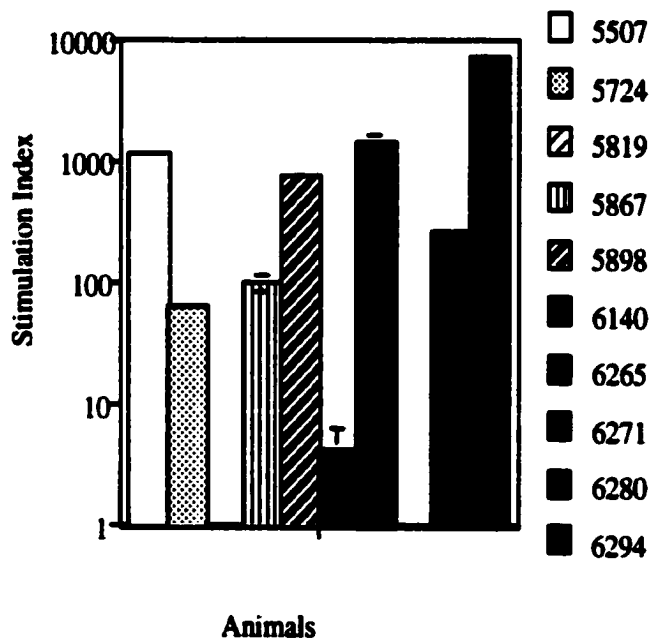
This study was the first investigation of the effects of a CFP based vaccine on oral challenge of cattle with *M. a. paratuberculosis*. It is very difficult to assess the protective efficacy of a vaccine for such a chronic disease. Notwithstanding the difficulties, there is evidence to suggest that the CFP vaccine has skewed the immune response toward a more activated and likely protective phenotype. Following vaccination and challenge, the animals in the CFP vaccinated group had T cell subset phenotypes that suggested increased activation as compared to the adjuvant group. Evidence for the protective

Figure 3.8. Expression of CD45R on WC1$^-$ γδ T Cells



Flow cytometry was used to analyze differences in CD45R expression on WC1$^-$ γδ T cells between the CFP vaccine group (purple) and the adjuvant group (green).

Figure 3.9. IFN-γ Production



IFN-γ released from *M. a. paratuberculosis* >10 kDa CFP stimulated PBMCs one week post-challenge. Data are presented as mean OD indices ± SEM.

effects of the activated populations of T cells will be investigated at the conclusion of this study.

Experimentally infecting cattle with *M. a. paratuberculosis* is very difficult. There is not much information regarding the experimental conditions that will result in a chronic infection that mimics the natural infection in cattle. We chose to infect the animals via the oral route, since that is the most likely route of natural infection. Previous experimental oral infections suggested that a high dose was equivalent to 10^8 colony forming units given on three consecutive days, whereas a low dose was equivalent to 10^6 colony forming units given on three consecutive days (3). We chose to give 10^7 colony forming units on three consecutive days. Although confirmation of infection may have to wait until the animals are necropsied at the conclusion of this study, there is evidence to suggest that these animals are infected. With the exception of one animal in the CFP vaccinated group, all of the animals had a positive DTH response to Johnin or one of the CFP skin test antigens post-challenge. As of 30 weeks post challenge only one animal has shown signs of shedding mycobacteria in feces, although subclinically infected animals have been shown to shed only intermittently (229). Additionally, 7 of the 9 animals produced in vitro IFN-γ in response to stimulation with *M. a. paratuberculosis* >10 kDa CFP.

CD62L is a cell surface molecule that is important for cell trafficking into lymph nodes (163, 268). Naïve or resting cells express high levels of this molecule and activated cells have little or no CD62L on their surfaces (163). Following vaccination, there were no significant differences in CD62L expression on $WC1^+$, $WC1^-$, $CD4^+$, or $CD8^+$ T cells between the CFP vaccinated and adjuvant groups, with one exception. The

CD8$^+$ T cells from the animals in the adjuvant group did have a slightly higher percentage of cells that were CD62L$^{neg/lo}$, when compared to the CFP vaccinated group (18% in the adjuvant group compared to 10%in the CFP group). These results were somewhat unexpected, as a previous study by our laboratory demonstrated that the percentage of WC1$^+$ γδ T cells that were of the CD62L$^{neg/lo}$ phenotype significantly increased following vaccination with Vira Shield® 5 (Chapter 4). This is however, a multivalent viral vaccine, which could stimulate T cells differently than the CFP vaccine used in this study. After challenge with *M. a. paratuberculosis*, a significantly higher percentage of WC1$^-$, CD4$^+$, and CD8$^+$ T cells from the animals in the CFP vaccinated group were of the more activated phenotype (CD62L$^{neg/lo}$) than the animals in the adjuvant group (WC1$^-$:52% in the CFP group compared to 33% in the adjuvant group, CD4$^+$:43% in the CFP group compared to 36% in the adjuvant group,CD8$^+$:23% in the CFP group compared to 18% in the adjuvant group). Roughly 252 days following the onset of this study, there was a large increase in the percentage of WC1$^+$, WC1$^-$, CD4$^+$, and CD8$^+$ T cells that were of the CD62L$^{neg/lo}$ phenotype in both the CFP vaccinated and adjuvant vaccinated groups. It is likely that the animals were exposed to some immunological challenge, as the animals are not housed in a pathogen free environment.

CD45R is important for T cell signaling and is classified by the number of molecules present on the cell surface (290). Like CD62L, CD45R is a cell surface molecule that is often associated with activation and memory. In mice, humans, and cattle, differential expression of the CD45 isoforms distinguish naïve (CD45R$^{mid/hi}$) and memory (CD45R$^{lo/neg}$) cells (144, 172, 195). The flow cytometric analysis of CD45R expression on T cell subsets, like CD62L, revealed significant differences between the

CFP vaccinated and adjuvant groups. Following vaccination, significantly higher percentages of both $WC1^+$ and $WC1^-$ γδ T cells from the CFP vaccinated group ($WC1^+$:21%, $WC1^-$: 14%) were of the $CD45R^{neg/lo}$ phenotype, as compared to the adjuvant group ($WC1^+$:12%, $WC1^-$:9%). Additionally, this trend continued following oral challenge. Similar results were seen in a previous experiment following vaccination with Vira Shield® 5 (Chapter 4), and indicate the activation of both $WC1^+$ and $WC1^-$ γδ T cells by the CFP vaccine. There were no significant differences in CD45R expression on $CD4^+$ or $CD8^+$ T cells. This somewhat unexpected, as CFP from *M. tuberculosis* have been shown to be recognized by murine $CD4^+$ T cells, in the mouse model of tuberculosis (242). As with CD62L, at the last timepoint there was a substantial increase in the percentage of $CD45R^{neg/lo}$ cells.

In addition to CD45R and CD62L, the expression of CD25 and CD44 was evaluated on the surface of $WC1^+$, $WC1^-$, $CD4^+$, and $CD8^+$ T cells. CD25 is the alpha-chain of the high affinity IL-2 receptor and is thus a marker of activation. The $CD4^+$ T cells were the only cell type studied that had greater than 5% that were positive for CD25. The expression of CD25 varied throughout the course of this study but at no timepoint did the T cells from the CFP vaccinated group have a significantly greater percentage of $CD25^+$ cells than the adjuvant group. Lymphocytes bind to hyaluronate on high endothelial venules via the CD44 molecule (16). CD44 has been shown to be highly expressed on human and murine activated and memory T cells (50, 101, 251). A previous study by our laboratory demonstrated that following vaccination, the circulating populations of $WC1^+$ and $WC1^-$ γδ T cells had less CD44 on their surfaces than before vaccination (Chapter 4). In this study there were no significant differences in CD44

expression between the CFP vaccinated group and the adjuvant group. This may indicate that the CFP vaccine did not sufficiently activate the T cell populations, although this is not consistent with the CD62L and CD45R results. It is important to consider that the activation marker expression on peripheral blood T cell populations may not be consistent with the expression on T cells within the gut. Therefore, conclusions drawn from the peripheral blood assessments must be confirmed with cells within the intestine.

Previous bovine mycobacterial vaccine studies have demonstrated that the production of IFN-γ following in vitro stimulation, after vaccination, is a good indicator of the protective efficacy of the vaccine (53, 308). The vaccine formulation given in this study did not induce a measurable in vitro IFN-γ response in any of the animals. There were however, strong in vitro IFN-γ responses following challenge. Despite the absence of in vitro IFN-γ production following vaccination, we can not rule out the potential protective effects of the CFP vaccine. The correlation between IFN-γ production following vaccination and protection from an oral challenge of *M. a. paratuberculosis* has not yet been established.

Although the protective efficacy of the CFP/DDA/CpG/IL-2 vaccine for Johne's disease has not yet been determined, some valuable information regarding the bovine immune response to *M. a. paratuberculosis* has been extracted from this study. Vaccination with the CFP/DDA/CpG/IL-2 vaccine induced significantly higher percentages of activated $WC1^+$, $WC1^-$, $CD4^+$, and $CD8^+$ T cells, as compared with the animals in the adjuvant group. Although there was statistically significant differences between the CFP and adjuvant vaccinated groups, the biological relevance to protection will be assessed at necropsy at the conclusion of this study.

Acknowledgments

The author would like to thank Dr. Oliver Turner for all of his contributions to this work; Brian Fraley for excellent technical assistance; and Dr. Joanne Turner for many stimulating intellectual conversations.

CHAPTER 4

Expression of Activation and Memory Markers on WC1$^+$ and WC1$^-$ γδ T Cells

Abstract

Understanding the immunological function of bovine γδ T cells is essential for evaluating the role that they play in the response to infectious agents and for determining the potential of targeting this population with vaccines. The differential expression of CD2 on bovine γδ T cells was used to define two distinct populations of cells in the peripheral blood. This study examined the age dependent changes of both $WC1^+$ and $WC1^-$ γδ T cells as well as differences in the expression of activation markers between these two populations. The expression of these activation markers was also evaluated following vaccination with Vira Shield® 5 (used to vaccinate against common viral bovine pathogens). Throughout the duration of this study, the $WC1^+$ γδ T cells represented a significantly higher percentage of the peripheral blood mononuclear cells than the $WC1^-$ γδ T cells. CD62L was expressed on all $WC1^+$ γδ T cells but only a subset of $WC1^-$ γδ T cells. Both $WC1^+$ and $WC1^-$ γδ T cells consistently expressed high levels of CD44. The majority of both $WC1^+$ and $WC1^-$ γδ T cells also expressed CD45R; however, more of the $WC1^+$ cells were $CD45R^{neg/lo}$. Following vaccination there was a significant decrease in the percentage of $WC1^+$ and $WC1^-$ γδ T cells that expressed CD44 and CD45R, but there was only a significant decrease in the percentage of $WC1^+$ γδ T cells that expressed CD62L. These data indicate significant differences in activation and memory marker expression on $WC1^+$ and $WC1^-$ γδ T cells, which adds to the growing evidence that there may be functional as well as phenotypic differences between these two populations of bovine γδ T cells.

Introduction

While γδ T cells are relatively minor lymphocyte populations in humans and mice, in ruminants γδ T cells are found in much higher numbers, particularly during the first few months of life (20, 137). The biological significance of the large numbers of γδ T cells present in ruminant species has not been determined, although several hypotheses have been generated (137). Numerous murine studies have shown that γδ T cells are important in the immune response to several pathogens (89, 201, 215, 260). Much less information is available regarding bovine γδ T cells, although there is evidence to suggest that bovine γδ T cells are important in the immune response to many bovine pathogens (90, 237, 239, 266, 299).

Two populations of γδ T cells have been identified in bovine peripheral blood. One population is described as $WC1^+/CD2^-/CD4^-/CD8^-$ and the other as $WC1^-/CD2^+/CD8^{+/-}$ (181). WC1 is a 215 kDa glycoprotein (184) with an unknown function but with homology to CD5 and CD6 (315). Despite the lack of information regarding specific functional differences between $WC1^+$ and $WC1^-$ γδ T cells, it has been shown that there are differences in their tissue distribution. A higher percentage of $WC1^+/CD2^-$ γδ T cells are found in the peripheral blood, whereas a higher percentage of $WC1^-/CD2^+$ γδ T cells are found in the spleen (181).

This study characterized age dependent changes of both the $WC1^+$ and $WC1^-$ γδ T cell populations and identified phenotypic differences in activation and memory marker expression between $WC1^+$ and $WC1^-$ γδ T cells. Additionally, the response of $WC1^+$ and $WC1^-$ γδ T cells to a typical bovine viral vaccine (Vira Shield® 5) was investigated. This study confirmed the peripheral age dependent decrease in the $WC1^+$ γδ T cell population

and demonstrated the relatively constant percentage of WC1$^-$ γδ T cells over the first year. Significant differences in activation and memory marker expression were found between WC1$^+$ and WC1$^-$ γδ T cells, but these differences did not appear to be age dependent. Additionally, changes in memory and activation marker expression were observed in both bovine γδ T cell populations following vaccination with Vira Shield® 5.

Material and Methods

Animals

Three-week-old calves were obtained from a local dairy (3 Holstein bulls, 1 Black Angus bull, and 1 Black Angus heifer). All animals were bottle fed and kept in indoor pens until they were eight weeks old. Thereafter the calves were moved to outdoor pens where they were fed grain and hay twice daily. The calves were vaccinated with Vira Shield® 5 (Grand Laboratories, Larchwood IA) when they were 8 weeks old as part of their normal care. Vira Shield® 5 contains chemically inactivated infectious bovine rhinotracheitis (IBR) virus, a cytopathic type 1 bovine virus diarrhea (BVD), a noncytopathic type 2 BVD virus, a parainfluenza III (PI_3) virus, and a bovine respiratory syncytial virus (BRSV). At the conclusion of this study animals were vaccinated a second time with Vira Shield® 5 to confirm the findings following the first vaccination.

5 healthy one-year-old female heifers were used in one of the experiments discussed in this study. These animals were kindly donated by XY Inc.

Surgical Removal of Prescapular Lymph Nodes

At 58 weeks of age the right cranial superficial prescapular lymph nodes were removed from each of the animals. Each animal was given 3000 mg of OxyCure 2000 (Vedco) and 2500 mg of Flunixamine by intramuscular injections. The right

shoulder area of each animal was shaved with a surgical blade and the animals were immobilized in a squeeze shoot. The animals were given about 500 mg of Lidocaine to anaesthetize the area. A 4 cm^2 section of the right cranial superficial lymph node was removed and placed on ice. The muscle and skin of each animal was sutured. One week following the second vaccination with Vira Shield® 5 the procedure was repeated, taking a section of the left cranial superficial prescapular lymph node from each animal.

Cell isolation

Jugular venipunctures were performed at regular intervals (3, 5, 7, 9, 11, 15, 19, 33 and 55 weeks of age). Thirty milliliters of whole blood was collected into sodium heparin vacutainers (VWR, Denver CO). The whole blood was diluted 1:1 in sterile phosphate buffered saline (Life Technologies, Grand Island NY) and layered onto an equal volume of Ficoll-Paque Plus (Pharmacia, Piscataway NJ). The samples were then centrifuged at 900 g for 30 minutes at 18°C and allowed to stop without a brake. The mononuclear cells were removed and washed three times in deficient RPMI (Irvine Scientific, Santa Ana CA) supplemented with 0.1% sodium azide. The cells were counted and adjusted to $1x10^7$ cells/ml. 200 µl of the cell suspensions were plated into 96 round bottom well plates prior to staining for flow cytometry.

Lymph node sections were squashed through a spleen screen to obtain single cell suspensions. The cells were then washed three times in deficient RPMI (Irvine Scientific) supplemented with 0.1% sodium azide. The cells were counted and adjusted to $1x10^7$ cells/ml. 200 µl of the cell suspensions were plated into 96 round bottom well plates prior to staining for flow cytometry.

Flow Cytometry

Three color flow cytometry was performed by staining peripheral blood mononuclear cells and lymph node cells with mouse anti-bovine CD2 -PE (BAQ95A) (VMRD Pullman WA) in combination with mouse anti-bovine δ -FITC (GB21A) (VMRD) antibodies. The CD2 and γδ antibodies were conjugated by Chromaprobe (Mountain View CA). The third color was either mouse anti-bovine WC1 (MCA1655) (Serotec, Raliegh NC), mouse anti-bovine CD62L (MCA1649) (Serotec), mouse anti-bovine CD25 (CACT116A)(VMRD), mouse anti-bovine CD44 (BAT31A) (VMRD), or mouse anti-bovine CD45R (MCA1650) (Serotec). All third color antibodies were stained with a secondary rat anti-mouse IgG1 PerCP antibody (Becton Dickinson, San Diego CA). The appropriate isotype controls were also performed for each animal at every time point. All staining steps were carried out at 4°C, in the dark, for 30 minutes and were followed by three washes with deficient RPMI with azide. After staining, the cells were resuspended in 500 μl of deficient RPMI plus azide and data were acquired using the FacsCalibur flow cytometer (Becton Dickinson). 50,000 cells were read for each individual animal/stain and data were analyzed using CellQuest software (Becton Dickinson). Lymphocytes were identified by forward /side scatter and specific staining was identified by setting quadrants or markers on isotype controls.

Statistical Analysis

Statistical analysis of data from flow cytometry was done by the Student's *t* Test.

Results

$WC1^+$ and $WC1^-$ bovine γδ T cells can be distinguished by the differential expression of CD2 ($WC1^+$ cells are negative for CD2 and $WC1^-$ cells are positive for

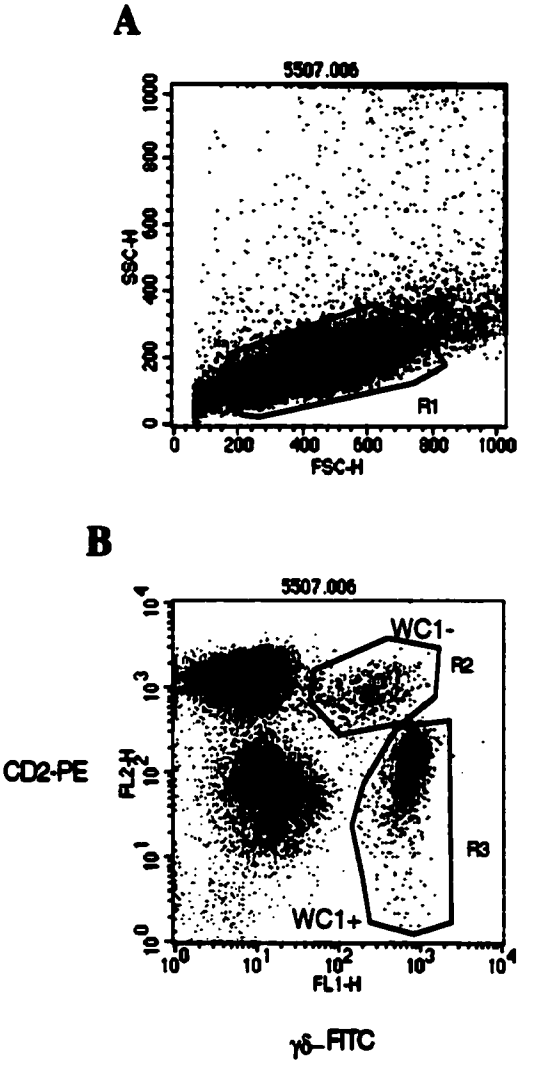
CD2) as shown in Figure 4.1. This differential expression of CD2 was used to determine the percent $WC1^+$ and $WC1^-$ bovine γδ T cells over time. The percentage of $WC1^+$ γδ T cells was high early in the lives of the cows but stabilized at about 10% by week 15 of the study, whereas the percentage of $WC1^-$ γδ T cells remained relatively constant throughout the 55 weeks of the study (Figure 4.2). The $WC1^+$ γδ T cells were significantly more prominent in the peripheral blood than the $WC1^-$ γδ T cells throughout the course of this study. Animals were vaccinated with Vira Shield® 5 when they were 8 weeks old. There were no significant changes in the percentages of $WC1^+$ or $WC1^-$ γδ T cells following vaccination. There was however, a significant increase in the percentage of $WC1^+$ γδ T cells at week 31 which was probably due to an unknown immunological challenge, as the animals were not kept in a pathogen free environment.

The animals were vaccinated a second time to confirm the changes seen post vaccination. There was a significant decrease in the percent $WC1^+$ γδ T cells one week following the second vaccination (Figure 4.3). There were no significant changes in the percentage of $WC1^-$ γδ T cells (Figure 4.3).

Expression of CD62L on $WC1^+$ and $WC1^-$ γδ T cells

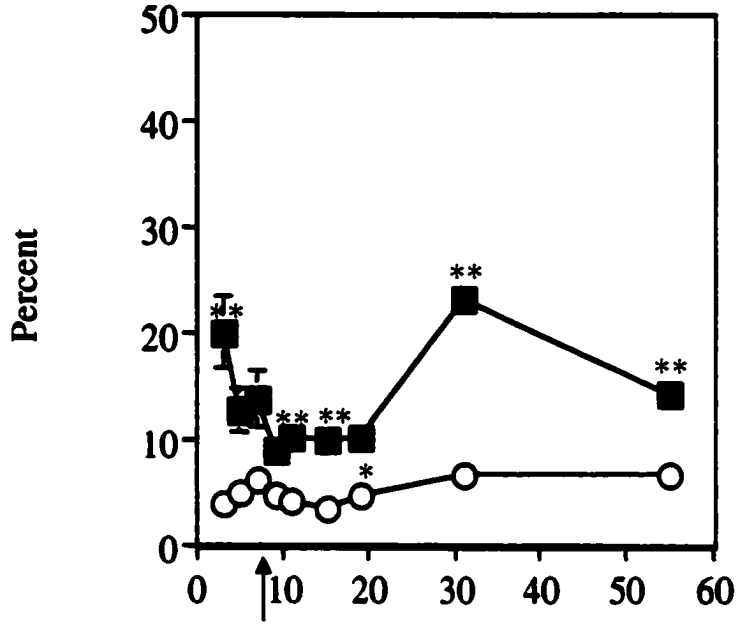
The expression of CD62L is associated with cell trafficking into the lymph nodes (163, 268), and is also an indicator of cell activation. Activated cells express very little CD62L ($CD62L^{lo}$) whereas resting cells express substantially more ($CD62L^{hi}$) (163). Typically, less than 10% of the $WC1^+$ γδ T cells were $CD62L^{lo}$ throughout this study (Figure 4.4), except when the animals were vaccinated with Vira Shield® 5 when there was a significant decrease in fluorescent intensity of CD62L on $WC1^+$ γδ T cells (Figure4.5A). This shift in fluorescence intensity suggests that the $WC1^+$ γδ T cells

Figure 4.1. Differential Expression of CD2 on WC1$^{+/-}$ γδ T Cells



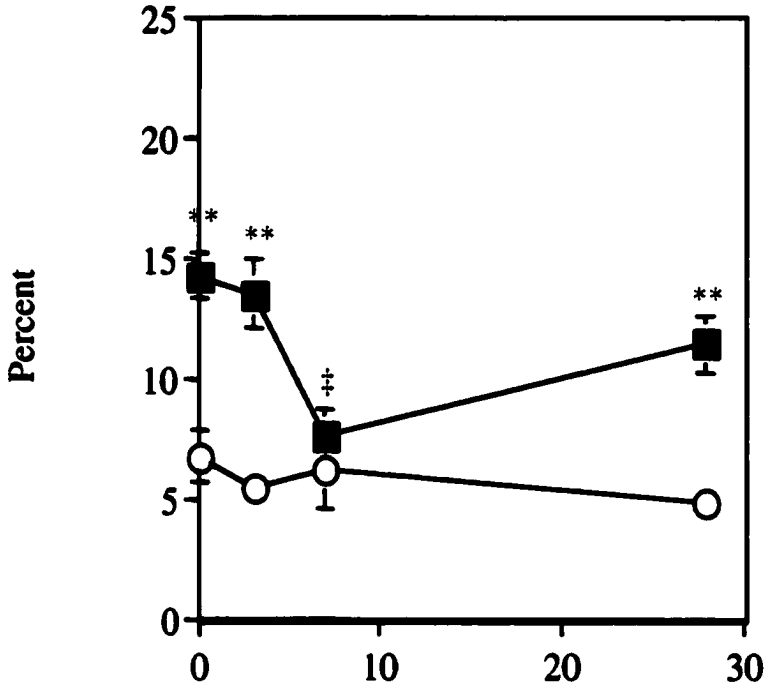
This Figure represents a typical dot plot generated during this study. Lymphocytes are gated based on their forward and side scatter profiles (A). PBMCs are stained with anti-CD2 PE and anti-γδ FITC. The WC1^{+} γδ T cells stain positively for the delta chain of the TCR but negatively for CD2, while WC1^{-} γδ T cells stain positively for the delta chain of the TCR and for CD2.

Figure 4.2. Percent WC1$^{+/-}$ γδ T Cells



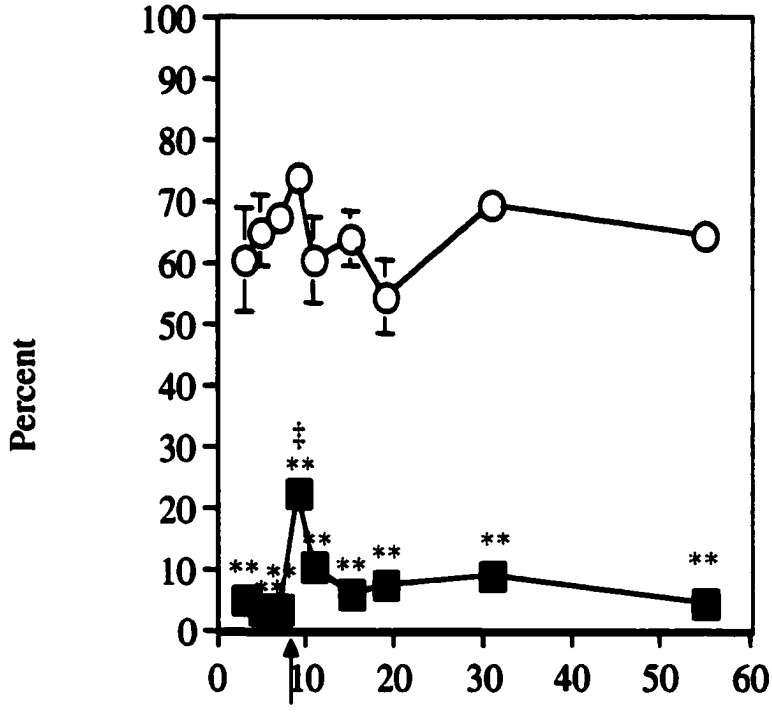
PBMCs from all five animals were stained with anti-bovine γδ FITC and anti-CD2 PE. The percentages of WC1$^+$ and WC1$^-$ γδ T cells were determined using CELLQuest software. Data represent the mean percentages from all five animals at each timepoint for both WC1$^+$ (closed squares) and WC1$^-$ (open circles) γδ T cells ± SE. Significant differences between the WC1$^+$ cells and the WC1$^-$ cells are shown as *= p value< 0.05 and **= p value< 0.005. ↑ = Animals vaccinated with Vira Shield® 5.

Figure 4.3. Percent WC1$^{+/-}$ γδ T Cells Following Second Vaccination



PBMCs from all five animals were stained with anti-bovine γδ FITC and anti-CD2 PE. The percentages of WC1$^+$ and WC1$^-$ γδ T cells were determined using CELLQuest software. Data represent the mean percentages from all five animals at each timepoint for both WC1$^+$ (closed squares) and WC1$^-$ (open circles) γδ T cells ± SE. Significant differences between the WC1$^+$ cells and the WC1$^-$ cells are shown as **= p value< 0.005. Significant differences in cell populations before and after vaccination are shown as ‡ = p value < 0.005.

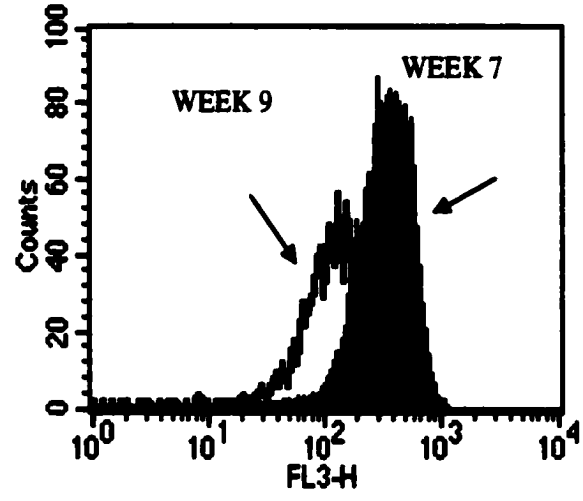
Figure 4.4. CD62L Expression on WC1$^{+/-}$ γδ T Cells



PBMCs from all five animals were stained with anti-bovine γδ FITC, anti-CD2 PE, anti-CD62L (followed by anti-IgG1 PerCP). The percentages of WC1^{+} and WC1^{-} γδ T cells that were also CD62L$^{neg/lo}$ were determined using CELLQuest software. Data represent the mean percentages from all five animals at each timepoint for both WC1^{+} (closed squares) and WC1^{-} (open circles) γδ T cells ± SE. Significant differences between the WC1^{+} cells and the WC1^{-} cells are shown as **= p value< 0.005. Significant differences in cell populations before and after vaccination are shown as ‡ = p value < 0.005. ↑= Animals vaccinated with Vira Shield® 5.

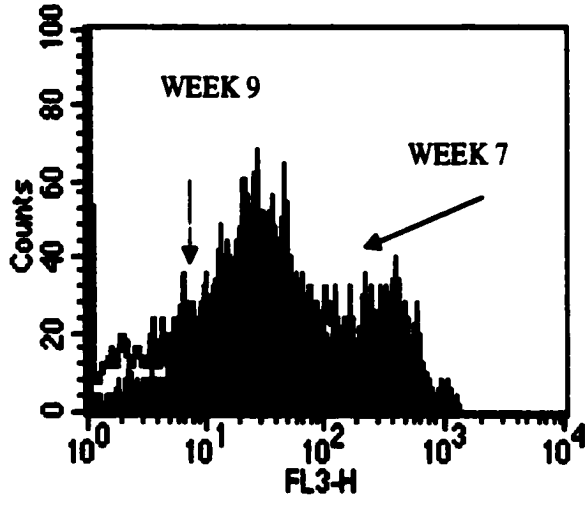
Figure 4.5. CD62L Expression on WC1$^{+/-}$ γδ T Cells

A



Fluorescence Intensity

B



Fluorescence Intensity

A representative histogram of CD62L expression on WC1$^+$ (A) and WC1$^-$ (B) γδ T cells before and after vaccination, shows the log fluorescence intensity of CD62L PerCP (x axis) and the number of cells (y axis).

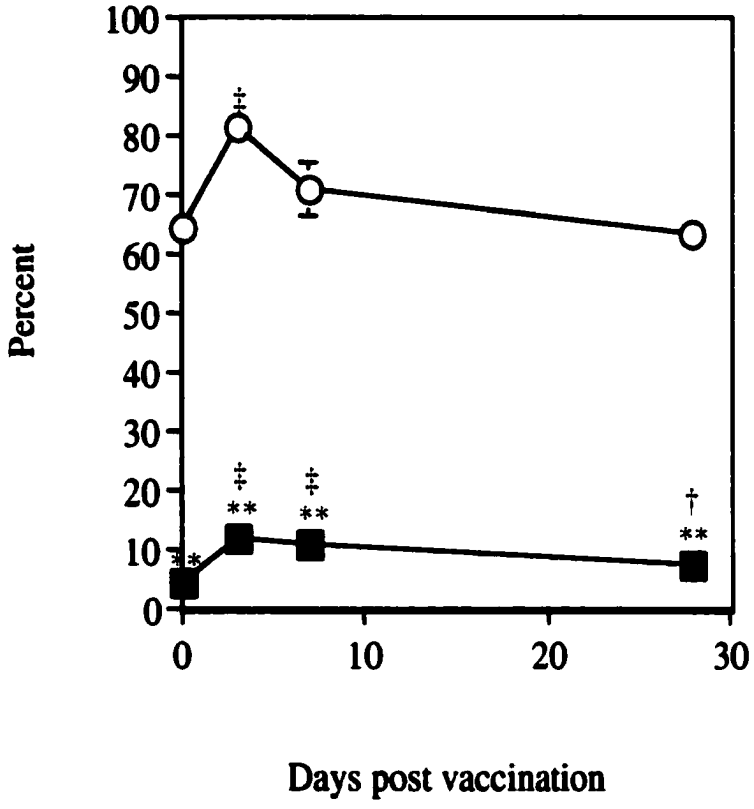
responded to the vaccine. In contrast to the WC1$^+$ γδ T cells, the WC1$^-$ γδ T cells had more variable expression of CD62L during the course of this experiment (Figure 4.4). The majority of the WC1$^-$ γδ T cells were of the CD62L$^{neg/lo}$ phenotype (Figure 4.5B). As with the WC1$^+$ γδ T cells, the WC1$^-$ γδ T cells down regulated CD62L expression after vaccination, although this was not statistically significant (Figure 4.4).

As previously stated, Vira Shield® 5 was administered a second time, at the end of this study, to confirm the changes observed post vaccination. After the second vaccination, there was a significant increase in the percentage of WC1$^+$(6%) and WC1$^-$ (17%) γδ T cells that were of the activated (CD62L$^{neg/lo}$) phenotype (Figure 4.6). The shift in fluorescence intensity was less apparent for the WC1$^+$ γδ T cells (Figure 4.7A) than on the WC1$^-$ γδ T cells (Figure 4.7B). In contrast to the first vaccination (Figure 4.4), the WC1$^-$ γδ T cells also showed a significant increase in the activated phenotype, following the second vaccination (Figure 4.6).

Percentage of WC1$^+$ and WC1$^-$ γδ T cells that express CD44

CD44 is a cell surface receptor that mediates binding of lymphocytes to high endothelial venules through its ligand, hyaluronate (16). In humans and mice it has been demonstrated that activated cells and memory cells express high levels of this cell surface antigen (50, 101, 251). Over the course of this study, a significantly higher percentage of WC1$^+$ γδ T cells expressed CD44 than the WC1$^-$ γδ T cells (Figure 4.8). Similar to what was observed with CD62L expression, there was also a shift in CD44 expression in both the WC1$^+$ and WC1$^-$ γδ T cells following Vira Shield® 5 vaccination at 8 weeks of age (Figure 4.8). The shifts in CD44 expression are also shown in Figures 4.9A and 4.9B.

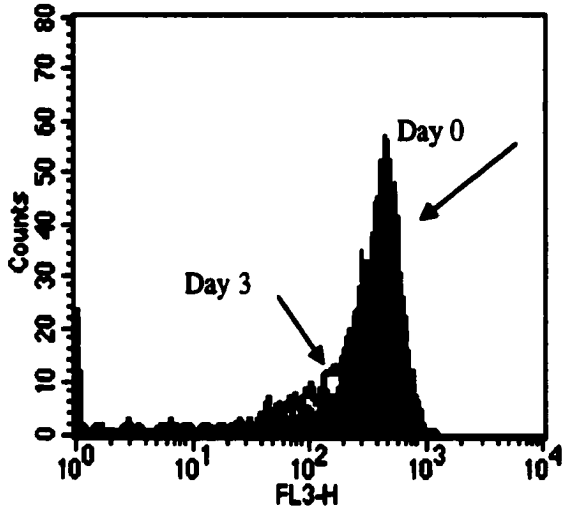
Figure 4.6. CD62L Expression of WC1$^{+/-}$ γδ T Cells Following Second Vaccination



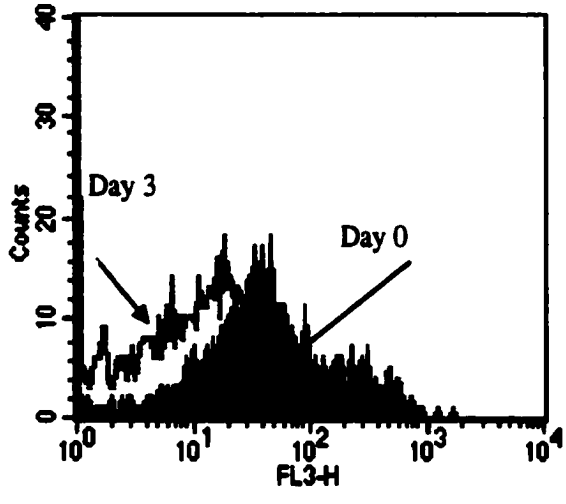
PBMCs from all five animals were stained with anti-bovine γδ FITC, anti-CD2 PE, anti-CD62L (followed by anti-IgG1 PerCP). The percentages of WC1^{+} and WC1^{-} γδ T cells that were also CD62L$^{neg/lo}$ were determined using CELLQuest software. Data represent the mean percentages from all five animals at each timepoint for both WC1^{+} (closed squares) and WC1^{-} (open circles) γδ T cells ± SE. Significant differences between the WC1^{+} cells and the WC1^{-} cells are shown as **= p value< 0.005. Significant differences in cell populations before and after vaccination are shown as † = p value < 0.05 and ‡ = p value < 0.005.

Figure 4.7. CD62L Expression on WC1$^{+/-}$ γδ T Cells Following Second Vaccination

A



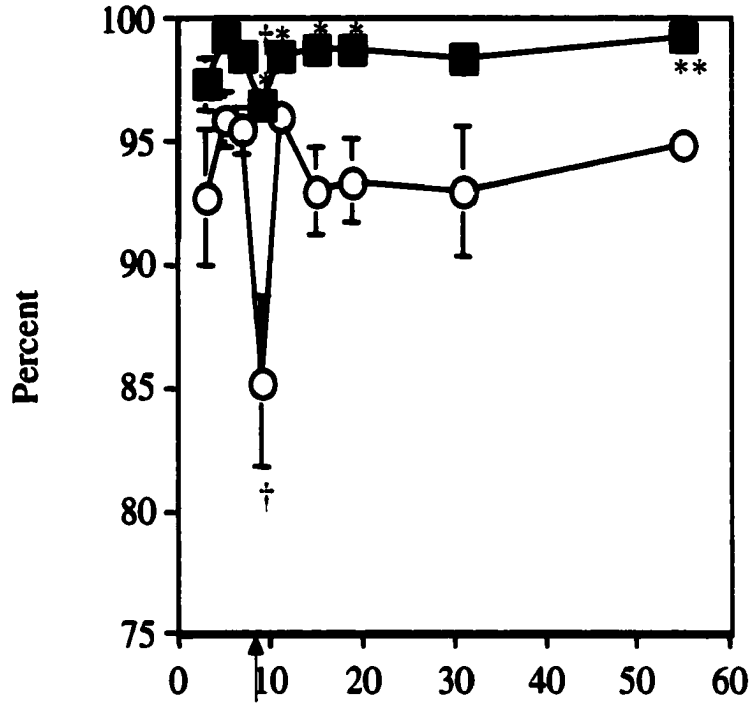
B



Fluorescence Intensity

A representative histogram of CD62L expression on WC1^{+} (A) and WC1^{-} (B) γδ T cells before and after the second vaccination, shows the log fluorescence intensity of CD62L PerCP (x axis) and the number of cells (y axis).

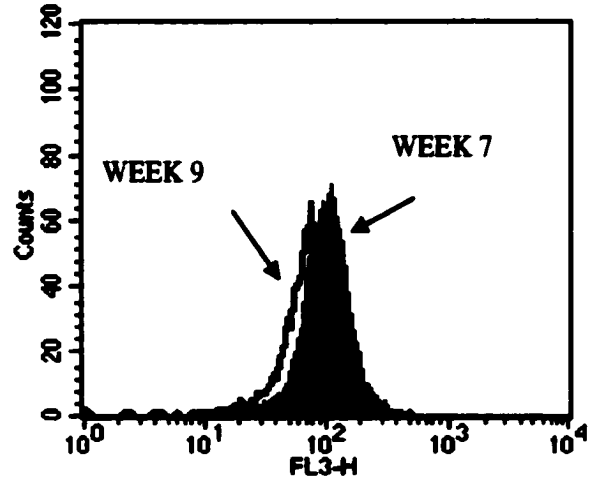
Figure 4.8. CD44 Expression on WC1$^{+/-}$ γδ T Cells



PBMCs from all five animals were stained with anti-bovine γδ FITC, anti-CD2 PE, anti-CD44 (followed by anti-IgG1 PerCP). The percentages of WC1^{+} and WC1^{-} γδ T cells that were also CD44^{mid} were determined using CELLQuest software. Data represent the mean percentages from all five animals at each timepoint for both WC1^{+} (closed squares) and WC1^{-} (open circles) γδ T cells ± SE. Significant differences between the WC1^{+} cells and the WC1^{-} cells are shown as *= p value< 0.05 and **= p value< 0.005. Significant differences in cell populations before and after vaccination are shown as †= p value < 0.05. ↑= Animals vaccinated with Vira Shield® 5.

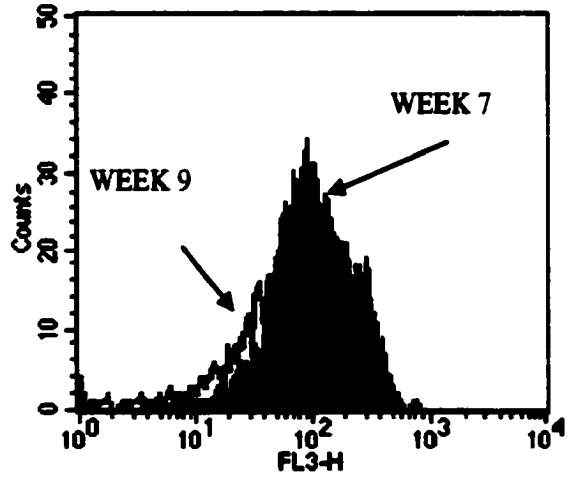
Figure 4.9. CD44 Expression on WC1$^{+/-}$ γδ T Cells

A



Fluorescence Intensity

B



Fluorescence Intensity

A representative histogram of CD44 expression on WC1$^+$ (A) and WC1$^-$ (B) γδ T cells before and after vaccination, shows the log fluorescence intensity of CD44 PerCP (x axis) and the number of cells (y axis).

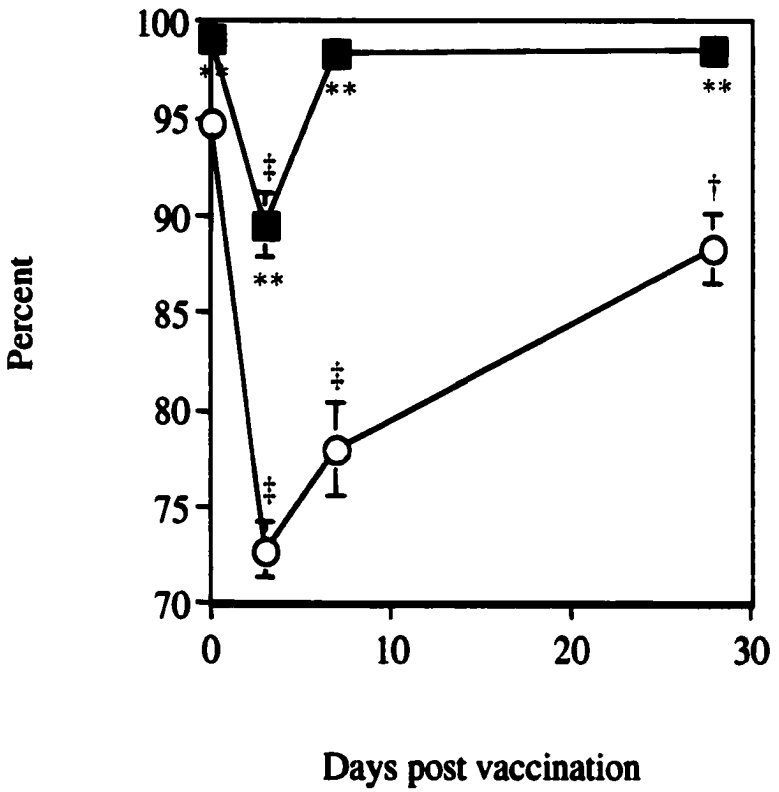
Following the second vaccination with Vira Shield® 5, again there was a significant decrease in the percentage of both $WC1^+$ (10%) and $WC1^-$ (22%) γδ T cells that were $CD44^{mid}$ (Figure 4.10). The decrease in the percentage of cells that were $CD44^{mid}$ resulted from, again, a decrease in the expression of CD44 on both $WC1^+$ (Figure 4.11A) and $WC1^-$ (Figure 4.11B) γδ T cells.

CD45R expression on $WC1^+$ and $WC1^-$ γδ T cells

CD45R is a cell surface molecule that is important for T cell signaling and can be differentially classified according to the number of molecules on the cell surface (290). In mice, humans, and cattle, differential expression of the CD45 isoforms distinguish naïve ($CD45R^{mid/hi}$) and memory ($CD45R^{lo/neg}$) $CD4^+$ T cells (144, 172, 195). $WC1^+$ γδ T cells had a significantly higher percentage of cells that were of the $CD45R^{neg/lo}$ phenotype than did $WC1^-$ γδ T cells (Figure 4.12). Following vaccination there was a significant increase in the percentage of both $WC1^+$ (28%) and $WC1^-$ (18%) γδ T cells that were of the $CD45R^{neg/lo}$ phenotype (Figure 4.12). Figure 4.13 shows the decrease in fluorescence intensity for CD45R on both $WC1^+$ (Figure 4.13A) and $WC1^-$ (Figure 4.13B) γδ T cells. Interestingly, following vaccination the percentage of $WC1^+$ cells with the activated/memory phenotype remained elevated for the duration of the study, whereas the percentage of $WC1^-$ cells with this phenotype returned to normal levels three weeks after vaccination (Figure 4.12).

Following the second vaccination, there was an increase in the percentage of both $WC1^+$ (20%) and $WC1^-$ (13%) γδ T cells that expressed the $CD45R^{neg/lo}$ phenotype (Figure 4.14). The decrease in fluorescence intensity of CD45R is shown for $WC1^+$ (Figure 4.15A) and $WC1^-$ (Figure 4.15B) γδ T cells.

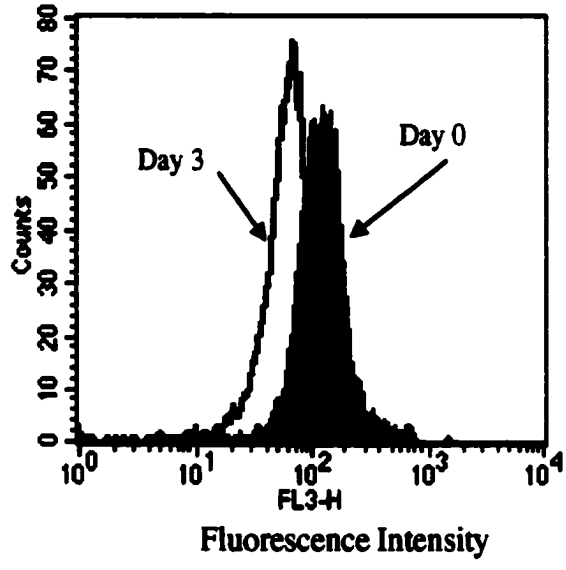
Figure 4.10. Expression of CD44 on WC1$^{+/-}$ γδ T Cells Following Second Vaccination



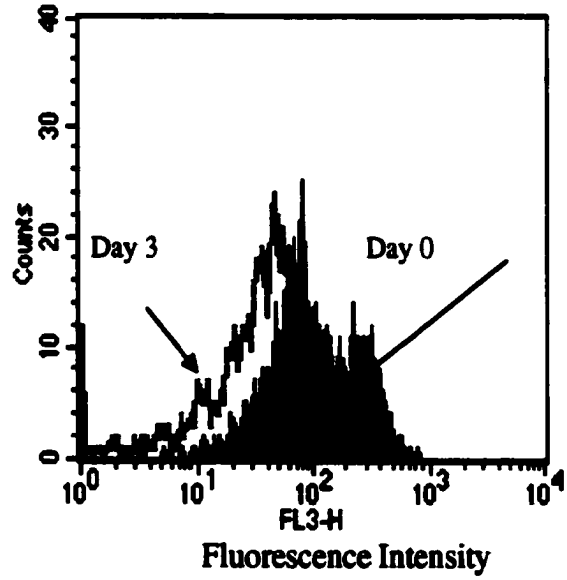
PBMCs from all five animals were stained with anti-bovine γδ FITC, anti-CD2 PE, anti-CD44 (followed by anti-IgG1 PerCP). The percentages of WC1^{+} and WC1^{-} γδ T cells that were also CD44^{mid} were determined using CELLQuest software. Data represent the mean percentages from all five animals at each timepoint for both WC1^{+}(closed squares) and WC1^{-} (open circles) γδ T cells ± SE. Significant differences between the WC1^{+} cells and the WC1^{-} cells are shown as **= p value< 0.005. Significant differences in cell populations before and after vaccination are shown as †= p value < 0.05 and ‡= p value < 0.005.

Figure 4.11. CD44 Expression on WC1$^{+/-}$ γδ T Cells Following Second Vaccination

A

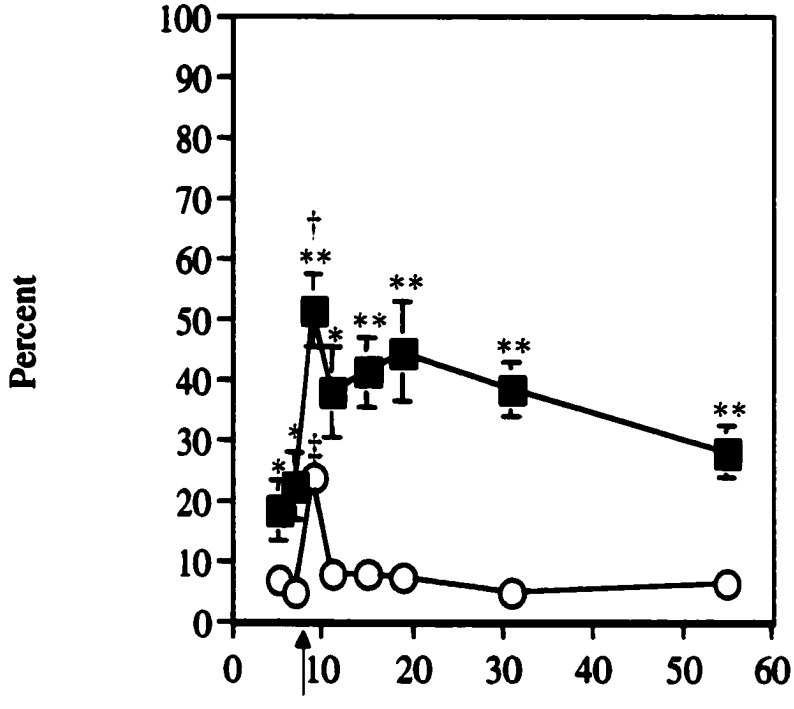


B



A representative histogram of CD44 expression on WC1$^+$ (A) and WC1$^-$ (B) γδ T cells before and after the second vaccination, shows the log fluorescence intensity of CD44 PerCP (x axis) and the number of cells (y axis).

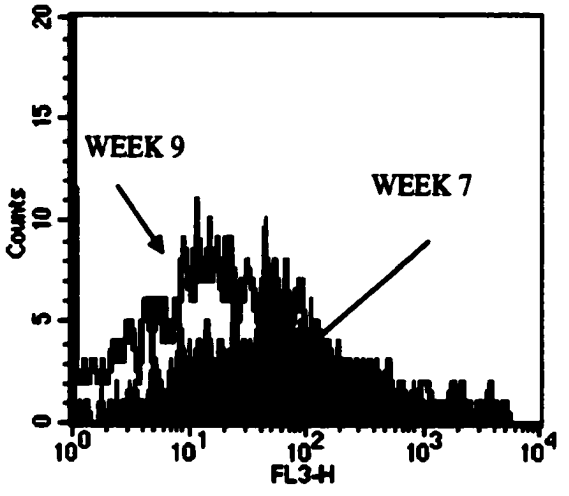
Figure 4.12. CD45R Expression on WC1$^{+/-}$ γδ T Cells



PBMCs from all five animals were stained with anti-bovine γδ FITC, anti-CD2 PE, anti-CD45R (followed by anti-IgG1 PerCP). The percentages of WC1^{+} and WC1^{-} γδ T cells that were also CD45R$^{neg/lo}$ were determined using CELLQuest software. Data represent the mean percentages from all five animals at each timepoint for both WC1^{+} (closed squares) and WC1^{-} (open circles) γδ T cells ± SE. Significant differences between the WC1^{+} cells and the WC1^{-} cells are shown as *= p value< 0.05 and **= p value< 0.005. Significant differences in cell populations before and after vaccination are shown as †= p value < 0.05 and ‡ = p value < 0.005. ↑ = Animals vaccinated with Vira Shield® 5.

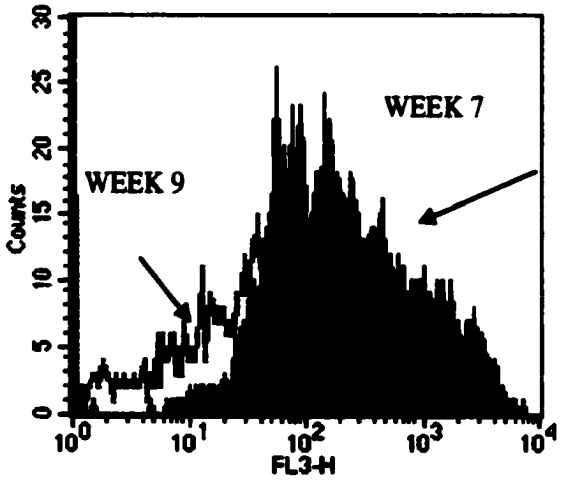
Figure 4.13. CD45R Expression on WC1$^{+/-}$ γδ T Cells

A



Fluorescence Intensity

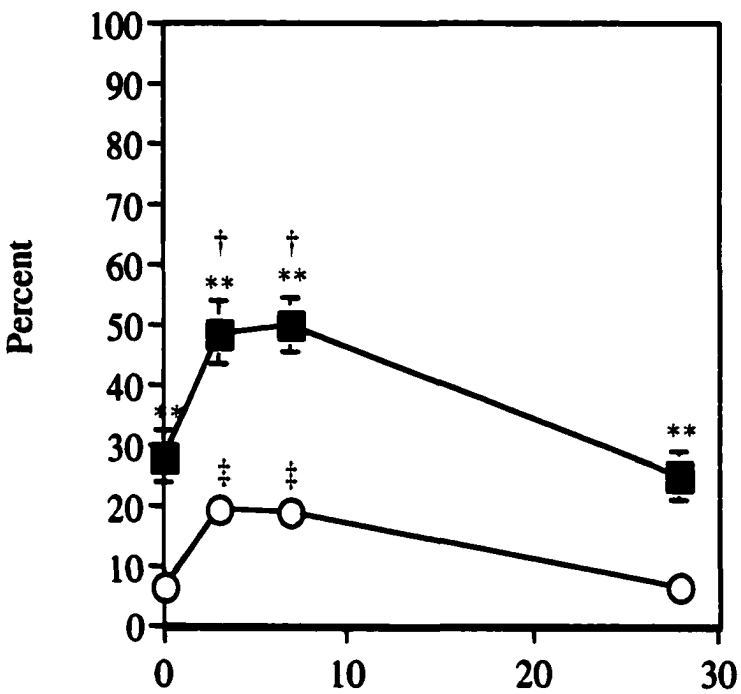
B



Fluorescence Intensity

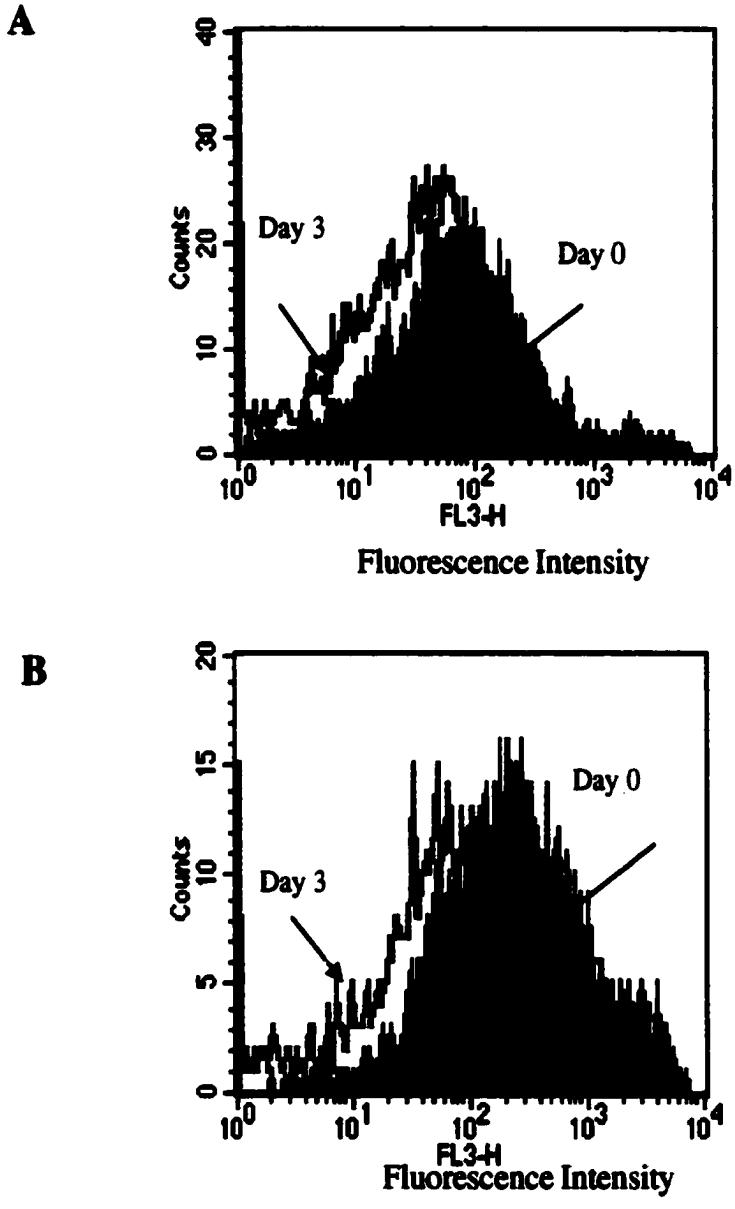
A representative histogram of CD45R expression on WC1$^+$ (A) and WC1$^-$ (B) γδ T cells before and after vaccination, shows the log fluorescence intensity of CD45R PerCP (x axis) and the number of cells (y axis).

Figure 4.14. CD45R Expression on WC1$^{+/-}$ γδ T Cells Following Second Vaccination



PBMCs from all five animals were stained with anti-bovine γδ FITC, anti-CD2 PE, anti-CD45R (followed by anti-IgG1 PerCP). The percentages of WC1^{+} and WC1^{-} γδ T cells that were also CD45R$^{neg/lo}$ were determined using CELLQuest software. Data represent the mean percentages from all five animals at each timepoint for both WC1^{+} (closed squares) and WC1^{-} (open circles) γδ T cells ± SE. Significant differences between the WC1^{+} cells and the WC1^{-} cells are shown as **= p value< 0.005. Significant differences in cell populations before and after vaccination are shown as †= p value < 0.05 and ‡ = p value < 0.005.

Figure 4.15. CD45R Expression on WC1$^{+/-}$ γδ T Cells Following Second Vaccination



A representative histogram of CD45R expression on WC1^{+} (A) and WC1^{-} (B) γδ T cells before and after the second vaccination, shows the log fluorescence intensity of CD45R PerCP (x axis) and the number of cells (y axis).

CD25 expression on WC1$^+$ and WC1$^-$ γδ T cells

CD25 is the alpha chain of the IL-2 high affinity receptor, and is a marker of activation. No more than 2% of either the WC1$^+$ or WC1$^-$ γδ T cells were positive for CD25 at any timepoint following the first (Figure 4.16) or the second (Figure 4.17) vaccination.

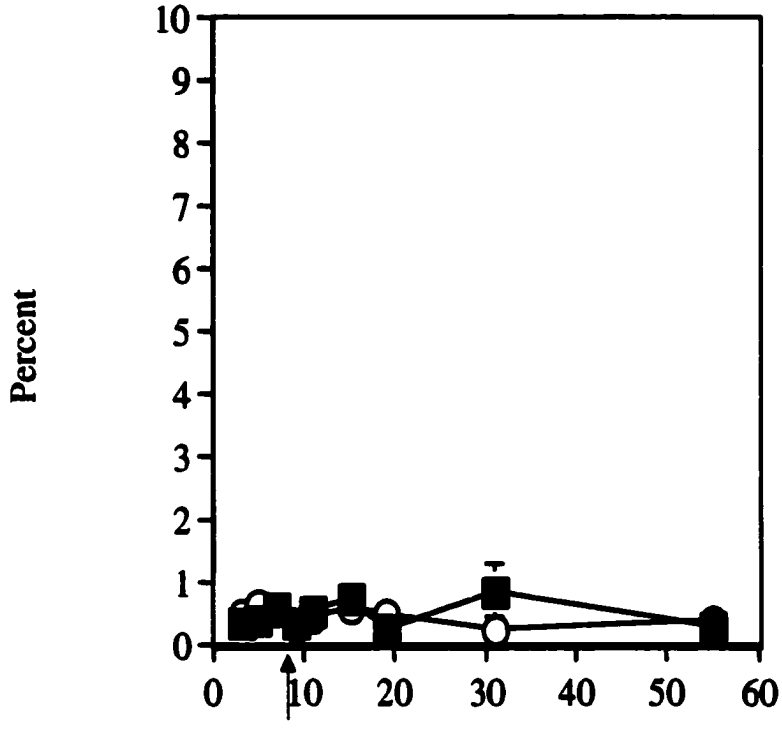
Expression of activation and memory markers on lymph node and γδ T cells pre and post the second vaccination

At the conclusion of this study a small section of the right prescapular lymph node was removed from each animal. The cells were isolated and analyzed by flow cytometry. The animals were vaccinated with Vira Shield® 5 and a section of the left prescapular lymph node from each animal was removed one week later. WC1$^+$ and WC1$^-$ γδ T cells both made up about 5% of the lymph node lymphocytes and there were no significant changes post vaccination (Figure 4.18).

WC1$^+$ γδ T cells had a significantly lower percentage of CD62L$^{neg/lo}$ lymph node cells than did the WC1$^-$ γδ T cell (Figure 4.19). Both subsets of γδ T cells had a significantly more activated phenotype following vaccination (Figure 4.19). Figure 4.20 shows the changes in expression of CD62L pre and post vaccination, for both WC1$^+$ (Figure 4.20 A) and WC1$^-$ (Figure 4.20B) γδ T cells. The percentage of CD62L positive WC1$^-$ γδ T cells is much higher in the lymph node (Figure 4.20B) than in the peripheral blood (Figure 4.7B).

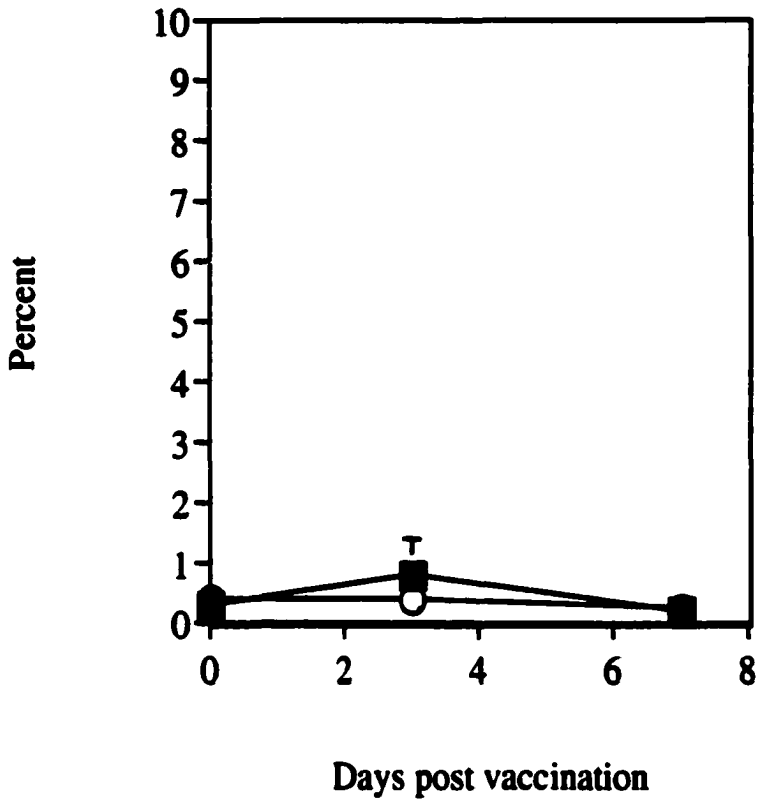
WC1$^+$ γδ T cells had significantly lower percentages of CD44^{mid} cells in the lymph node as compared to WC1$^-$ γδ T cells (Figure 4.21). Following vaccination, the WC1$^+$ γδ T cells had a significantly larger percentage of cells in the lymph node that

Figure 4.16. CD25 Expression on WC1$^{+/-}$ γδ T Cells



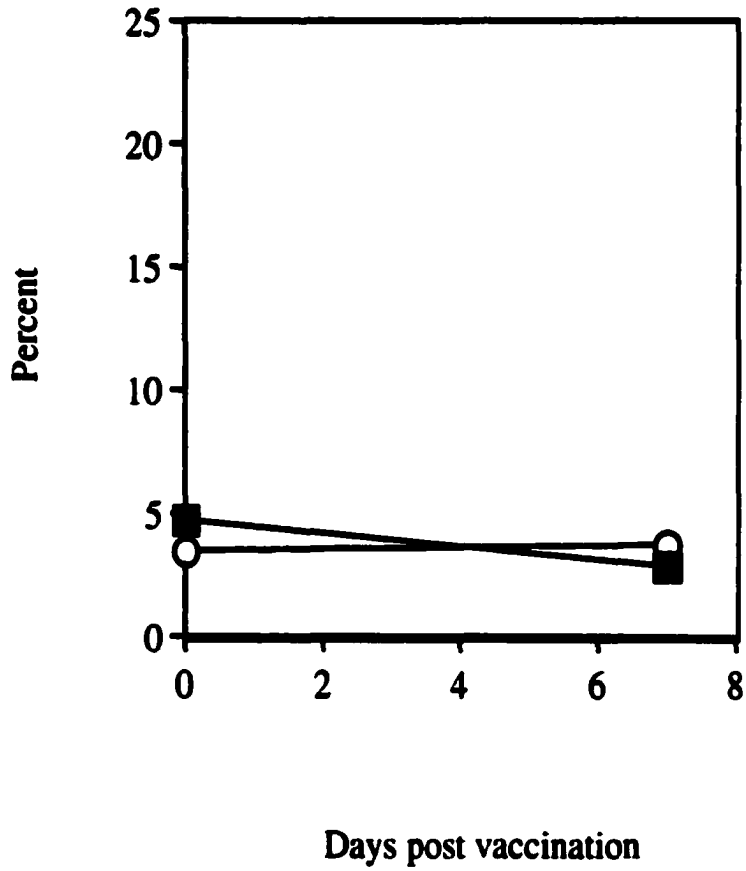
PBMCs from all five animals were stained with anti-bovine γδ FITC, anti-CD2 PE, anti-CD25 (followed by anti-IgG1 PerCP). The percentages of WC1$^+$ and WC1$^-$ γδ T cells that were also CD25$^+$ were determined using CELLQuest software. Data represent the mean percentages from all five animals at each timepoint for both WC1$^+$ (closed squares) and WC1$^-$ (open circles) γδ T cells ± SE. ↑ = Animals vaccinated with Vira Shield® 5.

Figure 4.17. Expression of CD25 on WC1$^{+/-}$ γδ T Cells Following Second Vaccination



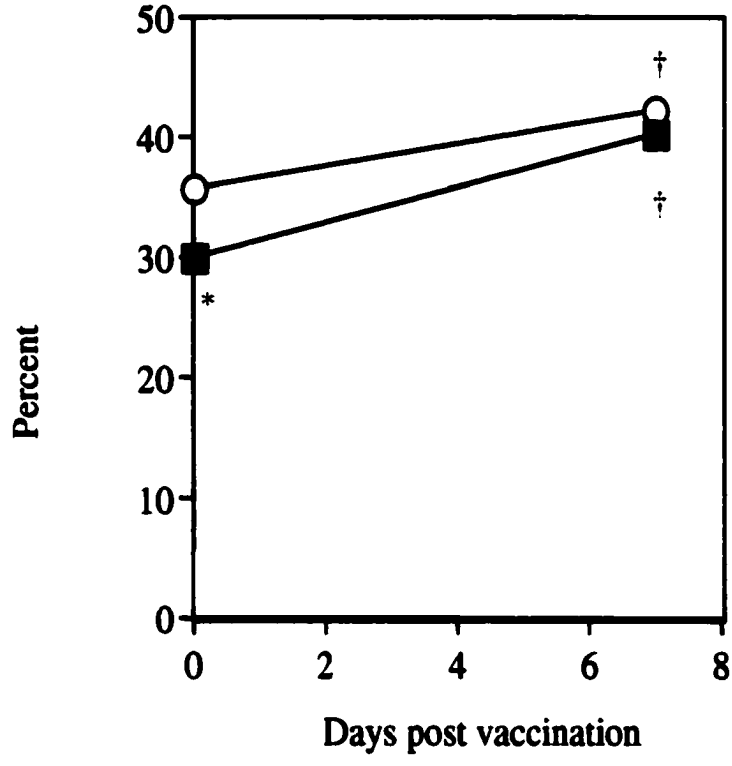
PBMCs from all five animals were stained with anti-bovine γδ FITC, anti-CD2 PE, anti-CD25 (followed by anti-IgG1 PerCP). The percentages of WC1^{+} and WC1^{-} γδ T cells that were also CD25^{+} were determined using CELLQuest software. Data represent the mean percentages from all five animals at each timepoint for both WC1^{+}(closed squares) and WC1^{-} (open circles) γδ T cells ± SE.

Figure 4.18. Percent WC1$^{+/-}$ γδ T Cells in Lymph Nodes



Cells from the prescapular lymph node from all five animals were stained with anti-bovine γδ FITC and anti-CD2 PE. The percentages of WC1^{+} and WC1^{-} γδ T cells were determined using CELLQuest software. Data represent the mean percentages from all five animals at each timepoint for both WC1^{+} (closed squares) and WC1^{-} (open circles) γδ T cells ± SE.

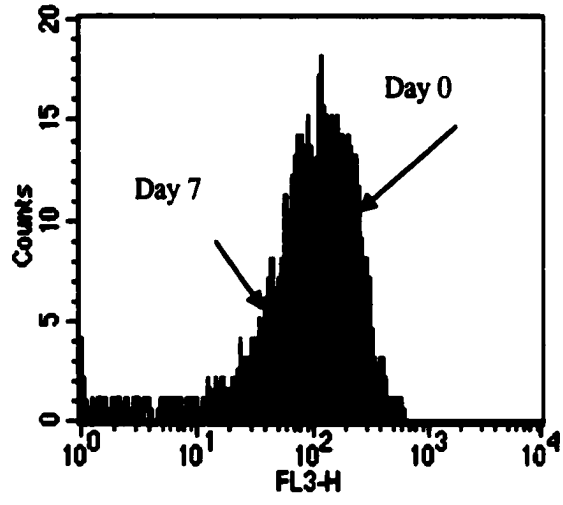
Figure 4.19. CD62L Expression on Lymph Node $WC1^{+/-}$ γδ T Cells



Cells from the prescapular lymph nodes from all five animals were stained with anti-bovine γδ FITC, anti-CD2 PE, anti-CD62L (followed by anti-IgG1 PerCP). The percentages of $WC1^+$ and $WC1^-$ γδ T cells that were also $CD62L^{neg/lo}$ were determined using CELLQuest software. Data represent the mean percentages from all five animals at each timepoint for both $WC1^+$(closed squares) and $WC1^-$ (open circles) γδ T cells ± SE. Significant differences between the $WC1^+$ cells and the $WC1^-$ cells are shown as *= p value< 0.05. Significant differences in cell populations before and after vaccination are shown as † = p value < 0.05.

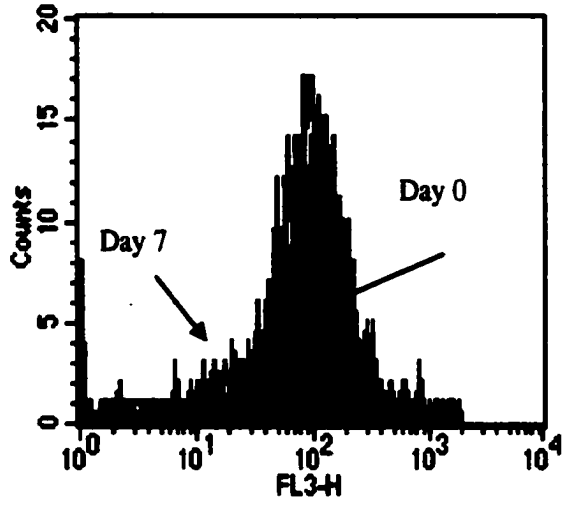
Figure 4.20. CD62L Expression on Lymph Node $WC1^{+/-}$ γδ T Cells

A



Fluorescence Intensity

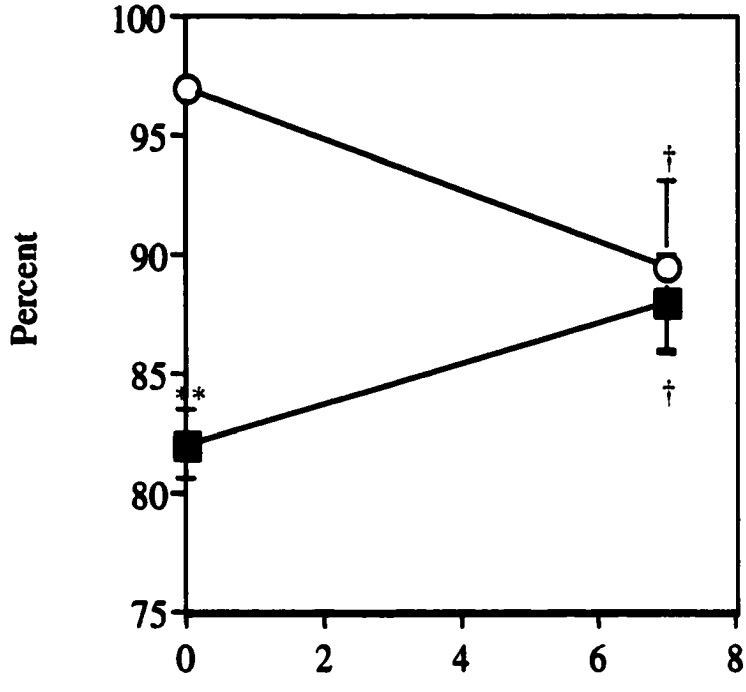
B



Fluorescence Intensity

A representative histogram of CD62L expression on $WC1^+$ (A) and $WC1^-$ (B) γδ T cells before and after the second vaccination, shows the log fluorescence intensity of CD62L PerCP (x axis) and the number of cells (y axis).

Figure 4.21. CD44 Expression on Lymph Node WC1$^{+/-}$ γδ T Cells



Cells from the prescapular lymph nodes from all five animals were stained with anti-bovine γδ FITC, anti-CD2 PE, anti-CD44 (followed by anti-IgG1 PerCP). The percentages of WC1$^+$ and WC1$^-$ γδ T cells that were also CD44^{mid} were determined using CELLQuest software. Data represent the mean percentages from all five animals at each timepoint for both WC1$^+$(closed squares) and WC1$^-$ (open circles) γδ T cells ± SE. Significant differences between the WC1$^+$ cells and the WC1$^-$ cells are shown as **= p value< 0.005. Significant differences in cell populations before and after vaccination are shown as †= p value < 0.05.

were $CD44^{mid}$ and the $WC1^{-}$ γδ T cells had a significantly smaller percentage of cells in the lymph node that were $CD44^{mid}$ (Figure 4.21), as compared to before vaccination.

$WC1^{+}$ γδ T cells had significantly higher percentages of $CD45R^{neg/lo}$ cells in the lymph nodes, as compared to $WC1^{-}$ γδ T cells (Figure 4.22). There were no significant changes of CD45R expression, on either $WC1^{+}$ or $WC1^{-}$ γδ T cells, following vaccination (Figure 4.22).

$WC1^{-}$ γδ T cells had significantly higher percentages of $CD25^{+}$ cells in the lymph node, as compared $WC1^{+}$ γδ T cells (Figure 4.23). There were no significant changes following vaccination (Figure 4.23).

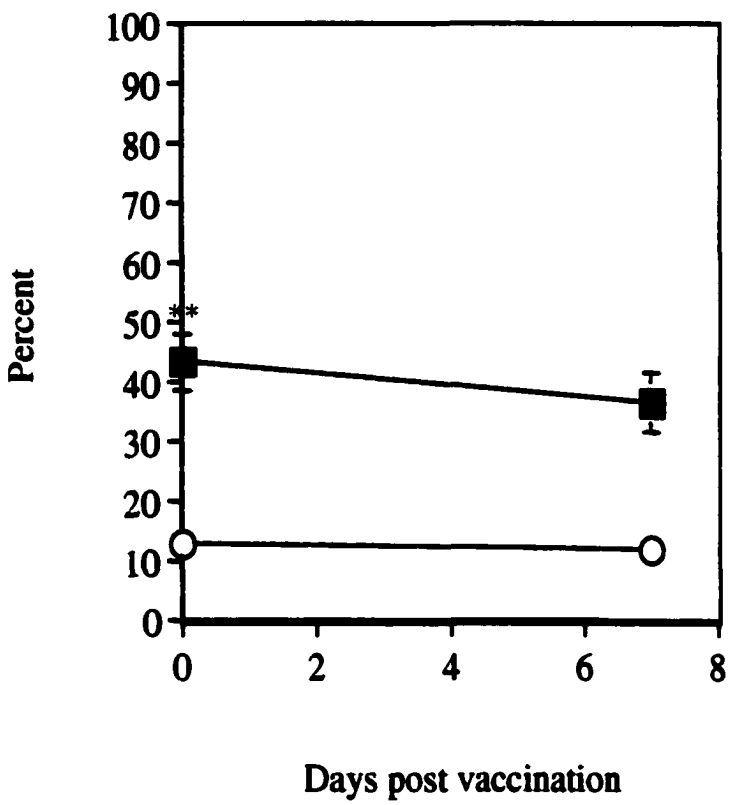
Three Color Flow Cytometry For WC1, γδ TCR, and CD2

Studies describing the phenotypes of $WC1^{+}$ and $WC1^{-}$ γδ T cells indicate that $WC1^{+}$ γδ T cells are $CD2^{-}$ and $WC1^{-}$ γδ T cells are $CD2^{+}$ (181). To confirm this observation, given that the differential expression of CD2 was used to identify $WC1^{+}$ and $WC1^{-}$ γδ T cells in this study, three color flow cytometry was performed on the PBMCs from 5 healthy, one-year-old cows. Cells were stained for WC1, γδ TCR, and CD2. Figure 4.24 shows that the $WC1^{+}$ cells are $CD2^{-}$ but the $WC1^{-}$ cells are CD2 variable.

Discussion

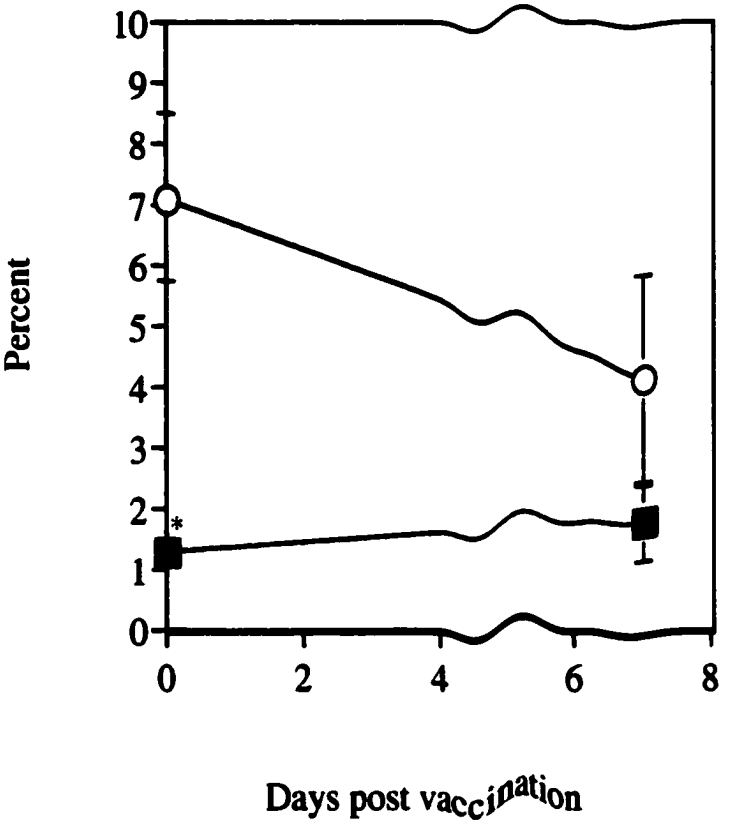
This study demonstrates that $WC1^{+}$ and $WC1^{-}$ γδ T cells differentially express cell surface molecules that indicate cellular activation or immunological memory. In particular, the $WC1^{+}$ γδ T cells specifically down regulate CD62L and CD45R following vaccine challenge, therefore indicating that the $WC1^{+}$ γδ T cells could be a potential target for vaccine design.

Figure 4.22. CD45R Expression on Lymph Node $WC1^{+/-}$ γδ T Cells



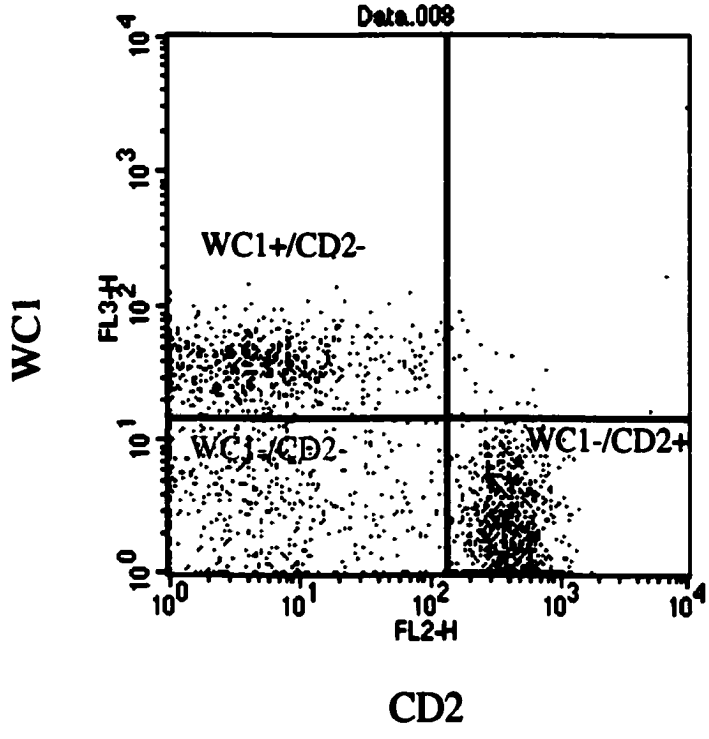
Cells from the prescapular lymph nodes from all five animals were stained with anti-bovine γδ FITC, anti-CD2 PE, anti-CD45R (followed by anti-IgG1 PerCP). The percentages of $WC1^+$ and $WC1^-$ γδ T cells that were also $CD45R^{neg/lo}$ were determined using CELLQuest software. Data represent the mean percentages from all five animals at each timepoint for both $WC1^+$(closed squares) and $WC1^-$ (open circles) γδ T cells ± SE. Significant differences between the $WC1^+$ cells and the $WC1^-$ cells are shown as **= p value< 0.005.

Figure 4.23. CD25 Expression of Lymph Node WC1+/- γδ T Cells



Cells from the prescapular lymph node from all five animals were stained with anti-bovine γδ FITC, anti-CD2 PE, anti-CD25 (followed by anti-IgG1 PerCP). The percentages of WC1+ and WC1- γδ T cells that were also CD25+ were determined using CELLQuest software. Data represent the mean percentages from all five animals at each timepoint for both WC1+ (closed squares) and WC1- (open circles) γδ T cells ± SE. Significant differences between the WC1+ cells and the WC1- cells are shown as * = p value < 0.05.

Figure 4.24. WC1 and CD2 Expression on γδ T Cells



Peripheral blood mononuclear cells were stained for WC1, CD2, and γδ TCR. CD2 and WC1 expression on γδ T cells is shown.

Very little information is known regarding the function of bovine γδ T cells, despite the fact that they make up such a large percentage of the peripheral blood mononuclear cells. It has however, been demonstrated that WC1$^-$ γδ T cells are much less efficient at accumulating at sites of inflammation than are WC1$^+$ γδ T Cells (317). Additionally, WC1$^+$ γδ T cells have been shown to be cytolytic and to be capable of producing numerous cytokines, including IFN-γ (48, 78). This study investigated age dependent changes in WC1$^+$ and WC1$^-$ γδ T cells, analyzed differences in activation/memory marker expression between the two populations, and evaluated the responses of WC1$^+$ and WC1$^-$ γδ T cells following vaccination. This study revealed several phenotypic differences between WC1$^+$ and WC1$^-$ γδ T cells that suggest distinct functional properties.

A recent study suggested that the majority of WC1$^-$ γδ T cells were deficient in CD62L expression (317). Since CD62L is an important molecule for homing to lymph nodes this observation implies that WC1$^-$ γδ T cells are not able to traffic to lymph nodes as efficiently as WC1$^+$ γδ T cells. Similar to the work by Wilson et al (317) this study demonstrated that the majority of WC1$^-$ γδ T cells did not express CD62L. In contrast to Wilson et al, this study also suggests that there is a significant population of WC1$^-$ γδ T cells that are CD62L positive (317). Disparity between results can perhaps be accounted for by the different monoclonal antibodies used. Our study used an anti-bovine CD62L antibody, whereas the study by Wilson et al (317) used an anti-human CD62L antibody that is cross reactive with the bovine molecule. We also demonstrate here that WC1$^+$ γδ T cells are consistently CD62L^{hi}. Interestingly, following vaccination the WC1$^+$ γδ T cells significantly down regulated CD62L expression whereas the WC1$^-$ γδ T cells did

not. This suggests that perhaps the WC1$^+$ γδ T cells are more responsive to antigenic challenge than WC1$^-$ γδ T cells.

In this study, we also observed changes in CD44 expression following vaccination with Vira Shield® 5. Studies of CD44 expression on murine and human CD4$^+$ T cells have revealed that the expression of this molecule is increased upon cell activation and is brighter on memory cells than naïve cells (50, 251). In contrast to observations in mice and humans, both WC1$^+$ and WC1$^-$ γδ T cells down regulated CD44 expression following vaccination. To the authors knowledge this is the first observation of a cell type down regulating CD44 expression following activation. CD44 facilitates the binding of lymphocytes to the high endothelial venules and therefore enables lymphocytes to leave circulation and enter into tissues. The advantage of moderately decreasing the expression of CD44 on activated γδ T cells is unknown. Alternatively, the observed decrease in CD44 expression could be a result of the activated (CD44^{hi}) cells leaving the peripheral blood to enter tissue. In either case, it is important to note that both WC1$^+$ and WC1$^-$ γδ T cells appeared to respond to vaccination.

This study demonstrated that although the majority of both WC1$^+$ and WC1$^-$ γδ T cells expressed some CD45R, a population of WC1$^-$ γδ T cells stained very brightly for CD45R. In contrast to Howard et al (144), who reported no CD45R staining on WC1$^+$ γδ T cells, we did indeed see substantial expression of this cell surface marker. Again, the disparity between these observations is likely due to differences in the monoclonal antibodies selected (this study used anti- CD2 and anti-δ chain antibodies to identify WC1$^+$ γδ T cell whereas the study by Howard et al used a monoclonal antibody to the BoWC1 antigen).

The nature of immunological memory suggests that a particular cell, a memory cell, exists for extended periods of time and is able to respond quickly to another challenge by the particular antigen that it recognizes. The WC1$^+$ γδ T cells shifted to the more activated phenotype, CD45R$^{neg/lo}$, following vaccination. This activated phenotype seemed to persist for at least 40 weeks following vaccination. It is reasonable to speculate, therefore, that this could indicate that WC1$^+$ γδ T cells are capable of immunological memory. This would obviously be important for the design of bovine vaccines, as one could include an antigen that would be recognized by the WC1$^+$ γδ T cells. Further experiments to address the long-term ability of WC1$^+$ γδ T cells to maintain a memory phenotype need to be carried out.

In order to conclusively demonstrate that WC1$^+$ cells are CD2$^-$ and WC1$^-$ cells are CD2$^+$, three color flow cytometry was performed on the peripheral blood of 5 healthy adult cows. The cells were stained for WC1, CD2, and the γδ T cell receptor. The results indicated that the WC1$^+$ cells were indeed CD2$^-$. There were however, two populations of WC1$^-$ cells, a CD2$^+$ and a CD2$^-$ population. Although the literature suggests that WC1$^-$ cells are CD2$^+$, there is no evidence that three color flow cytometry has been performed, that is staining for WC1, CD2, and γδ TCR (181). This does not change the results that were seen in this study but it does have implications on the interpretation of the results and on the nomenclature of the field. Alternatively, it is possible that there are only two populations, WC1$^+$/CD2$^-$ and WC1$^-$/CD2$^+$. There are numerous antibodies that recognize WC1 and since the different antibodies recognize different epitopes it is possible that an individual antibody to WC1 will not recognize all WC1$^+$ cells ((206). Additional studies

need to be performed to definitively characterize the individual populations of γδ T cells in the cow.

Acknowledgements

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CHAPTER 5

Antigen Recognition by Bovine γδ T Cells

Abstract

Cattle have very large circulating populations of γδ T cells but specific functions for these cells have not yet been clearly defined. There is however, evidence that human, murine, and bovine γδ T cells have a role in the immune response to mycobacteria. This study investigated the ability of bovine γδ T cells to expand and produce IFN-γ in response to stimulation with mycobacterial antigens. Bovine γδ T cells, isolated from the peripheral blood of healthy cows, expanded following in vitro stimulation with live mycobacteria, mycobacterial crude cell wall extract, and *M. bovis* culture filtrate proteins. In addition, purified γδ T cells, co-cultured with purified monocytes, produced IFN-γ in response to the mycolylarabinogalactan peptidoglycan fraction of the *M. tuberculosis* cell wall. These data indicate that components of the mycobacterial cell wall may be useful adjuvant components of subunit vaccines for bovine mycobacterial diseases.

Introduction

The genes for the γ chain of the TCR were discovered in 1984 while looking for the TCR genes of the α and β chains (247). Two years later the γδ T cell was identified (46). In the fifteen years since their discovery much research has been conducted to characterize the antigens that they recognize and to elucidate their functions. Populations of γδ T cells have been defined that have specific tissue tropisms and very limited diversity in the TCRs, indicating restricted antigen recognition (17, 150). Interestingly, γδ T cell populations with extremely diverse junctional regions have also been identified, indicating an ability to recognize a wide range of antigens (35). The requirement of antigen presentation to γδ T cells remains unclear (189, 238, 241, 253, 259, 309).

Moreover, γδ T cells have been demonstrated to recognize both protein antigens (38, 40, 117, 133, 249) and protease resistant antigens (81, 114, 203, 232, 283, 284).

Few experiments have been conducted to phenotypically and functionally characterize bovine γδ T cells. Despite this, the experiments that have been performed have revealed interesting and significant information. When compared to humans and mice, cattle and other ruminants have very large circulating populations of γδ T cells, while up to as many as 75% of the PBMCs (137). Bovine γδ T cells demonstrate cytolytic activity and express IL-2, IL-4, IL-10, IFN-γ, and TNF-α (48, 78). Additionally, a subset of bovine γδ T cells has been shown to efficiently migrate to sites of inflammation (317).

Several experiments have demonstrated the relevance of γδ T cells to mycobacterial diseases. Human, murine, and bovine γδ T cells have been shown to accumulate either in mycobacterial lesions, or lymph nodes (99, 126, 196). Human γδ T cells recognize both peptide and non-peptide mycobacterial antigens (118, 133, 231). Additionally, altered granuloma formation was evident following mycobacterial challenge in γδ KO mice and in γδ depleted SCID-bo mice (89, 266).

A CFP based vaccine for bovine tuberculosis provided some protection to animals experimentally infected with virulent *M. bovis*. Although the protection was not as great as that conferred by the BCG vaccine, animals that received the CFP vaccine had significantly less severe lesions than those animals in the negative control group. In addition, the CFP vaccine failed to induce significant cell mediated immune responses following vaccination. Given the large circulating population of bovine γδ T cells and the propensity of human and murine γδ to recognize mycobacterial antigens, this study

attempted to identify mycobacterial antigens that were recognized by γδ T cells from healthy cattle. This study demonstrates that while bovine γδ T cells expand following in vitro stimulation with various mycobacterial antigens, only a component of the mycobacterial cell wall sub-cellular fraction consistently elicits the production of a significant amount of IFN-γ. In addition, assays were developed for measuring intracellular IFN-γ production in bovine γδ T cells and for the quantitative real-time PCR analysis of IFN-γ, TNF-α, IL-4, IL-12, and MCP-1 mRNA expression in bovine γδ T cells.

Materials and Methods

Animals

All animals used in this study were between 6 months and 2 years old. The animals were housed in outdoor pens and fed hay and grain once daily. Five of the animals used in this study (three Holstein steers, one Black Angus steer, and one Black Angus heifer) were used as normal blood donors in this study and others. The remaining thirteen animals were non-pregnant heifers from XY Incorporated.

Peripheral Blood Mononuclear Cell Isolation

Depending on the experiment, between 120 and 250 ml of whole blood was collected into 8 ml CPT Vacutainer tubes with sodium heparin (Beckton Dickinson, Franklin Lakes, NJ). The tubes were centrifuged at 1800 RCF for 30 minutes at 20°C. The PBMCs were removed and washed once in PBS. The residual red blood cells were removed from the PBMCs by resuspending the PBMCs in Geys solution and incubating on ice for 5 minutes. The PBMCs were then washed twice in PBS, counted and resuspended in the appropriate media at the working concentration.

Antigen Preparation and Stimulation

Concanavalin A (Sigma, St. Louis MO), *M. tuberculosis* ManLAM (TB Contract, Fort Collins CO), and *M. tuberculosis* AraLAM (TB contract) were used at a concentration of 1 μg/ml. *M. tuberculosis* sub-cellular cell wall (TB contract), *M. a. paratuberculosis* > 10 kDa CFP (prepared as previously described in Chapter 3), *M. bovis* > 10 kDa CFP (prepared as previously described in Chapter 2), isopentenyl pyrophophate (IPP) (Sigma), *M. bovis* sub-cellular cell wall, *M. tuberculosis* total lipid extract (TB contract), *M. tuberculosis* mAGP (TB contract), soluble cell wall fraction of *M. bovis*, and proteinase K treated *M. bovis* sub-cellular cell wall were all used at a concentration of 10 μg/ml. Live *M. bovis* and live *M. a. paratuberculosis* were used at a multiplicity of infection (MOI) of 0.1.

M. bovis sub-cellular cell wall was prepared from a γ-irradiated whole cell pellet of *M. bovis* 862422. The cells were resuspended at 2 grams/ml in PBS containing DNase, RNase, PMSF, pepstatin A, and leupeptin. The cells were then broken by passing the suspension through a French Press pressure cell ten times. The unbroken cells were removed by centrifuging at 3000 x g for 5 minutes at 4°C. The supernatant was then centrifuged at 27,000 x g for one hour at 4°C. The cell wall pellet was then washed in PBS twice. The protein concentration of the crude cell wall was determined by the BCA assay (Pierce, Rockford Illinois). The crude cell wall was resuspended in PBS at 0.5 mg/ml and stored at -80°C.

SDS-soluble cell wall fraction of *M. bovis* was prepared from the sub-cellular cell wall extract. The sub-cellular cell wall pellet was resuspended in PBS with 2% SDS (1 mg/ml) and stirred at 37°C for twelve hours. The soluble cell wall was

collected from the supernatant after centrifuging at 27,000 x g for one hour. Another 2% SDS extraction was performed on the pellet for an additional four hours at 37°C. The soluble cell wall was collected from the supernatant after centrifuging at 27,000 x g for one hour. A final 2% SDS extraction was performed on the pellet for an additional twelve hours at 37°C. The soluble cell wall from the three individual 2% SDS extractions were pooled. The SDS was removed from the soluble cell wall fraction using Extracti gel columns (Pierce) and SDS-Out (Pierce) SDS precipitation kit. The protein concentration was determined by the BCA assay (Pierce) and adjusted to 0.5 mg/ml with PBS. 10 μg/ml of the soluble cell wall was incubated with bovine PBMCs for 60 hours to ensure that any residual SDS would not be toxic to the cells.

The proteinase K treated *M. bovis* cell wall was prepared from the *M. bovis* sub-cellular cell wall extract. The crude cell wall was digested with 50 μg, 100 μg, 200 μg, and 1000 μg of proteinase K at 65°C for 1 hours and the proteinase K was heat inactivated at 90°C for 5 minutes. The digestion of the protein was assessed by one-dimensional gel electrophoresis. All 4 concentrations of proteinase K resulted in complete digestion of proteins. For in vitro stimulation the crude cell wall sample that was digested with 1000 μg of proteinase K was used.

In Vitro Expansion of Bovine γδ T Cells:

PBMCs were obtained from 5 healthy cows, as described previously. PBMCs were resuspended in complete RPMI 1640 (RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, 0.12% β-Mercaptoethanol, 5% HEPES, 5% L-glutamine (200 mM), and 50 mg ampicillin). The cells were seeded at $1x10^6$ cells/ml into 6 well plates, 8 ml per well. Cells were stimulated with the following

antigens and controls: PBS, ConA, *M. tuberculosis* sub-cellular cell wall extract, *M. bovis* CFP, *M. tuberculosis* ManLAM, and IPP. At 0, 2, 4, and 6 days following stimulation cells were harvested with a cell scraper, and washed with D-RPMI + 0.1% sodium azide. The cells were stained with anti-bovine CD2-PE (BAQ95A) (VMRD, Pullman WA/Chromaprobe, Mountain View CA), anti-bovine δ TCR-FITC (GB21A) (VMRD/Chromaprobe), and appropriate isotype controls for 30 minutes at 4°C in the dark. The cells were washed twice in D-RPMI + 0.1% sodium azide and resuspended in 200 µl of D-RPMI + 0.1% sodium azide. The cells were analyzed using the FACSCalibur flow cytometer. Lymphocytes were gated by forward and side scatter profiles. Percentages of total γδ T cells were determined by CELLQuest, after setting quadrants based on the staining of the isotype controls.

Magnetic Bead Isolation of γδ T cells and Monocytes

PBMCs were resuspended in PBS at $2x10^6$ cells/ml. The cells were stained with either murine anti-bovine δ TCR FITC (VMRD/Chromaprobe), anti-bovine δ TCR biotin (VMRD/Chromaprobe), murine anti-bovine monocyte (BAQ151A VMRD) or murine anti-bovine monocyte FITC (VMRD/Chromaprobe) for 30 minutes at 4°C in the dark. The cells were then washed twice in PBS plus 2mM EDTA. The cells were incubated with anti-FITC, streptavidin, or anti-IgG1 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), for 15 minutes at 4°C in the dark. Anti-IgG1 PerCP (Beckton Dickinson, San Diego CA) was then added to the cells that were stained with anti-monocyte antibody and streptavidin RED670 (Gibco BRL) was added to the cells stained with anti- δ TCR biotin antibody and incubated for an additional 10 minutes. The cells were washed twice with PBS plus 2mM EDTA and resuspended to 500 µl/$1x10^8$

cells in PBS plus 2mMEDTA. The cell suspensions were then run over magnetic columns and the adherent cells were collected. Both the purified monocytes and the purified γδ T cells were analyzed for purity by flow cytometry.

Antigenic Stimulation of Purified γδ T Cells

PBMCs, purified monocytes, and purified γδ T cells were resuspended in complete RPMI 1640. PBMCs, purified γδ T cells, and purified monocytes were seeded into 96 well flat bottom plates at a concentration of $1x10^6$ cells/ml with 200 µl/well. For wells with both purified γδ T cells and purified monocytes 100 µl of each cell type was added at $2x10^6$ cells/ml. Initially these cells were stimulated with the following antigens and controls: PBS, *M. tuberculosis* sub-cellular cell wall extract, *M. bovis* CFP, *M. a. paratuberculosis* CFP, *M. tuberculosis* ManLAM, IPP, live *M. bovis*, and live *M. a. paratuberculosis*. All cultures were incubated at 37°C with 5% CO_2 for 48 hours. Recombinant bovine IL-2 (A generous gift from Neil Wedlock) was added to the culture media in subsequent experiments (60 U/ml). The antigens listed above, in addition to AraLAM, *M. bovis* sub-cellular cell wall extract, proteolytically digested *M. bovis* crude cell wall extract, SDS soluble *M. bovis* cell wall fraction, total lipid extract from *M. tuberculosis*, and mAGP from *M. tuberculosis* were used for stimulation in subsequent experiments with IL-2.

Stimulation of Purified γδ T Cells in a Transwell System

Purified γδ T cells ($4x10^6$ cells/ml) and purified monocytes ($1.33x10^6$ cells/ml) were resuspended in complete RPMI 1640 and 60 U/ml of recombinant bovine IL-2. 3 µm 12 -well Transwell plates were used separate the γδ T cells and the monocytes, whilst allowing media and molecules <3 µm in size to diffuse into both

compartments. 600 µl of the purified monocyte cell suspensions were plated in the outer wells and 200 µl of the purified γδ T cell suspensions were plated in the inner wells. The cells were stimulated with *M. tuberculosis* sub-cellular cell wall extract, which was added to the outer well, or left unstimulated. The plates were incubated at 37°C and 5% CO_2 for 48 hours.

IFN-γ ELISA

All samples for IFN-γ ELISAs were frozen at -80°C until they could be analyzed. Bovine IFN-γ EASIA kit was used to qualitatively measure the IFN-γ in the supernatants (Biosource, Belgium). The kit protocol was followed. Briefly, 100 µl of the tissue culture supernatant was added to the anti-IFN-γ coated wells. Positive and negative controls were supplied with the kit and added to anti-IFN-γ coated wells. 50 µl of incubation buffer was added to each well and the plates were incubated at room temperature on a horizontal shaker (700 rpm) for one hour. The plates were washed 3 times with 1x PBS/Tween. 100 µl of the working conjugate was added to each well and incubated at room temperature on a horizontal shaker (700 rpm) for one hour. The plates were washed 3 times with 1x PBS/Tween. 100 µl of the Chromogen (TMB) was added to each well and the plates were incubated at room temperature on a horizontal shaker (700 rpm) for 15 minutes. 200 µl of the Stop Solution was added to each well and the plate was read at 450 nm against a 630 nm reference filter. Positive samples had OD values that were greater than the average OD reading of the negative control plus 0.15. All results are reported as the mean OD reading ± SEM.

Intracellular Staining

PMBCs were purified from 5 normal cows, as described previously. For

each animal, PBMCs were seeded at $1x10^6$ cells/ml with 6 ml/well into 10, 6 well plates. 5 plates were harvested after 24 hours of stimulation and 5 plates were harvested after 48 hours of stimulation. Each antigen was added to 3 wells per cow, per timepoint, at the concentrations previously described. In this experiment the following antigens and controls were used: PBS, ConA, live *M. bovis*, live *M. a. paratuberculosis*, *M. tuberculosis* sub-cellular cell wall extract, *M. bovis* CFP, *M. a. paratuberculosis* CFP, ManLAM, AraLAM, and IPP. The Cytofix/Cytoperm Plus® kit was used for intracellular staining (Pharmingen, San Diego CA). After approximately 16 hours of stimulation, 4 µl of GolgiStop was added to each well of the 24 -hour plates. After an additional 8-10 hours of incubation with GolgiStop, the cells were harvested from each of the wells. The cells were washed twice in D-RPMI + 0.1% sodium azide. The cells were then stained for 30 minutes in the dark at 4°C with anti-bovine $CD2^-$PE (VMRD/Chromaprobe), anti-bovine δ -TCR-FITC (VMRD/Chromaprobe), and the appropriate isotype controls. The cells were washed in D-RPMI and incubated with the Cytofix/Cytoperm solution for 20 minutes at 4°C. The cells were then washed in 1x Perm/Wash solution. The cells were stained with anti-IFN-γ-APC (MCA1783) (Serotec/Chromaprobe) and the appropriate isotype controls for 30 minutes in the dark at 4°C. The cells were washed in the 1x Perm/Wash solution and resuspended in 200 µl of D-RPMI. The samples were analyzed with the FACSCalibur flow cytometer. After gating on the lymphocyte populations and setting the quadrants based on the isotype controls, the percentages of $CD2^+$ and $CD2^-$ γδ T cells that were also IFN-γ positive were determined by CellQuest Software.

Real Time PCR

TaqMan Primer and Probe Design

TaqMan primers and probes were designed for bovine IFN-γ, TNF-α, MCP-1, IL-4, and IL-12 using Primer Express v1.5 (Applied Biosystems, Foster City Ca). The sequences are as follows: IFN-γ forward primer (CAG CTC TGA GAA ACT GGA GGA CTT), IFN-γ reverse primer (GGC TTT GCG CTG GAT CTG), IFN-γ FAM labeled probe (AAA GCT GAT TCA AAT TCC GGT GGA TGA TCT), TNF-α forward primer (CGG TGG TGG GAC TCG TAT), TNF-α reverse primer (GGT TGT CTT CCA GCT TCA CA), TNF-α FAM labeled probe (CAA TGC CCT CAT GGC CAA CG), MCP-1 forward primer (CTA CTC ACA GTA GCT GCC TTC AG) MCP-1 reverse primer (CGA CTT GGG AGT TAA TTG CAT), MCP-1 FAM labeled probe (CGA GGT GCT CGC TCG CTC AGC CA), IL-4 forward primer (CTG CAG GGT TGG AAT TGA), IL-4 reverse primer (CCG CCC AGG AAT TTG TT), IL-4 FAM labeled probe (ATC TAC AGG AGC CAC ACG TGC TTG A), IL-12 forward primer (AAA GTC ACA TGC CAC AAG GA), IL-12 reverse primer (TAA CTG CAG GAC ACA AGA TGC), IL-12 FAM labeled probe (CGT CCG CGT GCA AGC CC).

Production of In Vitro Transcribed RNA

Forward and reverse primers were also designed for PCR cloning bovine IFN-γ, TNF-α, MCP-1, IL-4, and IL-12 into pCR®2.1-TOPO® (Invitrogen, Carlsbad CA). The sequences were designed from mRNA sequences and were designed to amplify the entire gene. The sequences are as follows: IFN-γ forward primer (GGC CTA ACT CTC TCC TAA ACA ATG), IFN-γ reverse primer (TAT TGC AGG CAG GAG GAC CAT TAC), TNF-α forward primer (GAG CAC CAA AAG CAT GAT CC),

TNF-α reverse primer (TCA CAG GGC GAT GAT CCC AA), MCP-1 forward primer (TCC TCT CGC TGC AAC ATG AA), MCP-1 reverse primer (TGC CAA CAG TCA GGT TTT GT), IL-4 forward primer (ATG GGT CTC ACC TAC CAG CT), IL-4 reverse primer (ACG CTT CAA CAC TTG GAG TA), IL-12 forward primer (ATG CAC CCT CAG CAG TTG GT), IL-12 reverse primer (CTG AGG TTT CAT ACT GAG GT).

cDNA for PCR amplification was obtained by stimulating bovine PBMCs with 1 μg/ml of ConA for 72 hours. After 72 hours of stimulation the PBMCs were harvested and pelleted in 1.5 ml centrifuge tubes and the RNA was isolated with TRIZOL® reagent (Life Technologies, Grand Island NY) according to the product insert. 677 μg of RNA was isolated and resuspended to 5 μg/ 60 μl. cDNA was obtained as previously described in Chapter 2.

Bovine IFN-γ, TNF-α, MCP-1, IL-4, and IL-12 genes were PCR amplified using 25 μl of PCR Master Mix (Promega, Madison WI), 2.5 μl of the forward primer (10 μm), 2.5 μl of reverse primer (10 μm), 10 μl of cDNA, and 10 μl of ultrapure H_2O. The PCR reaction conditions were as follows: 94°C/5 min., 30 cycles of 94°C/1 min., 50°C/2 min., and 72°C/3 min., 72°C/10 minutes. $MgCl_2$ concentrations were adjusted for the amplification of some of the genes. All PCR products were analyzed on 1.5% agarose gels. The TOPO TA Cloning kit® (Invitrogen) was used to clone the PCR products into pCR®2.1-TOPO®, as described in the product insert. Briefly, 4 μl of fresh PCR product was added to 1 μl of salt solution, and 1 μl of the TOPO® vector, gently mixed, and incubated at room temperature 5 minutes (the salt solution and TOPO® vector are supplied in the kit). The reaction was placed on ice and 2 μl was added to one vial of One Shot® Chemically Competent *E. coli*. The mixture was mixed gently and placed on

ice for 30 minutes. The cells were then heat-shocked for 30 seconds at 42°C and placed on ice. 250 µl of room temperature SOC medium was added and the cells were allowed to recover for one hour at 37°C. 10-50 ml of cells were plated onto TSA plates (40 µg/ml kanamycin and 40 µg/ml X-Gal) and incubated overnight at 37°C. Several white colonies were selected and placed into 5 ml of LB broth (40 mg/ml kanamycin). The cultures were placed at 37°C with shaking overnight. The plasmid DNA was isolated using Qiagen Mini-Prep (Qiagen), according to the product insert. Restriction digests confirmed that the correct insert was in the vector and in the correct orientation.

The mMessage mMachine™ high yield capped RNA transcription kit was used to transcribe RNA from the bovine cytokine clones, according to the product insert (Ambion, Austin Texas). Briefly, the plasmid DNA was linearized with a restriction enzyme that cut downstream of the insert. The linearized plasmid DNA was then precipitated and adjusted to a concentration of 1 µg/ml. 5 µl of nuclease free water, 2 µl of 10X reaction buffer, 10 µl of 2X NTP/Cap, and 2 µl of the enzyme mix (T7) was added to 1 µl of template DNA and incubated for 2 hours at 37°C. 1 µl of DNase 1 was then added and incubated for an additional 15 minutes at 37°C. Lithium chloride precipitation was used to purify the in vitro transcribed RNA. Briefly, 30 µl of nuclease free water and 25 µl of LiCl precipitation solution (included in mMessage mMachine Kit) was added to the transcription reaction and incubated for 30 minutes at -20°C. The samples were then centrifuged at 13,000 x g for 15 minutes at 4°C. The supernatant was removed and the pellet was washed with 1 ml of 70% ethanol. The samples were then centrifuged at 13,000 x g for 15 minutes at 4°C. The supernatant was removed and the pellets dried at room temperature. The pellets were resuspended in 50 µl of nuclease free

water and stored at –20°C. The integrity and size of the RNA transcripts were analyzed on 1.5% agarose gels with an RNA ladder (Ambion). The RNA was quantified by UV light absorbance (A_{260}).

Sample Preparation

Whole blood was collected from 5 healthy cows. The PBMCs were isolated and cultured in 6 well plates ($6x10^6$ cells/well). The cells were cultured in complete RPMI 1640. One 6 well plate of cells was seeded for each of the following antigens and controls: PBS, ConA, *M. tuberculosis* cell wall, *M. bovis* CFP, *M. a. paratuberculosis* CFP, live *M. bovis, and live M. a. paratuberculosis.* All plates were incubated for 48 hours at 37°C. The cells ($3.6x10^7$ cells/antigen and control) were harvested using a cell scraper and washed in D-RPMI + 0.1% sodium azide. The γδ T cells were isolated from each antigen stimulated cell population using magnetic beads, as previously described. The RNA was isolated from the γδ T cells using TRIZOL, according to the product insert.

Reverse Transcription of Samples and Standards

Working stocks of all in vitro transcribed RNA samples were made and 100 fold serial dilutions were made for the generation of standard curves. 12 µl of standards or samples were reverse transcribed as described in Chapter 2. Following the reverse transcription reaction 95 µl of nuclease free water was added to each sample (for a final volume of 120 µl of cDNA).

Real Time PCR Reaction

For the generation of standard curves 5 µl of cDNA, for each of the seven standards for each cytokine (IFN-γ, TNF-α, MCP-1, IL-4, and IL-12), was used

in the amplification reactions. For each of the samples 10 µl of cDNA was used in the amplification reactions. The cDNA from each of the standards and samples was placed into separate wells in a MicroAmp® Optical 96-well reaction plate. The samples were all plated in duplicate, one well for cytokine amplification and one well for 18s ribosomal controls. The 18s reagents were vic (fluorescent reporter dye) labeled and were purchased as a kit (Applied Biosystems). All working concentrations of TaqMan primers were 100 µM and the FAM (fluorescent reporter dye) labeled probes were 2.5 µM. 5 µl of forward primer, 5 µl of reverse primer, 5 µl of probe, 5 µl of nuclease free water, and 45 µl of TaqMan Universal PCR master mix (Applied Biosystems) was added to each well. Optical caps were placed on each well of the 96-well plate and the samples were run on the ABI PRISM 7700 (standard reactions conditions). Standard curves were generated by plotting the C_T values (cycle number at which there is a significant increase in the amplification of the target) on the y-axis and the log ng of standard on the x-axis. All samples were normalized to the 18s RNA using the delta C_T method, as described in the Users Bulletin #2 (Applied Biosystems).

Statistical Analyses

Statistical significance for the expansion study of bovine γδ T cells was determined by performing ANOVA and LSD using SAS statistics software. Production of IFN-γ was considered positive when above the assay cutoff (OD 450-630 nm of the negative control + 0.015).

Results

Bovine γδ T cell Expansion

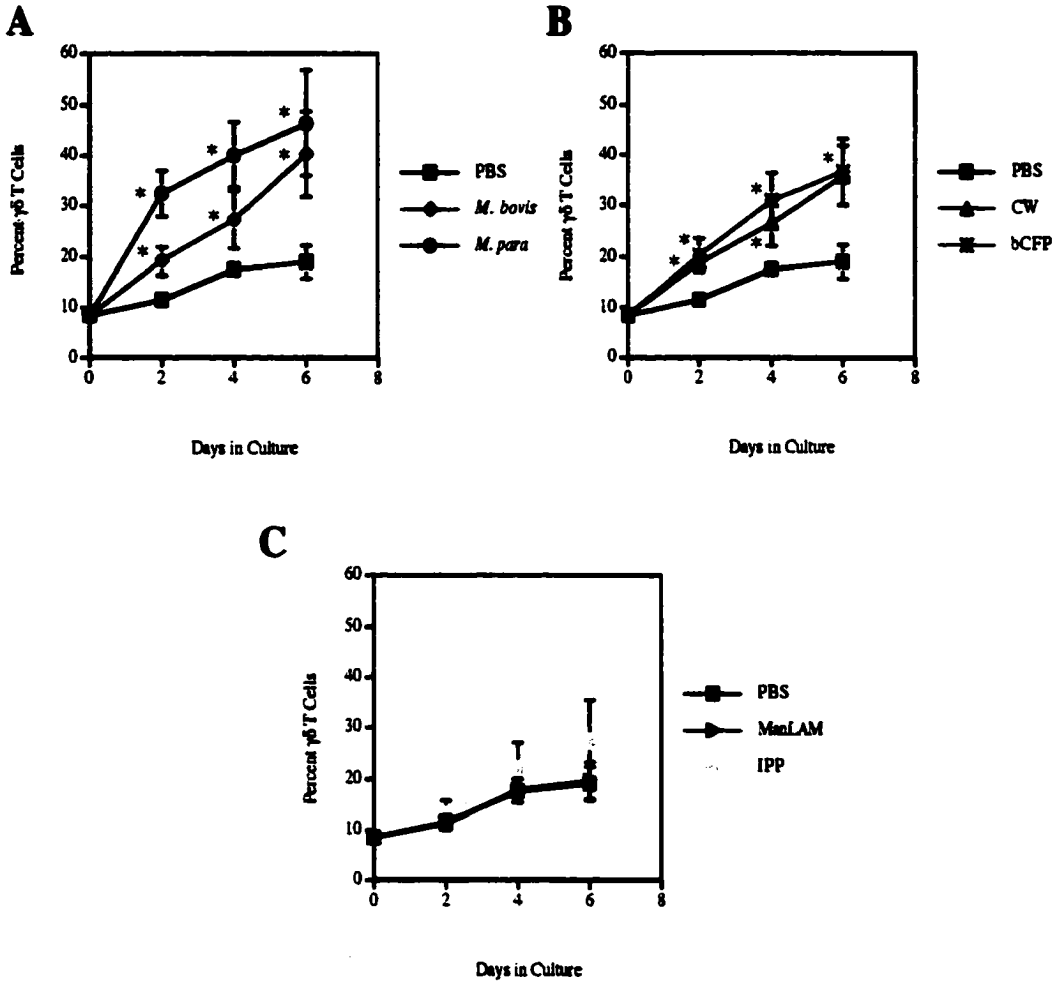
In order to determine if bovine γδ T cells recognized mycobacterial

antigens, PBMCs from healthy cows were cultured with live *M. bovis*, live *M. a paratuberculosis*, *M. tuberculosis* cell wall, *M. bovis* CFP, *M. tuberculosis* ManLAM, IPP, and PBS. The percent γδ T cells of total cultured cells were measured after 0, 2, 4, and 6 days in culture by flow cytometry. Bovine γδ T cells expanded significantly in response to stimulation with both live *M. bovis* and live *M. a. paratuberculosis* at all three timepoints (Figure 5.1A). In addition, bovine γδ T cells expanded in response to stimulation with *M. tuberculosis* cell wall at days 2 and 4 and to *M. bovis* CFP at all three timepoints (Figure 5.1B). There were however, no significant responses to either IPP or *M. tuberculosis* ManLAM (Figure 5.1C).

Requirements for IFN-γ Production by Bovine γδ T Cells

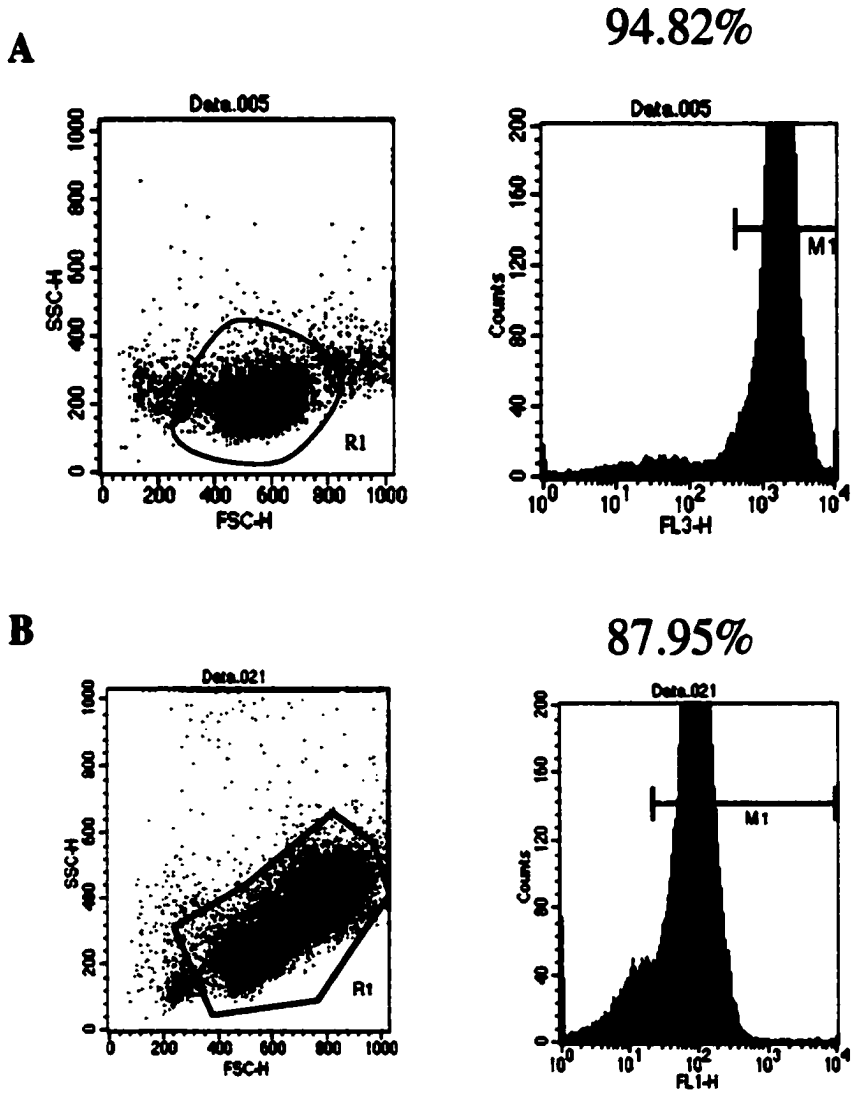
IFN-γ is a key cytokine in the protective immune response to mycobacteria (82, 110). Given the evidence that γδ T cells do not require antigen presentation for antigen recognition, the ability of purified bovine γδ T cells to produce this key cytokine in response to mycobacterial antigens was assessed (253, 259, 309). Bovine γδ T cells were purified from the peripheral blood of 5 healthy cows using magnetic beads and stimulated with several mycobacterial antigens and controls (ConA, *M. bovis* CFP, *M. tuberculosis* cell wall, *M. tuberculosis* ManLAM, IPP, live *M. a. paratuberculosis*, and live *M. bovis*). The purity of the bovine γδ T cell populations was assessed by flow cytometry and found to be consistently greater than 90% pure (Figure 5.2A). IFN-γ ELISAs were performed to detect the production of IFN-γ by purified γδ T cells in response to stimulation with the mycobacterial antigens listed previously. Figure 5.3 shows that no detectable IFN-γ was produced by purified γδ T cells in response to stimulation with any of the mycobacterial antigens tested.

Figure 5.1. Expansion of γδ T Cells



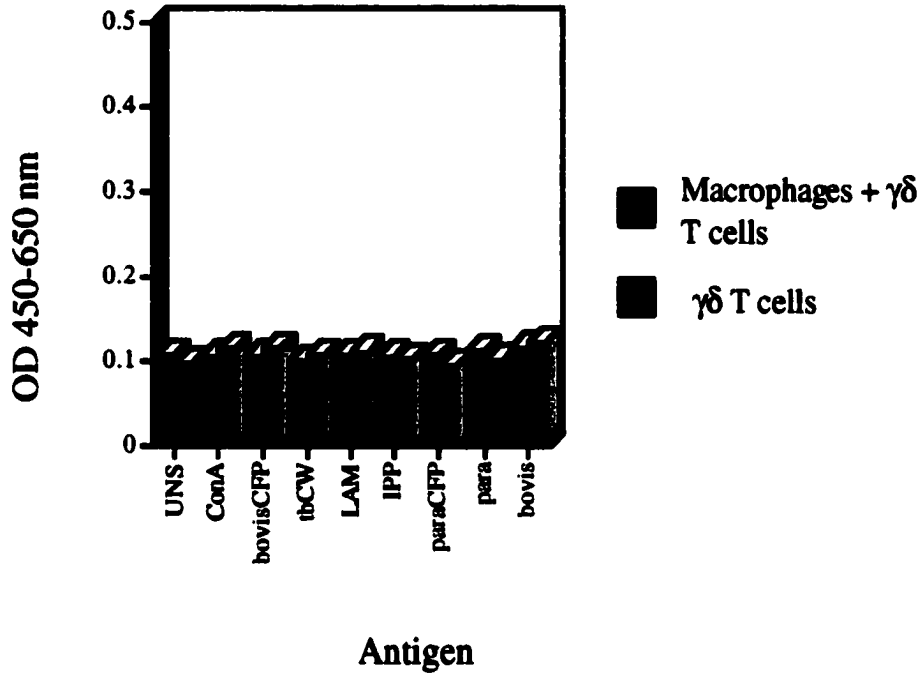
In vitro expansion of bovine γδ T cells following stimulation with live *M. bovis* and live *M. a. paratuberculosis* (A), cell wall and CFP (B), and ManLAM and IPP (C). Data are presented as mean percent γδ T cells from four normal cows ± SEM. Statistical significance was determined using ANOVA/LSD. * = p value < 0.05.

Figure 5.2. Purity of γδ T Cell and Monocyte Populations



Purity of γδ (A) and monocyte (B) populations following magnetic bead purification.

Figure 5.3. IFN-γ Production by γδ T Cells



IFN-γ production by purified γδ T cells and γδ T cells overlayed onto purified macrophages, following stimulation with mycobacterial and control antigens.

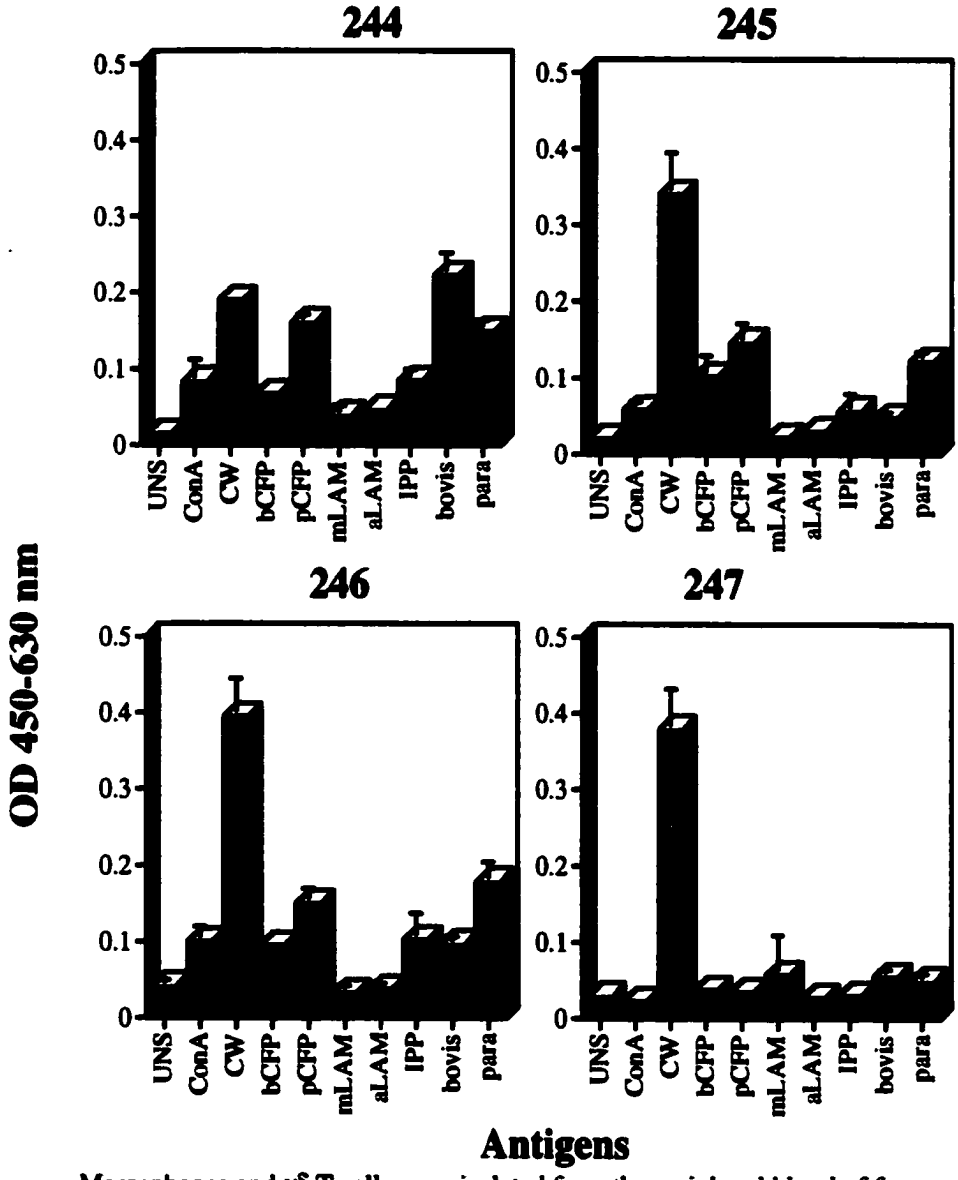
There is also evidence that γδ T cells require antigen presentation for antigen recognition (189, 238, 241). Therefore, γδ T cells were purified from the peripheral blood of 5 healthy cows and cultured in the presence of matched purified monocytes and the aforementioned mycobacterial antigens. The purity of the bovine monocyte populations, to be used as antigen presenting cells, was assessed by flow cytometry and found to be consistently greater than 85% pure (Figure 5.2B). As seen with the purified γδ T cells, purified γδ T cells cultured with purified monocytes as antigen presenting cells and mycobacterial antigens did not result in production of a detectable amount of IFN-γ in the culture supernatants (Figure 5.3).

This was not totally unexpected, as T cells need growth factors such as IL-2 for proliferation and cytokine secretion. The addition of IL-2 to the cultures containing purified γδ T cells and purified monocytes resulted in the production of IFN-γ from bovine γδ T cells (from 4 individual cows) in response to mycobacterial cell wall (Figure 5.4). This experiment has been repeated in 10 additional animals and consistently, the stimulation of purified γδ T cells cultured with purified monocytes, bovine IL-2, and *M. tuberculosis* cell wall resulted in significant IFN-γ secretion (Data not shown).

Bovine γδ T Cells Produce IFN-γ in Response to Stimulation with *M. bovis* Cell Wall and to Proteolytically Digested *M. bovis* Cell Wall but not the SDS Soluble Fraction of *M. bovis* Cell Wall

Since the production of IFN-γ by bovine γδ T cells was demonstrated in response to stimulation with *M. tuberculosis* cell wall, the sub-cellular cell wall fraction of a mycobacterial species that is relevant to cattle was harvested and tested for its ability to stimulate IFN-γ production by γδ T cells. γδ T cells and monocytes were purified from

Figure 5.4. IFN-γ Production by γδ T Cells

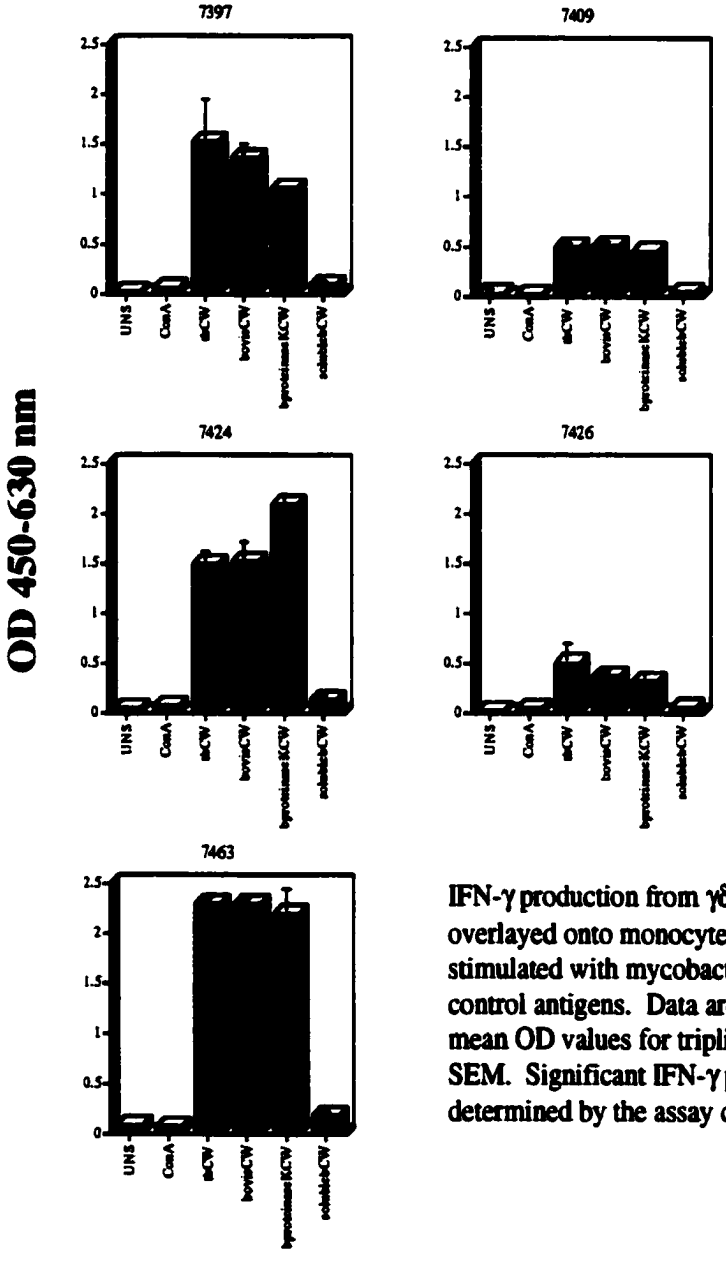


Macrophages and γδ T cells were isolated from the peripheral blood of four cows using magnetic beads. The macrophages and γδ T cells were incubated with the antigens listed above for 48 hours. The supernatants were frozen and then tested for the presence of IFN-γ. The data is presented as the mean OD 450-650 nm for triplicate wells ± SE. Significant IFN-γ production was determined by the assay cutoff ( ).

5 healthy cows and stimulated with the sub-cellular cell wall fraction from a virulent strain of *M. bovis*. Significant IFN-γ was detected in the culture supernatants stimulated with *M. bovis* cell wall, from all 5 animals (Figure 5.5). This experiment was repeated using 5 additional animals and the results showed identical trends (Figure 5.6). Purified monocytes alone were also tested to ensure that there were no contaminating T cells and significant amounts of IFN-γ were not produced (Data not shown).

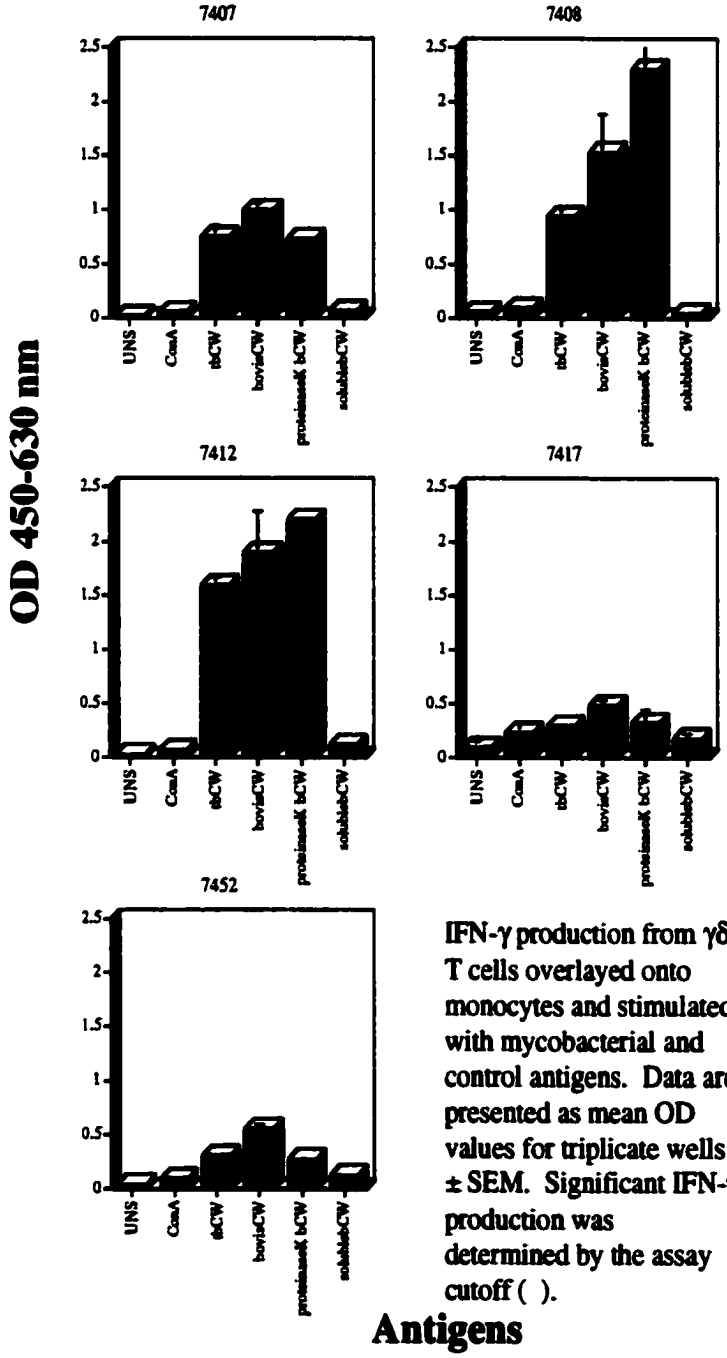
To determine if the IFN-γ stimulatory component of the *M. bovis* sub-cellular cell wall fraction was a protein both the SDS soluble cell wall fraction and a proteolytically digested cell wall fraction was tested in the IFN-γ culture assay. Figure 5.7 shows a silver stained SDS-PAGE gel of *M. bovis* sub-cellular cell wall, the SDS soluble sub-cellular cell wall, and the proteolytically digested sub-cellular cell wall from *M. bovis*. The sub-cellular cell wall and the SDS soluble cell wall fraction both contain a complex mixture of proteins (Figure 5.7, lanes 2 and 8) while the proteolytically digested sub-cellular cell wall contains no visible protein (Figure 5.7, lanes 3-6). Again, purified γδ T cells from 5 healthy cows were cultured with matched monocytes, IL-2, the SDS soluble sub-cellular cell wall fraction and the proteolytically digested sub-cellular cell wall fraction for 48 hours. The proteolytically digested sub-cellular cell wall stimulated culture supernatants from all 10 animals had significant amounts of IFN-γ, while none of the soluble sub-cellular cell wall stimulated culture supernatants from the 10 animals had any detectable amounts of IFN-γ (Figure 5.5). This experiment was repeated with 5 additional healthy cows and the result was identical (Figure 5.6). Purified monocytes alone were also tested to ensure that there were no contaminating T cells and significant amounts of IFN-γ were not produced (Data not shown). These results indicated that the

Figure 5.5. IFN-γ Production by γδ T Cells Following Stimulation with Mycobacterial Cell Wall



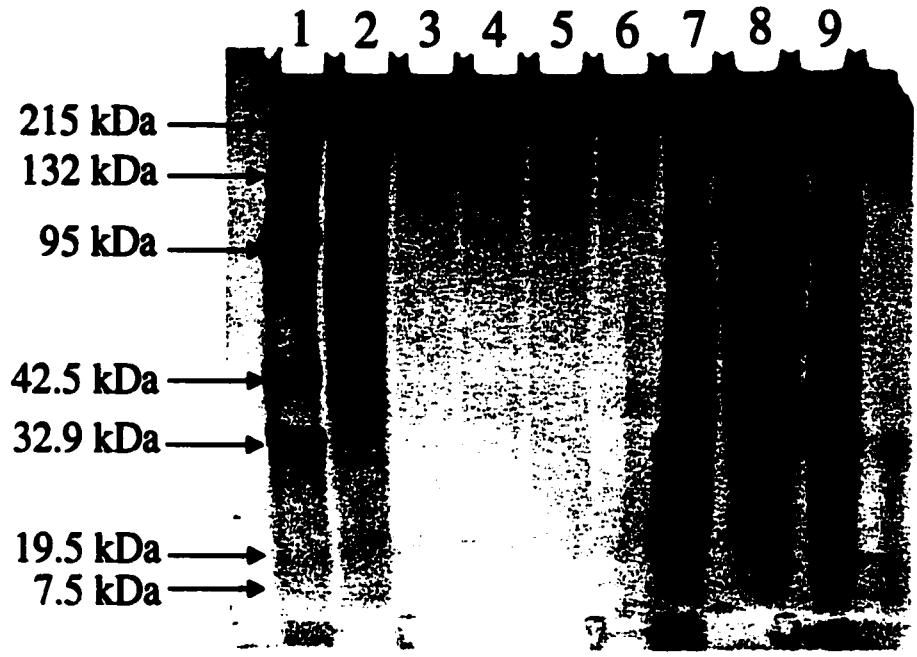
IFN-γ production from γδ T cells overlayed onto monocytes and stimulated with mycobacterial and control antigens. Data are presented as mean OD values for triplicate wells ± SEM. Significant IFN-γ production was determined by the assay cutoff ( )

Figure 5.6. IFN-γ Production by γδ T Cells Following Stimulation with Mycobacterial Cell Wall



IFN-γ production from γδ T cells overlayed onto monocytes and stimulated with mycobacterial and control antigens. Data are presented as mean OD values for triplicate wells ± SEM. Significant IFN-γ production was determined by the assay cutoff ( ).

Figure 5.7. SDS Polyacrylamide Gel of *M. bovis* Cell Wall



1. 7. &9: Kaleidoscope Protein Standards

2: *M. bovis* 863422 crude cell wall

3: *M. bovis* 862422 crude cell wall digest (50 ug proteinase K)

4: *M. bovis* 862422 crude cell wall digest (100 ug proteinase K)

5: *M. bovis* 862422 crude cell wall digest (200 ug proteinase K)

6: *M. bovis* 862422 crude cell wall digest (1000 ug proteinase K)

8: *M. bovis* 862422 soluble cell wall

10 µg of each sample was loaded into the wells of a 4-20% gradient polyacrylamide gel. The gel was run at 120 volts for 1 hour and 15 minutes and silver stained.

IFN-γ stimulatory component of the *M. bovis* sub-cellular cell wall was not likely a protein.

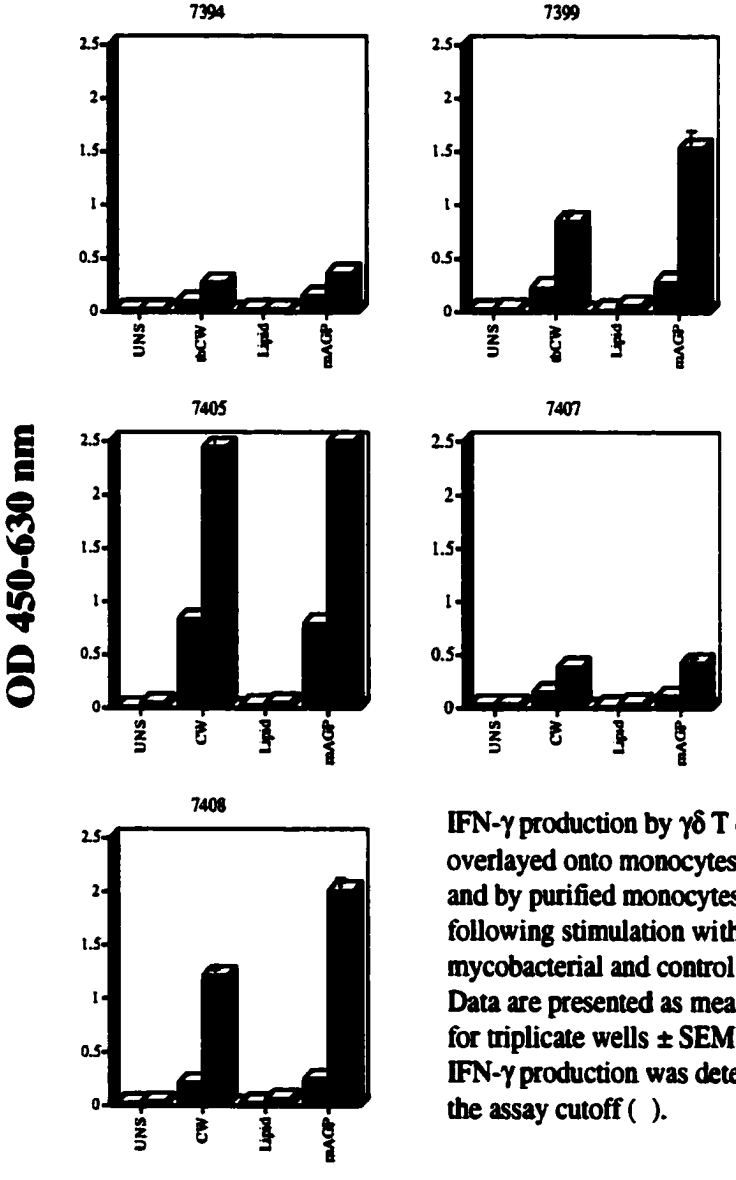
Bovine γδ T Cells Produce IFN-γ Following Stimulation with mAGP but not Total Lipid Extract from *M. tuberculosis*

Given that bovine γδ T cells produced significant IFN-γ following stimulation with proteolytically digested sub-cellular cell wall, the ability of the total lipid extract from *M. tuberculosis* and mycolylarabinogalactan peptidoglycan (mAGP) to stimulate the production of IFN-γ by γδ T cells was determined. γδ T cells and monocytes were purified from the peripheral blood of 5 healthy cows and stimulated with IL-2 and *M. tuberculosis* total lipid extract or IL-2 and mAGP. Significant IFN-γ responses were present in the mAGP stimulated macrophage alone culture supernatants but not in the *M. tuberculosis* total lipid stimulated culture supernatants (Figure 5.8). Purified monocytes alone were also tested to ensure that there were no contaminating T cells. Although in some of the mAGP stimulated culture supernatants there were significant amounts of IFN-γ, the levels were much lower when compared to the levels found in γδ and monocyte mAGP stimulated culture supernatants (Figure 5.8). These results indicate that bovine γδ T cells, when stimulated by mAGP, are capable of producing significant amounts of IFN-γ when cultured with IL-2 and monocytes.

Cell Contact is Necessary for IFN-γ Production by Bovine γδ T Cells

To determine whether direct monocyte γδ T cell contact was necessary for the production of IFN-γ, a Transwell system was used (Costar, Cambridge MA). γδ T cells and monocytes were purified from the peripheral blood of 10 healthy cows. The purified monocytes were cultured in the lower compartment of the Transwell and the

Figure 5.8. IFN-γ Production by γδ T Cells Following Stimulation with Mycobacterial Cell Wall Components



IFN-γ production by γδ T cells overlayed onto monocytes (blue bars) and by purified monocytes (grey bars) following stimulation with mycobacterial and control antigens. Data are presented as mean OD values for triplicate wells ± SEM. Significant IFN-γ production was determined by the assay cutoff ( ).

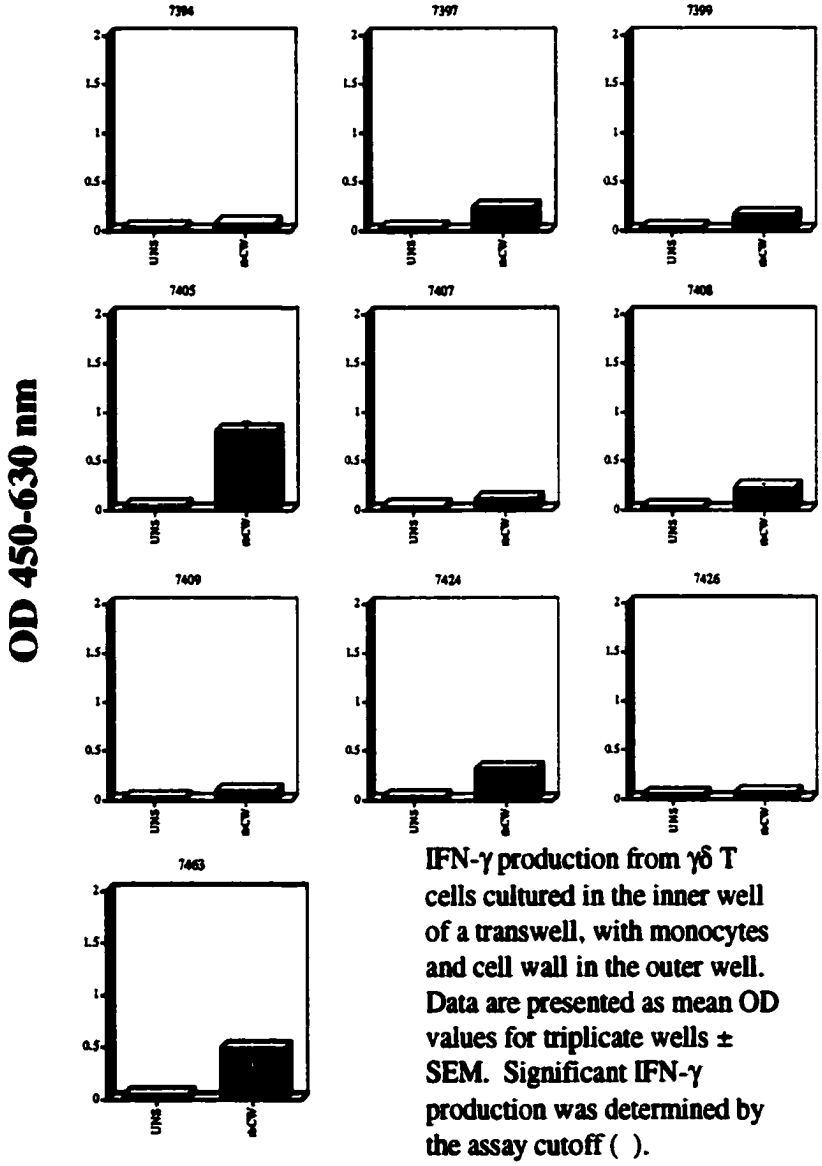
Antigens

purified γδ T cells were cultured in the upper compartment of the Transwell. A 3.0 μm microporous membrane filter separates the two compartments, allowing media and secreted components of the cells to freely move between the compartments, whilst preventing direct cell to cell contact of between the monocytes and the γδ T cells. *M. tuberculosis* sub-cellular cell wall was added to the lower compartment. IFN-γ was not detected in the sub-cellular cell wall stimulated culture supernatants in 5 out of the 10 animals (Figure 5.9). Significant levels of IFN-γ were detected in the sub-cellular cell wall stimulated culture supernatants of the other 5 animals (Figure 5.9). The significant levels of IFN-γ produced by 5 out of 10 animals were 2-3 fold less than the levels produced when cell to cell contact was not prevented (Data not shown). In addition, the monocyte populations for those cultures that produced IFN-γ were less pure than normal and were contaminated by γδ T cells (Data not shown). These results indicate, although not conclusively, that γδ T cell and monocyte cell contact is necessary for the production of IFN-γ.

Intracellular IFN-γ Detection in Bovine γδ T Cells

Detecting the presence of IFN-γ within a cell by flow cytometry eliminates the need to purify the γδ T cells and the monocytes from the peripheral blood, which can be tedious and sometimes result in cell populations that are not pure. PMBCs from 5 healthy cows were cultured with PBS, ConA, *M. tuberculosis* cell wall, *M. bovis* CFP, *M. a. paratuberculosis* CFP, *M. tuberculosis* ManLAM, *M. tuberculosis* AraLAM, IPP, live *M. bovis*, and live *M. a. paratuberculosis* for 24 and 48 hours. The addition of monensin to the culture 8 hours before analysis allows the intracellular accumulation of IFN-γ. γδ T cells were identified by flow cytometry and the percentage of γδ T cells that contained

Figure 5.9. IFN-γ Production by γδ T Cells in a Transwell System



IFN-γ production from γδ T cells cultured in the inner well of a transwell, with monocytes and cell wall in the outer well. Data are presented as mean OD values for triplicate wells ± SEM. Significant IFN-γ production was determined by the assay cutoff ( ).

intracellular IFN-γ was determined (Figure 5.10). Table 5.1 summarizes the percent increase in IFN-γ producing γδ T cells over the PBS stimulated cells for each of the antigens for each animal. We were unable to detect one antigen that consistently increased the intracellular IFN-γ detected within the γδ T cells (Table 5.1). For example, at the 48 hour timepoint, *M. tuberculosis* cell wall had virtually no effect on the IFN-γ accumulation within the γδ T cells of 4 animals but in one animal stimulation increased the intracellular IFN-γ by almost 25% (Table 5.1).

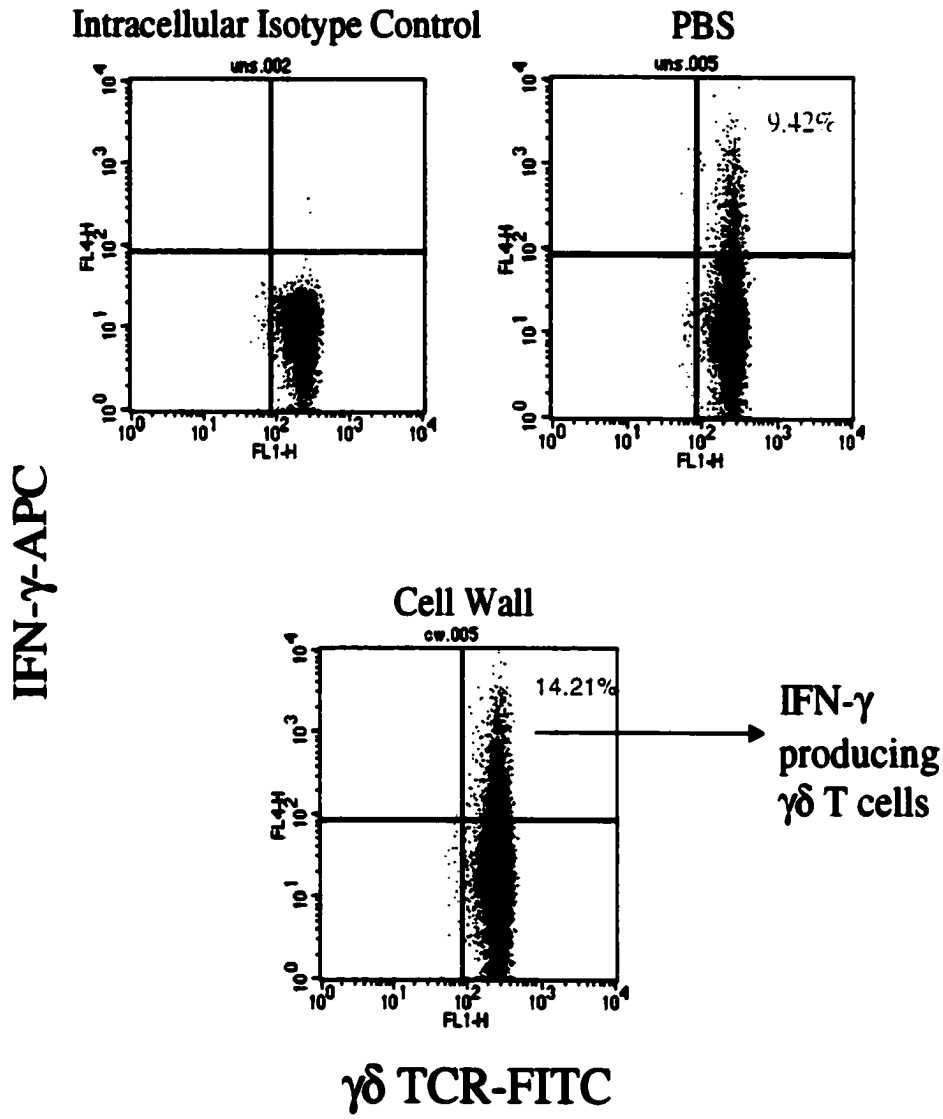
Real Time PCR Analysis of Bovine Cytokine Expression in γδ T Cells

Real time PCR was used to assess the effects of mycobacterial antigens on cytokine expression of bovine γδ T cells. In vitro transcribed RNA was made for bovine IFN-γ, TNF-α, MCP-1, IL-4, and IL-12 to generate standard curves. The in vitro transcribed RNA was reverse transcribed and amplified using bovine cytokine specific TaqMan primers and FAM labeled probes. The standard curves were generated by plotting the log nanograms of RNA versus the C_T values (Figures 5.11A-E). PBMCs were stimulated with ConA, *M. tuberculosis* cell wall, *M. bovis* CFP, *M. a. paratuberculosis* CFP, live *M. bovis*, and live *M. a. paratuberculosis* for 48 hours. Real time PCR analysis of the expression of bovine cytokines revealed no patterns of expression in response to stimulation with mycobacterial antigens (Data not shown). Some samples had very large increases in the amount cytokine specific RNA but it was not consistent between animals (Data not shown).

Discussion

This study provided useful information regarding the antigen recognition and function of bovine γδ T cells. Live mycobacteria, mycobacterial cell wall, and *M. bovis*

Figure 5.10. Intracellular IFN-γ Staining of γδ T Cells



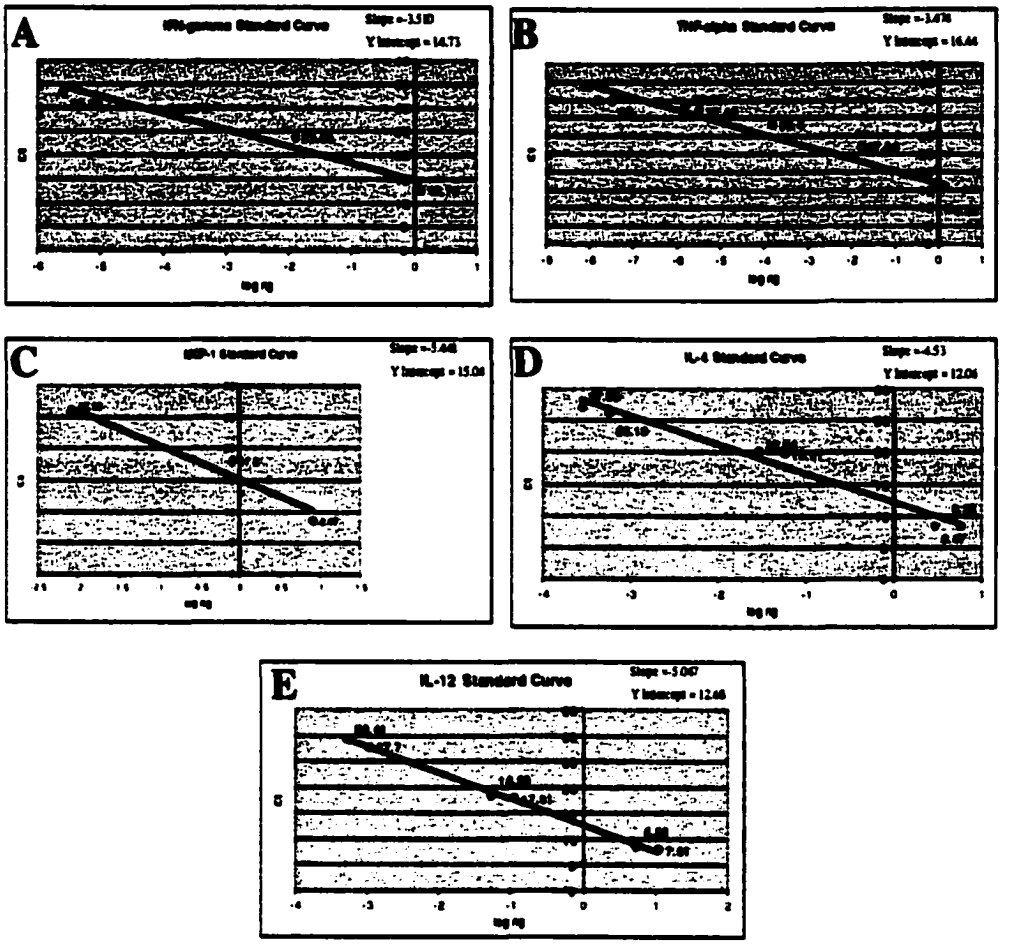
Intracellular IFN-γ staining in bovine γδ T cells. The percentage of IFN-γ positive γδ T cells was determined using CELLQuest software.

Table 5.1. Percent Increase of IFN-γ Positve γδ T Cells

Animal #	Antigen	24 hour % change from UNS	48 hour % change from UNS
244	conA	-1.05	-3.48
	cell wall	-3.38	-2.48
	bovis CFP	-0.27	-2.77
	para CFP	NA	NA
	manLAM	-2.34	-1.62
	araLAM	2.65	-2.21
	IPP	0	-2.67
	M. b ovis	0.52	-2.1
	M. para	-0.68	-2.22
245	conA	3.44	1.03
	cell wall	4.79	1.53
	bovis CFP	1.19	2.51
	para CFP	2.17	1.17
	manLAM	0.68	1.83
	araLAM	1.51	1.21
	IPP	1.69	1.89
	M. b ovis	5.69	1.3
	M. para	2.37	1.57
246	conA	2.57	-3.93
	cell wall	5.03	5.17
	bovis CFP	4.93	-0.5
	para CFP	5.75	0.83
	manLAM	6.13	3.5
	araLAM	3.86	6.27
	IPP	6.49	0.24
	M. b ovis	13.26	1.03
	M. para	7.15	0.21
247	conA	1.69	-2.65
	cell wall	2.44	0.66
	bovis CFP	2.66	0.31
	para CFP	4.41	0.83
	manLAM	6.12	1.39
	araLAM	3.89	2.81
	IPP	4.21	0.76
	M. b ovis	3.12	-1.26
	M. para	2.46	1.77
248	conA	-1.34	2.85
	cell wall	-1.56	24.71
	bovis CFP	2.59	4.25
	para CFP	2.43	16.46
	manLAM	2.45	2.77
	araLAM	1.13	1.61
	IPP	3.53	14.99
	M. b ovis	0.2	8.23
	M. para	1.04	6.77

Percent increase in intracellular IFN-γ detection over the unstimulated control for 5 healthy cows.

Figure 5.11. Real-Time PCR Standard Curves



Real-time PCR standard curves for bovine IFN-γ (A), TNF-α (B), MCP-1 (C), IL-4 (D), and IL-12 (E). Standard curves were generated by reverse transcribing dilutions of in vitro transcribed RNA and PCR amplifying with TaqMan primers and probes.

CFP significantly stimulated the in vitro expansion of bovine γδ T cells. In addition, the mAGP fraction of the *M. tuberculosis* cell wall was identified as a potent stimulus for the production of IFN-γ by γδ T cells. Moreover, monocyte and γδ T cell contact was shown to be a requirement for IFN-γ production.

The in vitro expansion of T cell populations in response to antigen is a useful way to identify potential antigens that are recognized by T cells. This study demonstrated that both live mycobacteria and sub-cellular fractions of mycobacteria, namely cell wall and CFP, were capable of inducing the in vitro expansion of bovine γδ T cells. This indicates that mycobacteria and mycobacterial components are potential antigens for bovine γδ T cells. The in vitro expansion of γδ T cells could also be explained by the in vitro death of another cell population. This could be addressed by using propidium iodide to determine the percentages of dead cells. Interestingly, there was no significant expansion following stimulation with either ManLAM or IPP. The lack of a response from the IPP stimulated cells was somewhat surprising since IPP has been identified as a human γδ T cell antigen (203, 283). The human γδ T cells that recognize IPP have a specific Vγ and Vδ gene segment, Vγ2Vδ2 (80, 232, 258, 284). Bovine γδ T cells may not have the specific gene segments that recognize IPP.

The ability to produce IFN-γ during a mycobacterial infection is essential to an effective host response (82, 110). This study demonstrated that γδ T cells, isolated from the peripheral blood of healthy cows, were able to produce significant amounts of IFN-γ following stimulation with mycobacterial cell wall (from both *M. tuberculosis* and *M. bovis*). In addition, bovine γδ T cells produced equal, if not greater, amounts of IFN-γ following stimulation with proteolytically digested mycobacterial cell wall. To confirm

that the immunostimulatory component of the cell wall was not likely a protein, bovine γδ T cells were stimulated with a SDS-soluble cell wall extract and no IFN-γ was produced. This suggests that a non-protein component of the cell wall induced the IFN-γ production. We cannot however, rule out a protein that is tightly associated and protected by the cell wall as the immunostimulatory component. Such a protein would likely be resistant to proteolytic digestion and may not be released from the cell wall following SDS extraction.

Since a protein component of the cell wall could not easily be identified as the immunostimulatory component, the total lipid extract and mAGP from *M. tuberculosis* was also tested for its ability to induce IFN-γ production by bovine γδ T cells. No significant amount of IFN-γ was produced following stimulation with the total lipid extract but the mAGP fraction did induce IFN-γ production. Although the individual components of mAGP (mycolic acids, arabinogalactan, and peptidoglycan) (32) were not tested, the peptidoglycan is the likely component that induced IFN-γ production by bovine γδ T cells. Mycolic acids would have been present in the total lipid extract of *M. tuberculosis* and since this fraction did not induce IFN-γ production, they are not likely the IFN-γ inducing component. Arabinogalactan has been demonstrated to be an immunosuppressor and is thus not likely to induce IFN-γ production by bovine γδ T cells (165, 202, 214). Peptidoglycan and or mAGP has been shown to induce the production of inflammatory cytokines (190, 213, 291, 322). Moreover, mAGP isolated from *M. tuberculosis* was shown to induce the production of TNF-α and this was dependent upon toll-like receptor-2 expression (298). It is therefore likely that the peptidoglycan component of mAGP induces the production and secretion of TNF-α, IL-1, and IL-12 by

bovine monocytes which then acts on the bovine γδ T cells to induce the production of innate IFN-γ. Further studies would be necessary to confirm this hypothesis.

This study also demonstrated that contact between monocytes and γδ T cells was necessary for IFN-γ production by bovine γδ T cells. Separating the two populations of cells completely ablated or significantly reduced the amount of IFN-γ produced by bovine γδ T cells. T cell and monocyte contact has been identified as essential for the production of TNF-α by *M. leprae* stimulated monocytes (250). Sampaio et al also demonstrated that the production of TNF-α was modulated by blocking antibodies to CD40L, CD69, and CD18 (250), which suggests that these cell surface molecules and their ligands may be crucial to this interaction.

Intracellular IFN-γ assays and real-time TaqMan PCR assays were also developed for bovine γδ T cells in this study. Unfortunately the results from both assays were very inconsistent and variable between the animals in the study. Bovine γδ T cells produce IFN-γ following stimulation with mycobacterial cell wall, as detected by IFN-γ ELISA. In contrast, intracellular IFN-γ was only detected in 2 out of the 5 animals after stimulation with mycobacterial cell wall. The protocol for intracellular staining (Pharmingen) suggests using antibodies directed against CD3 and or CD28 to stimulate cytokine production. This may be a way of enhancing the detection of intracellular IFN-γ. The real-time PCR assay worked when the in vitro transcribed RNA standards were used. Unfortunately, the RNA samples from the in vitro stimulated γδ T cells did not give results that were consistent between the animals that were tested. The inconsistent results could be attributed to insufficient quantities of cytokine specific RNA and degradation of RNA during isolation.

It is apparent from bovine mycobacterial vaccine trials (Chapters 2 and 3) that an adjuvant that strongly induces the production of IFN-γ by innate immune cells is needed. This study demonstrates that bovine γδ T cells, which make up a large percentage of circulating T cells, are capable of producing large quantities of innate IFN-γ when stimulated by the mAGP fraction of the mycobacterial cell wall. It may be useful to use the mAGP fraction of the mycobacterial cell wall as one of the adjuvant components of future CFP based bovine mycobacterial vaccines.

Acknowledgments

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CHAPTER 6

General Conclusions

Bovine tuberculosis is a serious threat to wildlife and to farmed animals, specifically cattle and deer. Currently there are no vaccines for bovine tuberculosis. The *M. bovis* BCG vaccine has been used experimentally and offers some protection against experimental challenge with virulent *M. bovis* (53, 54). The vaccine has not been used as a preventative measure for bovine tuberculosis because once vaccinated, animals cannot be distinguished from infected animals using the diagnostic test for bovine tuberculosis. A vaccine for bovine tuberculosis that offers some protection or provides a reduction in the severity of the disease without disabling the diagnostic test is desperately needed.

This study described the development and testing of a subunit vaccine for bovine tuberculosis in the natural host, cattle. The subunit vaccine was composed of CFP from virulent *M. bovis*, recombinant bovine IL-2, and MPL. Despite the fact that the CFP vaccine failed to induce a significant post-vaccination IFN-γ response to bovine PPD, it significantly reduced the lung lesion severity following challenge. Moreover, vaccinating animals with the CFP vaccine did not induce a DTH response to bovine PPD. This demonstrates that a subunit vaccine for bovine tuberculosis is capable of providing some protection without compromising the diagnostic test. Unfortunately, this vaccine also led to the increased extrathoracic spread of *M. bovis*. The working hypothesis to explain the increased dissemination is that the CFP vaccine induced a strong humoral response that led to the increased opsonization of *M. bovis* and the subsequent spread to extrathoracic lymph nodes. In the past few years several investigators have reevaluated the roles of B cells and antibodies in mycobacterial infections (41, 121, 155, 286), some even suggesting a role in protection (41, 286). The data from the CFP vaccine trial seems to suggest that although a significant amount of protection was seen concurrently with

strong humoral responses, the overall outcome of a strong antibody response is detrimental. As a whole, the results of the CFP vaccine are encouraging. The design of future CFP vaccines for bovine tuberculosis must include an adjuvant that will strongly induce post-vaccination cell mediated immune responses in cattle.

Johne's disease is another bovine mycobacterial disease that is a serious economic threat to the agriculture industry. Here in the United States, some areas have reported that up to 74% of their dairy herds have infected animals (289). Annually this disease costs the US between 200 million and 1.5 billion dollars (69, 226). Animals are infected shortly after birth and develop clinical signs of disease 3-5 years later (170, 279). Animals shed *M. a. paratuberculosis* in their feces up to 18 months prior to the onset of clinical symptoms. Because there is no vaccine for Johne's disease and because it is very difficult to diagnose animals in the pre-clinical stage, there is ample opportunity for shedding sub-clinical animals to infect young animals in the herd. A vaccine for Johne's disease would have an immense impact on the control of this disease.

This study described the development and testing of a subunit vaccine for Johne's disease. The vaccine components were similar to the components of the bovine tuberculosis subunit vaccine. *M. a. paratuberculosis* CFP, recombinant bovine IL-2, CpG motifs, and DDA were included in the vaccine formulation. Due to the chronic nature of this disease the assessment of the protective efficacy of this vaccine is ongoing and will not be concluded for several months. Preliminary data suggests that the CFP vaccine induced a higher percentage of $\alpha\beta$ and $\gamma\delta$ T cells with activated phenotypes, as compared to the adjuvant vaccine. It is therefore possible that a more vigorous cell mediated immune response was induced. Whether this will actually translate into

protection will be conclusively determined at the end of this study. As with the bovine tuberculosis vaccine, the CFP vaccine for Johne's disease failed to induce significant post-vaccination IFN-γ following stimulation with *M. a. paratuberculosis* CFP. Future CFP vaccines must contain an adjuvant that is capable of inducing strong post-vaccination cellular responses.

Despite the fact that γδ T cells were discovered more than fifteen years ago, they are still somewhat of an enigma. Interestingly, γδ T cells have been demonstrated to be relevant to mycobacterial diseases (89, 153, 196, 231). Bovine γδ T cells have not been as well studied however, it is known that cattle have a very high percentage of peripheral cells that have the γδ T cell receptor (137).

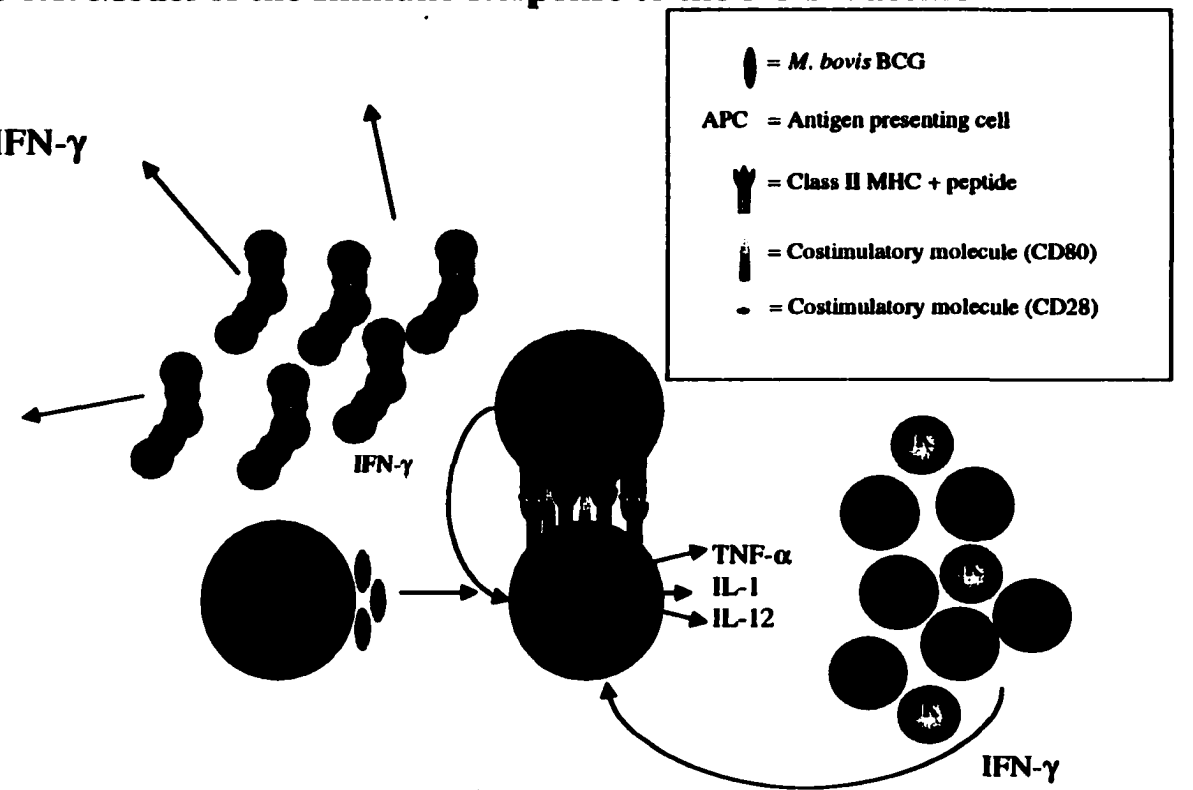
This study characterized the expression of activation and memory markers on bovine γδ T cells. Several phenotypic differences were identified between $CD2^+$ and $CD2^-$ γδ T cells. Moreover CD44, CD45R, and CD62L were identified as useful markers of activation on both $CD2^+$ and $CD2^-$ γδ T cells. These markers will be useful when investigating the activation of bovine γδ T cells during the course of disease or following vaccination.

Given that γδ T cells are such a prominent cell population in the cow, their ability to respond to stimulation with mycobacterial antigens was investigated. γδ T cells were shown to expand in response to stimulation with both live mycobacteria and to mycobacterial sub-cellular fractions. Interestingly there was no in vitro expansion of γδ T cells following stimulation with IPP, which has been identified as a human γδ T cell antigen. Moreover, we identified that the mAGP fraction of the mycobacterial cell wall induced bovine γδ T cell mediated production of IFN-γ.

The knowledge gained from the work described herein can be applied to the development of more effective bovine mycobacterial vaccines. Figures 6.0 and 6.1 describe a possible model to explain why the BCG vaccine protects better than the CFP vaccine for bovine tuberculosis. *M. bovis* BCG is taken up into antigen presenting cells, which then secrete TNF-α, IL-1, and IL-12. These cytokines act on various populations of immune cells to stimulate the production of IFN-γ by innate cells such as γδ T cells and NK cells. This study demonstrated that bovine γδ T cells produced IFN-γ when stimulated with mycobacterial cell wall and since the cell wall is a component of the BCG vaccine, IFN-γ production by bovine γδ T cells can be expected. The innate IFN-γ then acts on the antigen presenting cells, causing the induction of anti-microbial effects as well as increases in MHC Class II expression and costimulatory molecule expression. This enhances the antigen presentation of mycobacterial components to $CD4^+$ T cells. $CD4^+$ T cells that are capable of recognizing the peptides being presented by the antigen presenting cells can now become effector cells and secrete IFN-γ. Due to the large number of mycobacterial peptides that can be processed and presented from an entire bacterium, the BCG vaccine likely induces a very large effector $CD4^+$ T cell repertoire. A sufficient number of $CD4^+$ T cells with diverse T cell receptors will persist and become memory cells, capable of responding and producing IFN-γ quickly upon challenge with virulent *M. bovis*.

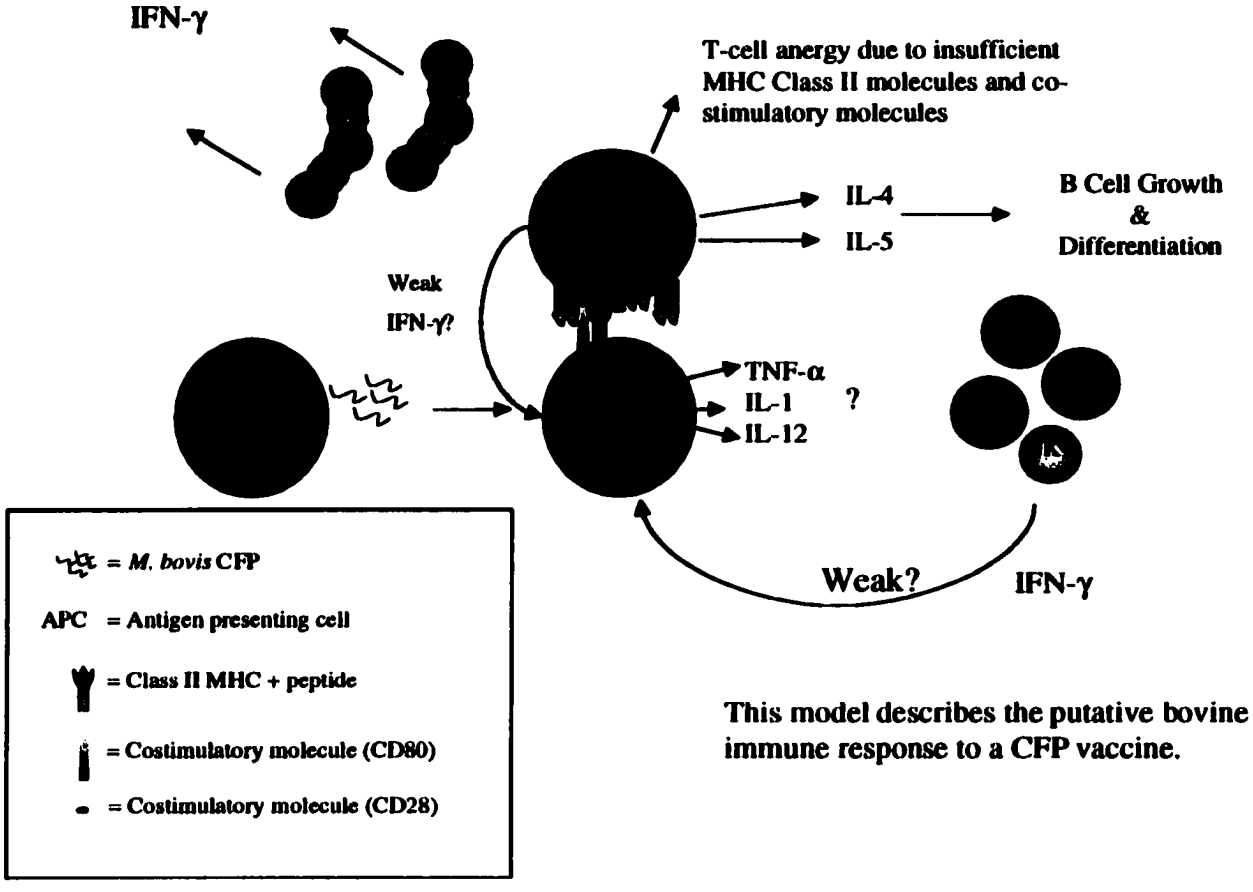
Figure 6.1 is a putative model for what is happening at the cellular level following vaccination with a CFP vaccine. At least a portion of the CFP vaccine is being taken up by the antigen presenting cells. Because one of the components of the CFP vaccine is an adjuvant (MPL), the antigen presenting cell should then secrete TNF-α, IL-1, and IL-12.

Figure 6.1. Model of the Immune Response to the BCG Vaccine



This model describes the putative bovine immune response to vaccination with *M. bovis* BCG.

Figure 6.2. Model of the Immune Response to the CFP Vaccine



This model describes the putative bovine immune response to a CFP vaccine.

It is possible that the cytokine secretion from antigen presenting cells that have taken up the CFP vaccine will not be as strong as the secretion following vaccination with the BCG vaccine. A weaker cytokine response from the antigen presenting cells could then lead to the recruitment of fewer innate cells such as γδ T cells and NK cells. In addition, we have shown that bovine γδ T cells do not produce significant amounts of IFN-γ in response to CFP, which suggests that the subsequent innate IFN-γ response will be weaker than the IFN-γ response following vaccination with BCG. A weaker innate IFN-γ response could lead to insufficient antigen presentation to $CD4^+$ T cells, resulting in a weaker specific IFN-γ response. Moreover, an insufficient IFN-γ response may cause inadequate upregulation of MHC class II and costimulatory molecules. This could then lead to the induction of anergy in $CD4^+$ T cell populations that are capable of responding to the CFP vaccine. This, taken together with the fact that the $CD4^+$ T cell repertoire that can respond to the CFP vaccine is more limited than the repertoire that can respond to the BCG vaccine, could explain the inadequate memory response seen following challenge. Moreover, the vaccine study demonstrated that the CFP vaccine induced a strong CFP specific antibody response. Perhaps as a consequence of the weak IFN-γ response, responding $CD4^+$ T cells are producing IL-4 and IL-5 and skewing the immune response to a more TH2 dominated response.

One possible solution to the less than sufficient post-vaccination IFN-γ responses seen with both the bovine tuberculosis and Johne's vaccines is a stronger adjuvant. There is precedence for adjuvants working well in small animal models but not in cattle (personal communication – Dr. Bryce Buddle). Given that this study demonstrated the strong induction of IFN-γ by mycobacterial cell wall stimulated bovine γδ T cells, I

propose that future CFP vaccines include at least a component of the mycobacterial cell wall as an adjuvant. It is likely that this strong adjuvant will help to skew the acquired response from a TH2 dominated to a TH1 dominated response.

References

1. 1986. Bergey's Manual of Systematic Bacteriology. Volume 2. Williams & Wilkins, Baltimore.

2. April 2000, posting date. Tuberculosis Fact Sheet. WHO. [Online.]

3. **Adams, J. L., M. T. Collins, and C. J. Czuprynski.** 1996. Polymerase chain reaction analysis of TNF-alpha and IL-6 mRNA levels in whole blood from cattle naturally or experimentally infected with *Mycobacterium paratuberculosis.* Canadian Journal of Veterinary Research. **60:**257-262.

4. **Adams, L. B., M. C. Dinauer, D. E. Morgenstern, and J. L. Krahenbuhl.** 1997. Comparison of the roles of reactive oxygen and nitrogen intermediates in the host response to *Mycobacterium tuberculosis* using transgenic mice. Tubercle and Lung Disease. **78:**237-246.

5. **Albertson, D. G., R. Fishpool, P. Sherrington, E. Nacheva, and C. Milstein.** 1988. Sensitive and high resolution in situ hybridization to human chromosomes using biotin labeled probes: assignment of the human thymocyte CD1 antigen genes to chromosome 1. EMBO Journal. **7:**2801-2805.

6. **Aldwell, F. E., D. L. Keen, V. C. Stent, A. Thompson, G. F. Yates, G. W. de Lisle, and B. M. Buddle.** 1995. Route of BCG administration in possums affects protection against bovine tuberculosis. New Zealand Veterinary Journal. **43:**356-359.

7. **Aldwell, F. E., A. Pfeffer, G. W. de Lisle, G. Jowett, J. M. Heslop, D. L. Keen, A. E. Thompson, and B. M. Buddle.** 1995. Effectiveness of BCG vaccination in protecting possums against bovine tuberculosis. Research in Veterinary Science. **58:**90-95.

8. **Aldwell, F. E., D. N. Wedlock, and B. M. Buddle.** 1996. Bacterial metabolism, cytokine mRNA transcription and viability of bovine alveolar macrophages infected with *Mycobacterium bovis* BCG or virulent *M. bovis*. Immunology and Cell Biology. **74:**45-51.

9. **Allison, J. P.** 1993. Gamma delta T-cell development. Current Opinions in Immunology. **5:**241-246.

10. **Andersen, P.** 1994. Effective vaccination of mice against *Mycobacterium tuberculosis* infection with a soluble mixture of secreted mycobacterial proteins. Infection and Immunity. **62:**2536-2544.

11. **Andersen, P., D. Askgaard, A. Gottschau, J. Bennedsen, S. Nagai, and I. Heron.** 1992. Identification of Immunodominant Antigens during Infection with *Mycobacterium tuberculosis*. Scandinavian Journal of Immunology. **36**:823-831.

12. **Andersen, P., D. Askgaard, L. Ljungqvist, M. W. Bentzon, and I. Heron.** 1991. T-cell proliferative response to antigens secreted by *Mycobacterium tuberculosis*. Infection Immunity. **59**:1558-1563.

13. **Andersen, P., and B. Smedegaard.** 2000. CD4(+) T-cell subsets that mediate immunological memory to *Mycobacterium tuberculosis* infection in mice. Infection and Immunity. **68**:621-629.

14. **Apostolou, I., Y. Takahama, C. Belmant, T. Kawano, M. Huerre, G. Marchal, J. Cui, M. Taniguchi, H. Nakauchi, J.-J. Fournie, P. Kourilsky, and G. Gachelin.** 1999. Murine natural killer cells contribute to the granulomatous reaction caused by mycobacterial cell walls. Proceedings of the National Academy of Sciences of the United States of America. **96**:5141-5146.

15. **Arbones, M. L., D. C. Ord, K. Ley, H. Ratech, C. Maynard-Curry, G. Otten, D. J. Capon, and T. F. Tedder.** 1994. Lymphocyte homing and leukocyte rolling and migration are impaired in L- selectin-deficient mice. Immunity. **1**:247-260.

16. **Aruffo, A., I. Stamenkovic, M. Melnick, C. B. Underhill, and B. Seed.** 1990. CD44 is the principal cell surface receptor for hyaluronate. Cell. **61**:1303-1313.

17. **Asarnow, D., W. Kuziel, M. Bonyhadi, R. Tigelaar, P. Tucker, and J. Allison.** 1988. Limited Diversity of γ/δ Antigen Receptor Genes of Thy-1+ Dendritic Epidermal Cells. Cell. **55**:837-847.

18. **Astarie-Dequeker, C., E. N. N'Diaye, V. Le Cabec, M. G. Rittig, J. Prandi, and I. Maridonneau-Parini.** 1999. The mannose receptor mediates uptake of pathogenic and nonpathogenic mycobacteria and bypasses bactericidal responses in human macrophages. Infection and Immunity. **67**:469-477.

19. **Attanasio, R., K. Pehler, and H. M. McClure.** 2000. Immunogenicity and safety of *Mycobacterium tuberculosis* culture filtrate proteins in non-human primates. Clinical and Experimental Immunology. **119**:84-91.

20. **Ayoub, I. A., and T. J. Yang.** 1996. Age-dependent changes in peripheral blood lymphocyte subpopulations in cattle: a longitudinal study. Developmental and Comparative Immunology. **20**:353-363.

21. **Baldwin, S. L., C. D'Souza, A. D. Roberts, B. P. Kelly, A. A. Frank, M. A. Lui, J. B. Ulmer, K. Huygen, D. M. McMurray, and I. M. Orme.** 1998. Evaluation of new vaccines in the mouse and guinea pig model of tuberculosis. Infection and Immunity. **66**:2951-2959.

22. **Baldwin, S. L., C. D. D'Souza, I. M. Orme, M. A. Liu, K. Huygen, O. Denis, A. Tang, L. Zhu, D. Montgomery, and J. B. Ulmer.** 1999. Immunogenicity and protective efficacy of DNA vaccines encoding secreted and non-secreted forms of *Mycobacterium tuberculosis* Ag85A. Tubercle and Lung Disease. **79:**251-259.

23. **Barlow, N. D.** 1994. Bovine tuberculosis in New Zealand: epidemiology and models. Trends in Microbiology. **2:**119-124.

24. **Barnes, P. F., A. B. Bloch, P. T. Davidson, and D. E. Snider, Jr.** 1991. Tuberculosis in patients with human immunodeficiency virus infection. New England Journal of Medicine. **324:**1644-1650.

25. **Barnes, P. F., D. Chatterjee, J. S. Abrams, S. Lu, E. Wang, M. Yamamura, P. J. Brennan, and R. L. Modlin.** 1992. Cytokine production induced by *Mycobacterium tuberculosis* lipoarabinomannan. Relationship to chemical structure. Journal of Immunology. **149:**541-547.

26. **Bassey, E. O., and M. T. Collins.** 1997. Study of T-lymphocyte subsets of healthy and *Mycobacterium avium* subsp. *paratuberculosis*-infected cattle. Infection and Immunity. **65:**4869-4872.

27. **Bean, A. G., D. R. Roach, H. Briscoe, M. P. France, H. Korner, J. D. Sedgwick, and W. J. Britton.** 1999. Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol *Mycobacterium tuberculosis* infection, which is not compensated for by lymphotoxin. Journal of Immunology. **162:**3504-3511.

28. **Beckman, E. M., S. A. Porcelli, C. T. Morita, S. M. Behar, S. T. Furlong, and M. B. Brenner.** 1994. Recognition of a lipid antigen by CD1-restricted αβ+ T cells. Nature. **372:**691-694.

29. **Behar, S. M., C. C. Dascher, M. J. Grusby, C.-R. Wang, and M. B. Brenner.** 1999. Susceptibility of mice deficient in CD1D or TAP1 to infection with *Mycobacterium tuberculosis.* Journal of Experimental Medicine. **189:**1973-1980.

30. **Bendelac, A., M. N. Rivera, S.-H. Park, and J. H. Roark.** 1997. Mouse CD1-Specific NK1 T Cells: Development, Specificity, and Function. Annual Reviews in Immunology. **15:**535-562.

31. **Berggren, S. A.** 1981. Field Experiment with BCG vaccine in Malawi. British Veterinary Journal. **137:**88-96.

32. **Besra, G. S.** 1998. Preparation of cell-wall fractions from mycobacteria. Methods in Molecular Biology. **101:**91-107.

33. **Bishop, J. M. G. a. P. J.** 1982. A tribute to Robert Koch's discovery of the tubercle bacillus, 1882. Tubercle and Lung Disease. **63:**3-17.

34. **Black, A. C.** 2000. Delayed Type Hypersensitivity: Current Theories with an Historic Perspective. Dermatology Online Journal. **5:**1-11.

35. **Bluestone, J., R. Cron, T. Barrett, B. Houlden, A. Sperling, A. Dent, S. Hedrick, B. Rellahan, and L. Matis.** 1991. Repertoire Development and Ligand Specificity of Murine TCR γδ T Cells. Immunological Reviews. **129:**5-33.

36. **Boismenu, R., L. Feng, Y. Y. Xia, J. C. Chang, and W. L. Havran.** 1996. Chemokine expression by intraepithelial gamma delta T cells. Implications for the recruitment of inflammatory cells to damaged epithelia. Journal of Immunology. **157:**985-992.

37. **Boismenu, R., and W. L. Havran.** 1997. An innate view of gamma delta T cells. Current Opinions in Immunology. **9:**57-63.

38. **Boom, W. H., K. N. Balaji, R. Nayak, K. Tsukaguchi, and K. A. Chervenak.** 1994. Characterization of a 10- to 14-kilodalton protease-sensitive *Mycobacterium tuberculosis* H37Ra antigen that stimulates human gamma delta T cells. Infection and Immunity. **62:**5511-5518.

39. **Boom, W. H., R. S. Wallis, and K. A. Chervenak.** 1991. Human *Mycobacterium tuberculosis*-reactive CD4+ T-cell clones: heterogeneity in antigen recognition, cytokine production, and cytotoxicity for mononuclear phagocytes. Infection and Immunity. **59:**2737-2743.

40. **Born, W., L. Hall, A. Dallas, J. Boymel, T. Shinnick, D. Young, P. Brennan, and R. O'Brien.** 1990. Recognition of Peptide Antigen by Heat Shock-Reactive γδ T Lymphocytes. Science. **249:**67-69.

41. **Bosio, C. M., D. Gardner, and K. L. Elkins.** 2000. Infection of B cell-deficient mice with CDC 1551, a clinical isolate of *Mycobacterium tuberculosis*: delay in dissemination and development of lung pathology Journal of Immunology. **164:**6417-6425.

42. **Bosio, C. M., and I. M. Orme.** 1998. Effective, nonsensitizing vaccination with culture filtrate proteins against virulent *Mycobacterium bovis* infections in mice. Infection and Immunity. **66:**5048-5051.

43. **Bothamley, G. H., and J. M. Grange.** 1991. The Koch phenomenon and delayed hypersensitivity: 1891-1991 [editorial]. Tubercle and Lung Disease. **72:**7-11.

44. **Bradbury, A., K. T. Belt, T. M. Neri, C. Milstein, and F. Calabi.** 1988. Mouse CD1 is distinct from and co-exists with TL in the same thymus. EMBO Journal. **7:**3081-3086.

45. **Brandt, L., M. Elhay, I. Rosenkrands, E. B. Lindblad, and P. Andersen.** 2000. ESAT-6 Subunit Vaccination against *Mycobacterium tuberculosis.* Infection and Immunity. **68:**791-795.

46. **Brenner, M. B., J. McLean, D. P. Dialynas, J. L. Strominger, J. A. Smith, F. L. Owen, J. G. Seidman, S. Ip, F. Rosen, and M. S. Krangel.** 1986. Identification of a putative second T-cell receptor. Nature. **322:**145-149.

47. **Brock, T. D.** 1999. Robert Koch, A Life in Medicine and Bacteriology. American Society for Microbiology, Washington.

48. **Brown, W. C., W. C. Davis, S. H. Choi, D. A. Dobbelaere, and G. A. Splitter.** 1994. Functional and phenotypic characterization of WC1+ gamma/delta T cells isolated from *Babesia bovis*-stimulated T cell lines. Cellular Immunology. **153:**9-27.

49. **Bruning-Fann, C., M. Chaddock, T. Cooley, J. Fierke, P. Friedrich, J. Lipe, S. M. Schmitt, M. Vanderklok, and N. Zauel.** 1999. Northeast Michigan Surveillance Activities for Bovine Tuberculosis in the Livestock and Free-Ranging Deer Populations.

50. **Budd, R. C., J. C. Cerottini, C. Horvath, C. Bron, T. Pedrazzini, R. C. Howe, and H. R. MacDonald.** 1987. Distinction of virgin and memory T lymphocytes. Stable acquisition of the Pgp-1 glycoprotein concomitant with antigenic stimulation. Journal of Immunology. **138:**3120-3129.

51. **Buddle, B. M., F. E. Aldwell, D. L. Keen, N. A. Parlane, G. Yates, and G. W. de Lisle.** 1997. Intraduodenal vaccination of brushtail possums with bacille Calmette-Guerin enhances immune response and protection against *Mycobacterium bovis* infection. International Journal of Tubercle and Lung Disease. **1:**377-383.

52. **Buddle, B. M., F. E. Aldwell, A. Pfeffer, G. W. De Lisle, and L. A. Corner.** 1994. Experimental *Mycobacterium bovis* infection of cattle: effect of dose of *M. bovis* and pregnancy on immune responses and distribution of lesions. New Zealand Veterinary Journal. **42:**167-172.

53. **Buddle, B. M., G. W. de Lisle, A. Pfeffer, and F. E. Aldwell.** 1995. Immunological responses and protection against *Mycobacterium bovis* in calves vaccinated with low dose of BCG. Vaccine. **13:**1123-1130.

54. **Buddle, B. M., D. Keen, A. Thompson, G. Jowett, A. R. McCarthy, J. Heslop, G. W. De Lisle, J. L. Stanford, and F. E. Aldwell.** 1995. Protection of cattle from bovine tuberculosis by vaccination with BCG by the respiratory or subcutaneous route, but not by vaccination with killed *Mycobacterium vaccae.* Research in Veterinary Science. **59:**10-16.

55. **Buddle, B. M., M. A. Skinner, and M. A. Chambers.** 2000. Immunological approaches to the control of tuberculosis in wildlife reservoirs. Veterinary Immunology and Immunopathology. **74:**1-16.

56. **Burke, R. M.** 1955. An Historical Chronology of Tuberculosis., second ed. Charles C Thomas, Springfield.

57. **Burrells, C., C. J. Clarke, A. Colston, J. M. Kay, J. Porter, D. Little, and J. M. Sharp.** 1999. Interferon-gamma and interleukin-2 release by lymphocytes derived from the blood, mesenteric lymph nodes and intestines of normal sheep and those affected with paratuberculosis (Johne's disease). Veterinary Immunology and Immunopathology. **68:**139-148.

58. **Burrells, C., C. J. Clarke, A. Colston, J. M. Kay, J. Porter, D. Little, and J. M. Sharp.** 1998. A study of immunological responses of sheep clinically-affected with paratuberculosis (Johne's disease). The relationship of blood, mesenteric lymph node and intestinal lymphocyte responses to gross and microscopic pathology. Veterinary Immunology and Immunopathology. **66:**343-358.

59. **Calabi, F., and C. Milstein.** 1986. A novel family of human major histocompatibility complex-related genes not mapping to chromosome 6. Nature. **323:**540-543.

60. **Carswell, E. A., L. J. Old, R. L. Kassel, S. Green, N. Fiore, and B. Williamson.** 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proceedings of the National Academy of Sciences of the United States of America. **72:**3666-3670.

61. **Caruso, A. M., N. Serbina, E. Klein, K. Triebold, B. R. Bloom, and J. L. Flynn.** 1999. Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis. Journal of Immunology. **162:**5407-5416.

62. **Castano, A. R., S. Tangri, J. E. Miller, H. R. Holcombe, M. R. Jackson, W. D. Huse, M. Kronenberg, and P. E. Petersen.** 1995. Peptide binding and presentation by mouse CD1. Science. **269:**223-226.

63. **Chambers, M. A., H.-M. Vordermeier, A. Whalen, N. Commander, R. Tascon, D. Lowrie, and R. G. Hewinson.** 2000. Vaccination of Mice and Cattle with Plasmid DNA Encoding the *Mycobacterium bovis* Antigen MPB83. Clinical Infectious Diseases. **30:**S283-287.

64. **Chan, J., X. D. Fan, S. W. Hunter, P. J. Brennan, and B. R. Bloom.** 1991. Lipoarabinomannan, a possible virulence factor involved in persistence of *Mycobacterium tuberculosis* within macrophages. Infection and Immunity. **59:**1755-1761.

65. **Chan, J., Y. Xing, R. S. Magliozzo, and B. R. Bloom.** 1992. Killing of virulent *Mycobacterium tuberculosis* by reactive nitrogen intermediates produced by activated murine macrophages. Journal of Experimental Medicine. **175:**1111-1122.

66. **Chatterjee, D., K. Lowell, B. Rivoire, M. McNeil, and P. J. Brennan.** 1992. Lipoarabinomannan of *Mycobacterium tuberculosis.* Journal of Biological Chemistry. **267:**6234-6239.

67. **Chiodini, R. J., and W. C. Davis.** 1992. The cellular immunology of bovine paratuberculosis: the predominant response is mediated by cytotoxic gamma/delta T lymphocytes which prevent CD4+ activity. Microbial Pathogenesis. **13:**447-463.

68. **Chiodini, R. J., H. J. Van Kruiningen, and R. S. Merkal.** 1984. Ruminant paratuberculosis (Johne's disease): the current status and future prospects. Cornell Veterinarian. **74:**218-262.

69. **Clarke, C. J.** 1997. The pathology and pathogenesis of paratuberculosis in ruminants and other species. Journal of Comparative Pathology. **116:**217-261.

70. **Clemens, D. L., and M. A. Horwitz.** 1995. Characterization of the *Mycobacterium tuberculosis* phagosome and evidence that phagosomal maturation is inhibited. Journal of Experimental Medicine. **181:**257-270.

71. **Clemens, D. L., and M. A. Horwitz.** 1996. The *Mycobacterium tuberculosis* phagosome interacts with early endosomes and is accessible to exogenously administered transferrin. Journal of Experimental Medicine. **184:**1349-1355.

72. **Cocito, C., P. Gilot, M. Coene, M. de Kesel, P. Poupart, and P. Vannuffel.** 1994. Paratuberculosis. Clinical Microbiology Reviews. **7:**328-345.

73. **Cockburn, A., Cockburn, E., Reyman, T.** (ed.) 1998 Mummies, Disease & Ancient Cultures., Second ed. Cambridge University Press, Cambridge.

74. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, 3rd, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, B. G. Barrell, and et al.** 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature. **393:**537-544.

75. **Collins, F. M.** 1984. Protection against mycobacterial disease by means of live vaccines tested in experimental animals., p. 747-840. *In* G. P. Kubica, and L. G. Wayne (eds), The Mycobacteria, a source book. Marcel Dekker, New York.

76. **Collins, F. M., J. R. Lamb, and D. B. Young.** 1988. Biological activity of protein antigens isolated from *Mycobacterium tuberculosis* culture filtrate. Infection and Immunity. **56:**1260-1266.

77. **Collins, M. R.** 1998. Accuracy and Interpretation method for the IDEXX ELISA for Bovine Paratuberculosis. 41st Annual American Association of Veterinary Laboratory Diagnosis.

78. **Collins, R. A., P. Sopp, K. I. Gelder, W. I. Morrison, and C. J. Howard.** 1996. Bovine gamma/delta TcR+ T lymphocytes are stimulated to proliferate by autologous *Theileria annulata*-infected cells in the presence of interleukin-2. Scandinavian Journal of Immunology. **44:**444-452.

79. **Connolly, J. M., T. H. Hansen, A. L. Ingold, and T. A. Potter.** 1990. Recognition by CD8 on cytotoxic T lymphocytes is ablated by several substitutions in the class I alpha 3 domain: CD8 and the T-cell receptor recognize the same class I molecule. Proceedings of the National Academy of Sciences of the United States of America. **87:**2137-2141.

80. **Constant, P., F. Davodeau, M. A. Peyrat, Y. Poquet, G. Puzo, M. Bonneville, and J. J. Fournie.** 1994. Stimulation of human gamma delta T cells by nonpeptidic mycobacterial ligands. Science. **264:**267-270.

81. **Constant, P., Y. Poquet, M. A. Peyrat, F. Davodeau, M. Bonneville, and J. J. Fournie.** 1995. The antituberculous *Mycobacterium bovis* BCG vaccine is an attenuated mycobacterial producer of phosphorylated nonpeptidic antigens for human gamma delta T cells. Infection and Immunity. **63:**4628-4633.

82. **Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell, and I. M. Orme.** 1993. Disseminated tuberculosis in interferon gamma gene-disrupted mice. Journal of Experimental Medicine. **178:**2243-2247.

83. **Cooper, A. M., J. Magram, J. Ferrante, and I. M. Orme.** 1997. Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with *Mycobacterium tuberculosis*. Journal of Experimental Medicine. **186:**39-45.

84. **Cooper, A. M., A. D. Roberts, E. R. Rhoades, J. E. Callahan, D. M. Getzy, and I. M. Orme.** 1995. The role of interleukin-12 in acquired immunity to *Mycobacterium tuberculosis* infection. Immunology. **84:**423-432.

85. **Cooper, A. M., B. H. Segal, A. A. Frank, S. M. Holland, and I. M. Orme.** 2000. Transient loss of resistance to pulmonary tuberculosis in p47(phox-/-) mice. Infection and Immunity. **68:**1231-1234.

86. **Corpa, J. M., V. Perez, M. A. Sanchez, and J. F. Marin.** 2000. Control of paratuberculosis (Johne's disease) in goats by vaccination of adult animals. Veterinary Record. **146:**195-196.

87. **Crowle, A. J., R. Dahl, E. Ross, and M. H. May.** 1991. Evidence that Vesicles Containing Living, Virulent *Mycobacterium tuberculosis* or *Mycobacterium avium* in Cultured Human Macrophages Are Not Acidic. Infection and Immunity. **59:**1823-1831.

88. **D'Souza, C., A. Cooper, A. Frank, S. Ehlers, J. Turner, A. Bendelac, and I. Orme.** 2000. A Novel Nonclassic beta2-Microglobulin-Restricted Mechanism Influencing Early Lymphocyte Accumulation and Subsequent Resistance to Tuberculosis in the Lung. American Journal of Respiratory Cell and Molecular Biology. **23:**188-193.

89. **D'Souza, C. D., A. M. Cooper, A. A. Frank, R. J. Mazzaccaro, B. R. Bloom, and I. M. Orme.** 1997. An anti-inflammatory role for gamma delta T lymphocytes in acquired immunity to *Mycobacterium tuberculosis*. Journal of Immunology. **158:**1217-1221.

90. **Daubenberger, C. A., E. L. Taracha, L. Gaidulis, W. C. Davis, and D. J. McKeever.** 1999. Bovine gamma delta T-cell responses to the intracellular protozoan parasite *Theileria parva*. Infection and Immunity. **67:**2241-2249.

91. **Davies, P. D. O.** (ed.) 1998 Clinical Tuberculosis., second ed. Chapman & Hall, London.

92. **Dawson, J., A. D. Sedgwick, J. C. Edwards, and P. Lees.** 1992. The monoclonal antibody MEL-14 can block lymphocyte migration into a site of chronic inflammation. European Journal of Immunology. **22:**1647-1650.

93. **De Libero, G., I. Flesch, and S. H. Kaufmann.** 1988. Mycobacteria-reactive Lyt-2+ T cell lines. European Journal of Immunology. **18:**59-66.

94. **de Lisle, G. W., P. Seguin, B. S. Samagh, A. H. Corner, and J. R. Duncan.** 1980. Bovine paratuberculosis I. A herd study using complement fixation and intradermal tests. Canadian Journal of Comparative Medicine. **44:**177-182.

95. **Delogu, G., A. Howard, F. M. Collins, and S. L. Morris.** 2000. DNA Vaccination against Tuberculosis: Expression of a Ubiquitin-Conjugated Tuberculosis Protein Enhances Antimycobacterial Immunity. Infection and Immunity. **68:**3097-3102.

96. **Denis, M.** 1991. Interferon-gamma-treated murine macrophages inhibit growth of tubercle bacilli via the generation of reactive nitrogen intermediates. Cellular Immunology. **132:**150-157.

97. **Diagbouga, S., J. P. Chiron, O. Sanou, and E. Ledru.** 2001. Alteration in CD29high CD4+ Lymphocyte Subset is a Common Feature of Early HIV Disease and of Active Tuberculosis. Scandinavian Journal of Immunology. **53:**79-84.

98. **Dinarello, C. A., J. G. Cannon, S. M. Wolff, H. A. Bernheim, B. Beutler, A. Cerami, I. S. Figari, M. A. Palladino, Jr., and J. V. O'Connor.** 1986. Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. Journal of Experimental Medicine. **163:**1433-1450.

99. **Doherty, M. L., H. F. Bassett, P. J. Quinn, W. C. Davis, A. P. Kelly, and M. L. Monaghan.** 1996. A sequential study of the bovine tuberculin reaction. Immunology. **87:**9-14.

100. **Dormandy, T.** 1999. The White Death: A History of Tuberculosis. New York University Press, New York.

101. **Dutton, R. W., L. M. Bradley, and S. L. Swain.** 1998. T cell memory. Annual Reviews in Immunology. **16:**201-223.

102. **Emery, D. L., J. H. Dufty, and P. R. Wood.** 1988. An analysis of cellular proliferation, and synthesis of lymphokines and specific antibody in vitro by leucocytes from immunized cattle. Veterinary Immunology and Immunopathology. **18:**67-80.

103. **Emoto, M., E. Yoshiko, I. B. Buchwalow, and S. H. E. Kaufmann.** 1999. Induction of INF-g-producing CD4+ natural killer T cells by *Mycobacterium bovis* bacillus Calmette Guerin. European Journal of Immunology. **29:**650-659.

104. **Emoto, M., E. Yoshiko, and S. H. E. Kaufmann.** 1997. Bacille Calmette Guerin and interleukin-12 down-modulate interleukin-4-producing CD4+ NK1+ T lymphocytes. European Journal of Immunology. **27:**183-188.

105. **Espinal, M. A., S. J. Kim, P. G. Suarez, K. M. Kam, A. G. Khomenko, G. B. Migliori, J. Baez, A. Kochi, C. Dye, and M. C. Raviglione.** 2000. Standard short-course chemotherapy for drug-resistant tuberculosis: treatment outcomes in 6 countries. Journal of the American Medical Association. **283:**2537-2545.

106. **Ferrari, G., H. Langen, M. Naito, and J. Pieters.** 1999. A coat protein on phagosomes involved in the intracellular survival of mycobacteria. Cell. **97:**435-447.

107. **Fisch, H., and G. E. Gifford.** 1983. In vitro production of rabbit macrophage tumor cell cytotoxin. International Journal of Cancer. **32:**105-112.

108. **Flegg, P. J., R. B. Laing, C. Lee, G. Harris, B. Watt, C. L. Leen, and R. P. Brettle.** 1995. Disseminated disease due to *Mycobacterium avium* complex in AIDS. QJM. **88:**617-626.

109. **Flesch, I. E., and S. H. Kaufmann.** 1991. Mechanisms involved in mycobacterial growth inhibition by gamma interferon-activated bone marrow macrophages: role of reactive nitrogen intermediates. Infection and Immunity. **59:**3213-3218.

110. **Flynn, J. L., J. Chan, K. J. Triebold, D. K. Dalton, T. A. Stewart, and B. R. Bloom.** 1993. An essential role for Interferon γ in Resistance to *Mycobacterium tuberculosis* infection. Journal of Experimental Medicine. **178:**2249-2253.

111. **Flynn, J. L., M. M. Goldstein, J. Chan, K. Triebold, K. Pfeffer, C. J. Lowenstein, R. Schreiber, T. W. Mak, and B. R. Bloom.** 1995. Tumor necrosis factor-alpha Is required in the Protective Immune Response Against *Mycobacterium tuberculosis* in Mice. Immunity. **2:**561-572.

112. **Flynn, J. L., M. M. Goldstein, K. J. Triebold, B. Koller, and B. R. Bloom.** 1992. Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. Proceedings of the National Academy of Sciences of the United States of America. **89:**12013-12017.

113. **Flynn, J. L., M. M. Goldstein, K. J. Triebold, J. Sypek, S. Wolf, and B. R. Bloom.** 1995. IL-12 increases resistance of BALB/c mice to *Mycobacterium tuberculosis* infection. Journal of Immunology. **155:**2515-2524.

114. **Fournie, J. J., and M. Bonneville.** 1996. Stimulation of gamma delta T cells by phosphoantigens. Research in Immunology. **147:**338-347.

115. **Francis, J.** 1958. Tuberculosis in Animal and Man. A study in Comparative Pathology. Cassell, London.

116. **Freidag, B. L., G. B. Melton, F. Collins, D. M. Klinman, A. Cheever, L. Stobie, W. Suen, and R. A. Seder.** 2000. CpG oligodeoxynucleotides and interleukin-12 improve the efficacy of *Mycobacterium bovis* BCG vaccination in mice challenged with *M. tuberculosis*. Infection and Immunity. **68:**2948-2953.

117. **Fu, Y. X., R. Cranfill, M. Vollmer, R. Van Der Zee, R. L. O'Brien, and W. Born.** 1993. In vivo response of murine gamma delta T cells to a heat shock protein-derived peptide. Proceedings of the National Academy of Sciences of the United States of America. **90:**322-326.

118. **Garcia, V. E., P. A. Sieling, J. Gong, P. F. Barnes, K. Uyemura, Y. Tanaka, B. R. Bloom, C. T. Morita, and R. L. Modlin.** 1997. Single-cell cytokine analysis of gamma delta T cell responses to nonpeptide mycobacterial antigens. Journal of Immunology. **159:**1328-1335.

119. **Gatfield, J., and J. Pieters.** 2000. Essential role for cholesterol in entry of mycobacteria into macrophages. Science. **288:**1647-1650.

120. **Gitnick, G.** 1984. Is Chrohn's disease a mycobacterial disease after all? Digestive Diseases and Sciences. **29:**1086-1088.

121. **Glatman-Freedman, A., and A. Casadevall.** 1998. Serum therapy for tuberculosis revisited: reappraisal of the role of antibody-mediated immunity against *Mycobacterium tuberculosis.* Clinical Microbiology Reviews. **11:**514-532.

122. **Gomes, M. S., S. Paul, A. L. Moreira, R. Appelberg, M. Rabinovitch, and G. Kaplan.** 1999. Survival of *Mycobacterium avium* and *Mycobacterium tuberculosis* in acidified vacuoles of murine macrophages. Infection and Immunity. **67:**3199-3206.

123. **Grange, J. M., J. Gibson, T. W. Osborn, C. H. Collins, and M. D. Yates.** 1983. What is BCG? Tubercle and Lung Disease. **64:**129-139.

124. **Greenwood, D.** 1997. Historical Introduction., p. 2-9. *In* L. H. O'Grady F, Finch RG, Greenwood D (ed.), Antibiotic and Chemotherapy., seventh ed. Churchill Livingstone, Edinburgh.

125. **Griffin, J. F., C. G. Mackintosh, L. Slobbe, A. J. Thomson, and G. S. Buchan.** 1999. Vaccine protocols to optimise the protective efficacy of BCG. Tubercle and Lung Disease. **79:**135-143.

126. **Griffin, J. P., K. V. Harshan, W. K. Born, and I. M. Orme.** 1991. Kinetics of accumulation of gamma delta receptor-bearing T lymphocytes in mice infected with live mycobacteria. Infection and Immunity. **59:**4263-4265.

127. **Griffin, J. P., and I. M. Orme.** 1994. Evolution of CD4 T-cell subsets following infection of naive and memory immune mice with *Mycobacterium tuberculosis.* Infection and Immunity. **62:**1683-1690.

128. **Grun, J. L., and P. H. Maurer.** 1989. Different T helper cell subsets elicited in mice utilizing two different adjuvant vehicles: the role of endogenous interleukin 1 in proliferative responses. Cellular Immunology. **121:**134-145.

129. **Gulle, H., L. M. Fray, E. P. Gormley, A. Murray, and K. M. Moriarty.** 1995. Responses of bovine T cells to fractionated lysate and culture filtrate proteins of *Mycobacterium bovis* BCG. Veterinary Immunology and Immunopathology. **48:**183-190.

130. **Gurunathan, S., D. M. Klinman, and R. A. Seder.** 2000. DNA VACCINES: Immunology, Application, and Optimization. Annual Reviews in Immunology. **18:**927-974.

131. **Haas, W., and S. Tonegawa.** 1992. Development and selection of gamma delta T cells. Current Opinions in Immunology. **4:**147-155.

132. **Hanby-Flarida, M. D., O. J. Trask, T. J. Yang, and C. L. Baldwin.** 1996. Modulation of WC1, a lineage-specific cell surface molecule of gamma/delta T cells augments cellular proliferation. Immunology. **88:**116-123.

133. **Haregewoin, A., G. Soman, R. C. Hom, and R. W. Finberg.** 1989. Human γδ+ T cells respond to mycobacterial heat-shock protein. Nature. **340:**309-312.

134. **Haring, C., J. Traum, F. Hayes, and B. Henry.** 1930. Vaccination of calves against tuberculosis with BCG. Hilgardia. **4:**307-394.

135. **Hart, P. D., J. A. Armstrong, C. A. Brown, and P. Draper.** 1972. Ultrastructural study of the behavior of macrophages toward parasitic mycobacteria. Infection and Immunity. **5:**803-807.

136. **Havlir, D. V., R. S. Wallis, H. Boom, T. M. Daniel, K. Chervenak, and J. J. Ellner.** 1991. Human Immune Response to *Mycobacterium tuberculosis* Antigens. Infection and Immunity. **59:**665-670.

137. **Hein, W. R., and C. R. Mackay.** 1991. Prominence of gamma delta T cells in the ruminant immune system. Immunology Today. **12:**30-34.

138. **Hess, J., and S. H. E. Kaufmann.** 1999. Live antigen carriers as tools for improved anti-tuberculosis vaccines. FEMS Immunology and Medical Microbiology. **23:**165-173.

139. **Hess, J., D. Miko, A. Catic, V. Lehmensiek, D. G. Russell, and S. H. E. Kaufmann.** 1998. *Mycobacterium bovis* bacille Calmette-Guerin strains secreting listeriolysin of *Listeria monocytogenes.* Proceedings of the National Academy of Sciences of the United States of America. **95:**5299-5304.

140. **Hetland, G., and H. G. Wiker.** 1994. Antigen 85C on *Mycobacterium bovis*, BCG and *M. tuberculosis* promotes monocyte-CR3-mediated uptake of microbeads coated with mycobacterial products. Immunology. **82:**445-449.

141. **Hoft, D. F., R. M. Brown, and S. T. Roodman.** 1998. Bacille Calmette-Guerin vaccination enhances human gamma delta T cell responsiveness to mycobacteria suggestive of a memory-like phenotype. Journal of Immunology. **161:**1045-1054.

142. **Horwitz, M. A., B. W. Lee, B. J. Dillion, and G. Harth.** 1995. Protective immunity against tuberculosis induced by vaccination with major extracellular proteins of *Mycobacterium tuberculosis.* Proceedings of the National Academy of Sciences of the United States of America. **92:**1530-1534.

143. **Howard, C. J., P. Sopp, J. Brownlie, K. R. Parsons, L.-S. Kwong, and R. A. Collins.** 1996. Afferent lymph veiled cells stimulate proliferative responses in allogeneic $CD4^+$ and $CD8^+$ T cells but not $\gamma\delta$ TCR^+ T cells. Immunology. **88:**558-564.

144. **Howard, C. J., P. Sopp, K. R. Parsons, D. J. McKeever, E. L. Taracha, B. V. Jones, N. D. MacHugh, and W. I. Morrison.** 1991. Distinction of naive and memory BoCD4 lymphocytes in calves with a monoclonal antibody, CC76, to a restricted determinant of the bovine leukocyte-common antigen, CD45. European Journal of Immunology. **21:**2219-2226.

145. **Huebner, R. E.** 1996. BCG vaccination in the control of tuberculosis. Current Topics in Microbiology and Immunology. **215:**263-282.

146. **Hughes, M. S., S. D. Neill, and M. S. Rogers.** 1996. Vaccination of the badger (Meles meles) against *Mycobacterium bovis*. Veterinary Microbiology. **51:**363-379.

147. **Hussain, R., A. Kifayet, and T. J. Chiang.** 1995. Immunoglobulin G1 (IgG1) and IgG3 antibodies are markers of progressive disease in leprosy. Infection and Immunity. **63:**410-415.

148. **Huygen, K., J. Content, O. Denis, D. L. Montgomery, A. M. Yawman, R. R. Deck, C. M. DeWitt, I. M. Orme, S. Baldwin, C. D'Souza, A. Drowart, E. Lozes, P. Vandenbussche, J. P. Van Vooren, M. A. Liu, and J. B. Ulmer.** 1996. Immunogenicity and protective efficacy of a tuberculosis DNA vaccine. Nature Medicine. **2:**893-898.

149. **Inoue, T., Y. Yoshikai, G. Matsuzaki, and K. Nomoto.** 1991. Early appearing gamma/delta-bearing T cells during infection with Calmette Guerin bacillus. Journal of Immunology. **146:**2754-2762.

150. **Itohara, S., A. Farry, J. Lafaille, M. Bonneville, Y. Takagaki, W. Haas, and S. Tonegawa.** 1990. Homing of a γ/δ Thymocyte Subset with Homogeneous T-cell Receptors to the Mucosal Epithelial. Nature. **343:**754-757.

151. **Izzo, A. A., and R. J. North.** 1992. Evidence for an alpha/beta T cell-independent mechanism of resistance to mycobacteria. Bacillus-Calmette-Guerin causes progressive infection in severe combined immunodeficient mice, but not in nude mice or in mice depleted of CD4+ and CD8+ T cells. Journal of Experimental Medicine. **176:**581-586.

152. **Jackman, R. M., S. Stenger, A. Lee, D. B. Moody, R. A. Rogers, K. R. Niazi, M. Sugita, R. L. Modlin, P. J. Peters, and S. A. Porcelli.** 1998. The Tyrosine-Containing Cytoplasmic Tail of CD1b Is Essential for Its Efficient Presentation of Bacterial Lipid Antigens. Immunity. **8:**341-351.

153. **Janis, E. M., S. H. Kaufmann, R. H. Schwartz, and D. M. Pardoll.** 1989. Activation of gamma delta T cells in the primary immune response to *Mycobacterium tuberculosis*. Science. **244:**713-716.

154. **Jardetzky, T. S., W. S. Lane, R. A. Robinson, D. R. Madden, and D. C. Wiley.** 1991. Identification of self peptides bound to purified HLA-B27. Nature. **353:**326-329.

155. **Johnson, C. M., A. M. Cooper, A. A. Frank, C. B. Bonorino, L. J. Wysoki, and I. M. Orme.** 1997. *Mycobacterium tuberculosis* aerogenic rechallenge infections in B cell- deficient mice. Tubercle and Lung Disease. **78:**257-261.

156. **Juste, R. A., J. F. Garcia Marin, B. Peris, C. S. Saez de Ocariz, and J. J. Badiola.** 1994. Experimental infection of vaccinated and non-vaccinated lambs with *Mycobacterium paratuberculosis*. Journal of Comparative Pathology. **110:**185-194.

157. **Kamath, A., H. T, H. Briscoe, and W. Britton.** 1999. Co-immunization with DNA vaccines expressing granulocyte-macrophage colony-stimulating factor and mycobacterial secreted proteins enhances T-cell immunity, but not protective efficacy against *Mycobacterium tuberculosis*. Immunology. **96:**511-516.

158. **Kamath, A. T., C. G. Feng, M. MacDonald, H. Briscoe, and W. Britton.** 1999. Differential Protective Efficacy of DNA Vaccines Expressing Secreted Proteins of *Mycobacterium tuberculosis*. Infection and Immunity. **67:**1702-1707.

159. **Kao, R. R., and M. G. Roberts.** 1999. A comparison of wildlife control and cattle vaccination as methods for the control of bovine tuberculosis. Epidemiology of Infection. **122:**505-519.

160. **Kaye, J., M. L. Hsu, M. E. Sauron, S. C. Jameson, N. R. Gascoigne, and S. M. Hedrick.** 1989. Selective development of CD4+ T cells in transgenic mice expressing a class II MHC-restricted antigen receptor. Nature. **341:**746-749.

161. **Kelley, D. K. P. a. R. H.** (ed.) 1995 The Women's Study Bible. Thomas Nelson Publishers, Nashville.

162. **Kindler, V., A. P. Sappino, G. E. Grau, P. F. Piguet, and P. Vassalli.** 1989. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. Cell. **56:**731-740.

163. **Kishimoto, T. K., M. A. Jutila, and E. C. Butcher.** 1990. Identification of a human peripheral lymph node homing receptor: a rapidly down-regulated adhesion molecule. Proceedings of the National Academy of Sciences of the United States of America. **87:**2244-2248.

164. **Klebanoff, S. J., M. A. Vadas, J. M. Harlan, L. H. Sparks, J. R. Gamble, J. M. Agosti, and A. M. Waltersdorph.** 1986. Stimulation of neutrophils by tumor necrosis factor. Journal of Immunology. **136**:4220-4225.

165. **Kleinhenz, M. E., J. J. Ellner, P. J. Spagnuolo, and T. M. Daniel.** 1981. Suppression of lymphocyte responses by tuberculous plasma and mycobacterial arabinogalactan. Monocyte dependence and indomethacin reversibility. Journal of Clinical Investigation. **68**:153-162.

166. **Kobayashi, M., L. Fitz, M. Ryan, R. M. Hewick, S. C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia, and G. Trinchieri.** 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. Journal of Experimental Medicine. **170**:827-845.

167. **Kormendy, B.** 1994. The effect of vaccination on the prevalence of paratuberculosis in large dairy herds. Veterinary Microbiology. **41**:117-125.

168. **Ladel, C. H., S. Daugelat, and S. H. Kaufmann.** 1995. Immune response to *Mycobacterium bovis* bacille Calmette Guerin infection in major histocompatibility complex class I- and II-deficient knock-out mice: contribution of CD4 and CD8 T cells to acquired resistance. European Journal of Immunology. **25**:377-384.

169. **Lambert, H. P.** 1960. The influence of chemoprophylaxis on immunity in experimental tuberculosis. American Review of Respiratory Disease. **82**:619.

170. **Larsen, A. B., R. S. Merkal, and R. C. Cutlip.** 1975. Age of cattle as related to resistance to infection with *Mycobacterium paratuberculosis*. American Journal of Veterinary Research. **36**:255-257.

171. **Launois, P., R. DeLeys, M. Niang, A. Drowart, M. Andrien, P. Dierckx, J. Cartel, J. Sarthou, J. Van Vooren, and K. Huygen.** 1994. T cell epitope mapping of the major secreted mycobacterial antigen 85A in tuberculosis and leprosy. Infection and Immunity. **62**:3679-3687.

172. **Lee, W. T., X. M. Yin, and E. S. Vitetta.** 1990. Functional and ontogenetic analysis of murine CD45Rhi and CD45Rlo CD4+ T cells. Journal of Immunology. **144**:3288-3295.

173. **Lefford, M. J.** 1975. Transfer of adoptive immunity to tuberculosis in mice. Infection and Immunity. **11**:1174-1181.

174. **Leveton, C., S. Barnass, B. Champion, S. Lucas, B. De Souza, M. Nicol, D. Banerjee, and G. Rook.** 1989. T-cell-mediated protection of mice against virulent *Mycobacterium tuberculosis*. Infection and Immunity. **57**:390-395.

175. **Liebana, E., A. Aranaz, M. Welsh, S. D. Neill, and J. M. Pollock.** 2000. In vitro T-cell activation of monocyte-derived macrophages by soluble messengers or cell-to-cell contact in bovine tuberculosis. Immunology. **100:**194-202.

176. **Liebana, E., R. M. Girvin, M. Welsh, S. D. Neill, and J. M. Pollock.** 1999. Generation of CD8(+) T-cell responses to *Mycobacterium bovis* and mycobacterial antigen in experimental bovine tuberculosis. Infection and Immunity. **67:**1034-1044.

177. **Lindblad, E. B., M. J. Elhay, R. Silva, R. Appelberg, and P. Andersen.** 1997. Adjuvant modulation of immune responses to tuberculosis subunit vaccines. Infection and Immunity. **65:**623-629.

178. **Little, D., H. M. Alzuherri, and C. J. Clarke.** 1996. Phenotypic characterisation of intestinal lymphocytes in ovine paratuberculosis by immunohistochemistry. Veterinary Immunology and Immunopathology. **55:**175-187.

179. **Lowrie, D. B., C. L. Silva, M. J. Colston, S. Ragno, and R. E. Tascon.** 1997. Protection against tuberculosis by a plasmid DNA vaccine. Vaccine. **15:**834-838.

180. **Lugosi, L.** 1992. Theoretical and methodological aspects of BCG vaccine from the discovery of Calmette and Guerin to molecular biology. A review. Tubercle and Lung Disease. **73:**252-261.

181. **Machugh, N. D., J. K. Mburu, M. J. Carol, C. R. Wyatt, J. A. Orden, and W. C. Davis.** 1997. Identification of two distinct subsets of bovine γδ T cells with unique cell surface phenotype and tissue distribution. Immunology. **92:**340-345.

182. **Mackaness, G. B.** 1967. The relationship of delayed hypersensitivity to acquired cellular resistance. British Medical Bulletin. **23:**52-54.

183. **Mackaness, G. B., and R. V. Blanden.** 1967. Cellular immunity. Progress in Allergy. **11:**89-140.

184. **Mackay, C. R., M. F. Beya, and P. Matzinger.** 1989. Gamma/delta T cells express a unique surface molecule appearing late during thymic development. European Journal of Immunology. **19:**1477-1483.

185. **Magnusson, M., &Bentzon, M.W.** 1958. Preparation of Purified Tuberculin RT 23. 19. World Health Organization.

186. **Maharais, G. G., P. J. Sabo, M. J. Hickey, D. C. Singh, and C. K. Stover.** 1996. Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. Journal of Bacteriology. **178:**1274-1282.

187. **Martin, S. W.** 1994. Livestock Disease Eradication: Evaluation of the Cooperative State-Federal Bovine Tuberculosis Eradication Program. National Research Council.

188. **Masood, E.** 1998. Outcry as "scientific" badger cull is launched to target TB. Nature. **394:**821.

189. **Matis, L., A. Fry, R. Cron, M. Cotterman, R. Dick, and J. Bluestone.** 1989. Structure and specificity of a class II MHC alloreactive γδ T cell receptor. Science. **245:**746-749.

190. **Mattsson, E., L. Verhage, J. Rollof, A. Fleer, J. Verhoef, and H. van Dijk.** 1993. Peptidoglycan and teichoic acid from Staphylococcus epidermidis stimulate human monocytes to release tumour necrosis factor-alpha, interleukin-1 beta and interleukin-6. FEMS Immunology and Medical Microbiology. **7:**281-287.

191. **McCluskey, R. T., and S. Cohen.** 1972. Mechanisms of delayed hypersensitivity. Pathobiology Annual. **2:**111-128.

192. **McCluskie, M. J., R. D. Weeratna, A. M. Krieg, and H. L. Davis.** 2000. CpG DNA is an effective oral adjuvant to protein antigens in mice. Vaccine. **19:**950-957.

193. **McDonough, K. A., Y. Kress, and B. R. Bloom.** 1993. Pathogenesis of Tuberculosis: Interaction of *Mycobacterium tuberculosis* with Macrophages. Infection and Immunity. **61:**2763-2773.

194. **Melsom, R., M. Harboe, B. Myrvang, T. Godal, and A. Belehu.** 1982. Immunoglobulin class specific antibodies to *M. leprae* in leprosy patients, including the indeterminate group and healthy contacts as a step in the development of methods for sero-diagnosis of leprosy. Clinical and Experimental Immunology. **47:**225-233.

195. **Michie, C. A., A. McLean, C. Alcock, and P. C. Beverley.** 1992. Lifespan of human lymphocyte subsets defined by CD45 isoforms. Nature. **360:**264-265.

196. **Modlin, R. L., C. Pirmez, F. M. Hofman, V. Torigian, K. Uyemura, T. H. Rea, B. R. Bloom, and M. B. Brenner.** 1989. Lymphocytes bearing antigen-specific gamma delta T-cell receptors accumulate in human infectious disease lesions. Nature. **339:**544-548.

197. **Modllin, R. L., C. Pirmez, F. M. Hofman, V. Torigian, K. Uyemura, T. H. Rea, B. R. Bloom, and M. B. Brenner.** 1989. Lymphocytes bearing antigen-specific γδ T-cell receptors accumulate in human infectious disease lesions. Nature. **339:**544-548.

198. **Mombaerts, P., J. Arnoldi, F. Russ, S. Tonegawa, and S. H. Kaufmann.** 1993. Different roles of alpha beta and gamma delta T cells in immunity against an intracellular bacterial pathogen. Nature. **365:**53-56.

199. **Moody, D. B., B. B. Reinhold, M. R. Guy, E. M. Beckman, D. E. Frederique, S. T. Furlong, S. Ye, V. N. Reinhold, P. A. Sieling, and R. L. Modlin.** 1997. Structural requirements for glycolipid antigen recognition by CD1b-restricted T cells. Science. **278:**283-286.

200. **Moore M, O. I., McCray E, Castro KG.** 1997. Trends in drug-resistant tuberculosis in the United States, 1993-1996. Journal of the American Medical Association. **278:**833-837.

201. **Moore, T. A., B. B. Moore, M. W. Newstead, and T. J. Standiford.** 2000. Gamma delta-T cells are critical for survival and early proinflammatory cytokine gene expression during murine Klebsiella pneumonia. Journal of Immunology. **165:**2643-2650.

202. **Moreno, C., A. Mehlert, and J. Lamb.** 1988. The inhibitory effects of mycobacterial lipoarabinomannan and polysaccharides upon polyclonal and monoclonal human T cell proliferation. Clinical and Experimental Immunology. **74:**206-210.

203. **Morita, C. T., E. M. Beckman, J. F. Bukowski, Y. Tanaka, H. Band, B. R. Bloom, D. E. Golan, and M. B. Brenner.** 1995. Direct presentation of nonpeptide prenyl pyrophosphate antigens to human gamma delta T cells. Immunity. **3:**495-507.

204. **Morley, P. S., W. P. Shulaw, B. Byrum, and L. Barber.** 1998. Comparison of Assays Used to Diagnose *Mycobacterium paratuberculosis* Infections in Cattle. 79th Meeting of Research Workers in Animal Diseases.

205. **Morris, S., C. Kelley, A. Howard, Z. Li, and F. Collins.** 2000. The immunogenicity of single and combination DNA vaccines against tuberculosis. Vaccine. **18:**2155-2163.

206. **Morrison, W. I., and W. C. Davis.** 1991. Individual antigens of cattle. Differentiation antigens expressed predominantly on CD4- CD8- T lymphocytes (WC1, WC2). Veterinary Immunology and Immunopathology. **27:**71-76.

207. **Muller, I., S. P. Cobbold, H. Waldmann, and S. H. Kaufmann.** 1987. Impaired resistance to *Mycobacterium tuberculosis* infection after selective in vivo depletion of L3T4+ and Lyt-2+ T cells. Infection and Immunity. **55:**2037-2041.

208. **Munk, M. E., A. J. Gatrill, and S. H. E. Kaufmann.** 1990. Target Cell Lysis and IL-2 Secretion by γ/δ T Lymphocytes After Activation with Bacteria. The Journal of Immunology. **145:**2434-2439.

209. **Murray, P., A. Aldovini, and R. Young.** 1996. Manipulation and potentiation of anti-mycobacterial immunity using recombinant bacille Calmette-Guerin strains that secrete cytokines. Proceedings of the National Academy of Sciences of the United States of America. **93:**934-939.

210. **Myrvang, B., T. Godal, D. S. Ridley, S. S. Froland, and Y. K. Song.** 1973. Immune responsiveness to *Mycobacterium leprae* and other mycobacterial antigens throughout the clinical and histopathological spectrum of leprosy. Clinical and Experimental Immunology. **14:**541-553.

211. **Myrvik, Q. N., E. S. Leake, and M. J. Wright.** 1984. Disruption of Phagosomal Membranes of Normal Alveolar Macrophages by the H37Rv Strain of *Mycobacterium tuberculosis.* American Reviews of Respiratory Disease. **129:**322-328.

212. **Nagai, S., H. G. Wiker, M. Harboe, and M. Kinomoto.** 1991. Isolation and Partial Characterization of Major Protein Antigens in the Culture Fluid of *Mycobacterium tuberculosis.* Infection and Immunity. **59:**372-382.

213. **Nagao, S., Y. Kawabata, M. Kitano, K. Suzuki, T. Nishikawa, and H. Takada.** 1995. Modification of delayed-type hypersensitivity reactions by muramyldipeptide in guinea pigs. FEMS Immunology and Medical Microbiology. **11:**231-245.

214. **Niinaka, T., S. Kishimoto, T. Aoki, H. Ikegami, and F. Ito.** 1975. Skin reaction, inhibition of macrophage migration, and lymphocyte transformation with tuberculin active peptide (TAP) and arabinogalactan obtained from tubercle bacilli. International Archives of Allergy and Applied Immunology. **49:**585-596.

215. **Ninomiya, T., H. Takimoto, G. Matsuzaki, S. Hamano, H. Yoshida, Y. Yoshikai, G. Kimura, and K. Nomoto.** 2000. Vgamma1+ gammadelta T cells play protective roles at an early phase of murine cytomegalovirus infection through production of interferon-gamma. Immunology. **99:**187-194.

216. **O'Donnell, M., A. Aldovini, R. Duba, H. Yang, A. Szilvasi, R. Young, and W. DeWolf.** 1994. Recombinant *Mycobacterium bovis* BCG secreting functional interleukin-2 enhances gamma interferon production by splenocytes. Infection and Immunity. **62:**2508-2514.

217. **Obasanjo, I. O., Y. T. Grohn, and H. O. Mohammed.** 1997. Farm factors associated with the presence of *Mycobacterium paratuberculosis* infection in

dairy herds on the New York State Paratuberculosis Control Program. Preventative Veterinary Medicine. **32:**243-251.

218. **Okamura, H., H. Tsutsi, T. Komatsu, M. Yutsudo, A. Hakura, T. Tanimoto, K. Torigoe, T. Okura, Y. Nukada, K. Hattori, and et al.** 1995. Cloning of a new cytokine that induces IFN-gamma production by T cells. Nature. **378:**88-91.

219. **Orme, I. M.** 1988. Characteristics and specificity of acquired immunologic memory to *Mycobacterium tuberculosis* infection. Journal of Immunology. **140:**3589-3593.

220. **Orme, I. M.** 1988. Induction of nonspecific acquired resistance and delayed-type hypersensitivity, but not specific acquired resistance in mice inoculated with killed mycobacterial vaccines. Infection and Immunity. **56:**3310-3312.

221. **Orme, I. M.** 1987. The kinetics of emergence and loss of mediator T lymphocytes acquired in response to infection with *Mycobacterium tuberculosis*. Journal of Immunology. **138:**293-298.

222. **Orme, I. M., and F. M. Collins.** 1984. Adoptive protection of the *Mycobacterium tuberculosis*-infected lung. Dissociation between cells that passively transfer protective immunity and those that transfer delayed-type hypersensitivity to tuberculin. Cellular Immunology. **84:**113-120.

223. **Orme, I. M., and A. M. Cooper.** 1999. Cytokine/chemokine cascades in immunity to tuberculosis. Immunology Today. **20:**307-312.

224. **Orme, I. M., E. S. Miller, A. D. Roberts, S. K. Furney, J. P. Griffin, K. M. Dobos, D. Chi, B. Rivoire, and P. J. Brennan.** 1992. T lymphocytes mediating protection and cellular cytolysis during the course of *Mycobacterium tuberculosis* infection. Evidence for different kinetics and recognition of a wide spectrum of protein antigens. Journal of Immunology. **148:**189-196.

225. **Orme, I. M., A. D. Roberts, J. P. Griffin, and J. S. Abrams.** 1993. Cytokine secretion by CD4 T lymphocytes acquired in response to *Mycobacterium tuberculosis* infection. Journal of Immunology. **151:**518-525.

226. **Ott, S. L., S. J. Wells, and B. A. Wagner.** 1999. Herd-level economic losses associated with Johne's disease on US dairy operations. Preventative Veterinary Medicine. **40:**179-192.

227. **Pal, P. G., and M. A. Horwitz.** 1992. Immunization with extracellular proteins of *Mycobacterium tuberculosis* induces cell-mediated immune responses and substantial protective immunity in a guinea pig model of pulmonary tuberculosis. Infection and Immunity. **60:**4781-4792.

228. **Pawankar, R. U., M. Okuda, K. Suzuki, K. Okumura, and C. Ra.** 1996. Phenotypic and molecular characteristics of nasal mucosal gamma delta T cells in allergic and infectious rhinitis. American Journal of Respiratory and Critical Care Medicine. **153:**1655-1665.

229. **Payne, J. M., and J. D. Rankins.** 1961. The pathogenesis of experimental Johne's disease in calves. Research in Veterinary Science. **2:**167-174.

230. **Pedrazzini, T., K. Hug, and J. A. Louis.** 1987. Importance of L3T4+ and Lyt-2+ cells in the immunologic control of infection with *Mycobacterium bovis* strain bacillus Calmette-Guerin in mice. Assessment by elimination of T cell subsets in vivo. Journal of Immunology. **139:**2032-2037.

231. **Pfeffer, K., B. Schoel, H. Gulle, S. H. Kaufmann, and H. Wagner.** 1991. Human gamma/delta T cells responding to mycobacteria. Behring Institute Mitteilungen:36-42.

232. **Pfeffer, K., B. Schoel, H. Gulle, S. H. Kaufmann, and H. Wagner.** 1990. Primary responses of human T cells to mycobacteria: a frequent set of gamma/delta T cells are stimulated by protease-resistant ligands. European Journal of Immunology. **20:**1175-1179.

233. **Pfuetze, K. H., M.M. Pyle, H.C. Hinshaw, and W.H. Feldman.** 1955. The first clinical trial of streptomycin in human tuberculosis. American Review of Tuberculosis. **71:**752-754.

234. **Pillai, S. D., K. W. Widmer, L. J. Ivey, K. C. Coker, E. Newman, S. Lingsweiler, D. Baca, M. Kelley, D. S. Davis, N. J. Silvy, and L. G. Adams.** 2000. Failure to identify non-bovine reservoirs of *Mycobacterium bovis* in a region with a history of infected dairy-cattle herds. Preventative Veterinary Medicine. **43:**53-62.

235. **Pizcueta, P., and F. W. Luscinskas.** 1994. Monoclonal antibody blockade of L-selectin inhibits mononuclear leukocyte recruitment to inflammatory sites in vivo. American Journal of Pathology. **145:**461-469.

236. **Pollock, J. M., and P. Andersen.** 1997. Predominant recognition of the ESAT-6 protein in the first phase of infection with *Mycobacterium bovis* in cattle. Infection and Immunity. **65:**2587-2592.

237. **Pollock, J. M., D. A. Pollock, D. G. Campbell, R. M. Girvin, A. D. Crockard, S. D. Neill, and D. P. Mackie.** 1996. Dynamic changes in circulating and antigen-responsive T-cell subpopulations post-*Mycobacterium bovis* infection in cattle. Immunology. **87:**236-241.

238. **Porcelli, S., M. Brenner, J. Greenstein, S. Balk, C. Terhorst, and P. Bleicher.** 1989. Recognition of cluster differentiation I antigens by CD4-CD8- cytolytic T lymphocytes. Nature. **341:**447-449.

239. **Quade, M. J., and J. A. Roth.** 1999. Antigen-specific in vitro activation of T-lymphocyte subsets of cattle immunized with a modified live bovine herpesvirus 1 vaccine. Viral Immunology. **12:**9-21.

240. **Rhodes, S. G., D. Gavier-Widen, B. M. Buddle, A. O. Whelan, M. Singh, R. G. Hewinson, and H. M. Vordermeier.** 2000. Antigen specificity in experimental bovine tuberculosis. Infection and Immunity. **68:**2573-2578.

241. **Rivas, A., J. Koide, M. Cleary, and E. Engleman.** 1989. Evidence for the involvement of the γδ T cell antigen receptor in cytotoxicity mediated by human alloantigen-specific T cell clones. Journal of Immunology. **142:**1840-1847.

242. **Roberts, A. D., M. G. Sonnenberg, D. J. Ordway, S. K. Furney, P. J. Brennan, J. T. Belisle, and I. M. Orme.** 1995. Characteristics of protective immunity engendered by vaccination of mice with purified culture filtrate protein antigens of *Mycobacterium tuberculosis*. Immunology. **85:**502-508.

243. **Rook, G. A., J. Steele, M. Ainsworth, and B. R. Champion.** 1986. Activation of macrophages to inhibit proliferation of *Mycobacterium tuberculosis*: comparison of the effects of recombinant gamma-interferon on human monocytes and murine peritoneal macrophages. Immunology. **59:**333-338.

244. **Rothel, J. S., S. L. Jones, L. A. Corner, J. C. Cox, and P. R. Wood.** 1990. A sandwich enzyme immunoassay for bovine interferon-gamma and its use for the detection of tuberculosis in cattle. Australian Veterinary Journal. **67:**134-137.

245. **Rudensky, A., P. Preston-Hurlburt, S. C. Hong, A. Barlow, and C. A. Janeway, Jr.** 1991. Sequence analysis of peptides bound to MHC class II molecules. Nature. **353:**622-627.

246. **Sager, P., Schalimtzek, M., and Moller Christensen, V.** 1972. A case of tuberculosis spondylosa in the Danish Neolithic Age. Danish Medical Bulletin. **19:**176-180.

247. **Saito, H., D. Kranz, Y. Takagaki, A. Hayday, H. Eisen, and S. Tonegawa.** 1984. Complete Primary Structure of a Heterodimeric T-cell Receptor Deduced from cDNA Sequences. Nature. **309:**757-762.

248. **Sakula, A.** 1983. BCG: who were Calmette and Guerin? Thorax. **38:**806-812.

249. **Salvetti, M., C. Buttinelli, G. Ristori, M. Carbonari, M. Cherchi, M. Fiorelli, M. G. Grasso, L. Toma, and C. Pozzilli.** 1992. T-lymphocyte reactivity to the

recombinant mycobacterial 65- and 70-kDa heat shock proteins in multiple sclerosis. Journal of Autoimmunity. **5**:691-702.

250. **Sampaio, E. P., R. B. Oliveira, J. Warwick-Davies, R. B. Neto, G. E. Griffin, and R. J. Shattock.** 2000. T cell-monocyte contact enhances tumor necrosis factor-alpha production in response to *Mycobacterium leprae*. Journal of Infectious Diseases. **182**:1463-1472.

251. **Sanders, M. E., M. W. Makgoba, S. O. Sharrow, D. Stephany, T. A. Springer, H. A. Young, and S. Shaw.** 1988. Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL1, CDw29, and Pgp-1) and have enhanced IFN-gamma production. Journal of Immunology. **140**:1401-1407.

252. **Saparov, A., F. H. Wagner, R. Zheng, J. R. Oliver, H. Maeda, R. D. Hockett, and C. T. Weaver.** 1999. Interleukin-2 expression by a subpopulation of primary T cells is linked to enhanced memory/effector function. Immunity. **11**:271-280.

253. **Schild, H., N. Mavaddat, C. Litzenberger, E. W. Ehrich, M. M. Davis, J. A. Bluestone, L. Matis, R. K. Draper, and Y. H. Chien.** 1994. The nature of major histocompatibility complex recognition by gamma delta T cells. Cell. **76**:29-37.

254. **Schlesinger, L. S.** 1993. Macrophage phagocytosis of virulent but not attenuated strains of *Mycobacterium tuberculosis* is mediated by mannose receptors in addition to complement receptors. Journal of Immunology. **150**:2920-2930.

255. **Schlesinger, L. S., C. G. Bellinger-Kawahara, N. R. Payne, and M. A. Horwitz.** 1990. Phagocytosis of *Mycobacterium tuberculosis* is mediated by human monocyte complement receptors and complement component C3. Journal of Immunology. **144**:2771-2780.

256. **Schlesinger, L. S., S. R. Hull, and T. M. Kaufman.** 1994. Binding of the terminal mannosyl units of lipoarabinomannan from a virulent strain of *Mycobacterium tuberculosis* to human macrophages. Journal of Immunology. **152**:4070-4079.

257. **Schmitt, S. M., S. D. Fitzgerald, T. M. Cooley, C. S. Bruning-Fann, L. Sullivan, D. Berry, T. Carlson, R. B. Minnis, J. B. Payeur, and J. Sikarskie.** 1997. Bovine tuberculosis in free-ranging white-tailed deer from Michigan. Journal of Wildlife Diseases. **33**:749-758.

258. **Schoel, B., S. Sprenger, and S. H. Kaufmann.** 1994. Phosphate is essential for stimulation of V gamma 9V delta 2 T lymphocytes by mycobacterial low molecular weight ligand. European Journal of Immunology. **24**:1886-1892.

259. **Sciammas, R., R. Johnson, A. Sperling, W. Brady, P. Linsley, P. Spear, F. Fitch, and J. Bluestone.** 1994. Unique antigen recognition by a herpesvirus-specific TCR-γ-δ cell. Journal of Immunology. **152:**5392-5397.

260. **Sciammas, R., P. Kodukula, Q. Tang, R. L. Hendricks, and J. A. Bluestone.** 1997. T cell receptor-gamma/delta cells protect mice from herpes simplex virus type 1-induced lethal encephalitis. Journal of Experimental Medicine. **185:**1969-1975.

261. **Sibley, L. D., L. B. Adams, and J. L. Krahenbuhl.** 1990. Inhibition of interferon-gamma-mediated activation in mouse macrophages treated with lipoarabinomannan. Clinical and Experimental Immunology. **80:**141-148.

262. **Sibley, L. D., S. W. Hunter, P. J. Brennan, and J. L. Krahenbuhl.** 1988. Mycobacterial lipoarabinomannan inhibits gamma interferon-mediated activation of macrophages. Infection and Immunity. **56:**1232-1236.

263. **Sieling, P. A., D. Chatterjee, S. A. Porcelli, T. I. Prigozy, R. J. Mazzaccaro, T. Soriano, B. R. Bloom, M. B. Brenner, M. Kronenberg, P. J. Brennan, and R. L. Modlin.** 1995. CD1-Restricted T Cell Recognition of Microbial Lipoglycan Antigens. Science. **269:**227-230.

264. **Sieling, P. A., D. Jullien, M. Dahlem, T. F. Tedder, T. H. Rea, R. L. Modlin, and S. A. Porcelli.** 1999. CD1 Expression by Dendritic Cells in Human Leprosy Lesions: Correlation with Effective Host Immunity. The Journal of Immunology. **162:**1851-1858.

265. **Skeen, M. J., and H. K. Ziegler.** 1995. Activation of gamma delta T cells for production of IFN-gamma is mediated by bacteria via macrophage-derived cytokines IL-1 and IL-12. Journal of Immunology. **154:**5832-5841.

266. **Smith, R. A., J. M. Kreeger, A. J. Alvarez, J. C. Goin, W. C. Davis, D. L. Whipple, and D. M. Estes.** 1999. Role of CD8+ and WC1- gamma/delta T cells in resistance to *Mycobacterium bovis* infection in the SCID-bo mouse. Journal of Leukocyte Biology. **65:**28-34.

267. **Snider, D. E., Jr., and W. L. Roper.** 1992. The new tuberculosis [editorial; comment]. New England Journal of Medicine. **326:**703-705.

268. **Spertini, O., G. S. Kansas, J. M. Munro, J. D. Griffin, and T. F. Tedder.** 1991. Regulation of leukocyte migration by activation of the leukocyte adhesion molecule-1 (LAM-1) selectin. Nature. **349:**691-694.

269. **St-Jean, G., and A. D. Jernigan.** 1991. Treatment of *Mycobacterium paratuberculosis* infection in ruminants. Veterinary Clinics of North America Food Animal Practice. **7:**793-804.

270. **Stabel, J. R.** 1998. Johne's disease: a hidden threat. Journal of Dairy Science. **81:**283-288.

271. **Stabel, J. R.** 1996. Production of gamma-interferon by peripheral blood mononuclear cells: an important diagnostic tool for detection of subclinical paratuberculosis. Journal of Veterinary Diagnostic Investigation. **8:**345-350.

272. **Stenger, S., K. R. Niazi, and R. L. Modlin.** 1998. Down-Regulation of CD1 on Antigen-Presenting Cells by Infection with *Mycobacterium tuberculosis.* The Journal of Immunology. **161:**3582-3588.

273. **Stout, R. D.** 1993. Macrophage activation by T cells: cognate and non-cognate signals. Current Opinions in Immunology. **5:**398-403.

274. **Stout, R. D., and K. Bottomly.** 1989. Antigen-specific activation of effector macrophages by IFN-gamma producing (TH1) T cell clones. Failure of IL-4-producing (TH2) T cell clones to activate effector function in macrophages. Journal of Immunology. **142:**760-765.

275. **Stover, C. K., P. Warrener, D. R. VanDevanter, D. R. Sherman, T. M. Arain, M. H. Langhorne, S. W. Anderson, J. A. Towell, Y. Yuan, D. N. McMurray, B. N. Kreiswirth, C. E. Barry, and W. R. Baker.** 2000. A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. Nature. **405:**962-966.

276. **Stuart, F., K. H. Mahmood, J. L. Stanford, and D. G. Pritchard.** 1988. Development of diagnostic tests for, and vaccination against, tuberculosis in badgers. Mammal Review. **18:**74-75.

277. **Sturgill-Koszycki, S., P. H. Schlesinger, P. Chakraborty, P. L. Haddix, H. L. Collins, A. K. Fok, R. D. Allen, S. L. Gluck, J. Heuser, and D. G. Russell.** 1994. Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science. **263:**678-681.

278. **Sugawara, I., H. Yamada, H. Kaneko, S. Mizuno, K. Takeda, and S. Akira.** 1999. Role of interleukin-18 (IL-18) in mycobacterial infection in IL-18-gene-disrupted mice. Infection and Immunity. **67:**2585-2589.

279. **Sweeney, R. W.** 1996. Transmission of paratuberculosis. Veterinary Clinics of North America Food Animal Practice. **12:**305-312.

280. **Sweeney, R. W., D. E. Jones, P. Habecker, and P. Scott.** 1998. Interferon-gamma and interleukin 4 gene expression in cows infected with *Mycobacterium paratuberculosis.* American Journal of Veterinary Research. **59:**842-847.

281. **Sweeney, R. W., R. H. Whitlock, and A. E. Rosenberger.** 1992. *Mycobacterium paratuberculosis* isolated from fetuses of infected cows not manifesting signs of the disease. American Journal of Veterinary Research. **53:**477-480.

282. **Sypek, J. P., S. E. H. Chung, J. M. Mayor, S. L. Subramanyam, D. S. Goldman, D. S. Sieburth, S. F. Wolf, and R. G. Schaub.** 1993. Resolution of cutaneous leishmaniasis: interleukin 12 initiates a protective T helper type 1 immune response. Journal of Experimental Medicine. **177:**1797-1802.

283. **Tanaka, Y., C. T. Morita, Y. Tanaka, E. Nieves, M. B. Brenner, and B. R. Bloom.** 1995. Natural and synthetic non-peptide antigens recognized by human gamma delta T cells. Nature. **375:**155-158.

284. **Tanaka, Y., S. Sano, E. Nieves, G. De Libero, D. Rosa, R. L. Modlin, M. B. Brenner, B. R. Bloom, and C. T. Morita.** 1994. Nonpeptide ligands for human gamma delta T cells. Proceedings of the National Academy of Sciences of the United States of America. **91:**8175-8179.

285. **Tascon, R. E., E. Stavropoulos, K. V. Lukacs, and M. J. Colston.** 1998. Protection against *Mycobacterium tuberculosis* infection by CD8+ T cells requires the production of gamma interferon. Infection and Immunity. **66:**830-834.

286. **Teitelbaum, R., A. Glatman-Freedman, B. Chen, J. B. Robbins, E. Unanue, A. Casadevall, and B. R. Bloom.** 1998. A mAb recognizing a surface antigen of *Mycobacterium tuberculosis* enhances host survival. Proceedings of the National Academy of Sciences of the United States of America. **95:**15688-15693.

287. **Ten Dam, H. G.** 1984. Research on BCG vaccination. Advances in Tuberculosis Research. **21:**79-106.

288. **Terhorst, C., A. van Agthoven, K. LeClair, P. Snow, E. Reinherz, and S. Schlossman.** 1981. Biochemical studies of the human thymocyte cell-surface antigens T6, T9 and T10. Cell. **23:**771-780.

289. **Thorne, J. G., and L. E. Hardin.** 1997. Estimated prevalence of paratuberculosis in Missouri, USA cattle. Preventative Veterinary Medicine. **31:**51-57.

290. **Trowbridge, I. S., and M. L. Thomas.** 1994. CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development. Annual Reviews in Immunology. **12:**85-116.

291. **Tsuji, S., M. Matsumoto, O. Takeuchi, S. Akira, I. Azuma, A. Hayashi, K. Toyoshima, and T. Seya.** 2000. Maturation of human dendritic cells by cell wall

skeleton of *Mycobacterium bovis* bacillus Calmette-Guerin: involvement of toll-like receptors. Infection and Immunity. **68:**6883-6890.

292. **Tuo, W., F. W. Bazer, W. C. Davis, D. Zhu, and W. C. Brown.** 1999. Differential Effects of Type I IFNs on the Growth of WC1- CD8+ γδ T Cells and WC1+ CD8- γδ T Cells In Vitro. The Journal of Immunology. **162:**245-253.

293. **Turner, J., C. D. D'Souza, J. E. Pearl, P. Marietta, M. Noel, A. A. Frank, R. Appelberg, I. M. Orme, and A. M. Cooper.** 2001. CD8- and CD95/95L-dependent mechanisms of resistance in mice with chronic pulmonary tuberculosis. American Journal of Respiratory Cell and Molecular Biology. **24:**203-209.

294. **Turner, J., A. A. Frank, J. V. Brooks, P. M. Marietta, and I. M. Orme.** 2001. Pentoxifylline treatment of mice with chronic pulmonary tuberculosis accelerates the development of destructive pathology. Immunology. **102:**248-253.

295. **Turner, O. C., A. D. Roberts, A. A. Frank, S. W. Phalen, D. M. McMurray, J. Content, O. Denis, S. D'Souza, A. Tanghe, K. Huygen, and I. M. Orme.** 2000. Lack of protection in mice and necrotizing bronchointerstitial pneumonia with brochiolitis in guinea pigs immunized with vaccines directed against the hsp60 molecule of *Mycobacterium tuberculosis.* Infection and Immunity. **68:**3674-3679.

296. **Ulmer, J., J. Donnelly, S. Parker, G. Rhodes, P. Felgner, V. Dwarki, S. Gromkowski, R. Deck, D. De Witt, A. Friedman, L. Hawe, K. Leander, D. Martinez, H. Perry, J. Shiver, D. Montgomery, and M. Liu.** 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science. **259:**1745-1749.

297. **Ulrichs, T., M. E. Munk, H. Mollenkopf, S. Behr-Perst, R. Colangeli, M. L. Gennaro, and S. H. Kaufmann.** 1998. Differential T cell responses to *Mycobacterium tuberculosis* ESAT-6 in tuberculosis patients and healthy donors. European Journal of Immunology. **28:**3949-3958.

298. **Underhill, D. M., A. Ozinsky, K. D. Smith, and A. Aderem.** 1999. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. Proceedings of the National Academy of Sciences of the United States of America. **96:**14459-14463.

299. **Ungar-Waron, H., J. Brenner, R. Paz, U. Moalem, and Z. Trainin.** 1996. gamma delta T-lymphocytes and anti-heat shock protein reactivity in bovine leukemia virus infected cattle. Veterinary Immunology and Immunopathology. **51:**79-87.

300. **van Pinxteren, L. A., J. P. Cassidy, B. H. Smedegaard, E. M. Agger, and P. Andersen.** 2000. Control of latent *Mycobacterium tuberculosis* infection is dependent on CD8 T cells. European Journal of Immunology. **30:**3689-3698.

301. **van Schaik, G., C. H. Kalis, G. Benedictus, A. A. Dijkhuizen, and R. B. Huirne.** 1996. Cost-benefit analysis of vaccination against paratuberculosis in dairy cattle. Veterinary Record. **139:**624-627.

302. **Vicari, A. P., and A. Zlotnik.** 1996. Mouse NK1.1+ T cells: a new family of T cells. Immunology Today. **2:**71-76.

303. **Vordermeier, H. M., N. Venkataprasad, D. P. Harris, and J. Ivanyi.** 1996. Increase of tuberculous infection in the organs of B cell-deficient mice. Clinical and Experimental Immunology. **106:**312-316.

304. **Waters, W. R., J. R. Stabel, R. E. Sacco, J. A. Harp, B. A. Pesch, and M. J. Wannemuehler.** 1999. Antigen-specific B-cell unresponsiveness induced by chronic *Mycobacterium avium* subsp. *paratuberculosis* infection of cattle. Infection and Immunity. **67:**1593-1598.

305. **Wedlock, D. N., F. E. Aldwell, D. M. Collins, G. W. de Lisle, T. Wilson, and B. M. Buddle.** 1999. Immune responses induced in cattle by virulent and attenuated *Mycobacterium bovis* strains: correlation of delayed-type hypersensitivity with ability of strains to grow in macrophages. Infection and Immunity. **67:**2172-2177.

306. **Wedlock, D. N., E. E. Doolin, N. A. Parlane, S. J. Lacy-Hulbert, M. W. Woolford, and B. M. Buddle.** 2000. Effects of yeast expressed recombinant interleukin-2 and interferon- gamma on physiological changes in bovine mammary glands and on bactericidal activity of neutrophils. Journal of Dairy Research. **67:**189-197.

307. **Wedlock, D. N., L. P. Goh, A. R. McCarthy, R. G. Midwinter, N. A. Parlane, and B. M. Buddle.** 1999. Physiological effects and adjuvanticity of recombinant brushtail possum TNF-alpha. Immunology and Cell Biology. **77:**28-33.

308. **Wedlock, D. N., B. Vesosky, M. A. Skinner, G. W. de Lisle, I. M. Orme, and B. M. Buddle.** 2000. Vaccination of cattle with *Mycobacterium bovis* culture filtrate proteins and interleukin-2 for protection against bovine tuberculosis. Infection and Immunity. **68:**5809-5815.

309. **Weintraub, B., M. Jackson, and S. Hedrick.** 1994. γ-δ T cells can recognize non-classical MHC in the absence of conventional antigenic peptides. Journal of Immunology. **153:**3051-3058.

310. **Wells, S. J., and B. A. Wagner.** 2000. Herd-level risk factors for infection with *Mycobacterium paratuberculosis* in US dairies and association between familiarity of the herd manager with the disease or prior diagnosis of the disease in that herd and use of preventive measures. Journal of the American Veterinary Medical Association. **216:**1450-1457.

311. **Wentink, G. H., J. H. Bongers, J. H. Vos, and A. A. Zeeuwen.** 1993. Relationship between negative skin test with Johnin after vaccination and post mortem findings. Veterinary Record. **132:**38-39.

312. **Wentink, G. H., J. H. Bongers, A. A. Zeeuwen, and F. H. Jaartsveld.** 1994. Incidence of paratuberculosis after vaccination against *M. paratuberculosis* in two infected dairy herds. Zentralbl Veterinarmed [B]. **41:**517-522.

313. **Wentink, G. H., V. P. Rutten, F. H. Jaartsveld, A. A. Zeeuwen, and P. J. van Kooten.** 1984. [Diagnosis of paratuberculosis in the preclinical stage]. Tijdschr Diergeneeskd. **109:**739-750.

314. **Wijngaard, P. L., N. D. MacHugh, M. J. Metzelaar, S. Romberg, A. Bensaid, L. Pepin, W. C. Davis, and H. C. Clevers.** 1994. Members of the novel WC1 gene family are differentially expressed on subsets of bovine CD4-CD8- gamma delta T lymphocytes. Journal of Immunology. **152:**3476-3482.

315. **Wijngaard, P. L., M. J. Metzelaar, N. D. MacHugh, W. I. Morrison, and H. C. Clevers.** 1992. Molecular characterization of the WC1 antigen expressed specifically on bovine CD4-CD8- gamma delta T lymphocytes. Journal of Immunology. **149:**3273-3277.

316. **Wiker, H. G., and M. Harboe.** 1992. The antigen 85 complex: A major secretion product of *Mycobacterium tuberculosis*. Microbiology Reviews. **56:**648-661.

317. **Wilson, E., M. K. Aydintug, and M. A. Jutila.** 1999. A circulating bovine gamma delta T cell subset, which is found in large numbers in the spleen, accumulates inefficiently in an artificial site of inflammation: correlation with lack of expression of E-Selectin ligands and L-selectin. The Journal of Immunology. **162:**4914-4919.

318. **Wolff, J., R. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani, and P. Felgner.** 1990. Direct gene transfer into mouse muscle in vivo. Science. **247:**1465-1468.

319. **Wood, P. R., L. A. Corner, J. S. Rothel, J. L. Ripper, T. Fifis, B. S. McCormick, B. Francis, L. Melville, K. Small, K. de Witte, and et al.** 1992. A field evaluation of serological and cellular diagnostic tests for bovine tuberculosis. Veterinary Microbiology. **31:**71-79.

320. **Woodruff, H., and T. Gregory.** 1929. The prevention of tuberculosis in cattle. Further investigations to determine the value of the BCG vaccine for the prevention of tuberculosis. Journal of the Council for Scientific and Industrial Research. **2:**1-14.

321. **Yamamoto, S., T. Yamamoto, S. Shimada, E. Kuramoto, O. Yano, T. Kataoka, and T. Tokunaga.** 1992. DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells and inhibits tumor growth. Microbiology and Immunology. **36**:983-997.

322. **Yang, S., R. Tamai, S. Akashi, O. Takeuchi, S. Akira, S. Sugawara, and H. Takada.** 2001. Synergistic effect of muramyldipeptide with lipopolysaccharide or lipoteichoic acid to induce inflammatory cytokines in human monocytic cells in culture. Infection and Immunity. **69**:2045-2053.

323. **Yu, C. Y., and C. Milstein.** 1989. A physical map linking the five CD1 human thymocyte differentiation antigen genes. EMBO Journal. **8**:3727-3732.

324. **Zhongming, L., A. Howard, C. Kelley, G. Delogu, F. Collins, and S. Morris.** 1999. Immunogenicity of DNA Vaccines Expressing Tuberculosis Proteins Fused to Tissue Plasminogen Activator Signal Sequence. Infection and Immunity. **67**:4780-4786.

325. **Zumla, J. M. G. A.** 2000. Advances in the management of tuberculosis: clinical trials and beyond. Current Opinions in Pulmonary Medicine. **6**:193-197.