Brucella abortus RB51 Vaccine: Testing its Spectrum of Protective and Curative Characteristics

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Abstract

Brucella abortus (BA) are gram-negative, facultative intracellular bacteria that cause abortions in cattle and debilitating illness in humans. The US is now virtually free of bovine brucellosis, but the disease is endemic in wildlife. The official brucellosis vaccine in the US is strain RB51 (RB51). It elicits protective cell-mediated immunity (CMI) against BA infections.

Mycobacterium avium subspecies paratuberculosis (MAP) causes paratuberculosis in ruminants. It is a slow growing intracellular parasite that requires CMI for its control, belongs to the genus *Mycobacterium*, and is closely related to *M. avium avium* (MA*)*.

Using RB51 as a vector that induces strong protective CMI may be useful to protect against MAP if it expresses MAP protective antigens. Therefore, MAP 85A and 35kDa proteins were expressed at low levels in RB51, and the immune responses elicited by these vaccines in BALB/c mice were evaluated. Strong anti-*Brucella* immunity was generated, but the anti-mycobacterial response was low. To evaluate protective efficacy, a BALB/c model using MA was developed. When mice were challenged with MA, protection was obtained in some experiments but was inconsistent. This may be due to the low expression of MAP antigens in RB51.

Another objective was to evaluate the effect of an ongoing *Brucella*-infection on the efficacy of RB51 vaccination, and whether vaccination of already infected animals could have a curative effect. Mice acutely or chronically infected with virulent BA, rapidly cleared the RB51 vaccine organisms, but there was no significant decrease in the number of virulent BA.

Brucella spp. have been developed as biological weapons, but there are no vaccines to protect humans. The development of a very attenuated protective vaccine is necessary to prevent human infections, as well as to protect wildlife. To generate such a vaccine, RB51 based vaccines were irradiated to render them non-replicative, but metabolically active. We demonstrated that in general, irradiated and non-irradiated RB51 vaccines remain protective at levels similar to those elicited by the live vaccines. Therefore, irradiation of strain RB51 is an effective means of attenuating the strain without affecting its protective characteristics, and could eventually be used as a vaccine for wildlife and humans.

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LIST OF ABBREVIATIONS

A5	*M. avium* A5
Amp	Ampicillin
APC	Antigen presenting cell
ATTC	American Type Culture Collection
BCG	*Bacillus* Calmette Guerin
BSL-3	Biosafety Level 3 laboratory
cDMEM	Complete Dulbecco's Modified Eagle Medium
CFU	Colony forming units
Cm	Chloramphenicol
CMI	Cell mediated immunity
ConA	Concanavalin A
CTL	Cytotoxic lymphocyte
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DTH	Delayed Type Hypersensitivity
ELISA	Enzyme Linked Immuno Sorbent Assay
Esat-6	Early Secreted Antigenic Target
FBS	Fetal Bovine Serum
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
HK	Heat killed
IR	Immune response
kDa	Kilodalton
KO	Knock-out
L. mono	*Listeria monocytogenes*
LPS	Lipopolysaccharide
LPA	Lymphocyte proliferation assay

MAP	*M. avium subsp. paratuberculosis*
MHC	Major Histocompatibility Complex
mg	milligram
MW	Molecular weight
MTB	*Mycobacterium tuberculosis*
NCBI	National Center for Biotechnology
NK	Natural Killer cell
O.a	*Ochrobactum anthropi*
OADC	Oleic Acid Dextrose Catalse supplement
pBB	Plasmid pBBR1MCS
PBS	Phosphate Buffer Saline
pg	picogram
pTB	Paratuberculosis
Rif	Rifampicin
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel
SOD	CuZnSOD
TBS	Tris Buffer Saline
Th1	T helper 1
Th2	T helper 2
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TSB	Tryptic Soy Broth
TSA	Tryptic Soy Agar
µg	microgram

CHAPTER 1

Literature Review

General Characteristics of the Genus *Brucella*

At the end of the 19th century, Dr. David Bruce an English military doctor in the island of Malta, first identified *Brucella* as disease causing microorganisms. Thus, the name given to the bacterium and one of the names of the disease caused by it: *Brucella* and Malta fever or brucellosis respectively. *Brucella spp*. were later isolated from aborted bovine fetuses by Dr. Bang, a Danish veterinarian, and therefore the test used to identify the disease as well as the disease in cattle are also known as Bang's test and Bang's disease (281). *Brucella spp.* are small, gram negative, non-motile, non-sporulating, microaerophilic coccobacilli. They are part of the α-2 subclass of proteobacteria and are genetically related to *Ochrobactum spp.*, *Agrobacterium spp.*, *Rhizobium spp*. and *Phylobacterium*. s*pp* (86, 410). It is believed that *Brucella* were originally a plant pathogen that jumped to mammalian hosts during its evolution (257, 335, 340, 410). The genus *Brucella* consists of seven highly related species (257), that due to their high genetic homology, have recently been proposed to be only biovars within one species (309). The species possess certain host specificity; *B. melitensis* infects mainly caprine and bovines, *B. abortus* affects bovines and some wildlife species such as elk and bisons, *B. ovis* infects sheep, *B. canis* infects dogs, *B. cetacea* or *B. pinnipedae* affect marine mammals, *B. suis* infects swine and *B. neotomae* has been isolated from a desert wood rat (86). The disease has been identified in wild and domestic animals worldwide. It is endemic in Middle Eastern, Latin American, Asian, African and some European countries (51, 138, 242, 336). Human infections have been described with *B. melitensis, B. suis, B. abortus* and occasionally with *B. canis* (433). The severity of the disease in humans is of decreasing magnitude respectively. Recently, human cases of brucellosis due to marine mammal *Brucella* species have been reported (368). The different species

of *Brucella* may be differentiated based on their host preference, phage specificity, dye sensitivity, CO_2 requirement, the type or proportion of surface antigens they possess (A or M) and colony morphology (86).

Most *Brucella spp.* contain two true chromosomes, both encoding fundamental genes. *B. abortus*, *B. melitensis*, *B. ovis*, *B. neotomae* and *B. suis* all have a chromosome of 2100 kb, and a smaller one of 1150 kb. *B. suis* 3 has only one chromosome of 3.1 Mb and *B. suis* 2 and 4 have two chromosomes of 1.85 and 1.35Mb respectively. The smaller chromosome appears to have been a plasmid acquired early during *Brucella's* evolution and crucial genes were then transferred onto it. DNA analysis among all these biovars indicates an approximate GC content of 58% and over 95% homology (309). The genome of *B. suis* (309) and of *B. melitensis* (94) have recently been sequenced and sequencing of the genome of *B. abortus* is in progress (340). *Brucella* organisms lack the classic virulence determinants such as exotoxins, plasmids, flagella, fimbriae, lysogenic phages, highly endotoxic LPS, cytolysins etc., that are usually associated with pathogenic bacteria (132, 309, 370). Instead, *Brucella's* virulence seems to rely on those genes that allow it to invade, resist killing and replicate inside phagocytes (66, 152, 335).

Brucella contains a complete *virB* operon that is highly homologous to that found in *Agrobacterium spp.* In this latter species the *virB* operon encodes for a type IV secretion system involved in DNA and protein transfer. No substrates for this secretion system have yet been identified in *Brucella,* but studies performed using deletion mutants for several of the genes in this operon have been shown that an intact VirB system is necessary for virulence and intracellular invasion (52, 66, 286, 385).

The lipopolysaccharide (LPS) outer layer of the bacterial cell wall is the major antigenic and toxic component of gram negative bacteria (363). It consists of a lipid A (major endotoxic portion of LPS), an oligosaccharide core, KDO (2-keto-3- deoxyoctonic acid), and an O-side chain (O-polysaccharide, O-antigen, O-chain), that is the major antigenic portion of the LPS (258, 378). The LPS and its O-side chain stimulate the production of antibodies by B cells by initiating a signal transduction cascade upon interaction with specific pattern recognition receptors (287). *Brucella's* LPS

characteristics differ from that of other gram negative bacteria in that it is less endotoxic. This feature is due to a modification in the lipid A fatty acid performed only by live virulent *Brucella,* and dependent on the presence of an intact BvrRS regulatory system. This system is required for the homeostasis of the outer membrane and is crucial for cellular invasion as it is involved in actin polymerization which is modulated during *Brucella* invasion (226). The structure of the O-chain in *Brucella* consists of repeating units of 4,6-dideoxy-4-formamido–α-D-pyranosyl linked by an α1-2 linkage. There are variations on the linkage of these chains depending on the LPS characteristics, yielding two strain classifications: A and M strains. A strains have homopolymers linked by an α 1-2 linkage, while M strains have an α 1-3 linkage between every fifth residue (139, 259). Not all *Brucella* species and strains possess the O-side chain. Those that do, have a "smooth" colony appearance and exclude the crystal violet dye. Smooth species include *B. melitensis, B. abortus, B. suis* and the marine mammal species *B. pinnipedae* and *B. cetacea*. Species and strains that lack the O-side chain are described as "rough" and stain purple with the crystal violet dye. Naturally rough species are *B. ovis* and *B. canis* (3, 86, 351). Smooth strains are regarded more virulent than rough strains, although this is not the case for all species; clearly, *B. ovis* and *B. canis* are virulent for sheep and dogs respectively, although they are not able to infect other species (86). Interestingly, *Brucella* species have 8-35 copies of the IS711 insertion sequence in their genomes. This sequence is inserted in several different genomic locations in different species and biovars thus allowing their identification (58, 165). *B. abortus* vaccine strain RB51 has one such insertion sequence disrupting the *wboA* gene which codes for a glycosyltransferase involved in the O-chain biosynthesis (420). This mutation is only partially responsible for the rough phenotype of the strain, as demonstrated by complementation experiments in strain RB51. The phenotype of the strain complemented with the *wboA* gene remains smooth, but the O-chain is expressed in the cytoplasm of the bacterium (420), indicating the existence of additional mutations affecting the transport of the O-side chain to its final location. The *wzt* gene of *Brucella* has been recently identified. This gene codes for a protein product that bears similarity to two-component

ABC transporters, and is required for the translocation of the O-chain across *Brucella's* inner membrane (76, 147). Interestingly, vaccine strain RB51 appears to have a mutation in this gene (R. Vemulapalli, S.M. Boyle, N. Sriranganathan and G.G. Schurig, Personal Communication). As mentioned above, the O-chain is the major antigenic determinant of *Brucella*, and therefore the presence of antibodies directed to this O-chain has been widely used as a positive marker for infection in diagnostic tests (137, 260, 296, 332, 377).

Animals and humans become infected with *Brucella* when their mucosal membranes or broken skin come in contact with infected secretions (milk, blood, uterine discharge) or aborted fetuses (200). In animals, brucellosis may range from no apparent signs of infection, to orchitis and decreased fertility in rams, to abortion in goats, cows, dogs and elk. In humans, brucellosis has many manifestations. It usually is associated with marked malaise, undulant fever, joint aches, and orchitis. It is known that *Brucella* species are able to colonize the human fetus but its correlation with abortion has not been clearly established. One study indicates that among a population of women in an area where brucellosis is endemic, those serologically positive to *Brucella* had abortion rates significantly higher than those serologically negative (194). *Brucella* infection has also recently been associated with premature delivery and lower birth weight in babies born to infected mothers (234). If the disease in humans is not treated appropriately with antibiotics, it results in chronic brucellosis with relapses of articular problems, neurobrucellosis and in some cases depression (346, 433).

In bovines, the susceptibility to infection depends on the route and dose of exposure, as well as the age and gestation stage of the animal. Adult cows are more sensitive to infection and among these, pregnant animals are the most susceptible (303). This susceptibility is thought to be related to the concentration of the sugar erythritol in the gravid bovine uterus (351). *Brucella* organisms invade the host through microabrasions in the skin or through mucous membranes and replicate in the retropharyngeal lymph nodes resulting in local lymphadenopathy, then they spread through the blood stream while replicating inside macrophages. Finally the infection is

established with preference for the bovine female genital tract tissues, uterine trophoblasts and fetus. Abortion typically occurs during the third trimester of gestation (86, 433). Although some damage to placental structures (namely necrosis of the cotyledons) has been described in cattle and mice experimentally infected with virulent *Brucella* (391), the exact pathogenesis of the abortion is not clearly understood. Birth of weak or non-viable calves, placental retention, mastitis and granulomas are also frequent findings of the disease. The infected animal will shed virulent *Brucella* through milk, as well as in afterbirth and aborted secretions (86). The aborted fetus will also be highly contaminated and the abomasal content one of the samples preferred for isolation of the bacterium.

The enormous cost of brucellosis to dairy, beef and goat industries, as well as its impact on public health, has prompted many countries to adopt brucellosis control and eradications programs (7, 51, 96, 336). In the USA, a brucellosis eradication program was established in 1954 with the aim of eradicating *B. abortus* infections from cattle. The program was successful and currently the USA is essentially free of cattle brucellosis. Unfortunately, the threat is far from over. Trade and wild animals continue to jeopardize the domestic animal population. Brucellosis is still endemic in bison, elk and feral swine that graze or inhabit the same pastures as cattle; raccoons and coyotes have also been shown to become infected with *Brucella,* although their role as reservoirs of the disease is not known (92, 203, 349). Sporadic human infections are still observed in high risk groups that come in contact with wild animals, such as hunters. Therefore, current efforts are aimed at developing effective vaccines and delivery methods to protect susceptible wildlife (92). Pivotal to the success of the eradication program has been the implementation of adequate diagnostic tests to identify infected animals and the development of efficient vaccines to protect against the disease. Originally, the American brucellosis eradication program involved the use of the smooth *B. abortus* derived attenuated vaccine Strain 19. This vaccine was switched to the rough *B. abortus* strain RB51 in 1996 (see brucellosis vaccines below), which has the advantage of not inducing O-side chain antibodies that interfere with the serological diagnostic tests (319).

In addition to the potential danger of re-introduction of brucellosis into cattle populations and its risk as a zoonosis, *Brucella* is also considered a class 3 biowarfare agent (180, 325) thus making the development of effective vaccines to protect humans an important future goal of some research institutions such as the US Department of Defense.

Immunology of *Brucella*

The mammalian host is equipped with various non-immune and immune mechanisms to combat invasion by foreign organisms. The first barrier against infection is a physico-chemical shield provided by the skin and mucous membranes. Specific mucosal IgA antibodies as well as lysozymes, and other enzymes provide some degree of protection against potential pathogens. Upon penetration of the bacteria into the host, an innate non-specific response is mounted. This response relies mainly on PMNs and non activated macrophages that attempt to destroy the invading organism. The polymorphonuclear (PMN) phagocytes circulate to protect the body and can ingest and destroy the invaders in a non-specific manner (301). The complement system can also eliminate microorganisms by deposition of proteins onto the bacterial surface that serve as opsonins, attract phagocytic cells, and form and assemble the membrane attack complex (MAC), which causes lysis of the targeted bacterium. The classic complement pathway and the lectin pathway but not the alternative complement pathway have been implicated in destruction of *Brucella* (130, 200). Microbes possess pathogen associated molecular patterns (PAMPs) that are recognized by macrophage pattern recognition receptors (PRR) (271, 272). Among these is CD14, a receptor that recognizes the lipid A portion of bacterial LPS when complexed with the LPS binding protein, a soluble PRR (272). Toll- like receptors (TLR), originally identified in *Drosophilla melanogaster*, are a highly conserved family of PRR expressed on the membrane on leukocytes and are involved in innate immunity (271). TLRs recognize a broad range of ligands including

LPS, dsRNA, CpG, and some bacterial proteins (271). Ligation results in signaling by the recruitment of the adaptor proteins MyD88 and TIRAP. This activation induces a signal transduction cascade that up-regulates NF-kB, resulting in the ultimate production of IL-12 and IL-8, and also the recruitment of NK and some γδ T cells (177). This pathway is believed to be the first event that drives the immune response towards a Th1 type cytokine profile, the key branch required for the establishment of protective immunity against intracellular pathogens (148). It has been suggested that the modification in the lipid A could allow *Brucella* to avoid interaction with the macrophage's Toll-like receptors (TLR) and therefore down regulate inflammatory responses mounted against it (329). However, it appears that *Brucella* is able to activate both TLR-4 and TLR-2 a process that leads to secretion of IL-12, TNF-α and IFN-γ, but purified LPS and lipid A are only able to stimulate TLR-4 (63, 177). Interestingly, when a CHO cell line was transfected with CD14 and TLR-4, marked differences in the effect of the lipid A moiety of smooth and rough *Brucella* were observed; smooth *Brucella* lipid A, as opposed to lipid A from rough strains, is more efficient at stimulating TLR-4 and driving dendritic cell maturation (63). Heat killed *Brucella* however, seem to only be able to stimulate TLR-2 in a process that is MyD88 dependent and that this process is involved in secretion of TNF-α by *Brucella* activated cells (177).

Brucella organisms are intracellular pathogens that actively seek and are able to thrive and replicate inside professional and non-professional phagocytes in their mammalian hosts (16, 66, 152, 312). *Brucella* is able to survive inside non-activated macrophages, the same cells that are ultimately responsible for its clearance (24, 152, 164). Different *Brucella* differ in their ability to invade and survive within macrophages, and this ability correlates with their virulence in vivo (24). Virulent *Brucella* appear to actively re-direct traffic through the intracellular compartment to end up in compartments associated with the endoplasmic reticulum and traffic within vacuoles of the autophagic system (81, 102, 152, 223, 359). It has been demonstrated that virulent *Brucella* actively prevent phago-lysosome fusion and do not acquire the late lysosomal markers (66, 202, 312, 322). Upon invasion, *Brucella* tends to bind preferentially to the basolateral plane

of the cell. It seems that recruitment of actin is also required for invasion in HeLa cells and membrane ruffling dependent on an intact *virB* operon takes place at the site of contact (158). Opsonized *Brucella* are internalized by complement and Fc receptors and recently have been shown to associate with the Scavenger Receptor A (SR-A) (62, 195) and some other yet unidentified receptors. Phagocytosis by M cells, macrophages and neutrophils is carried out by a zipper-like mechanism (2). The mechanisms for invasion seem to have important repercussions in the cellular localization of the ingested organism and its ultimate fate: opsonized bacteria are more efficiently killed and degraded by macrophages, with better fusion of the phagosome with the lysosome than those that are not opsonized (155).

Intracellular *Brucella* blocks or modifies several macrophage functions in order to avoid its destruction. Infection of monocytes with smooth *B. melitensis* protects infected cells from apoptosis, but rough strains fail to do so, which suggests a potential role of the O-polysaccharide in this event (131). Virulent *B. suis* is able to prevent macrophage apoptosis, not only of those macrophages containing live organisms but also of non-infected neighbors suggesting the presence of soluble mediators. This protection against apoptosis has been correlated with the up-regulation of *Brucella*'s A1 gene, a homologue of Bcl-2 family of anti-apoptotic genes, and these *Brucella* infected cells are also more resistant to Fas-ligand or IFN–γ induced apoptosis (155). Infection of a human macrophage cell line with live *B. suis,* has shown that these cells are able to produce IL-1, IL-6, and IL-8 but no TNF–α. However, infection with heat killed organisms induces the production the above cytokines but also of TNF–α indicating that *Brucella* actively blocks the production of this cytokine (65). This inhibition appears to be regulated by the *Brucella* outer membrane protein OMP25 (103). On the other hand, mouse cells of different lineage produce TNF–α very rapidly after infection with several strains of *Brucella*, a finding is corroborated by in-vivo experiments using BALB/c mice (23).

The interaction of the pathogen with PRR on APCs leads to secretion of chemokines that attract NK cells to the inflammation site and of IL-12 which promotes secretion of IFN-γ by these NK cells. This begins a positive feedback loop in which the

IFN-γ secreted by NK and T cells, together with IL-12 and other cytokines such as IL-18 (also secreted by APC), further increases IFN-γ production. IFN-γ has been shown to be one of the crucial protective cytokines for the control of brucellosis and other diseases caused by intracellular pathogens whereas IL-4 a Th2 cytokine, is associated with decreased protection (183, 263). Several studies have shown that *Brucella* infections induce secretion of IFN-γ and TNF-α but no IL-4 (4, 22, 23, 307).

In human infections, a fraction of lymphocytes bearing γδ markers becomes activated. This activation seems to be driven by a *Brucella* non-peptidic fraction of low molecular weight. This fraction is a potent stimulator of these lymphocytes directly activating them to secrete IFN–γ and TNF–α. TNF-α is a proinflammatory cytokine that drives the secretion of IL-12 and IFN–γ, directing an acquired secondary response of the Th1 phenotype by CD4+ and CD8+ lymphocytes (23, 177). In addition, activated γδ cells become directly cytotoxic against infected cells. This particular subset may be of crucial importance in the establishment of an adequate protection in the bovine species where up to 50% of circulating lymphocytes are of γδ phenotype (68, 70).

B lymphocytes will drive the humoral immune responses and antibody production by plasma cells. Antibodies are believed to confer reasonable or only marginal protection against brucellosis depending on the animal species affected (14, 15, 227, 331).

Immunity to *Brucella spp.* and to other intracellular pathogens requires CD4+ T cells of the Th1 cytokine profile and also activation of CD8+ CTLs (170, 263, 290, 291). Activation of T cells requires a double signal provided by the binding of the MHC molecule containing the antigenic peptide with the T cell receptor and the interaction of B7.1/B7.2 on the APC with CD28 on the lymphocyte (148).

Although *Brucella* are able survive inside resting macrophages, the destruction of at least some *Brucella* is crucial for activation of T cells. Activation of CD4+ cells is dependent on the presentation of short peptide sequences by MHC-II molecules on APCs (21). Exogenous antigens, resulting from the endocytosis of a foreign organism are degraded by enzymes contained in the phago-lysosome. The MHC-II molecules are synthesized in the endoplasmic reticulum (ER) where the complex is assembled and the

fully assembled complex is then transported to the Golgi apparatus where the exogenous peptides are loaded onto these molecules. The MHC-II:peptide complexes are then transported to the cell surface where they are presented to CD4+ cells (148). CD4+ cells (T helper cells) have been classified into two subsets depending on the types of cytokines they secrete. T helper 1 cells (Th1), secrete IFN-γ and IL-2 among other cytokines. T helper 2 cells (Th2) secrete mainly IL-4 and IL-10 (432).

Antigen presentation to CD8+ cells is carried out in the context of MHC-I molecules which present endogenous antigens (90). Cytosolic proteins are degraded by the proteasome, a barrel shaped enzymatic complex located in the cytoplasm of eukaryotic cells, and the degraded peptides are then actively translocated into the endoplasmic reticulum by an ATP dependent transporter associated with TAP molecules (217). The MHC-I-peptide complex is then assembled and continues its path to the Golgi apparatus and finally onto to the cell surface where it is presented to CD8+ cells (90, 218). This compartmentalization of the two pathways suggests that the localization of the infecting organism will determine the ultimate recruitment of effector cells. Extracellular organisms mainly induce MHC-II restricted peptides which are recognized by T helper cells, while viral peptides, which use the cellular machinery, are presented in an MHC-I context to cytotoxic lymphocytes (217). Intracellular bacteria that enter into the host's cytosol, such as *Shigella*, induce MHC-I presentation to CD8+ cells. Other intracellular bacteria such as *Brucella* and *Mycobacterium,* remain in compartments separate from the cytosol and induce MHC-II restricted peptides but are also able to induce presentation to CD8+ cells indicating that some exogenous peptides do reach the cytosol (21, 279). The process by which these exogenous antigens are presented in an MHC-I context is not clearly understood. Some theories propose the "leakage" of peptides from the ER into the cytoplasm, where they are then processed by the proteasome and continue in the MCH-I path, but recently the active role of dendritic cells in this cross priming has become apparent. These cells are able to translocate exogenous antigens from the endocytic to the cytosolic compartment of the cells, where they are processed by the proteasome and assembled into MHC-I molecules to be presented to CD8+ T cells (217).

The importance of T cells for protection against brucellosis has been demonstrated in passive transfer experiments. In an experiment by Araya and Winter (15), transfer of T cells was shown to significantly protect against *Brucella* challenge. Jimenez de Bagues et al., (186) performed a similar experiment in which T cells from mice vaccinated with either vaccine strain 19 or vaccine strain RB51 (described below), were able to induce significant protections against challenge with virulent *Brucella*. It has also been shown that vaccine strain RB51 induces cytotoxic lymphocytes that secrete IFN-γ and are able to kill infected macrophages (170).

IFN-γ is a type II IFN secreted by Th1 CD4+ T cells, NK cells, CD8+ cytotoxic cells, B cells and other antigen presenting cells (350). It interacts with the IFN-γ receptor and signals through the Jak/Stat signaling pathway. Binding of IFN-γ with its receptor triggers a conformational change in this structure that results in the autophosphorylation of Jak2 which in turn transphosphorylates Jak1. Once Jak1 is activated, it phosphorylates specific tyrosines in the IFN-γ receptor chain 1 that provide the attachment site for a Stat-1 homodimer. This molecule is then phosphorylated and it dissociates from the receptor, translocating into the nucleus where it binds specific GAS sequences in the DNA where it promotes the transcription of different genes and of other transcription factors that will drive the IFN-γ induced responses. The role of IFN-γ in protection is several-fold: it induces the up-regulation of cell surface MHC-I molecules which results in efficient induction of cell mediated immunity and recognition of foreign peptides by cytotoxic lymphocytes. IFN-γ also up-regulates chaperones involved in the assembly of the MHC-I:peptide complex and it induces the replacement of some of the proteasomal units for other "immune subunits" which increase the quantity and repertoire of the peptides generated for MHC-I loading. It also increases the quantity of MHC-II: peptide molecules displayed on the surface of APC and is able to induce expression of MHC-II molecules in non-professional phagocytes. It increases expression of the MHC-II chains and of lysosomal proteases involved in the degradation of peptides to be loaded onto MHC-II. All these events ultimately make the APC more efficient in presenting antigens to T and B cells (90). IFN-γ also increases the pinocytic and phagocytic activity of

macrophages and the bactericidal ability of macrophages by up-regulating the oxidative burst mechanism (NADPH-dependent phagocyte oxidase), up-regulating lysosomal enzymes and inducing the production of nitric oxide (NO) and the generation of reactive nitrogen intermediates (RNI) (149, 213, 350). The importance of IFN-γ in protection against virulent *Brucella* has been demonstrated in several systems (23, 128, 183, 263, 291, 413). It is mainly thought to act through the activation of macrophages making them more efficient at killing intracellular *Brucella,* as only macrophages activated by the IFN–γ, as opposed to their non-activated counterparts, are able to efficiently kill intracellular parasites including *Brucella* (24, 149, 184, 224). During intracellular infections, IFN-γ and TNF-α induce Interferon regulatory factor 1 (IRF-1), which activates macrophages. This was shown in studies using IRF-1-deficient (IRF-$1^{-/-}$) mice that are more susceptible to BCG infection than wild type mice, and die when infected with virulent *B. abortus* but not when infected wit attenuated strains (199).Therefore, IFN-γ is crucial during the very early stages of infection, but its importance is somewhat diminished during the latency phase and in chronic stages (24, 122, 149, 184). It is thought that during the later stages of *Brucella* infection, CD8+ T cells play a fundamental role in controlling the infection by killing infected macrophages (263).

IL-10 is an anti-inflammatory cytokine secreted by T cells and macrophages. It interacts with the IL-10 receptor and like IFN-γ, signals through the Jack/Stat signaling pathway. It down-regulates inflammatory responses by modulating the expression of cytokines, cell surface molecules and of soluble mediators (254, 280). IL-10 inhibits the production of IL-1α, IL-1β, IL-6, IL-12, IL-18, GM-CSF and TNF among others, which has profound effect on inflammatory responses. It also inhibits the secretion of most of the inducible chemokines in activated macrophages and up-regulates expression of chemotactic molecules. It exerts an indirect effect on IFN-γ by down-regulating IP-10, a protein induced by IFN-γ that attracts Th1 cells. IL-10 modulates cytokine production by different mechanisms including destabilization of mRNA, inhibition of mRNA degradation, enhancing expression of cytokine antagonists and down-regulating the expression of accessory molecules required for the function of these cytokines (254, 264).

The importance of IL-10 in regulating inflammatory responses has been demonstrated in several models. Administration of IL-10 rescues BALB/c mice from LPS induced endotoxic shock by down regulating TNF-α (144, 235). IL-10 -/- mice or mice treated with anti-IL-10 antibodies are much more susceptible to endotoxin induced lethality that normal mice and have severe inflammatory responses (42, 206). Additionally, human production of IL-10 during septicemia is associated with the intensity of the inflammatory response and with the outcome of the disease (278).

Brucella infections lead to secretion IL-10 but it has been shown that IL-10 production does not down-regulate the production of IFN-γ during *Brucella* infections (127, 129, 307). The initial identification of IL-10 in *B. abortus* infected mice was thought to be detrimental for protection against this pathogen (127, 129). However, analysis of the cytokine profile induced by vaccination with strain RB51 indicates that although IL-10 is produced at high levels after vaccination with this strain, mice are nevertheless protected against challenge (307). Therefore, the role of IL-10 during *Brucella* vaccination of infection may be to regulate the inflammatory response.

Protective Antigens of *Brucella spp.*

O-side chain

This oligosaccharide chain is the major antigenic determinant of *Brucella spp.* (139, 252, 258). Animals infected with smooth *Brucella* strains develop strong antibody responses directed against this antigen (283, 327, 378). Although cell mediated responses are the main protective immune mechanism against *Brucella* infections (432), a certain role for antibodies has been established. Araya et al., (15) demonstrated that passive transfer of immune serum from strain 19 or strain 2308 inoculated mice conferred protection against challenge with virulent *Brucella*. The role of antibody mediated protection is apparently dependent on the animal host and the *Brucella* species used. Protection against *B. melitensis* in goats apparently requires a certain level of O-chain specific antibodies since strain VTRM1, a rough mutant derived from *B. melitensis,*

failed to induce protection when administered as a single to dose to pregnant goats (110). The protective role of the O-chain antibody responses is further emphasized by the increased protection observed in mice vaccinated with a *B. abortus* RB51 strain expressing the *wboA* gene (described below) relative to strain RB51 not expressing the O–chain. Mice vaccinated with this strain were significantly better protected against challenge with virulent *B. abortus* strain 2308 than mice vaccinated with strain RB51, and also developed protective immunity to challenge with virulent *B. melitensis* (414, 417). However, in bovines the role of O antibodies is not completely clear, but appears to be of little importance (163, 282).

Superoxide dismutases

Superoxide dismutases (SOD) are metallo-enzymes that catalyze the dismutation of superoxide into hydrogen peroxide and molecular oxygen, thus preventing damage by these reactive oxygen species (ROS). Superoxides are the byproducts of aerobic respiration produced in multiple reactions, including the production of microbicidal ROS during the respiratory burst in the phago-lysosomes of infected macrophages (39). SODs are considered major virulence determinants in many bacterial species because they allow the bacterium to resist the bactericidal activity of superoxide radicals (184). *Brucella* Cu/ZnSOD, encoded by the *sodC* gene, is located in the periplasmic space of *Brucellae* (375). This gene is highly conserved among *Brucella* biovars (372). Studies using *B. abortus sodC* deletion mutants have been somewhat controversial. One study indicates that Cu/ZnSOD deletion mutants are able to survive inside J774 and HeLa cells (388), but show decreased survival in the spleens of BALB/c mice. In contrast a second study found no difference in the same mouse model between wild-type and mutant strains (216). In either case, Cu/ZnSOD mutants were still able to establish a chronic infection in the mouse. *Brucella* Cu/ZnSOD has been shown to be a protective antigen under several experimental conditions. Recombinant *E. coli* expressing *Brucella* Cu/ZnSOD and strain RB51 over-expressing SOD have been demonstrated to elicit strong protective responses in mice against challenge with virulent *Brucella* (299, 300). Also DNA

vaccines and *O. anthropi* expressing *Brucella* SOD are able to induce protection against challenge with virulent *Brucella* in mice (169, 299).

Outer membrane proteins

The outer membrane proteins (OMP) of *Brucella* are a group of immunogenic proteins that, as their name indicates, are located in the outer membrane of the organism (77). Serological analysis of mice and human sera indicated that infected individuals can mount responses to these antigens. Some of these proteins have shown some vaccine potential. The OMP31 has been shown to induce protection against *B. ovis* challenge (120). A deletion mutant of OMP25, has been shown to be attenuated in mice and to induce some protection against *B. melitensis* challenge in goats (105).

Heat Shock Proteins

Heat shock proteins (Hsp) are cytoplasmic proteins induced during stress periods. These proteins serve as chaperones aiding in the folding, assembly and transport of proteins. Several Hsp of *Brucella* have been analyzed, among them the GroES/EL antigen (20, 333). These proteins have been analyzed for protective efficacy in recombinant *E. coli* vectors, DNA vaccines as well as by over-expressing them in *Brucella* (220, 416). No clear protective responses have been observed. In the case of GroEL, serological and cytokine responses of the Th1 type were obtained using a DNA vaccine model but no protection was elicited (220). The same occurred with over-expression of the gene in vaccine strain RB51 (R. Vemulapalli, G.G Schurig, Personal communication). The HtrA protein, a member of the serine proteases, has also been analyzed, although it was found to be important for virulence and intracellular survival, its role in protection could not be clearly established (330).

L7/L12

This ribosomal protein has been studied in several vector and vaccination models. It has been described as a T-cell reactive antigen. Antibody and delayed type

hypersensitivity (DTH) responses to this protein have been demonstrated in cattle and mice vaccinated with strain 19 or infected with virulent strain 2308 respectively. Vaccination of mice using L7/12 protein in a DNA vaccine and also as a fusion protein has conferred protection against *B. abortus* challenge in mice (207, 292).

Existing and Tested Brucellosis Vaccines

Strain 19

B. abortus Strain 19 was one of the first vaccine strains used to fight brucellosis. It was developed and identified almost by accident during the 1920s. Anecdotal reference indicates that a culture of *B. abortus* was left on the counter for several years; upon retesting the strain for virulence, it was found to be attenuated in mice (9). Subsequent cattle trials showed that this live attenuated vaccine was able to confer protection in cattle against bovine brucellosis (7, 228, 379). The mechanism of attenuation of the strain is still not well understood but may be related to a decreased capacity to utilize erythritol (121). This strain was and still is widely used in brucellosis eradication programs outside the US (7, 138, 404). Although the strain confers protection against virulent *B. abortus* in cattle, it has the disadvantage of being fairly virulent to humans and of producing O-chain antibodies that interfere with diagnostic tests making it hard to differentiate infected and vaccinated animals (352).

H38

This is a *B. melitensis* based formalin killed vaccine used for vaccination of goats and sheep. Because it is made of killed smooth *B. melitensis*, vaccinated animals develop O-chain antibodies that interfere with diagnosis. It confers variable protection and induces local reactions at the inoculation site (249, 352).

B. suis strain 2

This vaccine, an attenuated smooth strain derived from biovar 1 of *B. suis,* was originally developed in China. It has been used with varying degrees of successes to vaccinate sheep and swine (47, 266). Although it does induce the development of O-chain antibodies that interfere with the serological diagnosis of the disease, these antibodies seem to decline and disappear by one year post vaccination (266).

Strain 45/20

This vaccine has also been used in brucellosis control programs (9). It is an attenuated live strain derived from *B. abortus.* It has the disadvantage of serological interference and more importantly, when administered as a live vaccine it reverts to virulent *in-vivo,* therefore it needs to be used as a bacterin, making it less effective (352).

Rev1

This vaccine is a spontaneous mutant derived from *B. melitensis* (352). It confers protection against *B. melitensis* infection in goats and is widely used in the caprine industry in endemic areas (7). It also protects cattle, but because it is a smooth strain, it has the disadvantage of strongly interfering with diagnostic tests (352). Vaccination of goats with the full dose of the vaccine results in abortion; therefore, vaccination with a reduced dose has been proposed. However, vaccination with a reduced dose fails to induce adequate levels of protection. Also, the degree of immunogenicity and residual protection of the vaccine shows marked differences depending on the batch and source of the vaccine being used (45). Rev1 induces protection in lambs against *B. ovis* challenge, but the vaccine is still virulent in this species as cases of post vaccinal epididymitis have been observed (48). The vaccine is virulent for humans and several cases of Rev1 induced brucellosis have been reported (28, 35, 46, 200). An additional concern is that this strain is resistant to streptomycin, one of the antibiotics used to treat brucellosis (352).

Vaccine strain RB51

B. abortus vaccine strain RB51 is a naturally rough derivative of *B. abortus* strain 2308 (351). This strain is attenuated in mice and cattle and is able to induce protection in cattle against challenge with virulent *B. abortus* at a level similar to that conferred by Strain 19 (69, 109, 186, 228, 294, 307). It is thought that this strain possesses at least two mutations in its LPS biosynthetic pathway. One of these has been traced to the presence of an IS711 element in the *wboA* gene responsible for a glycosyltransferase involved in the synthesis of the O-chain (420). Complementation studies using the *wboA* gene have shown that the strain maintains the rough phenotype but is able to produce O-side chain that remains in the cytoplasm of strain RB51, indicating that at least one other mutation in the LPS synthesis pathway has occurred (417). Recently, other genes involved in *Brucella's* LPS biosynthesis have been described (76). Among them is the *wzt* gene that codes for an ABC type transporter which is involved in the translocation of the O-side chain across the inner membrane of *Brucella* (147). A mutation in the *wzt* gene has been identified in strain RB51 (R. Vemulapalli, SM. Boyle, N. Sriranganathan and G.G. Schurig, Personal Communication), which may account for the cytoplasmic location of the O-side chain in strain RB51wboA. Vaccination with strain RB51 does not induce antibodies to the smooth LPS's O-chain thus, it does not interfere with the diagnostic tests for brucellosis used in the field (351, 377, 379, 380). Some studies using monoclonal antibodies have detected traces of smooth LPS in strain RB51, but there is no evidence of O-side chain antibody induction by RB51 vaccination (78).

Vaccination with strain RB51 induces cell mediated immunity with a polarized type 1 cytokine profile that is accepted as the desired type to induce protection against intracellular parasites (170, 183, 416). Pasquali et al., (307) have reported that in mice vaccinated with strain RB51, the vaccine organisms are observed in the spleen 6 days post vaccination with a peak at 18 days and a continuous decline until complete clearance by day 42. Cytokine profiles of mice vaccinated with strain RB51 show higher levels of IFN-γ than non-vaccinated/challenged mice and that IL-4 is not detected. This finding has

also been reported by He et al., (170) who did not detect this Th2 associated cytokine in response to vaccination with strain RB51.

It has been shown that vaccination with strain RB51 or infection with virulent *Brucella* also induces the production of IL-10 (307, 413). Although the presence of IL-10 has been associated with increased susceptibility to infection with *Brucella spp.* (23), mice vaccinated with strain RB51 are protected against *B. abortus* infection; therefore, the role of IL-10 in this case might be to limit the elicitation of an exacerbated immune response (307, 413).

Finally, vaccination with strain RB51 induces CD8+ T cells with cytotoxic activity against *Brucella* infected macrophages. These cytotoxic cells play a fundamental role in controlling the infection not only because of the direct killing effect on infected cells but also by secretion of IFN-γ which further increases the anti-*Brucella* response (263),(170). As discussed above, IFN-γ's role in protection is mainly through the activation of macrophages making them more efficient APC and increasing their bactericidal capacity. It is thought that during the later stages of infection, CD8+ T cells play a fundamental role in controlling the infection by killing infected macrophages (263).

Experimental Vaccines

Killed and subunit vaccines

Vaccination attempts using heat killed or otherwise dead *Brucella* vaccines without strong adjuvants have been unsuccessful. It is thought that killed organisms are not able to induce the appropriate immune response required for protection against intracellular organisms. Similarly, subunit vaccines have been unable to induce high levels of antigen expression and induce protection against virulent *Brucella* challenge (253, 282, 352).

Viral vector vaccines

Vaccinia virus as well as the insect baculovirus have been used as vectors to express *Brucella* antigens, including CuZnSOD, a protective antigen (20, 27). Although *Brucella* proteins were expressed in these systems, protection against virulent challenge was not obtained.

***Ochrobactrum anthropi* vector vaccine.**

This gram negative soil bacterium is the closest genetic relative of *Brucella* (410). It has been used to express *B. abortus* protective antigens. Vaccination of mice with *O. anthropi* expressing *Brucella* Cu/ZnSOD induces immune responses specific to *Brucella* SOD of a mixed Th1/Th2 profile, with high IFN-γ but also high IL-4 levels. These vaccines proved to be non-protective unless they were co-administered with the genetic adjuvant CpG which switched the cytokine profile to a Th1 type without IL-4 production (169). These results emphasize the importance of using the appropriate vector to induce a protective immune response.

DNA vaccines

Protection results obtained using vaccination with naked DNA vaccines expressing *Brucella* antigens, have been somewhat controversial. Some groups have reported no protection against *Brucella* challenge in mice, whereas others report success. Onate et al (299) have induced protection against challenge with virulent *Brucella* by vaccinating with a DNA vaccine expressing Cu/ZnSOD. Velikovsky et al, (412) demonstrated protection in a DNA vaccine system expressing *B. abortus* lumazine synthase gene. The L7/L12 protein gene has also been expressed as a DNA vaccine inducing protection against challenge (207). However, expression of *Brucella* GroEL in a DNA vaccine model elicited Th1 cytokine profiles with high IFN-γ but was unable to induce protection against challenge (220). Clearly the selection of the antigen to be expressed and the route, dose, and frequency of immunization, has crucial importance for the outcome of the experiments.

Recombinant strain RB51 vaccines

Homologous over-expression of a protective antigen in strain RB51 has been shown to be an effective means of inducing protective immunity against brucellosis. Studies have demonstrated that compared to strain RB51, recombinant strain RB51 over-expressing homologous *B. abortus* Cu/ZnSOD (approximately 10 times the normal level), induces significantly increased protection against challenge with a virulent *B. abortus* strain in BALB/c mice (418). This increased protection may be attributed to the higher IFN-γ levels found in splenocytes of mice vaccinated with this strain, but unpublished data in our laboratory has shown that at least part of the superior protective efficacy may be attributed to increased CTL activation.

Complementation of strain RB51 with a functional copy of the *wboA* gene fails to revert the strain to a smooth phenotype. Although evidence of O-chain production is observed, the O-chain remains intracytoplasmic and is not transported to the outer membrane. Mice vaccinated with this construct develop O-chain antibodies and are almost completely protected against infection with virulent strain 2308 (414, 417).

As mentioned above, vaccine strain RB51 induces a polarized immune response characterized by high levels of IFN–γ and no IL-4 (170, 307), this feature makes it an attractive candidate for the expression of protective antigens belonging to other intracellular pathogens which require this type of immune pattern for protection. Therefore, expression of heterologous antigens has also been considered as means of increasing protection by strain RB51. *E. coli* and mycobacterial antigens have been successfully expressed in *B. abortus* strain RB51. Although the expression levels of the heterologous protein has been variable, vaccination with these constructs induces antibody responses directed to the heterologous gene product (306, 416, 419).

In general, in order to be effective, vaccines designed to protect against brucellosis should elicit a biased Th1 type immune responses with high levels of IFN–γ, little or no IL-4 and polarized IgG2a antibodies as a consequence of the Th1 response.

Elicitation of protective cytotoxic lymphocytes is also desirable. In addition, vaccines should not interfere with the diagnostic methods used in the field, should be attenuated or completely avirulent and ideally, confer protection to a variety of diseases simultaneously and if they are intended for livestock use, they must also be cheap.

General Characteristics of the Genus *Mycobacterium*

Mycobacteria are aerobic, gram positive, acid fast rods that belong to the order Actinomycetales. These organisms are widely distributed in water and soil environments as well as in several animal hosts. Many of the strains are non pathogenic to domestic species while others are able to infect and cause severe morbidity/mortality in several hosts. *M. tuberculosis* accounts for the largest number of deaths worldwide due to an infectious organisms (135, 301). *M. bovis* is responsible for bovine tuberculosis and is also able to infect humans causing a tuberculosis-like disease virtually undistinguishable from that caused by *M. tuberculosis* (345). *M. avium avium*, originally described in poultry, was considered non-pathogenic to humans but within the last decade has been implicated as a member of the *Mycobacterium avium* complex (MAC) that accounts for severe infections and mortality in AIDS and other immunocompromised patients (123).

M. avium subsp. paratuberculosis

*M. avium subsp. paratuberculosis (*MAP*)* and *M. avium avium* strains are very closely related, sharing between 75% to 99% identity depending on the specific DNA region analyzed (34). Although the strains are genetically very similar, they exhibit profound differences. Among them are the very different laboratory growth characteristics, in which MAP requires almost 6 times longer than its parent strain to grow, MAP's dependency of mycobactin J supplementation (211) and also the pathogenesis and clinical signs of the diseases caused by both organisms.

MAP has long been associated with a chronic disease in cattle, caprine and ovine populations where it causes paratuberculosis or Johne's diseases. Johne's disease is the endemic, chronic, granulomatous enteritis caused by MAP (381). It affects ruminants although it is believed to also be spread by rabbits (38, 91). Controversial data has also implicated it as a possible cause of Crohn's disease in humans (71, 75, 134, 171, 179, 191, 277). The disease is economically important in cattle, goat and sheep industry due to decreased production, reduced slaughter value and replacement cost of infected animals (80).

The incubation period of paratuberculosis is very long; cattle become infected as neonates and rarely become infected after 6 months of age. Clinical signs only appear after 2 years, the peak being at 4-7 years; it is not uncommon for clinical signs to first appear in animals over 10 years of age and even then, the onset of the disease is rather inconspicuous, characterized only by intermittent diarrhea and deterioration of body condition even though the animal shows good appetite (405). It is a bigger concern in dairy herds than in beef herds not due to differential susceptibilities, but because dairy cows are kept for longer periods of time and dairy management practices favor the spread of the disease (405).

Identification of infected animals is further complicated by the lack of effective diagnostic methods (80, 353). The definitive diagnosis of paratuberculosis is made by fecal culture and isolation of MAP, but this proves to be very challenging since the organism may take from several weeks up to six months to grow in laboratories. Moreover, many of the isolation methods actually inhibit the growth of these fastidious bacilli in the laboratory (190). PCR methods based on amplification of the IS900 sequence of MAP have been used (326), but carry the disadvantage of not being able to discriminate between viable and non- viable organisms. Furthermore, it has been reported that certain mycobacterial species other than MAP also test positive for this marker (114). In this case though, the definitive diagnosis can be achieved by combining PCR information with MAP's slow growth, phenotypical and staining characteristics as well as its dependency on mycobactin J supplementation. It should be noted that this mycobactin

dependence is lost after several passages outside of the host (211). One final and major diagnostic problem is that infected animals do not constantly shed the organisms in their feces (426).

MAP like other mycobacteria, and like *B. abortus,* is an intracellular pathogen able to survive inside macrophages and prevent phago-lysosome fusion (143, 175, 251). The molecular mechanisms involved in the inhibition process are not yet known. Compared to other mycobacterioses, very little is known about the pathogenesis, immune mechanisms and molecular genetics of MAP (426). Only a few immunogenic proteins have been identified in this species (32). It is of interest that most of these proteins are secreted and their homologs in *M. tuberculosis* apparently constitute protective antigens.

In the United States, the prevalence of paratuberculosis infection in slaughtered cows ranges from 2-18% and only a few herds have a Johne's free status. National herd prevalence is estimated at 20-22%, with some states having 1/3 of their herds infected (284). The economic losses associated with paratuberculosis are estimated to be $1.5 billion/year. From a public health perspective, it has been shown that the organism is able to survive the standard pasteurization procedures of commercial dairies in the United Kingdom, but this has not yet been reported in the US (153, 374).

In April 2002, the USDA introduced the Uniform Program Standards for the Voluntary Bovine Johne's Disease Control Program. This program is based on 3 essential areas: education, management, and herd testing and classification. The official tests are fecal and tissue culture, DNA probe assay, and histological analysis. A screening methods, an USDA approved ELISA test, with a sensitivity of approximately 25% in non-clinical animals and 85% in clinical animals and with 98-99% specificity is being used (402, 403) .

There are currently only a few vaccines to control Johne's disease. The live attenuated strain 316F of MAP is available in New Zealand for vaccination of cattle, sheep and goats. Countries such as Iceland, Norway and The Netherlands vaccinate calves less than 30 days old with a heat killed MAP vaccine (159, 265). Vaccination is not able to reduce the number of infections but it is still economically advantageous since

it delays the onset of clinical signs and decreases shedding of the organisms thus reducing contamination of pastures and spread of the disease (189). Unfortunately, vaccination alone as presently practiced will not prevent transmission to susceptible animals, therefore hygienic and management practices remain crucial (79). Vaccination with the current commercial vaccines is discouraged in the US because they induce a local reaction at the inoculation site, and more importantly, interfere with the tests for bovine tuberculosis. In some states, vaccination is allowed on a case by case basis in severely infected herds, but the vaccine can only be obtained through the state veterinarian (402, 403).

Clearly, an effective vaccine that does not interfere with diagnosis of bovine tuberculosis and paratuberculosis would be of great value in this control program. Unfortunately, the development of effective vaccines for this disease is hampered by the lack of suitable animal models. The most widely used models to replicate the clinical signs of paratuberculosis in laboratory animals are immunocompromised strains such as Beige/SCID, nude or thymectomized mice (268, 373). This underscores the importance of T cells in the control of the disease, but it also makes it impossible to evaluate vaccine candidates in animals so severely immunocompromised.

Immunology of *Mycobacterium*

Protection against mycobacteria depends on the induction of specific cell-mediated immune responses (301). Like *Brucella*, *Mycobacterium spp.* can survive and replicate inside non activated macrophages. Therefore, one of the key elements in the protective response against this pathogen is the activation of antimycobacterial functions of the macrophages (83, 187).

The uptake of mycobacteria by the host's macrophages involves several receptors on these macrophages. Complement Receptors (CR) bind different complement components; CR1 binds C3b and C4b and mediates the phagocytosis of bound particles. CR2 and CR3 bind C3bi. Bacteria can activate the alternative pathway of the complement

cascade which results in their opsonization with C3b and C3bi. Opsonized bacteria then bind to CR1, CR3 and CR4 and are efficiently phagocytosed. It has been shown that pathogenic mycobacteria enhance their phagocytosis by recruiting the complement component C2a to form C3 convertase which in turn produces C3b, without requiring the early components of the complement pathway. Other receptors are also involved in mycobacterial uptake; virulent mycobacteria are internalized by mannose receptors which interact with mycobacterial lipoarabinomannan (LAM). CD14, a receptor for gram negative LPS can also bind MTB LAM and a Scavenger Receptor which bind gram negative LPS and also gram positive lipoteichoic acid (117). The relative abundance of mycobacterial uptake mechanisms is a reflection of the organism's intracellular niche. Intracellular *M. avium* is able to prevent phagosome maturation into phagolysosome and actively increases phagosomal pH, which allows it to survive, replicate and establish a long term infection within these cells (175, 288). As described before, macrophages activated by cytokines are able to control intracellular parasites much more efficiently than their resting counterparts. Fusion of the phagosome with the lysosome provides one of the first killing mechanisms. It is believed that endosomal enzymes such as hydrolases coupled with a decreased pH environment in this compartment are effective degraders of mycobacteria. It has been shown that certain pathogenic mycobacterial species (*M. tuberculosis* and *M. avium*), are able to actively prevent phagolysosome fusion despite acquiring LAMP-1 markers (88, 175, 311). The mechanisms of how mycobacteria are able to achieve this are not clearly understood but it is believed that there is selective inhibition of fusion with endosomal vacuoles; this may be directed by the recruitment of the coat protein TACO (tryptophane, aspartate containing coat protein) in a process that requires cholesterol (311).

Free radicals may also provide protection against mycobacteria. Macrophages activated by TNF-α and IFN-γ release high levels of NO and other reactive intermediates through the NO synthase 2 (NOS_2) pathway (83). In mice, these components play a crucial role in protection against acute and chronic infections. One of the first toxic oxygen species proposed to have a role in killing intracellular mycobacteria was

hydrogen peroxide, which is released through the oxidative burst (241). The significance of this event in resistance to mycobacterial infections is somewhat controversial. In a study by Saunders et al., (342) mice nasally infected with MAC strains exhibited an increase in the number or inflammatory cells in the lungs, as well as increased cytotoxicity and release of H_2O_2 and NO, however, no bactericidal activity was observed.

Activation of an innate immune response through signaling via Toll like receptors (TLR) has also been shown for mycobacteria (244, 376). Certain mycobacterial components are able to interact with distinct TLR populations and this ligation induces a signal transduction cascade leading to the activation the NF-kβ pathway for the release of IL-1RB via the adapter protein MyD88 (376). It has been shown that *M. tuberculosis* (MTB) can simultaneously signal through TLR-2 and TLR-4, but seem to mediate different aspects of the immune response. It has been shown that binding of TLR-2 by lipoproteins leads to secretion of TNF-α and host cell apoptosis (318, 396, 401). The exact role of TLR-4 in mycobacterial infections is currently the subject of some debate in which some groups indicate that TLR-4 is required for protection, whereas other groups indicate that it plays no role in mouse resistance to mycobacteria (357, 376, 396).

As mentioned above, the cytokine profile emerging during the host/pathogen interaction has a pivotal role in the outcome of infections due to intracellular parasites. As occurs with *Brucella*, resistance against mycobacterial infections requires the elicitation of a Th1 type cytokine profile (88, 100, 178, 187). After phagocytosis of *Mycobacterium* by macrophages or dendritic cells, IL-12 is produced and this leads to the release of IFN-γ by NK cells. IL-12 has been shown to be very important in resistance to mycobacterial infections, especially in susceptible mouse strains such as BALB/c, were administration of IL-12 significantly decreases the number of recovered mycobacteria (434). Mycobacteria are strong inducers of IL-12, therefore during a mycobacterial infection IFN-γ is always produced (187, 360). The final outcome of the infection is not dependent on the presence of IFN-γ but rather on the level of production. In this sense it has been shown that both genetically resistant and genetically susceptible mice produce IFN-γ during mycobacterial infections, however the levels of IFN-γ produced in

susceptible strains are much lower and is a reflection of the lower levels of IL-12 and IL-18 secreted by these mice (201). It has also been shown that mice and humans with active tuberculosis have decreased IFN–γ activity. This decrease has been traced to a decreased expression of the cyclic adenosine 5'-monophosphate response element-binding protein (CREB) which binds to the IFN-γ promoter. The reduction in CREB molecules leads to reduced IFN-γ production. Also, IFN-γ activated macrophages are unable to kill virulent MTB, as MTB is able to prevent the association of STAT1 with CREB which disrupts the of the signal transduction cascade elicited by binding of IFN-γ to the IFN-γ receptor, and therefore, the IFN-γ mediated responses are not activated (339, 390).

IL-18 (formerly known as interferon gamma inducing factor, IGIF), has been recently identified as an important Th1 cytokine. It is produced by monocytes and is a strong inducer of secretion of IFN-γ by CD4+ and NK cells. Several mouse studies have shown that it plays an important role in resistance against mycobacterial infections (74).

Another cytokine with a major role in the outcome of the immune response against intracellular parasites is IL-4, one of the heralds of the Th2 cytokine profile. This has been demonstrated in *Brucella* studies using *O. anthropi* SOD, in which vaccination with this strain induced the generation of a non protective mixed Th1/Th2 response with secretion of IFN-γ but also of IL-4. Once the immune response was switched towards IFN-γ secretion without IL-4, protection was achieved (169). However, the role of IL-4 in tuberculosis is somewhat less defined. It has been shown that resistance to tuberculosis requires IFN-γ and the elicitation of a Th1 type response. Since infection with mycobacteria is a strong inducer of IL-12, IFN-γ is always produced by immuno-competent individuals. Kobashashi et al., (201) reported that genetically susceptible mice exhibit a decreased expression of IL-12, IGIF (IL-18), and decreased IFN-γ without an increase in IL-4; therefore, a decrease in these cytokines does not induce a Th2 response. It appears that IL-4 does not play an important role in decreasing protection against mycobacterial infections. It has been shown that that the presence of IL-4 does not significantly impair clearance of *M. avium* from the lungs of infected mice, and that

deletion of genes associated with Th2 responses (IL-4 receptor chain KO, IL-13 KO and Stat-6 KO) does not lead to increased resistance to airborne MTB infection in these KO mice compared to wild-type controls (188). In tuberculosis patients, IL-4 is either not detected or it is at very low levels. Some reports indicate that a strong Th2 response is not associated with increased susceptibility to tuberculosis, but may be associated with toxicity induced by TNF-α (172).

TNF -∝ has several roles in the immune control and pathogenesis of mycobacterial disease. It is believed to be mainly related to its involvement in macrophage activation (87). TNF-∝ acts in a synergistic way with IFN–γ to increase expression of NOS_2 and is crucial for the formation of the granuloma associated with granulomatous mycobacterioses (13, 362). It affects migration of cells to the granuloma as well as the expression of chemokines, adhesion molecules and chemokine receptors. TNF-α appears to be a crucial cytokine for the maintenance of a latent tuberculosis infection, and in its absence uncontrolled dissemination of the disease occurs (54). TNF-α has been implicated as one of the cytokines involved in the destruction of lung tissue of infected individuals, but contradicting results have shown that it may act as a protective element for the lungs during chronic infections (256, 365).

As discussed above, IL-10 is an anti-inflammatory cytokine produced by macrophages and T cells. It acts as a down regulator of the immune response by downgrading macrophage activation and IL-12 production, therefore decreasing IFN-γ release. The effects of IL-10 on resistance to mycobacterial infections have been somewhat inconsistent. One study showed that mice with deletions in the IL-10 gene do not have significantly higher rates of clearance of MTB infections than wild type mice, suggesting that IL-10 does not play a significant role in decreasing resistance (188); however, another study showed that mice with deletions in the IL-10 gene have increased resistance to *M. bovis* BCG infections, suggesting that IL-10 may decrease resistance to this attenuated strain (264). As described before, IL-10 exerts a significant anti-inflammatory effect through a variety of mechanisms, including the down regulation of TNF-α. It is thought that the role of IL-10 is directed at limiting the damage induced by

pro-inflammatory cytokines during the immune response against an invading pathogen (254, 280).

CD4+ T cells

This subset of lymphocytes are one of the most important cell types involved in protection against mycobacterial diseases (126). Since mycobacteria are intracellular parasites that reside in macrophages, many of their antigens are presented in an MHC-II context to CD4+ T cells (376). These cells have been identified as crucial for the control of mycobacterial diseases in mice studies using CD4 gene knock-out models, CD4+ T cell depletion models, transgenic mice deficient in MHC-II chains, and by the observation of the outcome of AIDS in humans, in which patients have compromised CD4+ T cell responses (89, 344, 347). CD4+ cells produce IFN-γ and are involved in the activation of APC by the interaction of the CD4+ TCR with the MHC-II-peptide complex presented by the APC, and the interaction of the CD40 molecule on APCs with the CD40L on T cells. They are also involved in the very important stage of priming and maintaining the activation of CD8+ cytotoxic lymphocytes. The importance of CD4+ cells in the control of tuberculosis has recently been proposed to also be IFN-γ independent. A CD4+ T cell depletion study, using mice chronically infected with MTB, has shown that the disease is reactivated despite the presence of IFN-γ and of reactive nitrogen intermediates (89, 347). Interestingly, virulent mycobacteria have evolved mechanisms to decrease expression of MHC-II molecules on the surface of antigen presenting cells, in a process that is regulated by the 19 kDA lipoprotein antigen (141).

CD4+ cells are important in regulating the function of B lymphocytes by the secretion of cytokines such as IL-4, IL-2, IL-5 and IL-13, that collectively stimulate B cell proliferation, antibody secretion, antibody synthesis, promote immunoglobulin class switching, and stimulate eosinophils to secrete IgE (89). Although the role of antibodies in mycobacterial control has been controversial and generally disregarded, there is some evidence of a role for antibodies in protection (89).

CD8+ cells

Cytotoxic CD8+ lymphocytes are considered crucial for the destruction of cells infected by intracellular parasites, bacterial or viral (126, 217). Antigen specific CD8+ T cells can secrete cytokines of the Th1 profile (TNF-α and IFN-γ) which are potent activators of the mycobactericidal mechanisms of macrophages. Recently a role for NK cells in the specific regulation of CD8+ cells has been demonstrated. NK cells regulate the frequency and cytolytic capacity of IFN-γ secreting CD8+ cells in a process dependent on the secretion of IFN-γ, IL-15 and IL-18 by the NK cells (407). In humans, the crucial role of CD8+ cells in the control of mycobacteria through cytokine secretion as well as cytotoxicity has been demonstrated (73). However, the role of cytotoxic lymphocytes in the control of mycobacteria in mice is still debated (367). It has been shown that the lungs of TB infected mice contain increased numbers of IFN-γ secreting CD8+ cells (126), and mycobacteria specific CD8+ cells have been identified, but their killing capacity remains to be determined in vivo (366). Other studies have identified TB specific CD8+ CTL in the lungs of infected mice that are able to lyse infected macrophages through a perforin dependent pathway (354). CD8+ cells exert direct cytolytic activity via the perforin/granzyme pathway or through Fas/FasL induced apoptosis which releases bacteria from infected cells for direct killing or for uptake by activated macrophages. However, the significance of this event in protection is unclear as Fas, perforin and granzyme gene knock-out mice are not more susceptible during an acute infection to MTB than wild-type mice, suggesting that this event may be more important for control during the chronic stages of the disease (84, 214). The mechanism of mycobacterial antigen presentation in an MHC-I context is not clear yet. Some antigens such as the 85A complex have been identified as being presented in an MHC-I context but it is not clear how they gain access to the MHC-I pathway (366). It is believed that proteins secreted by the parasite within the phagolysosome may translocate or diffuse through a pore into the cytosol compartment where they are processed by the proteasome and continue on the MHC-1 path (192, 279). mycobacteria also use a non-classical presentation to CD8+ cells, by CD1 molecules. These are non-polymorphic

antigen presenting molecules that are divided into 4 classes: CD1a, b, c and d. They are structurally somewhat similar to MHC-I molecules, but their antigen presenting groove is deeper. The main difference is that CD1 molecules are able to present lipids or glycolipids (192, 315, 348, 398, 400). Recently, Schiable et al., (348) have reported a new method of mycobacterial antigen presentation to CD1b and CD8+ cells. They have demonstrated that apoptosis of *M. tuberculosis* infected cells, releases antigens that are captured by dendritic cells, which in turn present these antigens to T cells. This two step approach of antigen presentation appears to be crucial for the induction of cytotoxic lymphocytes during MTB infections, by allowing the immune system to by-pass the down regulation of MHC molecules observed in cells infected with MTB.

Protective Antigens of Mycobacteria

85 complex

Analysis of culture filtrate supernatants of mycobacterial broth culture has shown the presence of several proteins including proteins of the 85 complex. This protein complex is the main component of the culture filtrate and is represented by three proteins, namely 85A, 85B and 85C, with molecular weights ranging between 30-32kDa. These proteins are encoded by three highly homologous genes, located at different sites in the mycobacterial genome (98). The 85A protein is a 32 kDa fibronectin-binding protein that has mycolyl transference activity and is involved in cell wall synthesis. It is a major antigenic determinant of mycobacteria. It induces antibody as well as cell mediated responses as demonstrated in mouse and human studies evaluating the development of antibody, DTH and cytokine responses to 85A (25, 26, 97, 246, 273, 358, 366, 411).

Esat-6

The 6 kDa Esat-6 protein (Early Secreted Antigenic Target) is fairly conserved among all *M. tuberculosis* complex mycobacteria but is absent in *M. bovis* Bacille Calmette–Guerin (BCG) (145, 222). This protein of unknown function is secreted early

in MTB cultures by a mechanism that is still unclear since Esat-6 lacks a traditional secretion signal sequence (145), but that has recently been proposed to be part of a gram positive secretion system (302). Esat-6 is a potent T-cell antigen (267, 289) and its vaccine potential has been studied for MTB. In a study using purified Esat-6 protein in an adjuvant formulation, clear T cell responses and protective immunity were obtained in mice. A separate study demonstrated that immunization with just a single epitope of this protein is sufficient to generate protection in mice (57, 293). Comparison of the genomes of *M. tuberculosis, M. bovis* and *M. bovis* BCG has identified several regions of difference between these species. One of these regions, RD1 (Region of Difference 1), is absent in all *M. bovis* BCG strains. The gene that encodes the Esat-6 protein (*esat-6* or *esx*) is located in this region. In *M. tuberculosis*, this gene is located within a cluster of genes that has been duplicated 5 times (cluster 1-5). BCG lacks all clusters while *M. avium* and MAP lack cluster 1(145). This similarity between species suggests that the deletion of cluster 1 occurred before their differentiation, and suggested that the absence of this region could have diagnostic potential to discriminate between mycobacterial species. Indeed, it has been shown that cattle infected with *M. bovis* but not with non-tuberculous mycobacteria respond to Esat-6 (61). Similarly, only humans infected with MTB and not with MAC strains show PPD and IFN–γ positive responses to Esat-6 (222).

35 kDa protein

This protein is a major structural membrane protein that is present in several mycobacterial species but is absent from *M. bovis* and *M. tuberculosis* (395, 427). The 35 kDa protein is recognized by the sera almost 90% leprosy patients, and stimulation with purified 35 kDa protein induces strong IFN-γ and DTH responses in *M. leprae* sensitized animals (394), and is also recognized by sera of *M. avium* infected mice and guinea pigs (395). When used in skin tests, it discriminates between guinea pigs sensitized with *M. leprae* and *M. tuberculosis* (394). Crohn's disease patients and cattle infected with Johne's disease have antibodies that recognize this protein (33, 276, 395). It is hypothesized that the MAP 35 kDa protein plays a role in the invasion of bovine

intestinal tract epithelial cells and that it represent a virulence factor for this species (33). Because the 35kDa protein is absent from the genomes of *M. bovis* and *M. tuberculosis*, it has been proposed as a diagnostic antigen for the identification of MAP infected cattle (108, 358). Vaccination studies using this protein in DNA vaccine constructs, have shown that it induces protection against *M. avium* and also against *M. leprae* in mice (238, 239).

Superoxide dismutases

Three types of SODs with different metal cofactors have been described in mycobacteria: Cu/ZnSOD, FeSOD and MnSOD. The latter two have different secondary and tertiary structures and localization within the cells (241). In MAP, a secreted MnSOD (*sodA*) was identified, and secretion of the protein correlates with virulence of the species (225). A MAP Cu/Zn SOD (*sodC),* that is structurally different from MnSOD, has also been described (104). Unlike *Brucella's* Cu/ZnSOD which contains a signal sequence for its periplasmic location (375), MAP's Cu/ZnSOD contains a putative signal sequence for its export from the cell and it is hypothesized that the location of the enzyme correlates with the function of protection against the oxidative burst in macrophages (101, 104).

General Rationale of this Research

Brucellosis is a zoonotic disease that is endemic worldwide causing considerable monetary losses in cattle industries and is also an important public health concern. The U.S. is now virtually free of cattle brucellosis, but the disease remains endemic in wildlife providing a possible means of re-introduction of the disease into cattle populations and humans. Therefore, the development of effective, attenuated vaccines that can be used for the protection of wildlife is important.

As a zoonotic agent *Brucella* does infect humans, and since *Brucella* organisms have been developed as biological weapons, the threat of deliberate infections with this

agent makes it imperative to develop effective human vaccines as well. The current official vaccine in the US for the control of brucellosis in cattle is strain RB51 (319). This strain has been shown to elicit strong cell mediated responses to *B. abortus*, which are able to control the infection by this intracellular pathogen (307, 414, 418).

Paratuberculosis is a disease that also causes vast economic losses in cattle industries. There are currently very few vaccines available to protect against this disease, and these have varying degrees of efficacy. Therefore, the development of an effective alternative to the existing vaccines is an important goal of researchers.

Members of the genus *Mycobacterium* are also intracellular parasites that require strong cell mediated immunity for their elimination from the host. Although there are no studies directly comparing the immune responses generated in response to *Brucella* and to mycobacterial infections, some similarities are evident. In both cases the induction of cell mediated immunity of the Th1 cytokine profile with secretion of IFN-γ and TNF-α are essential for the control of these intracellular pathogens. Both *Brucella* and *Mycobacterium spp.* are able to replicate inside macrophages within phagosomal vesicles and prevent phagolysosome fusion, although they replicate in different compartments (88, 152, 175, 212, 313). Therefore, the activation of cytotoxic lymphocytes able to destroy infected cells is important for the control of both diseases. Brucellosis and tuberculosis are diseases that can be acute and may persist in chronic forms affecting various organs with the formation of local granulomas. Latent persistence of *Brucella* many years after exposure has been described (133). Therefore, it seems likely that generating a Th1 inducing vaccine based on one of these organisms expressing antigens from the other, may lead to the elicitation of an adequate immune response against both.

Chapter 2 of this dissertation is focused on the development of a recombinant strain RB51 based vaccine expressing antigens from *M. avium subsp. paratuberculosis*.

Chapter 3 is focused on a) the development of a mouse model to evaluate protection against MAP; b) the evaluation of the immune responses in mice elicited by the

recombinant vaccines developed in Chapter 2; and c) test the ability of the recombinant vaccines to develop protective immunity against challenge using this model.

Chapter 4 studies the possibility of generating fully attenuated RB51 vaccines with the potential to be used in susceptible animal populations and eventually in humans, as well as evaluates the curative potential of vaccination with strain RB51 on an ongoing *Brucella* infection.

CHAPTER 2

Use of *B. abortus* Strain RB51 as a Vector for the Expression of Heterologous Mycobacterial Genes

Introduction

B. abortus is a gram negative, facultative intracellular bacterium that is able to replicate inside macrophages and to infect a variety of mammalian hosts (24, 148, 152, 257). It is a very important zoonotic agent world wide because humans may become infected by contact with secretions, including milk from an infected animal (51, 346). In cattle, infections with *B. abortus* result in infertility and abortions (85, 294, 351, 364). *B. abortus* vaccine strain RB51 is a natural rough mutant derived from virulent *B. abortus* strain 2308 (351). Strain RB51 has been demonstrated to induce significant protection against infection with *B. abortus* in mice and cattle (69, 303, 304, 351, 378, 392). Mice vaccinated with strain RB51 develop protective cell mediated responses while remaining negative in the serological diagnostic tests traditionally used to diagnose the disease (307, 351). The cytokine profile of vaccinated mice indicates high levels of IFN-γ and very little or no IL-4, and the antibody subtype is polarized to IgG2a antibodies (307, 418, 419). All this indicates a polarization towards a Th1 type of response, pivotal for the control of infections with intracellular bacteria.

B. abortus vaccine strain RB51 has been widely used to vaccinate cattle as a prophylactic measure against infection with *B. abortus* and is the current official vaccine in the USA and in several other countries (404, 419). The immunological characteristics described in Chapter 1, and the fact that it is a cattle vaccine already approved and in use, makes vaccine strain RB51 a good vector candidate to induce protection against other intracellular bacteria that require cell mediated responses with secretion of IFN-γ for their control.

Brucella spp. lacks natural plasmids but can be transformed with the pBBRMCS series of plasmids (112, 416). These plasmids are broad host range, low copy number plasmids that contain a multiple cloning site and each plasmid in the series confers resistance to a specific antibiotic. Plasmid pBBR1MCS confers chloramphenicol resistance and has been widely documented as a vehicle for introducing functional genes into *B. abortus* (416, 418). Vemulapalli et al., (416) have shown that *B. abortus* strain RB51 is able to express heterologous antigens from *E. coli* and *M. bovis* when the genes were encoded in this plasmid; mice vaccinated with these recombinant strain RB51 vaccines were able to mount serological responses to the heterologous antigens.

M. avium subsp. paratuberculosis (MAP) like *Brucella spp.,* is an intracellular parasite that infects cattle worldwide (426). There are currently only very few vaccines against paratuberculosis. Due to the unique characteristics of this organism, the available vaccines are not designed to protect against infection, but rather to decrase shedding of the pathogen and delay the onset of clinical signs (189). As intracellular organisms, both *Brucella* and MAP require similar mechanisms of immune control. Both pathogens are able to replicate within phagosomal vesicles inside macrophages (88, 152, 175, 212, 313). The development of cell mediated immune responses with CD4+ T cell of a Th1 profile, secretion of IFN-γ and TNF-α, cytokines that are able to activate the microbicidal mechanisms of macrophages, is essential to control both pathogens. In both cases CD8+ cytotoxic lymphocytes play an important role in the destruction of infected cells (73, 88, 148, 170).

Because the control both diseases requires the induction of similar immune responses, we considered using vaccine strain RB51 as a vaccine vector to express antigenic epitopes of MAP in order to induce a cell mediated immune response directed to both, *Brucella* and MAP.

The genes for the 85A and for the 35kDa proteins of MAP were selected for introduction into strain RB51. As described below, the *esat-6* gene of *M. tuberculosis* (MTB) was selected to express MAP proteins as fusions with MTB Esat-6 protein.

Secreted mycobacterial culture filtrate proteins constitute important mycobacterial protective antigens. These proteins induce T cell responses and are protective against tuberculosis in several experimental models (11, 12, 49). The 85A protein is a member of the 85 complex proteins, a group of secreted proteins that represent a major component of the extracellular proteins found in mycobacterial culture filtrates. 85A is a 32kDa fibronectin-binding protein, that possesses mycolyl transferase activity and is involved in cell was synthesis. 85A is encoded by the *fbpA* gene which is highly conserved among mycobacterial species (17, 98, 274, 310, 328, 369). It is a major antigenic determinant of mycobacteria which has been shown to induce antibody as well as cell mediated responses in several animal models (25, 95, 97, 366). The 85A protein has been shown to stimulate cell mediated, antibody and protective responses to tuberculosis in a variety of animal models when used in DNA vaccines, recombinant vaccines or in homologous over-expression in BCG vaccine (26, 95, 97, 151, 196, 246). Vaccination with a DNA vaccine expressing *M. bovis* 85A has been shown to be protective against *M. avium* infection in mice, demonstrating that this may be a protective antigen and also that heterologous protection is possible (411). It was therefore hypothesized that production of the MAP 85A protein in strain RB51 could result in a protective vaccine.

The mycobacterial 35 kDa protein is a structural membrane protein of *M. leprae* (429) This protein has been shown to have high homology to a similar protein in *M. avium,* and to be absent from the genomes of *M. bovis* and *M. tuberculosis* (395). In MAP, it is expressed at higher levels under the low oxygen and high osmolality conditions found within the bovine intestinal tract, and it is presumed to play a role during invasion of MAP into intestinal cells. Because of this, it has been proposed to be a virulence factor for this species (29, 33, 36). The 35 kDa protein is a strong antibody inducer in leprosy infected humans and in mice and humans infected with the *Mycobacterium avium* complex (MAC) (*M. avium avium* and *M. intracellulare),* as is the case of AIDS patients (204, 238). Also, human Crohn's disease patients and cattle infected with MAP have antibodies directed to this protein (276, 383, 393, 427). A DNA vaccine expressing the 35 kDa protein of *M. leprae* was shown to be protective against

leprosy in mice (59). In a separate experiment, a DNA vaccine expressing the *M. avium* 35 kDa protein was shown to induce strong IFN-γ and antibodies responses and to be protective against *M. avium* in mice (238).

Esat-6 is a highly conserved 6 kDa protein that has been identified in almost all *M. tuberculosis* complex species with the notable exception of *M. bovis* BCG (157, 317). This protein has been shown to be highly immunogenic; it is thought that variations within this family of proteins may contribute to the antigenic variation of mycobacteria which allows *M. tuberculosis* to evade the host's immune system (236). Its function remains unknown, but it is secreted early in MTB cultures by a mechanism that is unclear as Esat-6 lacks traditional secretion signal sequences (289). It has been shown that cattle infected with TB but not with non-tuberculous mycobacteria respond to this protein (6). Similarly, only humans infected with TB and not with MAC strains show PPD and IFN–γ positive responses when stimulated with Esat-6 (399).

Unpublished data in our research group (N. Sriranganathan, S.M Boyle and G. G. Schurig, Personal Communication), has shown that the expression level of certain heterologous proteins in strain RB51 is enhanced when they are produced as fusions with the MTB Esat-6 protein. Therefore, expression of MAP antigens could also be increased when expressed as fusions with Esat-6.

Rationale and Hypothesis

B. abortus vaccine strain RB51 induces strong cell mediated immunity to *Brucella* antigens, with secretion of IFN-γ and TNF-α, and activation of cytotoxic lymphocytes and this immune response is protective against challenge with virulent *Brucella* in mice (308, 351). Strain RB51 has also been used as a vector to express heterologous antigens (416). We hypothesize that strain RB51 may be used as a vector to express heterologous antigens of MAP.

Objectives

1) To determine the feasibility of expressing heterologous MAP genes in vaccine strain RB51 and RB51SOD.
2) To determine whether the expression levels of these MAP genes vary when expressed as fusion proteins in *Brucella*.

Material and Methods

Bacterial strains and antibodies

B. abortus strains RB51 and RB51SOD are part of our collection of reference strains and have been described elsewhere (351, 416, 417). *Brucella* organisms were grown in TSB and TSA media by incubation in a 37ºC shaking incubator or at 37ºC in a stationary incubator supplemented with 5% CO_2. When appropriate, the following antibiotics were added to the media: chloramphenicol 30μg/ml and ampicillin 100 μg/ml (Sigma, St. Louis MO). All *Brucella* cultures and manipulations were carried out in a BSL-3 facility at the CMMID, VMRCVM of Virginia Tech by certified personnel. The *E. coli* competent strains DH5α and BL21DE3LysS were purchased from Invitrogen (Carlsbad, CA). A field isolate of MAP, was kindly provided by Dr. R. H. Whitlock (University of Pennsylvania). This strain was grown in Middlebrook 7H9 agar plates or 7H11 broth supplemented with glycerol, malachite green, OADC enrichment media and mycobactin J. Plates and liquid cultures were incubated at 37ºC for up to 6 months.

Polyclonal antibodies to the 35 kDa protein, and anti-85A sera generated by inoculation of mice with *M. bovis* 85A antigen as a fusion with maltose binding protein (MBP-85A) were part of our sera collection.

Plasmids

A list and characteristics of the plasmids used in this project is presented in Table 2.1. (Amp: Ampicillin, Cm: Chloramphenicol)

Table 2.1: Characteristics of the plasmids used throughout this study.

Name	Source	Function	Resistance
pCR2.1	Invitrogen	Clone PCR amplified fragments.	Amp
pRSETA	Invitrogen	Expression of recombinant proteins. Adds an amino terminal polyhistidine tag.	Amp
pBBR1MCS (pBB)	Collection	*E.coli-Brucella* shuttle vector, stable in *Brucella spp.*	Cm
pBBgroE	Collection	pBB plasmid containing *Brucella* groEL/ES gene promoter sequence.	Cm
pBBSODss	Collection	pBB plasmid containing *Brucella* Cu/ZnSOD gene promoter, signal sequence and first 48 amino terminal aminoacids.	Cm
pBBSOD	Collection	pBB plasmid containing *Brucella* Cu/ZnSOD gene including signal sequence, under its own promoter.	Cm
pBBgroEesat-6:TB85A	Collection	pBBgroE plasmid containing *M. tuberculosis* Esat-6 protein gene fused to the amino terminal portion of MTB 85A gene.	Cm
pBBsodssEsat-6	Collection	PBB plasmid containing *Brucella* Cu/ZnSOD promoter, signal sequences and first 48 aminoacids fused to the amino terminal portion of MTB Esat-6 protein gene.	Cm
pBBpTB85A	This Study	pBBgroE plasmid containing MAP 85A protein gene expressed under the *Brucella groE* promoter.	Cm
pBB35	This Study	pBBgroE plasmid containing MAP 35 kDa protein gene expressed under the *Brucella groE* promoter.	Cm
pBBSODpTB85A	This Study	pBBSOD plasmid containing *Brucella* Cu/ZnSOD gene expressed under its own	Cm

		promoter and MAP 85A protein gene expressed under the *Brucella groE* promoter.	
pBBSOD35	This Study	pBBSOD plasmid containing *Brucella* Cu/ZnSOD gene expressed under its own promoter and MAP 35 kDa protein gene expressed under the *Brucella groE* promoter.	Cm
pBBesat-6 pTB85A	This Study	pBBgroE plasmid containing *M. tuberculosis* Esat-6 protein gene fused to the amino terminal portion of MAP 85A gene.	Cm
pBBSODpTB85A	This Study	pBBSOD plasmid containing *Brucella* Cu/ZnSOD gene expressed under its own promoter and *M. tuberculosis* Esat-6 protein gene fused to the amino terminal portion of MAP 85A gene expressed under the *Brucella groE* promoter.	Cm
pBBsodsspTB 85A	This Study	PBB plasmid containing *Brucella* Cu/ZnSOD promoter, signal sequences and first 48 aminoacids fused to the amino terminal portion of MAP 85A protein gene.	Cm

Amplification of MAP genes

All recombinant DNA procedures were carried out according to standard molecular biology protocols (338). All PCR reactions were carried out using either Taq DNA polymerase or pfu DNA polymerases (Promega, Madison, WI) that have proof-reading activity.

Extraction of template DNA from *M. avium* was carried out as described by Whipple et al.,(424) with modifications. An aliquot of frozen MAP cells were washed with TEN buffer (50 mM Tris-HCl pH8.0, 100 mM EDTA, 150 mM NaCl), killed by incubation in an 80ºC water bath for 1 hour and then resuspended in 500 µl of TEN buffer. Lipase (Sigma, St Louis, MO) was added (8.000U) and cells were incubated for 2 hours at 37 ºC, followed by the addition of Lysozyme (5 mg/ml) (Sigma, St Louis, MO)

and 2 hrs incubation at 37ºC. Proteinase K (Sigma, St Louis, MO) and SDS (Fisher, Fair Lawn, NJ) were added (2mg/ml and 1% respectively), followed by 18h incubation at 65ºC. 0.4 volumes of 5M potassium acetate were added and the suspension was placed on ice and centrifuged. DNA was precipitated from the supernatant by phenol-cloroform-isoamylalcohol and the DNA pellets were dissolved in TE buffer or water (424).

The MAP 85A and 35 kDa protein genes were amplified from *M. avium subsp. paratuberculosis* genome by PCR. For the 85A gene, the primers were designed based on the published sequence of MAP 85A gene. Primers for the 35 kDa gene were designed based on the gene sequence of its *M. leprae* homolog. Restriction sites at each end of the genes were included for their easy cloning in subsequent steps. Primers designed using the Lasergene Primerselect Software including restriction sites identified using the Lasergene Mapdraw software are shown in Table 2.2. Enzymes that did not cut within each gene's ORF and that were also present in the multiple cloning site of the plasmid vectors were used. Template DNA from MAP was obtained as described above. The amplified fragments were cloned into plasmid pCR2.1 generating plasmids pCRpTB85A, and pCR35. Amplification of the MAP specific IS900 was used to confirm the presence of MAP DNA template.

Expression of MAP ***genes*** **in *E.coli***

To replicate the plasmids generated above, competent *E. coli* DH5α cells were transformed with these constructs by the heat shock method (338). Briefly, 25 µl aliquots of DH5α competent cells were incubated on ice for 30 minutes with 2.5 µl of the plasmid ligation mixture (approximately 2.5 ng DNA). The tubes were then heat shocked at 42°C for 45 seconds and immediately incubated for 2 minutes on ice. Next, 600 µl of SOC media (Invitrogen, Carlsbad, CA), were added to each tube and the tubes were then incubated at 37°C for 1 hour. After this incubation step, the transformed cells were plated onto antibiotic containing plates and incubated overnight at 37°C. Plasmid containing *E. coli* cells were selected by resistance to antibiotics. Individual colonies were selected and subcultured into TSB with appropriate antibiotics. Plasmids were extracted from liquid

cultures using a commercial plasmid extraction kit (Qiagen, Valencia, CA), and digested with appropriate restriction enzymes to excise each gene.

Table 2.2: PCR primers used to amplify MAP genes.

Gene	Forward primer	Restriction sites	Reverse primer	Restriction sites
pTB85A	GGATCCTCAAGAAAGC GGCCGAGCGGACGA	*BamHI*	TCTAGAGGCGTCATGCT CGGTATTTGGTTAGGTG	*Xba1*
pTB35	GGATCCCACGAAAGG ATCACGATGACGTCG	*BamHI*	TCTAGACCGCTCGGTAC TCACTTGTACTCATGG	*Xba1*
IS900	GAAGGGTGTTCGGGG CCGTCGCTTAGG	*none*	GGCGCTTGAGGTCGATC GCCCACGTGAC	*none*

Primers and restriction sites for PCR amplification of MAP. Restriction sites are shown underlined.

Generation of pBB plasmids expressing MAP genes

Recombinant pBB plasmids containing MAP genes were constructed by digesting the pCR2.1 plasmids containing the MAP 85A or 35 kDa protein genes with *BamHI* and *XbaI* restriction enzymes to excise each gene along with its RBS and stop codon. Digested fragments were ligated into plasmid pBBgroE previously digested with the same enzymes using T_4 DNA ligase (Promega, Madison, WI), generating plasmids pBBpTB85A and pBB35 (Table 2.1). The MAP genes were expressed under the *Brucella groE* promoter. Double construct plasmids expressing *B. abortus* Cu/ZnSOD and MAP 85A or the 35kDa protein genes were generated by digesting plasmid pBBSOD and plasmids pBBpTB85A and pBB35 with *SpeI* and *XbaI* (Promega, Madison, WI). These enzymes excise the complete MAP genes along with the *groE* promoter. The whole gene and promoter were then cloned into pBBSOD downstream from the *Brucella* SOD gene *(sodC)*, and ligated as above generating plasmids pBBSODpTB85A and pBBSOD35 (Table 2.1). The plasmids were amplified in *E.coli* DH5α cells, extracted as above and

then transformed into strain RB51 competent cells as previously described (243), generating RB51pTB85A, RB5135, RB51SOD35 or RB51SODpTB85A (Table 2.4). Briefly, a single colony of strain RB51 was inoculated into 10 mL of TSB broth and incubated for 30 h at 37°C. Two mL of this culture were used to inoculate 380 mL of TSB and the culture was incubated at 37°C until it reached 70-75 Klett units. The culture was then centrifuged at 5000g for 6 minutes at 4°C and the pellet was washed with 380 mL of ice cold distilled water. The washing step was repeated and the final pellet was resuspended in 500 μL ice cold distilled water. The suspension was then aliquoted into 100 μl volumes and stored at -80°C. For transformation, 10 μl of plasmid DNA (containing approximately 0.5 μg of plasmid) were gently added to 100 μl of competent cells. The cell-plasmid DNA mixture was incubated on ice for 30 minutes and then transferred into a pre-chilled 1 mm gap electroporation cuvette and electro-pulsed with 625V for 10 milliseconds using a BTX model ECM 630 electroporator (BTX, San Diego, CA). The suspension was immediately transferred into 900 μl of TSB broth supplemented with 10 mM $MgCl_2$ and 10mM $MgSO_4$ and incubated for 24 hours at 37°C in a shaking incubator. The samples were then plated onto antibiotic containing plates and incubated at 37°C +5% CO_2. Recombinant colonies were selected by their antibiotic resistance and the correct orientation of the gene with regards to the promoter was confirmed by restriction enzyme analysis and sequencing, confirming that the correct genes had been cloned in the right frame for protein translation. The general cloning scheme is presented in Figure 2.1.

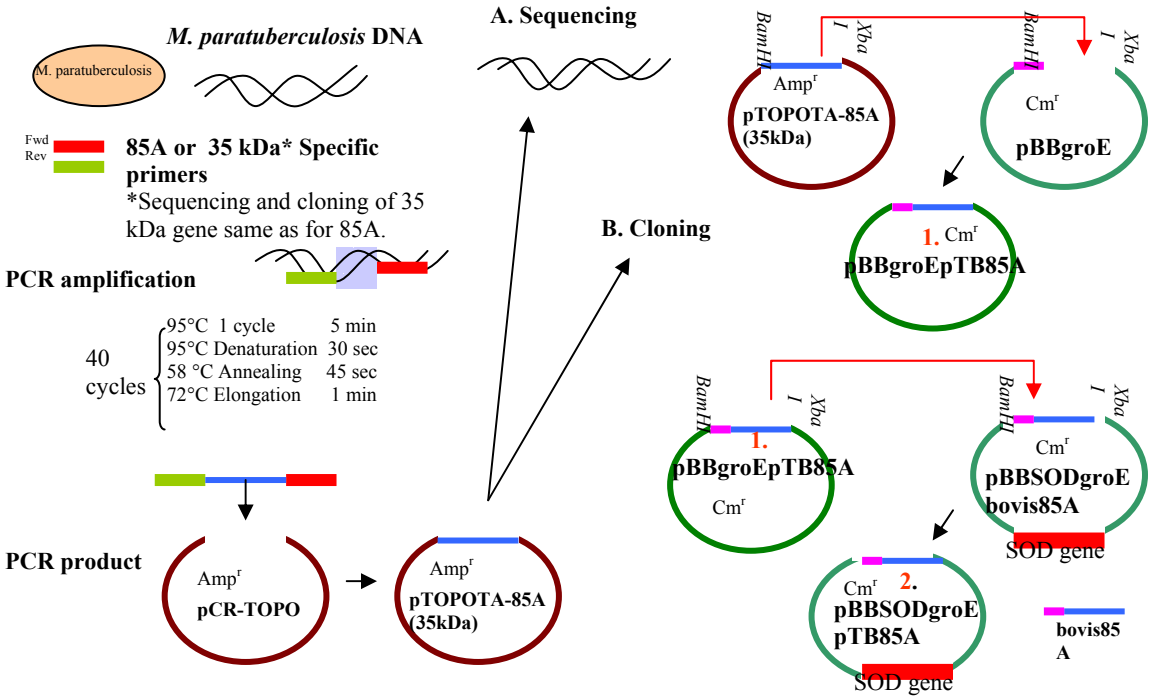

Figure 2.1: General cloning scheme used to generate recombinant strain RB51 based vaccines expressing MAP antigens as single or double constructs.

To generate constructs where the MAP proteins are expressed as a fusion with the MTB Esat-6 protein, the plasmid pBBgroEEsat-685A was used. This plasmid encodes the whole MTB Esat-6 protein without its stop codon expressed under the *B. abortus groE* promoter. It carries restriction sites that allow cloning of the chosen antigens as fusions with the Esat-6 protein. PCR primers were designed to amplify the MAP 85A and 35kDa genes without signal sequences or start codon and simultaneously encoding restriction sites. The pBBgroEEsat-685A plasmid contains restriction sites that allows in frame cloning of genes as fusion with Esat-6. Since all the enzymes present in its MCS cut within the coding regions of MAP 85A and of 35 kDa protein genes, the forward primers were designed to encode the blunt enzyme, *EcoRV* (Table 2.3). This enzyme is not included in the MCS of pBBgroEEsat-6, but the MCS contains *SmaI*, another blunt end enzyme. Therefore, digestion of pBBgroEEsat-6 with *Sma1* (Promega, Madison, WI) and of the primer amplified products with *EcoRV* (Promega, Madison, WI) allowed the ligation of two blunt ends. The primer design ensured that the genes were cloned in frame and that after ligation the *SmaI* and *EcoRV* sites were destroyed. The reverse primer was designed to include the *XbaI* restriction site after the stop codon of either the 85A or the 35 gene (Table 2.2). Plasmids pBBgroEEsat-6pTB85A or pBBgroEEsat-635 (Table 2.1) were generated by first amplifying the MAP genes from pBBgroEp85A and pBBgroE35 using these primers. The PCR products were checked for size using a UV trans-illuminator and amplified products were digested directly in the PCR tube. The plasmid pBBgroEEsat-6pTB85A was digested with *SmaI* and *XbaI*. The restriction fragments were resolved in agarose gels and bands of the appropriate size were extracted as described above. Digested pBBEsat-6 plasmid and digested PCR products were ligated by overnight incubation at 14ºC with T4 DNA ligase (Promega, Madison, WI). An identical approach was used to generate a fusion of the 85A and 35 kDa genes with the signal sequence and first 48 amino acids of *B. abortus* Cu/ZnSOD by cloning the genes into plasmid pBBSODss generating pBBSODssEsat-6pTB85A. A double construct plasmid expressing *B. abortus* Cu/ZnSOD and MAP 85A genes as a fusion with Esat-6 was generated by digesting plasmid pBBSOD and plasmid pBBEsat-6pTB85A with *SpeI*

and *XbaI* (Promega, Madison, WI). These enzymes excise the complete mycobacterial fusion gene along with the *groE* promoter. The whole gene and promoter were then cloned into pBBSOD downstream from the *Brucella* SOD gene *(sodC)*, and ligated as above generating plasmid pBBSODEsat6pTB85A. Correct recombinant plasmids were determined by restriction digestion analysis using *SmaI* and *XbaI* enzymes. Correct plasmids were linearized because the *SmaI* site was destroyed.

These plasmids were then electroporated in competent *B. abortus* strain RB51 as described above, generating strains RB51Esat-6pTB85A, RB51SODEsat-6pTB85A and RB51SODsspTB85A (Table 2.4).

Western Blot Analysis

To detect expression of the heterologous proteins in strain RB51, SDS-PAGE was performed as previously described (209). Whole cell extracts of recombinant *E. coli* or strain RB51 cells expressing MAP antigens were prepared by growing these cells on TSA plates containing antibiotics. The cells were then harvested and for RB51 strains, killed by incubation at 70°C for 45 minutes. The bacterial suspensions were then washed twice with 10 mM Tris-HCl, pH 8.0, and the concentration was adjusted to 10% transmittance at 525 nm. A 50 mL aliquot of each bacterial suspension was centrifuged and the pellet was resuspended in 500 µl of Tris-HCl buffer. The antigens were aliquoted into 100 µl stored at -20°C. Prior to their use, the samples were mixed with an equal volume of 2X Laemmli buffer (Sigma, St. Louis, MO) and boiled for 5 minutes. Twenty µl of the antigens were resolved in 12.5% polyacrylamide gels. The gels were then stained with Coomassie Brilliant Blue or transferred onto nitrocellulose membranes using a TransBlot semi-dry system (Bio-Rad, Hercules, CA) for Western Blot analysis. The membranes were blocked for 3 hours using 2% Bovine Serum Albumin (Sigma, St. Louis, MO) and incubated overnight with 85A or 35 kDa specific antisera. The membranes were then washed 5 times using TBS-Tween20 pH 7.4, wash buffer, and incubated with anti-mouse HRPO conjugated secondary antibody (Cappel-ICN, Irvine, CA) for 1 hour and washed 5 times as above. The blots were then developed using α-chloro-naphtol substrate or TMB

substrate (Sigma, St. Louis, MO). To determine whether these recombinant proteins could be found in the bacterial culture supernatants, mid-log phase cultures of RB51 vaccine strains were centrifuged, the pellets discarded and the supernatants were precipitated by an overnight incubation at 4ºC with Trichloroacetic acid (TCA) to a final concentration of 20%. The samples were again centrifuged and the pellets were washed with acetone and finally resuspended in Laemmli buffer (Sigma, St Louis MO). The concentration of the precipitated proteins was determined by the Bradford assay (56), using a protein concentration kit (BioRad, Hercules, CA) and western blot analysis was performed as above.

Table 2.3: PCR primers used to generate MAP 85A and 35 kDA protein antigens as fusions with Esat-6 protein.

Gene	Forward primer*	Restriction sites	Reverse primer*	Restriction sites
Esat-6::pTB85A	GGGATATCGGTCTGCC GCTGGAGTACCTGC	*EcoRV*	GGTCTAGACGGCGTCAT GGTCGGTATTGG	*Xba1*
Esat-6::35	GGGATATCCAGAATGA GTCTCAAGCACT	*EcoRV*	GGTCTAGACCGCTCGGT ACTCACTTGTAC	*Xba1*
Sodss::pTB85A	GGGATATCGGTCTGCC GCTGGAGTACCTGC	*EcoRV*	GGTCTAGACGGCGTCAT GGTCGGTATTGG	*Xba1*

* Restriction sites are shown underlined. Two extra bases were included at the start of each primer to protect the restriction sites.

Table 2.4 :Vaccine strains used throughout this study.

Vaccine	Characteristics
RB51	*B. abortus* strain RB51, official brucellosis vaccine
RB51SOD	Strain RB51 overexpressing *Brucella* Cu/ZnSOD.
RB51pTB85A	Strain RB51 expressing MAP 85A protein.
RB51SODpTB85A	Strain RB51 simultaneously expressing MAP 85A protein and overexpressing *Brucella* Cu/ZnSOD.
RB5135	Strain RB51 expressing MAP 35kDa protein.
RB51SOD35	Strain RB51 simultaneously expressing MAP 35kDa protein and overexpressing *Brucella* Cu/ZnSOD.
RB51Esat-685A	Strain RB51 expressing MAP 85A protein as a fusion with MTB Esat-6.
RB51SODEsat-685A	Strain RB51 simultaneously expressing MAP 85A protein as a fusion with MTB Esat-6 and overexpressing *Brucella* Cu/ZnSOD.
RB51SODsspTB85A	Strain RB51 expressing MAP 85A protein as a fusion with *Brucella* Cu/ZnSOD signal sequence.

Analysis of recombinant protein expression in *E. coli* and purification of recombinant proteins

Recombinant MAP 85A and 35 kDa proteins were prepared by amplification of the genes from MAP genomic DNA using the forward primers shown in Table 2.5 and the reverse primers shown in Table 2.3. The amplified fragments were cloned into plasmid pCR2.1 (Invitrogen, Carlsbad, CA) and subcloned into plasmid pRSETA (Invitrogen, Carlsbad, CA), to generate pRSETApTB85A or pRSET35. This vector expresses the protein product as a fusion with a His tag sequence in the amino terminal portion. BL21DE3LysS/ cells were chemically transformed with plasmids pRSETpTB85A or pRSETS35 as described above and grown in ampicillin containing media. The recombinant proteins were purified by affinity chromatography using Talon

metal affinity resin columns (BD Bioscience, Palo Alto, CA), under denaturing conditions (423). Briefly, recombinant *E. coli* were grown overnight in 150 mL TSB media. The cells were then pelleted by centrifugation at 6000xg for 15 minutes and the pellet was resuspended in 10 mL of denaturing binding buffer (20 mM Sodium Phosphate, 8M urea, 500 mM NaCl pH 7.8). Cell lysis was aided by flash freeze/thaw 3 times in a dry ice/ethanol bath followed by sonication for 3x10 seconds. The sample was centrifuged for 20 minutes at 3,000 xg at 4°C and the clarified supernatant was applied to a resin column previously equilibrated with wash buffer and incubated for 20 minutes at room temperature with gentle agitation. The sample was then centrifuged at 700 xg for 5 minutes and the supernatant was removed. Ten resin bed volumes of wash buffer were added and the sample was incubated for 10 minutes and centrifuged as above; the supernatant was removed and one volume of binding buffer was added to the column and the sample was allowed to flow through by gravity. The column was then washed twice with 5 mL of denaturing wash buffer pH 6.0 (20 mM sodium Phosphate, 8M urea, 500 mM NaCl) and the gravity flow through was stored for analysis. Next, 4 mL of denaturing wash buffer pH 5.3 (20 mM sodium Phosphate, 8M urea, 500 mM NaCl) were added and the column was treated as above. Finally, the His-tagged proteins were eluted using 5 mL of wash buffer pH 4.0 (20 mM sodium Phosphate, 8M urea, 500 mM NaCl), and 1 mL aliquots of the flow through were collected. The presence of tagged purified proteins in the different fractions was analyzed by SDS-page and western blotting using anti-His tag antibodies provided by the manufacturer, as well as anti-85A and anti-35kDa polyclonal antibodies. Fractions containing the recombinant proteins were pooled and dialyzed overnight at 4°C against 10mM Tris pH 8.0, 0.1% Triton-X-100 to remove urea. Protein concentration was determined by the Bradford method using a BioRad protein concentration kit (BioRad, Hercules, CA), as described above. Purified *Brucella* Cu/ZnSOD protein samples were part of our stocks and had been purified from recombinant *E. coli* DH5α pBS/SOD cells using an anion exchange column (HiTrap:Q; Pharmacia Biotech) as previously described (418).

Table 2.5: PCR primers used to generate MAP 85A and 35 kDA protein antigens to be expressed in *E. coli*.

Gene	Forward primer
85A	5'ACTAGTTCGCGCCCCGGTCTGC3'
35 KDA	5'ACTAGTATGACGTCGGCTCAAAATGAGTCTC AA3'

Results

Amplification of MAP 85A and 35 kDa genes

MAP genes were amplified by PCR as described in methods obtaining products of approximately 1Kb size (Figure 2.2). The amplified genes were sequenced at the Virginia Tech Core Laboratory Facility. Sequence analysis using BLAST alignment software confirmed the amplification of MAP 85A gene. The amplified sequence was identical to the previously published MAP 85A gene (GenBank accession number: AF280067) and was 99% identical to its homolog in *M. avium* (GenBank: D78144). It also revealed 84% identity to *M. bovis* 85A (GenBank X53034) and to MTB 85A (GenBank: U47335). The amplified sequence for the 35 kDa protein gene revealed 99 % identity to *M. avium* 35 kDa protein gene (GenBank: U43835), at the time, no matches to published MAP sequences were found. Shortly after Banasure et al, (29) used a similar approach and published the complete sequence of MAP 35 kDa protein gene (GenBank:AJ250887), which reveales 99 % identity to our amplified sequence.

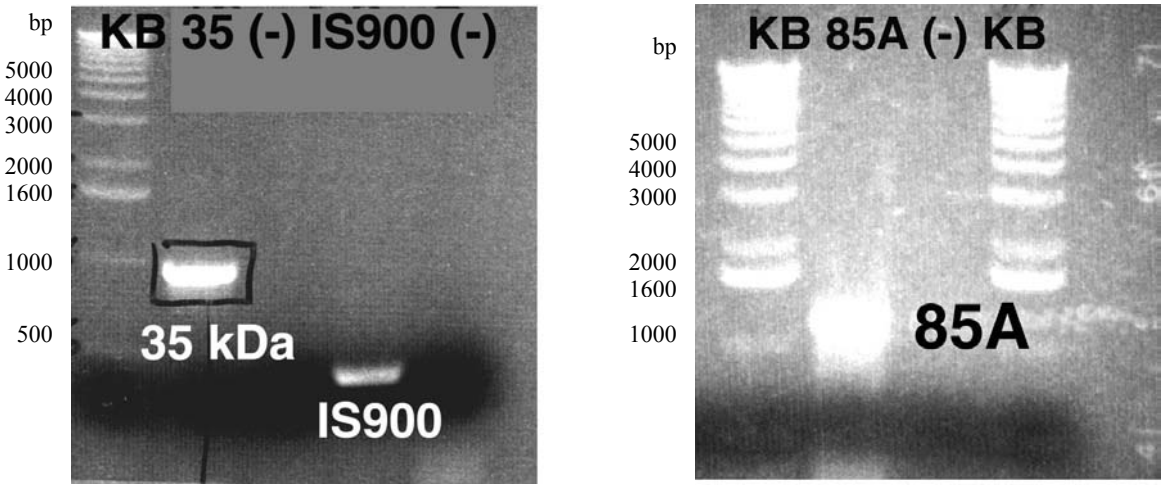

Figure 2.2: PCR products obtained from *M. avium subsp. paratuberculosis* DNA templates.

a): Amplification of the 35 kDa protein gene, and amplification of IS900 as a marker for *M. avium subsp. paratuberculosis*.

b): Amplification of the 85A gene.

Expression of the heterologous proteins in *E. coli* and *B. abortus* vaccine strain RB51

Expression of the recombinant MAP proteins in *E.coli* BL21DE3LysS/pRSET pTB85A or BL21DE3LysS/pRSETpTB35 cells was assessed by western blot using a rabbit his tag antisera (Invitrogen, Carlsbad, CA), as well as specific 85A and 35 kDa protein antisera. Low levels of expression of 85A and 35 kDa proteins were detected in *E. coli* (data not shown). Most of the recombinant proteins were located in insoluble fractions that only moderately solubilized in urea. These recombinant proteins were purified by affinity chromatography under denaturing conditions and the purified proteins were used as antigens in subsequent experiments.

Expression of the homologous and heterologous proteins in recombinant *E.coli* BL21DE3LysS, *E. coli* DH5α and strain RB51 cells was evaluated by 12% SDS-PAGE with Coomassie Blue staining or Western Blotting using 85A and 35 kDa specific antisera after heat induction at 42ºC for 45 minutes. Although low levels of expression of the 85A protein were detected in western blot assay in *E. coli* pBBpTB85A and in *E. coli* pBBSODpTB85A using an 85A specific antibody, very low expression was detected in strains RB51pTB85A and RB51SODpTB85. No expression of the 35 kDa protein was observed in *E. coli* pBB35 or pBBSOD35 or in strains RB5135 or RB51SOD35 when blots were reacted with 35 kDa specific antibodies.

Recombinant RB51 strains transformed with pBB plasmids encoding MAP antigens did not exhibit phenotypical differences in colony morphology compared to strain RB51. However, these strains did exhibit slower growth and required on average 6 days to achieve normal colony size.

The low level of expression observed in the recombinant RB51 strains forced us to create new constructs with better expression of the heterologous genes. Higher expression levels, especially of secreted proteins, have been shown to correlate with increased protection. It has been previously reported that expression of mycobacterial proteins in heterologous vectors is very low (210). However, we had observed an

increase in expression of mycobacterial antigens when they are expressed as fusion proteins with either MBP or Esat-6. It is for this reason, the constructs expressing the MAP antigens as fusions with MTB Esat-6 protein or with *Brucella* Cu/ZnSOD signal sequence were created as described in methods. The generation of fusion proteins with the Cu/ZnSOD signal sequence was carried out to additionally direct the protein product to the periplasmic space of *Brucella*. We have observed that over-expression of Cu/ZnSOD in strain RB51 leads to its leakage into the culture supernatant and provides increased protection against *Brucella* challenge; therefore, targeting this location could also lead to leakage of the recombinant fusion protein and could potentially better stimulate the immune response of vaccinated mice.

SDS-PAGE-Western Blot analysis of *E. coli* recombinant cells expressing MAP 85A as a fusion with Esat-6 demonstrated production of the recombinant protein when the blot was reacted with 85A specific antibody. The recombinant protein was observed in strain RB51Esat-6pTB85A but expression by RB51SODEsat-6pTB85A was low (Figure 2.3 and Figure 2.4 respectively). No 85A expression was detected in *E. coli* or in strain RB51 when the proteins were engineered as fusions with SOD signal sequence and first aminoacids in plasmid pBBSODsspTB85A (data not shown).

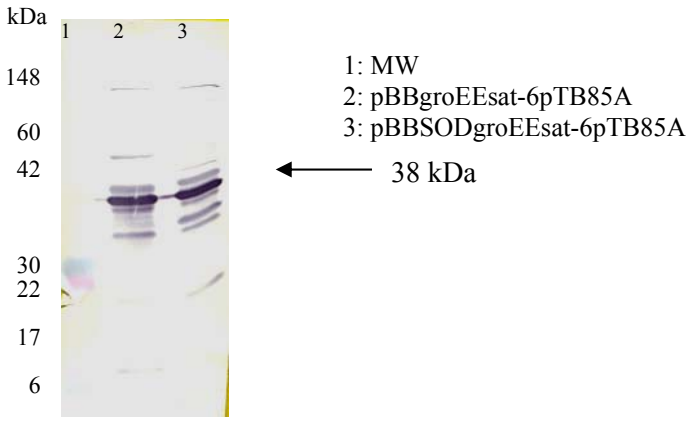

Figure 2.3: Western Blot of recombinant *E. coli* DH5A expressing MAP 85A as a fusion with Esat-6 protein.

Whole cell *E. coli* antigens were resolved in a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The blot was reacted with 85A specific antisera. The arrow indicates the position of the Esat-685A protein.

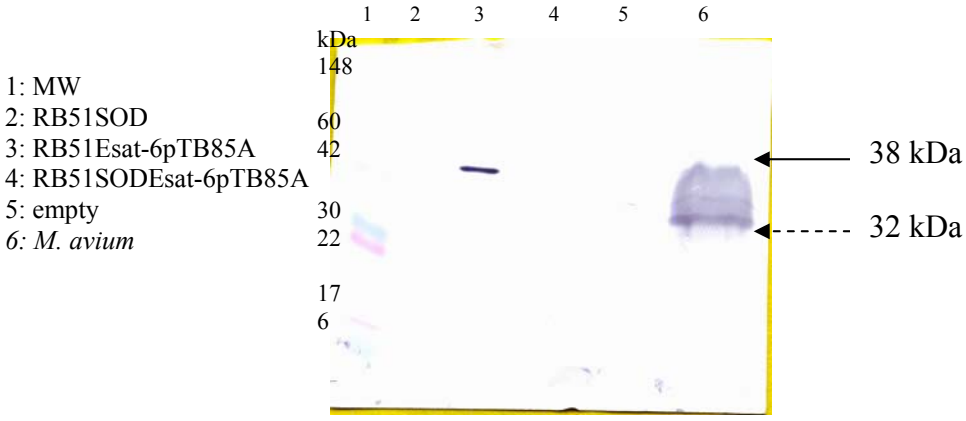

Figure 2.4: Western Blot analysis of strain RB51 based vaccines.

Whole cell lysates of RB51 recombinant strains were resolved on a 12% SDS-PAGE gel and reacted with 85A antisera. The solid arrow indicates the position of the Esat-6pTB85A band (approximately 38 kDa). The dashed arrow indicates the position of 85A in *M. avium* (32 kDa).

TCA precipitation of mid-log culture supernatants of all vaccine constructs failed to detect the recombinant MAP proteins. As expected, only *B. abortus* Cu/ZnSOD was detected in the culture supernatant of the RB51SOD control; data not shown.

Discussion

In our study we were able to express MAP 85A and 35 kDa protein in *E.coli* at low levels as insoluble proteins. This finding has been reported for the expression of heterologous proteins in *E. coli* where the protein product undergoes improper folding that leads to its accumulation in inclusion bodies (261). This is especially common for expression of mycobacterial proteins which typically accumulate in insoluble aggregates within the cytoplasm (361, 425), and has also been recently shown for the expression of the 35 kDa protein of MAP in *E. coli* (36).

Expression of MAP proteins in strain RB51 was very low but an increase in expression was observed when the 85A protein was fused to MTB Esat-6 protein (Figure 2.4). Based on our results we concluded that *B. abortus* vaccine strain RB51 is able to express heterologous antigens from *M. avium subsp. paratuberculosis* but expression is quite low. This low level of expression may ultimately result in non protective strains due to low levels of presentation of the heterologous antigen to the immune system.

There are several explanations for the lack of expression of MAP proteins in *Brucella*. Some mycobacterial proteins undergo post-translational modifications such as lipidation and glycosylation (101, 215) but this is not the case for the proteins selected in this research. *Brucella*'s genome has a GC content of approximately 58% (94, 309) whereas mycobacterial GC content is approximately 65% (31). This different nucleotide composition may account for differences in codon usage between *Brucella* and mycobacteria which may lead to the depletion of some of the tRNA pools required for synthesis of mycobacterial proteins in *Brucella*. In this regard, Lakey et al., (210) have

reported that increased expression of mycobacterial genes can be obtained in *E. coli* when codons of low use were replaced for codons normally used in *E. coli*.

Decreased expression of heterologous genes in *Brucella* has also been shown by Reichow et al., (321). In their study, expression of *B. anthracis* Protective Antigen in strain RB51 was very low until the *pag* gene for this antigen was modified to adapt to the codon usage in *Brucella*, obtaining higher expression levels. Therefore, future studies involving expression of mycobacterial genes in *Brucella* should focus on the engineering of the heterologous genes to use a *Brucella* friendly codon bias and therefore increase expression.

Another potential explanation for the lack or very low expression of the heterologous genes is that the protein product may be toxic or otherwise deleterious for *Brucella,* which would reduce its expression in the bacterium. Expression of the recombinant proteins in strain RB51 slowed growth on solid media but had otherwise no other obvious phenotypic effects.

Interestingly, expression of the 85A protein increased when the genes were expressed as fusions with the *M. tuberculosis* Esat-6 protein, but this was not observed when 85A protein was expressed as a fusion with SOD signal sequence, indicating that this may be a phenomenon specific for Esat-6. The fact that the 85A protein was not detected in strain RB51SODsspTB85A was not due to the "export" outside the cell as the protein was not detected in culture supernatants.

The phenomenon of increased expression of proteins when expressed as fusions has been previously reported. Lee et al., (221) increased the tandem multimeric expression of peptides when they were fused to an RNA binding protein that increased stability of the construct by covalent linkage of disulfide bonds. Expression of heterologous proteins in *E. coli* as fusions with a different gene product has been demonstrated to be an effective means of stabilizing protein expression by mechanisms that are not completely clear. Fusion proteins become resistant to protein degradation in some cases by promoting the formation of inclusion bodies or by promoting stable folding of the protein (261). It has also been demonstrated that the level of expression of

fusion proteins is dependent on the protein or peptide chosen to act as the fusion carrier (55, 356, 422). It is possible that similar processes could contribute to stabilization of heterologous proteins in *Brucella spp.*

When culture supernatants of the different vaccine candidates were analyzed by western blotting, Cu/ZnSOD was only detected in the supernatant of strain RB51SOD. No other proteins were detected in the culture supernatants of any of the recombinant strains. The finding of Cu/ZnSOD in the culture supernatant of strain RB51SOD has been previously observed in our laboratory. No secreted proteins have been identified in *Brucella* but a potential secretion system, the virB operon, has been recently identified and shown to be important for virulence of the bacterium; no substrate has yet been characterized for it (52, 286, 385). The presence of Cu/Zn SOD in the supernatant of this culture is thought to be due to the high accumulation of SOD in the periplasmic space of *Brucella* that promotes "leakage" of the protein into the supernatant. The absence of Cu/ZnSOD in the culture supernatants of strains RB51SODpTB85A and RB51SODesat6pTB85A is interesting. It is possible that the simultaneous expression of MAP 85A protein with over-expression of Cu/ZnSOD reduces the amount of the SOD protein produced in strain RB51. Western blot analysis did indicate and increased amount of SOD in whole cell lysates of strain RB51SOD compared to strain RB51, but the exact amount of protein was not determined. Expression of the heterologous protein may abrogate the leakage of Cu/ZnSOD, by direct binding or by interfering with the transport of the protein to the periplasmic space of strain RB51. Another possible explanation is that the heterologous proteins may be expressed in an insoluble form in strain RB51. As mentioned above, this was observed when MAP 85A and 35 kDA proteins were expressed in *E.coli*. If the heterologous proteins are indeed expressed as insoluble proteins or inclusion bodies, they may well interfere with the normal protein synthesis and general transport mechanisms in strain RB51. This would prevent the accumulation of the over-expressed Cu/ZnSOD in the periplasmic space thus preventing is "leakage" into the culture supernatant. Although the mycobacterial 85A proteins are secreted in *Mycobacterium spp*., as expected no 85A proteins were detected in culture supernatants

of our recombinant vaccine strains. The low expression levels as well as the lack of an active protein secretion system in *Brucella* made the possibility of secretion of the antigen very unlikely.

Further research should be carried out to determine the exact location and solubility of the heterologous proteins as well as of Cu/ZnSOD in these strains. The effect of different fusion protein carriers and of switching codon usage towards *Brucella* preferential may improve the level of expression and solubility of these recombinant proteins. The generation of strain RB51 based vaccines with increased expression of soluble proteins may potentially increase the immunogenic and protective potential of the vaccines.

CHAPTER 3

Evaluation of the mouse immune responses to recombinant strain RB51 vaccines expressing antigens of *M. avium subsp. paratuberculosis*.

Introduction

M. avium subsp. paratuberculosis (MAP) is the causative agent of paratuberculosis, also known as Johne's disease, an endemic disease that affects cattle, sheep and goats worldwide (123, 167). The organism is a member of the genus *Mycobacterium* and is closely related to its parent strain *M. avium avium,* sharing over 95% of genomic identity (198). Although these species are genetically very close, and both are acid fast intracellular rods with typical mycobacterial cell wall structure, they do possess some striking phenotypic differences (198). For example, MAP's dependence on mycobactin J supplementation when grown *in vitro*, and its extremely slow growth in laboratory conditions, where it may take up to 6 months for colonies to develop compared to 10-14 days for *M. avium* (211). Another difference is their host species preferences. *M. avium* was initially isolated from birds and was thought to be confined to a very limited number of species, but with the AIDS epidemic it has gained public health importance and has become an important secondary disease in immunocompromised individuals (123). MAP on the other hand, remains strongly linked to ruminants where it causes severe clinical signs, but recent findings have shown a potential link to Crohn's disease in human (75, 171, 275, 383).

In general, MAP infections are characterized by the formation of granulomas, which are a localized cellular immune response, in which macrophages and lymphocytes enclose an area of infected cells and of inflammatory infiltrates in an attempt to limit the dissemination of the organisms. Many mycobacterial organisms are killed within these structures, but as described in Chapter 1, virulent mycobacteria have developed strategies

to avoid or block the host's immune response and are able to survive and replicate inside them. Granulomas do cause considerable tissue damage and impairment of the function of the affected organs which correlates with the severity of the disease (40, 150, 192).

Regarding pathogenesis in cattle, MAP is associated with progressive chronic granulomatous enteritis that leads to wasting of the affected animal and to its eventual death (405). *M. avium* infections in cattle are generally subclinical with diffuse granulomas, *M. avium* infections in birds cause a granulomatous avian tuberculosis (156, 389). In immunocompetent humans, *M. avium* shows mainly pulmonary manifestations but some cases of lymphadenitis have also been described. In immuno-compromised individuals the clinical manifestations range from diffused pneumonia to generalized disseminated disease (123). These clinical signs tend to correspond with the infection route for both species. Infection with *M. avium* is thought to occur primarily via the inhalation route, whereas the oral route is believed to be the main portal of infection for MAP (123, 211, 405).

A strain RB51 based vaccine expressing protective MAP antigens appears to be an attractive approach for protection against both infections, i.e. *B. abortus* and MAP. Vaccination of cattle against brucellosis using strain RB51 is carried out on heifers between 4-8 months of age; vaccination before 3 months of age does not appear to induce protective immunity against brucellosis (314). Because calves become infected with MAP as neonates, the animals would already be infected by the time they are first vaccinated against brucellosis. Therefore, the ultimate goal of this research is to generate an RB51 based vaccine that would provide protection against MAP exposure in susceptible animals not by preventing infection, but by lengthening the time until clinical signs appear and/or decreasing the fecal shedding of organisms and contamination of pastures. Studies in New Zealand, in which vaccination of lambs already infected with MAP with a commercial *M. paratuberculosis* vaccine was carried out, have demonstrated that this is a feasible approach (159). In fact, vaccination with an effective strain RB51 based vaccine expressing MAP antigens could be used in combination with an early MAP vaccine and serve as the booster dose. In this case, the use of a multi-

valent strain RB51 vaccine able to protect against brucellosis and MAP, is economically desirable and immunologically sensible since both diseases are caused by intracellular parasites that require the elicitation of strong cell mediated immunity in order to control and clear the infection.

Rationale and Hypothesis

The genomes of MAP and its parent strain *M. avium avium* have been sequenced and have been shown to share over 95% genomic identity. A comparison of almost half of the genome of both species revealed only 27 genes that are present in MAP but absent in *M. avium* (34). Therefore, in principle, a vaccine able to protect against MAP should also have a high probability to protect at least partially against *M. avium*. The unique genes in each organism may account for the phenotypical differences between both strains, including MAPs requirement for mycobactin J supplementation, its extremely slow growth, the preferential tissue tropism and the clinical signs of the diseases (405). The differences in pathogenesis and growth characteristics have lead to the development of very different animal models to study these diseases. With regards to *M. avium,* several mouse models have been developed and are designed to replicate the aerosol route of exposure favored by this organism. Many aerosol models as well as mouse species have been characterized for *M. avium* (40, 74). On the other hand, the study of MAP's pathogenesis has been restricted to the natural hosts of the organism such as cattle, sheep and goats (159, 265, 382). Very few mouse models have been developed mainly due to MAP's inability to infect most mouse strains and the very long time required to establish this infection (408, 409). This obstacle has been overcome by the use of severely immunocompromised mouse models, including the Beige, Nude and SCID mouse strains as well as thymectomized and/or irradiated mice (268-270, 343, 373). Most mouse strains are resistant and therefore the use of severely immunocompromised mice has been standard procedure when clinical signs and the visualization of pathology are required (270, 373). Clearly, it is impossible to evaluate immune responses and vaccine efficacy in these models. BALB/c mice have been

described as being susceptible to infection with MAP (386),(72), but in these studies neonate or infant mice (3-5 day old) were used for infection. In these cases the number of cfu /spleen slowly increased up to 8 months post infection. This neonate model is a candidate for the evaluation of vaccines but does present several drawbacks. The immune responses of neonate mice have not been characterized. Even if mice are vaccinated as adults, another concern is the length of time it takes until and clearance results can be obtained. In addition, the endpoint to evaluate clearance of MAP from the spleens of vaccinated mice would have to be several months post vaccination. Moreover, MAP is an extremely slow growing organism in laboratory conditions and would require several months until results can be obtained.

Part of the research effort in this project relates to the development of a *B. abortus* strain RB51 based vaccine expressing antigens of MAP to induce immunity specifically directed to this and other closely related mycobacteria. The use of the natural host as models to study MAP infections is also impractical since cattle studies are very expensive and time consuming. The cheaper sheep and goat models are not adequate for the present study because vaccine strain RB51 does not induce protection against *Brucella* infection in these animal species (107, 185). Therefore the development of a suitable animal model to evaluate the elicitation of immune responses and protection was an important goal of this research.

The MAP genes expressed in the recombinant strain RB51 based vaccine strains constructed in Chapter 2 share over 98% identity to their *M. avium* homologs; therefore we decided to evaluate the feasibility of using a model of *M. avium* challenge instead of MAP to assess the potential protective efficacy against MAP. Several mouse models for *M. avium* have been described, but most of them involve aerosol challenge via the nasal or iv route (124, 125, 154, 201, 342). Since the *Brucella* models are based on the use of BALB/c mice and intraperitoneal challenge with virulent organisms followed by determination of splenic clearance (186, 428), we decided to evaluate the feasibility of using the genetically susceptible BALB/c mouse model (201) with *M. avium* challenge in a protocol similar to that used for the evaluation of *Brucella* vaccines. If such a model

can be developed, vaccines with potential to protect against MAP may give some protection against *M. avium* or vice-versa.

This chapter is divided into three sections, the first part evaluates whether BALB/c mice can be infected with *M. avium,* as a possible model for vaccine evaluation by checking for reduced infection in vaccinated mice. This model has the distinct advantage of being much faster than using MAP because it takes less than two weeks for the organism to grow in laboratory conditions and also, potentially provides a more general model for the evaluation of vaccines against *Mycobacterium spp.*, not just restricted to MAP. The second section evaluates the immune responses elicited by the recombinant strain RB51 based vaccines expressing MAP antigens developed in Chapter 2, and the third section uses the above model to test if these strain RB51 based vaccines expressing MAP antigens induce protection against *M. avium* challenge.

If a reduction in the number of splenic organisms is observed, these potentially protective vaccines could then in the future be evaluated for protection against MAP in a suitable animal model.

We hypothesize that vaccination with strain RB51 based vaccines expressing MAP antigens can induce protective immunity against challenge with *M. avium*.

Objectives

1) Establish a mouse model to evaluate protection against *M. avium* challenge by recombinant vaccine strain *B. abortus* RB51 expressing antigens from *M. avium subsp. paratuberculosis.*

2) Analyze the humoral and cell mediated immune responses of mice vaccinated with these recombinant vaccines.

3) Test the protective capacity of these vaccines against challenge with *M. avium* in the developed model.

Material and Methods

Animals, bacterial strains and antigens: Female, 4-5 week old BALB/c mice purchased from Charles Rivers Laboratories (Wilmington, MA), were used at 6-8 weeks of age for all experiments. The animals were housed in groups of 3-5 in micro-isolation cages at the BSL-3 facility of the VMRCVM. All animal procedures were performed according to the IACUC guidelines, with supervision by the university veterinarian..

Vaccine strains *B. abortus* RB51, RB51SOD, RB51pTB85A, RB51SODpTB85A, RB5135, RB51SOD35, RB51Esat-6pTB85A and RB51SODEsat-6pTB85A, were described in Chapters 1 and 2. *B. abortus* strain 2308 is a virulent smooth strain widely used in *Brucella* challenge models (15). *Mycobacterium avium* strain A5 was kindly provided by Dr. J. Falkinham III of Fralin Biotechnology Center, Virginia Tech. This strain, isolated from a human AIDS patient (421), was grown in Middlebrook 7H9 agar plates or 7H11 broth (BD, Sparks, MD), supplemented with glycerol and OADC enrichment media (BD, Sparks, MD). Plates and liquid cultures were incubated at 36ºC for 8-14 days. *Ochrobactum anthropi* 49237SOD (O.a SOD) is part of our collection and has been described elsewhere (169). This strain carries a pBBR1MCS plasmid encoding the *B. abortus sodC* gene and expresses *B. abortus* Cu/ZnSOD protein.

Recombinant *B. abortus* Cu/ZnSOD and purified recombinant MAP 85A were generated as described in Chapter 2. *M. avium* culture supernatant proteins were obtained by centrifugation from an *M. avium* culture at mid-log phase. The supernatant was then precipitated using trichloroacetic acid at a 20% final concentration and incubated overnight at 4°C. The sample was then centrifuged and the pellet was washed with acetone and resuspended in Laemmli Buffer (232). Protein concentration was determined by the Bradford method using a BioRad protein assay kit following manufacturer's instructions (BioRad, Hercules CA). The genetic adjuvant phosphothioate CpG: 5'-TCC ATG ACG TTC CTG ATG CT-3' was custom made at Genosys (Sigma, St. Louis, MO).

Vaccination studies: Mice were inoculated intraperitoneally with 100 μl of saline (0.85% NaCl) containing 2-4 x10^8 cfu of the experimental vaccine strains RB51pTB85A, RB51SODpTB85A, RB5135, RB51SOD35, RB51Esat-6pTB85A or RB51SODEsat-6pTB85A. Vaccination with *B. abortus* RB51 and RB51SOD was also included as controls.

Serological analysis: Serum samples were collected by retro orbital venipuncture under local anesthesia. Western Blot analysis was performed as previously described (209). Briefly, protein samples in 2X Laemmli buffer were resolved on a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Transfer efficiency was determined by Ponceau S staining and the membranes were incubated in blocking buffer (2% BSA/Tris-HCl) for at least 3 hours. The membranes were then incubated overnight with serum at an appropriate dilution followed by 5 washing steps (Tris-HCl/Tween-20 0.05%). The membranes were then incubated with anti-mouse secondary antibodies conjugated with HRPO (Cappel ICN, Irvine, CA), for 1 hour. The membranes were washed as above and the reaction was detected by incubation with a-chloro-naphtol/H_2O_2 or TMB substrate (Sigma, St. Louis, MO).

Antibody levels were determined by ELISA. Briefly, 96 well NUNC Maxisorp plates (Fisher, Fair Lawn NJ) were coated overnight at 4ºC with 0.5 μg of purified recombinant *Brucella* Cu/ZnSOD protein, 0.5 μg/well whole cell *Brucella* antigen, 0.1 μg/well of purified *Y. enterocolitica* LPS or 1 μg/well of *M. avium* culture supernatant TCA precipitated proteins (see Chapter 2), in carbonate coating buffer (pH 9.6). The plates were then blocked using 2% BSA in TBS at room temperature for 1 hour and the serum samples diluted 1:1000 in blocking buffer were added in duplicate and incubated at room temperature for 3 hours. After washing 5 times (TBS/Tween-20) the secondary HRPO conjugated antibodies (anti-mouse whole IgG, anti mouse IgG_1, anti-mouse IgG_{2a} or anti mouse IgG_3) (Cappel ICN, Irvine, CA), were added at appropriate concentrations and the plate was incubated at room temperature for 1 hour. Following the incubation step, the plates were washed 5 times with wash buffer and 100 μl /well of TMB substrate

(Sigma, St. Louis, MO), were added and the plates were incubated in the dark for 30 minutes and stopped by addition of 100 μl/well of 10 mM sulphuric acid. Optical density readings were determined using a Versamax plate reader (Molecular Devices, San Diego CA) set at 450 nm and the readings were adjusted with a blank of diluent buffer.

Uptake of *M. avium* A5 by J774A.1 macrophages: To determine whether *M. avium A5* organisms are phagocytosed by the J774A.1 macrophage like cell line (ATCC# TIB-67, Manassas, VA), 75 cc tissue culture flasks were seeded with J774A.1 cells in DMEM media (Fisher, Fair Lawn, NJ) supplemented with 10% sterile fetal bovine serum (Sigma, St. Louis MO) heat inactivated 45 minutes at 60ºC (cDMEM), cells were grown to confluency in a 37ºC, 5% CO_2 incubator. The cells were then infected with a 100:1 ratio of live *M. avium A5* (288), in cDMEM. The exposed cells were then incubated for 30 minutes, 1, 2, 4, 6 hours and overnight at 37ºC +5% CO_2. After each time point cells were recovered by using a cell scraper, centrifuged at 1200 rpm for 10 minutes and the pellet was washed 5 times with media to remove non-internalized mycobacteria. After the final wash, the cells were centrifuged, resuspended in 1 ml of media and aliquots from each test flask were smeared onto glass slides and heat fixed. The cells were then stained with Ziehl-Nielsen reagent (Sigma, St. Louis MO) as previously described (233), to identify the presence of acid-fast rods. Stained preparations were observed under a microscope with immersion oil. Acid fast rods stained red on a green background.
In a parallel experiment, 24 well tissue culture plates were seeded 1 ml cDMEM containing $5x10^5$ cells and incubated until confluency in a 37°C + 5% CO_2 incubator. Cells were then infected with *M. avium* in 1 ml cDMEM at a 100:1 ratio for 6-12 hours. After incubation the wells were washed 5 times with media and 200 μl of lysis buffer were added to each well (0.1% Triton X-100 in PBS) and incubated at room temperature for 20 minutes. Cells were then lysed by repeated pipetting, serially diluted and plated onto Middlebrook 7H11 plates. Cell lysis was confirmed by visual inspection of the plate under a microscope.

***M. avium* model:** In order to evaluate the vaccine potential of the strain RB51 based vaccines expressing MAP antigens described in Chapter II, an infection model in female, 6-8 week old BALB/c mice, using *M. avium* strain A5 via the intraperitoneal (i.p) route was evaluated. This mouse strain was selected because it is genetically susceptible to *M. avium* infection and is the standard strain used to evaluate *Brucella* vaccines (14, 125, 183, 186, 205, 253, 341). The first step was to determine the necessary dose of *M. avium* required to establish an infection with *M. avium* strain A5 in these mice, and evaluate its duration in the spleens of infected mice. Similar to the *Brucella* model, vaccination induced protection would be evidenced as a decreased number of splenic *M. avium* colonies in the vaccine groups compared to the saline controls. To determine the appropriate dose required to establish an infection, groups of 12 mice each were infected by i.p injection with $1x10^7$, $1x10^8$ or $1x10^9$ cfus of *M. avium* strain A5 in 100 µl of saline buffer (0.85% NaCl). The challenge doses were briefly sonicated 5 times for 20 seconds before inoculation in order to disperse aggregates, and the infection dose was retrospectively corroborated by plating onto Middlebrook 7H11 plates. The animals were visually monitored daily to determine signs of disease. Three mice per group were sacrificed 3, 6, 8 and 10 weeks post infection. Their spleens were removed, serially diluted and plated onto Middlebrook 7H11 plates and incubated at 36°C for up to 14 days for cfu determination. An additional group of 3 mice was infected i.p with $1x10^9$ cfu of *M. avium* A5 and one year post infection the mice were sacrificed and their spleens were plated as above.

Attenuation of *M. avium* A5 by gamma irradiation: Because there are currently no clearly protective *M. avium* vaccines, vaccination with irradiated *M. avium* was tested to determine whether irradiated *M. avium* could protect against challenge with live *M. avium A5*. The rationale behind this approach is that homologous vaccination with live organisms should confer the highest degree of protection because it exposes the immune system to the complete array of antigens present in the challenge strain. Research carried out in Chapter 4 indicated that irradiation of a live vaccine strain abrogated its replication

while preserving the vaccine's protective capacity. By abrogating replication, irradiation fully attenuated the strain but retained its metabolic activity, preserving the ability to stimulate the immune system, a characteristic lost in killed vaccines. To determine the radiation dose required to abrogate replication of *M. avium,* a 1 ml -80°C deep frozen/thawed vial of *M. avium* strain A5 was aliquoted into 5 microcentrifuge tubes. All tubes were irradiated in a Co source gamma irradiator (JL Shepperd and Associates, San Fernando, CA) with 198,000 rads, 396,000 rads, 297,000 rads and 594,000 rads (30, 45, 60, and 90 minutes in the irradiation chamber respectively), one non irradiated aliquot was used as control. After irradiation, the contents of the vials were serially diluted, plated onto Middlebrook 7H11 plates supplemented with glycerol and OADC (BD, Sparks, MD) and incubated at 36ºC for up to 20 days to determine viability. The presence of metabolic activity in the irradiated samples was determined by the Alamar Blue Reduction Assay (5). This assay relies on the reduction of the Alamar Blue dye by live cells. Live cells have a reduced internal environment that donates electrons to the dye, inducing a change of color that can be measured fluorometrically. Briefly, 100 µl aliquots from each of the irradiated samples, containing approx 1 $x10^9$ organisms were dispensed in triplicate onto Falcon 96 well flat bottom tissue culture plates (BD Labware, NJ). Aliquots of non irradiated and heat killed *M. avium* A5 were used as positive and negative controls respectively. The plate was incubated at 37ºC for 30 minutes and then 10 µl of Alamar Blue reagent (Biosource Int., Camarillo, CA) were added to each well. The plate was further incubated for 12 hours and the fluorometric reading was determined using a CytoFluor II fluorescence multiwell plate reader (Perspective Biosystem, Framingham MA) set to Excitation: 530/30, Emission: 590/30, and gain: 35. The readings were adjusted with a blank of media alone and the results are presented as average of the triplicate wells.

Another potential positive control was sought by vaccinating mice with the commercially available cattle *M. paratuberculosis* inactivated vaccine (Mycopar®, Fort Dodge Animal Health) or with gamma irradiated inactivated *M. avium* strain A5. Groups of 5 BALB/c mice each were vaccinated i.p with 100 µl of a 1:100 dilution of the

Mycopar vaccine, $1x10^9$ cfu of irradiated *M. avium A5* (594,000 rads see above) or saline control. Four weeks post vaccination all animals were challenged as above using live *M. avium* A5, and all animals were sacrificed 3 weeks later for splenic cfu determination.

Protection studies: Groups of 5 BALB/c mice each were vaccinated as described above. Six weeks post vaccination all animals were challenged i.p with $1x10^9$ cfu of *M. avium* A5. Three weeks post vaccinations mice were sacrificed by CO_2 inhalation and spleen suspensions were serially plated onto Middlebrook media for cfu determination.
One experiment designed to analyze the potential contribution of *Brucella* SOD in protection against *M. avium* challenge was performed by vaccinating groups of 5 BALB/c mice each with saline or with the vaccine strains RB51, RB51SOD, *O. anthropi* 237SOD or *O. anthropi*237SOD with the co-administration of the genetic adjuvant CpG. The dose of the vaccines were 2-4 $x10^8$ cfu i.p, and CpG was administered i.m at a dose of 10 nmols 4 hour prior to vaccination and 10 nmols at the time of vaccination. Six weeks later all animals were challenged with *M. avium* A5 as described before.

Cytokine analysis: For cytokine analysis mice were sacrificed at various time points post infection by CO_2 inhalation. The spleens were aseptically removed and disintegrated into single cell suspensions using a metal mesh screen. Red blood cells were lysed with ACK buffer (150 mM NH4Cl, 1 mM KCO3, 0.1 mM EDTA pH 7.3) and the splenocytes were resuspended in media (RPMI +10% heat inactivated fetal bovine serum + 1% penicillin-streptomycin) and dispensed in 1 ml aliquots containing $5x10^6$ cells/well into 24 well Falcon tissue culture plates (3 mL aliquots of these cell suspensions were reserved for lymphocyte proliferation assays) (351). Stimulating antigens were added to each well in 1 ml volumes at the following concentrations: ConA (Sigma, St. Louis, MO) 10 μg/well, heat killed RB51 $1x10^7$ cells /well, *M. avium* A5 $1x10^8$ cells/well, MAP 85A or *B. abortus* Cu/Zn SOD recombinant proteins 10 μg/well, media alone was used as the negative control. The plates were then incubated at 37°C + 5% CO_2 for 5 days as previously described (418). Culture supernatants were then collected and frozen at -80°C

until used in an ELISA based commercial mouse Th1/Th2 cytokine kit (Ebioscience, San Diego, CA) following manufacturer's recommendations. Briefly, Nunc Maxisorp 96 well plates were coated overnight at 4°C with IL-2, IL-4, IL-10 or IFN-γ capture antibodies at appropriate dilutions. The wells were then washed with PBSTween-20, blocked and standards and culture supernatant samples were added undiluted in triplicate in 100 µl volumes. The plates were incubated at room temperature for 2 hours, washed 5 times, incubated for 1 hour with 100 µl of cytokine specific antibodies. After washing as before, the plates were incubated for 30 minutes with avidin-HRP antibody. After a final wash (7x) the samples were incubated with TMB substrate solution. The color development was stopped after 15 minutes using 50 µl of 2N H_2SO_4 and the plates were read with an ELISA plate reader (Molecular Devices, Sunnyvale, CA) at 450 nm.

Lymphocyte proliferation Assay: 100 µl of the splenocyte suspensions reserved in the cytokine assay were added to the wells of a 96 well Falcon (Fisher, Fair Lawn NJ) flat bottom tissue culture plate at a concentration of $5x10^5$ cells well. One hundred µl of the stimulating antigens solutions described above were added in triplicate to the wells. The plate was then incubated at 37°C +5%CO_2. Cell proliferation was evaluated using the ^3H thymidine incorporation assay and also the Alamar Blue Reduction assay. For the thymidine uptake assay the plates were incubated for 54 hours and then pulsed with 100 µCi ^3H thymidine /well. The plates were then incubated at 37°C +5% CO2 for further 18 hours and then harvested using a plate harvester (Packard, Meriden CT) and read in a scintillation counter (Packard, Meriden CT) to determine counts/minute/well. The Alamar Blue Assay was performed by adding 10 µl of Alamar blue dye to each well after 56 hours of incubation and the plate was incubated for another 18 hours before reading in a fluorometer as described above.

Vaccine attenuation studies: Groups of 15 mice each were vaccinated with the above vaccine constructs. Two, four and six weeks post vaccination five mice/group were

sacrificed by CO_2 inhalation and their spleens were removed, serially diluted and plated onto TSA/Cm plates for cfu determination.

Statistical Analysis: Analysis of Variance (ANOVA) with post analysis using Dunnet's test or Dunn's non-parametric analysis were performed using SAS statistical software or InSTAT software. Comparisons between two groups were performed with the Student's T-test. Unless otherwise stated the level of statistical significance was set at 0.05.

Results

Development of a *M. avium* mouse model

Infection of BALB/c mice with $1x10^7$, $1x10^8$ or $1x10^9$ cfus of *M. avium A5* did not induce visible signs of disease at any of the time points analyzed. Upon necropsy enlarged spleens with significantly increased weights were observed in all groups. Larger spleens were observed in the groups infected with the higher *M. avium* doses of $1 x10^8$ and $1x10^9$ cfu (Figure 3.1 and Figure 3.2). The spleens remained enlarged throughout the experimental period. One year post infection with $1x10^9$ cfus of *M. avium* A5, the spleens remained significantly enlarged but no *M. avium* organisms were recovered (Appendix A).

Figure 3.1: Effect of *M. avium* infection on the size of the spleens of BALB/c mice.

Spleens from an *M. avium* infected mouse (on top of a U.S. 25¢ coin) and from a saline normal mouse (on top of a U.S. 5¢ coin) three weeks post infection with $1x10^9$ cfu of *M. avium* A5 are shown.

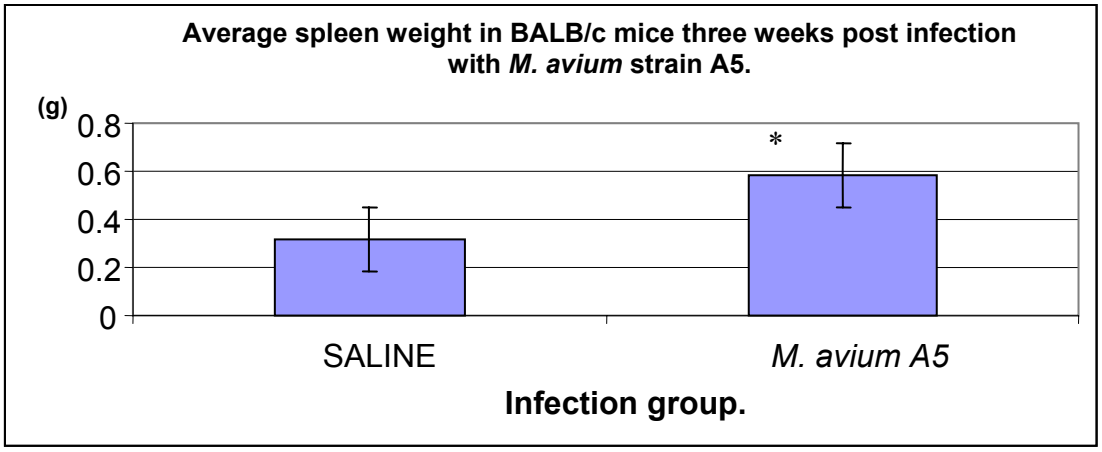

Figure 3.2: Average spleen weight in BALB/c mice three weeks post infection with *M. avium* A5 strain A5.

Mice were infected i.p with $1x10^9$ cfus of *M. avium* strain A5. The columns represent the average splenic weight, and error bars indicate the standard deviations. n=5, * $p<0.05$

A dose dependent decrease in the number of organisms recovered from the spleens was observed at each time point (Figure 3.3). Infection with the lower dose of $1x10^7$ only induced transient colonization of the spleen, with no organisms being recovered after 8 weeks of infection. The higher doses were able to establish colonization but a dramatic decrease in the number of organisms recovered at each timepoint was observed. Very few organisms (less than 100) were detected at the 10 weeks time point. The rate of clearance, as determined by analysis of the slope of the clearance curves, was not significantly different between the different doses ($p>0.05$). The *M. avium* cfu count in the spleen never surpassed the injected dose at any of the time points checked.

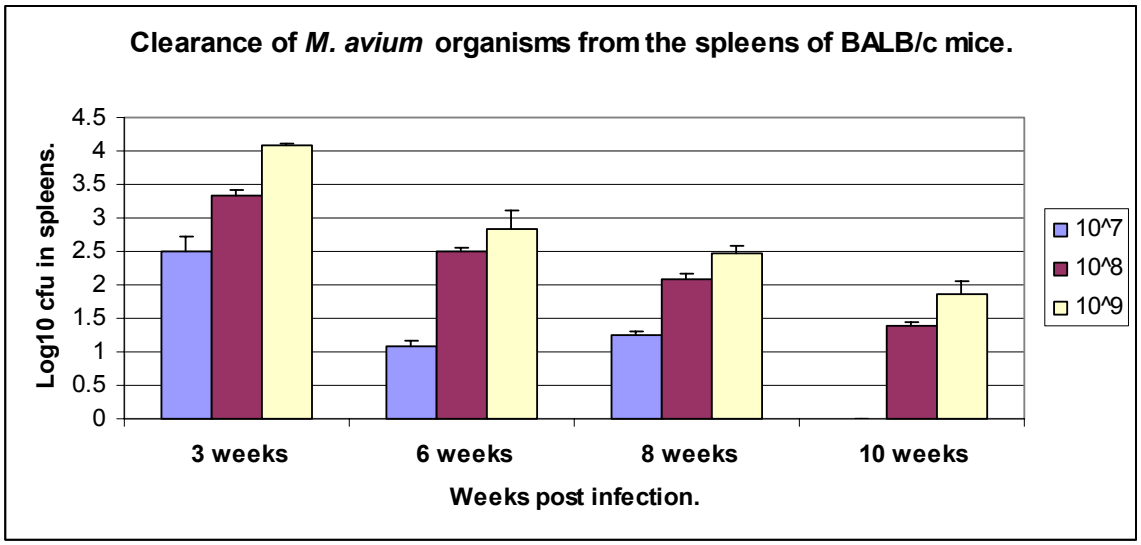

Figure 3.3: Clearance of *M. avium* organisms from the spleens of BALB/c mice.

Groups of 12 mice each were infected intraperitoneally with three doses of *M. avium* A5. At different times post challenge, three mice/ group were sacrificed and the spleens were plated for cfu determination. Columns represent the average Log10 *M. avium* cfu in the spleens of 3 mice/group, the bars represent standard error.

M. avium infected mice also developed low antibody levels to *M. avium* culture supernatant proteins. At 3 weeks post infection, the antibody levels were higher in animals inoculated with the higher doses and these titres decreased by 6 weeks post infection (Figure 3.4).

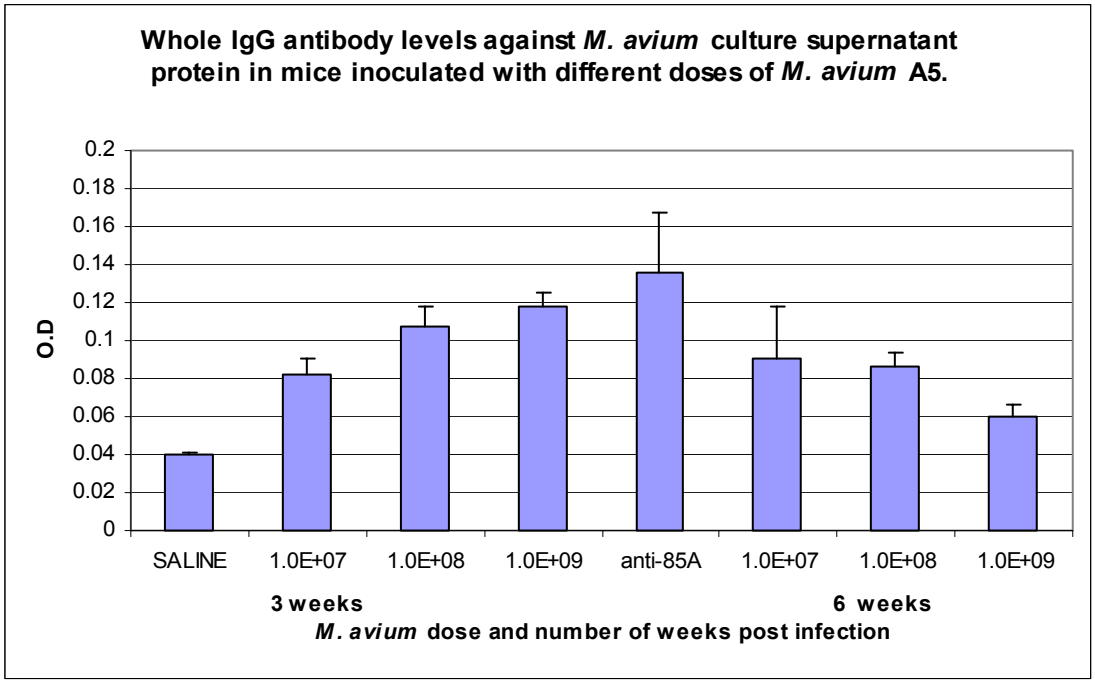

Figure 3.4: Whole IgG antibody levels against *M. avium* culture supernatant protein in mice inoculated with different doses of *M. avium* A5.

Sera were obtained from infected mice three and six weeks post i.p infection with $1x10^7$, $1x10^8$ or $1x10^9$ cfus of *M. avium* A5. Antibody titres to *M. avium* culture supernatant proteins were determined by ELISA. Sera from a mouse hyperimmunized with purified MAP 85A protein (anti-85A) was used as a positive control.

Western blot and ELISA analysis indicated the development of very low levels of antibodies to MAP 85A protein (data not shown). No 35 kDa protein specific antibodies were detected (Figure 3.5a), but unexpectedly, western blot analysis of the sera from the *Mycobacterium avium* infected mice showed antibodies to *Brucella* Cu/ZnSOD (Figure 3.5a and Figure 3.5b).

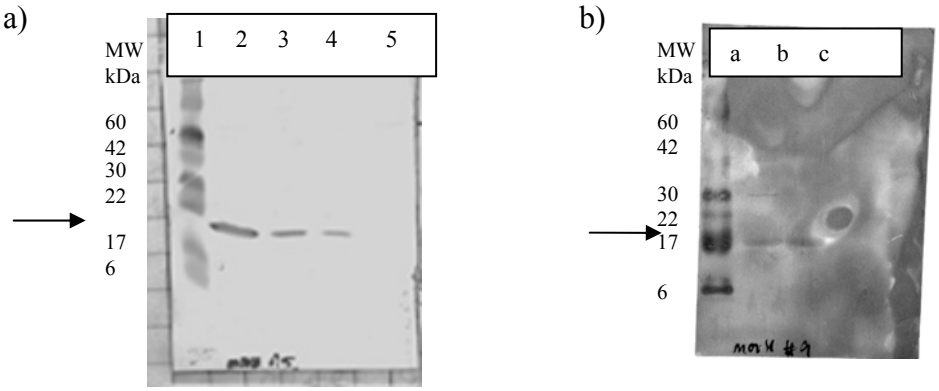

Figure 3.5: Development of *Brucella* Cu/ZnSOD specific antibodies in mice infected with *M. avium*.

a) Western Blot analysis was performed using vaccine constructs as antigens and sera from an *M. avium* A5 infected mice, 3 weeks post infection (the arrow points to *Brucella* Cu/ZnSOD antigen)

Lane 1: Molecular weight marker

Lane 2: RB51SOD

Lane 3: RB5135

Lane 4: RB51SOD35

Lane 5: purified recombinant MAP 35 kDa protein.

b) Western Blot analysis using the same serum sample against purified recombinant *B. abortus* Cu/ZnSOD as antigen.

Lane a: Molecular weight marker

Lanes b and c: purified recombinant *Brucella* Cu/ZnSOD

Macrophage uptake of *M. avium* A5:

In order to test whether *M. avium* A5 organisms are internalized by the macrophage-like J774A.1 mouse cell line, cells were infected with a 100:1 ratio of live *M. avium A5* to cells (288). The infected cells were incubated in a 37°C +5% CO_2 incubator. After extensive washing to remove extracellular bacteria, the cells were stained with Ziehl-Nielsen reagents to determine internalization of the acid fast rods. After 2 hours of incubation some intracellular rods were observed, with most intracellular rods observed after 6 hours of incubation (Figure 3.6). Upon staining, acid fast rods associated with macrophages were observed. No free *M. avium* organisms were observed indicating that the washing procedure had eliminated most if not all non-internalized organisms.

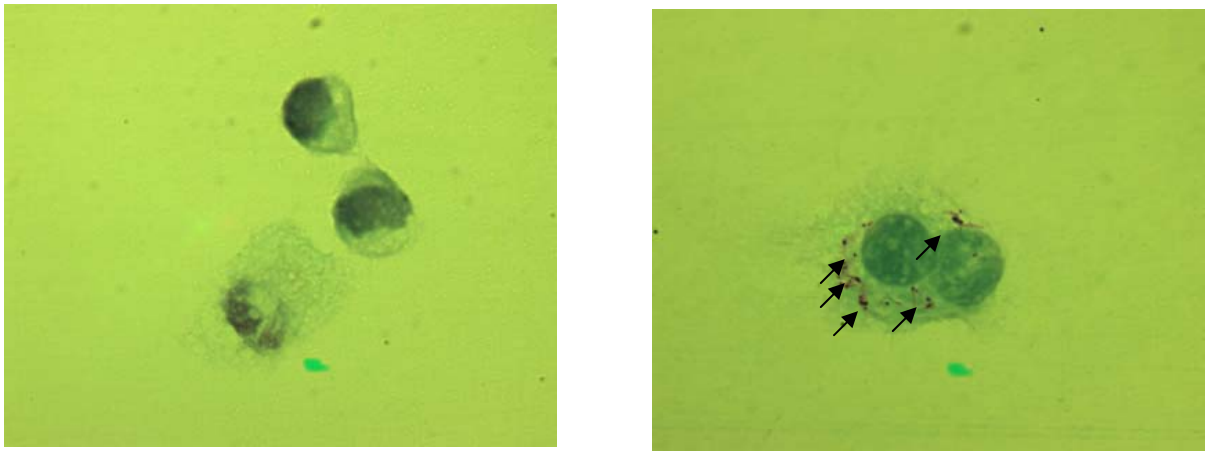

Figure 3.6: Uptake of *M. avium* A5 organisms by J774A.1 macrophage-like cells after 12 hours incubation.

Infection was carried out at a ratio of 100:1 (bacteria: J774A.1 cells) for 12 hours, washed 5 times, heat fixed and stained with acid fast stain. Left panel, negative control. Right panel, *M. avium* organisms are indicated by arrows.

To evaluate the rate of infection, 5 x10^6 J774A.1 cells/well were seeded onto 24 well tissue culture plates, a 100:1 ratio of live *M. avium* A5 organisms: cells were added to each well and 12 hour post infection the cells were lysed with Triton X-100 solution and plated onto Middlebrook plates to quantify viable *M. avium*. The results indicated an average recovery of *M. avium* organisms of 1.06 x10^6 cfu/ well. The proportion of infected cells and/or number of intracellular *M. avium*/cell was not determined.

From the experiments of spleen clearance and macrophage invasion we concluded that *M. avium A5* is able to infect and temporarily colonize spleens when injected i.p at the higher doses of 1x10^8 and 1x10^9, although the number of organisms sharply decreases after 3 weeks and are never higher than the original inoculum. Based on these observations we decided to use the higher dose of infection as our challenge dose with the endpoint three weeks post challenge.

To determine the cytokine profile of mice acutely or chronically infected with *M. avium* A5, mice were vaccinated with 1x10^9 cfu of *M. avium* and cytokine levels in splenocyte culture supernatants were determined 3 weeks (acute) and one year (chronic) post infection as described in methods. The analysis of the cytokine response mounted by BALB/c mice acutely infected with *M. avium* indicated strong IFN-γ secretion upon stimulation with heat killed *M. avium* and also to MAP 85A purified protein. This pattern is expected in mycobacterial infections as mycobacteria are strong inducers of IL-12 which drives IFN-γ secretion. Secretion of IFN-γ was also detected in splenocyte culture supernatants after stimulation with heat killed *B. abortus* RB51 and with purified *Brucella* Cu/ZnSOD (Figure 3.7). This result is also consistent with the observation of antibodies to Cu/ZnSOD in mice infected with *M. avium* (Figure 3.5) and suggests certain level of cross-reactivity between *Brucella* whole cell and purified CuZnSOD antigen and *Mycobacterium*. IL-10 was only detected after ConA stimulation and was lower in infected mice (Figure 3.8). IL-2 levels in infected mice were either higher or

equal to saline controls (Figure 3.9). IL-4 cytokine concentrations in splenocyte culture supernatants were below the detection level of our system.

Cytokine analysis from splenocyte culture supernatants of mice undergoing a chronic infection (one year post infection) with *M. avium* showed similar responses to those observed during an acute infection, namely a polarization towards a Th1 type response, as indicated by a strong IFN-γ response and lack of IL-4 response upon stimulation with heat killed *M. avium*. Elevated levels of IFN–γ were also detected when stimulated with heat killed strain RB51 and with purified *B. abortus* Cu/Zn SOD protein (Figure 3.12). Similar to what was observed during an acute infection, IL-4 cytokine levels were below the detection limit of the assay (4 pg/ml). IL-10 and IL-2 responses were low, similar to the responses observed in acutely infected mice (Figure 3.10 and Figure 3.11)

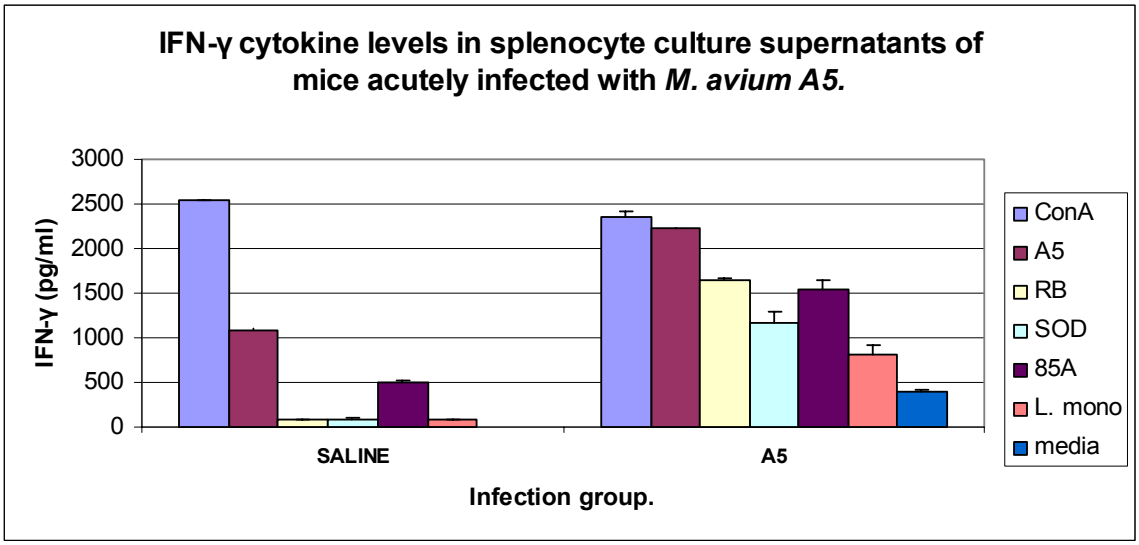

Figure 3.7: IFN-γ cytokine levels in splenocyte culture supernatants of mice acutely infected with *M. avium* A5.

Splenocytes of mice acutely infected with *M. avium* (3 weeks post infection) and saline controls were stimulated for 5 days with the following antigens. ConA= 10 μg/ml Concanavalin A, A5= $1x10^6$ cfu heat killed *M. avium* A5, RB= $1x10^6$ cfu heat killed RB51, SOD= 10 μg/ml recombinant *B. abortus* Cu/ZnSOD, L. mono= $1 x10^6$ cfu heat killed *Listeria monocytogenes*. (pooled 3 mice/group). Sensitivity of the assay >8 pg/ml.

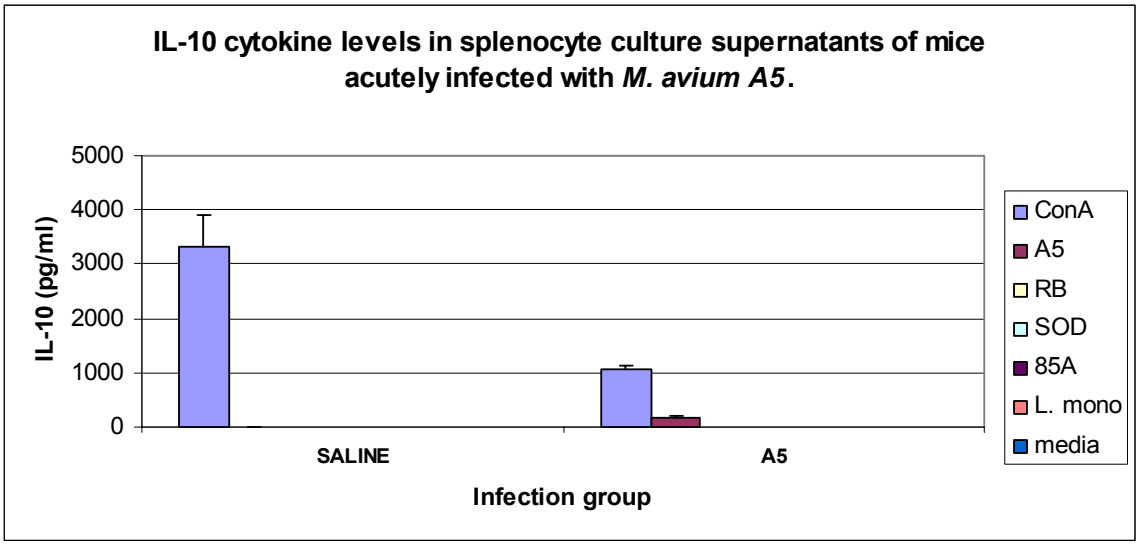

Figure 3.8: IL-10 cytokine levels in splenocyte culture supernatants of mice acutely infected with *M. avium* A5.

Splenocytes of mice acutely infected with *M. avium* (3 weeks post infection) and saline controls were stimulated for 5 days with the following antigens. ConA= 10 μg/ml Concanavalin A, A5= $1x10^6$ cfu heat killed *M. avium* A5, RB= $1x10^6$ cfu heat killed RB51, SOD= 10 μg/ml recombinant *B. abortus* Cu/ZnSOD, L. mono= $1 x10^6$ cfu heat killed *Listeria monocytogenes* (pooled 3 mice/group). Sensitivity of the assay >15 pg/ml.

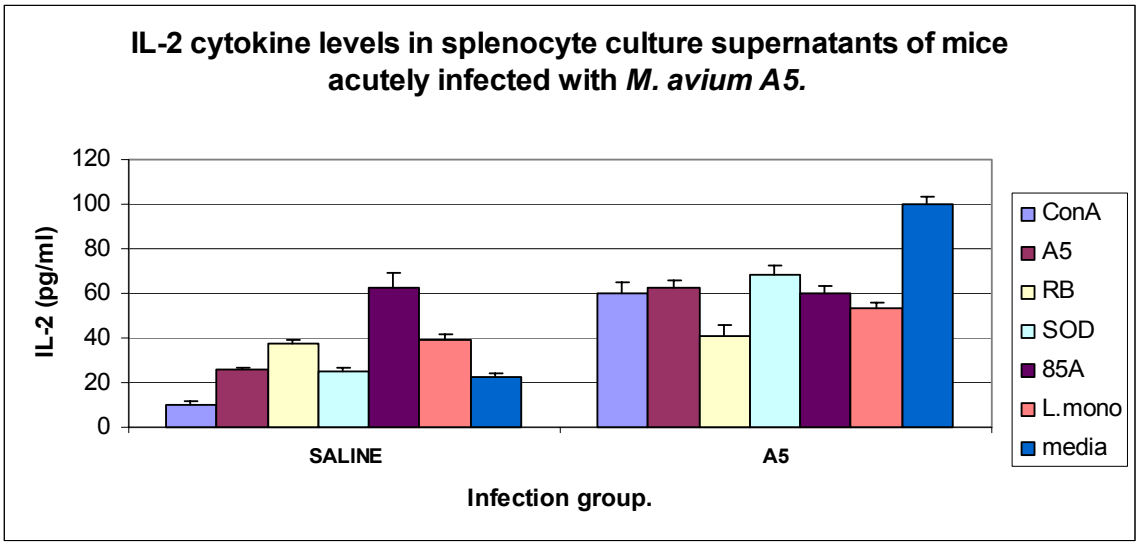

Figure 3.9: IL-2 cytokine levels in splenocyte culture supernatants of mice acutely infected with *M. avium A5*.

Splenocytes of mice acutely infected with *M. avium* (3 weeks post infection) and saline controls were stimulated for 5 days with the following antigens. ConA= 10 μg/ml Concanavalin A, A5= $1x10^6$ cfu heat killed *M. avium* A5, RB= $1x10^6$ cfu heat killed RB51, SOD= 10 μg/ml recombinant *B. abortus* Cu/ZnSOD, L. mono= $1 x10^6$ cfu heat killed *Listeria monocytogenes*. (pooled 3 mice/group) Sensitivity of the assay >2 pg/ml.

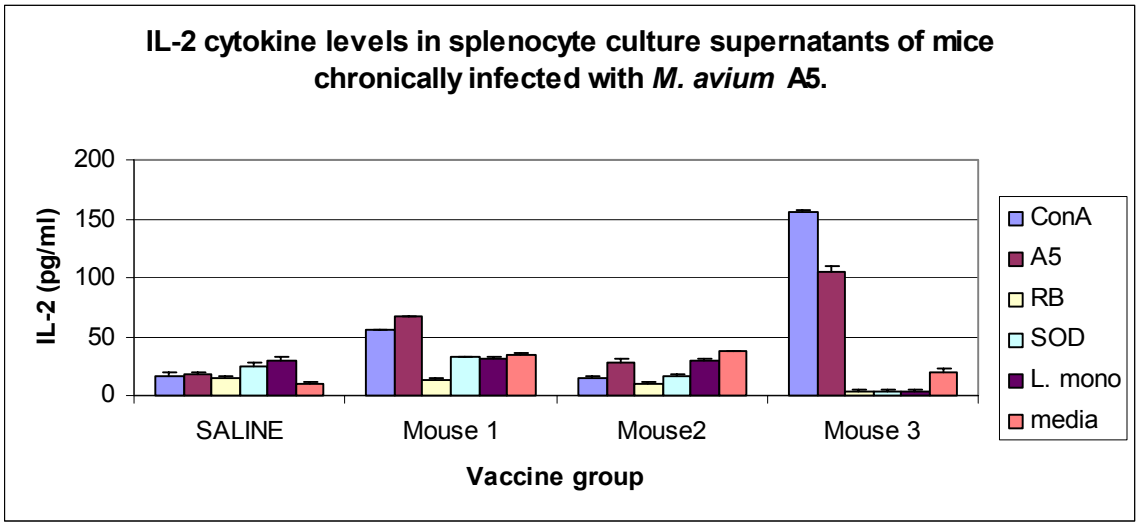

Figure 3.10: IL-2 cytokine levels in splenocyte culture supernatants of mice chronically infected with *M. avium A5*.

Splenocytes of mice chronically infected with *M. avium* (1 year post infection) were stimulated for 5 days with the following antigens: ConA= 10 µg/ml Concanavalin A, A5= $1x10^6$ cfu heat killed *M. avium* A5, RB= $1x10^6$ cfu heat killed RB51, SOD= 10 µg/ml recombinant *B. abortus* Cu/ZnSOD, L. mono= 1 $x10^6$ cfu heat killed *L. monocytogenes*. Sensitivity of the assay >2 pg/ml.

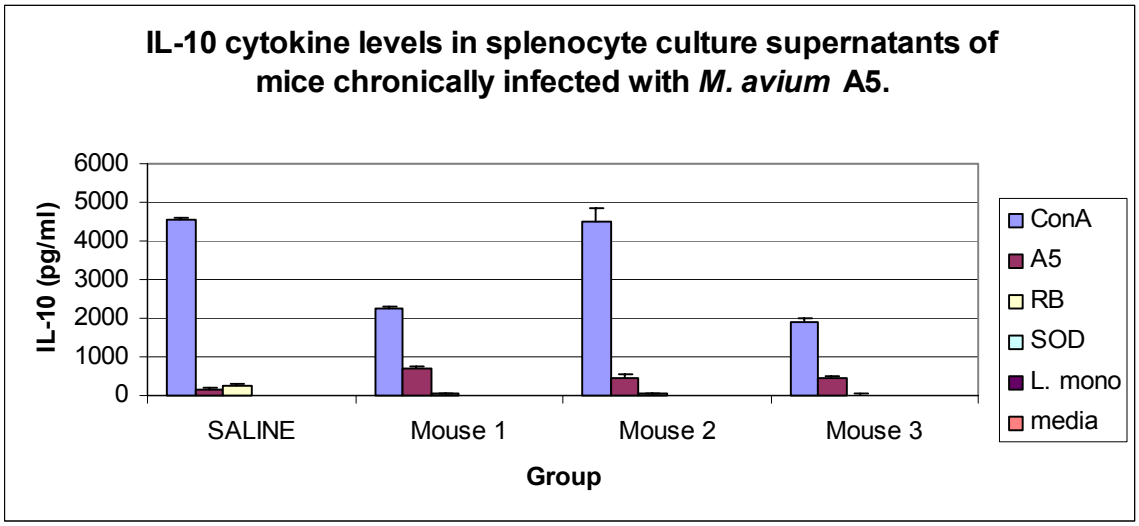

Figure 3.11: IL-10 cytokine levels in splenocyte culture supernatants of mice chronically infected with *M. avium* A5.

Splenocytes of mice chronically infected with *M. avium* (1 year post infection) were stimulated for 5 days with the following antigens: ConA= 10 μg/ml Concanavalin A, A5= $1x10^6$ cfu heat killed *M. avium* A5, RB= $1x10^6$ cfu heat killed RB51, SOD= 10 μg/ml recombinant *B. abortus* Cu/ZnSOD, L. mono= $1 x10^6$ cfu heat killed *Listeria monocytogenes*. Sensitivity of the assay 15 pg/ml.

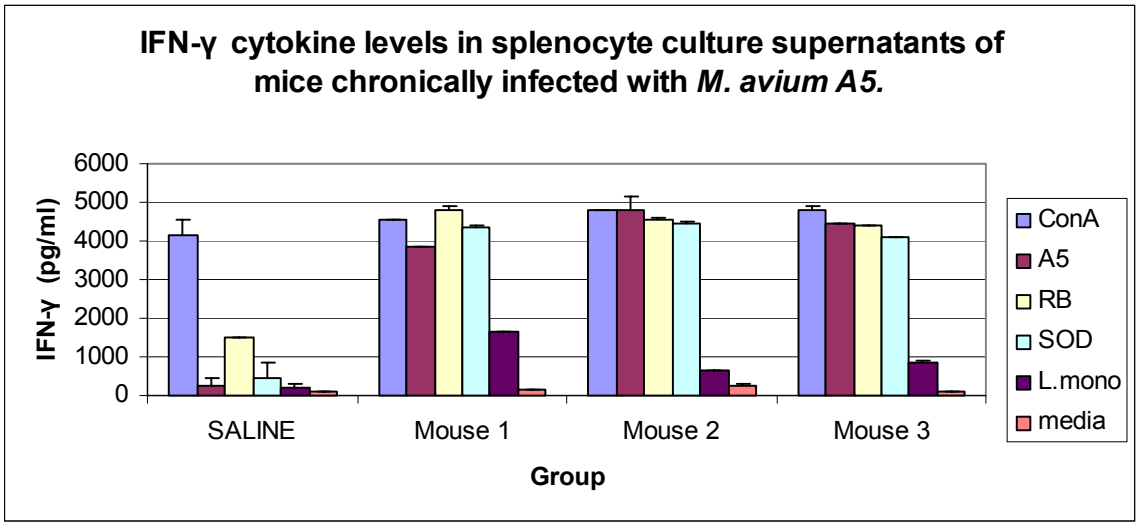

Figure 3.12: IFN-γ cytokine levels in splenocyte culture supernatants of splenocytes of mice chronically infected with *M. avium* A5.

Splenocytes of mice chronically infected with *M. avium* (1 year post infection) were stimulated for 5 days with antigens. ConA= 10 μg/ml Concanavalin A, A5= $1x10^6$ cfu heat killed *M. avium* A5, RB= $1x10^6$ cfu heat killed RB51, SOD= 10 μg/ml recombinant *B. abortus* Cu/ZnSOD, L. mono= $1 x10^6$ cfu heat killed *Listeria monocytogenes*. Sensitivity of the assay 8 pg/ml.

Consistent with the cytokine results, lymphocyte proliferation assays using ^{3}H thymidine incorporation as well as Alamar Blue reduction assay indicated that splenocytes of chronically infected mice proliferated when stimulated with ConA and also with heat killed *M. avium.* Proliferation to heat killed RB51 was observed in splenocytes of one of the mice when analyzed with ^{3}H thymidine incorporation assay and in two of the mice when the Alamar Blue reduction assay was used (data not shown).

Attenuation of *M. avium* strain A5 by gamma irradiation:

A linear radiation dose dependent reduction in the number of colony forming units was observed upon plating irradiated cultures onto Middlebrook plates. No organisms were recovered from samples irradiated with 594,000 rads (Figure 3.13). This results suggests that the damage inflicted by this dose of gamma radiation on the mycobacterial DNA and DNA repair mechanisms was sufficient to abrogate *in-vitro* replication.

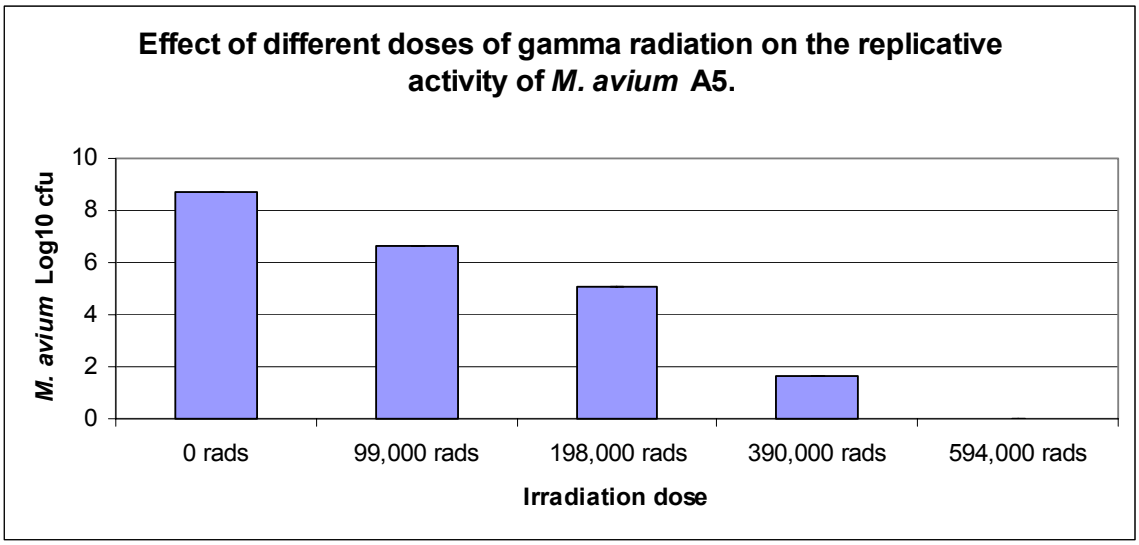

Figure 3.13: Effect of different doses of gamma radiation on the replicative activity of *M. avium* A5.

Samples were irradiated with 0 rads (negative control), 99,000 rads; 198,000 rads; 390,000 rads and 594,000 rads, and were cultured on Middlebrook 7H11 agar plates for up to 20 days. The columns represent the average Log10 cfu recovered after irradiation.

In order to determine whether the lack of *in-vitro* replication observed in *M. avium* cells irradiated with 594,000 rads was due to severe damage to the bacteria's replication and not due to cell death, an Alamar Blue Reduction assay was performed on the irradiated samples. The assay indicated no significant reduction in the metabolic activity of the irradiated cultures compared to the metabolic activity of non-irradiated live *M. avium* (Figure 3.14). Heat killed organisms exhibited the same metabolic activity of media alone. This result suggests that this dose of gamma irradiation does not significantly affect the viability of the irradiated organisms and that irradiated *M. avium* is metabolically active despite being non-replicative. This finding is significant because it is believed that live vaccines are able to induce effective protective immunity to intracellular pathogens (352).

The uptake of irradiated *M. avium* by J774.1 macrophages was also evaluated as described above. No differences in uptake were observed between irradiated and non irradiated organisms. (Data not shown).

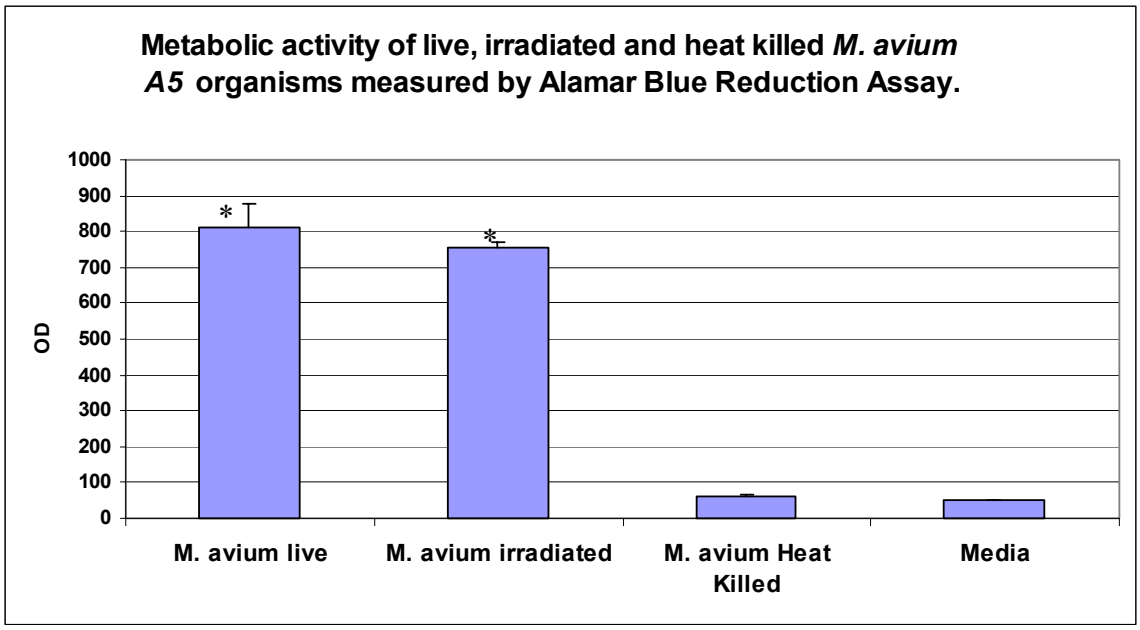

Figure 3.14: Metabolic activity of live, irradiated and heat killed *M. avium* A5 organisms as measured by Alamar Blue Reduction Assay.

Alamar Blue Reduction assay was performed on *M. avium* samples irradiated with 594,000 rads. Media alone was used as a negative control. Columns indicate the average fluorescence of 3 samples and the error bars represent the standard deviations. (*$p<0.05$ compared to media control).

As mentioned in the introduction, there are no protective *M. avium* vaccines available to use in mouse models. Two experiments were carried out in an attempt to generate a positive control for protection against *M. avium* challenge. In the first experiment, homologous vaccination of BALB/c mice with gamma irradiated, non-replicative *M. avium* followed by homologous challenge with live virulent *M. avium* was performed (Figure 3.15). This approach was selected because it was hypothesized that irradiated *M. avium* would be able to present the largest repertoire of mycobacterial antigens to the immune system and would likely provide protection against homologous challenge. The irradiated vaccine did not protect against homologous challenge with live *M. avium*. This result was unexpected since some protection was expected with the homologous vaccination.

In light of the results obtained with homologous challenge, a second attempt at identifying a positive control for vaccination was carried out using the anti-*M. paratuberculosis* commercial vaccine Mycopar® (Fort Dodge Animal Health). This vaccine is a killed bacterin in an oil suspension and is the only commercially available vaccine in the USA for vaccination of cattle. Three groups of five BALB/c mice each were vaccinated i.p with saline, *M. avium* A5 irradiated with 594,000 rads or with 50 µl of a 1:100 dilution of the Mycopar® bacterin s.c in the scruff of the neck. Four weeks post vaccination all animals were challenged i.p with *M. avium* as described before.

No local reactions were observed at the inoculation sites in the mice vaccinated with the Mycopar® vaccine neither post vaccination nor post challenge with *M. avium*.

At necropsy enlarged spleens were observed in all animals from the saline and Mycopar® vaccine groups, and in 2/5 mice from the irradiated *M. avium* group. Statistical analysis indicated no significant differences between the vaccine groups and the saline control group, indicating no protection (Figure 3.16).

Serological analysis indicated that mice vaccinated with irradiated *M. avium* do develop antibodies to *M. avium* culture supernatant proteins of predominantly an IgG2a isotype. Mice vaccinated with the Mycopar® vaccine however, did not develop antibodies to these culture supernatant proteins suggesting that the Mycopar® bacterin

may not be immunogenic in mice at this dose and route of inoculation (Figure 3.17, Figure 3.18 and Figure 3.19).

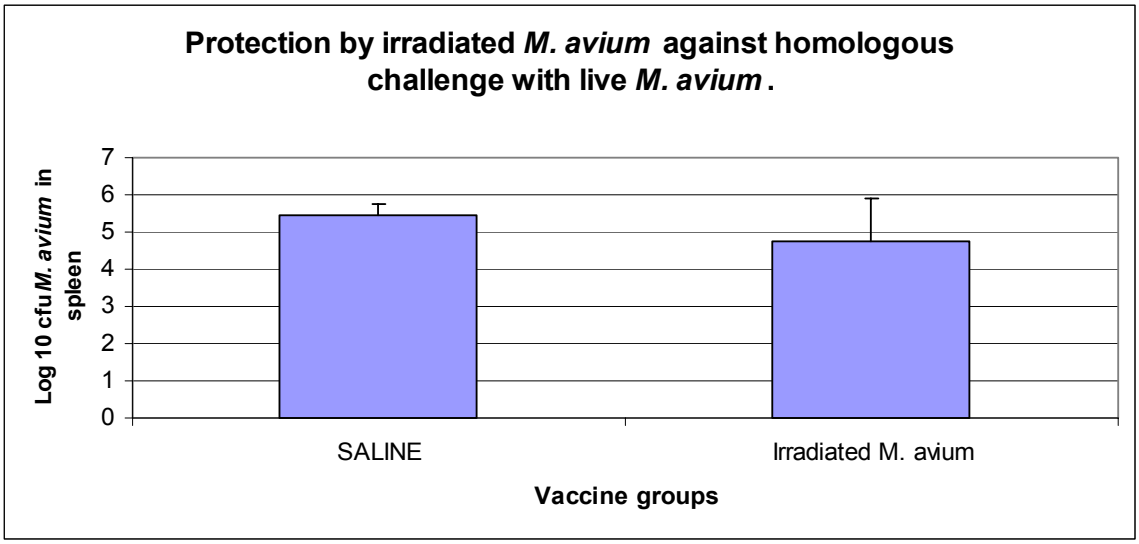

Figure 3.15: Protection by irradiated *M. avium* against homologous challenge with live *M. avium*.

BALB/c mice were vaccinated intraperitoneally (i.p) with $1x10^9$ cfus of irradiated (594,000 rads) *M. avium*. Six weeks post vaccination mice were challenged with $1x10^9$ cfus of live non-irradiated *M. avium*. Protection was determined 3 weeks post challenge by calculating the reduction in splenic cfus in the vaccine group compared to the saline control. Results are expressed as the average Log10 cfu/ group (n=5). No significant differences were observed ($p>0.05$).

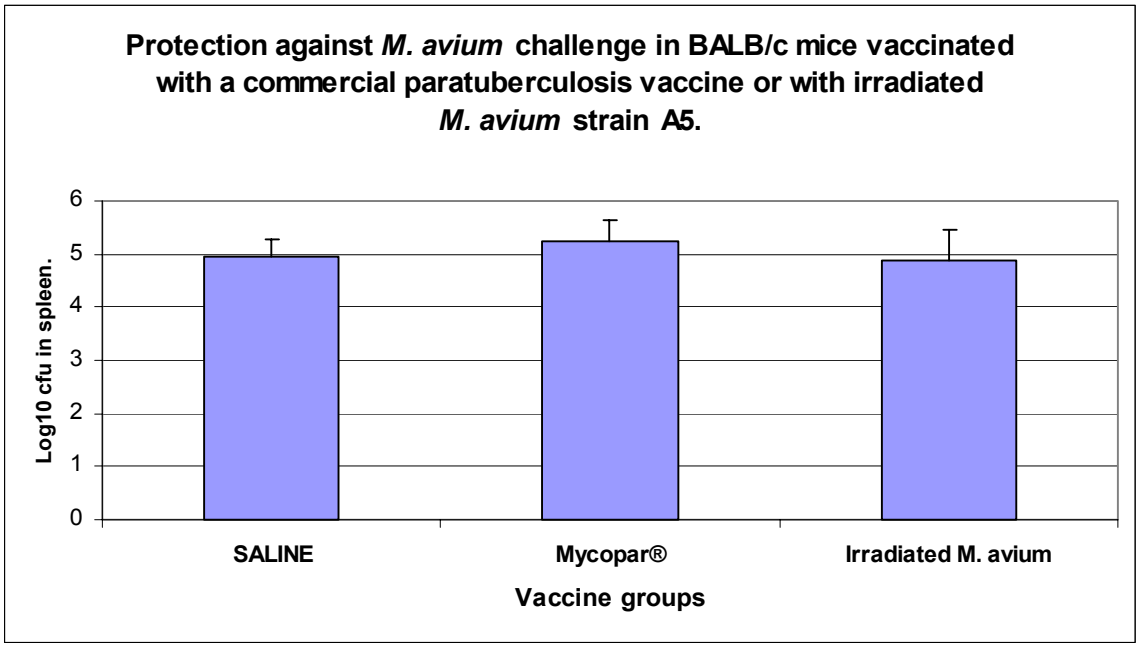

Figure 3.16: Protection against *M. avium* challenge in BALB/c mice vaccinated with a commercial paratuberculosis vaccine or with irradiated *M. avium* strain A5.

BALB/c mice were vaccinated i.p with 1 x10^9 cfus of irradiated (594,000 rads) *M. avium* A5 or with 50 µl of Mycopar® bacterin s.c. Four weeks post vaccination all animals were challenged with 1 x10^9 cfu of live *M. avium* A5. Protection was determined 3 weeks post challenge by calculating the reduction in splenic cfus in the vaccine groups compared to the saline controls. Columns represent the average *M. avium* Log10 splenic cfu/group (n=5) and the error bars are the standard deviations ($p>0.05$).

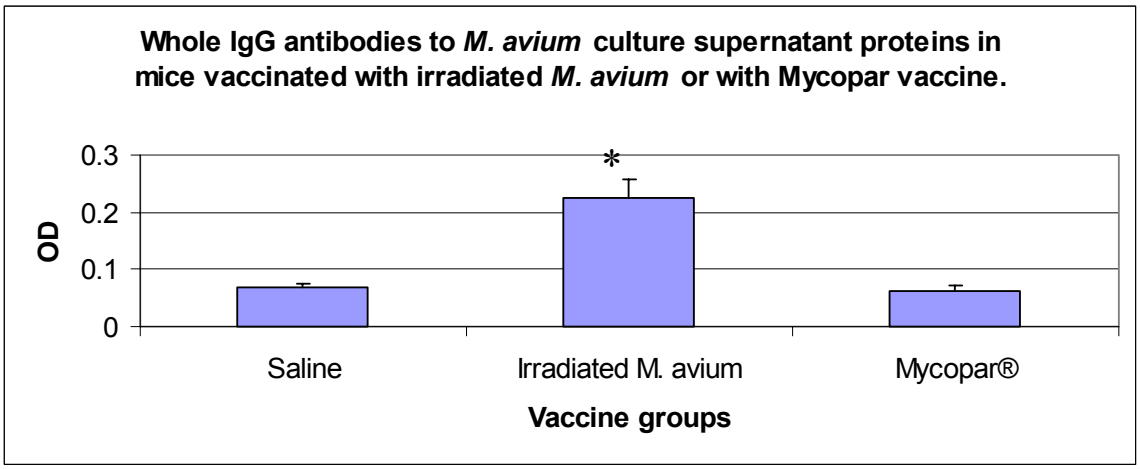

Figure 3.17: Whole IgG antibodies to *M. avium* culture supernatant proteins in mice vaccinated with irradiated *M. avium* or with Mycopar® vaccine.

BALB/c mice were vaccinated i.p with 1 x10^9 cfus of irradiated (594,000 rads) *M. avium* A5 or with 50 µl of Mycopar® bacterin s.c. Serum samples obtained four weeks post vaccination were analyzed by ELISA using *M. avium* culture supernatant protein antigen. Columns represent the average of 5 mice/group. The error bars are the standard deviations (* $p<0.05$).

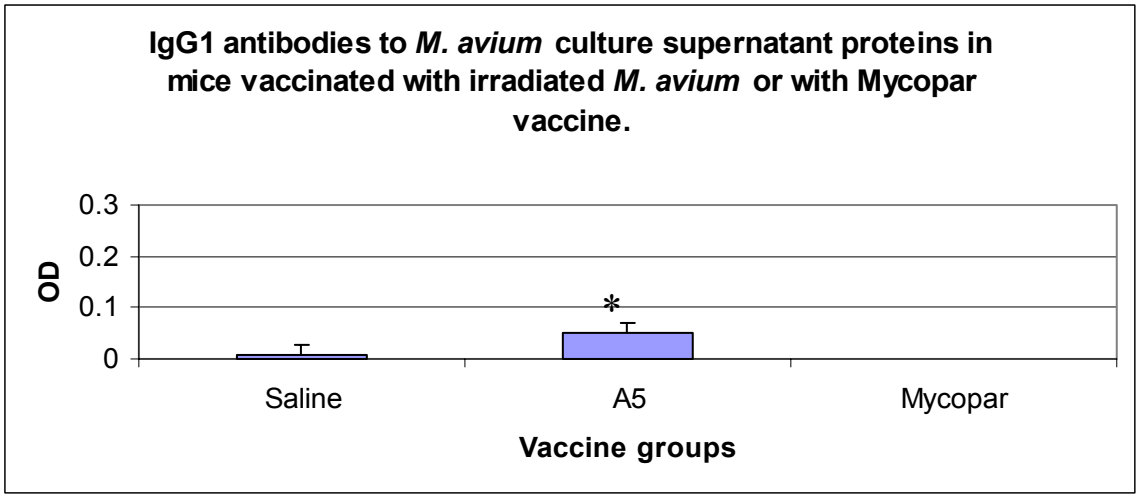

Figure 3.18: IgG1 antibodies to *M. avium* culture supernatant proteins in mice vaccinated with irradiated *M. avium* or with Mycopar® vaccine.

BALB/c mice were vaccinated i.p with 1 x10^9 cfus of irradiated (594,000 rads) *M. avium* A5 or with 50 µl of Mycopar® bacterin s.c. Serum samples obtained four weeks post vaccination were analyzed by ELISA using *M. avium* culture supernatant protein antigen. Columns represent the average of 5 mice/group. The error bars are the standard deviations (* $p<0.05$).

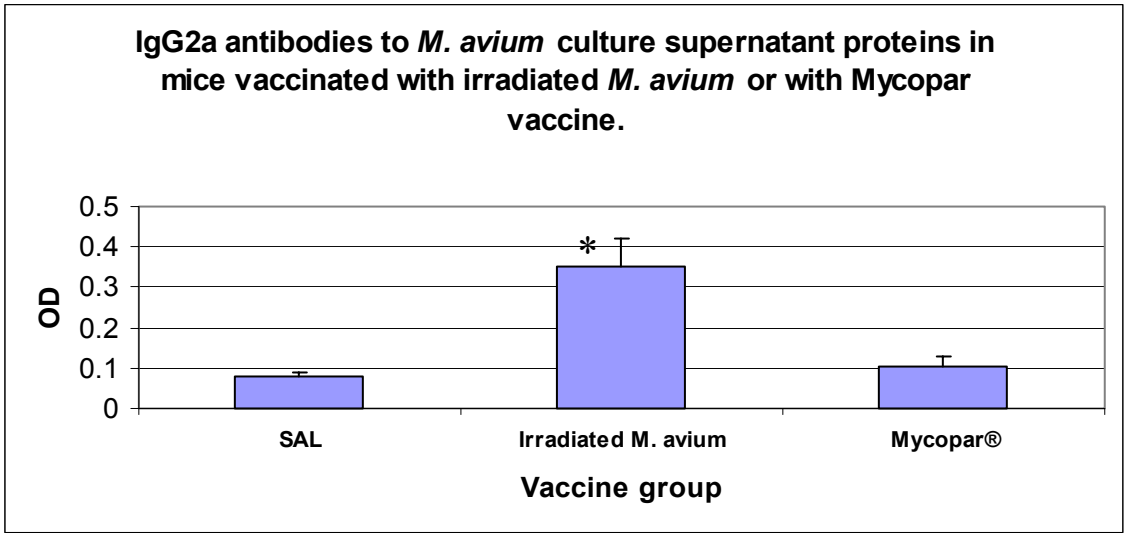

Figure 3.19: IgG2a antibodies to *M. avium* culture supernatant proteins in mice vaccinated with irradiated *M. avium* or with Mycopar® vaccine.

BALB/c mice were vaccinated i.p with 1 x10^9 cfus of irradiated (594,000 rads) *M. avium* A5 or with 50 µl of Mycopar® bacterin s.c. Serum samples obtained four weeks post vaccination were analyzed by ELISA using *M. avium* culture supernatant protein antigen. Columns represent the average of 5 mice/group. The error bars are the standard deviations (* $p<0.05$).

Evaluation of vaccine candidates for immune response induction and protection against *M. avium* challenge

Antibody Responses

To evaluate the immune responses to the experimental RB51 vaccines expressing MAP antigens in mice, BALB/c mice were vaccinated i.p with 2-4 x10^8 cfu of recombinant RB51 vaccines. Five weeks post vaccination serum samples were collected and the presence of antibodies to the MAP proteins and to whole cell strain RB51 antigen were determined by Western Blot and ELISA.

Western blot analysis indicated the development of very low levels of antibodies to MAP 85A protein (data not shown). No 35 kDa protein specific antibodies were detected (data not shown).

No 85A specific antibodies were detected by ELISA to purified recombinant MAP 85A in the sera from mice vaccinated with strain RB51 based vaccines expressing MAP 85A. All vaccine constructs expressing MAP antigens elicited the development of significant levels of whole IgG antibodies to whole cell *Brucella* antigens. The antibody levels were not significantly different between the vaccine groups, with a predominantly IgG_{2a} isotype (Figure 3.20 and Figure 3.21). IgG_1 antibody levels to *Brucella* whole cell antigens were below our detection limit. This indicates that the mice did respond to the vaccine vector but not to the MAP antigens. As expected, no O-chain antibodies were detected in mice vaccinated with any of the constructs indicating that expression of the heterologous antigens does not affect the rough phenotype of strain RB51 (data not shown).

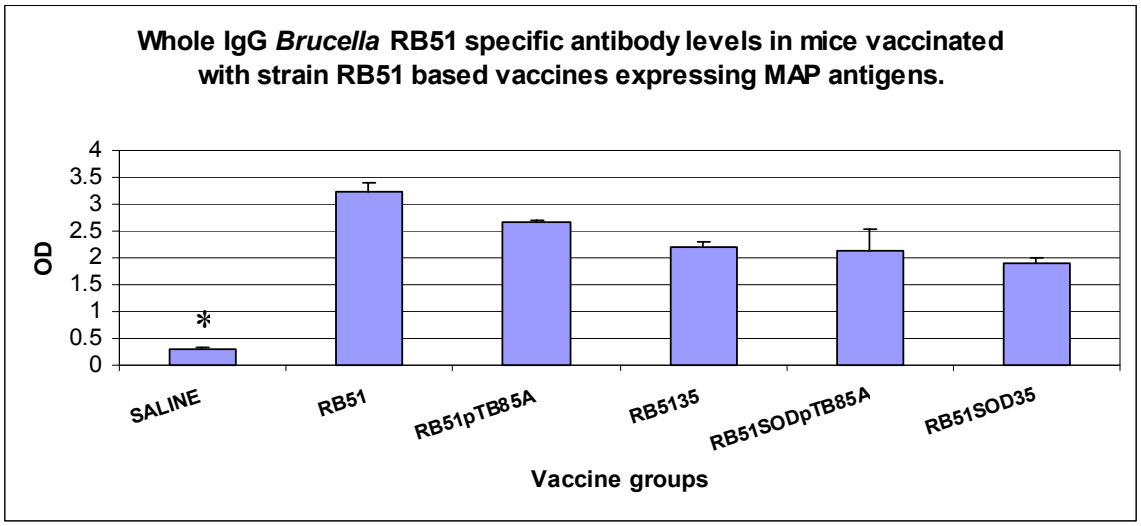

Figure 3.20: Whole IgG *Brucella* RB51 specific antibody levels in mice vaccinated with strain RB51 based vaccines expressing MAP antigens.

Serum samples obtained five weeks post vaccination with strain RB51 based vaccines were analyzed by ELISA using whole cell strain RB51 antigen. Columns represent the average of 3 mice/group. The error bars are the standard deviations (* $p<0.05$).

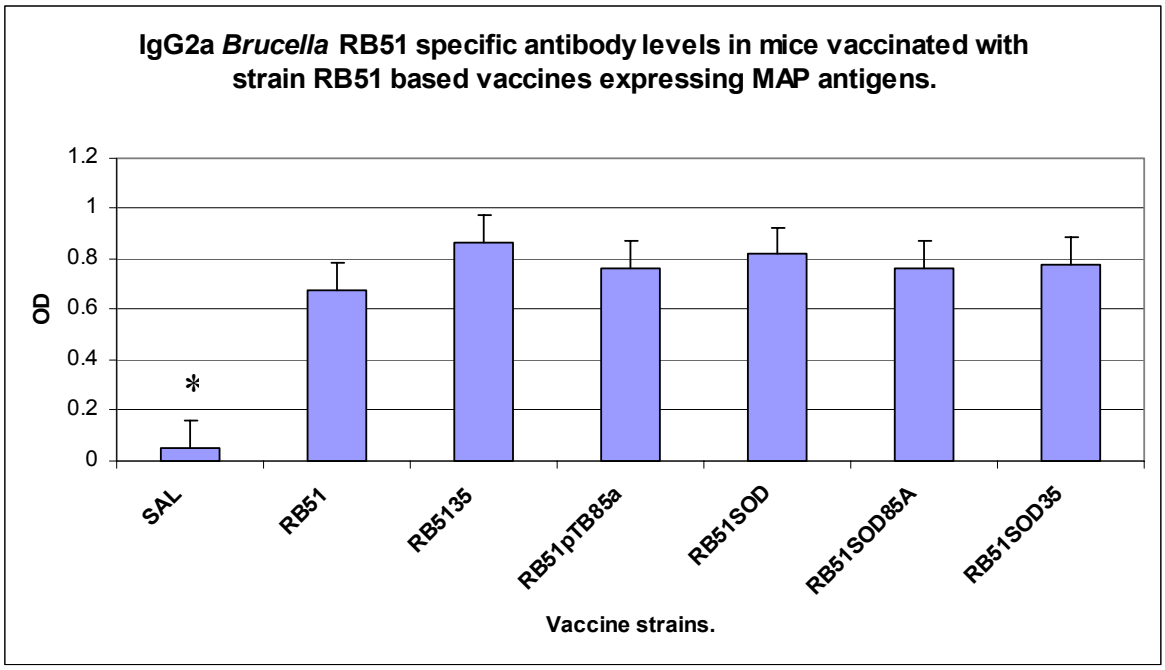

Figure 3.21: IgG2a *Brucella* RB51 specific antibody levels in mice vaccinated with strain RB51 based vaccines expressing MAP antigens.

Serum samples obtained five weeks post vaccination with strain RB51 based vaccines were analyzed by ELISA using whole cell strain RB51 antigen. Columns represent the average of 3 mice/group. The error bars are the standard deviations (* $p<0.05$).

The lack of antibody responses to the MAP antigens in mice vaccinated with the vaccine recombinants, and the low level of protection obtained (see Protection) could be due to the low expression levels of the 85A protein in strain RB51. Therefore, vaccine constructs expressing higher levels of the 85A protein were generated by fusing MAP 85A to the carboxy terminal region of the *M. tuberculosis* Esat-6 protein. This approach significantly improved expression of the recombinant protein in strain RB51 (see Chapter 2). The serological responses to these new vaccine constructs were evaluated as before. Again, no specific antibody responses to MAP 85A were detected in mice vaccinated with recombinant RB51 vaccines expressing the 85A protein as a fusion with Esat-6 (not shown). Mice vaccinated with these constructs did elicit strong *Brucella* RB51 antibody responses with IgG1 and IgG2a profiles similar to those elicited by mice vaccinated with recombinant strains expressing non-fusion MAP antigens. Antibody levels of the IgG3 isotype, another subclass of IgG were also evaluated and were determined to be significantly higher than the values obtained in the saline control group (Figure 3.22-Figure 3.24).

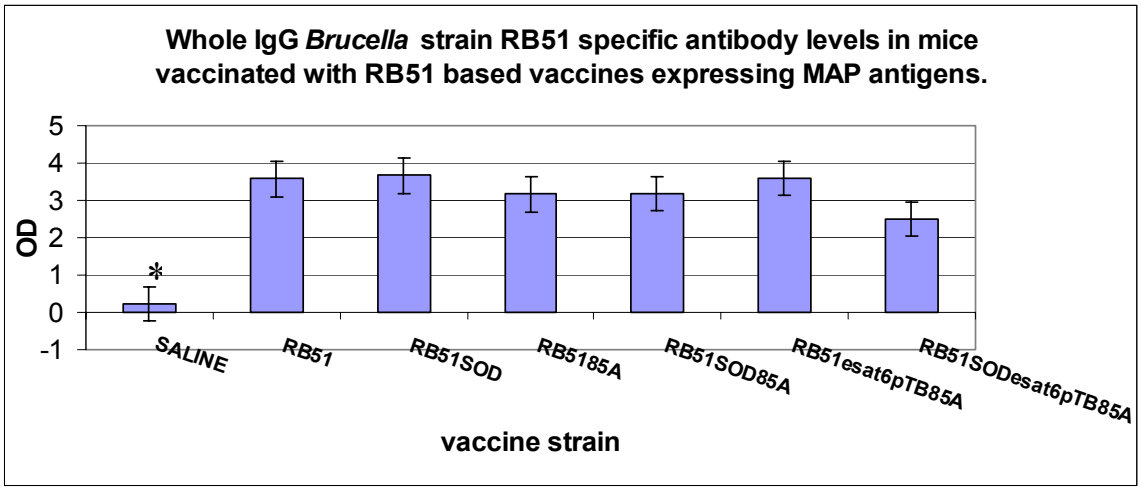

Figure 3.22: Whole IgG *Brucella* strain RB51 specific antibody levels in mice vaccinated with recombinant RB51 based vaccine strains expressing MAP antigens.

Serum samples obtained five weeks post vaccination with RB51 based vaccines were analyzed by ELISA using whole cell strain RB51 antigen. Columns represent the average OD of 3 mice/group. The error bars are the standard deviations (*<0.05).

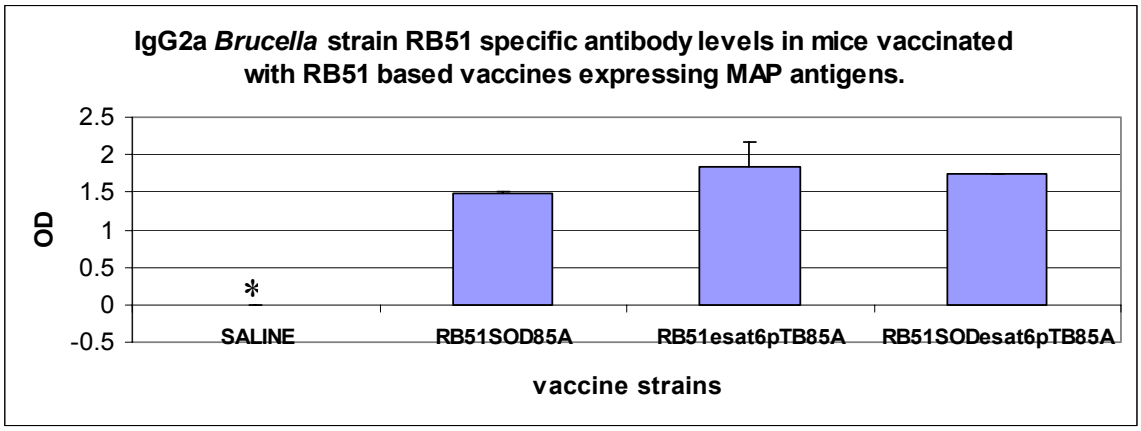

Figure 3.23: IgG2a *Brucella* strain RB51 specific antibody levels in mice vaccinated with strain RB51 based vaccines expressing MAP antigens.

Serum samples obtained five weeks post vaccination with RB51 based vaccines were analyzed by ELISA using whole cell strain RB51 antigen. Columns represent the average OD of 3 mice/group. The error bars are the standard deviations ($p<0.05$).

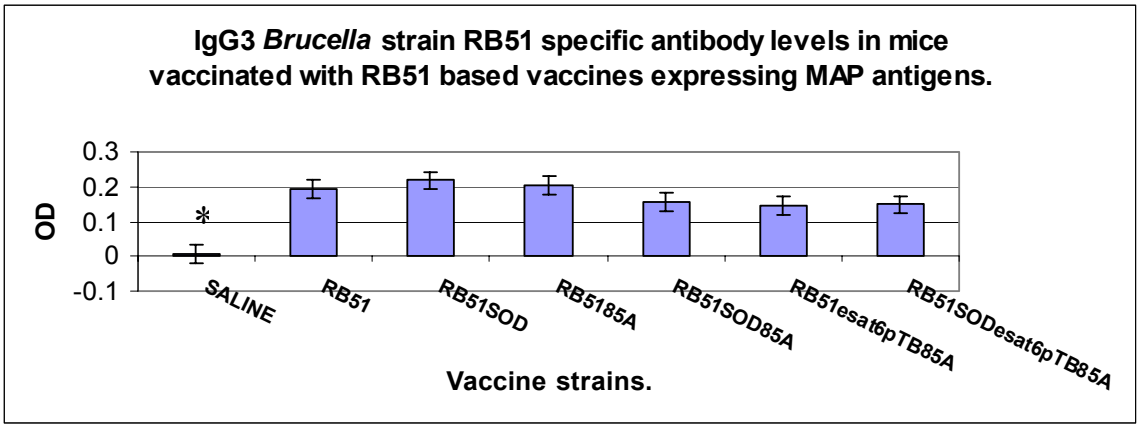

Figure 3.24: IgG3 *Brucella* strain RB51 specific antibody levels in mice vaccinated with RB51 based vaccines expressing MAP antigens.

Serum samples obtained five weeks post vaccination with RB51 based vaccines were analyzed by ELISA using whole cell strain RB51 antigen. Columns represent the average of 3 mice/group. The error bars are the standard deviations ($p<0.05$).

Cell mediated responses

Cell mediated responses were evaluated five weeks post vaccination with strain RB51 based recombinant vaccines expressing MAP antigens. Cytokine levels in splenocyte culture supernatants were determined by ELISA after 5 days of stimulation using a variety of antigens (Figure 3.25-Figure 3.38) as previously described (169, 418). The results are summarized in Table 3.1.

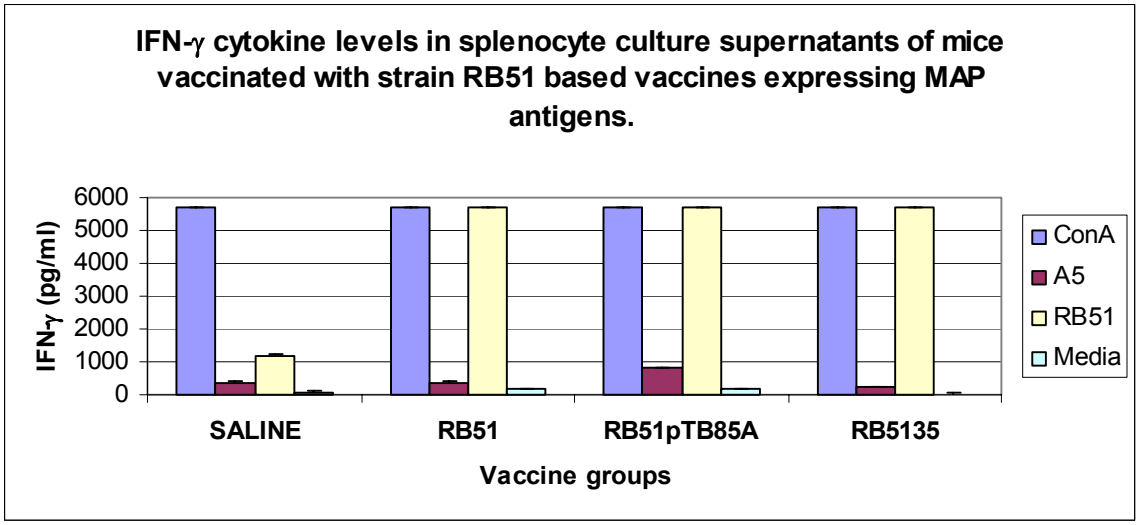

Figure 3.25: IFN-γ cytokine levels in splenocyte culture supernatants of mice vaccinated with strain RB51 based vaccines expressing MAP antigens.

Splenocytes were obtained 5 weeks post vaccination of BALB/c mice with strain RB51 based vaccines (n=3). Splenocyte cultures were stimulated for 5 days with the following antigens: ConA= 10 μg/ml Concanavalin A, A5= $1x10^6$ cfu heat killed *M. avium* A5, RB= $1x10^6$ cfu heat killed strain RB51.

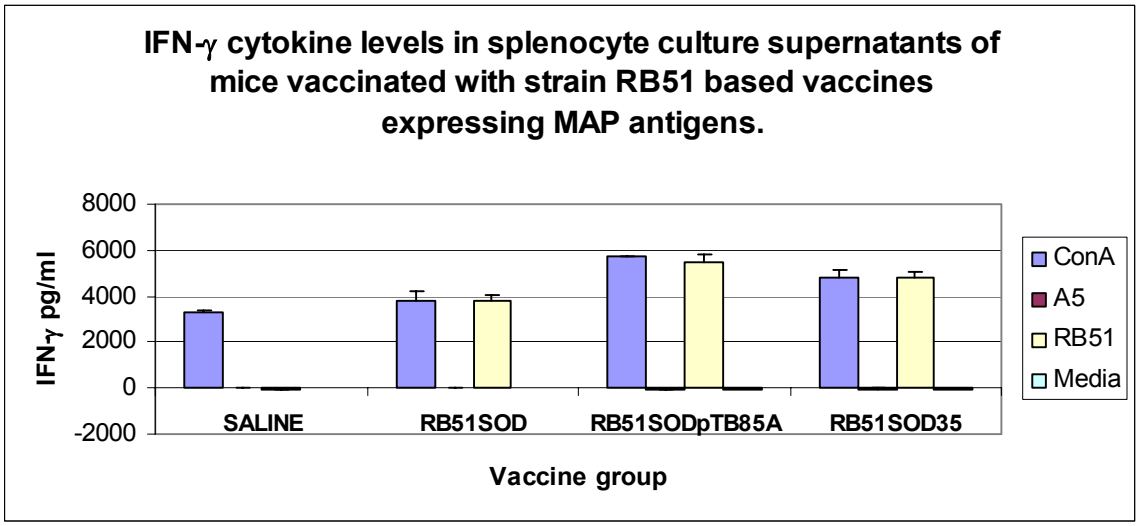

Figure 3.26: IFN- γ cytokine levels in splenocyte culture supernatants of mice vaccinated with strain RB51 based vaccines expressing MAP antigens.

Splenocytes obtained 5 weeks post vaccination with strain RB51 based vaccines were stimulated for 5 days with the following antigens: ConA= 10 μg/ml Concanavalin A, A5= $1x10^6$ cfu heat killed *M. avium* A5, RB= $1x10^6$ cfu heat killed strain RB51.

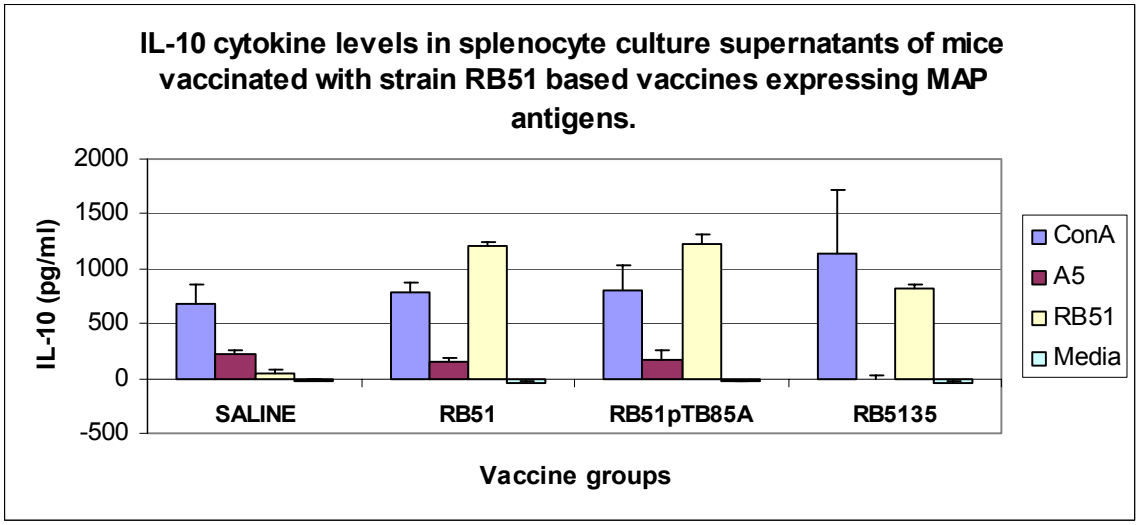

Figure 3.27: IL-10 cytokine levels in splenocyte culture supernatants of mice vaccinated with strain RB51 based vaccines expressing MAP antigens.

Splenocytes obtained 5 weeks post vaccination with strain RB51 based vaccines were stimulated for 5 days with the following antigens: ConA= 10 μg/ml Concanavalin A, A5= $1x10^6$ cfu heat killed *M. avium* A5, RB= $1x10^6$ cfu heat killed strain RB51.

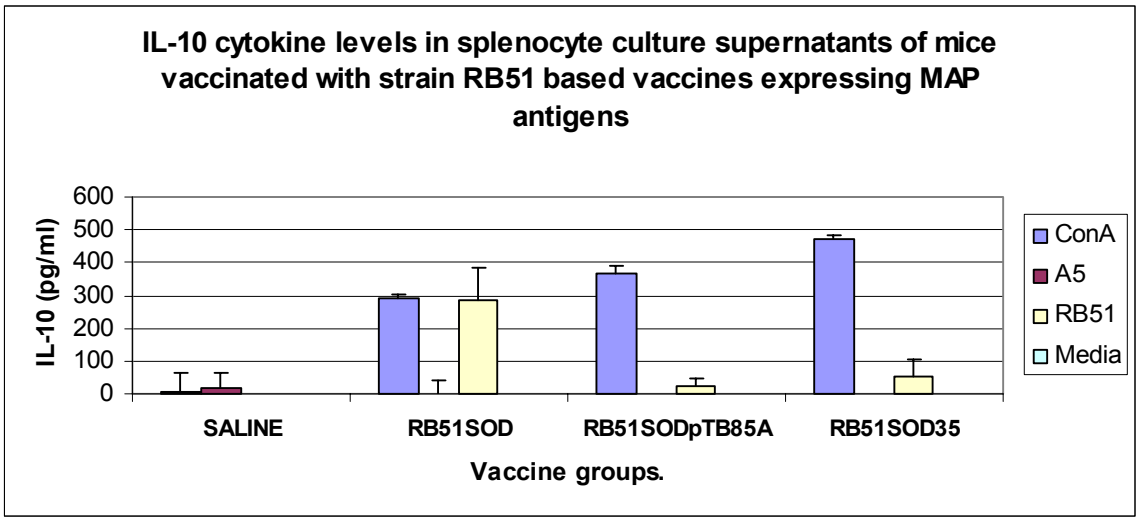

Figure 3.28: IL-10 cytokine levels in splenocyte culture supernatants of mice vaccinated with strain RB51 based vaccines expressing MAP antigens.

Splenocytes obtained 5 weeks post vaccination with strain RB51 based vaccines were stimulated for 5 days with the following antigens: ConA= 10 μg/ml Concanavalin A, A5= $1x10^6$ cfu heat killed *M. avium* A5, RB= $1x10^6$ cfu heat killed strain RB51.

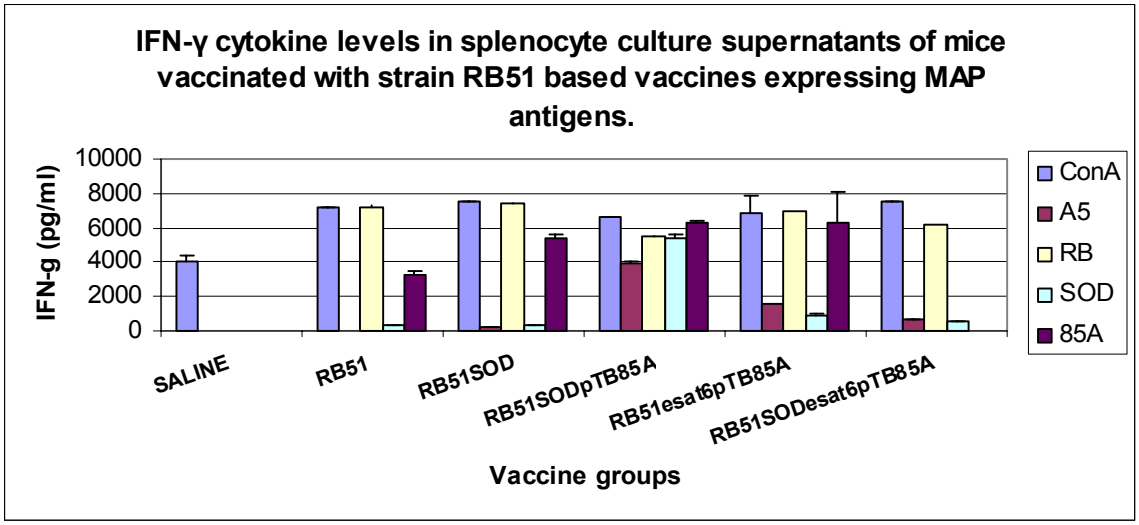

Figure 3.29: IFN-γ cytokine levels in splenocyte culture supernatants of mice vaccinated with strain RB51 based vaccines expressing MAP antigens.

Splenocytes obtained 5 weeks post vaccination with strain RB51 based vaccines were stimulated for 5 days with the following antigens: ConA= 10 μg /ml Concanavalin A, A5= $1x10^6$ cfu heat killed *M. avium* A5, RB= $1x10^6$ cfu heat killed strain RB51.

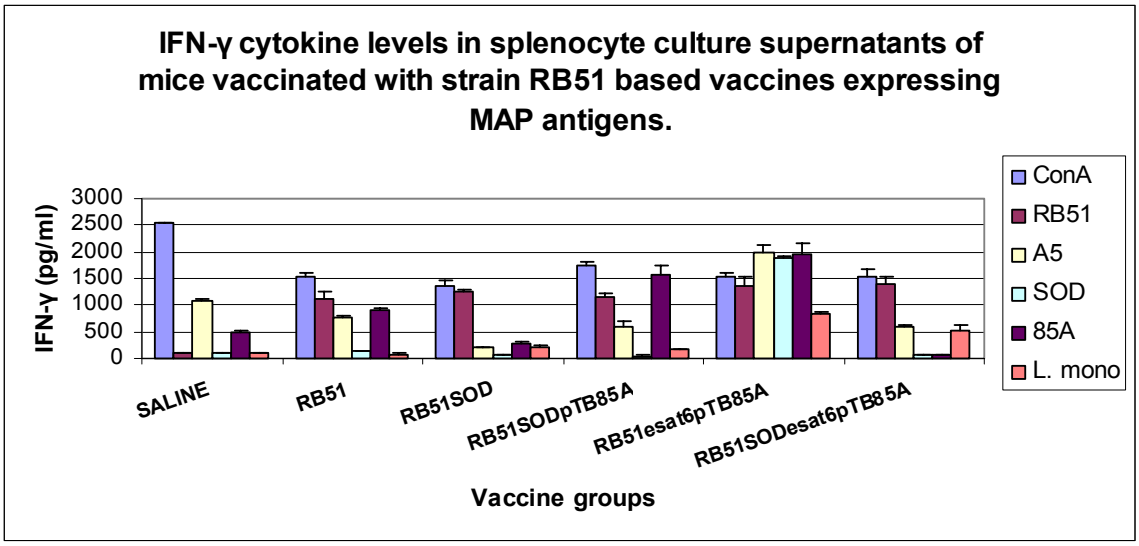

Figure 3.30: IFN-γ cytokine levels in splenocyte culture supernatants of mice vaccinated with strain RB51 based vaccines expressing MAP antigens.

Splenocytes obtained 5 weeks post vaccination with strain RB51 based vaccines were stimulated for 5 days with the following antigens: ConA= 10 μg /ml Concanavalin A, A5= $1x10^6$ cfu heat killed *M. avium* A5, RB= $1x10^6$ cfu heat killed strain RB51, L. mono= $1 x10^6$ cfu heat killed *Listeria monocytogenes*.

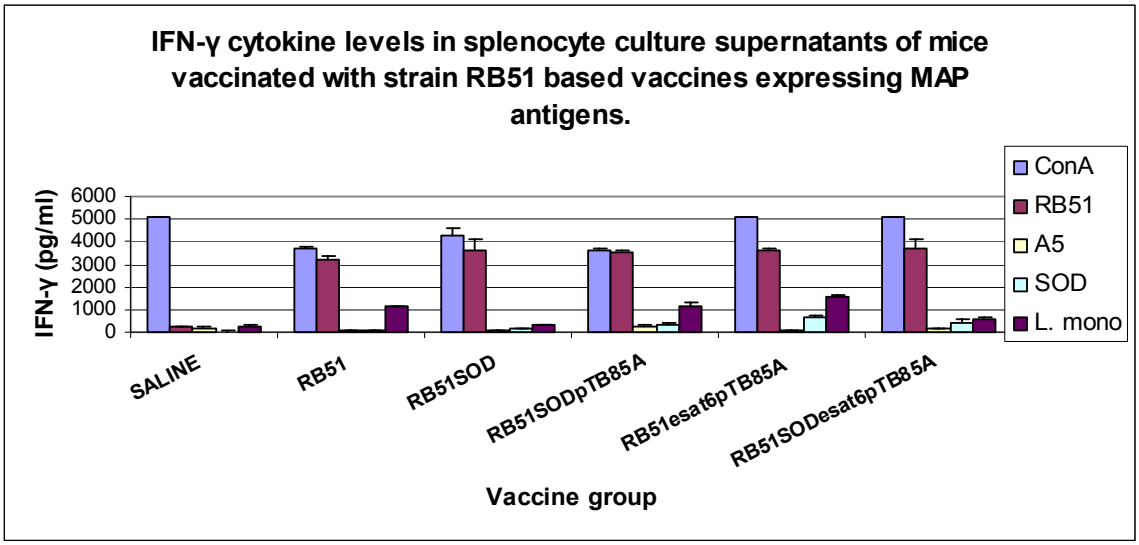

Figure 3.31: IFN-γ cytokine levels in splenocyte culture supernatants of mice vaccinated with strain RB51 based vaccines expressing MAP antigens.

Splenocytes obtained 5 weeks post vaccination with strain RB51 based vaccines were stimulated for 5 days with the following antigens: ConA= 10 μg /ml Concanavalin A, A5= $1x10^6$ cfu heat killed *M. avium* A5, RB= $1x10^6$ cfu heat killed strain RB51, L. mono= $1 x10^6$ cfu heat killed *Listeria monocytogenes*.

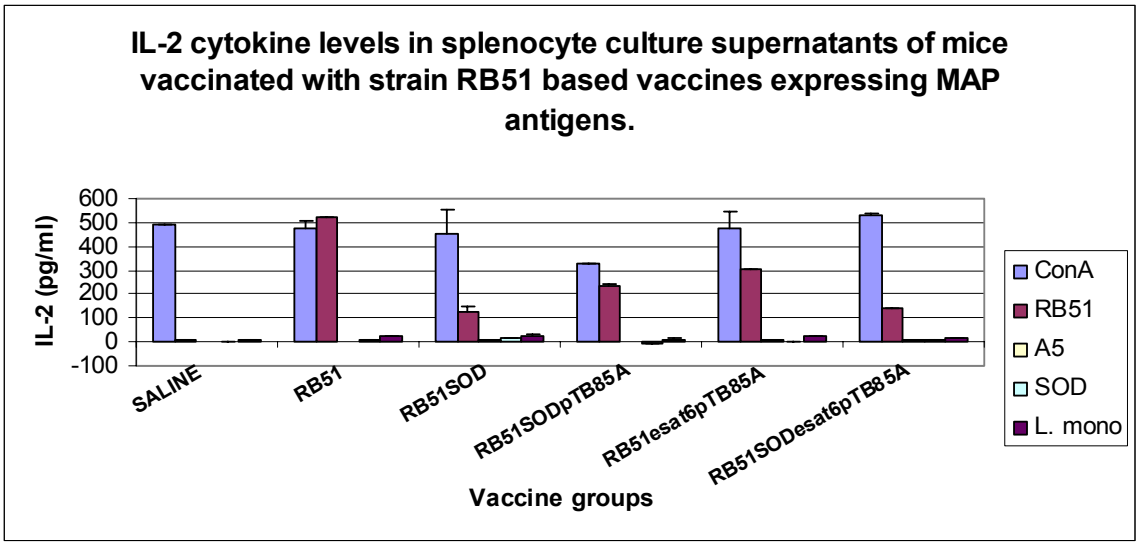

Figure 3.32: IL-2 cytokine levels in splenocyte culture supernatants of mice vaccinated with strain RB51 based vaccines expressing MAP antigens.

Splenocytes obtained 5 weeks post vaccination with strain RB51 based vaccines were stimulated for 5 days with the following antigens: ConA= 10 μg /ml Concanavalin A, A5= $1x10^6$ cfu heat killed *M. avium* A5, RB= $1x10^6$ cfu heat killed strain RB51, L. mono= $1 x10^6$ cfu heat killed *Listeria monocytogenes*.

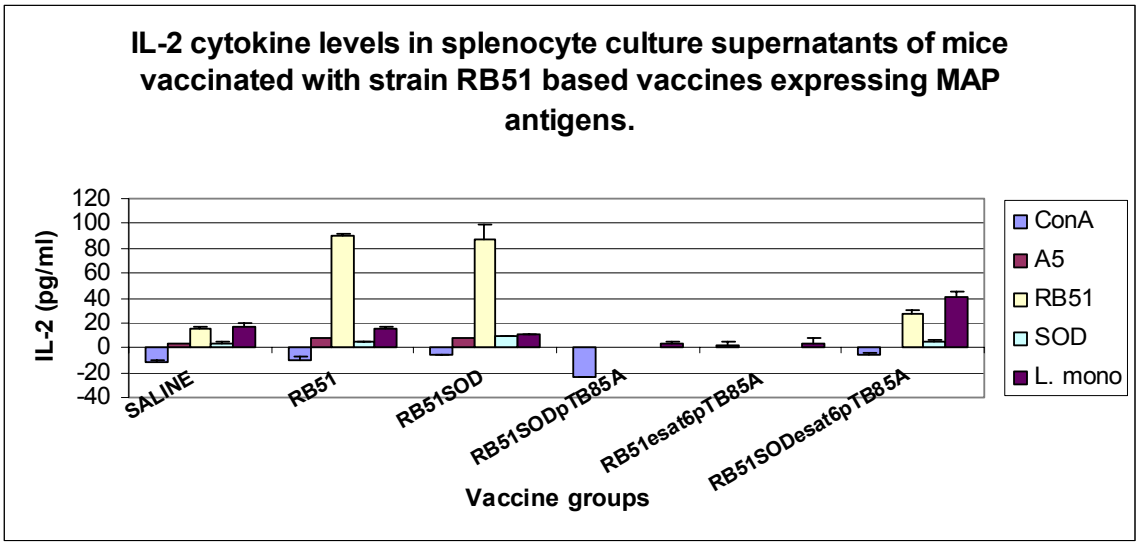

Figure 3.33: IL-2 cytokine levels in splenocyte culture supernatants of mice vaccinated with strain RB51 based vaccines expressing MAP antigens.

Splenocytes obtained 5 weeks post vaccination with strain RB51 based vaccines were stimulated for 5 days with the following antigens: ConA= 10 μg /ml Concanavalin A, A5= $1x10^6$ cfu heat killed *M. avium* A5, RB= $1x10^6$ cfu heat killed strain RB51, L. mono= $1 x10^6$ cfu heat killed *Listeria monocytogenes*.

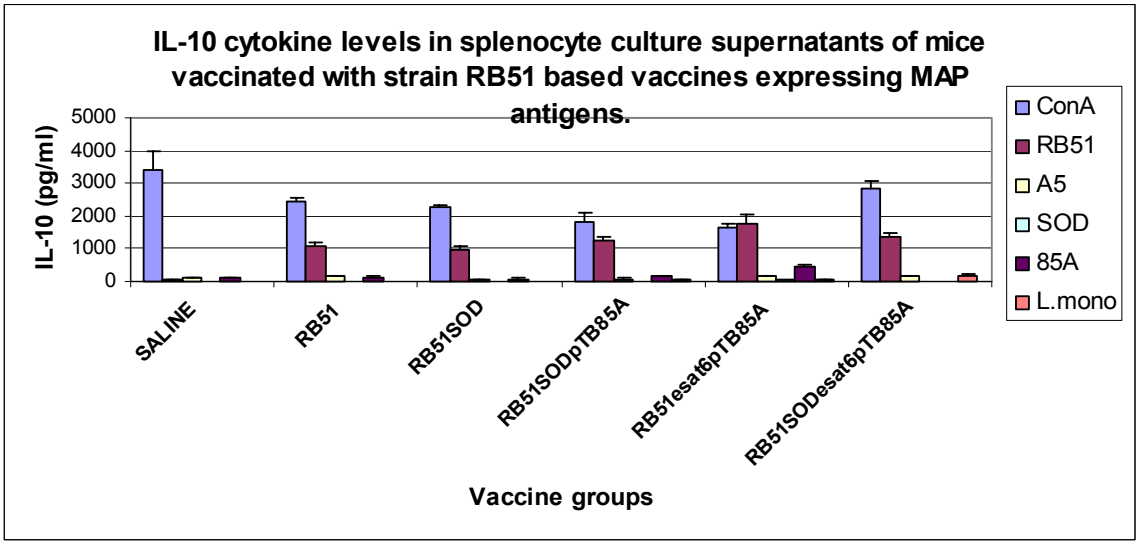

Figure 3.34: IL-10 cytokine levels in splenocyte culture supernatants of mice vaccinated with strain RB51 based vaccines expressing MAP antigens.

Splenocytes obtained 5 weeks post vaccination with strain RB51 based vaccines were stimulated for 5 days with the following antigens: ConA= 10 μg /ml Concanavalin A, A5= $1x10^6$ cfu heat killed *M. avium* A5, RB= $1x10^6$ cfu heat killed strain RB51, L. mono= $1 x10^6$ cfu heat killed *Listeria monocytogenes*.

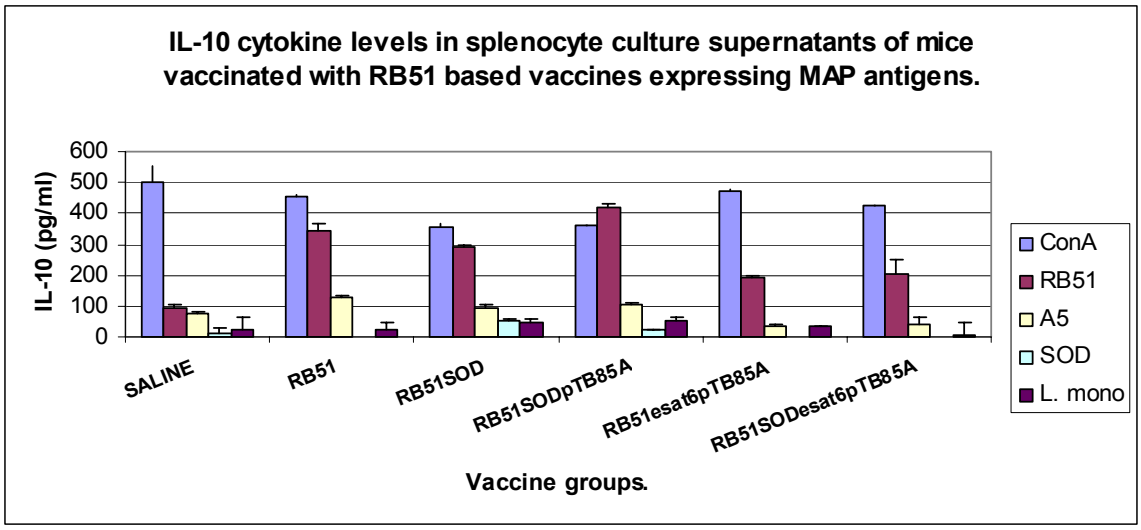

Figure 3.35: IL-10 cytokine levels in splenocyte culture supernatants of mice vaccinated with strain RB51 based vaccines expressing MAP antigens.

Splenocytes obtained 5 weeks post vaccination with strain RB51 based vaccines were stimulated for 5 days with the following antigens: ConA= 10 μg /ml Concanavalin A, A5= $1x10^6$ cfu heat killed *M. avium* A5, RB= $1x10^6$ cfu heat killed strain RB51, L. mono= $1 x10^6$ cfu heat killed *Listeria monocytogenes*.

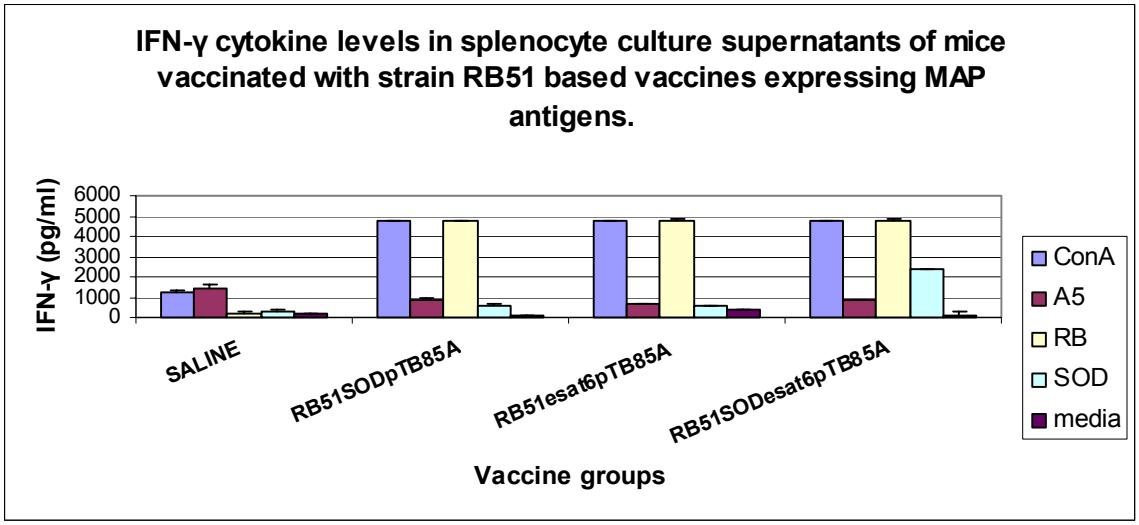

Figure 3.36: IFN-γ cytokine levels in splenocyte culture supernatants of mice vaccinated with strain RB51 based vaccines expressing MAP antigens.

Splenocytes obtained 5 weeks post vaccination with strain RB51 based vaccines were stimulated for 5 days with the following antigens: ConA= 10 μg /ml Concanavalin A, A5= $1x10^6$ cfu heat killed *M. avium* A5, RB= $1x10^6$ cfu heat killed strain RB51.

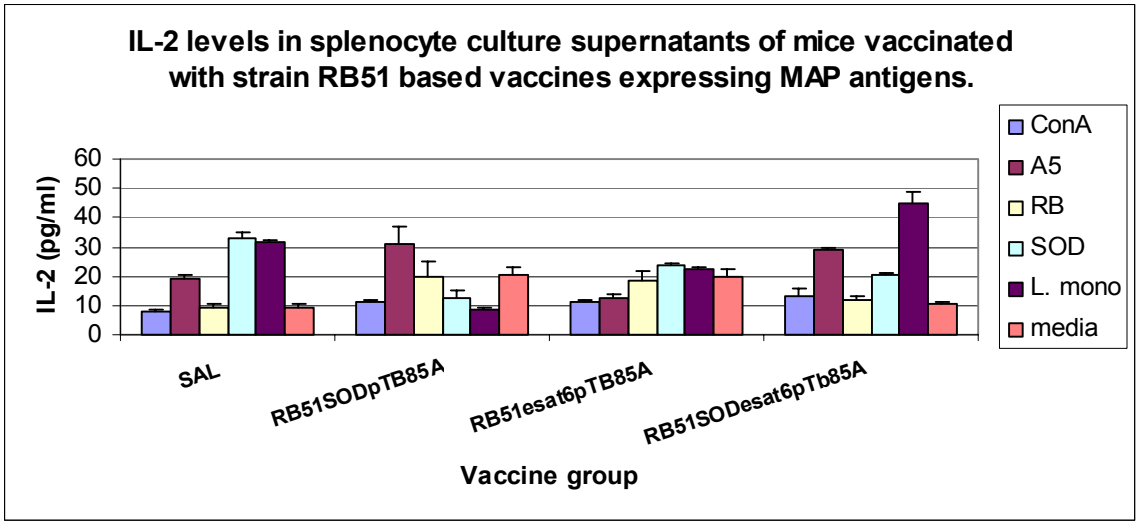

Figure 3.37: IL-2 levels in splenocyte culture supernatants of mice vaccinated with strain RB51 based vaccines expressing MAP antigens.

Splenocytes obtained 5 weeks post vaccination with strain RB51 based vaccines were stimulated for 5 days with the following antigens: ConA= 10 μg /ml Concanavalin A, A5= $1x10^6$ cfu heat killed *M. avium* A5, RB= $1x10^6$ cfu heat killed strain RB51, L. mono= $1 x10^6$ cfu heat killed *Listeria monocytogenes*.

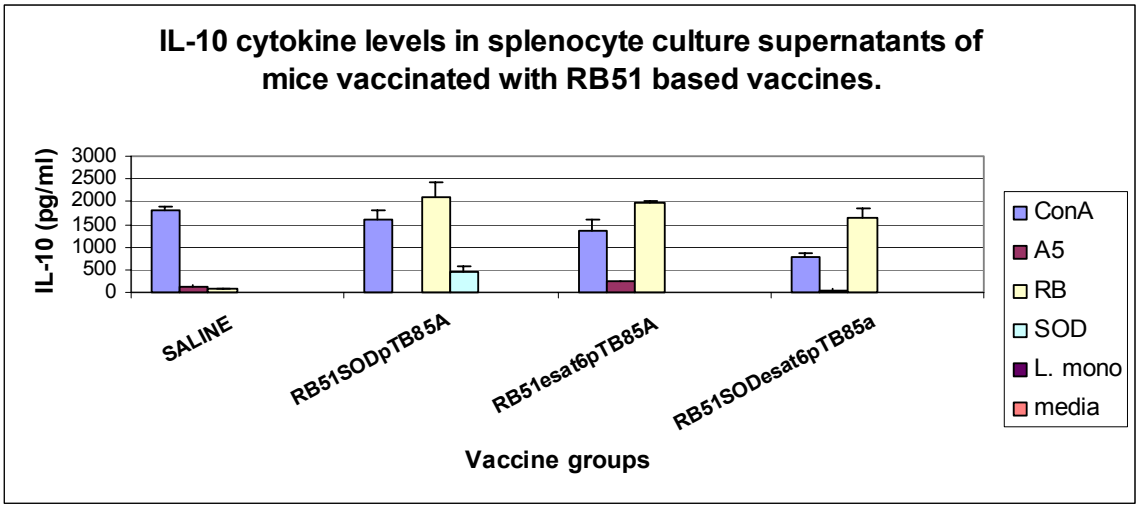

Figure 3.38: IL-10 cytokine levels in splenocyte culture supernatants of mice vaccinated with strain RB51 based vaccines expressing MAP antigens.

Splenocytes obtained 5 weeks post vaccination with strain RB51 based vaccines were stimulated for 5 days with the following antigens: ConA= 10 μg /ml Concanavalin A, A5= $1x10^6$ cfu heat killed *M. avium* A5, RB= $1x10^6$ cfu heat killed strain RB51, L. mono= $1 x10^6$ cfu heat killed *Listeria monocytogenes*.

In general, high levels of IFN-γ were produced in response to stimulation with heat killed strain RB51 in all groups vaccinated with recombinant strain RB51 based vaccines (Figure 3.25, Figure 3.26, Figure 3.29, Figure 3.30, Figure 3.31, Figure 3.36 and Summary Table 3.1). IFN-γ was also detected in culture supernatants after stimulation with heat killed *M. avium* in vaccine groups RB51SODpTB85A, RB51Esat-6pTB85A and, RBSODEsat-6pTB85A in some of the experiments (Figure 3.29, Figure 3.30, Figure 3.31, Figure 3.36 and Summary Table 3.1). Interestingly, no IFN-γ was detected in culture supernatants of splenocyte culture supernatants of the RB51SOD group after stimulation with purified Cu/ZnSOD (Figure 3.26, Figure 3.29, Figure 3.30 and Figure 3.31). IFN-γ was detected in response to Cu/Zn stimulation in the splenocyte culture supernatants of groups RB51SODpTB85A, RB51Esat685A and RB51SODEsat6-85A in some experiments (Figure 3.29, Figure 3.30 and Figure 3.36).

IL-2 was found to be produced in response to stimulation with heat killed strain RB51 by splenocytes from mice vaccinated with all vaccine groups in almost all experiments (Figure 3.32, Figure 3.33, Figure 3.37 and Summary Table 3.3). Only vaccine strain RB51SODpTB85A did not induce IL-2 production in one experiment (Figure 3.33), but this may have been due to a technical problem in just that one case.

IL-10 was produced in response to heat killed strain RB51 in culture supernatants from mice vaccinated with all vaccine groups in almost all experiments (Figure 3.27, Figure 3.28, Figure 3.34, Figure 3.35, Figure 3.38 and Summary Table 3.2). It was not produced in response to stimulation with heat killed *M. avium* in any of the groups.

IL-4 was never detected in the splenocyte culture supernatants of mice vaccinated with any of the RB51 based vaccines tested.

These results confirm that strain RB51 is a strong inducer of IFN-γ responses and polarization towards a Th1 cytokine profile, since in all experiments IL-4 was below the detection limit of our system. IL-10 was consistently secreted in response to heat killed strain RB51 in all groups. These results are consistent with previously published reports on cytokine profiles induced by this vaccine strain, and confirm that expression of heterologous antigens does not significantly alter the induction of *Brucella* specific

immune responses by vaccine strain RB51 (308). However, cytokine responses to heat killed *M. avium* were only detected in a few experiments, suggesting that the recombinant vaccines were not able to stimulate a specific response to the heterologous antigens.

Table 3.1: Summary of IFN-γ responses to stimulation with various antigens.

Strain	FIGURE	IFN- γ RESPONSE TO:				
		RB51	A5	SOD	85A	L. mono
RB51	**3.22**	+	-	**NA**	**NA**	**NA**
RB51	**3.26**	+	-	-	+	**NA**
RB51	**3.27**	+	-	-	+	-
RB51	**3.28**	+	-	-	**NA**	+
RB51SOD	**3.23**	+	-	**NA**	**NA**	**NA**
RB51SOD	**3.26**	+	-	-	+	**NA**
RB51SOD	**3.27**	+	-	-	-	-
RB51SOD	**3.28**	+	-	-	-	-
RB51 pTB85A	**3.22**	+	-	**NA**	**NA**	**NA**
RB51SOD pTB85A	**3.23**	+	-	**NA**	**NA**	**NA**
RB51SOD pTB85A	**3.33**	+	-	-	**NA**	**NA**
RB51SOD pTB85A	**3.26**	+	+	+	+	**NA**
RB51SOD pTB85A	**3.27**	+	-	-	+	-

Strain	FIGURE	IFN- γ RESPONSE TO:				
		RB51	A5	SOD	85A	L. mono
RB51SOD pTB85A	**3.28**	+	-	-	**NA**	+
RB51Esat-6 pTB85A	**3.33**	+	-	-	**NA**	**NA**
RB51Esat-6 pTB85A	**3.26**	+	+	+	+	**NA**
RB51Esat-6 pTB85A	**3.27**	+	+	+	+	+
RB51Esat-6 pTB85A	**3.28**	+	+	-	**NA**	+
RB51SOD Esat-6 pTB85A	**3.33**	+	+	+	**NA**	**NA**
RB51SOD Esat-6 pTB85A	**3.26**	+	+	+	-	**NA**
RB51SOD Esat-6 pTB85A	**3.27**	+	-	-	-	-
RB51SOD Esat-6 pTB85A	**3.28**	+	-	-	-	-
RB5135	**3.22**	+	-	**NA**	**NA**	**NA**
RB51SOD35	**3.23**	+	-	**NA**	**NA**	**NA**

Results are compared to saline controls and are presented by experiment. NA: not analyzed.

Table 3.2: Summary of IL-10 responses to stimulation with various antigens.

Strain	FIGURE	IL-10 Response to				
		RB51	A5	SOD	85A	L. mono
RB51	**3.24**	+	-	**NA**	**NA**	**NA**
RB51SOD	**3.25**	+	-	**NA**	**NA**	**NA**
RB51 pTB85A	**3.24**	+	-	**NA**	**NA**	**NA**
RB51SOD pTB85A	**3.25**	-	-	**NA**	**NA**	**NA**
RB51SOD pTB85A	**3.35**	+	-	+	**NA**	-
RB51SOD pTB85A	**3.31**	+	-	-	-	-
RB51SOD pTB85A	**3.32**	+	-	-	**NA**	-
RB51Esat-6 pTB85A	**3.35**	+	-	-	**NA**	-
RB51Esat-6 pTB85A	**3.31**	+	-	-	+	**NA**
RB51Esat-6 pTB85A	**3.32**	+	-	-	**NA**	-

Strain	FIGURE	IL-10 Response to				
		RB51	A5	SOD	85A	L. mono
RB51SOD **Esat-6 pTB85A**	**3.35**	+	-	-	**NA**	-
RB51SOD **Esat-6 pTB85A**	**3.31**	+	-	-	-	-
RB51SOD **Esat-6 pTB85A**	**3.32**	+	-	-	**NA**	-
RB5135	**3.24**	+	-	**NA**	**NA**	**NA**
RB51SOD35	**3.23**	+	-	**NA**	**NA**	**NA**

Results are compared to saline controls and are presented by experiment.
NA: not analyzed.

Table 3.3: Summary of IL-2 responses to stimulation with various antigens.

STRAIN	FIGURE	IL-2 Response to				
		RB51	A5	SOD	85A	L. mono
RB51	**3.29**	+	-	**NA**	**NA**	**NA**
RB51	**3.30**	+	-	-	**NA**	-
RB51SOD	**3.29**	+	-	-	**NA**	-
RB51SOD	**3.30**	+	-	-	**NA**	-
RB51SOD pTB85A	**3.34**	+	+	-	**NA**	+
RB51SOD pTB85A	**3.29**	+	-	-	**NA**	-
RB51SOD pTB85A	**3.30**	-	-	-	**NA**	-
RB51Esat-6 pTB85A	**3.34**	+	-	-	**NA**	-
RB51Esat-6 pTB85A	**3.29**	+	-	-	**NA**	-
RB51Esat-6 pTB85A	**3.30**	+	-	-	**NA**	-
RB51SOD Esat-6 pTB85A	**3.34**	+	-	-	**NA**	-

STRAIN	FIGURE	IL-2 Response to				
		RB51	A5	SOD	85A	L. MONO
RB51SOD **Esat-6 pTB85A**	**3.29**	+	-	-	**NA**	-
RB51SOD **Esat-6 pTB85A**	**3.30**	+	-	-	**NA**	+

Results are compared to saline controls and are presented by experiment.
NA: not analyzed.

Lymphocyte proliferation assays using ^{3}H thymidine incorporation or Alamar Blue dye reduction assay showed that all vaccine groups had marked proliferation towards Concanavalin A non-specific stimulation and to heat killed strain RB51 antigen stimulation. Although proliferation to purified recombinant *B. abortus* Cu/ZnSOD and MAP 85A or with heat killed *M. avium* was observed, these results were not significant when compared to proliferation to *L. monocytogenes*, suggesting a non-specific event (data not shown).

Protection

In the first experiment, groups of 5 mice were vaccinated with RB51 strains expressing MAP 85A and 35 kDa antigens. Three weeks post challenge with *M. avium* ($1x10^{9}$ cfu) the animals were sacrificed and protection was evaluated by determining the number of *M. avium* cfus in the spleens of vaccinated animals compared to saline controls. Only vaccine strain RB51 expressing MAP 85A antigen conferred significant protection ($p<0.05$) against virulent mycobacteria (Figure 3.39).

In a second experiment no protection was obtained with any of the vaccine strains analyzed (Figure 3.40). Although a trend towards protections is seen in groups RB51SOD and RB5185A and RB51SOD85A these results were not statistically significant. Very high intragroup variability was observed and this may in part be responsible for the lack of significance. Similar to the previous experiment, no protection was observed with vaccines expressing the MAP 35 kDa protein.

Partial repetition of this experiment again showed no protection and is presented in Figure 3.41.

In the fourth experiment irradiated *M. avium* (594,000 rads) was included as a potential positive protection control for *M. avium* challenge in mice (Figure 3.42). In this experiment, strain RB51SODpTB85A induced significant protection ($p<0.05$) against challenge with *M. avium* compared to the saline group. Again, very high intra group variation with a decrease in *M. avium* cfus recovered from the spleen of all vaccine

groups was observed, but the decrease was only significant for RB51SODpTB85A. Vaccination with irradiated *M. avium* failed to induce protection against homologous challenge with live *M. avium*.

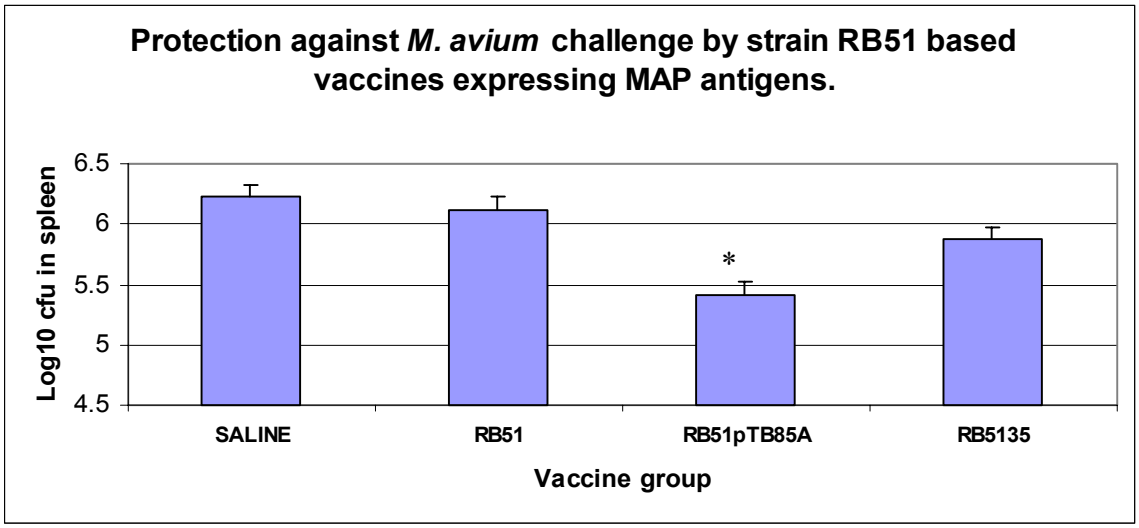

Figure 3.39: Protection against *M. avium* challenge induced by strain RB51 based vaccines expressing MAP antigens.

BALB/c mice were vaccinated with recombinant strain RB51 vaccines expressing MAP antigens and challenged 6 weeks post vaccination with $1x10^9$ cfu of *M. avium*. Protection was determined 3 weeks post challenge by calculating the reduction in splenic *M. avium* cfus in the vaccine groups compared to the saline control. Results are expressed as the average Log10 cfus of *M. avium* A5 in the spleens of 5 mice/group. The error bars represent the standard error *$p<0.05$.

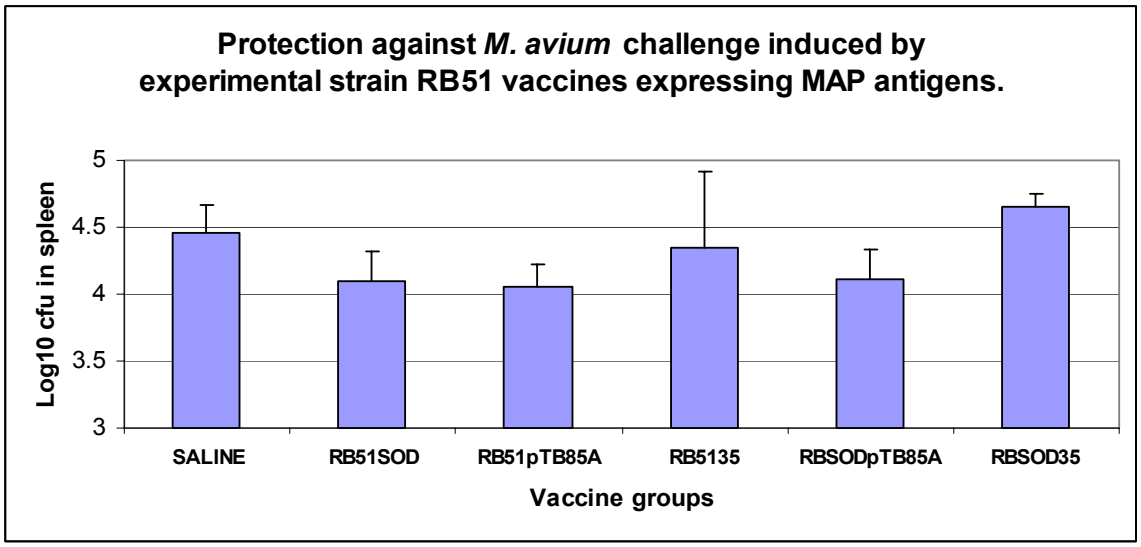

Figure 3.40: Protection against *M. avium* challenge induced by experimental strain RB51 vaccines expressing MAP antigens.

BALB/c mice were vaccinated with recombinant strain RB51 vaccines expressing MAP antigens and challenged 6 weeks post vaccination with $1x10^9$ cfu of *M. avium*. Protection was determined 3 weeks post challenge by calculating the reduction in splenic *M. avium* cfus in the vaccine groups compared to the saline control. Results are expressed as the average Log10 cfus of *M. avium* A5 in the spleens of 5 mice/group. The error bars represent the standard error.

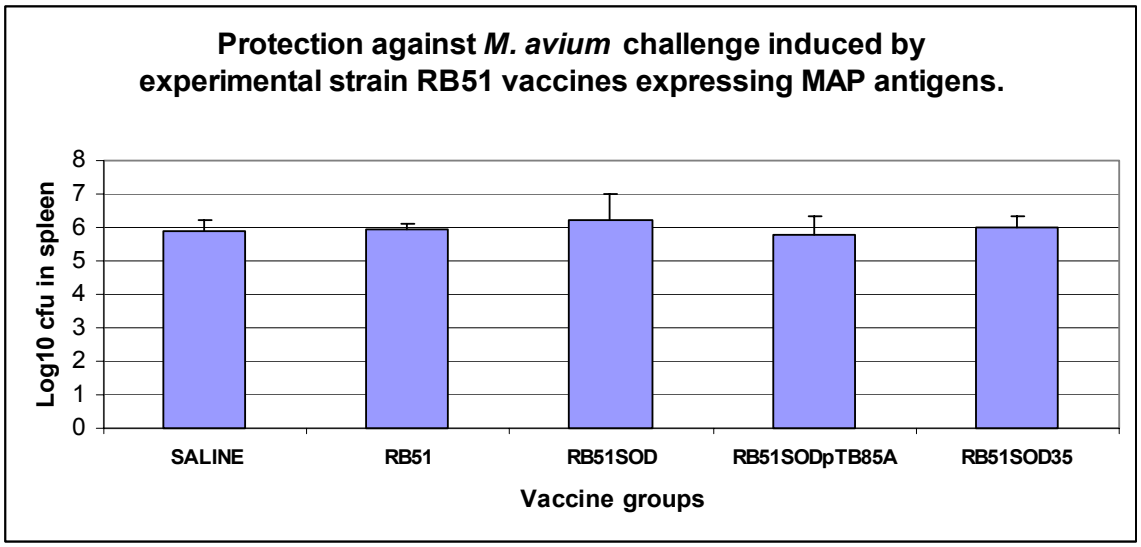

Figure 3.41: Protection against *M. avium* challenge induced by experimental strain RB51 vaccines expressing MAP antigens.

BALB/c mice were vaccinated with recombinant strain RB51 vaccines expressing MAP antigens and challenged 6 weeks post vaccination with $1x10^9$ cfu of *M. avium*. Protection was determined 3 weeks post challenge by calculating the reduction in splenic *M. avium* cfus in the vaccine groups compared to the saline control. Results are expressed as the splenic average Log10 cfus of *M. avium* A5. The error bars represent the standard deviations. Originally the experiment was designed with 9 mice/ group. Due to flooding of the cages the saline and RB51SOD35 groups ended up with only 6 mice/group and the RB51SOD group with 8 mice.

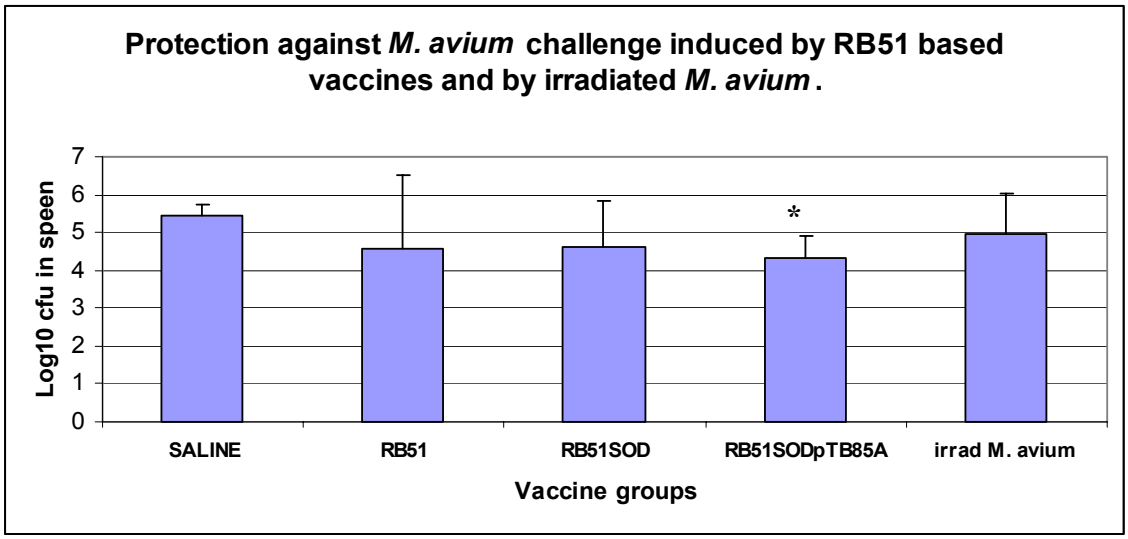

Figure 3.42: Protection against *M. avium* challenge induced by strain RB51 based vaccines and by irradiated *M. avium*.

BALB/c mice were vaccinated with recombinant strain RB51 vaccines expressing MAP antigens or with irradiated *M. avium* (described in Figure 3.16), and challenged 6 weeks post vaccination with $1x10^9$ cfu of *M. avium*. Protection was determined 3 weeks post challenge by calculating the reduction in splenic *M. avium* cfus in the vaccine groups compared to the saline control. Results are expressed as the splenic average Log10 cfus of *M. avium* A5 in the spleens of 5 mice/group. The error bars represent the standard deviations. (*$p<0.05$ compared to saline control).

As mentioned in the previous chapter, the expression levels of the MAP antigens in the original constructs were very low. Since the inconsistent or low levels of protection afforded by these vaccines could be related to the low levels of expression of the heterologous proteins in strain RB51, new RB51 strains expressing MAP 85A protein as a fusion with the Esat-6 protein of *M. tuberculosis* were created. These strains exhibited increased expression compared to that of the original strains in which the heterologous MAP proteins were produces as non fusion antigens (see Chapter 2). To determine whether increased expression of the 85A antigen could lead to consistent and increased protection, groups of 5 mice each were vaccinated and challenged with *M. avium A5* as described before.

Two experiments were carried out in order to test various vaccines with (Figure 3.44) or without (Figure 3.45) boosting with $1x10^7$ cfu of each vaccine candidate. This reduced booster dose was used because research presented in Chapter 4 indicated that boosting with the complete dose of vaccine strain RB51 may result in mortality. No significant protection was observed in any of the groups, including a lack of protection with strains RB51pTB85A and RB51SODpTB85A which contradict the results described in Figure 3.39 and Figure 3.32 in which these strains expressing MAP 85A protein as non fusion antigens did confer protection.

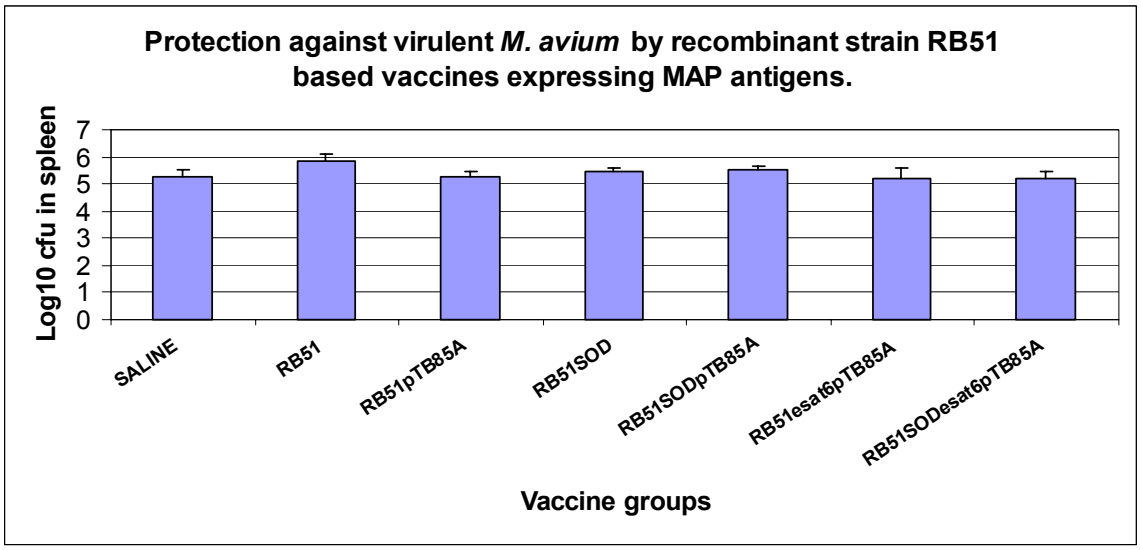

Figure 3.43: Protection against *M. avium* challenge by recombinant strain RB51 based vaccines expressing MAP antigens.

For this experiment, BALB/c mice were vaccinated with recombinant strain RB51 vaccines expressing MAP antigens and re-vaccinated 5 weeks post vaccination with a reduced dose of the vaccine (1 x10^7 cfus). Protection was determined 3 weeks post challenge by calculating the reduction in splenic *M. avium* cfus in the vaccine groups compared to the saline control. Results are represented as average *M. avium* Log10 cfus in the spleens of 5 mice/group. The error bars represent the standard deviations. No protection was observed in any of the groups ($p>0.05$).

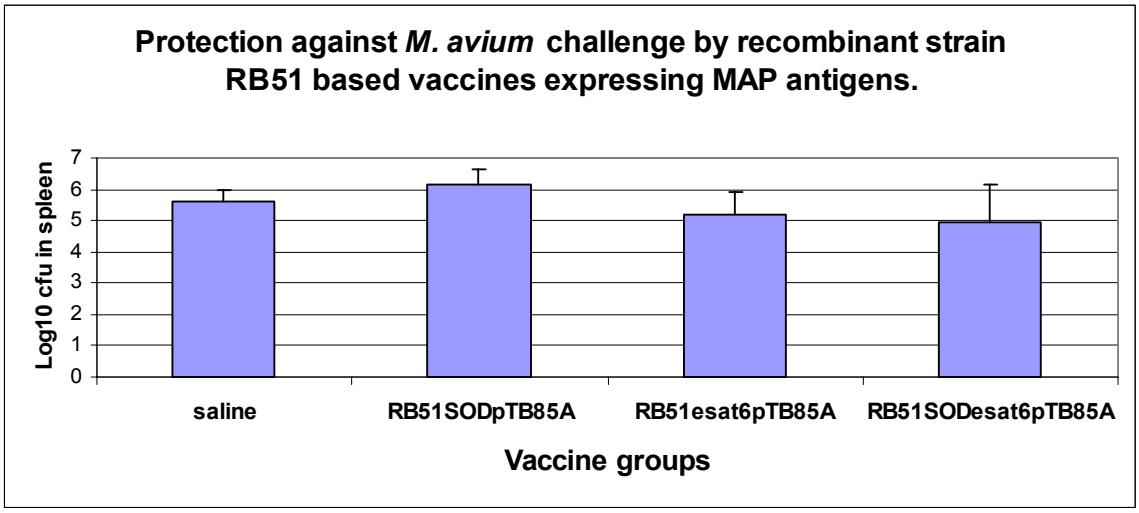

Figure 3.44: Protection against *M. avium* challenge by recombinant strain RB51 based vaccines expressing MAP antigens.

BALB/c mice were vaccinated with recombinant strain RB51 vaccines expressing MAP antigens and challenged 6 weeks post vaccination with $1x10^9$ cfu of *M. avium*. Protection was determined 3 weeks post challenge by calculating the reduction in splenic *M. avium* cfus in the vaccine groups compared to the saline control. Results are expressed as the splenic average Log10 cfus of *M. avium* A5. Results are represented as average *M. avium* Log10 cfu in the spleens of 5 mice/group. The error bars represent the standard deviations. No protection was obtained in any of the vaccine groups ($p>0.05$).

Protection against *M. avium* challenge by *B. abortus* Cu/ZnSOD

The presence of *Brucella* Cu/ZnSOD cross-reactive antibodies in the sera of *M. avium* infected mice was an unexpected result (Figure 3.5). Also, a non-significant trend to protection was observed in mice vaccinated with strain RB51SOD in some of our experiments. This suggested a possible protective role for *B. abortus* Cu/ZnSOD against *M. avium* infection. In order to further explore the potential protective ability of *B. abortus* Cu/ZnSOD against *M. avium* challenge, vaccination of BALB/c mice was performed using a recombinant *O. anthropi* strain that expresses *B. abortus* SOD (*O. anthropi SOD)*, with or without co-administration of CpG. This approach was chosen because *O. anthropi* is the closest genetic relative to *Brucella spp.* (410) but does not provide protection against *Brucella* challenge in the absence of CpG. He et al., (170) have demonstrated that *O. anthropi SOD* is able to induce significant protection against challenge with virulent *B. abortus* when the immune response to it is switched to a Th1 profile using CpG genetic adjuvant.

Contrary to our previous experiments (Figure 3.40, Figure 3.41, Figure 3.42 and Figure 3.43), strain RB51SOD did induce significant protection against *M. avium* challenge. In fact, significant protection against challenge with *M. avium* was obtained with all vaccine strains expressing (SOD $p<0.05$) (Figure 3.45). This result suggests that *Brucella* Cu/ZnSOD induces immune responses that appear to provide some degree of cross-protection against mycobacterial challenge, as suggested by our previous findings of SOD cross reactive antibodies in sera of *M. avium* infected mice, as well as the secretion of IFN-γ by splenocytes from mice acutely or chronically infected with *M. avium,* when stimulated with heat killed strain RB51 or with purified *Brucella* SOD. However, contrary to what has been previously observed in protection against *Brucella* challenge, where protective immunity was only achieved when the immune profile elicited by the strain was switched to a Th1 type; it appears that *O. anthropi* expressing *Brucella* SOD is able to induce protection against mycobacterial challenge independently of the co-administration of the genetic adjuvant CpG.

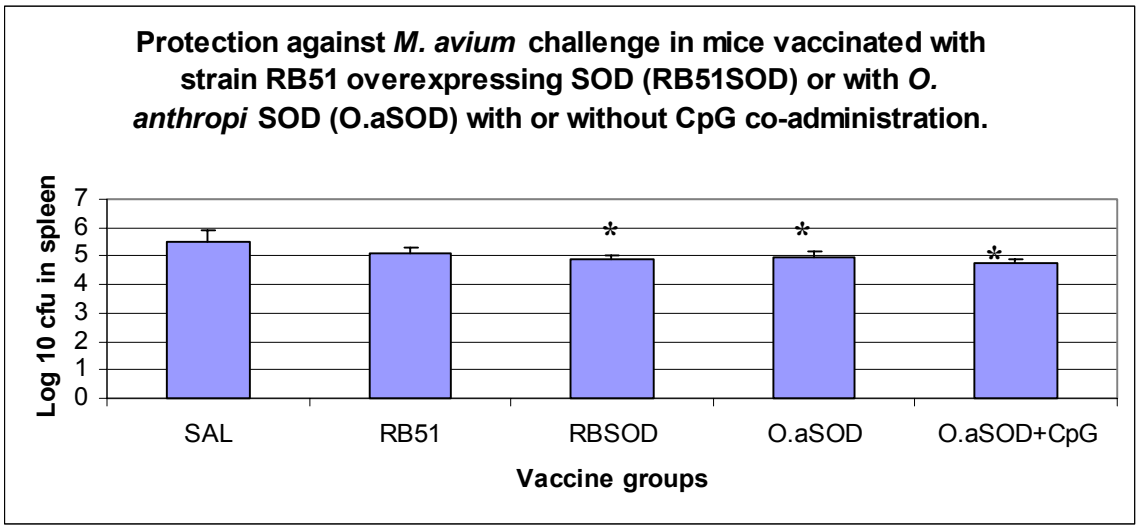

Figure 3.45: Protection against *M. avium* challenge in mice vaccinated with recombinant vaccine strain RB51 overexpressing SOD (RB51SOD) or with *O. anthropi* SOD (O.aSOD) with or without CpG co-administration.

BALB/c mice were vaccinated with strain RB51SOD or with recombinant *O. anthropi* expressing *Brucella* Cu/ZnSOD with or without co-administration of CpG as described in methods. Mice were challenged 6 weeks post vaccination with $1x10^9$ cfu of *M. avium*. Protection was determined 3 weeks post challenge by calculating the reduction in splenic *M. avium* cfus in the vaccine groups compared to the saline control. Results are expressed as the splenic average Log10 cfus of *M. avium* A5. Results are represented as average *M. avium* Log10 cfu in the spleens of 5 mice/group. The error bars represent the standard deviations (* $p<0.05$ compared to saline control).

Attenuation of the RB51 based vaccine strains

Vaccine strain RB51 has been demonstrated to be attenuated in mice and to be cleared from the spleens of vaccinated mice by 6 weeks post vaccination (351). To determine the virulence level of the experimental strain RB51 based vaccines expressing MAP antigens, mice were vaccinated as above and sequentially sacrificed to determine splenic clearance of the vaccine organisms. No significant differences ($p>0.05$) were observed in the clearance rates at 2 and 4 weeks post vaccination between any of the vaccine groups (Figure 3.46). No vaccine organisms were recovered six weeks post vaccination in the spleens of mice from any of the groups. These results are similar to what has been described for strain RB51, which is completely cleared from the spleens of vaccinated mice by six weeks post vaccination (307, 351), and indicate that expression of the heterologous MAP antigens in strain RB51 does not alter its attenuation characteristics. In a separate experiment, mice vaccinated with strain RB51pTB85A also cleared all vaccine organisms by week 6. The presence of the plasmid was tested in this strain by plating the spleen suspensions onto TSA and TSA/Cm plates. No differences were observed between the number of cells that grew on TSA v/s TSA/Cm plates, indicating that the plasmid is stable (Data not shown).

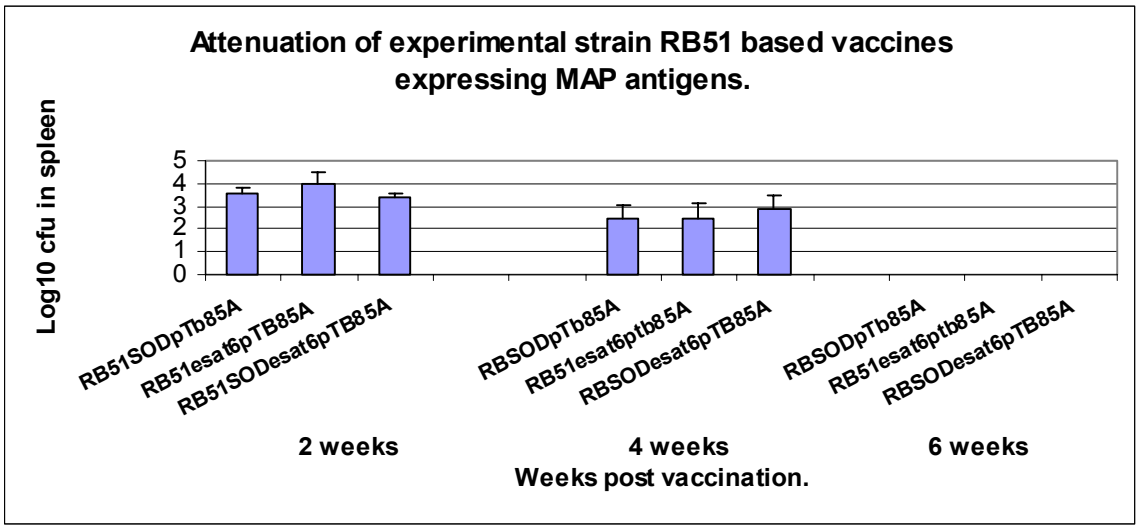

Figure 3.46: Attenuation of experimental strain RB51 vaccines expressing MAP antigens.

Mice were vaccinated as described in methods, and splenic clearance of the vaccine strains was determined 2, 4 and six weeks post vaccination. Results are expressed as the average Log10 cfus in the spleens of 5 mice/group.

Protection against *B. abortus* 2308

To determine whether the experimental *B. abortus* strain RB51 based vaccines expressing MAP antigens are still protective against *B. abortus* challenge, groups of 5 mice each were vaccinated with the different vaccine constructs expressing the 85A antigen and were challenged with virulent *B. abortus* strain 2308 as described in methods. Strains RB51, RB51Esat-6pTB85A, RB51SODpTB85A conferred significant protection against challenge with virulent *Brucella* when compared to the saline control group ($p \leq 0.05$). RB51pTB85A protected at a p level of 0.08. Although the splenic cfu levels in RB51SODEsat-6pTB85A were much lower that those in the saline control group, due to the high standard deviation in this group, the result was not statistically significant (Figure 3.47).

In general these results suggest that expression of the heterologous antigen in strain RB51 does not significantly affect its protective efficacy against virulent *B. abortus* infection. These results correlate with the observation that these vaccines elicit strong IFN-γ responses and antibody polarization towards IgG2a, a profile that has been correlated with protection against *Brucella* infections (307, 418).

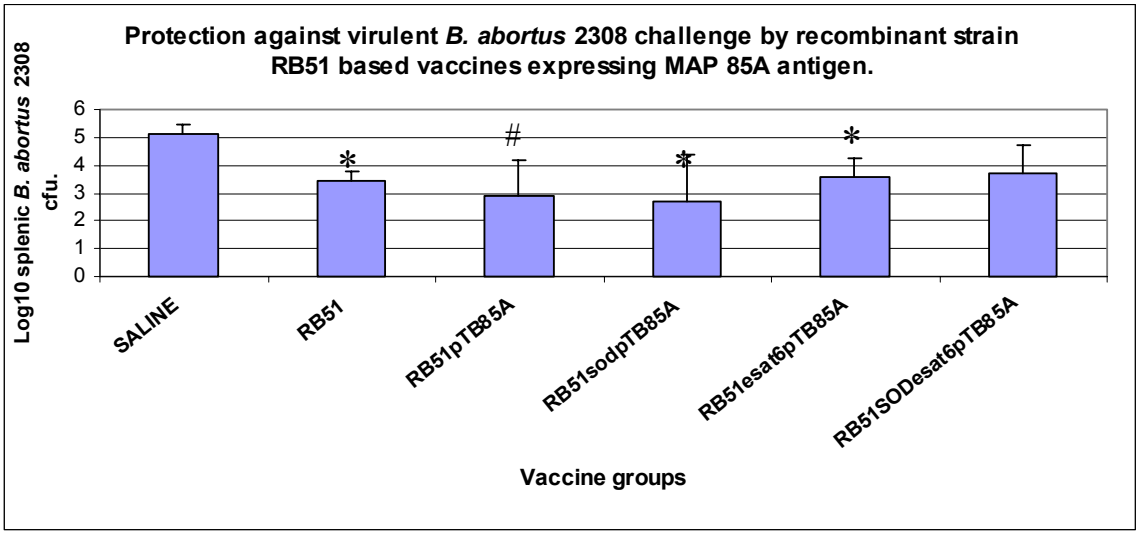

Figure 3.47: Protection against virulent *B. abortus* 2308 challenge by recombinant strain RB51 based vaccines expressing MAP 85A antigen.

BALB/c mice were vaccinated with strain RB51 vaccines expressing MAP 85A antigen and challenged 6 weeks post vaccination with virulent *B. abortus* strain 2308. Results are represented as average *B. abortus* strain 2308 Log10 cfu in the spleens of 5 mice/group, two weeks post challenge. The error bars represent the standard deviations * $p<0.05$; #$p<0.08$.

Discussion

B. abortus and *M. avium subsp. paratuberculosis* (MAP) are intracellular pathogens that cause significant morbidity in domestic animals. Brucellosis is also a major zoonosis that affects humans worldwide. The zoonotic potential of MAP is still debated but some reports suggest that it may be involved in Crohn's disease in humans (24, 51, 171, 193, 353, 405).

Immune control of infections caused by intracellular pathogens such as *Mycobacterium spp., Brucella spp.*, *Toxoplasma spp.* and *L. monocytogenes* is mediated by T-cell immune mechanisms. Several mouse models deficient in T cells and/or deficient in CMI related cytokines have underscored the importance of this branch of the immune system (14, 128, 263). Therefore, in order to be protective against intracellular pathogens, vaccines must elicit strong cell mediated responses. It has been widely documented that vaccine strain RB51 induces protection against challenge with virulent *B. abortus* in mice and cattle (69, 70, 109, 186, 351, 428), and this protection is achieved by specific stimulation of cell mediated responses involving CD4+ as well as CD8+ T cell populations (170, 308, 364).

The purpose of this research was several fold; first, to develop a mouse model suitable for the evaluation of the protective potential against MAP of vaccine candidates; second, to evaluate the potential of vaccine strain RB51 expressing MAP antigens to induce immune responses to MAP; and third, to test the protective efficacy of recombinant strain RB51 based vaccines expressing MAP antigens in this model.

As mentioned before, most of the mouse model developed to study MAP infections require the use of severely immunocompromised animals (268, 343, 373). Other paratuberculosis models have the disadvantage of requiring very long time to obtain results (72, 386). Using female BALB/c mice we attempted to develop a model that would serve as a first screen to select for potentially effective protective vaccines that could eventually be tested against paratuberculosis infection in an appropriate host.

As mentioned in Chapter 2, the MAP genes selected to be expressed in vaccine strain RB51 show very high homology to their homologs in *M. avium* ((29, 98). We hypothesized that if a protective immune response is mounted against these MAP antigens in mice vaccinated with the recombinant vaccines, this response would probably also protect against *M. avium* challenge; and vice versa, if the vaccine protects against *M. avium* challenge it would probably have similar or enhanced effects against a MAP challenge. Using *M. avium* strain A5 as the challenge strain in BALB/c mice presents definite advantages compared to the use of MAP. In the first place, adult mice can readily be infected with this species. This has been demonstrated in several studies, that have been mostly restricted to the aerosol or i.v challenge route because this is a model typically used to mimic the pulmonary infections associated with AIDS in humans (124, 125, 201). The other advantage is that *M. avium* is a strain that can be easily grown in laboratory conditions and requires much shorter incubation periods than MAP (430).

The first step in developing this model was to determine the dose of *M. avium* A5 required to establish an infection in BALB/c mice, and an appropriate post challenge endpoint to determine clearance of mycobacteria. To do this we decided to follow the *Brucella* model for challenge experiments in which protection is assessed by determining the load of virulent *Brucella* organisms in the spleens of infected mice, and comparing it to the bacterial load in the spleens of vaccinated mice challenged with the same virulent strain. BALB/c mice were infected with $1x10^7$, $1x10^8$ or $1x10^9$ cfu of *M. avium* strain A5 via the i.p route. Infection with these different doses of *M. avium* did not cause clinical signs of disease but did induce marked splenomegaly. The spleens were enlarged to more than twice the size of the controls, a finding that has previously been reported in wild type BALB/c mice after i.v infection with high doses of virulent *M. avium*, and has been linked to the effects of TNF-α (100).The spleens remained enlarged one year post infection but no *M. avium* organisms were recovered at that time. Mice infected with these three doses of *M. avium* showed a linear dose dependent rate of infection, and an amost linear time and dose dependent rate of clearance (Figure 3.3). Mice infected with $1x10^7$ cfu were able to completely clear the infection within 10 weeks. *M. avium*

organisms were still recovered from the spleens of mice infected with $1x10^8$ and $1x10^9$ cfu 10 weeks post infection. However, the infection level was very low and reflected the original dose of infection; i.e., mice infected with $1x10^9$ cfus had approximately 1 log higher splenic *M. avium* cfus than mice infected with $1x10^8$ cfus. In all cases, the number of bacteria recovered from the spleens never surpassed the original inoculum and exhibited a sharp decrease over time. Analysis of the slope of the clearance curves indicated that the rate of clearance of *M. avium* infection was not significantly different between the different doses, suggesting that over time all *M. avium* organisms would have been eliminated, even in the groups infected with the highest dose. However, it is possible that if the groups had been followed for a longer period of time, different rates of clearance would have been observed as a result of a break in the balance between the pathogen and the immune response that controls it. Such a profile is typically observed in cases of reactivation of latent infections (301). We demonstrated that the high dose of *M. avium* ($1x10^9$) is able to establish colonization of the spleen at a level that remains high for a longer period of time. Ten weeks post infection a few organisms (less than 100 bacteria) were recovered from the spleens of mice infected with this dose (Figure 3.3). Although this number is very low and there is a clear trend towards clearance of infection, it is possible that some organisms may remain inside different organs establishing a long term chronic infection. For our challenge experiments we decided to use the higher dose of infection ($1x10^9$ cfus) because higher splenic infection loads were obtained with it; since the number of mycobacteria decreased very quickly post infection, the 3 week time point was selected as the end point for our challenge experiments.

Our results are somewhat different to what has been previously reported by Orefici et al., (87). In their study, they report that an infection with *M. avium* persisted for more than 70 days at high levels in BALB/c mice. This difference is probably due to the use of different *M. avium* strains since different strains exhibit marked differences in virulence (341). Our results suggest that although BALB/c mice are susceptible to infection with *M. avium* A5, these mice are able to control the infection. It has been previously shown that although BALB/c mice are considered susceptible to infection

with different strains of *M. avium*, they are able to control infection in organs and peritoneum through B and T cells (100).

Despite the clear splenomegaly observed in mice chronically infected with *M. avium* (one year post infection), in our study we were not able to recover any *M. avium* organisms from the spleens of these mice. Splenic enlargement has been correlated with bacterial colonization (307), therefore it is possible that live mycobacteria were present in the spleens but our recovery technique failed to detect them. A possible explanation is that mycobacterial infections are characterized by the formation of granulomas in infected tissues (125, 362). These granulomas are the host's attempt to limit the spread of the invading mycobacteria by walling off the infected cells, and are composed of CD4+ and CD8+ T cells, B cells, fibroblasts and other cell types that are surrounding infected macrophages. Not all mycobacteria are killed within these granulomas; virulent mycobacteria have developed several evasion and survival mechanisms that allow them to survive within these structures which ultimately provide a protective niche for the bacteria. It is possible that the enlarged spleens observed after acute and chronic infections with *M. avium* are a reflection of the granulomatous response to the pathogen. Future studies should focus on the histological analysis of the spleens and other organs of acutely and chronically infected animals to determine the overall cellular composition, and the localization and persistence of immune cells in the organs of these animals.

Several studies have demonstrated the uptake of *Mycobacterium spp.* by J774A.1 cells (230, 245, 255, 288). To determine whether the *M. avium* strain A5 used in our study is phagocytosed by eukaryotic cells, we performed an *in-vitro* infection of the J774A.1 macrophage like cell line by incubation with *M. avium*. Intracellular mycobacteria were observed as early as 2 hours post infection indicating effective uptake of this strain by J774A.1 cells. This result suggests that *M. avium A5* may survive in the spleens of infected mice organisms by infecting macrophages, which is similar to *Brucella* infection.

In our experiments, we observed that mice acutely or chronically infected with *M. avium* developed strong IFN-γ and no detectable IL-4 responses to heat killed *M. avium,* heat killed *Brucella* and to purified *Brucella* Cu/ZnSOD. This profile of high IFN-γ in the absence of IL-4 suggests a strong polarization towards a Th1 cytokine profile, which is typically associated with protection against intracellular pathogens, and in this case was associated with the elimination of *M. avium* from the spleens of infected animals. Secretion of IFN-γ was also observed when splenocytes were stimulated with heat killed *L. monocytogenes*. This response appears to be non-specific and may be a reflection of the strong immune stimulation induced by mycobacteria. mycobacteria are strong inducers of IL-12, therefore during a mycobacterial infection IFN-γ is always produced (187, 360). As mentioned in Chapter 1, the final outcome of the mycobacterial infection is not only dependent on the presence of IFN-γ but also on the level of production of this cytokine (201). Together with the production of IFN-γ, the lack of IL-4 is consistent with the induction of an immune response of the Th1 profile. However, IL-4 does not appear to play an important role in decreasing protection against mycobacterial infections (188).

The splenocytes of mice chronically or acutely infected with *M. avium* secrete very low levels of IL-10 in response to *M. avium*. IL-10 is another cytokine associated with the Th2 profile and has strong anti-inflammatory activity. The effects of IL-10 on resistance to mycobacterial infections have been somewhat inconsistent. One study showed that mice with deletions in the IL-10 gene do not have significantly higher rates of clearance of MTB infections than wild type mice, suggesting that IL-10 does not play a significant role in decreasing resistance (188); however, another study showed that mice with deletions in the IL-10 gene have increased resistance to *M. bovis* BCG infections, suggesting that IL-10 may decrease resistance to this attenuated strain (264). As described in Chapter 1 the role of IL-10 is thought to be directed at limiting the damage induced by pro-inflammatory cytokines during the immune response mounted against an invading pathogen (254, 280).

The observation that mice infected with *M. avium* exhibit significant splenomegaly, coupled with the low levels of IL-10 secretion detected in these mice

suggest that in the absence of IL-10, the inflammatory response mounted against *M. avium* is not downregulated and results in persistent inflammation of the spleen. However, in order to confirm this, *in situ* expression of other pro-inflammatory cytokines such as TNF-α should be evaluated. Additionally, histological analysis of the spleens of infected mice could provide valuable information regarding the cellular composition, presence of granulomas and distribution of inflammatory cells in the organ.

Unexpectedly, serological analysis of the immune responses mounted by mice infected with *M. avium* revealed the presence of antibodies directed to *Brucella* Cu/ZnSOD (Figure 3.13a and b). These antibodies were only transiently detected in the sera of mice infected with the higher dose of *M. avium* and disappeared by 6 weeks post infection. The specificity of these antibodies was demonstrated by western blot analysis of whole cell extracts from strains RB51 or RB51SOD, as well as by reacting the sera obtained from mice infected with *M. avium* to purified recombinant *B. abortus* Cu/ZnSOD. Bacterial superoxide dismutases are part of the general oxidative stress response mechanism and are highly conserved in nature. *Brucella* possesses several SOD species (39) and Cu/Zn SOD is highly conserved among *Brucella* species (372). It has a periplasmic location and is encoded by the *sodC* gene (375). MAP and *M. avium* possess several superoxide dismutases (SOD) including a Cu/ZnSOD and a MnSOD (104, 119, 225). The genomes of *B. suis* (309) and of *B. melitensis* (93, 94) have been sequenced and published, and the genomes of *M. avium* and *M. avium paratuberculosis* are being sequenced (309). BLAST comparison of the *Brucella sodC* gene sequence to published sequences indexed in the PUBMED database revealed identity (100%) to only *B. suis* and *B. melitensis sodC* genes. The *M. avium* preliminary genomic sequence data was obtained from The Institute for Genomic Research website at http://www.tigr.org/. Comparison of the *sodC* gene of *Brucella* with this database yielded no significant matches at the genetic level (highest score=120, E=4.0, P=0.98 with contig:3273:m_*avium*). When a BLASTx comparison of the translated SodC protein of *Brucella* with this contig was performed, 30% identity and 45% positive substitutions in frame +1/+2 were detected to a 133 aminoacid length segment of the potential protein

product in contig:3273:m_*avium* ($E=2.6x10^{-7}$). BLAST search of the translated contig revealed no matches of the potential protein product with *Brucella* sequences. An extra attempt to find the potential gene responsible for the cross-reactive antibodies was carried out by PCR amplification. PCR primer pairs designed to amplify within the coding sequence of *B. abortus sodC* were used to test *M. avium* A5 and MAP genomic DNA templates (Forward primer: 5'AATGGCCTTACGGTTTCTC3', Reverse primer: 5'GCGGCTCAGGCTTATCG3'). No target sequences were amplified. We therefore conclude that the cross reacting antibodies may be directed to conformational epitopes in *B. abortus* Cu/Zn SOD. The limited protein similarity of *Brucella* SOD to the product encoded in contig:3273 appears to support this.

The phenomenon of cross reactivity between unrelated species has been reported in several systems. In a study of the plant Cowpea Mosaic Virus, Olszewska et al (298) detected cross reacting antibodies to the human Measles Virus. These antibodies, directed to a peptide of the measles virus Fusion protein, were found to cross-react with a conformational B cell epitope of a subunit of a coat protein of the cowpea mosaic virus. Furthermore, passive transfer of these cross reacting antibodies was able to confer resistance against challenge with measles.

As mentioned above, cytokine analysis of splenocyte culture supernatants from mice acutely and chronically infected with *M. avium* did show IFN-γ production in response to heat killed strain RB51 and also to *Brucella* Cu/ZnSOD. Because induction of cytokine responses requires T cell involvement, which recognize short peptide sequences, this response may be more specific than just isolated conformational epitopes. Cross reactivity at the T cell level has been shown in viral infections in humans. Nigels et al., (285), demonstrated cross reactivity in MHC-1 restricted CD8+ T cell responses between epitopes of the unrelated human papillomavirus and the human coronavirus. Mason (240), has proposed that this T cell cross-reactivity is a common event, part of the natural adaptation of the immune system that allows it to expand the repertoire of responding cells available for future infections. Because there are far more potential antigenic epitopes in nature than the T cell repertoire with which an individual is born

with, this theory is appealing. Our finding of SOD cross-reactive antibodies and IFN-γ induction in mice infected with *M. avium* upon stimulation with recombinant *Brucella* SOD, or with heat killed strain RB51, suggests that these events may be the consequence of such cross reactions. Bacterial SODs, and likely their conformational epitopes, are highly conserved among prokaryotes (39); therefore, it seems natural that the immune system would be able to develop a response to these conserved antigens as a means of broadening protection against a variety of potential pathogens.

Although the 85A and 35 kDa proteins have been described to be immunogenic in mice, and mice vaccinated with our purified recombinant 85A and 35 kDa proteins did develop antibodies to these antigens, vaccination with strain RB51 based vaccines expressing MAP antigens elicited only very low antibody responses to the recombinant 85A antigen and no detectable antibodies to 35kDa protein antigen.

The low immune response elicited to the heterologous protein could be due to the low expression levels of the foreign antigen observed in strain RB51 (see Chapter 2). However, we did expect to see higher 85A specific antibodies in the groups vaccinated with strain RB51Esat-6pTB85A, since western blot analysis revealed higher expression of the fusion protein in this strain. Improper folding, improper expression or inadequate solubility of the mycobacterial proteins may have also occurred in RB51 strains expressing these mycobacterial proteins, and this may in turn be responsible for the lack of specific antibody responses, as these protein products may not have been adequately presented or may have not been able to stimulate the correct repertoire of immune cells, or the antibodies induced may not have been able to recognize the recombinant antigen used in our assay. Additionally, these heterologous proteins may exert a toxic effect in strain RB51 and therefore, their expression is down regulated or eliminated. Recombinant RB51 strains expressing MAP 85A, 35 kDa protein or the fusion protein Esat-6pTB85A did exhibit slower growth than strain RB51 suggesting that these proteins exert a toxic but not lethal effect on *Brucella*, which may account for a down-regulation in expression. If this is the case, this down-regulation should be protein specific since antibodies directed to the mycobacterial Hsp65 and to *E. coli* Beta galactosidase have been detected

in mice vaccinated with strain RB51 expressing these heterologous genes (416). Another possible explanation is that the plasmid may have been eliminated from the recombinant strains. However, this does not seem to be the case since the vaccine organisms recovered from the spleens of vaccinated mice during the attenuation studies, conserved the plasmid as evidenced by their ability to grow on antibiotic containing plates. The purified proteins used as antigens in our assays were produced by heterologous expression in *E. coli*. As discussed in Chapter 2, expression of these recombinant proteins in *E. coli* was mainly in insoluble fractions that required denaturation with urea in order to purify them. It is possible that the expression in the heterologous *E. coli* vector and/or the denaturation of the protein altered the conformation of the protein product destroying the key epitopes to which antibody responses against the native protein are directed. In this regard, it has been shown that heterologous expression of mycobacterial proteins is low, may lead to improper folding of the proteins and typically results in expression of the heterologous proteins in inclusion bodies; therefore, it has been proposed that efficient expression of mycobacterial proteins requires a mycobacterial vector (36, 166, 429).

Stimulation of splenocytes from mice vaccinated with recombinant RB51 strains expressing MAP antigens with heat killed *M. avium,* did not induce consistent cytokine responses. Cytokine analysis of splenocyte culture supernatants of mice vaccinated with strain RB51 based vaccines expressing MAP antigen, showed that IFN-γ was secreted in response to *M. avium* stimulation in a few experiments, however in these cases, IFN-γ secretion did not correlate with protection as demonstrated by the results obtained with strains RB51esat-6pTB85A and RB51SODesat-6pTB85A which induced IFN-γ secretion and no detectable levels of IL-4 in 3 out of 4 experiments, and 2 out of 4 experiment respectively, but never induced protection against challenge. This finding is in agreement with reports that show that the elicitation of Th1 type cytokine responses with high IFN-γ secretion may not necessarily correlate with protection (150, 176). Inconsistent responses were also observed when cells were stimulated with purified recombinant 85A which came from different purification batches. These results may again be due to the low

expression levels of the recombinant proteins in strain RB51, but in the latter case, may also be due to the fact that purified 85A was obtained from recombinant *E.coli*. Some *E. coli* proteins may have contaminated the protein preparations and may have acted as non-specific stimulators.

Because there are currently no effective vaccines for protection against *M. avium* challenge in mice, there is no positive control for our protection experiments. In an attempt to generate a positive control for the vaccine experiments, the effect of gamma irradiation on *M. avium* A5's replication ability and metabolic activity was determined. Irradiation of bacterial cultures has long been used as a decontamination method in the food industry and causes non-reversible changes in the DNA strands that render the irradiated bacteria non-replicative (355). It has also been used in vaccine models. For example, vaccination of mice with an irradiated arthrospore vaccine against coccidiomycosis reduced the pathologic lesions in mice challenged with *Coccidioides immitis* (316). Also, inoculation of mice with irradiated *Toxoplasma gondii* tachyzoites has revealed that serological and cytokine responses are mounted to Toxoplasma antigens indicating that irradiation of microorganisms may be an adequate means for reducing virulence while maintaining the immunological properties of the vaccine (173).

The rationale for the use of irradiated organisms is the thought that homologous vaccination should present the highest number of immunogenic and protective antigens to the immune system therefore inducing immune responses to the largest number of antigens. It has been widely documented that only live vaccines elicit protective immunity against intracellular organisms (186, 219), by abrogating the replication of *M. avium* its virulence would be eliminated. However, since irradiated strains would still be metabolically active, they would mimic the dynamic behavior of live vaccines and therefore would be effective and could be safely used as a vaccine. As shown in our results, irradiation of *M. avium* with high doses of gamma irradiation effectively obliterated its replication capacity while retaining metabolical activity as determined by plating and by the Alamar Blue reduction assay respectively.

Contrary to our expectations, vaccination with irradiated *M. avium* conferred no protection against homologous challenge with live *M. avium* (Figure 3.7). The reasons for this lack of protection are not clear, but since the exact effect of irradiation on mycobacteria*'s* phenotype is not known, it is possible that some important immunogenic and protective antigen pathways are altered or destroyed by the irradiation process. It has been shown that most of the protective antigens of mycobacteria are actually secreted proteins (11, 12); irradiation may damage the mycobacterial secretion mechanisms preventing secretion of these protective antigens. Additionally, it has been shown that radiation damage is dose dependent. In our experiments we used a 198,000 rads (30 minutes) increment of the radiation dose between the last dose in which *M. avium* organisms still replicated (396,000 rads), and the dose that resulted in complete abrogation of replication, 594,000 rads (90 minutes). Although the irradiated cells were still metabolically active as determined by the Alamar Blue Reduction assay, the optimal radiation dose that completely abrogates replication, while still maintaining immunogenicity and the protective capabilities of the irradiated vaccine, may lie somewhere in between. Future studies should evaluate the effect of radiation dose using smaller radiation increments between 396,000 rads and 594,000 rads. Additionally, the ability of the irradiated *M. avium* strain to secrete antigens should also be analyzed. An additional attempt to try to identify a positive control using the commercial paratuberculosis vaccine Mycopar®, failed to induce protection against challenge with *M. avium.* This vaccine was selected because it is the only commercially available vaccine to vaccinate against paratuberculosis, but no reports have been published regarding its protective efficacy in mice. Mice vaccinated with Mycopar® vaccine developed significantly lower antibody levels to *M. avium* culture supernatant proteins than mice vaccinated with the irradiated strain. This result suggests that the commercial vaccine, at the dose used in our experiment, was not able to induce an immune response comparable to that induced by the irradiated strain.

The lack of a positive control is a major weakness of the model we tried to establish and suggests that this model is not useful. Nevertheless, faced with major time

constraints we decided to proceed using the model and focused on immune responses induced by the vaccines as well as studying a possible protective effect.

Protection experiments in which mice were vaccinated with recombinant RB51 strains expressing MAP antigens and challenged with *M. avium* revealed very high intragroup variation. Although the experimental conditions were maintained constant, the protection levels were not consistent. Some protection was observed when mice were vaccinated with RB51SODpTB85A or RB51pTB85A but these results were not always achieved. The lack of protection could be due to the low expression levels of the 85A protein in strain RB51. The role of the 85A protein in inducing proliferative responses has been found to be dose dependent, where very low doses (100-1000 ng of purified 85A) of the antigen inhibit proliferative responses (41), while higher doses elicit delayed type hypersensitivity (146). In order to test whether efficacy could be improved by increasing the expression levels of the MAP 85A antigen in strain RB51, constructs expressing higher levels of 85A as a fusion with MTB Esat-6 protein were used. Contrary to our expectations and consistent with the serology results, vaccination with these constructs failed to increase protection against *M. avium* challenge. Increased expression of the 85A antigen in strain RB51esat-6pTB85A was observed when whole cell lysates were analyzed using an 85A specific antisera (Chapter 2), however, as discussed above, the protein may be produced in insoluble fractions or in inadequate conformation that renders it non-immunogenic.

The high intragroup variability observed in the *M. avium* protection experiments contrasts with the little variation observed in *Brucella* challenge experiments. *Mycobacterium* organisms tend to aggregate when in suspension. It is possible that because of this phenomenon, and despite using sonication to disperse aggregates in the challenge suspensions, the challenge dose was not identical for all mice within the same group, and therefore different clearance results were obtained. Another technical factor that could account for the variability in the splenic *M. avium* count, is the bacterial

isolation method used in our experiments. To determine clearance of *Mycobacterium*, the spleens of vaccinated/ challenged mice were macerated and serially diluted before plating onto Middlebrook plates. As mentioned above, mycobacterial infections induce the formation of granulomas in infected tissues and organs. It is possible that the isolation method failed to detect all *Mycobacterium* organisms due to an inability to effectively disrupt some granulomas and release al mycobacterial cells. The use of additional or alternative bacterial determination methods such as the radiometric BACTEC system may be of value for future experiments. Additionally the high intragroup variability obtained in our experiments may be a consequence of the mycobacterial strain used. As mentioned above, *M. avium* strains vary greatly in regards to their virulence in different mouse strains (341). The use of strains with higher virulence as our challenge strain may better reveal the protective efficacy of the RB51 based anti-mycobacterial vaccines and may lead to more consistent results. Finally, the challenge dose may be too high and therefore protection is masked by the high bacterial load in the challenge dose. In this case, a reduction in the challenge dose or a change in the timing of clearance determination could allow better assessment of the vaccines.

The lack of a positive control in our experiments, limits our possibility to interpret negative results. It is quite possible that the *M. avium* strain or the challenge model we have developed to evaluate protection are not suitable for vaccine evaluation. If the model is actually suitable, a possibility for the lack of protection induced by these vaccines could lie in the fact that we are only expressing one mycobacterial antigen. Expression of only one antigen in strain RB51 may not be enough to induce effective protective immunity against *M. avium* A5. Future studies should focus on the possibility of generating a vaccine expressing several heterologous protective antigens.

Revaccination of mice with a reduce dose of the RB51 based vaccines was carried out in one experiment, although no protection was observed in this experiment, it is possible that additional booster doses could enhance protection.

The antigens selected for expression in strain RB51 have been described as protective based on in-vitro studies where 85A is shown to stimulate T cell repertoires and induce IFN-γ secretion. Many reports have demonstrated that 85A is a strong inducer of Th1 responses and that it is recognized by infected patients. Protection results have been carried out mostly using this antigen in isolation, mainly as DNA vaccines or as a pulsing antigen. When protective efficacy of the 85A antigen has been evaluated, the results have been somewhat scattered. In a vaccination study using dendritic cells loaded with antigen 85A, Gonzalez-Juarrero et al., (150) showed that significant infiltration of IFN-γ secreting CD8+ CD4+ T cells occurred in the lungs of vaccinated mice compared to the lungs of the controls, however this approach failed to protect against aerosol challenge with *M. tuberculosis*. In a study using a self-destructing *L. monocytogenes* vaccine expressing 85A Miki et al., (246) showed protection comparable to that induced by BCG vaccination. DNA vaccination using different plasmid vectors have shown to be protective against *M. tuberculosis* challenge (151, 237, 387), but apparently, the induction of immune responses requires more than just the expression of the right antigen in order to be protective. It has been shown that DNA vaccination with 85A is protective when the encoded 85A antigen is secreted but not when the antigen is not secreted (26). In a gene gun bombardment model of tuberculosis in guinea pigs, Sugawara et at (384) demonstrated that vaccination with a DNA vaccine expressing the MTB 85A antigen only conferred protection when a booster dose was administered; however only partial protection was induced as the bacterial load was decreased in the lungs of vaccinated animals, but vaccination could not protect against hematogenous dissemination into the spleen and other organs. DNA vaccination has also been shown to be protective against *M. avium* when the 85A antigen from *M. bovis* was used (411) showing that vaccination with this antigen can confer protection against challenge with heterologous mycobacteria. Recently, Dhar et al., (97) reported that recombinant BCG vaccines over-expressing different components of the 85A complex, elicit increased antibody production when 85A or 85C are expressed, but only over-expression of 85A induced cell mediated responses; unfortunately no protection experiments have been reported yet.

The role of the 35 kDa protein antigen in protection is not clear. Several studies have reported that animals and humans with active non-tuberculous mycobacterial disease have high titres of antibodies directed to this antigen (395, 427). Because the 35 kDa antigen is absent in *M. tuberculosis* and *M. bovis*, it has been proposed as a diagnostic candidate for paratuberculosis disease in cattle (358). DNA vaccination with a construct expressing *M. avium* 35 kDa protein induced significant protection against M. leprae foot-pad infection (239), and also against *M. avium* (238). However, no studies have been published using the 35 kDa protein in vaccine models other than DNA vaccines. In our experiments no protection was observed when mice were vaccinated with strains RB5135 or RB51SOD35. Again, the expression levels may not have been sufficient to stimulate an adequate protective response or the model may be unsuitable.

Protection against mycobacterial challenge has recently been shown to be more complex than previously thought. Some immunogenic antigens that elicit strong Th1 type responses have been shown to be deleterious to protection *in vivo*. Hovav et al., (176) showed that the *M. tuberculosis* 27 kDa antigen induces strong cell mediated immunity, as measured by specific DTH responses, high levels of IFN-γ secretion and elicitation of IgG2a specific antibodies. However, when this lipoprotein antigen was expressed in a DNA vaccine, not only the vaccine was not protective, but it significantly increased the mycobacterial load in vaccinated animals. Moreover, this antigen completely abrogated the protective response afforded by BCG vaccination when the purified protein was added to a BCG vaccine dose. The 19 kDa lipoprotein antigen of *M. tuberculosis* has also been shown to be immunogenic and to stimulate CD8+ cells to secrete IFN-γ in large quantities, but when used in vaccination experiments, despite generating strong antigen specific Th1 response, it failed to decrease the bacterial load in the lungs of infected mice (141, 250). This suggests that although the elicitation of a strong Th1 response is crucial to control infection with mycobacteria, the elicitation of such response as measured by our current *in vitro* and/or *in-vivo* tests does not necessarily correlate with protection against mycobacteria. These findings further emphasize the complexity that lies in the design of efficient vaccines to protect against mycobacterial diseases.

In our protection studies, vaccination with strain RB51SOD appeared to induce a tendency towards protection against *M. avium* challenge. Although this decrease in mycobacterial colonization of the spleens was not statistically significant, the tendency was observed throughout the experiments. To further examine the possible role of *Brucella* CuZnSOD in protection against *M. avium*, vaccination with recombinant *Ochrobactum anthropi* expressing *Brucella* CuZnSOD followed by challenge with *M. avium* was performed. This approach was selected because He et al., (169) have shown that this vaccine strain, when used in combination with CpG, is able to induce protection against virulent *Brucella* challenge. The CpG motifs are DNA sequences that contain an unmethylated CpG dinuclotide flanked by two purines (5') and two pyrimidines (3'). This type of sequences are frequently found in bacterial DNA, but vertebrate DNA contains a lower than expected amount of CpG motifs, and those present are found to be methylated, which are not immunostimulatory. Oligonucleotides containing CpG can stimulate an innate response through binding to the TLR-9. Ligation of this receptor induces a signal transduction cascade that results in the induction of NF-kB resulting in secretion of TNF-α, IL-10, IL-6 and IL-12 among other (37, 437).

As seen in Figure 3.45, significant protection was obtained when mice were vaccinated with strain RB51SOD or with *O. anthropi* SOD with or without addition of CpG. This result, although different to our previous experiments in which no statistically significant protection was achieved, is in concordance with the cytokine profile observed in mice acutely or chronically infected with *M. avium* as they do mount cytokine secretion in response to SOD. The results again suggest that *Brucella* Cu/ZnSOD may induce protection against *M. avium* infection and possibly against MAP. It also raises the possibility that the cross reactive epitope/s present in *M. avium* may be part of a highly protective antigen found in *Mycobacterium*. The fact that statistically significant protection was obtained in the group vaccinated with strain RB51SOD may be a reflection of the decrease in intragroup variability observed in this experiment compared to the previous experiments. This may be due to a more homogenous dose of the vaccine

and/or of the challenge strain in this particular experiment; it could also be due to a more efficient recovery of *M. avium* from the spleens of these mice. The finding of protection in the group vaccinated with *O. anthropi* SOD without the use of the CpG adjuvant is interesting as it contrasts with the lack of protection induced by this strain against *Brucella* challenge in the absence of CpG. Future experiments using vaccination of BALB/c mice with purified *Brucella* Cu/ZnSOD or a DNA vaccine expressing *Brucella* SOD (299), and also with the *O. anthropi* vector without over-expression of *Brucella* SOD may provide a better understanding of this phenomenon, as they would indicate whether *O. anthropi* it-self contains additional antigens that can provide certain level of protection against mycobacteria.

The low or non existent immune response to the mycobacterial antigens observed in mice vaccinated with recombinant RB51 vaccines expressing MAP antigens was not due to loss of immunogenicity of the strain. Compared to saline control mice, mice vaccinated with the experimental vaccines did develop antibody responses to *Brucella* RB51 antigens which were of mainly an IgG2a isotype with low levels of IgG1 antibodies (mostly below the detection limit of our system). This isotype profile is consistent with what has been previously reported for serological responses to *Brucella spp.* in general (378) and for strain RB51 in particular (415-417), and suggests the development of a response eschewed towards a Th1 phenotype which is not affected by the expression of the heterologous gene. This polarization towards IgG2a is likely the result of the IFN-γ induced by vaccination with these RB51 based vaccines, which promotes secretion of this immunoglobulin isotype (53).

As described in Chapter 1, it has been previously shown that strain RB51 is a strong inducer of IFN-γ without inducing secretion of IL-4 (128, 170, 263, 307). IFN-γ is produced by T cells (CD4+ and CD8+), NK cells and also by antigen presenting cells (350). In vaccinated animals, strain RB51 organisms interact with pathogen recognition receptors on the surface of macrophages. Campos et al.,(63), have shown that strain RB51 signals through Toll-like receptor 4 (TLR4) to induce secretion of TNF-α and IL-12. TLR4 is the major LPS signaling receptor and LPS induces the interaction between

TLR4 with CD14, a GPI linked cell surface glycoprotein (182). The secretion of chemokines and of IL-12 by the infected macrophages results in the recruitment of NK cells to the inflammation site. IL-12 promotes secretion of IFN-γ by the NK cells. This starts a positive feedback loop in which the IFN-γ secreted by NK and T cells, together with IL-12 and other cytokines such as IL-18, further increases IFN-γ production. This is the first event that drives the immune response towards a Th1 phenotype. Binding of IFN-γ to its receptor induces a pleiotropy of responses which ultimately lead to enhancement of the macrophage's phagocytic and antigen presenting capacity,

The cytokine profiles found in mice vaccinated with the recombinant RB51 based vaccines expressing MAP antigens revealed secretion of high levels of IFN-γ and undetectable levels of IL-4 in response to stimulation with heat killed strain RB51. Our results are consistent with observations in studies analyzing the cytokine profiles induced in mice after vaccination with strain RB51, which have shown that splenocytes of mice vaccinated with strain RB51 produce IFN-γ in response to *Brucella* stimulation but IL-4 secretion is not induced (170, 307). The response to stimulation with purified *Brucella* Cu/ZnSOD was also inconsistent. In our experiment we did not detect IFN-γ in the splenocyte culture supernatants of strain RB51 or RB51SOD vaccinated mice. It has been previously shown that mice vaccinated with this RB51SOD do mount strong IFN-γ responses to purified SOD (418), therefore the lack of detection of this cytokine in our system was probably due to technical failure rather than an actual biological result.

Another cytokine associated with the Th2 profile is IL-10 (24). High levels of this cytokine were detected in the culture supernatants of splenocytes from our vaccinated mice upon stimulation with heat-killed strain RB51. Baldwin et al., (128) showed that this cytokine is produced in higher quantities by BALB/c mice than C57/BL6 mice and mainly by CD4+ cells. It has been suggested that IL-10 is deleterious to protection against infection with *B. abortus* strain 2308 (127). In agreement with what has been reported by Pasquali et al., (307), our results show that despite the production of IL-10, mice vaccinated with strain RB51 are protected against challenge with virulent *Brucella*. Velikovsky et al., (413) have also reported production of IL-10 when they evaluated

protective vaccination with purified lumazine synthase with different adjuvant formulations and challenge with *B. abortus* 544. In contrast, Hoover et al., (174) showed that a *B. melitensis* purine auxotrophic mutant induced protection against systemic *B. melitensis* infection but failed to reduce the number of organisms recovered from the lungs of infected mice. They propose that a vaccine that selectively increases IFN-γ without IL-10 induction would better protect against *Brucella*. These results suggest that although IL-10 may to some degree decrease the protective response against *Brucella* challenge, IL-10 appears to play an important role in protective immunity against *Brucella* infection by limiting the "intensity" of the response geared by IFN-γ (307).

In general, the recombinant RB51 based strains expressing MAP antigens remained protective against *Brucella* infection indicating that expression of heterologous antigens does not significantly impair the ability of vaccine strain RB51 to mount protective immunity to *Brucella*. This was also confirmed by the clear polarization towards a Th1 cytokine profile observed when splenocytes from mice vaccinated with these recombinant strains were stimulated with heat killed *B. abortus*. Only vaccine strain RB51SODesat6pTB85A failed to induce protection against *Brucella* challenge. Decreased numbers of *Brucella* organisms were detected in the spleens of these animals, but the high intragroup variability lead to a non-significant result. This observation is different from what is normally observed in *Brucella* challenge studies, where typically intragroup variability is not a concern. This result could be due to technical artifact in which the *Brucella* organisms were not accurately detected or an error in the vaccination or challenge doses.

Vemulapalli et al., (418) have previously demonstrated that strain RB51SOD induces higher protection against virulent *Brucella* challenge than its parent strain RB51. In our studies, compared to strains RB51pTB85A and RB51esat-6pTB85A, vaccination with strains RB51SODpTB85A or RB51SODesat-6pTB85A which simultaneously over-expressed *Brucella* Cu/ZnSOD and expressed the MAP antigen did not result in increased protection against challenge with virulent *Brucella*. It is possible that co-expression of antigens abrogates this increased protection possibly by competition for the same

processing pathways or, if the heterologous protein is being expressed in an insoluble fraction or inclusion bodies, by interfering with the normal protein synthesis mechanisms of strain RB51. We have observed that *Brucella* SOD is found in culture supernatants only of strains that over-express SOD. It is possible that the increased protection elicited by RB51SOD is due to this "leakage" of SOD and that co-expression of 85A may limit this process.

The expression of foreign antigens did not affect the attenuation level of the strains, since all vaccine strains were cleared from mice by 6 weeks post vaccination, the same a mount of time required for clearance of vaccine strain RB51 (417).

In conclusion, these experiments suggest that vaccine strain RB51 is able to express mycobacterial antigens, without significantly impairing its ability to stimulate strong *Brucella* specific cell mediated immunity and its protective efficacy against *Brucella* challenge. However, the lack of consistent protection against mycobacterial challenge induced by the recombinant strains expressing MAP antigens indicates that a major increase in protection levels will be necessary in order to advocate these approaches as practical.

CHAPTER 4

Expanding the Versatility of *B. abortus* Vaccine Strain RB51 and Recombinants thereof

Introduction

Brucellosis is a zoonotic disease that affects animals and humans worldwide. Among humans the disease has a strong professional clustering (veterinarians, farm, and slaughterhouse workers) (260). *Brucella spp.* are also among the organisms classified as potential biological weapons (325). However, there are no approved human brucellosis vaccines, and most of the effective *Brucella* vaccines available to protect animals, such as Strain 19 or Rev1, are considered too virulent to be used in humans (45).

Control of brucellosis in domestic animals has been shown to be an important tool to reduce transmission of the disease to human population (248, 334). Calf hood vaccination of susceptible animals has been shown to be an effective approach for increasing resistance among the susceptible population (69, 295, 296). *B. abortus* vaccine strain RB51 is an attenuated natural mutant derived from the virulent parent strain *B. abortus* 2308, and has been shown to provide protection against challenge with virulent *Brucella* in mice and cattle (106, 109, 307, 351, 418). This vaccine is the official vaccine strain for the bovine brucellosis control and eradication programs in the USA, Mexico, and Chile (231, 319, 324). In its current state, it is used to immunize heifers before first mating in order to provide protection against infection and abortion during pregnancy. Clearly, the pregnant animal is the most susceptible to *Brucella* infection and to its consequences. Vaccination with the attenuated *B. abortus* strain RB51 has proven to be safe and effective for most cattle target groups (303), and several studies have determined that vaccination of pregnant heifers with vaccine strain RB51 is safe (106, 304, 337).

Unfortunately, vaccination of pregnant cattle or bison with the full dose of the vaccine can lead to colonization of the placenta that can occasionally result in abortion with fetal colonization by strain RB51 (303, 305, 406), or shedding of the vaccine organisms through milk (404). The mechanism by which strain RB51 is able to colonize the fetus or placenta is not understood. It has been shown that the strain is unable to replicate inside endoplasmic reticulum of infected cells and is also unable to prevent phagolysosome fusion the way virulent strain 2308 does (16). Vaccination of adult animals has proven to be effective (294), but the current recommendation is to avoid vaccination during pregnancy. If vaccination of pregnant animals is necessary, some control programs recommend that it be carried out with a reduced dose of the vaccine (16).

Recently, a report of adverse reactions to accidental inoculation with vaccine strain RB51 in humans has been published (19). However, the many cases of accidental human exposure and the scarcity of information about adverse reactions to this vaccine suggest that the strain has low virulence in humans (1).

The development of a *Brucella* vaccine with low virulence and with high protective efficacy has been the long-standing goal of *Brucella* vaccine researchers. Such vaccines could also be used to immunize animal groups where vaccination with the regular vaccines through traditional routes is not possible. These vaccines could potentially be used for human vaccination especially in high risk groups such as veterinarians and army personnel.

Killed or subunit *Brucella* vaccines are not virulent but, as reviewed in the introduction, are not effective in eliciting a protective response (253, 352). Viable organisms are able to replicate and to re-direct trafficking into specialized cellular compartments and this differential localization of the organism seems to be important for the establishment of an adequate immune response. In this sense, it is known that *Brucella* prevents phagolysosome fusion and replicates in a compartment that resembles the endoplasmic reticulum (ER) (140, 313). This location may explain *Brucella's* efficient capacity to stimulate CD8+ cytotoxic lymphocytes (170). These CD8+ cells recognize antigens presented in an MHC-I context, a pathway elicited by endogenous

antigens that are processed in the ER (162). Exogenous molecules that have been degraded by lysosomal enzymes are presented to CD4+ T helper cells in the context of MHC-II molecules. Although these pathways are well compartmentalized, exogenous molecules can and are presented in an MHC-1 context to CD8+ cells (208, 431). Both CD4+ and CD8+ cells are triggered by *Brucella* infection or vaccination with viable vaccines and both are believed to be important for the control and elimination of the infection (14, 24, 142, 170, 291).

One of the methods of decontamination traditionally used in the food industry is gamma radiation (355). The ionizing radiation from gamma rays affects the genetic material of eukaryotic and prokaryotic cells. The DNA damage induced by radiation can be classified into several types: physical double strand breaks due to the effect of the ionizing radiation with free radicals that lead to deletions and translocations in the chromosomal strand; single strand breaks, and base pair substitutions due to the conversion of pyrimidines into uracil. The damage is dose dependent and generally non-reversible (247).

Irradiation of vaccines has been carried out in the past to vaccinate against fungal, parasitic, and bacterial diseases with various degrees of success. Vaccination of mice with an irradiated arthrospore vaccine against coccidiomycosis reduced the pathologic lesions in mice challenged with *Coccidioides immitis* (316). A study evaluating protection afforded by an irradiated *Salmonella* vaccine indicated significantly increased survival rates in animals vaccinated with irradiated vaccine compared to non-vaccinated controls (99). Another study using *Burholderia mallei* showed no differences in proliferative and cytokine responses between vaccination with irradiated and non-irradiated organisms. The cytokine profile elicited by the irradiated vaccines resembled a Th1 phenotype, mixed IgG1 and IgG2a immunoglobulin isotypes were induced by the irradiated vaccine, but no protection against *B. mallei* challenge was obtained (10). Inoculation of mice with irradiated *Toxoplasma gondii* tachyzoites has revealed the elicitation of IFN-γ responses in vaccinated mice similar those elicited by the live organism, and that adequate serological responses are mounted by vaccinated animals

indicating that irradiation of microorganisms may be an adequate means for reducing or eliminating virulence while maintaining the immunological properties of a live vaccine (173).

With the aim of generating a *Brucella* vaccine that is effective but completely attenuated by abrogating its replication capacity, we irradiated different recombinant RB51 based vaccine strains. We hypothesize that irradiated, non-replicative organisms are still able to confer protection against challenge with virulent strains.

Another frequently encountered problem when vaccinating animals in endemic areas with high prevalence of the disease, is the possibility that the animals may already be infected by the time of vaccination. This is a particularly important concern when oral route mass vaccination of wildlife is considered. The impact of an ongoing *Brucella* infection on the effectiveness of the vaccine in mice has not been studied. Anecdotal evidence by field veterinarians suggests that emergency vaccination of cattle in a farm presenting severe abortion rates tends to decrease the number of abortions, but no evidence has been published to support this claim. Some vaccination studies using strain RB51 have been carried out in cattle populations with high and low prevalence of infection demonstrating that vaccination with strain RB51 constitutes an effective tool for decreasing infection rates, seroconversion and abortion in susceptible animals, but to our knowledge, no studies have been performed with already infected animals (228).

Cheers and Pagram (67) reported in 1979 that mice infected with Strain 19 and super infected with the same strain were able to control the infection, and the number of colonizing organisms were decreased by up to three fold, until an equilibrium was reached. This is an interesting finding but hard to analyze since in that experiment it was not possible to distinguish between primary and secondary infection. Interestingly, the macrophages of *Brucella* infected mice are activated, and in one study tit was shown that they are able to non-specifically protect against infection with *Listeria* (43, 67).

Vaccination post exposure to an infectious disease has been used as a therapeutic tool in a variety of viral and bacterial diseases. Post exposure human vaccination against

rabies using vaccine strain Fuenzalida-Palacios is still used in many parts of the world (50). The potential "curative" effect of vaccination has also been demonstrated in mice infected with *M. tuberculosis* when they have been vaccinated post infection with a DNA vaccine expressing MTB Hsp65 antigen (229). In those animals a significant decrease in the load of *M. tuberculosis* organisms recovered from the lungs after vaccination compared to the saline controls was observed. Additionally, as mentioned in the previous chapter, vaccination against paratuberculosis seems to rely in this principle. In a study analyzing lambs infected with *M. avium susbsp. paratuberculosis,* vaccination with a live attenuated vaccine reduced the mycobacterial burden and the severity of the lesions in comparison to non vaccinated controls (159).

To evaluate whether a pre-existing *Brucella* infection can be treated by vaccination with *B. abortus* strain RB51, we vaccinated mice that were acute or chronically infected with virulent strain *B. abortus* 2308 with strain RB51 and evaluated the clearance of both, the virulent as well as the vaccine organisms.

Hypothesis

- Irradiated, non-replicative strain RB51 based vaccines are able to protect BALB/c mice against challenge with virulent *Brucella*.
- Vaccination with strain RB51 decreases the infection load in animals already infected with virulent *Brucella*.

Objectives

1) Evaluate the effect of gamma irradiation on the immunological and protective properties of *B. abortus* strain RB51 based vaccines.

2) Evaluate whether vaccination of *Brucella* infected mice with strain RB51 based vaccines exerts a therapeutic effect.

Material and Methods

Mice and bacterial strains: female, 6-8 week old BALB/c mice were purchased from Charles Rivers Laboratories (Wilmington, VA). The animals were housed in micro isolation cages under BSL-3 containment in groups of up to 5 mice/cage. All animal handling experiments were carried out under IACUC and university guidelines with supervision by the university veterinarian.

Vaccine strains RB51, RB51SOD and RB51SODwboA are part of our culture collection and were described in detail in Chapter 1. Virulent *B. abortus* strain 2308, a smooth virulent strain that is widely used in *Brucella* challenge models (15, 428), is also part of our culture collection.

For serological analysis and protection studies, mice were vaccinated i.p with 2-4x10^8 cfu of *B. abortus* RB51 based vaccine strains. Mice were bled by retro-orbital venipuncture under local anesthesia 5 weeks post vaccination. Unless otherwise indicated, mice were challenged i.p six weeks post vaccination with 2-4 x10^4 cfus of *B. abortus* 2308.

Irradiation of *Brucella* organisms: A 1 ml deep frozen -80°C /thawed vial of vaccine strain RB51 containing approximately 2 x10^{11} organisms/ml was aliquoted into 5 microcentrifuge tubes. The tubes were irradiated in a Co source gamma irradiator (JL Shepperd and Assoc, San Fernando CA) with 198000 rads, 297000 rads or 396000 rads (30, 45, 60 minutes in the irradiation chamber respectively). After irradiation, the contents of the vials were serially diluted, plated onto TSA plates and incubated at 37ºC +5% CO_2 for up to 8 days to determine viability.

Metabolic activity of irradiated organisms: Metabolic activity was determined using the Alamar Blue Reduction Assay (5) described in Chapter 3. Briefly, 100 µl aliquots from each of the above irradiated strain RB51 cultures, containing approx 1 x10^9 cfus were dispensed in triplicate onto Falcon 96 well flat bottom tissue culture plates (BD

Labware, NJ). Aliquots of non irradiated and heat killed strain RB51 were used as positive and negative controls respectively. The plate was incubated at 37ºC + 5% CO_2 for 30 minutes and then 10 µl of Alamar Blue reagent (Biosource Int., Camarillo CA) were added to each well. The plate was further incubated for one hour and the fluorometric reading was determined using a CytoFluor II fluorescence multiwell plate reader (Perspective Biosystem) set to Excitation: 530/30, Emision:590/30 and gain: 35. The results were adjusted with a blank of media, and are presented as the average of triplicate wells.

Determination of duration of metabolic activity: A 500 µl aliquot of vaccine strain RB51 was irradiated with 396,000 rads (60 minutes) and stored at 4ºC. Alamar Blue reduction assay was performed as described above at time points 0 h, 18 h, 36 h, 96 h and 14 days post irradiation. Live non-irradiated strain RB51 and heat killed strain RB51 also stored at 4ºC were used as positive and negative controls respectively.

Serological responses of mice vaccinated with irradiated and non-irradiated *B. abortus* vaccine strain RB51 and recombinants thereof: Antibody titres to *Brucella* antigens were determined by ELISA. Briefly, 96 well NUNC Maxisorp plates were coated overnight at 4ºC with 0.5 µg/well of whole cell strain RB51 antigen, purified recombinant *Brucella* Cu/ZnSOD protein or *Yersinia enterocolitica* O:9 LPS in coating buffer (carbonate pH 9.6). The plates were then blocked using 2% BSA (Sigma, St. Louis, MO) in TBS at room temperature for 1 hour and the serum samples were added in duplicate at a 1:1000 dilution and incubated at room temperature for 3 hours. After washing 5 times (TBS/Tween-20) the secondary HRPO conjugated antibodies (anti-mouse whole IgG, anti mouse IgG1, anti-mouse IgG2a, or anti-mouse IgG3) (Cappel-ICN, Irvine, CA) were added at appropriate concentrations and the plate was incubated at room temperature for 1 hour followed by another washing step. One hundred µl/well were added of TMB substrate and the plates were incubated in the dark for 30 minutes followed by the addition of 100 µl/well of 10 mM sulphuric acid. Optical density

readings were obtained using a Titertek ELISA plate reader (Molecular Devices, Sunnyvale, CA) set at 450 nm and readings were adjusted with a blank of diluent.

Protection induced by irradiated and non-irradiated *B. abortus* vaccine strain RB51 and recombinants thereof: Seven groups of 5 female, 6-8 week old BALB/c mice each, were vaccinated i.p with 2-4 $x10^8$ cfu of *B. abortus* vaccine strains RB51, RB51SOD, RB51SODwboA, irradiated strain RB51, irradiated strain RB51SOD, irradiated strain RB51SODwboA and saline control. The irradiated vaccines were obtained by dividing the vaccine aliquots into two portions. One half was used to inoculate mice with the live vaccine and the other half was irradiated with a 396,000 rad dose (60 minutes). The vaccination dose was retrospectively corroborated by serially plating of the non-irradiated aliquot onto TSA or TSA/Cm plates and incubation at 37ºC +5% CO_2. Inactivation and metabolic activity of the irradiated vaccines was determined by plating onto TSA plates and using the Alamar Blue reduction assay. Six weeks post vaccination all mice were challenged i.p with 2-4 $x10^4$ cfu of virulent *B. abortus* strain 2308. Two weeks post challenge, the mice were sacrificed by CO_2 inhalation and their spleens were removed for splenic clearance determination by serial dilution and plating onto TSA plates for cfu determination.

Homologous (anti-RB51) protection by vaccine strain RB51: Groups of 10 mice each were vaccinated as above with 2-4 $x10^8$ cfus of *B. abortus* strain RB51 or saline control. Six weeks later all animals were challenged i.p with the same dose of strain RB51. Five or 10 days post challenge 5 mice/group were sacrificed and their spleens were plated for cfu determination.

Therapy studies: Groups of 5 mice each were infected i.p with 2-4 10^4 cfu of *B. abortus* 2308, 2 or 5 weeks post infection (acute or chronic infection respectively) the mice were vaccinated i.p with $1x10^7$ or 2-4 $x10^8$ cfu of vaccine strains RB51, RB51SOD, RB51SODWboA, or saline control. Two weeks post vaccination the animals were

sacrificed and splenic clearance was determined by plating spleen dilutions onto TSA and TSA/Rifampin (TSA/Rif) plates. Only strain RB51 organisms are able to replicate in the presence of the antibiotic rifampicin (351), allowing the differentiation between the virulent *B. abortus* strain 2308 and the vaccine strain. The results were calculated as follows:

splenic strain 2308 cfus = (#cfu on TSA plates) –(# cfu on TSA/Rif plates)

The phenotype of the colonies was confirmed by Crystal Violet staining of individual colonies using the standard method (233).

Statistical analysis: For comparisons between vaccine and saline groups statistical analysis were performed by Analysis of Variance and Dunnet's test at a confidence level of 0.05. In the irradiated vaccine experiment, comparisons between irradiated and non irradiated vaccines and also to saline were carried out using the Fisher LSD test. Statistical analysis was performed using SAS statistical software.

Results

Effect of gamma irradiation on *B. abortus* RB51

A dose dependent reduction in the number of viable *Brucella* recovered after 30, and 45 minutes of irradiation was observed. No organisms were recovered after irradiation for 60 minutes (equivalent to 396,000 rads) Figure 4.1, and for 90 minutes (594,000 rads; data not shown).

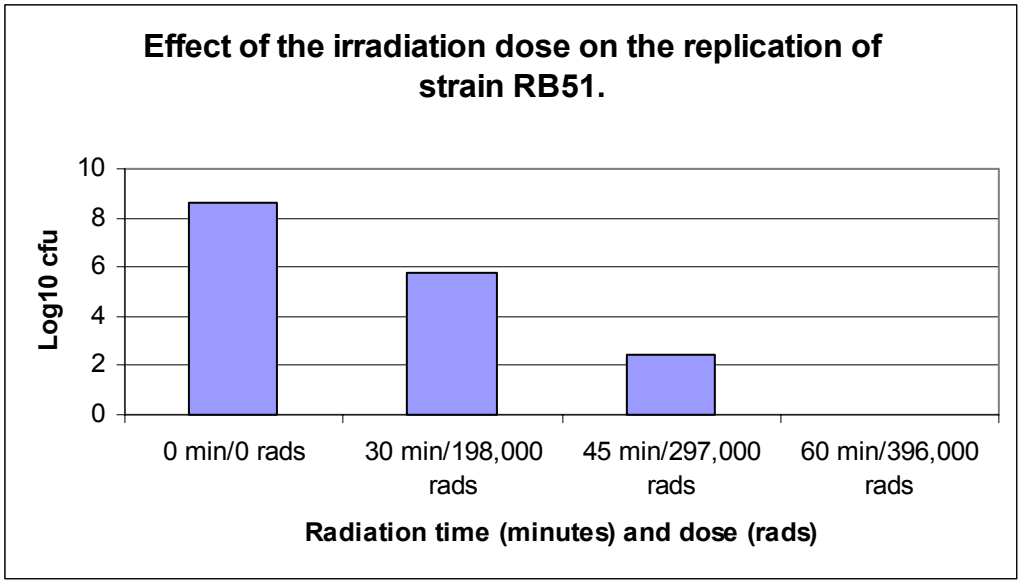

Figure 4.1: Effect of irradiation dose on the replication of strain RB51.

One ml aliquots containing 5 x10^8 cfu RB51 were irradiated with different radiation doses and subsequently plated for cfu determination. The columns represent the Log10 cfus. The X axis shows the irradiation times and dose.

Effect of irradiation on the metabolic activity of *B. abortus* RB51

B. abortus strain RB51 organisms (5 x10^8 cfu) were irradiated with 198,000; 297,000; or 396,000 rads. The metabolic activity of the irradiated strain was analyzed using the Alamar Blue Reduction assay. Non-irradiated and heat killed *B. abortus* strain RB51 were used as positive and negative controls respectively. As shown in Figure 4.2, the irradiated organisms exhibit metabolical activity rates comparable to that of the non irradiated controls, as represented by the reduction of the Alamar Blue dye. On the other hand, heat killed strain RB51 showed activity comparable to the activity of media alone. These results indicate that the abrogation of replication observed in aliquots irradiated with 396,000 rads was not due to the death of the irradiated vaccine. This is an important consideration since in general, live but not killed vaccines are able to induce protection against *Brucella* challenge (253).

The metabolic activity of strain RB51 irradiated for up to 60 minutes was maintained at levels comparable to those of the non-irradiated controls when stored at 4ºC for up to 96 hours post irradiation, and was still significantly higher than the activity of the heat killed negative control 2 weeks post irradiation (data not shown).

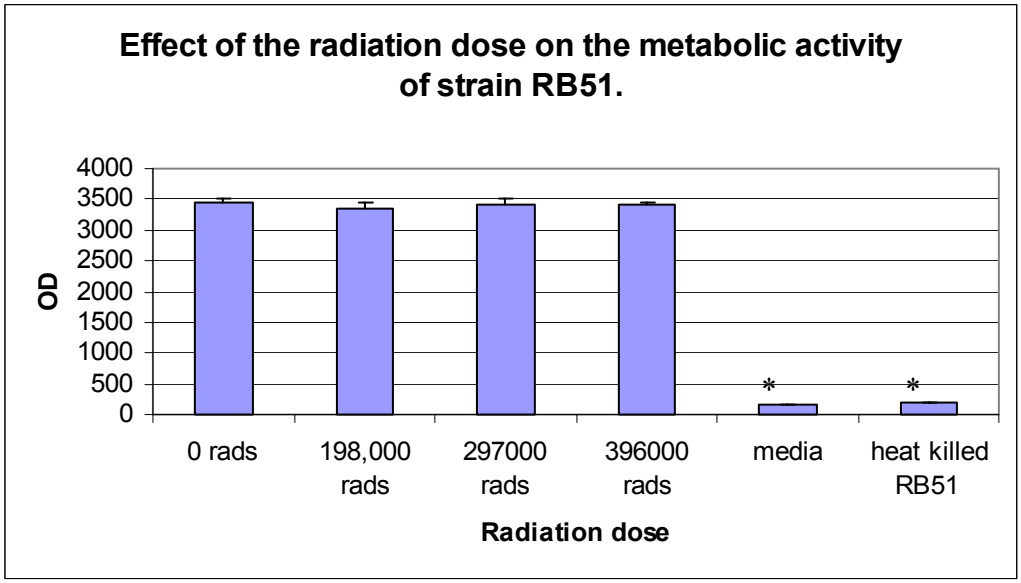

Figure 4.2: Effect of the radiation dose on the metabolic activity of strain RB51.

5 x10^8 cfu of strain RB51 were irradiated with 198,000; 297,000 or 396,000 rads (30 min, 45 min and 60 min respectively) The metabolic activity was determined by the Alamar Blue reduction assay. A non irradiated aliquot (0 rads) was used as a positive control. Heat killed strain RB51 and media alone were used as negative controls. Results are the average fluorescence and the error bars are standard deviations (*$p<0.05$).

Serological responses of mice vaccinated with irradiated *B. abortus* RB51 based vaccine strains

To evaluate the production of antibodies to the O-side chain of *Brucella*, sera from vaccinated mice was analyzed in ELISA using *Y. enterocolitica* 0:9 LPS which cross reacts with *Brucella* O-side chain (197).

Vaccination with irradiated and non irradiated strains of RB51 did not significantly affect antibody production. As expected, only BALB/c mice vaccinated with 2-4 x10^8 cfu of irradiated or non irradiated *B. abortus* strain RB51 vaccine strains expressing the *wboA* gene developed O-chain antibodies. Isotype analysis showed IgG2a subclass antibodies while IgG1 antibody production was below our detection limit. No O-chain antibodies were detected in the other vaccine groups, indicating that irradiation does not alter the rough phenotype of strain RB51. No differences in the levels of whole IgG antibodies were detected between irradiated and non irradiated vaccines, but IgG2a levels were lower in the non irradiated RB51SODwboA group. Figure 4.3 and Figure 4.4. These antibody levels declined over time and by 4 months post vaccination were not detectable (data not shown).

Production of anti-*Brucella* Cu/ZnSOD antibodies was also measured by ELISA using purified recombinant *B. abortus* Cu/ZnSOD (Figure 4.5).

Mice vaccinated with strains RB51SOD and RB51SODwboA developed SOD antibodies that were significantly higher than in the saline control group. Irradiation of the vaccine did not have a significant effect on anti-SOD antibodies in mice vaccinated with strain RB51SODwboA but a significant decrease in the level of SOD antibodies in mice vaccinated with irradiated strain RB51SOD were observed. To determine whether the lack of SOD antibodies elicited by strain RB51SOD vaccination was due to a decreased expression of SOD in the irradiated strain, whole cell antigens prepared from the irradiated and non irradiated RB51SOD strains were analyzed by western blot to detect expression of SOD. Over-expression of the SOD protein was detected in both irradiated and non irradiated RB51SOD strains when compared to the whole cell strain

RB51 antigen (data not shown). It has been observed that over-expression of *Brucella* Cu/Zn SOD in strain RB51 leads to its "leakage" into the extracelluar environment. This phenomenon is thought to be important in the increased protection elicited by this strain. TCA precipitation of culture broth from a sample inoculated with irradiated strain RB51SOD, and incubated for 48 hours also revealed the presence of SOD (Data not shown).

Antibody responses to whole cell strain RB51 antigen was evaluated by ELISA (Figure 4.7 and Figure 4.8). The results indicate that all vaccine groups, including irradiated RB51SOD developed high levels of antibodies to strain RB51. Mice vaccinated with irradiated and non irradiated vaccines developed similar, high antibody levels to strain RB51 that were predominantly of the IgG2a isotype. IgG1 levels were very low and not significantly different than the saline control.

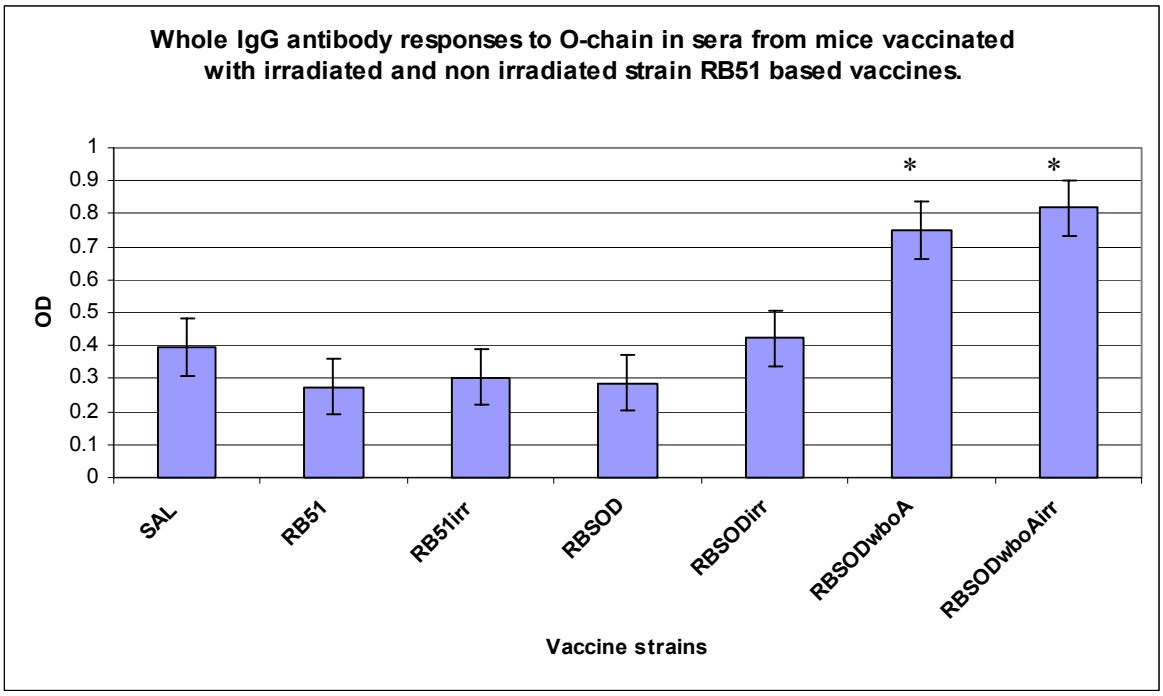

Figure 4.3: Whole IgG antibody responses to O-chain in sera from mice vaccinated with irradiated and non irradiated strain RB51 based vaccines.

Columns represent the average OD values of 3 mice/group, five weeks post vaccination with irradiated and non irradiated strain RB51 based vaccines. Error bars are the standard deviations (*$p<0.05$).

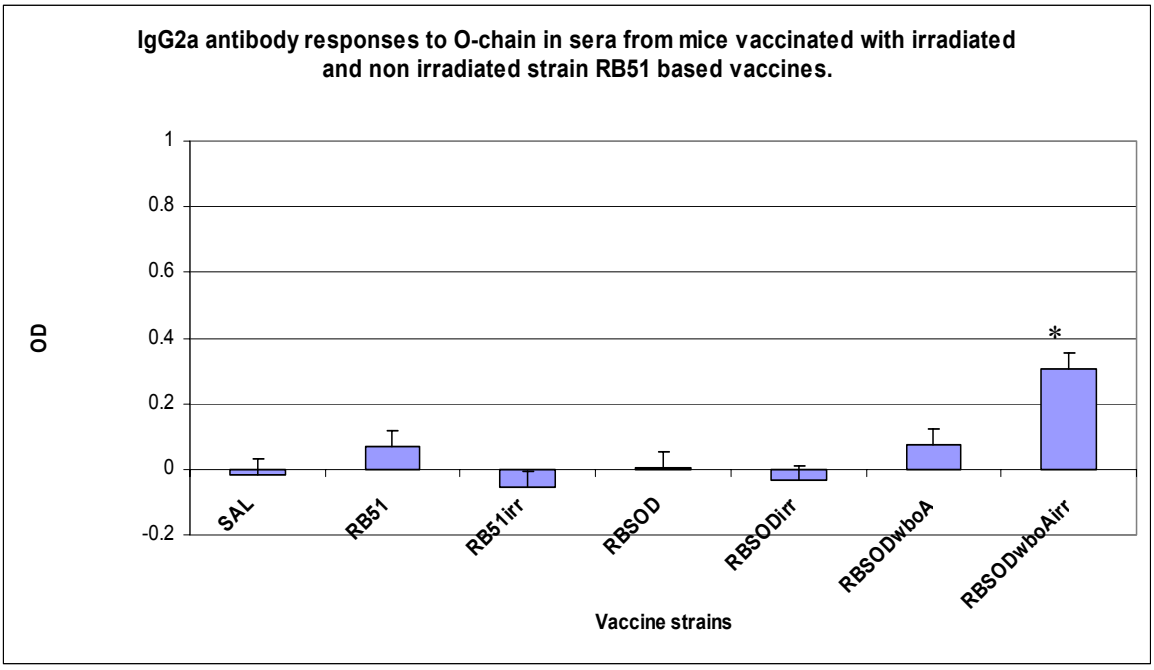

Figure 4.4: IgG2a antibody responses to O-chain in sera from mice vaccinated with irradiated and non irradiated strain RB51 based vaccines.

Columns represent the average OD values of 3 mice/group, five weeks post vaccination with irradiated and non irradiated strain RB51 based vaccines. Error bars are the standard deviations (*$p<0.05$).

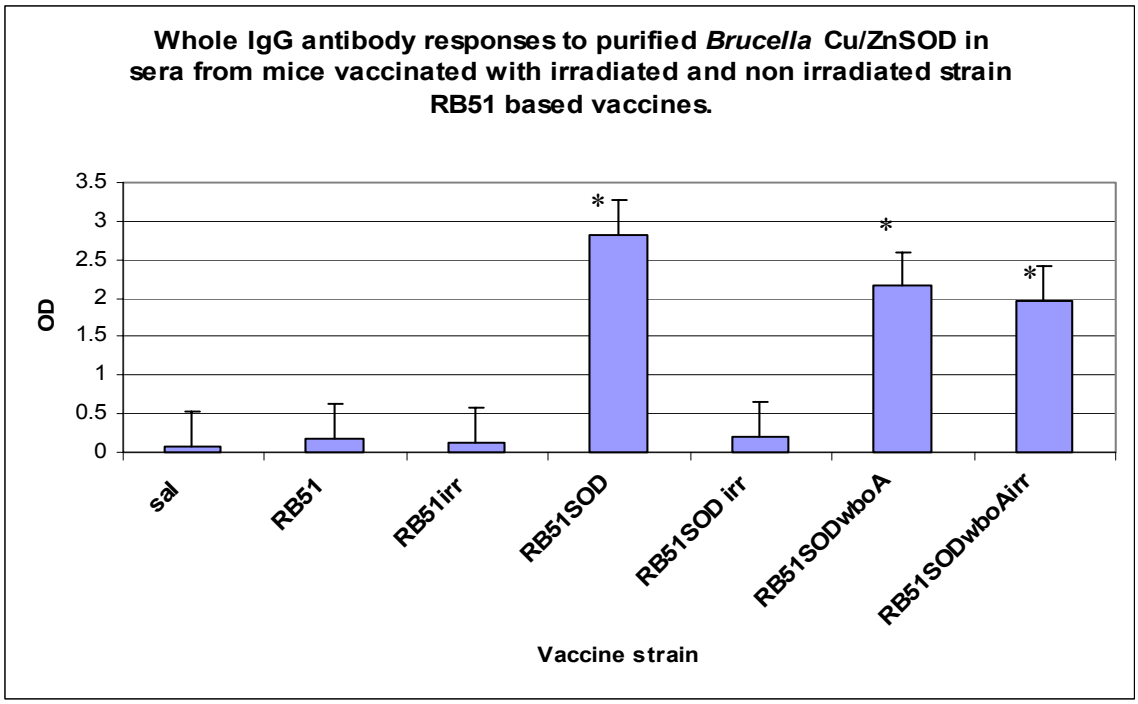

Figure 4.5: Whole IgG antibody responses to purified *Brucella* Cu/ZnSOD in sera from mice vaccinated with irradiated and non irradiated strain RB51 based vaccines.

Columns represent the average OD values of 3 mice/group, five weeks post vaccination with irradiated and non irradiated strain RB51 based vaccines. Error bars are the standard deviations (*$p<0.05$).

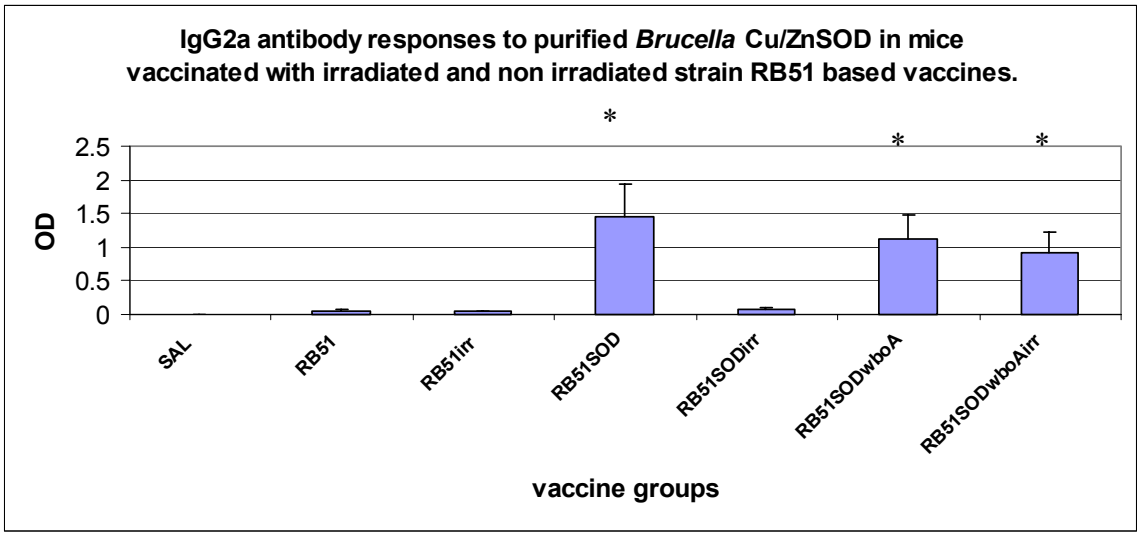

Figure 4.6: IgG2a antibody responses to purified *Brucella* Cu/ZnSOD in mice vaccinated with irradiated and non irradiated strain RB51 based vaccines.

Columns represent the average OD values of 3 mice/group, five weeks post vaccination with irradiated and non irradiated strain RB51 based vaccines. Error bars are the standard deviations (*$p<0.05$).

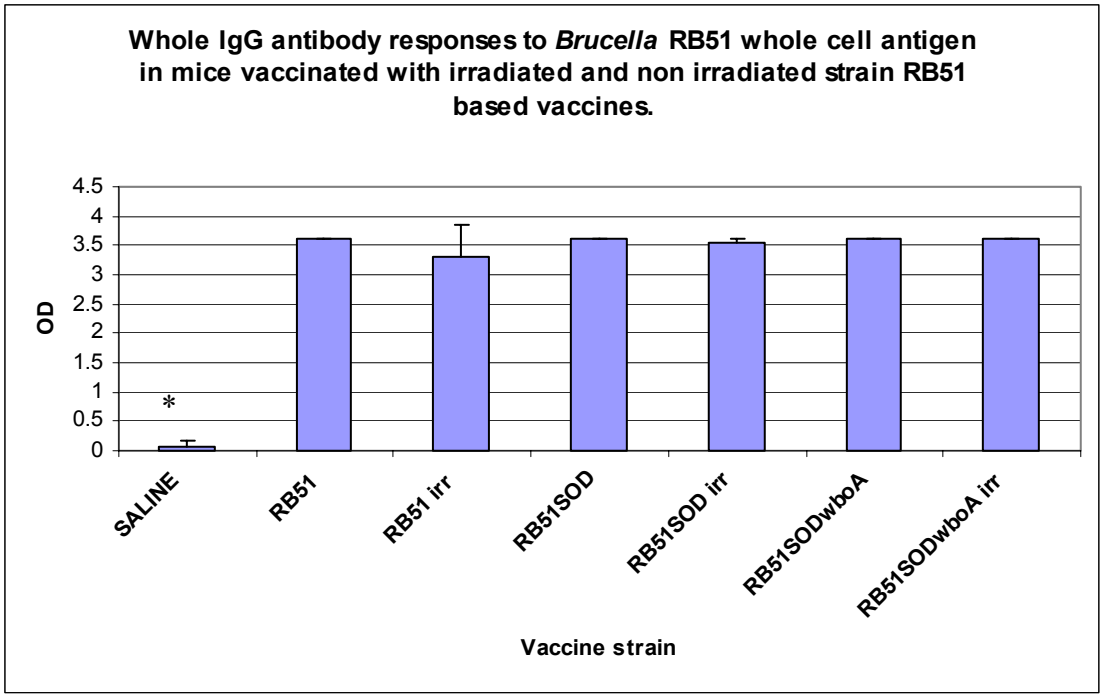

Figure 4.7: Whole IgG antibody responses to *Brucella* RB51 whole cell antigen in sera from mice vaccinated with irradiated and non irradiated strain RB51 based vaccines.

Columns represent the average OD values of 3 mice/group, five weeks post vaccination with irradiated and non irradiated strain RB51 based vaccines. Error bars are the standard deviations (*$p<0.05$).

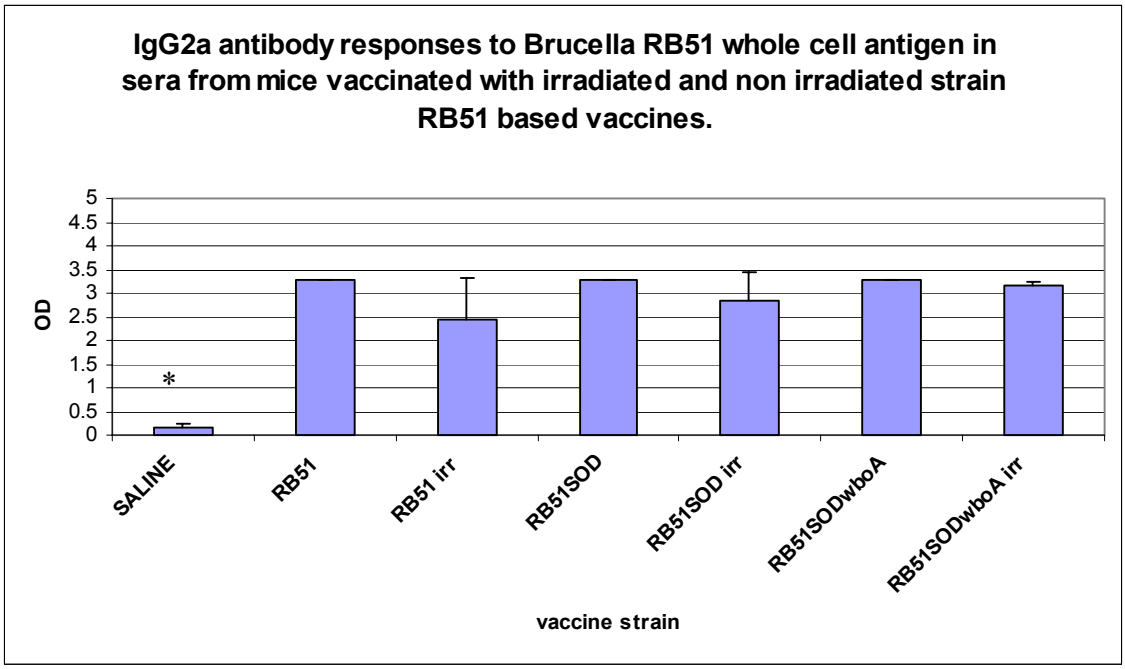

Figure 4.8: IgG2a antibody responses to *Brucella* RB51 whole cell antigen in sera from mice vaccinated with irradiated and non irradiated strain RB51 based vaccines.

Columns represent the average OD values of 3 mice/group, five weeks post vaccination with irradiated and non irradiated strain RB51 based vaccines. Error bars are the standard deviations (*$p<0.05$).

Protection by irradiated *B. abortus* strain RB51 based vaccines

Vaccination of BALB/c mice with 2-4 x10^8 cfu of irradiated (396,000 rads) or non irradiated RB51, RB51SOD and RB51SODwboA induced significant protection against challenge with virulent *B. abortus* strain 2308 when compared to saline control group. Irradiation of the vaccine strains did not significantly affect their protective ability compared to the non-irradiated counterpart. Irradiated and non-irradiated strains RB51SODwboA provided increased protection against challenge. No colonies were recovered from the spleens of 3/5 (mean cfu group: 0.8) and 2/5 (mean cfu group:4.4) mice vaccinated with non-irradiated and irradiated RB51SODwboA respectively. Only strain RB51SOD lost its protective ability after irradiation (Figure 4.9).

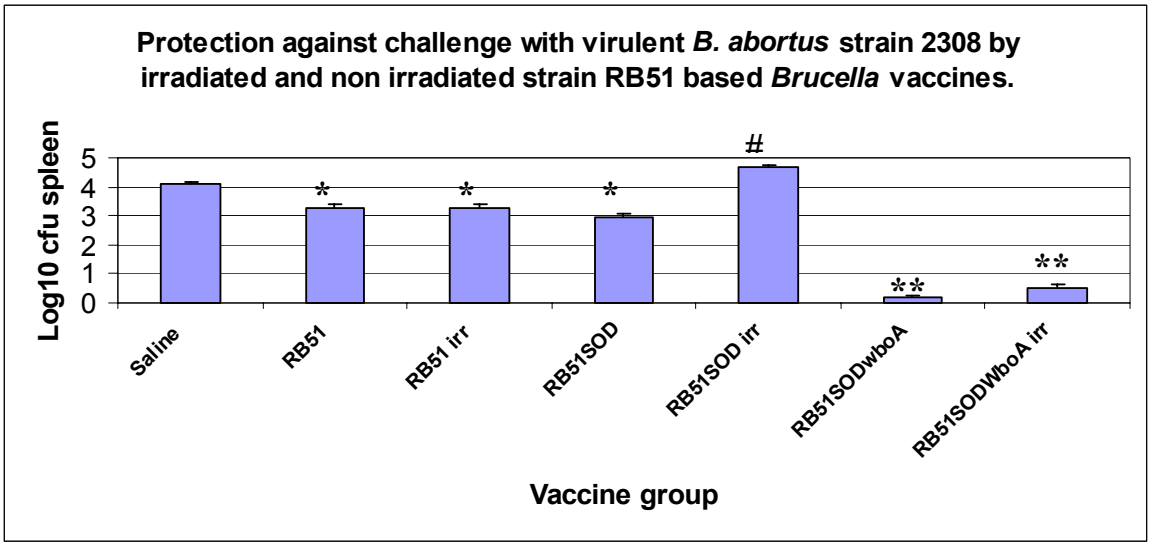

Figure 4.9: Protection against challenge with virulent *B. abortus* strain 2308 by irradiated and non irradiated strain RB51 based *Brucella* vaccines.

The columns represent the mean of Log10 cfu/spleen, the bars represent the SEM. Comparisons were performed between vaccine groups and saline and also between each pair of vaccines. (*$p<0.05$ and **$p<0.001$ compared to saline control), (# $p>0.05$ compared to saline and $p<0.05$ compared to RB51SOD).

Homologous protection by *B. abortus* vaccine strain RB51

In a pilot experiment designed to determine the appropriate dose of strain RB51 vaccine required for boosting strain RB51 vaccinated mice, groups of five BALB/c female mice were vaccinated i.p with 2-4 $x10^8$ cfu of RB51 strain (full dose). Six weeks post vaccination the animals were boosted with the same dose of strain RB51 and splenic cfu were determined 5 and 10 days later as described above (Figure 4.10).

Vaccination with *B. abortus* strain RB51 conferred significant protection against homologous challenge, with all vaccine organisms being cleared from the spleens by 10 days. Unexpectedly, 4/10 mice from the group that was vaccinated and challenged with strain RB51 died within 48 hours of boosting. No animals died in the saline/ one dose of vaccine strain RB51 group. Because of these results, subsequent studies that required boosting with strain RB51 vaccines were performed with a reduced dose of 2-4 x 10^7 cfu, which did not result in mortality.

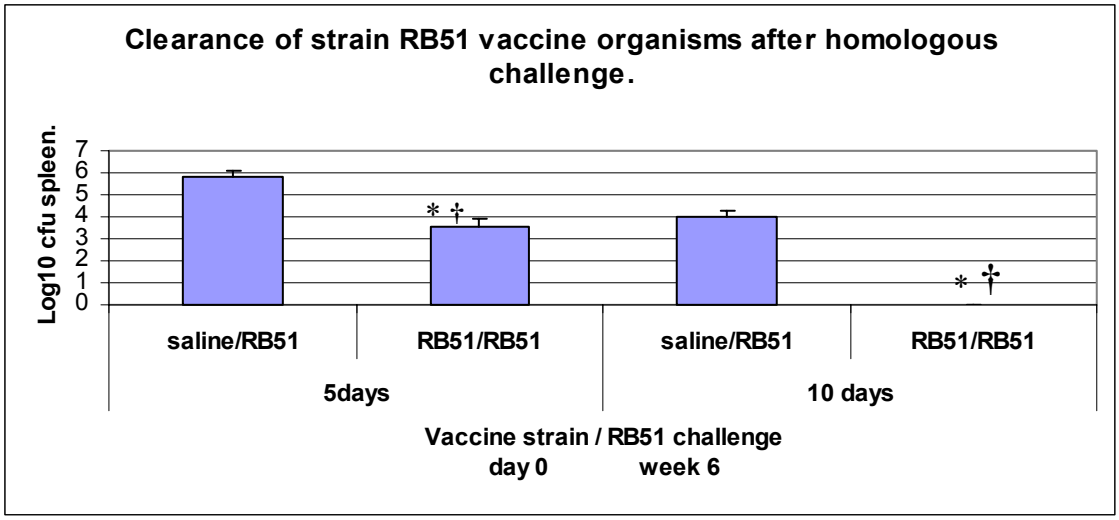

Figure 4.10: Clearance of strain RB51 vaccine organisms after homologous challenge.

Mice were vaccinated with 2-4 x10^8 cfus of vaccine strain RB51 or saline and 6 weeks post vaccination all animals were re-vaccinated (challenged) with 2-4 x10^8 cfus of the vaccine. Clearance of the vaccine was determined by splenic cfu determinations 5 and 10 days post the second dose. Results are expressed as the average Log10 cfu per group. The error bars are the standard deviations (*= $p<0.05$ compared to the respective saline control groups; †= animal deaths).

Therapeutic effect of vaccination with strain RB51

With the aim of determining a potential curative effect of strain RB51 vaccination on mice already infected with a virulent strain of *Brucella*, mice infected with *B. abortus* strain 2308 for 2 weeks (acute) or 5 weeks (chronic) were vaccinated i.p with strain RB51. Mice were killed 14 days post vaccination and their spleens were plated for cfu determination. Because of the results obtained in the booster experiment described above, vaccination with a reduced dose of strain RB51 (2-4 $x10^7$ cfu) was chosen initially.

The number of strain RB51 organisms recovered following vaccination of mice acutely infected with *B. abortus* 2308 was very low as determined by growth on rifampin plates and crystal violet staining. Although a reduction in the number of virulent *B. abortus* 2308 organisms recovered from the spleens of those mice was observed, these results were not significant when compared to the cfu in spleens from the non vaccinated control.

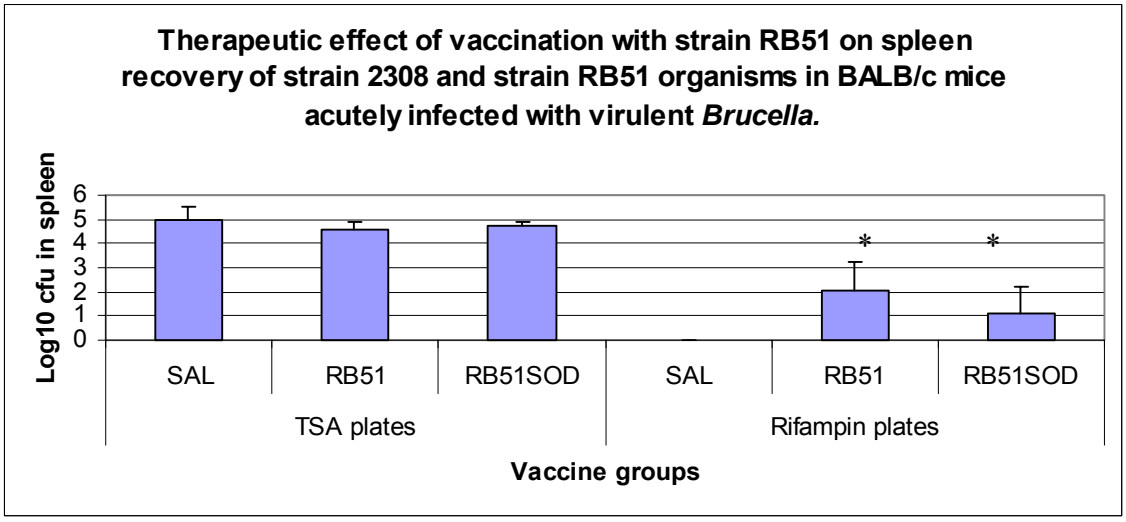

Figure 4.11: Therapeutic effect of vaccination with strain RB51 on spleen recovery of strain 2308 and strain RB51 organisms in BALB/c mice acutely infected with virulent *Brucella*.

Mice acutely infected with virulent *B. abortus* strain 2308 were vaccinated with a reduced dose of strain RB51 ($1x10^7$ cfus). Splenic clearance of the virulent and vaccine strains was determined two weeks post vaccination. The X axis shows the vaccine strains used a well as the media used for plating. Results are expressed as the average Log10 cfu per group (n=5). The error bars are the standard deviations (*$p<0.05$, compared to the same groups grown on TSA plates).

In order to determine whether protection could be increased by vaccination with the full dose of the vaccine, a pilot experiment was performed to determine the effect of vaccinating with a full dose (2-4 $x10^8$ cfu) of strain RB51 on mice previously infected with virulent *B. abortus* strain 2308. Two groups of 2 mice each were infected i.p with 2-$4x10^4$ cfu of *B. abortus* 2308. Two or 4 weeks post infection the animals were vaccinated i.p with a full dose of strain RB51 and the animals were monitored for signs of disease. No animals died or showed signs of sickness. Therefore vaccination of BALB/c mice infected with strain 2308 with full dose of strain RB51 at 2 or 4 weeks post strain 2308 infection does not induce the adverse effects observed when revaccinating strain RB51 vaccinated mice (see Figure 4.10).

In light of these results, and in order to determine whether a curative effect could be achieved by increasing the vaccination dose, the therapeutic experiment was repeated this time using vaccination with a full dose of strain RB51.

Vaccination of acutely infected mice (2 weeks post infection), with the full dose of strain RB51 vaccine did not induce clinical signs of disease. Similar to what was observed in the previous experiment, a dramatic decrease in the number of strain RB51 organisms recovered from the spleens of vaccinated animals was observed (data not show), but the number of strain 2308 organisms showed only a slight not significant decrease (Figure 4.12).

Vaccination of chronically infected mice with strain RB51 did not induce adverse effect on the animals. No *B. abortus* strain RB51 organisms were recovered from the spleens of these mice. A slight decrease in the number of virulent strain 2308 organisms was observed in the group vaccinated with strain RB51SOD but this result was not significant when compared to the number of strain 2308 organisms recovered from the saline control group (Figure 4.13). This data suggests that chronic infection with strain 2308 induces a sterile immunity against vaccine strain RB51 re-infection that does not seem to affect the original *B. abortus* 2308 infection level.

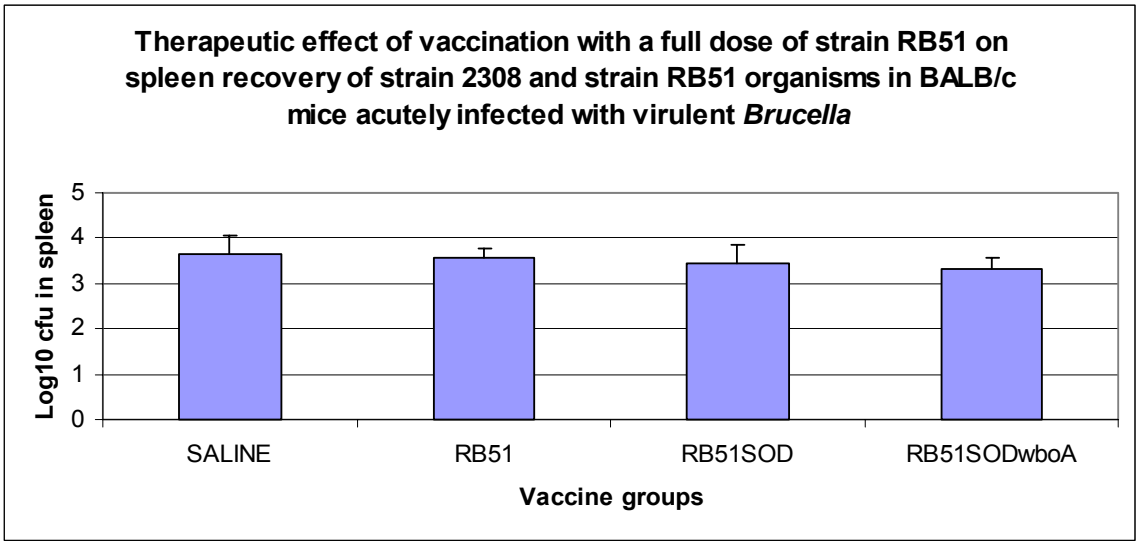

Figure 4.12: Therapeutic effect of vaccination with a full dose of strain RB51 on spleen recovery of strain 2308 and strain RB51 organisms in BALB/c mice acutely infected with virulent *Brucella*.

Mice acutely infected with virulent *B. abortus* 2308 were vaccinated with the full dose of strain RB51 (2-4 x10^8 cfus). Splenic clearance of the virulent organisms was determined two weeks post vaccination by plating onto TSA and TSA/Rif media and subtracting the growth in TSA/Rif plates from the total growth in TSA plates, as detailed in methods. Results are expressed as the average *B. abortus* 2308 Log10 cfu per group. The error bars are the standard deviations (*$p<0.05$, compared to the same groups grown

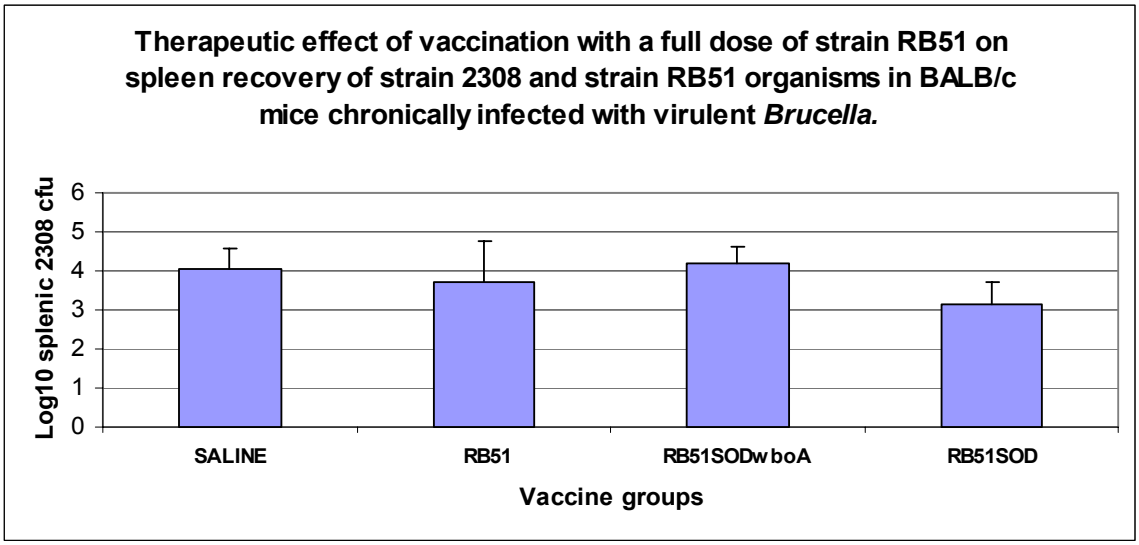

Figure 4.13: Therapeutic effect of vaccination with a full dose of strain RB51 on spleen recovery of strain 2308 and strain RB51 organisms in BALB/c mice chronically infected with virulent *Brucella*.

Mice chronically infected with virulent *B. abortus* 2308 were vaccinated with the full dose of strain RB51 (2-4 x10^8 cfus). Splenic clearance of the virulent organisms was determined two weeks post vaccination. Results are expressed as the average *B. abortus* 2308 Log10 cfu per group. The error bars are the standard deviations.

Discussion

The purpose of these studies was to explore the feasibility of expanding the versatility of vaccine strain RB51.

B. abortus vaccine strain RB51 is an attenuated natural mutant derived from the virulent parent strain *B. abortus* 2308, and has been shown to provide protection against challenge with virulent *Brucella* in mice and cattle (106, 109, 307, 351, 418). Vaccination with the attenuated *B. abortus* strain RB51 has proven to be safe and effective for most cattle target groups (303). Unfortunately, vaccination of pregnant cattle or bison with the full dose of the vaccine can lead to colonization of the placenta that can occasionally result in abortion with fetal colonization by strain RB51 (303, 305, 406), or shedding of the vaccine organisms through milk (404). The development of a strain RB51 vaccine that is even more attenuated, but retains its protective efficacy, is a useful goal of this research group.

Another incentive for the development of such a vaccine is the fact that although cattle brucellosis has been virtually eliminated in the U.S.A, the disease remains endemic in wildlife (113, 181, 203). This is of particular importance for cattle ranchers that are close to national parks. Bison and other wildlife infected with *Brucella* could be the means of reintroduction of the disease into previously free herds, due to sharing of pastures (113). Individual vaccination of wild bison and other feral animals is impractical. Therefore, several groups have explored the feasibility of mass vaccination via the oral route (82, 109, 113, 297). This approach could be very useful to protect wildlife, but carries the disadvantage that it is not possible to determine the gestational status of the vaccinates, the vaccine dose ingested, nor to control the ingestion of the vaccine by non-target species. Studies carried out in coyotes (203) and other wild species have shown that although strain RB51 can be transiently recovered from these animals, it does not pose any clinical danger to them (113). This is not the case for all species; pregnant reindeer are extremely susceptible to strain RB51 vaccination, and accidental

exposure to the vaccine, may lead to abortion in these animals (44). Therefore the development of an avirulent protective strain that could be used to immunize these target groups would be of great value for the complete eradication of the disease.

Finally, brucellosis is a zoonotic disease. It is endemic worldwide with especially high levels in some Middle Eastern, African, Asian and Latin American countries (30, 96, 242, 320, 336, 346). *B. melitensis* especially, can cause a disease that may lead to severe impairment of the quality of life of affected people (8, 116, 320). It has also been developed for use as a biological weapon in several countries (168, 325). There are currently no vaccines to protect humans against infection. In general, the current vaccines are still considered too virulent, and their efficacy questionable, to be acceptable for human use (18, 35, 161). Vaccine strain RB51 is not approved for human use. Although many cases of accidental inoculation have been reported world wide, most of the cases have not shown clinical signs (1). Recently a report of adverse reactions in apparently immunocompetent patients has been published (18), but the incidence of these cases is low. Again, the development of an attenuated vaccine would prove to be very valuable for vaccination of animals with decreased risk to the vaccine handler.

Irradiation has been described as a means for attenuating vaccines (99). Studies carried out with irradiated *Toxoplasma gondii* and *Burholderia mallei* have shown the elicitation of cytokine responses in vaccinated mice similar those elicited by the live organism (10, 173). In one study, where vaccination with irradiated *Coccioides immitis* arthrospores was used, the irradiated organisms did confer significant protection against coccidiomycosis challenge in mice (316).

In our study, irradiation of strain RB51 vaccine with 396,000 rads rendered it non-replicative, but left it metabolically active, as determined by the Alamar Blue Reduction assay. The metabolic activity was maintained for several days after irradiation when the irradiated vaccines were stored at 4°C. This finding has practical applications because it provides a larger window of opportunity for vaccination, as the vaccines could be irradiated days before their use. Because in our protection studies vaccination with the

irradiated strains was performed immediately after irradiation, further studies should be carried out to determine how late post irradiation vaccination can take place, without significantly decreasing the protective efficacy of the vaccine.

Vaccination with irradiated and non-irradiated RB51 strains induced clear antibody responses to whole cell *B. abortus* strain RB51 antigens with a polarized IgG_{2a} response (Figure 4.7and Figure 4.8). Contrary to what was observed by Amemiya et al., (10), irradiation of the vaccine did not have a significant effect on the isotype subclass or antibody levels elicited in vaccinated mice. However, the serological response to purified *Brucella* Cu/ZnSOD did show clear differences. Mice that received the irradiated RB51SOD vaccine developed significantly lower SOD antibodies than those that received the non irradiated strain (Figure 4.4). Because this lower antibody response could be due to a lack of SOD expression in the irradiated strain, whole cell antigens of both the irradiated and the non irradiated RB51SOD strains were analyzed by western blot using *Brucella* SOD specific anti-sera. The analysis revealed the presence of increased levels of SOD in irradiated strain RB51SOD compared to strain RB51. Although the irradiated strain did seem to have increased levels of SOD compared to strain RB51, irradiation may have induced changes that affected either the location or solubility of the protein, which could have great impact on the immunogenicity of this protective antigen. Irradiation induces the formation of reactive species such as hydroxyl and hydrated electrons that are able to change the redox state of the metal core in metallo-proteins (60), Cu/ZnSOD is a metallo-protein and it may be more affected by gamma irradiation than other non-metal containing *Brucella* proteins. Further research is needed to characterize the protein expression pattern in irradiated strain RB51 based vaccines. Western blot analysis of the culture supernatant from liquid media inoculated with irradiated strain RB51SOD, revealed the presence of SOD. This result should be interpreted with caution. Because the effects of irradiation on the strain are not understood, it is quite possible that at least some of this SOD is being released by dying cells. The lack of response to the SOD antigen appears to be a unique feature of mice vaccinated with the irradiated RB51SOD, and is not a generalized response to all

Brucella antigens, since mice vaccinated with irradiated strain RB51SOD do mount antibody responses to strain RB51 whole cell antigen, at levels comparable to the non irradiated strains. Interestingly, the decreased antibody response to SOD in mice vaccinated with irradiated strain RB51SOD was not observed in mice vaccinated with irradiated strain RB51SODwboA. Strain RB51SODwboA was generated by complementation of the rough RB51 strain with the *wboA* gene which resulted in a strain that synthesizes the O-side chain but it remains intracytoplasmic. This strain simultaneously overexpresses *Brucella* Cu/ZnSOD. The apparent "rescue" of the SOD immunogenicity induced by intracytoplasmic expression of the O-side chain in irradiated strain RB51SODwboA compared to irradiated strain RB51SOD, suggests that the O-side chain of *Brucella* may provide a potential "protective" effect on SOD. The intracytoplasmic O-side chain present in strain RB51SODwboA may directly bind or interact with the SOD protein, which in turn may stabilize the protein and/ or may promote its transport to the periplasmic space. A possible interaction of intra-cytoplasmic *Brucella* O-side chain with *B. anthracis* protective antigen has been observed in *Brucella* (A. Bandara, S.M. Boyle, N. Sriranganathan and G. G. Schurig, Personal Communication). Immunoprecipitation analysis using antibodies directed to the *Brucella* O-side chain and to *Brucella* SOD, as well as electron microscopy studies may help to elucidate the potential role of the O-side chain on the location and expression of SOD in live and irradiated cells.

In order to analyze antibody responses to *Brucella* O-side chain, an ELISA test using purified *Y. enterocolitica* O:9 LPS was performed. The serological cross reactivity between the *Y. enterocolitica* O:9 LPS and *Brucella* O-side chain has been documented (197). As expected, only mice vaccinated with irradiated and non irradiated strain RB51SODwboA developed antibodies to the O-side chain (Figure 4.3).This result confirms that vaccine strain RB51 does not induce antibodies to this O-side chain and that irradiation does not affect its rough phenotype. Mice vaccinated with strain RB51SODwboA do develop antibodies to this intracytoplasmic O-side chain. However, the antibody response decreases over time and no antibodies to the O-side chain were

detected 4 mice post vaccination. Interestingly, much higher levels of IgG2a antibodies to O-side chain were observed in the group vaccinated with irradiated RB51SODwboA. This result suggests that irradiation of the strain may somehow increase the presentation of the O-side chain to the immune system. As discussed above, in this irradiated strain the O-side chain may interact or bind to SOD, it is possible that if this case, the SOD protein may serve as a hapten increasing the immunogenicity of the O-chain.

In our study, with the exception of the irradiated RB51SOD vaccine strain, vaccination with irradiated and non irradiated strain RB51 based vaccines induced protection similar to that elicited by the non-irradiated controls. The lack of protection afforded by irradiated strain RB51SOD corresponds with the lack of SOD antibody response observed in the same vaccine group. As mentioned above, western blot analysis does indicate that the irradiated strain over-expresses Cu/ZnSOD but the location of the SOD in this strain was not analyzed. It is possible that irradiation prevents transport of the protein to the periplasmic space or alters the solubility of the protein thus affecting its appropriate presentation to the immune system. It is notable that vaccination with strain RB51SOD expressing the *wboA* gene (RB51SODwboA), induced almost sterile protection against challenge with virulent strain 2308 which, although slightly decreased by irradiation, was not significantly different than the protection afforded by the non irradiated strain. In fact, less than ten *B. abortus* 2308 organisms were recovered from the spleens of mice vaccinated with these strains. This result again highlights the apparent role of the *Brucella* O-side chain on rescuing the protective efficacy of SOD in the irradiated strain RB51SODWboA. To clarify the role of the O-side chain in the restoration of strain RB51SOD's protective capacity, future studies evaluating protection and cytokine induction by irradiated and non irradiated strains RB51SOD, RB51SODwboA and RB51wboA (without over-expression of SOD) should be performed.

Overall, irradiation does appear to be a feasible means for attenuating strain RB51 vaccines without compromising the efficacy of the vaccine, but the impact of irradiation

on the protective efficacy should be determined for each individual vaccine strain. This result has important implications for mass vaccination of wildlife as it reduces the risks associated with the unintentional inoculation of non-target species. Additionally, it provides an important step in the development of a fully attenuated brucellosis vaccine for use in humans.

The preservation of strain RB51's protective efficacy after irradiation, contrasts with the results obtained after irradiation of *M. avium* A5 cells (Chapter 3). Vaccination with irradiated *M. avium* did not induce protection against homologous challenge. The reasons behind this difference are not known, but the irradiation dose used to abrogate replication in strain RB51 vaccines was considerably lower that the dose used for *M. avium*. It is possible that the lower radiation dose abrogated replication but preserved the correct expression of protective antigens in *Brucella*. It could be speculated that irradiation of *M. avium* affected the protein secretion mechanisms in this strain, and since most of mycobacterial protective antigens, this could have significantly decreased its protective capacity. It is interesting that irradiation of strain RB51SOD also reduced its protective efficacy, and in this strain the accumulation of SOD in the periplasmic space and its "leakage" has been proposed to be important in protection.

The commercial RB51 vaccine used for cattle vaccination is provided as a lyophilized product that should be reconstituted shortly before vaccination. If irradiation of the lyophilized vaccine does not affect its efficacy, mass irradiation of the vaccine vials could be carried out saving time, money and increasing safety of the vaccine. It has been shown that lyophilization per se does not have significant detrimental effects on vaccine strain RB51 (64). In Appendix B, we describe a series of experiments designed to determine whether irradiation of the lyophilized commercial product affects the vaccine's efficacy. A vial of the commercial vaccine strain RB51 was irradiated with 396,000 rads before reconstituting it with the diluent provided by the manufacturer. One immediate difference on the effect of irradiation effects on the lyophilized commercial product, was the finding that a dose of 396,000 rads did not completely inactivate the strain ($6x10^2$ live bacteria/ml were recovered). This may be due to the physical conditions in which the

bacterial organisms are presented (dry versus suspended in liquid). The freeze-dried vaccine stock does not allow circulation of the bacteria during irradiation and this may result in zones within the vaccine vial that receive higher or lower irradiation doses. In the irradiation experiment described above, the strains were in suspension and in that case, fluid dynamics allow all organisms to circulate in the vial and receive the complete dose of radiation.

Mice vaccinated with these lyophilized commercial vaccines mounted strong antibody responses to whole cell *Brucella* antigens that were predominantly of the IgG2a isotype, suggesting the elicitation of a Th1 profile. Similar to our previous results, irradiation of the commercial strain RB51 vaccine did not reduce or alter the antibody responses to whole cell *Brucella* antigen.

Vaccination of mice with the irradiated and non-irradiated commercial vaccines as well as with our RB51 reference strain, induced cell mediated responses measured by proliferation to antigenic stimulation as well as by secretion of cytokines. Proliferation to heat killed strain RB51 was observed in mice vaccinated with both our live reference RB51 strain as well as with the commercial non irradiated vaccine. No significant proliferation was observed in the mice vaccinated with the irradiated commercial vaccine. This result contrasts with what was observed by Amemiya et al., (10), who reported that splenocytes of mice vaccinated with irradiated *Burkholderia mallei* showed proliferation levels that were not different from that of their negative controls. These different results may be due not only to the different bacterial species analyzed but also to the vaccination strategy used. In our study we only vaccinated once, whereas Amemiya et al., performed booster vaccinations. Additionally, in their system irradiation of liquid stocks was performed. Regarding cytokine production, all groups vaccinated with strain RB51 developed high levels of IFN-γ in response to stimulation with heat killed strain RB51, with higher responses in the irradiated group. As described in Chapters 1, 2 and 3, this cytokine is considered crucial for protection against *Brucella* infections because it enhances the bactericidal mechanisms that allow macrophages to kill internalized *Brucella* (149). Interestingly, the levels of IL-2 induced by the irradiated commercial

vaccine were not significantly different from the levels induced by the live commercial vaccine in which proliferation and protection was observed. IL-4 cytokine levels were below our detection limit in all groups. However, when IL-10 cytokine levels were analyzed, much lower levels of this cytokine were detected in splenocyte culture supernatants of from the group vaccinated with irradiated commercial strain RB51. This general pattern of cytokine production by mice vaccinated with strain RB51 has also been reported by Pasquali et al., (307) who have shown that high IFN-γ responses with no IL-4 are induced by vaccination with strain RB51. They have also shown significant induction of IL-10 in vaccinated mice which contrary to previous reports (127), does not correlate with a decrease in protection.

Contrary to our previous results, challenge with virulent *B. abortus* strain 2308 revealed significant protection only in mice inoculated with the live vaccine groups, at levels not significantly different from each other. The high level of IFN-γ and low level of IL-10 observed in the irradiated group did not correlate with the proliferation results or with protection. This result is interesting as in our previous experiment we have shown that irradiation of our reference strain RB51 vaccine did not significantly affect protection. As discussed in the previous chapter, the induction of IL-10 appears to be necessary for the induction of an effective protective immunity against *Brucella*. Although this is a cytokine that down-regulates the immune response, it appears to be crucial in maintaining the appropriate balance required for protection (308). Clearly high levels of IFN-γ were induced by vaccination with the irradiated commercial vaccine, but in the absence of appropriate levels of IL-10, protection was not obtained. Additionally, irradiation of the lyophilized commercial vaccine may have significantly impaired the secretion of other cytokines or molecular pathways that were not evaluated in this research but are important for the induction of protection against intracellular organism. Several reports have demonstrated that although the elicitation strong IFN-γ based Th1 polarized immune responses are necessary to induce protection against intracellular pathogens, the elicitation of these responses are not necessarily indicative of protection. As mentioned before, several vaccine studies involving mycobacterial antigens have

shown specific induction of strong Th1 responses without significant protection and in some cases even with an increase in infection load as demonstrated when the MTB 27 kDa antigen was used (176). Additionally, a DNA vaccine encoding *Brucella* GroEL also failed to induce protection despite the generation of IgG2a specific antibodies and IFN-γ in response to immunization (220).

Control of *B. abortus* infections has also been shown to be mediated by CD8+ cytotoxic lymphocytes (CTL). Vaccination with strain RB51 has been shown to induce CD8+ CTLs that are able to specifically lyse *Brucella* infected macrophages (170). The induction of CD8+ CTLs in mice vaccinated with the irradiated commercial strain RB51 was not analyzed in this research, but may have been affected by irradiation and may to some degree explain the lack of protection against challenge despite the generation of IFN-γ. The mechanism by which vaccine strain RB51 is able to induce cytotoxic lymphocytes is not clear. Some *Brucella* antigens must enter the endogenous pathway in order to be presented in an MHC-I context to CD8+ cells. Irradiation may have somehow impaired this process. Further studies are necessary to establish whether CTL responses are affected in mice vaccinated with the irradiated commercial strain and also to analyze the intracellular traffic of the irradiated vaccine organisms, as the intracellular fate of *Brucella* could have an impact on the type of immune response induced by it.

The lack of proliferation and protection elicited by the irradiated lyophilized commercial vaccine could also be due to differences in the vaccine dose. Because the replicative capacity of the irradiated strain is lost, retrospective confirmation of the vaccine dose is not possible, and therefore, mice vaccinated with the irradiated strain may have received a much lower vaccine dose.

A second objective in this chapter was to evaluate the effect of an ongoing *Brucella* infection on the efficacy of strain RB51 vaccination, and to determine whether vaccination of already infected animals could have some curative effect. Vaccination post

exposure to an infectious disease has been used as a therapeutic tool in a variety of viral and bacterial diseases (50, 159, 229).

In order to test whether this could also be true for vaccination with strain RB51, BALB/c mice were infected with virulent *B. abortus* strain 2308. To mimic vaccination of animals acute or chronically infected with *Brucella*, mice were vaccinated 2 or 5 weeks post infection and the number of *Brucella* cfus was determined by plating onto TSA and TSA/rifampin plates as described in methods. This approach was chosen because strain RB51 is resistant to rifampin but strain 2308 is not, and therefore only strain RB51 organisms can grow in this media (351). Vaccination of mice acutely infected with virulent *B. abortus* strain 2308 with a reduced dose of strain RB51 ($1x10^7$ cfus), revealed a rapid and significant decrease in the number of strain RB51 colonies recovered, but there was not a significant decrease in the number of virulent *B. abortus* strain 2308 organisms remaining in the spleen. To determine whether increasing the vaccine dose could lead to better protection, acutely infected mice were vaccinated with the full dose of the vaccine ($2\text{-}4\ x10^8$ cfus). Again, a slight, non significant decrease in the number of virulent *B. abortus* strain 2308 organisms in the spleens of the vaccinated groups was observed. A significant decrease in the number of vaccine organisms recovered from these mice was observed.

Chronic infection was slightly different. In this case, vaccination not only did not decrease the number of *B. abortus* 2308 organisms recovered from vaccinated animals compared to those that received saline, but chronically infected animals completely eliminated vaccine strain RB51 organisms. All the colonies were smooth upon crystal violet staining confirming them as *B. abortus* strain 2308. This result contrasts with the reports by Lowrie et al., (229) who vaccinated mice previously infected with *M. tuberculosis* with vaccine constructs expressing Hsp65 and by Ha et al., (160) who combined antibiotic therapy with DNA vaccination. In both cases, vaccination significantly reduced the load of virulent organisms. Despite these findings, and in accordance to what we observed, other therapeutic models have also failed to induce a curative effect. Lowrie et al., (229) in the same study described above, failed to obtain the

therapeutic effect when DNA vaccines expressing Hsp70 or Esat-6 protein instead of Hsp65 were used to treat an ongoing MTB infection. Turner et al., (397) reported that vaccination of mice infected with MTB with an 85A subunit vaccine or with a DNA vaccine expressing 85A fails to decrease the bacterial load. Significantly, in some cases re-exposure to mycobacterial antigens such as vaccination with BCG, has been reported to induce an immune response characterized by elevated levels of TNF-α and IL-6 that increase the size of pre-existing granulomas and tissue damage without decreasing the mycobacterial load (256).

The lack of recovery of vaccine strain RB51 organisms from the spleens of chronically infected mice, as well as the sharp decrease in the number of RB51 cells recovered from acutely infected mice, suggests that *B. abortus* 2308 provides very strong protective immunity against strain RB51, but this immunity is selective since the number of strain 2308 organisms did not decrease after vaccination with strain RB51. Contrary to many other pathogens that down regulate the immune responses that may be detrimental to their survival in the host, *Brucella* infected mice develop strong Th1 responses with secretion of high levels of IFN-γ (307). The RB51 vaccine organisms were cleared very quickly by the immune response elicited by *B. abortus* strain 2308 and were probably unable to further stimulate the anti-*Brucella* immune response and had no influence on the established strain 2308 infection. One possible explanation for the effective elimination of strain RB51 without affecting the established strain 2308 infection is the timing of the cytokine secretion. When a naïve animal is infected with *Brucella*, the organisms are internalized by the host's macrophages. Because there has been no pre-exposure to *Brucella*, IFN-γ has not been produced and therefore the macrophages are not activated and are not able to kill a significant number of *Brucella* thus, the infection with the virulent strain is established. During this initial event, as described in Chapter 1, the interaction of *Brucella* with Toll like receptors induces the secretion of pro-inflammatory cytokines such as TNF-α and IL-12, which eventually lead to the production of IFN-γ. Additionally, although resting macrophages are not able to efficiently destroy *Brucella*, some organisms are killed and presented to CD4+ and CD8+ cells. Therefore, when

vaccination post infection with *B. abortus* strain 2308 was performed, the vaccine organisms likely encountered macrophages that had already been activated by the IFN-γ produced in response to the virulent strain and these activated macrophages and T cells, quickly eliminated the vaccine organisms. This too may explain why mice chronically infected with *Brucella* were more efficient at clearing strain RB51 organisms than acutely infected mice. Cell mediated responses require 4 weeks time to be fully generated (14), when acutely infected mice were vaccinated, the vaccine organisms did not encounter a fully developed anti-*Brucella* cell mediated immune response and were able to survive longer. It has been shown that IFN-γ is crucial to the control of *Brucella* during the first week of infection but its importance is greatly decreased as the chronic stage progresses (263). The reasons for the difference may lie in that during the initial stages of infection, *Brucella* are still being internalized by macrophages. It is at this stage that IFN-γ induced activation of the macrophage's phagocytic, bactericidal and antigen presentation capabilities would have the most impact on destruction of the invading organisms. During the chronic stages, *Brucella* organisms have already been established in their intracellular niche, and therefore the CD8+ cytotoxic lymphocyte responses become important for their destruction (291).

The question of how the *Brucella* infection becomes chronic when a clearly protective immune response is mounted against it remains to be answered. Virulent organisms replicate inside the macrophages that are not able to kill them. Two important considerations arise: first, how do *Brucella* organisms prevent activation of the infected macrophage by IFN-γ and second, how does it resist killing by cytotoxic lymphocytes.

Infected macrophages still display and present antigens in MHC-I and MHC-II molecules indicating that down regulation of the presentation pathway at this level, is not involved in this resistance (262, 371). One possibility that has not been explored is that *Brucella* may decrease expression of the IFN-γ receptor in infected macrophages or actively block the signal transduction cascade, induced by IFN-γ, which culminates in the activation of the phagocyte's bactericidal properties. This has been shown for *M. tuberculosis* infections. Virulent mycobacteria reduce the activity of the IFN-γ promoter

by reducing expression of cyclic adenosine 5'-monophosphate response element-binding proteins (CREB) which bind to the promoter to drive transcription. Also, MTB actively blocks the interaction of the CREB and STAT-1 adaptor molecules, blocking the IFN-γ induced macrophage activation, preventing the induction of its mycobactericidal activity (390).

A possible mechanism in which virulent *Brucella* are able to avoid the cytotoxic effect of CD8+ cells has been demonstrated for *B. suis*. This species actively prevents apoptosis by the release of a putative soluble factor and which disrupts IFN-γ and also Fas mediated apoptosis (155).

In this chapter we have also demonstrated that challenge with the full dose of the RB51 vaccine in mice vaccinated with strain RB51, leads to the complete clearance of the vaccine organisms within 10 days post homologous challenge. As described above, during the first exposure to strain RB51, IFN-γ secretion and activation of macrophage's bactericidal mechanisms along with the induction of *Brucella* specific CD4+ and CD8+ T cells occurs. Therefore, the immune response to the homologous challenge is very strong probably because challenge with the same organism naturally presents the same complement of antigens to which the original immune response was directed. However, the immune response mounted against the homologous strain RB51 challenge can result in the death of 40% of the mice within 2 days. Death is likely the result of endotoxic shock, due to the quantities of lipid A in the LPS released during the immune mediated destruction of the challenge strain.

This finding is different than what was observed in mice already infected with strain 2308; vaccinating infected mice with strain RB51 did not lead to death, even though the vaccine organisms were rapidly eliminated. LPS triggered endotoxic shock is mediated by pro-inflammatory cytokines. As described in Chapter 1, the lipid A component of the gram negative LPS signals through TLR-4 in association with CD14. Binding of the LPS molecule to the receptor activates a signal transduction pathway that results in translocation of NF-kB into the nucleus and transcription of cytokine genes,

mainly TNF-α, IL-1β and IL-6 (182, 271, 436). It has been shown that *B. suis*, a smooth strain whose LPS contains the O-side chain, can down-regulate the production of TNF-α (103), and that rough *Brucella* LPS, which lacks the O-side chain, induces higher amounts of TNF-α, and IL-6 than smooth LPS (323). Therefore, the effective immunity induced by vaccination with the rough RB51 strain, coupled with the massive release of rough LPS by the dying vaccine organisms, could lead to a much larger inflammatory response than that generated in mice previously infected with the smooth *B. abortus* 2308 strain. In these animals, infection with the smooth strain may have downregulated the production of TNF-α, resulting in the clearance of strain RB51 without endotoxicity.

In brucellosis control and eradication programs, the elimination of infected animals is paramount for success. Ideally, infected animals would never be vaccinated as they would have already been eliminated from the herd. However, due to monetary constraints this is not always the case, especially in developing countries where state sponsored reimbursement of culled stock does not occur. Vaccination of the uninfected cattle mass is still important, and infected animals may be unknowingly vaccinated. In cattle, the effect of a previous infection with smooth virulent *Brucella* on strain RB51 vaccination remains to be studied. Field data seem to agree with what was observed during vaccination of mice infected with a virulent strain, as there are no published reports of cattle death following vaccination with strain RB51, even in areas of high prevalence of the disease where vaccination of infected animals is likely to have taken place. Our study suggests that vaccination of infected animals would not have a detrimental effect, but would not provide additional benefits.

The differences in the responses observed between the vaccination of animals previously "infected" with strain RB51 or with strain 2308 highlights some other interesting points. Vaccination with strain RB51 vaccine does not lead to sterile immunity when mice are challenged with strain 2308. On the other hand, "vaccination" with strain 2308 does provide sterile immunity against challenge with strain RB51. The difference between both strains is the presence of O-side chain on the surface of the

parent strain. Since strain RB51 lacks this O-chain in theory, the humoral immune response generated to the O- antigen in mice infected with strain 2308 should play a very small role if any in specific protection. However, some reports suggest that the O-chain may be involved in T cell responses. It has been shown that *Brucella* LPS can directly bind to MHC-II molecules in B cells and may play a direct or indirect role in T cell activation (118, 136).

The potential protective role of the O-side chain in *Brucella* infections in mice is further emphasized in the protection experiments in which strain RB51SODwboA has been used. This vaccine strain provides almost sterile protection against challenge with virulent strain 2308 (this study, (414, 417). In this strain, the O-side chain remains intra-cytoplasmic so the vaccine strain retains its rough phenotype (417) but induction O-chain antibodies in mice is still observed (at least for a few months). These antibodies may be involved at least partially in the increased protection since some level of protection by O-chain antibodies has been demonstrated in the mouse (111, 253, 435).

In summary, in chapter we have demonstrated that in mice infected with virulent *B. abortus*, vaccination with strain RB51 does not induce clearance of the established infection. Furthermore, the immune response generated to the ongoing *Brucella* infection is able to completely eliminate the vaccine organisms.

We have also shown that irradiation of vaccine strain RB51 may be a suitable means of attenuating the vaccine strain without significantly compromising its protective efficacy. This finding has practical implications as it provides the basis for the generation of fully attenuated, protective vaccines to protect wildlife.

CHAPTER 5

Summary and Discussion

The research presented in this dissertation was designed to evaluate the potential of vaccine strain *B. abortus* strain RB51 expressing antigens of *M. avium subsp. paratuberculosis* (MAP) to induce protection against heterologous challenge using *M. avium*. The goal of this approach was two-fold: first, to produce a mouse model that would allow identification of vaccine candidates with protective potential against MAP infection; and second, to produce a recombinant strain RB51 vaccine able to induce protection against *M. avium* (as model for MAP) by expressing MAP antigens, while retaining or improving its protective efficacy against *Brucella*. Additionally, alternative approaches for the use of strain RB51 vaccine to increase its versatility in protection against *B. abortus* were evaluated.

In Chapter 2, vaccine strain RB51 was used as a vector to express the MAP antigens 85A or 35 kDa protein either alone (RB51pTB85A and RB5135), or with the simultaneous over-expression of *B. abortus* Cu/ZnSOD (RB51SODpTB85A and RB51SOD35). The 85A protein has been shown to be a protective antigen in several *M. tuberculosis* challenge systems. The 35 kDa protein was chosen because at the time of the initiation of this research, antibodies that cross reacted with *M. leprae* had been described in MAP infected cattle, indicating that it was an immunogenic antigen but the gene had not yet been described in MAP. Over-expression of the CuZnSOD (*sodC*) gene was performed because previous reports had indicated that the homologous over-expression of this protective antigen in strain RB51 leads to increase protection against *Brucella* challenge.

The expression of protective or immunogenic mycobacterial antigens in strain RB51 was expected to generate a dual vaccine that would induce protection against *Mycobacterium* as well as against *Brucella* challenge. This approach makes biological sense. Both *Mycobacterium spp.* and *Brucella spp.* are intracellular pathogens able to replicate and survive inside macrophages. Control of both diseases requires the involvement of cell mediated immune responses with strong IFN-γ secretion required for the activation of macrophages to enable them to kill the infecting bacteria. Strain RB51 is a strong inducer of such an immune response, therefore presenting mycobacterial antigens to the immune system in a context that already gears it towards the right type of immunity, could potentially lead to increased protection to this heterologous antigen.

Cloning and sequencing of the MAP 85A and 35 kDa protein antigens of MAP revealed high identity to the genes in *M. avium*. These proteins were expressed in *E. coli* as insoluble proteins. Expression of the heterologous antigens in strain RB51 proved to be very low. However, when the 85A protein was produced as a fusion with the MTB Esat-6 protein the level of expression was increased. The increased expression of 85A as a fusion with Esat-6 could be due to stabilization of the protein.

This approach proved that vaccine strain RB51 is able to express these heterologous antigens albeit at low levels. However, the low level of expression is an important drawback to the use of these strain RB51 based vaccines to protect against mycobacterial disease. Because the elicitation of adequate immune responses is to a certain degree dependent on the dose of the antigen, low levels of expression can lead to low or no protection against challenge with the virulent organism. The low expression of the heterologous proteins in *Brucella* could be due to many factors. Among these are the potential toxicity of the foreign proteins, which would force the *Brucella* cells to down-regulate their expression; improper folding of the proteins, which would target them for degradation or would be produced as insoluble aggregates that may not efficiently stimulate the immune system; and also, the fact that codon usage between *Brucella* and *Mycobacterium* exhibit some important differences and may mean that the use of relatively rare tRNAs in *Brucella* could lead to their rapid depletion resulting in

decreased protein expression. Future studies should focus on the engineering of heterologous genes to be expressed in *Brucella,* with *Brucella's* preferential codon usage.

In Chapter 3, the immune responses and protection elicited by the vaccine strains expressing MAP antigens created in Chapter 2 were evaluated in BALB/c mice.

To evaluate protection by these vaccines, a suitable mouse model had to be developed. MAP is an extremely slow growing organism in laboratory conditions, and MAP models have required the infection of neonate BALB/c mice where infection takes many months to become established, making the use of this strain impractical for vaccine evaluation. *M. avium avium* is the parent strain of MAP and both strains are genetically very closely related. The MAP genes we expressed in strain RB51 share over 99% identity with the homologs in the parent strain. *M. avium* is able to infect adult BALB/c mice and is less fastidious in laboratory growth requirements. In order to evaluate the protective efficacy of the recombinant vaccines, a challenge model using *M. avium* was developed to screen for those vaccines with the most protective potential.

Infection of BALB/c mice with high doses of *M. avium* (1×10^9) via the i.p route is able to establish a transient colonization of the spleen. Although infection induced no clinical signs of disease, infected mice developed a severe splenomegaly that remained for more that one year post infection. The splenomegaly observed in these mice may be due to the presence of mycobacterial granulomas in the spleens of infected mice. Aditionally, the splenomegaly may also be a reflection of increased TNF-α in these infected animals which would not be down-regulated by the anti-inflammatory cytokine IL-10, as IL-10 secretion was determined to be very low. Chronically and acutely infected mice develop strong IFN-γ responses to *Mycobacterium* antigen stimulation and also to heat killed strain RB51. As described in Chapter 1, this response is expected as mycobacteria are strong inducers of IL-12 and TNF-α which leads to secretion of IFN-γ. The response to *Brucella* antigens suggests the presence of cross-reactive antigen between these species.

The recombinant strains induced very low levels of antibodies specific to the heterologous proteins in mice. On the other hand strong *Brucella* specific antibody responses were detected. These were mainly of the IgG2a phenotype that is indicative of a Th1 profile. These strains also stimulated antigen specific secretion of IFN-γ and undetectable levels of IL-4 upon stimulation with heat killed strain RB51, or with purified *Brucella* Cu/ZnSOD. Secretion of the Th2 associated cytokine IL-10 was also detected in mice vaccinated with these recombinant strains. These results indicate that expression of the heterologous MAP antigens in strain RB51 does not alter the ability of the vaccine strain to induce strong cell mediated immunity of a Th1 cytokine profile to *Brucella*. The elicitation of Th1 responses is thought to be crucial for protection against *Brucella*. Although IL-10 is cytokine associated with a Th2 profile, IL-10 was also detected in our vaccine groups. This finding has been reported in vaccination with strain RB51 and during infection with virulent *Brucella*. Initially, the identification of IL-10 in virulent *B. abortus* 2308 infected mice, lead to the conclusion that IL-10 favored infection. Clearly this is not the case. In our study we show significant protection against *Brucella* challenge despite the elicitation of high IL-10 levels by vaccine strain RB51. Therefore, IL-10's role in *Brucella* infection/protection may be a regulatory one to control an excessive inflammatory immune response generated by the host. Although no 85A or 35 kDa protein specific antibodies were detected in vaccinated mice, the recombinant strains did stimulate IFN-γ response to heat killed *M. avium* in some experiments.

The recombinant RB51 vaccines induced protection against *Brucella* challenge at levels similar to those induced by vaccine strain RB51, indicating that the expression of the heterologous antigens does not significantly affect the protective efficacy of the strain. The recombinant strains also showed attenuation similar to strain RB51 indicating that expression of these heterologous genes did not increase virulence of the vaccine strains.

Despite obtaining some IFN-γ secretion in response to *M. avium* antigen stimulation in some experiments, no overall significant protection was observed against

M. avium challenge. Protection was observed in some experiments when strains RB51SODpTB85A or RB51pTB85A were used but it was not consistent between experiments, and very high intra-group variability was observed. Contrary to our expectations, increasing expression of 85A in strain RB51 by generating a fusion with the Esat-6 protein failed to induce protection. Vaccination with recombinant strains RB5135 and RB51SOD35 did not induce protection or elicit antibodies to the heterologous protein. The reason for the lack of protection elicited by these vaccines is not completely clear. The low level of expression of the heterologous proteins in strain RB51 is likely a major contributing factor, but also improper folding or conformation of the recombinant and fusion proteins in strain RB51 could also be involved. These proteins may be expressed as inclusion bodies or as otherwise insoluble proteins that may not be adequately presented to the immune system.

One of the concerns regarding our mouse model is the lack of positive protective controls. In an attempt to generate such a control, homologous vaccination with irradiated *M. avium* was performed in the thought that *M. avium* would be the best candidate to induce protection against itself. Since we had observed that irradiation of RB51 vaccine strains did not impair their ability to induce protection, and that vaccination with strain RB51 induced sterile protection against homologous challenge (Chapter 4), this approach was thought to be workable. No protection was elicited by the irradiated *M. avium* against homologous challenge with non irradiated *M. avium.* This result could be due to an excessive radiation dose and future studies using smaller radiation dose increments may be of value. Additionally, most of the protective antigens in mycobacteria are secreted proteins, irradiation may have damaged the secretion pathways in *M. avium* abrogating secretion of these protective proteins. This result also highlights the issue that the precise mechanisms of attenuation induced by gamma irradiation and its effects on the irradiated strains are not known and are virtually impossible to predict. It also indicates that the effect of irradiation on the attenuation and protective capacity should be evaluated for each individual vaccine strain of interest as it may not be the solution for all vaccines.

Another concern is that very high intra-group variability was detected after vaccination with the strain RB51 based vaccines expressing MAP antigens and challenge with *M. avium*; this may account for the lack of statistical significance in some of the experiments. As demonstrated in Chapter 3, the *M. avium A5* strain chosen in our study is able to infect BALB/c mice, but has been described as being less virulent than other *M. avium* strains. The use of highly virulent strains may be of value in future experiments. Finally, since no positive protection controls are available, it is possible that the model is ultimately not useful. Therefore, the development of suitable animal models remains an important goal in paratuberculosis research.

One interesting finding of this research was the fact that short lasting cross reactive antibodies to *Brucella* Cu/Zn SOD were observed in *M. avium* infected mice. We also observed that mice acutely or chronically infected with *M. avium* were able to secrete IFN-γ when stimulated with strain RB51 or with purified *Brucella* Cu/ZnSOD. Attempts to identify a *sodC* gene in *M. avium* with higher homology to *Brucella* s*odC* than the previously identified gene, proved unsuccessful. Partial identities to an unidentified potential protein in the genome of *M. avium* suggest the possibility that these aminoacid segments could be conformational epitopes, and that the cross- reactive antibodies are directed to them. These amino acid sequences may also be recognized by specific T cell repertoires involved in protection. Further studies are needed to identify the precise identity of these peptides and also the T cell populations specific for them. If such populations are indeed found, new generations of vaccines could be engineered to target these populations by the delivery of specific epitopes.

Consistent with this finding, a non-significant trend towards protection against *M. avium* challenge was observed when mice were vaccinated with strain RB51 over-expressing *Brucella* Cu/ZnSOD (RB51SOD). In order to further explore this phenomenon, the isolated contribution of *Brucella*'s Cu/Zn SOD was evaluated by vaccination with an *O. anthropi* strain that expresses *Brucella* Cu/ZnSOD. We found that mice vaccinated with strain RB51SOD or with *O. anthropi* SOD with or without

concomitant addition of the genetic adjuvant CpG were significantly protected against challenge with *M. avium* compared to the saline and strain RB51 control groups. This finding is interesting as it suggests heterologous protection generated by *B. abortus* Cu/ZnSOD against *M. avium* infection. However, in order to fully understand the role of Brucella Cu/ZnSOD in protection against *M. avium*, studies using vaccination of BALB/c mice with purified *Brucella* Cu/ZnSOD or a DNA vaccine expressing *Brucella* SOD (299), and also with the *O. anthropi* vector without over-expression of *Brucella* SOD are needed, as they would indicate whether *O. anthropi* it-self contains additional antigens that can provide protection against mycobacteria.

An additional goal of this research was to expand the versatility of *B. abortus* vaccine strain RB51 in protection against brucellosis by decreasing its virulence, while retaining its ability to protect against *Brucella*. This is an important concern because in the long term, eradication of the disease will require control of the infection among wildlife where targeted vaccination is impossible. In mass vaccination campaigns it is very difficult to prevent inoculation of non-target species that may be very susceptible to the vaccine. For these groups, as well as for the potential use of the vaccine in human subjects, the generation of protective but very attenuated strains is of great importance.

Irradiation of *B. abortus* RB51 abrogated its replication and therefore its virulence, while retaining the metabolic activity; this metabolic activity remained active for several days. Vaccination with the irradiated RB51 strains in general, induced the elicitation of *Brucella* specific antibodies of predominantly IgG2a phenotype which is associated with a Th1 profile. Challenge of vaccinated animals revealed that the strains elicited significant protection at levels not significantly different to those attained with the non-irradiated vaccines. This is a very encouraging finding because it proves that irradiation of strain RB51 vaccine can be used as a means of increasing its attenuation without impairing its protection against *Brucella* infection. Another important finding derived from this experiment was the confirmation that vaccine strain RB51SODwboA induces almost sterile immunity against challenge with virulent *Brucella* and that this

protection was not lost by irradiation. The increased protection induced by this strain, and the fact that it remained equally protective after irradiation, makes it a very promising vaccine candidate. Further studies should be carried out to determine the exact serological response and protective efficacy of this vaccine in bovines. Because the main difference between vaccine strain RB51 and its parent strain 2308 is the presence of the O-chain antigen in the latter, the increased protection by strain RB51SODwboA highlights the potential importance of O-chain specific antibodies in protection in the mouse model. On the other hand, the role of antibodies in protection against *Brucella* infections in cattle seems to be minor. This does not necessarily mean that this vaccine would not exhibit increased protection in cattle. It has been shown that the *B. abortus* smooth LPS is able to directly bind to MHC-II molecules in B cells and this may play a role in the induction of strong Th1 responses.

Vaccination with RB51 strains expressing the O-side chain does have the potential draw back of serological interference with diagnostic tests, but according to our study, the O-side chain specific antibodies elicited in mice after vaccination tend to decline and disappear after a few months. Also, a pilot experiment, were calves were vaccinated with this strain showed that no O-chain specific antibodies were developed (G.G. Schurig, Personal Communication). Even if the vaccine induces O-chain specific antibodies, the merit of vaccination with this highly protective strain, especially in areas with high prevalence of the disease, may by-pass the diagnostic disadvantage. Several ELISA format assays and polarized fluorescence assays have been developed that may allow discrimination of infected v/s vaccinated animals. Once the prevalence is decreased to levels where eradication is within reach, the vaccination strategy could then be changed to strain RB51 which will not interfere with the diagnosis.

Irradiation did not have the same effect on the lyophilized vaccine as it did on the liquid preparations. After irradiation, the lyophilized vaccine still contained a small number of viable organisms. This difference could be due to the fact that in the lyophilized vaccine the organisms are in a solid state, and therefore areas that receive higher radiation doses can be present. On the other hand, irradiation of liquid suspensions

would be more homogeneous as fluid dynamics would allow most organisms to receive a similar radiation dose. Although the irradiated vaccine was able to induce high antibody levels to *Brucella* antigens and high levels of IFN-γ without IL-4 secretion, the strain was not able to confer protection against challenge with virulent *Brucella*. In our study, the only difference in the cytokine profile generated by this irradiated vaccine was a decreased level of IL-10 production. This preliminary result suggests that the role of IL-10 in protection against *Brucella* may be more important than previously thought, and may not just be a side effect of infection with an intracellular parasite trying to prevent its elimination, but an active requirement for protection against infection. However, since in this study only a few cytokines were analyzed, it is possible that irradiation interferes with the secretion of other cytokines that are important for protection against *Brucella* challenge. Also, protection against *Brucella* infections is also dependent on cytotoxic lymphocytes it is possible that this branch of immunity may have been affected by irradiation. Finally, the difference in protection may also be a consequence of a lower vaccination dose. Irradiation significantly impaired replication therefore retrospective confirmation of the vaccine dose could not be carried out. Further studies should be conducted to analyze the impact on the protective efficacy of irradiation of vaccine stocks prior to lyophilization, as this could be an alternative means of mass producing attenuated vaccines.

In this research, we also evaluated the effect of a previous ongoing *Brucella* infection on the efficacy of strain RB51 vaccination, and the potential curative effect of vaccination. This is a concern especially if as mentioned above, vaccination of wildlife in an area where the brucellosis is endemic is sought, or if vaccination of cattle in areas of high prevalence is undertaken. In both cases, there is a high probability that the inoculation of already infected animals will take place. To evaluate this, we mimicked the conditions of vaccination of animals that are acutely or chronically infected with virulent *B. abortus* 2308. Vaccination of acutely infected mice with strain RB51 induced a non-significant trend to reduction in the number of strain 2308 organisms recovered, but more importantly showed a dramatic decrease in the number of strain RB51 vaccine organisms

remaining in the spleens. Vaccination of chronically infected mice did not seem to promote clearance of the infection instead, complete clearance of the vaccine strain was observed. Since it has been shown that cell mediated immunity is generated 4 weeks post infection with virulent *Brucella*, vaccination during the chronic stages of infection would encounter robust humoral as well as cell mediated responses, that are able to quickly eliminate the vaccine organisms. It is notable that despite the generation of an effective protective immune response against *Brucella* by the active strain 2308 infection, the virulent strain 2308 organisms are not eliminated by it. This highlights the importance of *Brucella*'s niche in its survival. Although macrophages are responsible for the elimination of this organism, virulent *Brucella* are able to survive and replicate inside them and, as shown in our study, resist the effects of the immune response mounted by the host. Part of this resistance may be related to the timing of the encounter of *Brucella* with the host's macrophages. In the case of infection of an unvaccinated animal, the host's macrophages have not yet been activated by IFN-γ and are not able to destroy the organisms, whereas in our study, when previously infected animals were vaccinated with strain RB51, the vaccine organisms encountered activated macrophages that efficiently destroyed them. It has been shown that *Brucella* does not down regulate the expression of MHC molecules on the cell surface but the LPS of smooth *Brucella* is able to bind to MHC-II molecules potentially providing a physical barrier to antigen presentation. Another mechanism has been shown for *B. suis* which is able to prevent Fas and IFN-γ mediated apoptosis of the infected macrophages by an unidentified soluble mediator.

In our study we also show that full dose revaccination of mice previously vaccinated with strain RB51 elicits a very strong immune response that can kill mice, most likely due to endotoxic shock. The surviving mice eliminated strain RB51 organisms indicating sterile protection against homologous challenge. This observation lead us to recommend that all experiments that require boosting with strain RB51 should be carried out using a reduced dose of the vaccine.

In summary, this work indicates that,

- *Brucella* can express heterologous antigens of *Mycobacterium spp*., but vaccination with the recombinant strains may not protect against challenge with *M. avium*, probably due to the low levels of heterologous antigens produced in these strains.
- *M. avium* infected mice develop antibody and cell mediated immune responses to *Brucella* Cu/Zn SOD which can lead to a certain degree of protection against *M. avium* infection.
- Irradiation of live *Brucella* vaccines may provide an effective means of attenuation while retaining their protective efficacy.
- Vaccination of infected animals with strain RB51 does not induce a curative effect in fact; it results in complete clearance of the vaccine strain.

CHAPTER 6

Conclusions and future studies

In this dissertation we have shown that vaccine strain RB51 can be used as a vector for the expression of heterologous antigens from MAP. However, vaccination with these strains does not lead to consistent levels of protection against *M. avium* challenge. Therefore, a major increase in the protective efficacy will be necessary in order to advocate these approaches as practical.

One of the first steps in this regard, will be the generation of RB51 strains with increased expression of the heterologous antigens. One approach is to engineer synthetic MAP antigens using *Brucella's* preferential codon usage. This approach has been used to express *B. anthracis* antigens in strain RB51 and has resulted in increased expression of the heterologous antigen compared to expression of the native gene.

Additionally, the heterologous protein product can be targeted to different location in strain RB51. Thus, selective targeting of the heterologous antigen to *Brucella* outer membrane using the *B. abortus* 18 kDa outer membrane protein signal sequence or to the periplasmic space by engineering the gene to include *Brucella* Cu/ZnSOD signal sequence has the potential to more effectively induce presentation of the heterologous genes to immune cells.

Expression of the whole mycobacterial protein may not be necessary to induce protective immunity. Therefore, plasmid constructs expressing only selected epitopes of the mycobacterial antigens will be evaluated. In this sense, several immunogenic and protective epitopes of different antigens have been identified in mycobacteria (115, 250, 267, 293). This approach has some definite advantages, single protective epitopes or a combination of a few epitopes can be expressed. Due to their small size, codon replacement to *Brucella's* codon usage would be simpler, and replication of the plasmid and transcription of the gene may be more efficient. Expression of only selected epitopes also has the advantage of by-passing the potential toxicity induced by mycobacterial proteins in strain RB51. Additionally, these epitopes could also be targeted to specific

locations by engineering them to be expressed with different signal sequences as described above.

Different plasmid vectors will also be used to express these antigens or epitopes. Currently, new vectors derived from plasmid pBBR1MCS are being developed. These plasmids express significantly higher levels of homologous and heterologous proteins in *Brucella*. Therefore, the use of these vectors to express MAP antigens in strain RB51 can potentially increase the expression levels in our recombinant vaccines.

Future studies will also be carried in order to refine the mouse model used to evaluate protection. In our experiments very high intragroup variability was observed. This could be due to the use *M. avium* strain A5 which is not as virulent in BALB/c mice as other *M. avium* strains. Therefore, different *M. avium* strains will be compared in BALB/c mice and a strain that induces consistent infection levels will be selected. Also, alternative challenge routes can be evaluated.

The lack of a positive control of protection against *M. avium* challenge still limits the interpretation of our negative results. Therefore, irradiation of *M. avium* A5 as a means to generate an attenuated vaccine to protect against homologous infections will be repeated using several irradiation doses, spanning from 396,000 rads until the 594,000 rad dose we used. The lowest dose that abrogates replication will be used in vaccination experiments. Most of mycobacterial protective antigens are secreted proteins; therefore, protein secretion will be evaluated in irradiated and non-irradiated *M. avium* to determine whether the lack of protection observed in mice vaccinated with *M. avium* irradiated with a dose of 594,000 rads was due to abrogation of the secretion of these proteins. An additional approach will be to use mice that have already cleared the infection as controls, as these mice have already developed protective immunity against *M. avium*.

One interesting finding of this research was the identification of cross reactive antibodies to *Brucella* Cu/Zn SOD in *M. avium* infected mice. Also, mice acutely or chronically infected with *M. avium* were able to secrete IFN-γ when stimulated with strain RB51 or with purified *Brucella* Cu/ZnSOD. When the potential role of *Brucella*'s

Cu/Zn SOD on protection against *M. avium* was evaluated by vaccination with an *O. anthropi* strain that expresses *Brucella* Cu/ZnSOD, mice vaccinated with strain RB51SOD or with *O. anthropi* SOD with or without concomitant addition of the genetic adjuvant CpG were significantly protected against challenge with *M. avium* compared to the saline and strain RB51 control groups. This finding is interesting as it suggests heterologous protection generated by *B. abortus* Cu/ZnSOD against *M. avium* infection. However, in order to clarify the role of *Brucella* SOD in protection, vaccination with strain RB51SOD, purified *Brucella* Cu/ZnSOD or with a DNA vaccine expressing this protein (299), as well as with strains *O. anthropi* and *O. anthropi*SOD with and without the addition of the genetic adjuvant CpG, can provide better insight into this phenomenon.

In our research we have also demonstrated that irradiation of vaccine strain RB51 can be used as an effective means of attenuating the strain without significantly compromising its protective efficacy. Future studies using vaccination with irradiated and non irradiated strains will be carried out to characterize the serological and cytokine immune response induced by these strains. Analysis of the amount and location of SOD in irradiated strain RB51SOD, and the cytokine pattern induced by vaccination with irradiated and non irradiated strain RB51SOD, should help in the understanding of the reasons behind the lack of protection induced by the irradiated vaccine.

Finally, we have shown that vaccination of infected animals with strain RB51 does not induce a curative effect. In fact, it results in complete clearance of the vaccine strain. The cytokine patterns induced after infection and before and after vaccination will be analyzed.

Additional studies focusing on the effect of intracellular *Brucella* on IFN-γ signaling in infected cells will be studied, as this could provide answers to how *Brucella* is able to persist and establish chronic infections even though strong cell mediated immunity is induced.

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APPENDIX A

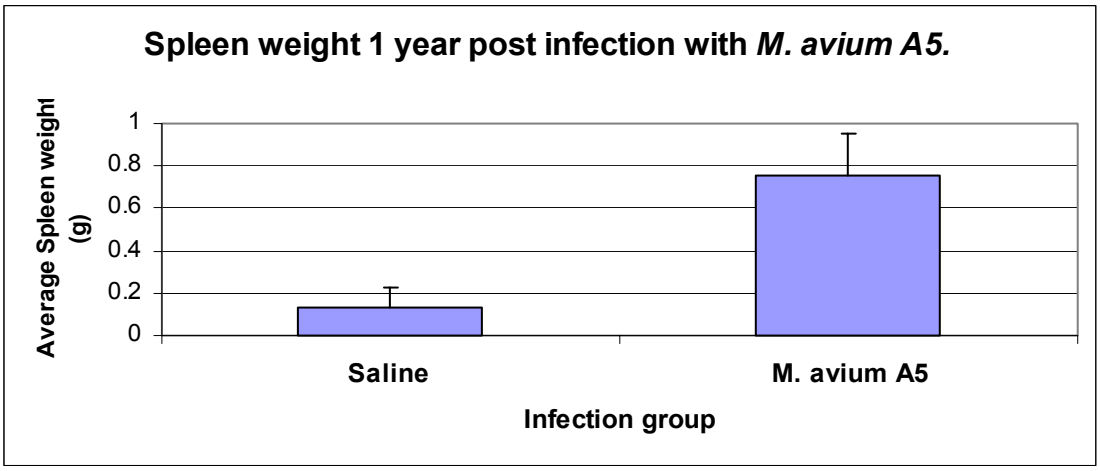

Figure A.1: Spleen weight 1 year post infection with *M. avium* A5.

Columns represent the average spleen weight of 3 BALB/c mice/group one year post infection with $1x10^9$ cfu of live *M. avium A5*. Error bars represent the standard deviations.

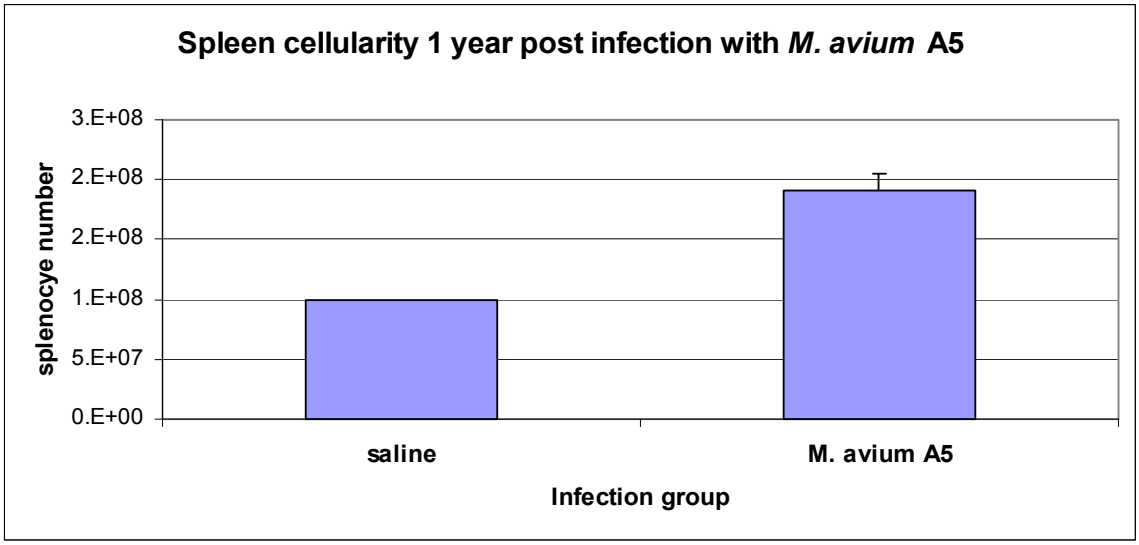

Figure A.2: Spleen cellularity 1 year post infection with *M. avium* A5.

Columns represent the average splenic cellularity in 3 BALB/c mice/group one year post infection with $1x10^9$ cfu of live *M. avium A5* as determined by Trypan Blue Exclusion method. Error bars represent the standard deviations.

APPENDIX B

Effect of gamma irradiation on commercial *B. abortus* RB51

As a continuation of the research presented in Chapter 4, the effect of irradiating the commercial vaccine strain RB51 was determined. The rationale for this approach is that if the protective efficacy of the lyophilized commercial vaccine is not altered by irradiation, mass irradiation of the commercial vaccine could be used as an inexpensive means of attenuating the product.

Mice and bacterial strains: female, 6-8 week old BALB/c mice purchased from Charles Rivers Laboratories (Wilmington, VA). The animals were housed in micro isolation cages under BSL-3 containment in groups of up to 5 mice/cage. All animal handling experiments were carried out under IACUC and university guidelines with supervision by the university veterinarian.

Vaccine strains RB51 is part of our culture collection and was described in detail in Chapter 1. Virulent *B. abortus* strain 2308, a smooth virulent strain that is widely used in *Brucella* challenge models (15, 428), is also part of our culture collection. Commercial strain RB51 vaccine was purchased from Colorado Serum Co, (Denver, CO) as a lyophilized product and was stored at 4ºC.

For serological analysis and protection studies, mice were vaccinated i.p with 2-4x10^8 cfu of *B. abortus* RB51 based vaccine strains. Mice were bled by retro-orbital venipuncture under local anesthesia 5 weeks post vaccination. Unless otherwise indicated, mice were challenged i.p six weeks post vaccination with 2-4 x10^4 cfus of *B. abortus* 2308.

Irradiation of *Brucella* organisms: Commercial lyophilized vaccine strain RB51 was irradiated in a Co source gamma irradiator (JL Shepperd and Assoc, San Fernando

CA) with 396,000 rads and was resuspended post irradiation in the vaccine diluent provided by the manufacturer. A second vial of non irradiated commercial lyophilized strain RB51 vaccine was used as control. Viability of the irradiated vaccine was determined plated onto TSA plates and incubated at 37ºC +5% CO_2 for up to 8 days. The metabolic activity of the irradiated strain was determined using the Alamar Blue Reduction Assay described in Chapter 4.

Serological responses of mice vaccinated with irradiated and non-irradiated *B. abortus* commercial vaccine strain RB51: Antibody titres to *Brucella* antigens were determined by ELISA. Briefly, 96 well NUNC Maxisorp plates were coated overnight at 4ºC with 0.5 μg/well of whole cell strain RB51 antigen as described in detail in Chapter 4.

Cytokine analysis: For cytokine analysis mice were sacrificed 4-6 weeks post infection by CO_2 inhalation. Splenocyte suspensions (pooled 3 mice/group) were obtained as described in Chapter 3. Stimulating antigens were added to each well in 1 ml volumes at the following concentrations: ConA (Sigma, St. Louis MO) 10 μg /well, heat killed strain RB51 $1x10^7$ cells /well or *B. abortus* Cu/Zn SOD recombinant protein 10 μg /well, media alone was used as the negative control. The plates were then incubated at 37°C + 5% CO_2 for 5 days. Culture supernatants were then collected and frozen at -80°C until analyzed. Cytokine production was determined using an ELISA based commercial mouse Th1/Th2 cytokine kit (Ebioscience, San Diego, CA) following the manufacturer's recommendations as described in Chapter 3. All samples were run in triplicate.

Lymphocyte proliferation Assay: 100 μl of the splenocyte suspensions reserved from the cytokine assay were added in triplicate to the wells of a 96 well Falcon (Fisher, Fair Lawn NJ) flat bottom tissue culture plate at a concentration of $5x10^5$ cells well. One hundred μl of the stimulating antigens solutions described above were added to the wells. The plate was then incubated for at 37°C +5%CO_2 for 54 hours and then pulsed with 100 μCi ^3H thymidine /well. The plates were then incubated at 37°C +5% CO_2 for further 18

hours and harvested using a plate harvester (Packard, Meriden CT) and read in a scintillation counter (Packard, Meriden, CT) to determine counts/minute/well.

Protection induced by irradiated and non-irradiated commercial *B. abortus* vaccine strain RB51: To determine whether an irradiated commercial RB51 vaccine is able to confer protection against virulent challenge, 4 groups of 5 female, 6-8 week old BALB/c mice each, were vaccinated with 2-4 $x10^8$ cfu of vaccine strains RB51 (reference stock), live commercial vaccine strain RB51, commercial vaccine strain RB51 irradiated with 396,000 rads as described above or saline. The vaccination dose and inactivation of replication of the irradiated vaccine was determined as above. Six weeks post vaccination all groups were challenged with virulent *B. abortus* strain 2308 and two weeks post challenge, the mice were sacrificed and splenic clearance of virulent organisms was determined.

Statistical analysis: For comparisons between vaccine and saline groups statistical analysis were performed by Analysis of Variance and Dunnet's test at a confidence level of 0.05. Statistical analysis was performed using SAS statistical software.

Results

To evaluate whether commercial lyophilized vaccine stocks remain protective after irradiation, two strain RB51 vaccine vials were purchased from Colorado Serum Co. Based on our previous findings, one lyophilized vial was irradiated for 60 minutes while the other vial was not irradiated. The vials were then reconstituted with 10 ml of the vaccine diluent provided by the manufacturer. Irradiation of lyophilized commercial strain RB51 with 396,000 rads was not sufficient to completely abrogate replication and $6x10^2$ cfu/ml were recovered post irradiation. No differences in the metabolic activity measured by Alamar Blue assay were detected between irradiated and non irradiated commercial RB51 vaccines or to our reference RB51 strain (data not shown).

Serological responses of mice vaccinated with irradiated *B. abortus* RB51 vaccine strains

Groups of 5 mice each were vaccinated i.p with 2-4 x10^8 cfus of irradiated or non-irradiated RB51 commercial vaccines, as well as with RB51 reference strain or with saline control, mice were bled five weeks post irradiation and antibody titres to whole cell *Brucella* RB51 antigens were analyzed by ELISA.

Vaccination of mice with the irradiated and non irradiated commercial strains induced high *Brucella* specific antibody levels of predominantly IgG_2a isotype (Figure B.1 and Figure B.2). IgG1 antibodies were very low and not significantly different than saline (data not shown).

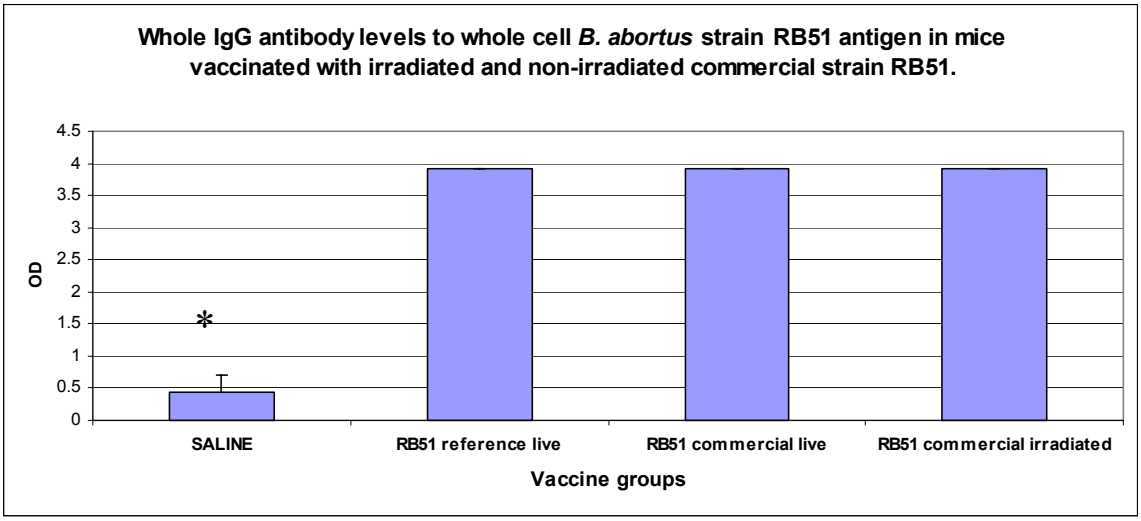

Figure B.1: Whole IgG antibody levels to whole cell *B. abortus* strain RB51 antigen in mice vaccinated with irradiated and non-irradiated commercial strain RB51.

Sera collected 5 weeks post vaccination was analyzed by ELISA using whole cell strain RB51 antigen. Results represent the mean of 3 mice/ group, and the error bars are the standard deviations (*$p<0.05$).

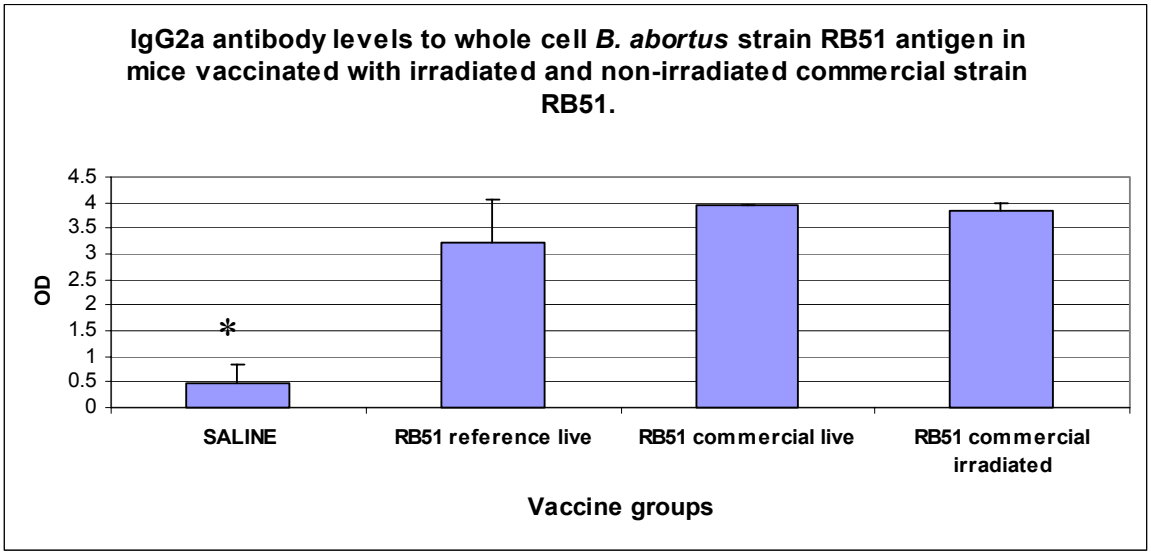

Figure B.2: IgG2a antibody levels to whole cell *B. abortus* strain RB51 antigen in mice vaccinated with irradiated and non-irradiated commercial strain RB51.

Sera collected 5 weeks post vaccination was analyzed by ELISA using whole cell strain RB51 antigen. Results represent the mean of 3 mice/ group, and the error bars are the standard deviations (*$p<0.05$).

Cell mediated responses of mice vaccinated with irradiated *B. abortus* RB51 vaccine strains

Evaluation of the cell mediated immunity induced by vaccination with irradiated commercial and reference RB51 strains was performed five weeks post vaccination. Cytokine analysis was determined by ELISA using pooled splenocyte culture supernatants after 5 days incubation with various stimulating antigens (Figure B.3, Figure B.4, Figure B.5). The results are shown as pg of cytokines /ml of supernatant.

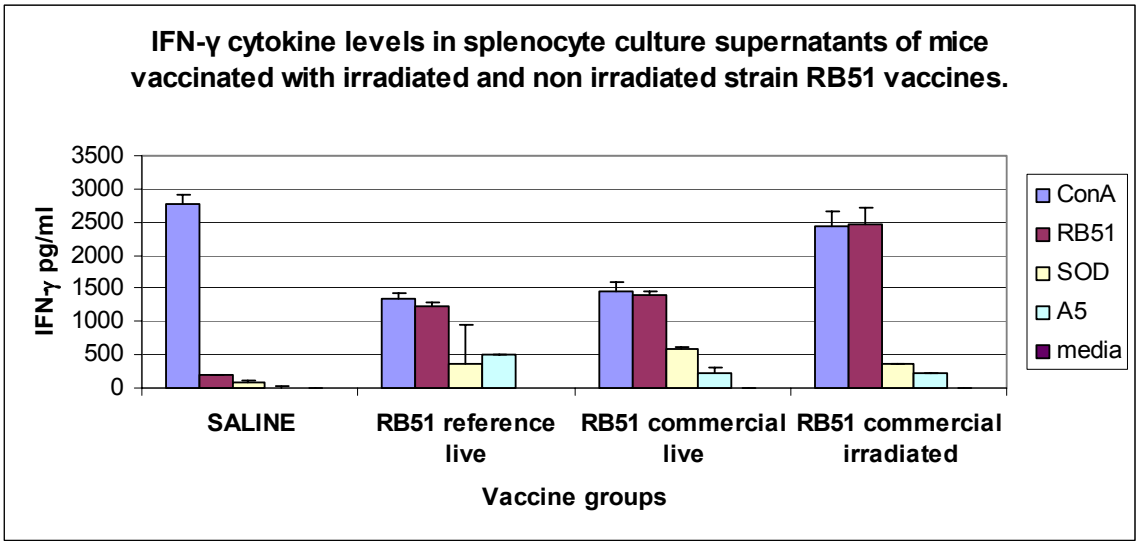

Figure B.3: IFN-γ cytokine levels in splenocyte culture supernatants of mice vaccinated with irradiated and non irradiated strain RB51 vaccines.

Splenocytes were stimulated for 5 days with antigens. Concanavalin A (ConA) = 10 μg /ml, heat killed *M. avium* A5 (A5) = $1x10^6$ cfu, heat killed RB51 (RB) = $1x10^6$ cfu, recombinant *B. abortus* Cu/ZnSOD (SOD) = 10 μg /ml. (Sensitivity of the assay >8 pg/ml).

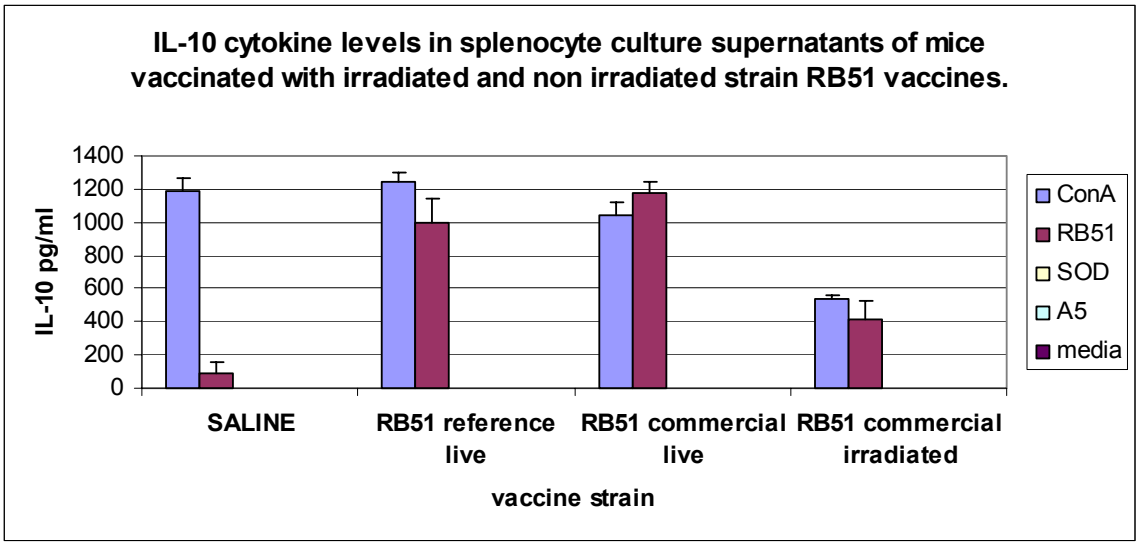

Figure B.4: IL-10 cytokine levels in splenocyte culture supernatants of mice vaccinated with irradiated or non irradiated strain RB51 vaccines.

Splenocytes were stimulated for 5 days with antigens. Concanavalin A (ConA) = 10 μg /ml, heat killed *M. avium* A5 (A5) = $1x10^6$ cfu, heat killed RB51 (RB) = $1x10^6$ cfu, recombinant *B. abortus* Cu/ZnSOD (SOD) = 10 μg /ml. (Sensitivity of the assay >15 pg/ml).

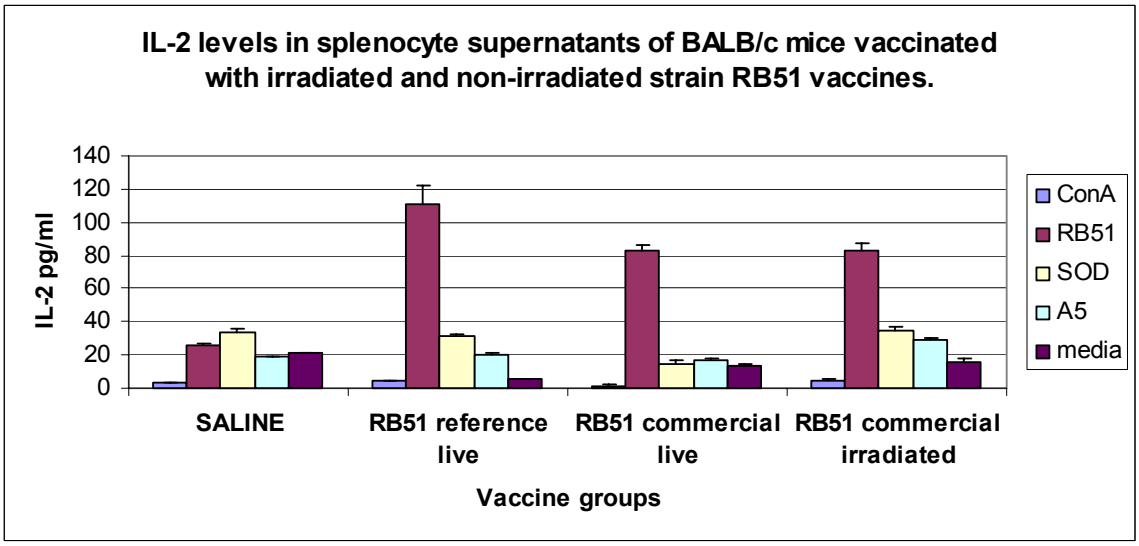

Figure B.5: IL-2 levels in splenocyte culture supernatants of mice vaccinated with irradiated or non irradiated strain RB51 vaccines.

Splenocytes were stimulated for 5 days with antigens. Concanavalin A (ConA) = 10 μg /ml, heat killed *M. avium* A5 (A5) = $1x10^6$ cfu, heat killed RB51 (RB) = $1x10^6$ cfu, recombinant *B. abortus* Cu/ZnSOD (SOD) = 10 μg /ml.

These results show that mice vaccinated with irradiated and non irradiated strains are able to mount strong cell mediated responses as determined by cytokine secretion, and that irradiation did not affect RB51's ability to elicit cell mediated immunity. All vaccine groups secreted high quantities of IFN-γ and IL-2. IL-10 was detected in high levels in the splenocyte supernatants of mice vaccinated with the live vaccines, but was lower in culture supernatants from the group vaccinated with irradiated RB51. No IL-4 was detected in culture supernatants from any of the groups.

The ability to proliferate upon stimulation with various antigens by splenocytes from mice vaccinated with the above vaccines was evaluated by determining ^{3}H thymidine incorporation in splenocyte cultures from vaccinated mice using the lymphocyte proliferation assay (Figure B.6).

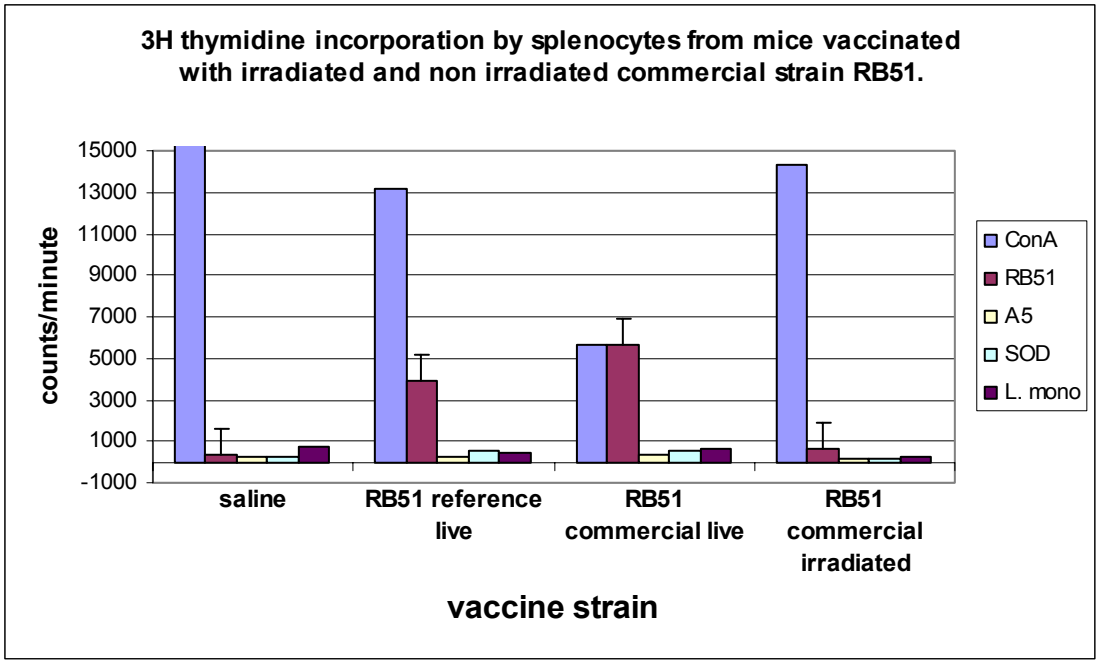

Figure B.6: ^{3}H thymidine incorporation by splenocytes from mice vaccinated with irradiated and non irradiated commercial strain RB51.

Splenocytes suspensions were stimulated with ConA= 1 µg /well; Heat killed RB51= $1x10^{6}$/well; Heat killed *M. avium* A5=$1x10^{7}$/well; recombinant *B. abortus* Cu/ZnSOD= 1 µg /well and heat killed L. monocytogenes =$1x10^{6}$/well. Results are expressed as total counts per minute (cpm) – cpm of media negative control.

The proliferation results indicate significant proliferation by splenocytes from mice vaccinated with live strain RB51 vaccines to stimulation with heat killed strain RB51. This proliferation was greatly decreased in the group vaccinated with the irradiated strain to a level no different than proliferation induced by stimulation with media alone.

Protection against challenge with virulent *B. abortus 2308* in mice vaccinated with irradiated *B. abortus* RB51 vaccine strains

To evaluate protection conferred by irradiated and non-irradiated commercial strain RB51, BALB/c mice were vaccinated as described before. All mice were challenged with virulent *B. abortus* 2308 six weeks post vaccination and splenic clearance of strain 2308 organisms was determined 2 weeks post challenge (Figure B.7).

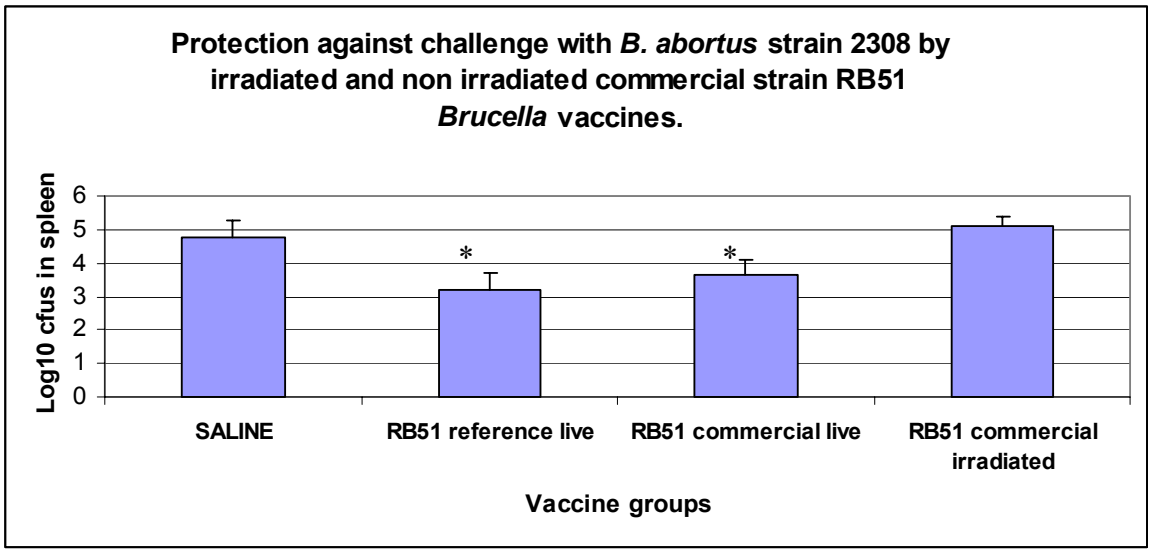

Figure B.7: Protection against challenge with *B. abortus* strain 2308 by irradiated and non irradiated commercial strain RB51 *Brucella* vaccines.

Lyophilized commercial *B. abortus* RB51 vaccine was irradiated as described in methods and was reconstituted in the vaccine diluent post irradiation. BALB/c mice were vaccinated and challenged with virulent strain 2308. Bars represent the mean Log10 *B. abortus* 2308 cfu in the spleens of 5 mice/group two weeks post challenge, and the bars are the standard deviations (*$p<0.05$).

Vaccination with both live RB51 vaccines induced equivalent and significant protection against challenge with virulent *B. abortus* 2308. Irradiation of the lyophilized commercial vaccine removed this protective effect.

VITA

ANDREA PAZ CONTRERAS ROJAS

Dr. Andrea Contreras was born not too long ago, in the beautiful city of Valdivia, in the south of Chile. She attended primary and secondary school in her home town, with only a brief interruption when she attended primary school in Compton, Berkshire in England. After finishing high school in Valdivia, she enrolled in the Biochemistry program at the Universidad Austral de Chile. Quickly regaining her senses, she decided to become a veterinarian instead. She earned the degree of Licenciado en Medicina Veterinaria and the Doctor of Veterinary Medicine degree (Medico Veterinario) from Universidad Austral de Chile in January, 1997. She worked at the brucellosis diagnostic laboratory of the Instituto de Microbiologia, at the same university until the fall of 1998, when she enrolled in the Doctor of Philosophy program at the VA-MD Regional College of Veterinary Medicine, Department of Biomedical Sciences and Pathobiology. She joined the *Brucella* research group under the supervision of Dr. Gerhardt Schurig.

Her interest in veterinary medicine and *Brucella* research runs in her genes. She is the daughter of veterinarians. Her mother, Ximena Rojas is a brucellosis researcher in Chile. Her father Pedro Contreras is a large animal researcher and clinician who contracted brucellosis while delivering the calf of a particularly uncooperative cow.

She is married to Jochen Rode, and is currently trying to learn german, with seems to be the motto: “varying degrees of success”.