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Evaluation of granulysin and perforin as candidate biomarkers for protection following vaccination with *Mycobacterium bovis* BCG or *M. bovis* ΔRD1

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Evaluation of granulysin and perforin as candidate biomarkers for protection following vaccination with *Mycobacterium bovis* BCG or *M. bovis* ΔRD1

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This dissertation is dedicated to my wonderful and always supporting wife Juliana and my daughters Mariana and Carolina

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Evaluation of granulysin and perforin as candidate biomarkers for protection following vaccination with *Mycobacterium bovis* BCG or *M. bovis* ΔRD1

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The development of improved vaccines against tuberculosis (TB) is directly linked to the investigation of new and better correlates of protection after vaccination against TB. Cloning and characterization of bovine homologues of the antimicrobial protein granulysin (Bo-lysin) and perforin by our group could be used as potential biomarkers for TB vaccination efficacy. In the present study we examined the kinetics of granulysin, perforin, IFNγ and Fas-L responses to *Mycobacterium bovis* purified protein derivative (PPD) stimulation by peripheral blood mononuclear cells from *M. bovis ΔRD1*-, BCG- and non-vaccinated cattle. Gene expression profiles following PPD stimulation showed significant increases in transcripts for granulysin and IFNγ in both $CD4^+$ and $CD8^+$ T cells in BCG-vaccinated as compared to non-vaccinated animals. Perforin and IFNγ examined by flow cytometry, showed a difference of 1-2% more PPD-specific cells in BCG-vaccinated than non-vaccinated animals. In the vaccine trial, granulysin and perforin were significantly increased in both vaccine groups as compared to control after vaccination and challenge. IFNγ expression was increased only after vaccination and secretion was higher in the control, as compared to both vaccine groups demonstrating no correlation with protection upon vaccination. In summary, results shown here provide evidence that granulysin and perforin are prospective candidates as biomarkers of protection after vaccination against TB.

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TB	Tuberculosis
XDR-TB	Extremely drug resistant tuberculosis
BCG	*Bacille of Calmette and Guérin*
PPD	Purified Protein Derivative
RD1	Region of Difference 1
DC	Dendritic cell
CR3	Complement receptor 3
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule 3 grabbing non-integrin
MBL	Mannose Binding Lectin
LAM	Lipoarabinomannam
TLR	Toll like receptor
MHC	Major Histocompatibility Complex
Mtb	*Mycobacterium tuberculosis*
CTL	Cytotoxic T lymphocyte
WC1	Workshop Cluster 1
NK	Natural Killer
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
PBMC	Peripheral Blood Mononuclear cells

CHAPTER 1
INTRODUCTION

Tuberculosis: still a major public health problem

Tuberculosis (TB) remains a major public health problem due to the increasing numbers of reported cases throughout the US and worldwide. Several factors have been involved in the increased rates of TB worldwide including (i) HIV co-infection, (ii) emergence of drug resistant strains including extremely drug-resistance TB (XDR-TB), (iii) immigration from high prevalence countries (related to US) and (iv) variable efficacy of the BCG (*Bacille of Calmette-Guérin*) vaccine still extensively used worldwide. According to CDC and WHO reports, it is estimated that more than 15 million people get infected with *Mycobacterium tuberculosis*, and 10% of these will develop TB, worldwide [1]. Each year it is estimated that more than 2 million individuals die due to infection; there were 9.2 million new cases of TB, where 0.7 million are HIV –positive cases, with an estimate of more than 50% of HIV-infected patients also TB positive [2]. The drug resistance factor involves both primary resistance cases in people infected with a strain that is already resistant, and individuals that acquire resistance during therapy. Treatment of TB cases is based on the use of a cocktail of drugs including isoniazid, rifampin, pyrazinamide, ethambutol, rifabutin and rifapentine. Besides these drugs several other have been introduced in the cocktail such as streptomycin, cycloserine and levofloxacin. The treatment lasts for at least 6 months but can be longer (up to 12 months) depending on the strain and resistance profile of the strain infecting the patient [1, 2]. Multidrug resistance represents 0.5 million cases in 2006, besides that, XDR-TB cases represent 16,000 cases from the period of 2007-2008 and treatment can be for up to 2 years [2].

Another factor contributing to the increased TB rates in US is immigration of people from countries where TB has high incidence such as India, Africa and China. In 2006, data presented by CDC comparing TB rates by race/ethnicity shows that for every 100.000 people, from a total of 35 cases, 15 are from people of Asian origin and 20 are from people from other origins (Hispanics, white, American Indians and African Americans) [1, 2].

BCG, a *Mycobacterium bovis* attenuated strain, is the only currently licensed vaccine against TB and has been used in more than 100 million people worldwide. BCG was implemented initially on 1921 and since then it had its success, but also a reason for a lot of controversy in terms of continuing its use or not. The efficacy of the vaccine goes from 0-80% in several clinical trials. Factors involved in this extreme variability include, but are not limited to, the use of different strains worldwide, conservation and administration of the vaccine, pre-exposure to environmental mycobacteria and loss of BCG genes over the year due to multiple passages (genetic drift). So far, several new vaccine candidates are in phase I and II trials, but have not progressed to the point of offering a better response than BCG.

Tuberculosis in bovine and humans: a zoonotic perspective

In addition to increasing rates of human TB, bovine TB can be considered an important reemerging disease. The cattle pathogen, *Mycobacterium bovis,* presents disease characteristics that parallel the human pathogen *M. tuberculosis,* including disease pathogenesis and the host immune response. *M. bovis* is not an exclusive pathogen of cattle but is able to infect and cause disease in all mammals. *M. tuberculosis* was originally thought to be a pathogen of humans, but outbreaks in elephants in 2001 and 2003 in a Swedish zoo [3] added this species to the list of susceptible hosts. The close interrelationships of humans with cattle in various settings world wide present a potential risk for infection between the 2 species. Similarly to bovine, elephants in zoos can be potential sources of tuberculosis to zoo keepers and to visitors. Bovine tuberculosis in the US has been restricted to cases in California, Arizona, New Mexico, Texas, Minnesota and Michigan. Cases in Minnesota and Michigan are mainly due to the presence of whitetail deer, a natural reservoir of *M. bovis* [4, 5]. Sources of *M. bovis* in the other states are also related to wildlife or to farmed game animals. Control of wildlife species such as deer is done every year around farms with positive cases of bovine TB, but the effort is contained by the number of animals that show up the year after. Also, control of bovine TB in the mentioned states is done by quarantine and slaughtering of the positive animals. Infections with *M. bovis* in humans were reported in New York, where a total of 35 cases were due to consumption of contaminated unpasteurized cheese from Mexico [6].

TB in cattle is a major livestock problem in UK, where the incidence of bovine TB increases an average of 18% per year. As observed in the US, the increasing numbers of bovine TB cases in UK are thought to be mainly due to a wildlife reservoir, particularly the Eurasian badger. In New Zealand the brush tail possum is reported to be the wildlife reservoir of *M. bovis* [7]. The policies involving quarantine and slaughtering, and the hunting seasons do not control the rising cases of bovine TB. In addition to better control measures in a variety of species, there is a major need for improved vaccines and also better diagnostic test(s) to discriminate infected from vaccinated cattle. Bovine TB is diagnosed based on comparing skin thickness after intradermal injection of both bovine and avium PPD in different spots. Once increased thickness is observed in the bovine PPD site, the animal is subjected to further testing.

Mycobacterium tuberculosis* and *Mycobacterium bovis

1-Taxonomy, description and growth requirements

Mycobacterium tuberculosis and *M. bovis* are in the family Mycobacteriaceae, genus *Mycobacterium*. They are characterized as being aerobic, non-spore forming, non-motile, rod shaped, and need a specific staining procedure to be visualized. Although they are considered Gram positive (G^+), an acid fast staining is required due to their high content of lipids in the cell wall. *Mycobacterium* species present very complex growth requirements, but can readily access ammonia, amino acids as sources of nitrogen and carbon dioxide in the presence of salts. The growth is relatively slow, taking more than

20 hours for a next generation to form, and colonies are observed usually after several days to 6 weeks of culture [8]. The most common media used to grow *Mycobacterium spp*. is Middlebrook 7H11 that includes inorganic compounds with essential amino acids and growth stimulating inorganic salts as well as vitamins. Glycerol or pyruvate is provided as a source of carbon and energy, and sodium citrate is converted to citric acid. Other compounds included on the 7H11 are selective agents such as Malachite green (BD Pharmingen, CA).

Mycobacterium species can survive for weeks to several months in the environment when protected from sunlight, but are easily killed by temperatures over 65°C/30 minutes or by UV light from the sun or by chemical compounds such as chloride [9]. Some species like *M. tuberculosis* are able to survive for a long time in the environment, and the presence of a living cell is important to allow pathogen replication. *Mycobacterium spp.* is composed of several microorganisms that have different requirements, being either obligate intracellular pathogens, opportunist or saprophytes [8]. Example of saprophytic *Mycobacterium spp.* are the ones in the *M.avium* complex, usually isolated from water, soil and plants [10] which represent a potential problem for vaccination as will be discussed later.

According to Pfyffer et al. (2003) [8], close to 100 species of mycobacteria have been described. Classification is based on groups, starting with the *M. tuberculosis* complex, which includes *M. tuberculosis, M. bovis, M. africanum, M. microti* and *M. canettii*. *M. leprae*, the causative agent of leprosy or Hansen's diseases, is considered to be in a separate group of mycobacterium due to its characteristic inability to grow *in*

vitro. Leprosy is characterized by a chronic, granulomatous and debilitating disease, involving skin lesions and peripheral neuropathy [8]. The remaining mycobacteria of the family Mycobacteriaceae include human and non- human pathogens such as *M. avium complex, M. marinum* and *M. smegmatis.*

2-The *Mycobacterium spp.* genome and the RD1 (Region of Difference-1)

The *Mycobacterium tuberculosis* genome was published in 1998 based on the H37Rv strain (a virulent lab strain). It was a very important step for the TB vaccine development field. The genome comprises 4.4 Mb that encode around 4,000 genes, with a high G+C content of over 65% [11]. The *M. bovis* genome was published in 2003 showing that it was 99.95% identical to *M. tuberculosis*. Comparisons between *M. bovis* and *M. tuberculosis* showed 11 deletions in *M. tuberculosis*. The sequence of genes missing in *M. tuberculosis* corresponds to a unique locus in *M. bovis* called TbD1 [12] . Several other differences are described between the *M . tuberculosis* and *M. bovis*. Among the genes present in *M. tuberculosis* are the genes that encode PE and PPE, named after their conserved proline-glutamate (PE) or proline-proline-glutamate (PPE) residues near the N terminus of the predicted proteins. PE and PPE maybe responsible for host and tissue tropism difference between the 2 strains since PE was shown to bind fibronectin [13]. PE and PPE were shown to be expanded in slow growing mycobacteria and seem to be functionally linked to the ESAT-6 secretion system (ESX) [14]. Comparison of the BCG vaccine with virulent strains showed that BCG was lacking a

region called Region of Difference 1 (RD1). A comparison among all BCG strains with the wild type *M. bovis* revealed that Regions of Difference encompass a total of 129 open reading frames. However, only the RD1 region is absent in all BCG strains, being composed of 9 genes which include 2 important secreted proteins, ESAT-6 and CFP-10 [11, 15]. These 2 antigens were shown to be potent T cell stimulators and may be linked to spread of the organism. It was further shown that 6 different ESAT-6 proteins are present in *M. tuberculosis* and missing in *M. bovis,* potentially impacting the antigen load [12]. On the other hand the bovine bacillus presents 2 other serodominant proteins called MPB70, a filtrate protein, and MPB 83, a glycosylated cell wall-associated protein [12].

Further characterization of the RD1 region and CFP-10: ESAT-6 protein-protein interaction studies revealed that RD1 encodes a specialized secretion system in mycobacteria, named ESX-1 (ESAT-6 secretion system). A close analysis of the genome revealed that there are 5 clusters containing members of the CFP-10 and ESAT-6 family of proteins, all very important T cell antigens, as well as other secreted proteins involved on several metabolic pathways [14]. These 5 clusters also encode secretion systems named from ESX-2 to ESX-5. The genes encoded in the RD1 region, specially the CFP-10:ESAT-6 complex, are involved in a series of events as reviewed by Ganguly et al., (2008) [16]. These events include effects on pathogenesis and virulence such as cell lysis, granuloma formation, cytokine suppression, phagosomal maturation arrest and interaction with Toll-like receptors; and effects on immunomodulation such as down regulation of macrophage cell signaling and effects in the DC-T cell interaction. An important immunomodulatory effect of the CFP-10:ESAT-6 complex is the down regulation of

Reactive Oxidative Species (ROS). Modulation of the ROS response ultimately results on inhibition of NF-kB activation [16].

The dual role of CFP-10:ESAT-6 complex on virulence and stimulation of the immune response suggests an important source for vaccine development and new diagnostic tests using ESAT-6. The secreted protein ESAT-6 was also evaluated as a potential diagnostic to discriminate infected from vaccinated animals and may potentially be used for humans [17-20].Taken together, alterations or deletions in the genome of each strain, are important findings that will impact the understanding of evolution, pathogenesis and immune response, development of better vaccines and diagnostic tests. One of the strains used in our studies is based on a deletion of the RD1 region (ΔRD1) on a *M. bovis* strain.

Mycobacterium tuberculosis and *Mycobacterium bovis* pathogenesis

Both *M. tuberculosis* and *M. bovis* present similar pathology. *M. tuberculosis* was recently described also as a pathogen of elephants [3, 21, 22], while *M. bovis* is a pathogen for mammalian species [23]. Both species are carried in airborne particles generated by infected persons or infected animals. Once these droplets containing the pathogen are inhaled, they go to the alveoli and are engulfed/ phagocytized by resident macrophages. It is now clear how many bacilli are required to lead to TB infection. In cattle, it is suggested that less than 5 bacilli would be enough to result in infection but in experimental models, aqueous suspensions of 10^4 cfu are used [24]. It is unknown how

many bacilli are required to establish infection in humans. As few as 10 bacilli maybe enough to provide infection. Facts such as droplet size and breathing frequency of the person being infected, have to be considered [25].

Besides resident macrophages, dendritic cells lying on the trachea may also be the first ones to come in contact with the pathogen. Initial interaction of the pathogen with these 2 cell types is performed through several receptors. Much of the immune response will depend on the receptors used by *Mycobacterium spp.* Among the receptors used, the mannose receptors are a potential safe route to facilitate intracellular survival. Complement receptors (CR3) are also very advantageous for *Mycobacterium* since it does not induce the release of oxygen intermediates [26]. Other receptors are DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin), surfactant protein A (Sr-A), Class A scavenger receptor, mannose binding lectin (MBL) and dectin -1 [27-29]. Internalization of the pathogen is followed by phago-lysosomal fusion, destruction of bacteria, processing and presentation of antigens to T cells. However, this process does not always occur. *Mycobacterium spp.* is able to adapt the macrophage environment by inhibiting phago-lysosomal fusion, more specifically inhibiting trafficking events that modulate the endosomal/phagosomal maturation. A close analysis of the mycobacterial phagosome showed that it has an elevated pH, lack of ATPases and late endosomal and lysosomal markers such as Rb7 or LAMP1 [30, 31] and tryptophan-aspartate containing coat protein (TACO), normally removed from the phagosome before fusion with the lysosome [32]. The mechanism involved in the inhibition of the phagolysosomal fusion starts with the prevention of the generation of PI3P

kinase (phosphatidylinositol 3 –phosphate) by a mycobacterial phosphatase called SapM [33] and inhibition of recruitment of Ca^{++} influx by LAM (lipoarabinomannam). This mechanism was observed when comparing killed and live mycobacteria internalized by macrophages [34]. The potential influence on trafficking and phagolysosomal fusion affects the presentation of antigen that would potentially activate cytotoxic T cells [35].

Infection of human macrophages by *M. tuberculosis*, results in the secretion of TNFα, IL-1, IL-6, IL-8, RANTES and MCP-1, that would potentially induce the recruitment of T cells to the site of infection and to the granuloma. IL-10 is also secreted by infected macrophages, potentially suppressing the Th1 response needed to control the infection [7, 36]. Another consequence of trafficking influence in the cell by Mycobacteria, is the reduced expression of MHC-II potentially linked to stimulation via TLR-2 [37, 38].

The interaction of mycobacteria with macrophages and dendritic cells is the object of intense studies due to the consequences of infection in these 2 different cells. Dendritic cells (DC) are the most potent antigen presenting cell and responsible for initiating the immune response. DC are able to uptake mycobacteria via DC-SIGN, a receptor used by the pathogen to evade the immune system [29, 39-41]. Following uptake, DC of both humans and bovine mature, increase their expression of MHC-II and co-stimulatory molecules CD80-CD86. Infected human DC secret several cytokines such as IL-12, TNFα and IL-1, but bovine infected DC secrete mainly IL-12 [7]. Differences exist between bovine DC and macrophages. It was demonstrated that bovine dendritic cells are more permissive for *M. bovis* replication than macrophages, but as far as

cytokines, they release more IL-12 and induce a better immune T cell proliferation than macrophages [36, 42]. In this work, DC and macrophages were treated with IFNγ to evaluate a potential decrease of *M. bovis* viability. Only macrophages had their number of acid fast bacilli decreased upon IFNγ treatment. Such observations result in a concern that DC would be major reservoirs of *M. bovis in vivo.* By the same time, a low bacterial turnover in DC would be reflecting in the constant availability of antigens to be presented to T cells.

Granuloma: a hallmark of tuberculosis

After the inhalation of aerosol containing *Mycobacterium tuberculosis* / *M. bovis*, an initial host-cell interaction of either macrophages or DC will provide the first line of defense against infection by secreting cytokines and triggering of chemoattractant mechanisms [43, 44].The interaction of pathogen with the different host cells will determine the outcome of the disease by either controlling or clearing it, or resulting in disease progression. Granuloma morphology is characterized by a central necrotic core surrounded by concentric layers of macrophages, epithelioid cells, multinucleated giant cells and lymphocytes [45-48].

Granuloma distribution presents variability depending on the species. In naturally infected cattle, lesions are most frequently found in the dorso-caudal region, close to the pleural surface. This lesion location seems to be the most distal end and the longest route in the lungs, probably related to the size of the droplets containing the pathogen and the

topographical orientation that influences the air flow in lung [49]. In humans, it was shown that lesions are more frequently found in the apical region of the lung [50]. Besides lungs, lesions are also frequently located in the lymph nodes responsible for draining the region, such as the mediastinal lymph node. Several studies have also found involvement of the retropharyngeal, submandibular and parotid lymph nodes as well as tonsil and pharynx, suggesting that the pathogen may be excreted from the surface of these tissues [49].

The center of the granuloma is characterized by an area of necrosis where mycobacteria may still be intact and be a potential infection source for other macrophages. The real battlefield in the granuloma happens in the surroundings where pathogen and immune cells come together. The inner cell layer of the human granuloma does not contain $CD8^+$ T cells, but these cells are found on the granuloma periphery surrounded by APC and B cells [51]. B cell aggregates were found in both humans and mice, where B cells in different activation stages are observed [52]. Similar observations in bovine showed that B cells were present but more restricted to outer fibrotic capsular wall of advanced lesions [53, 54]. Granulomas in mice can be characterized by not being organized and lacking the classical structure, observed in other species [55]. In calves, mice, rats, guinea-pigs and hamsters, the initial lesion formation is performed by migration of neutrophils, γδ T cells and NK cells that either kill macrophages or produce IFNγ [56]. From these innate immunity players, γδ T cells are the ones that accumulate in abundant numbers early in infection, in both cattle and mice [57, 58]. These events culminate with further migration of $CD4^+$ and $CD8^+$ T cells. In a study where bovine

granulomas were analyzed for the cell composition, the authors demonstrated that cell composition is dependent on the stage of the granuloma. Lesions that are in development have more abundant γδ T cells than advanced lesions, but as happens in humans, lymph node granulomas of unknown duration have around 80% of $CD4^+$ and $CD8^+$ T cells [59].

Among the cytokines that exert important functions in granuloma formation is TNFα. This cytokine can promote beneficial or detrimental effects on the granuloma. Increased blood flow and coagulation upon TNFα action will ultimately result in tissue necrosis [49]. Secretion of TNFα seems to be detrimental for the Th1 response and favors a Th2, characterized by the presence of IL-4. This last cytokine has been involved in the formation of so called cavitation in tuberculosis, a phenomenon that occurs mostly in humans [60]. Cavitation was also observed in guinea-pigs, rabbits and primates but not in cattle. It is hypothesized that cattle may develop cavitation, but it is not observed due to the detection and culling of the animals [49]. The cavitation process is a consequence of liquefaction via a process facilitated by metalloproteinase enzymes that act on solid caseous material [61, 62]. Bovine granulomas are classified in four stages according to their cellular composition and degree of necrosis [7]. Stage 1 granulomas are characterized by not having necrosis and consisting of primarily epithelioid cells, few giant cells and some lymphocytes. Stage 4 granulomas are the ones where extensive necrosis is observed, large number of epithelioid cells surrounded by $WC1^+$ T cells and B cells [57]. In experimental infections and vaccine trials using the bovine model, animals receive a score based on their lung radiographic evaluation, where score 0 has no lesions

and score 5 has gross coalescent lesions [63]. More details of the immune response against myobacterial infection are discussed in subsequent sections.

The immune response against *Mycobacterium spp.* infection

The initial response against TB infection is characterized by the engagement of *Mycobacterium spp.*(Mtb) ligand with Toll like receptors (TLR). *Mycobacterium spp.* has a broad range of ligands able to induce activation of different TLRs. Among the proteins that are TLR-2 agonist are the secreted 19 KDa lipoprotein, lipoarabinomannan and phosphatidyl-myo-inositol. Interaction between TLR-2 and the Mtb 19KDa lipoprotein results in production of TNFα, IL-12 and nitric oxide production in macrophages [64]. The same 19 KDa lipoprotein either from *M.tuberculosis* or *M. bovis* BCG, was shown to promote macrophage apoptosis. Macrophage apoptosis can be considered an evasion mechanism as a way to promote disseminated mycobacterial infection [65]. Apoptosis of infected macrophages can also induce $CD8^+$ T cell stimulation via cross-priming. It was shown that apoptotic vesicles from macrophages could be internalized by bystander DCs and further activates CTL [66]. There are reports that apoptosis may be present in more than 50% of macrophages in the granuloma [67]. TLR-4 deficient mice did not exhibit any compromised responses against TB, unless a high dose of *Mycobacterium* was used [68]. Besides TLR-2 and TLR-4, TLR-9 was also responsible for inducing IL-12 secretion by DCs, but the same was not observed in macrophages, where TLR-2 was the

main inducer [69]. The lack of activity by both TLR in mice seems to be linked to tuberculosis progression [70] .

1-γδ T cells

These cells are early participants in the immune response against *Mycobacterium* infection and are recruited promptly to the infected lung. In the presence of phosphoantigens and IL-2, these cells proliferate and respond to mycobacteria infected cells by secreting IFNγ and being cytotoxic [71, 72]. In humans a population designated Vγ9Vδ2 appears to be the main population involved in mycobacteria response and is present in circulating blood. Besides the secretion of IFNγ, these cells secrete TNFα and induce IL-15 production by DCs [65]. IL-15 can further induce proliferation of $CD8^+$ T cells and activate NK cells. Mouse γδ T cells also proliferate 1 week after infection with *Mycobacterium spp.*

Bovine γδ T cells differ from mouse and human γδ T cells, initially related to numbers encountered in blood. They correspond to approximately 2-7% in both humans and mice, 10-15% in cattle, but can be up to 40% in young calves from the total PBMC population [73]. A role for the high number of these cells in young calves is related to a possible early mechanism to produce Th1 cytokines [74]. Bovine γδ T cells use another nomenclature, based on the Workshop Cluster 1 (WC1), and are then classified as $WC1^+$ γδ T cells and $WC1^-$ γδ T cells. The first population is the predominant population on

bovine blood from young and adult ruminants, and $WC1^-$ γδ T cells are predominantly located in spleen and mammary gland [75].

Upon infection of cattle with *M. bovis* by intranasal route, there is an initial decrease in the size of the population of the $WC1^+$ γδ T cells due to migration to the infection sites, with a subsequent expansion in the circulation [76]. Analysis of the bovine skin (after DTH) or granulomas identified $WC1^+$ γδ T cells as one of the primary cells that are present [57, 77]. The rapid migration and expansion upon infection may be considered as a primary effort to control infection against *M. bovis*. Even though it has been demonstrated that these cells can produce IFNγ and TNFα, the fast response observed upon infection might be related to the production of chemokines and other cytokines that promote recruitment of other T cells. Depletion of $WC1^+$ γδ T cells had minor effect on infection, mainly related to the anatomy of the granuloma [73], and probably related to the lack of TNFα that was secreted by this cell type. Another study showed that besides differences in the granuloma, there was an initial decrease of IFNγ with a potential to compromise the Th1 bias upon infection with mycobacteria in favor of a Th2 response [78].

2- NK cells

NK cells together with γδ T cells are important players of the innate immune response. NK cells are large granular lymphocytes, bone marrow derived, with the ability

to lyse tumors cells, viral and bacterial infected cells [79-81]. NK cells are characterized by expressing activating and inhibitory receptors, where action of a single or a combination of receptors will determine the outcome of the interaction of the NK cells with possible targets [82]. NK cells are able to distinguish normal healthy cells from abnormal cells by using a mechanism that is dependent on the expression of MHC-I molecules on cell surface. Activation or deactivation is dependent on expression of several inhibitory receptors on the surface called KIR –killer cell Immunoglobulin like receptors [83]. NK cells are not restricted to bone marrow, but are also encountered in other organs. The origin of NK in cattle is unknown, but in mice and humans they originate from an hematopoietic stem cell that colonizes liver, thymus, bone marrow and spleen [84]. Different locations of the NK cells suggest an explanation why a diversity of markers is needed in order to get a purified population from blood [84, 85].

Besides being important in the innate immune response, the interaction of NK cells with antigen presenting cells such as DC, provides a link between innate and adaptive immune response [86, 87]. Bovine NK cells are present in higher number in neonates (≥ 10%) than in adults and the number decreases with age [88], similar to what has been observed in humans [89]. NK cells are an important source of IFNγ in the immune response against pathogens such as *M. tuberculosis* [90]. Upon infection of mice with *M. tuberculosis*, there was a substantial increase of NK cells in the lung in the initial 3 weeks [91]. Recruitment to lymph nodes was shown to be dependent on chemokine receptor CXCR3 and CD62L-dependent mechanisms following activation by DC or adjuvant [92].

Bovine NK cells have been the subject of many recent studies. A lot of progress in the characterization and understanding of activator/inhibitory conditions has been clarified in the last 5 years. Much of the progress on the phenotypic and functional characterization of bovine NK cells was advanced by the characterization of a surrogate marker called NKp46 [82, 93] where the use of an antibody to NKp46 provides more than 98% pure NK cells [42]. NK cells are very important in the response of bovine neonates against infection with *M. bovis*. The higher number of NK cells on animals of young age seems to provide a mechanism by which the innate immune response is able to compensate for the immature state of the adaptive system in these animals. It is demonstrated that neonatal vaccination of cattle and humans enhances the protection of the BCG vaccine as compared to adults [7]. Bovine NK cells were demonstrated to be able to restrict the replication of *M. bovis* in infected bovine macrophages dependent on activation by IL-12 and IL-15 [81]. They also enhance production of IL-12 and NO by infected macrophages, when they were previously incubated with IL-2 [94]. The production of IL-12 can serve to stimulate the production of more IFNγ and further activate macrophages for the killing of intracellular pathogen.

Furthermore, it is not known if different populations of NK cells can induce different responses according to the tissue they are homing. NK cells, as $CD8^+$ T cells are characterized by the production of granule proteins such as perforin and granulysin. Production and release of granules in infected cells induce lyses or apoptosis and killing of intracellular pathogens such as *Mycobacterium sp*. and viruses [79, 95-100]. It is well described that there is a cross talk between dendritic cells (DCs) and NK cells, and

reciprocal activation via different cytokines such as type I interferon. The cross talk is fundamental to link innate and cell immunity.

3-$CD4^+$ and $CD8^+$ T cells response against *Mycobacterium spp.*

Requirements for T cell mediated immunity against tuberculosis are well defined. Both $CD4^+$ and $CD8^+$ T cells are essential to effectively control infection with *M. tuberculosis* or *M. bovis* shown by the lack of $CD4^+$ T cells in HIV positive patients and the use of knock –out mice [7, 101]. $CD4^+$ T cells are major producers of IFNγ, able to activate macrophages and induce mycobacterial killing intracellularly [58, 102]. Besides the production of IFNγ, both T cell populations have been shown to have important cytotoxic function, by releasing granzymes, perforin, granulysin, and by expressing Fas-L. The T cell involvement on the response against tuberculosis in cattle is characterized by a sequential mechanism initiated by the migration of γδ T cells, followed by $CD4^+$ T cells and later on increased involvement of $CD8^+$T cells [76, 103]. Both $CD4^+$ and $CD8^+$ T cells are activated by *M. tuberculosis* and *M. bovis*. $CD4^+$ T cells respond to MHC-II carrying mycobacterial antigens and $CD8^+$ T cells respond upon engagement with MHC-I. Several alternative pathway theories to explain peptide load to MHC-I have been generated. A recent review by Vyas et al., (2008) [104], suggests that soluble antigens may be loaded to MHC-I by a process involving leaking of phagosome vesicles. Activation of $CD8^+$ T cells has been described after treatment with mycobacterial soluble

antigens such as bovine PPD [76, 105] resulting in proliferation and IFNγ production by $CD8^+$ T cells. In summary, interplay involving $\gamma\delta^+$, $CD4^+$ and $CD8^+$ T cells is essential for the immune response against mycobacterial infection.

Mechanism of intracellular *Mycobacterium* killing

1-The $P2X_7$ purinergic receptor

Mycobacteria are particularly resistant to intracellular killing mechanisms and only succumb *in vitro* to the effects of granulysin or activation of the infected cell through the $P2X_7$ purinergic receptor [106, 107]. Binding of ATP to $P2X_7$ purinergic receptor induces macrophage apoptosis and mycobacteria killing. It seems that ATP accumulates in the extracellular inflammatory fluid in a concentration sufficient to stimulate $P2X_7$ receptor . Several sources of ATP are reported including release from necrotic cells, secretory granules from CTLs and export via plasma membrane ABC transporters . Macrophages are also able to produce ATP that can be used in an autocrine fashion by further activating themselves and induce a microbicidal activity [108]. The ATP release from macrophages was also shown to be a consequence of *M. tuberculosis* infection [109]. Even though no exact measure of ATP in the extracellular fluid has been done, experimental evidence of ATP function is demonstrated by using 3mM of ATP. The concentration used on *in vitro* studies has to be evaluated carefully. Studies using human macrophages exposed to degranulating T cells or lysed bystander cells failed to

control mycobacterial growth and mice lacking $P2X_7$ receptor ($P2X_7$ -/-) that had the same bacterial counts as wild type mice [110].

The signaling cascade of $P2X_7$ purinergic receptor is not fully understood, but it was shown that several events are induced upon activation. Upon ATP-$P2X_7$ engagement, activation of Caspase-8 by epithelial membrane protein -2, induces macrophage apoptosis independent of events that result in mycobacteria killing [111, 112]. This last pathway is shown to be induced by activation of Phospholipase D (PLD) and phosphatidic acid. This results in phagosome-lysosome fusion (P-L) and /or modulation of the phagosome pH within the cells and killing of the resident mycobacteria [111, 113]. A more recent observation shows that autophagy together with apoptosis may be directly linked to the ATP-mediated killing of mycobacteria [112].

Other effects were also observed after ATP treatment of macrophages. In addition to apoptosis and mycobacteria killing, upon ATP binding, $P2X_7$ turns into a promiscuous channel that is involved on different functions: increase of intracellular Ca^{++} by allowing extracellular Ca^{++} influx in the cell; increased permeability to molecules with size up to 900Da; and release of K^+ and other molecules [107, 109, 114, 115]. It is not clear if Ca^{++} influx is an event independent of P-L fusion and mycobacteria killing [113], but it was shown that ATP-mediated killing of mycobacteria in *M. tuberculosis* infected macrophages and BCG infected human macrophages was dependent of Ca^{++} influx event. The role of $P2X_7$ was also demonstrated in bovine where alveolar macrophages and peripheral blood-derived macrophages had increased $P2X_7$ mRNA expression upon infection with *M. bovis* and responded to ATP treatment by decreased

viability of mycobacteria. Such characteristic was not observed when inducing complement mediated lysis of macrophages or cross-linking CD95 (Fas-L) [107].

In addition to the above described effects of $P2X_7$ activation, T cells seem to respond to ATP binding on the receptor by also forming a promiscuous pore that ultimately can induce cell death [116]. Macrophages treated with concentrations lower than 3mM were shown to release IL-1β or result in multinucleated cells [117-119]. When added, all ATP- $P2X_7$ binding effects seem to converge on an effort to eliminate cells infected with microorganisms that can potentially evade the immune system, and may act concomitant to the action of IFNγ and nitric oxide. To better understand the effects of $P2X_7$ activation, please refer to Illustration 1 (page 24).

2-Nitric oxide

Nitric oxide (NO) is another very important molecule in the cell mediated response against mycobacterial infection. Nitric oxide is a product of, but not restricted to, macrophages activated either by cytokines or bacterial products such as LPS or both. It is derived from L-arginine and enzymatic activity of the nitric oxide synthase (NOS). NO has several other functions, not being restricted to the killing of pathogens. It can be produced in response to tumors, necrosis and functions in other cells as a molecule that

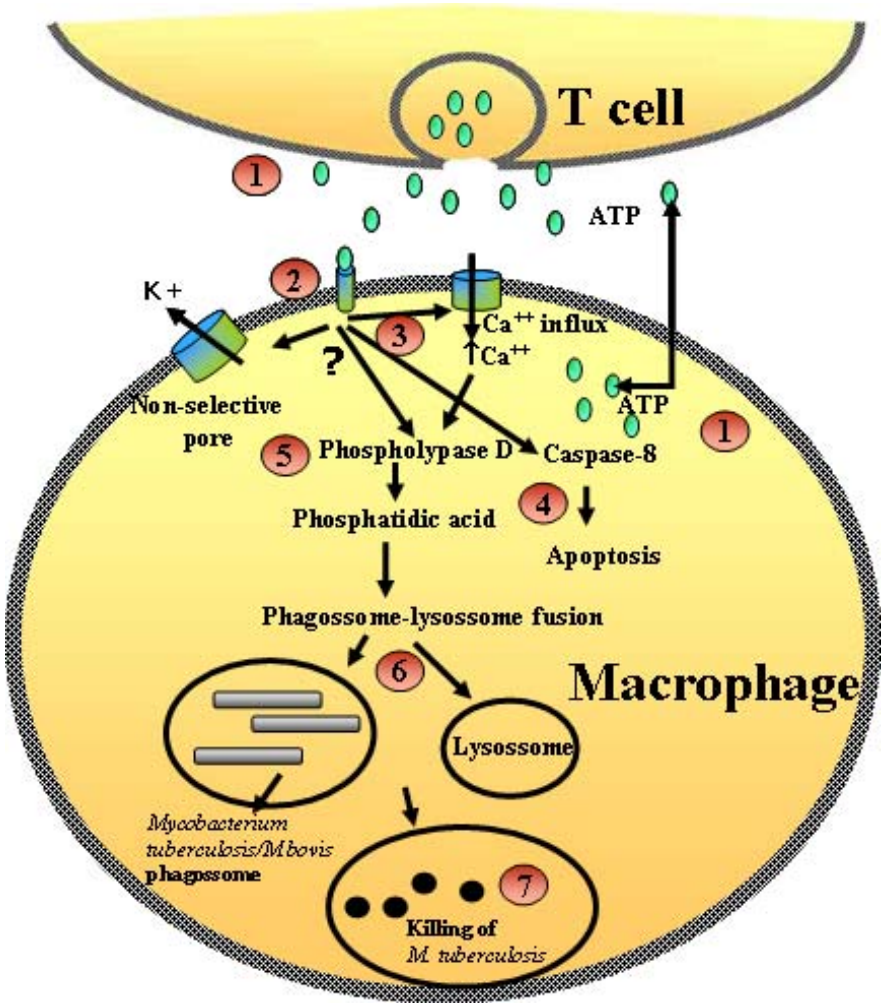

Illustration 1: The $P2X_7$ pathway. **1**.ATP is released in the extracellular environment through T cell secreted granules, or necrotic cells, or exported by plasma membrane ABC transporters or from the own infected macrophage. **2**. ATP binds to $P2X_7$ receptor. **3**. A signaling cascade starts by inducing and influx of extracellular Ca^{++} and increases the intracellular Ca^{++}. **4**. Macrophage apoptosis is induced via activation of caspase-8. **5**. Non-selective pores are created on the cell membrane and Phospholypase D (PLD) pathway is activated. **6**. PLD activation leads to phosphatidic acid increase and phagolysosome fusion (P-L). **7**. Mycobacteria are killed in response to acidification and P-L fusion. Macrophage apoptosis and killing of intracellular mycobacteria can now be engulfed by Dendritic cells and prime T cells via cross presentation. Adapted from references [107, 111, 113, 120].

stimulates cytokine production such as IL-1, IL-6, IL-10 and IL-12 [121]. NO activation conditions changes depending on the species. In mice and humans, IFNγ associated with a response to LPS seems to be enough to elicit an antimicrobial effect [122]. In cattle, IFNγ alone was not able to induce or induced low levels of NO in bovine monocyte-derived macrophages and alveolar macrophages, but NO was observed when cells were treated with IFNγ and exposed to LPS [123]. In humans, NO is not induced by either IFNγ and/or LPS. On the other hand, it has been shown that IL-4 and anti-CD23 or IFNα/β were more successful on inducing NO [124]. Please refer to illustration 2 (pg. 34) for better understanding of this mechanism.

3-Granulysin

Granulysin was initially identified in effector T cells by subtractive hybridization [125]. Granulysin is a cationic protein with broad spectrum antimicrobial activity, including gram negative and gram positive bacteria, protozoa, fungi, and parasites [95, 99, 106, 126, 127] One of the most important antimicrobial activities associated with granulysin is the ability to directly kill mycobacteria *in vitro* [128]. Granulysin is expressed by various human effector populations including $CD4^+$, $CD8^+$ $WC1^+\gamma\delta$ T cells and NK cells [125, 129]. The molecular structure of granulysin indicates that it is a member of the saposin-like protein family [130] and is characterized by having a five helix bundle as demonstrated for other proteins of the same family [131]. Much of the

lytic activities of granulysin were demonstrated based on the use of peptides derived from the granulysin sequence [126, 127, 132]. These peptides were demonstrated to have high cytotoxic/broad spectrum antimicrobial activity against both gram positive and gram negative bacteria [97, 106, 133]. Homologues of granulysin have been characterized for several species, including pigs, horses and cattle [79, 95, 134]. The gene for granulysin has not been identified in any rodent species to date, restricting the use of gene deficient animals for *in vivo* studies. The bovine homologue of granulysin, based on nucleotide and amino acid sequences, exhibits a relatively high degree of conservation of functional domains with the other granulysin homologues, including a predicted 5 helix bundle and demonstrated antimicrobial activity against several types of bacteria such as *E. coli* and *M. bovis* BCG [79].

Importantly, cytotoxic activity by T lymphocytes against *M. bovis* BCG infected macrophages correlates to increased expression of granulysin in human T cells isolated from the peripheral blood of PPD reactive subjects [135]. Successful chemotherapy in pediatric tuberculosis patients has also been correlated with increased expression of granulysin by peripheral blood leukocytes [97, 136]. *Mycobacterium leprae* (*M. leprae*) specific $CD4^+$ T cell clones express greater amounts of granulysin following *in vitro* antigen exposure while greater levels of perforin are expressed by *M. leprae* specific $CD8^+$ T cell clones [137]. Expression of granulysin by $CD4^+$ T lymphocytes in contact with *M. leprae* infected macrophages in that study was associated with containment of the leprosy lesion. The human Vγ9δ2 gamma delta T cell subset proliferates dramatically in response to mycobacterial antigens. The proliferative capacity of this gamma delta T

cell subset however, correlates to establishment of chronic tuberculosis infection while expression of IFNγ and granulysin by the same cells correlate to recovery [136]. To better understand the granulysin mechanism of release and action, please refer to Illustration 3 (pg 35).

Other mechanisms involved in cell mediated immunity against mycobacterial infection

1- Fas ligand (Fas-L)

Fas-L is a type II transmembrane protein of 40 KDa that belongs to the tumor necrosis factor superfamily (TNF). Fas-L is one of the effector mechanism involved in target cell killing by T cells and NK cells, together with the release of cytotoxic granules which include granulysin, perforin and granzymes [138]. Besides being encountered as a transmembrane protein, Fas-L can also be found as a soluble factor. This soluble factor exists as a trimer [139, 140] and can be either cleaved from a transmembrane Fas-L by metalloproteinases or released as a soluble from T cell granules [140]. Coupling of Fas-L –Fas triggers the so called death-inducing signaling complex (DISC) and recruitment of the adapter molecule Fas associated protein with death domain (FADD). This will further recruit procaspase 8 and induce apoptosis of the cell [138, 141-143]. Besides eliminating potentially harmful cells, Fas-L is also involved on the control of other cells, such as B or T cells. This process is important in limiting excess proliferation of these cells in the

periphery after elimination of an antigen [138]. It was shown also that Fas-L-Fas is involved on the negative selection that occurs in the thymus, but this process also involves other TNF-Receptors such as TNF-R1 and TNF-R2 [144, 145].

The activation of the surface expression of Fas-L in T cells is dependent on TCR engagement with MHC and participation of co-stimulatory molecules CD80/CD86-CD28. Expression of Fas-L upon engagement of these receptors-co-receptors is dependent on the binding of the nuclear factor of activated T cells (NFAT) to the Fas-L gene [146]. Several other proteins have been shown to be involved on Fas-L transcription including NFkB and proteins from the interferon transcription family such as IRF-1 and IRF-2 [147].

2- Granzymes and Perforin

The granzyme family is composed of a series of proteins with serine protease characteristics. Granzymes are classified in various categories by their activities, and their presence is variable depending on the species. Granzymes A, B, H, K and M were found in humans and granzymes A, B, C, D, E, F, G, K, L, M and N were found in mice. Granzymes A and B are the most abundant granzymes and are by consequence the most studied [148]. The action of granzymes was shown to be dependent of perforin, where it creates a pore on the plasma membrane of the target cells allowing granzymes and granulysin to enter the cells, but it is also proposed that granzymes can enter the cell via endosomes, a charge based process, where further action of perforin allows it to be

released into the cytoplasm [149, 150]. It is also proposed that granzyme B uses the mannose-6 phosphate receptor as a way to enter the cell but there is extensive debate on proving this concept [151].

Granzyme B is present in CTL and NK cell granules where it is responsible for inducing apoptosis via proteolysis of several substrates. Granzyme B uses both caspase activation and mitochondrial permeabilization to induce apoptosis of the cell. The Caspase mediated pathway starts with the cleavage of pro-caspase 8 by Granzyme B after aspartic acid residues [152]. The process that results in mitochondrial damage is performed by granzyme B action on the *Bid* (BH3 Interacting Domain Death Agonist Protein) protein with further oligomerization of *Bax* (Bcl-2 Associated X Protein) and *Bak* (Bcl-2 Homologous Antagonist-Killer Protein) in the outer mitochondrial membrane. This results on the release of cytochrome C and assembly of the apoptosome [153]. Granzyme B apoptosis induction seems to be preferentially done by cleaving caspases in mice as in humans, but both caspase and *Bid* cleavage processes are used [154].

Granzyme A is also responsible for inducing apoptosis, but via a slower process than Granzyme B. The process maybe related to different substrates used by granzyme A in comparison to granzyme B. Depolarization of the mitochondrial membrane has also been shown, which results in a rapid increase of reactive oxygen species and killing of the cell [155]. In regard to the other granzymes of mice or humans not much is known, but some findings have been very interesting showing target cell killing by a pathway independent of granzyme A or B. Granzyme H has been shown to induce chromosome condensation and nuclear fragmentation together with mitochondrial depolarization and

production of reactive oxygen species, all independent of using caspase or *Bid* as substrates [156]. The divergent pathways used by granzymes in different species cannot be considered as independent events, but maybe better understood as a complex mechanism of action, where different granules can exert the same function at the same time in a redundant way to control infection and protect other cells from being infected.

The last granule protein discussed here is perforin, an essential lymphocyte effector molecule. The lack of this molecule in both mice and humans results in drastic consequences with severe immune deficiencies or disorders in the lymphoid and myeloid system [157]. As mentioned before, perforin facilitates the entry of granzymes and granulysin into the target cells by forming pores in the cell membrane [158, 159]. Perforin is around 67 KDa, and its expression is regulated by lymphocyte activation receptors and cytokines [160]. Biosynthesis and storage of perforin have interesting characteristics. Once synthesized, it is carried by calreticulin to lysosomes, where an acidic pH promotes cysteine protease cleavage, when it is then further released. The whole process of transporting perforin and safe-guarding it promotes the safety of the cells that produce it [161]. Once released from the T/NK cell, perforin will encounter a neutral pH that will release it from its carrier, and promote aggregation and action in the target cell membrane by a Ca^{++} dependent mechanism [162, 163]. After multimerization and pore forming, granzymes and granulysin can use these pores as a passage tunnel to the cell cytoplasm. The granzyme A and B, as well as perforin pathways are shown on Illustration 3 (Page 35).

Measurement of immune correlates of protection following vaccination

The development of better vaccines against TB culminates with the development of reliable prognostic indicators of a protective immunity following vaccination. A more recent definition of correlates of protection is called biomarkers. Biomarkers are defined as a set of characteristics that objectively measures and evaluates as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention [164]. The biomarker term does not need to constitute a single molecule but a combination of other indicators that will classify an individual of a group. The current biomarkers used to evaluate protection upon vaccination against TB are IFNγ secretion and leukocyte proliferation and increasing evidence demonstrates that they are not adequate [136, 165-168]. Tests used to discriminate between BCG-vaccinated and *M. tuberculosis* infected individuals also need to be developed and improved for rapid identification of infected subjects with high specificity. The worldwide used tuberculin skin test does not discriminate between subjects vaccinated with BCG and infected with Mtb [169]. A new test based on the measurement of the response against 2 *Mycobacterium spp.* secreted proteins (ESAT-6 and CFP-10) present a potential solution for this problem [170].

The frequent lack of association between protection from challenge and the secretion of IFNγ or leukocyte proliferation following *in vitro* antigen exposure, indicates the need to identify additional prognostic indicators. Other potential correlates under

evaluation are related to the analysis of cytokines secreted by T cell sub-populations after antigen specific stimulation [164]. It is clear that protection after vaccination from TB cannot be measured by a single marker. Instead several markers added to the current IFNγ have to be used. In our approach, we are examining the potential use of granulysin and perforin as potential biomarkers of protection in association with IFNγ.

BCG and other vaccines against tuberculosis

BCG has a long history as a vaccine given to neonates, due to several advantages such as protecting against milliary tuberculosis. However, BCG presents variable efficacy, it does not provide protection against adult lung tuberculosis, and cannot be used in immunocompromised patients [171, 172]. Efforts are in progress to develop new vaccines against TB, based either on pre-exposure to environmental mycobacteria or a boosting effect later in life with previous BCG vaccination. The use of DNA vaccines has shown promising results either in bovine or mice as potent adjuvants [166, 173] (Appendix 3). DNA vaccines elicited both cell-mediated and humoral responses to encoded antigens and are an option as a boosting strategy such as in immunocompromised individuals [174, 175]. Vaccination in cattle with BCG and co-administration of rFLT-3L and rGM-CSF enhanced $CD4^+$ T cell response [176]. Some problems exist related to DNA vaccines in large animals, such as high amounts of DNA delivered in multiple doses [177]. Another potential strategy using BCG is based on priming with BCG and boosting with a modified vaccinia virus that express

mycobcaterial antigen Ag85A [178-181]. This vaccine is currently in Phase II clinical trials in humans in Africa. Work published by our group showed enhanced protection when a DNA vaccine based on CD80 and CD86 or ESAT-6/CFP-10 was co-administered with BCG [166, 182].

Several mycobacterial mutants have been shown to be an option for vaccination. Auxotroph mutants of *M. bovis* or *M. tuberculosis* constructed based on deletion of genes that abrogate growth of the pathogen, but maintains its effect as a potent vaccine. Auxotroph mutants of *M. tuberculosis* showed protection in mice and guinea-pigs upon challenge with virulent *M. tuberculosis* [183, 184] or *M. bovis* [185]. More recently, an autoxtrophic vaccine candidate, characterized by lacking RD1 and the panthothenate pathway (Δ*RD1*Δ*panCD*), and designated $mc^2$6030, failed to protect in our bovine model. The same strain tested in monkeys also did not elicit protection [63]. In our studies we are using another potential vaccine candidate called *M. bovis* Δ*RD1* (Appendix2). The strain has the same characteristics of the gold standard BCG, and it is expected to have same vaccine efficacy as BCG. If successful, the strain will be used for further deletions and tested on our bovine model. Overall, vaccines that elicit similar response in bovine neonates and primates can be considered as a potential vaccine candidate for children.

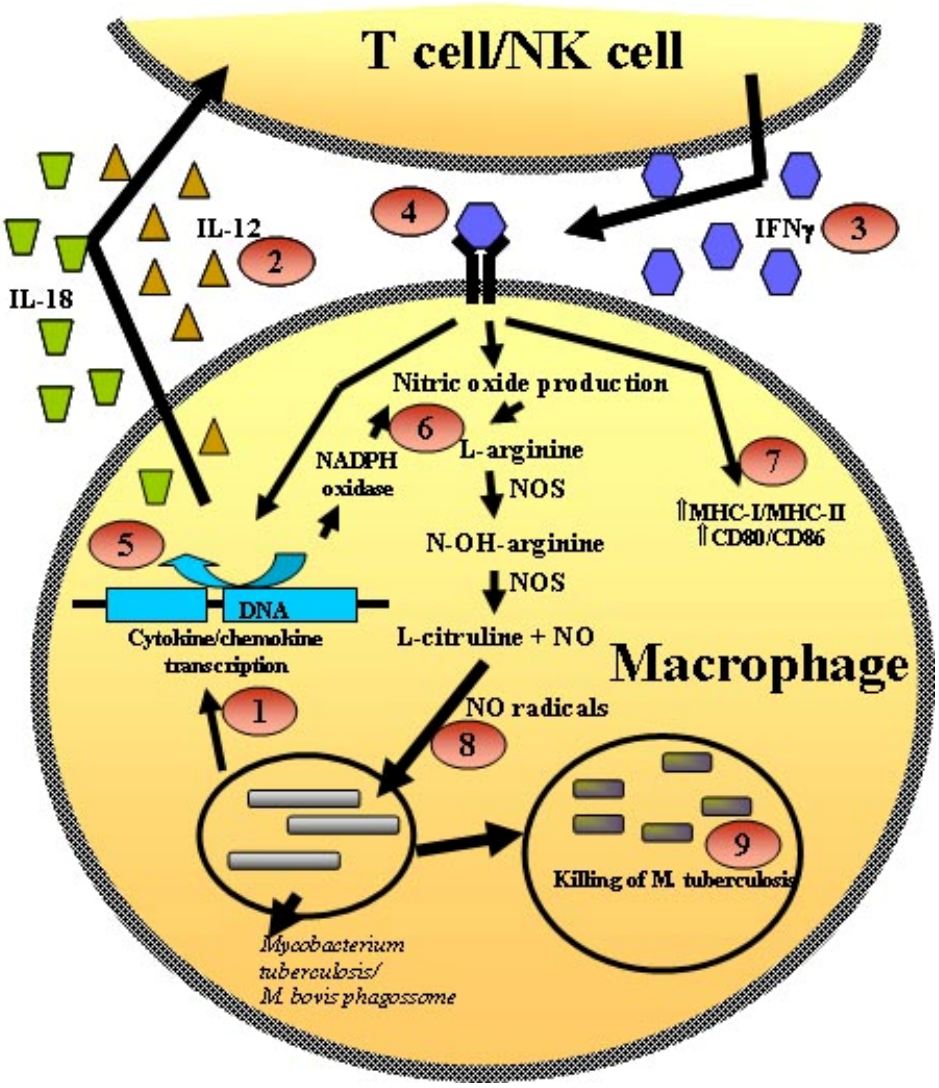

Illustration 2: Nitric oxide production pathway. (1) Uptake of mycobacteria by macrophages and/or Dendritic cells will result in cytokine, chemokine gene expression, such as IL-12 and IL-18. (2) In response to IL-12 and/or IL-18, T cells and NK cells will produce IFNγ, a potent macrophage activator (3). (4) Binding of IFNγ to the receptor (IFNGR1/IFNGR2) results in the activation of several pathways including IFNγ and NADPH protein complex gene expression (5) and increased expression of MHC-I, MHC-II and co-stimulatory molecules (7). (6) Formation of the NADPH complex induces the production of Nitric Oxide radical by activation of the enzyme Nitric oxide synthase and further mycobacterial death (8-9). Adapted from references [42, 64, 94, 121, 186-193].

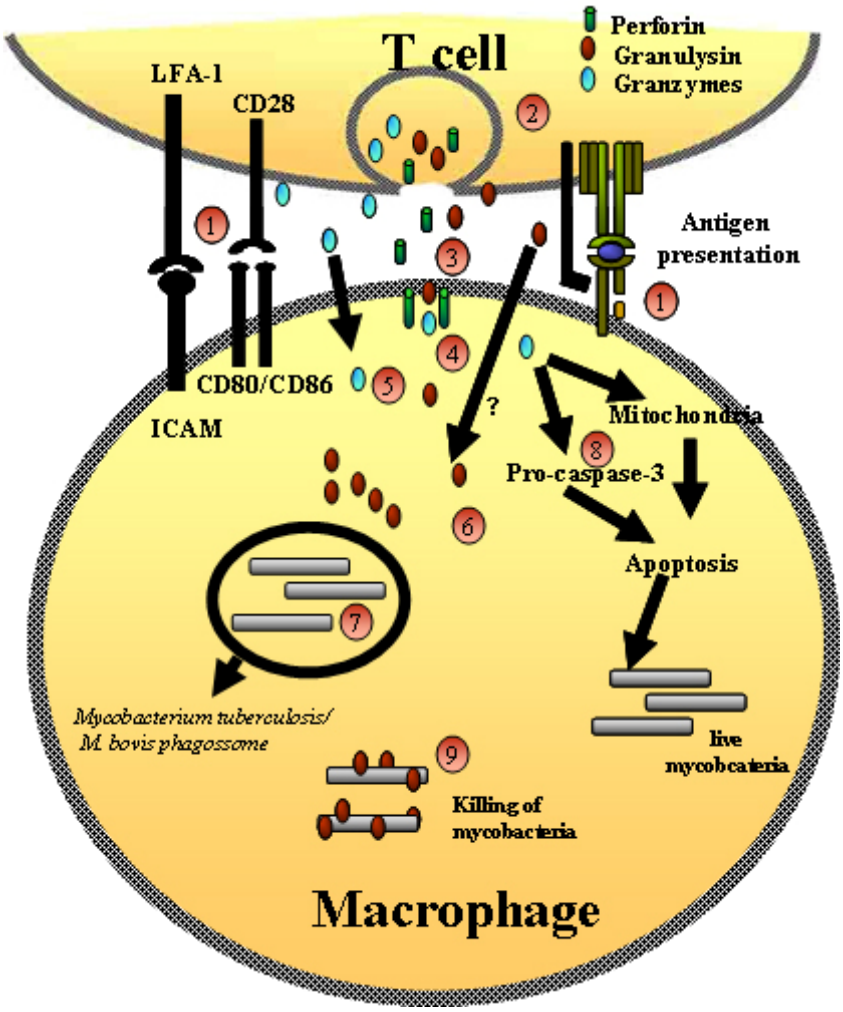

Illustration 3: Cytotoxic granule release pathway. (1) Upon the encounter of a T cell with the macrophage and formation of the immunological synapse, T cells ($CD4^+$ or $CD8^+$) recognize the antigen via their MHC-II or MHC-I (depicted on this cartoon). (2) The further activation of the T cells induces the release of cytotoxic granules on the immunological synapse clef. (3) Perforin, a pore forming granule promotes a passage for granzymes and granulysin to enter the cell. (4) Potential pathway involving entrance of granzymes and granulysin into the target cell cytoplasm (in this case, a macrophage) independent of perforin were shown for granzymes (5) and may possibly be involved on granulysin (6). The action of granzymes will ultimately result in apoptosis induction by cleavage o pro-caspases or damage of the mitochondrial membrane (8). Granulysin once inside the cell will promote killing of *Mycobacterium tuberculosis* /*M. bovis* (9). The combined action of all cytotoxic granules will result in the killing of the macrophage and of the pathogen. Cartoon adapted from information from the following references [81, 95, 98, 106, 126, 128, 153, 157, 160, 161, 194-203].

Animal models to study tuberculosis and to evaluate vaccines

Several animal models are used to study human tuberculosis, including mice, guinea-pigs, rabbits, cattle and monkeys, but each model has its own limitation. Guinea-pigs and rabbits are good models to study TB pathogenesis, mimicking several aspects of the pathology present in humans such as cavitation [204]. The problem with these 2 models is that they are highly susceptible to fatal disease [55, 205]. Taking in consideration the cost of the animal model, mice are the ones most affordable and easy to handle. Other advantages of using mice are the number of reagents available and the genetic manipulation that can be done on this species such as gene knockout [206, 207]. The disadvantages of using mice include their natural resistance to TB, differences in granuloma formation which is characterized by lack of organization and classical structure [55]; different repertoire of ligands for γδ T cells, lack of group 1 CD1 molecules and lack of the gene that encodes granulysin [207]. Due to the natural resistance of mice to TB, latency studies are difficult to perform, unless combined with chemotherapeutic treatment [208-210]. In experimentally infected mice, the pathogen replicates for 2-3 weeks and then persists to a constant titer (between 10^4 and 10^6 cfu) for up to 12 months [55, 211].

Following the use of mice as a model, guinea-pig and non-human primates are the next ones in the progression to human trials. Guinea-pigs are less expensive as compared to non-human primates, but the lack of reagents, and special requirements regarding

husbandry of these animals, are some of the limitations of their use. Guinea-pigs have been extensively used to test vaccine potency and biological standardization of tuberculin before human testing, as well as the testing of new drugs against TB [205, 210]; and are excellent models to study pathogenesis and the TB pathology, since they have several aspects that are similar to humans. Rabbits also follow the line of guinea-pigs, with similar pathology to humans but they also lack immunological reagents, and require more space and care as compared to mice and guinea-pigs [210].

Another potential model that could be used to study pathogenesis, vaccine efficacy and reactivation, is the bovine model. As discussed earlier, cattle present several similarities to humans in terms of the immune response, pathogenesis, and the variable efficacy of BCG [49, 212, 213]. Other characteristics are the characterization of bovine homologues of granulysin, present in humans and absent in mice, and perforin [79], as well as vaccine trials of vaccine candidates that show similar results in bovine and in non-human primates [63]. The use of the bovine model is further strengthened by results presented in the current work, where potential new correlates of protection have been evaluated. The primate model is the best model to be used, since it is the one that more closely resembles what happens in human TB. The main problem with the use of non-human primates is the cost for both acquisition and maintenance of the animals, as well as ethical considerations.

The neonate immune response and the bovine neonate model

Neonates are the first target for BCG vaccination and a complete understanding of the immune response in these animals needs to be carefully evaluated. The study of vaccines in neonate humans becomes difficult, since there are no reliable correlates of protection and studies that could evaluate natural exposure to mycobacteria would take several years to be done and most important of all, is the ethical consideration. Neonatal cattle offer several advantages such as exposure to environmental mycobacteria early in life, immunocompetency at birth and the ability to challenge the animals [213]. Evidence suggests that neonate cattle have higher number of NK cells and γδ T cells in the total T cell population when compared to adult cattle [81]. Bovine TB is caused by *M. bovis* which is characterized by similar pathology and immune responses when compared with *M. tuberculosis* infection in humans. A study in dairy calves showed a strong cell-mediated immune response upon vaccination with BCG, as generated in humans at comparable stages of development. The same study in calves showed comparable cell-mediated immune response with adults [214]. The experimental use of BCG vaccination in cattle also showed a variable efficacy as observed in innumerable human studies [165]. As occurs in humans, TB was demonstrated to be localized in the respiratory tract with a very slow course of infection when cattle were aerosol-inoculated [215]. TB in humans can be caused by both *M. tuberculosis* and by *M. bovis* (pathogen of cattle), but as observed in humans, there is no cavitation present in bovine TB infections [212].

Therefore, there is also a concern about vaccination strategies in cattle that can prevent human infections in places where both species are in more close contact. It is not fully understood if the immune response in the lungs of cattle is different than the one in humans, since there might be differences in the specific T cell populations such as γδ T cells [212, 213].

Hypothesis, specific aims, rationale and significance of our studies

It is our hypothesis that **Granulysin expression patterns in different T cell populations is affected by vaccination against tuberculosis (TB) and after challenge with virulent *M. bovis*, and that granulysin expression will positively correlate with protection against TB.** To provide evidence and give support to our hypothesis we divided this study in three specific aims: (1) **Characterize the kinetics and specific activation conditions of Bo-lysin (bovine granulysin) expression in activated bovine lymphocyte populations in parallel with perforin, IFNγ and Fas-L; (2) Evaluate the potential use of granulysin as a correlate of protection following vaccination with *Mycobacterium bovis* BCG and *M. bovis* ΔRD1, and challenge with virulent *M. bovis;* (3) Examine the expression of granulysin in parallel with IFNγ, perforin and Fas-L at the sites of infection of *M. bovis*.**

The significance and rationale of our studies is initially based on the importance of TB as a public health problem, the variable protection elicited by BCG and the lack of

vaccines that can surpass BCG's efficacy. One important gap in the field of vaccine development for TB is the lack of correlates of protection other than IFNγ and T cell proliferation assays. Once better correlates of protection following vaccination for TB are characterized, vaccine trials can be shortened, the challenge with virulent strains can be avoided and multiple vaccines can be tested. As discussed in this manuscript, it is not clear if cytotoxic granules such as granulysin and perforin have the potential for being correlates of protection. Reports of granulysin gene in humans and in bovine, but the lack of granulysin gene in rodent species, suggests the bovine model can be used to examine this molecule as a correlate of protection and discards the possibility of granulysin gene knock-out mice. There is limited information on granulysin kinetics and regulation in the different T cell populations, especially in the bovine model. Activation requirements will be important in determining its use as a correlate of protection after vaccination against TB as compared to other important mediators of Th1 response and responsible for mycobacteria infected cell death, such as IFNγ, perforin and Fas-L.

Nevertheless, studies looking at a vaccine response at different time points post-vaccination/pre-challenge and post-challenge are difficult to be performed due to the requirement of BSL-3 facilities and trained people to perform these studies. At the National Animal Disease Center in Ames, IA, we have the proper facilities and trained people to make these studies possible.

CHAPTER 2
MATERIALS AND METHODS

Animals, immunization and challenge procedures

All immunizations and challenge procedures were conducted at the National Animal Disease Center, Ames, IA and were approved by the Institutional Animal Care and Use Committee of the National Animal Disease Center. BCG- and non-vaccinated animals were obtained as previously described [216]. The vaccine trial consisted of the following groups: non-vaccinated (n=4), 1.8 X 10^5 cfu BCG-Danish vaccinated (n=4) and 1.8 X 10^5 cfu of *M. bovis* ΔRD1 vaccinated (Ravenel strain) (n=4). Vaccine groups were vaccinated at 2 weeks of age and were challenged by aerosol inoculation as previously described [63, 215] with 1.6 X10^3 cfu of *M. bovis* originally isolated from a white-tailed deer in Michigan [5]. All procedures were performed in BSL-3 facilities following strict BSL-3 safety protocols.

Isolation of Peripheral Blood Mononuclear cells (PBMC) and stimulation conditions.

Peripheral blood mononuclear cells (PBMC) were isolated as described previously [216] from 4 healthy bovine donors and from 4 *M. bovis* BCG-vaccinated animals. The different T cell populations- CD4, CD8 and γδ T cells- were enriched using an AutoMACS (Miltenyi Biotech, Bergisch Gladbach, Germany) cell separation system and antibodies to bovine anti-CD4, CD8 and γδ T cells (VMRD, Pullman, WA) and

magnetic bead-conjugated rat anti-mouse IgG1 and rat anti-mouse IgG2 as appropriate. Monocytes were sorted by the same method using human anti-CD14 microbeads, and were cultured for 12 h, with 1400U/ml of recombinant human GM-CSF prior to adding the T cells. Each T cell population was analyzed for purity by flow cytometry as shown in Figure 1. For the antigen specific stimulation, bovine macrophages were pulsed with 5μg/ml of *M. bovis* PPD for 8h, prior to addition of purified T cells (10^6 cells/ml). T cells were exposed to PPD pulsed macrophages (1:5 ratio) for different time points (0, 4, 12, 24, 48 and 72 hours) and then were harvested for RNA extraction and cDNA synthesis. Mitogen stimulation was performed by adding 10ng/ml of phorbol myristic acetate (PMA; Sigma Chemical, St. Louis, MO) and 1 μg/ml of ionomycin (Sigma) to the T cells [79]. Mitogen-stimulated T cells were harvested at the same time points as for the antigen specific treatment. For the vaccine trial, blood samples were collected at 3, 2 and 1 week before challenge and 2 weeks after challenge. PBMCs were purified from 50 ml of whole blood from 12 animals (4 from each treatment group: BCG vaccinated n=4, ΔRD1 n=4 and non-vaccinated controls n=4) by Accuprep (Accurate Chemical, Westbury, NY) density gradient. Purified PBMCs were plated at 10^6 cells/ml of complete RPMI 1640 supplemented with 10% FBS, 2 mM L glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and penicillin-streptomycin (Life Technologies, Grand Island, NY). Cells were either non-stimulated or stimulated with 20μg/ml of bovine PPD, or mitogen stimulated with 10ng/ml of PMA and 1 μg/ml of Ionomycin, for 5 days. Cells for FACS analysis and for mRNA extraction were harvested on each of the time points.

Vaccine efficacy evaluation

Vaccine efficacy was measured by using different parameters: bacterial recovery, mean disease score and lung radiographic analysis as previously described [63]. Briefly, following necropsy at approximately 4.5 months after challenge, gross pathology was evaluated in the lungs and lymph nodes. For quantitative assessment of mycobacterial burden, tracheobronchial lymph nodes were removed, weighed, and homogenized in phenol red nutrient broth using a blender (Oster, Shelton, CT). Logarithmic dilutions (10^{0}-10^{-9}) of homogenates in PBS were plated in 100 µl aliquots plated on Middlebrook 7H11 selective agar plates (Becton Dickinson, San Diego, CA) and incubated for 8 weeks at 37°C. Data are presented as mean (± standard error) cfu per gram of tissue. To provide additional information regarding the extent of the lung lesions, we individually radiographed each lung as described [63, 166]. Affected areas were divided by total lung area then multiplied by 100 to determine percent affected lung. Using combined data from each lung lobe, results for individual animals are presented as the mean (± standard error) percentage of affected lung.

Flow cytometric analysis (FACS)

Cells were treated with brefeldin A (BD Pharmingen, San Diego, CA) during the last 5 h of culture prior to FACS analysis. Surface labeling was performed using the

following bovine specific antibodies: anti-CD3ε, CD4, CD8 or γδ TCR (MM1A, CACT138A, CACT80C and GB21A, VMRD) and secondary PE –conjugated antibodies rat anti-mouse IgG1 and rat anti-mouse IgG2^{a+b} (BD Biosciences, , San Diego, CA). Intracellular staining was performed by using Cytofix/cytoperm reagents (BD Biosciences) according to manufacturers protocol and antibodies to bovine IFNγ-FITC (Serotec, Raleigh, NC) or an antibody to human perforin, cross reactive to bovine perforin (BD biosciences) [79]. Samples were fixed using 4% buffered paraformaldehyde prior to collection with a FACScan flow cytometer (BD Biosciences). Analysis was performed using CellQuest Software (BD biosciences). Flow cytometric analysis of perforin and IFNγ was based on gating on T cell subset.

IFN-γ ELISA

ELISA for IFN-γ was measured as published before by Waters et al. (2007)[63]. Briefly, PBMC were seeded at 5 x 10^5 cells in a total volume of 200 μl per well and were further stimulated with 10 μg/ml *M. bovis* PPD or medium alone (no stimulation) for 48 h at 38°C in 5% CO_2. Culture supernatants were harvested and stored at -80°C until thawed for analysis by an ELISA kit (Bovigam, Prionics AG). Concentrations of IFN-γ in test samples were determined by comparing absorbances of test samples with absorbance's of standards within a linear curve fit. Mean IFN-γ concentrations (ng/ml) produced in 48-hr

cultures in response to antigen minus concentrations in non-stimulated cultures (i.e., Δ IFN-γ) are presented.

RNA extraction and Real- time RT-PCR

RNA extraction was performed using the RNeasy extraction kit (Qiagen, Valencia, CA) and cDNA synthesis was performed as previously described [216, 217]. Differences in gene expression were determined by using bovine specific primers and probes for Granulysin [216], IFNγ forward 5'- CAG AAA GCG GAA GAG AAG TCA GA -3' reverse 5'- CAG GCA GGA GGA CCA TTA CG -3' and probe TCT CTT TCG AGG CCG GAG AGC ATC A, perforin, forward 5'-TTC GCC GCC CAG AAG AC -3', reverse 5'-CAC TCC ACT AAG TCC ATG CTG AA -3', and probe 5'-ACC AGG ACA ACT ACC G -3'labeled with FAM, Fas-L, forward 5'- CAA GGT CTT ACT CCA GGA ACT TTA GG-3', reverse 5'- TTC ATC ATC TTC CCC TCA TCA GT-3', and probe 5'- ACC CCC AGG ACA TG-3' (Applied Biosystems, Foster City, CA). Gene expression was determined using an ABI PRISM 7000 sequence detector (Real-Time PCR Core Facility, UTMB, Galveston, TX). Equivalent RNA template was based on the amplification of ribosomal RNA.

Antibody production

To generate monoclonal antibodies (mAb) to bovine granulysin, four Balb/c mice (Jackson Laboratories, Bar Harbor, ME) were immunized with granulysin peptide conjugated to keyhole limpet hemocyanin (KLH). Non-conjugated peptide, peptide conjugated to KLH, and peptide conjugated to bovine serum albumin were purchased from Sigma-Genosys. The peptide sequence of bovine granulysin (IQHLMDKLGDQPDENTC) used to generate the mAb is conserved among the bovine granulysin 62 and 89 variants [79]. Mice were immunized i.m. with 3 doses of 25 μg of peptide conjugated to KLH, mixed equally with Incomplete Freund's Adjuvant (IFA) and with 1 dose of peptide mixed equally with IFA. Sera from immunized mice was obtained by using the submandibular bleeding method with a lancet [218], and screened for specificity to granulysin peptides conjugated to BSA by ELISA. Splenocytes from mice were fused with mouse myeloma CRL 1580 (ATCC, Manassas, VA) using polyethylene glycol and fusions selected by growth in HAT media (Sigma, St. Louis, MO). Hybridoma populations were screened for immunoglobulin production by ELISA. Purified immunoglobulin was analyzed for granulysin specificity by ELISA and immunoblot using lysates from PBMC.

Western blotting

PBMC or purified $CD4^+$ and $CD8^+$ T cells (2 x 10^6 cells) isolated from PBMC were stimulated with 20μg/ml of bovine PPD or left unstimulated and cell pellets were harvested at 0, 3, 5 and 10 days after stimulation. Purity of both T cell populations was examined by flow cytometry and is shown in Figure 1. Total cell pellets were frozen at -80°C until used for Western blotting. Detection of granulysin was performed by using an antibody to bovine granulysin (anti-granulysin) and an anti mouse- IgM-HRP conjugated antibody. Total protein from each sample was measured by BCA protein assay kit (Pierce, Rockford, IL) and β-actin was used as loading control by using a β-actin antibody (Sigma-Aldrich).

Tissue analysis

Mediastinal lymph node from Control non-vaccinated (n=4), *M bovis* BCG-vaccinated (n=4), and *M bovis* ΔRD1-vaccinated (n=4) animals were collected in RNAse free conditions at necropsy, snap frozen in liquid nitrogen and stored at -80C. Collected tissues were γ-irradiated at 3000 rads on dry ice for safety issues. Total RNA was isolated from approximately 1 gram of tissue using Qiagen RNA extraction kit (Qiagen, Valencia, CA). Resulting mRNA was used for cDNA synthesis and Gene expression was measured

for bo-lysin, perforin, IFNγ and Fas-L by Quantitative Real-Time PCR. Relative expression on each tissue was calculated using the ribosomal RNA as the internal control.

Statistics

Statistical analysis was performed using GraphPad Prism 4 ® (La Jolla, CA). Data were analyzed by 1 way ANOVA for significant effects and significant differences among individual treatment groups were determined using a Student's t test. A Spearman Correlation was used to analyze cytokine gene expression with the different pathology parameters in the different vaccine groups. Values for $p < 0.05$ were considered significant.

CHAPTER 3

KINETICS AND SPECIFIC ACTIVATION CONDITIONS OF BOLYSIN EXPRESSION IN ACTIVATED BOVINE LYMPHOCYTE POPULATIONS IN PARALLEL WITH PERFORIN, IFNγ AND FASL

INTRODUCTION

The increasing rates of TB worldwide and the variable efficacy of the current vaccine against TB demand the development of better vaccines against TB. The lack of a vaccine that can be used in HIV infected patients also adds to the request for a better vaccine that can be used in this increasing population. The lack of correlates of protection better than IFNγ also demands the characterization and development of better tools to evaluate vaccine candidates against TB [219-221]. In our first aim we are looking at the kinetics of the cytotoxic granule proteins granulysin and perforin in parallel with the surrogate marker IFNγ and also Fas-L. The characterization of granulysin in the bovine species together with the important antimicrobial properties exhibited by granulysin against *M. tuberculosis* and *M. bovis*, suggests its potential as a correlate of protection following vaccination against these pathogens [79].

Further understanding of the kinetics of gene and protein expression of bovine granulysin in the different T cell populations will yield important information to be applied in the evaluation of vaccine trials in both bovine and humans.

RESULTS

Granulysin is upregulated in $CD4^+$ and $CD8^+$ T cells after vaccination with BCG

Bovine granulysin was previously demonstrated by our laboratory to be expressed in bovine $CD4^+$T cells, $CD8^+$Tcells and $WC1^+\gamma\delta$ T cells, including BCG-specific $CD4^+$T cells cultured with PPD-pulsed macrophages from vaccinated animals [216] and in granulomas from *M. bovis* infected cattle [79]. Based on these observations we sought to determine expression kinetics and antigen specificity of this anti-mycobacterial molecule relative to other effector molecules in different T cell populations from vaccinated and non-vaccinated animals. Upon stimulation with bovine PPD-pulsed macrophages, granulysin expression was observed as early as 24 h post stimulation in $CD4^+$T cells and as early as 12 h in $CD8^+$T cells in BCG-vaccinates (Fig. 2A-2B). Expression of granulysin mRNA upon an antigen specific stimulation was significantly increased (> 50 fold difference) in BCG-vaccinates as compared to non-vaccinates at 48h in $CD4^+$T cells ($P<0.05$). Similar results were found when examining $CD8^+$T cells, where granulysin expression was significantly greater (> 50 fold at 12h to 90 fold difference at 48h) in BCG-vaccinates as compared to non-vaccinates ($P<0.05$). In comparison to granulysin expression, perforin was expressed later (72h) in all T cell populations upon antigen stimulation. These findings corroborate our previous findings that perforin appears to be a late activation molecule in cattle [79], being transcribed 3-5 days after antigen stimulation in BCG-vaccinates (Fig. 2A and Fig. 3). Granulysin transcripts were not

significantly expressed in γδ T cells obtained from BCG-vaccinates (Fig. 2C). IFNγ was significantly higher in BCG vaccinates as compared non-vaccinates in both $CD4^+$ and $CD8^+$ T cells from 4 to 72 hours of antigen stimulation (*P<0.05*) (Fig. 2A-2B). Fas-L was observed at or below baseline levels (< 1fold) in all T cell populations.

Polyclonal stimulation with PMA and Ionomycin significantly increased perforin, IFNγ and Fas-L expression by PBMC from BCG- and non-vaccinates. As we have previously observed in bovine $CD4^+$ T cells [216] granulysin mRNA expression upon polyclonal stimulation was lower than PPD-elicited responses in all tested T cell populations evaluated (Fig. 2A-2C).

Granulysin, perforin and IFNγ proteins increase upon vaccination with BCG

To better correlate the results from our gene expression analysis with cell-associated protein expression for select markers, we used flow cytometry to measure the percentage of cells that were either perforin and/or IFNγ positive following PPD stimulation. Cells that were positive for perforin and/or IFNγ increased over time post activation (Fig. 3). The majority of the antigen-specific perforin-positive cells were detected only at 5 days following stimulation but no significant differences were observed between BCG vaccinated and non-vaccinated animals. In contrast, IFNγ-positive cells were present relatively early after stimulation (1 day of stimulation), comprising more than 4% of the total $CD8^+$ T cell population at 5 days. Even though we

did not observe statistically significant differences between BCG vaccinated and non-vaccinated animals, a careful comparison between animal groups showed that there was a difference of 1-2% at 5 days of stimulation, which corresponds to a relatively high number of antigen specific T cells (Fig. 3A). Such differences are depicted on representative flow plots comparing intracellular perforin and IFNγ in purified $CD8^{+}$T cells after 5 days of stimulation (Fig. 3B).

To further correlate gene expression profile of granulysin with protein, we used an immunoblot approach to detect granulysin in PBMC lysates previously stimulated with bovine PPD and compared that to the gene expression profile of granulysin in non-stimulated and antigen-specific stimulated PBMCs. To further examine the variability in granulysin expression between BCG vaccinated and non-vaccinated animals we analyzed protein expression in cell lysates from 3 BCG vaccinated and 3 untreated animals (Figure 4). Previous reports in humans evaluating granulysin expression in purified $CD4^{+}$T cells [195] or PBMCs [222] indicated that granulysin was expressed relatively late following activation (no earlier than 5 days after IL-2 stimulation). We observed both granulysin precursors and cleaved active proteins of 15 and 9 KDa respectively present at initial time points in freshly harvested cells (zero hours post-stimulation) in 2 of 3 BCG vaccinated animal. The 9 KDa molecule was detected with more intensity at 3-5 days of antigen-specific stimulation in vaccinated animals. The 15 KDa noncleaved molecule was also detected in untreated (non-vaccinated) animals between 3 to 5 days of antigen specific stimulation. One animal produced granulysin protein at all time points including conditions without exogenous stimulations, similar to BCG-vaccinated animals. In this

animal the 9KDa molecule was also produced at 3 days independent of stimulation and disappeared thereafter with continued expression of the 15 KDa isoform. Granulysin gene expression based on stimulated PBMC was similar among the 3 non-vaccinated animals independent of stimulation, not exceeding 7 fold increases at 5 days. Fold induction (mRNA) was significantly higher *($P < 0.001$)* at 3 and 5 days of stimulation with PPD when compared to non-stimulated controls on BCG-vaccinated animals. Even though we observed differences in bovine granulysin gene expression, protein detected by immunoblot in whole PBMC did not show any conclusive difference between BCG vaccinated and non-vaccinated animals. As reported previously, NK cells constitutively express granulysin independent of stimulation [79]. Moreover, the active 9KDa portion was always present in BCG vaccinated animals, increasing after antigen-specific stimulation, suggesting that the initial participation of NK cells contribute to baseline expression of the active bo-Lysin molecule.

To elucidate a possible masking of our results by bovine NK cells, we used highly purified T cells in our immunoblots. Before sorting pure T cells, we analyzed the number of NK cells by a FACS surface staining with an antibody to NKp46, a surrogate marker for NK cells [82, 93, 223]. We observed that NKp46 cells were approximately 6% (± 1) of the total lymphocyte number (data not shown), which is in agreement with the expected number for adult bovine reported by others [88]. As observed in Figure 4 the 15KDa isoform of the molecule was observed in both BCG vaccinated and non-vaccinated animals but the 9 KDa portion was observed mostly in BCG vaccinated animals upon stimulation with PPD upon 5 days of stimulation. One of the BCG

vaccinated animals had both isoforms present at all time points including in the non-stimulated treatment, suggesting possible pre-activation or sensitization with an antigen, even though band intensity was higher upon PPD stimulation in this particular animal. Once both $CD4^+$ and $CD8^+$ T cells are analyzed on each animal, no major differences in terms of expression were observed between both populations of cells demonstrating the potential of $CD4^+$ T cells for being cytotoxic. One interesting observation is that in most of the animals, in both T cell populations, increasing amounts of the 9 KDa bo-Lysin were observed once the pro-active portion of the molecule increased which is in accordance with previous observations [129]. In summary, even though the active 9 KDa bo-Lysin was observed in one of the non-vaccinated animals, these results suggest that granulysin is a molecule predominantly produced in BCG vaccinated animals but not in untreated animals upon recall response with the specific antigen PPD.

DISCUSSION

Investigation of new and better correlates of protection after vaccination against animal and human forms of TB is key to the development of vaccines that are more effective than BCG. The bovine model has been shown to be useful for vaccine protection studies against TB having comparable findings to studies conducted in non-human primates [63]. In this study a new candidate vaccine for TB ($mc^2$6030) evaluated in both bovine neonates and non-human primates demonstrated that IFNγ levels post challenge (60 days) did not correlate with a protective response.

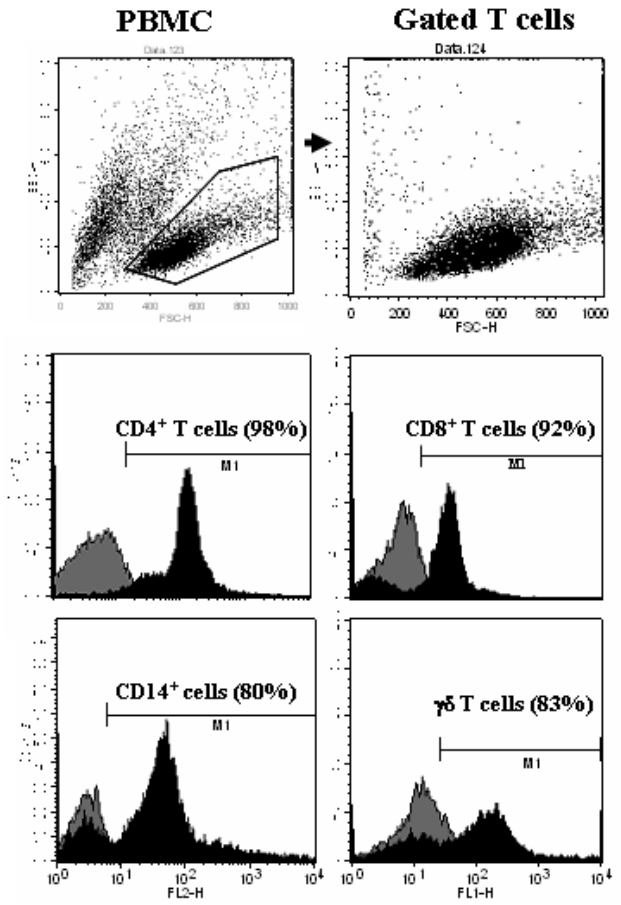

FIGURE 1. Isolation and purification of leukocyte populations. Bovine PBMC were separated by accuprep gradient and monocytes and T cells were purified by AutoMACS ® using specific antibodies to bovine CD14, CD4, CD8 and γδ T cells. Figure demonstrates light scatter properties before and after sorting, and further purity analysis of mononuclear cell subsets.

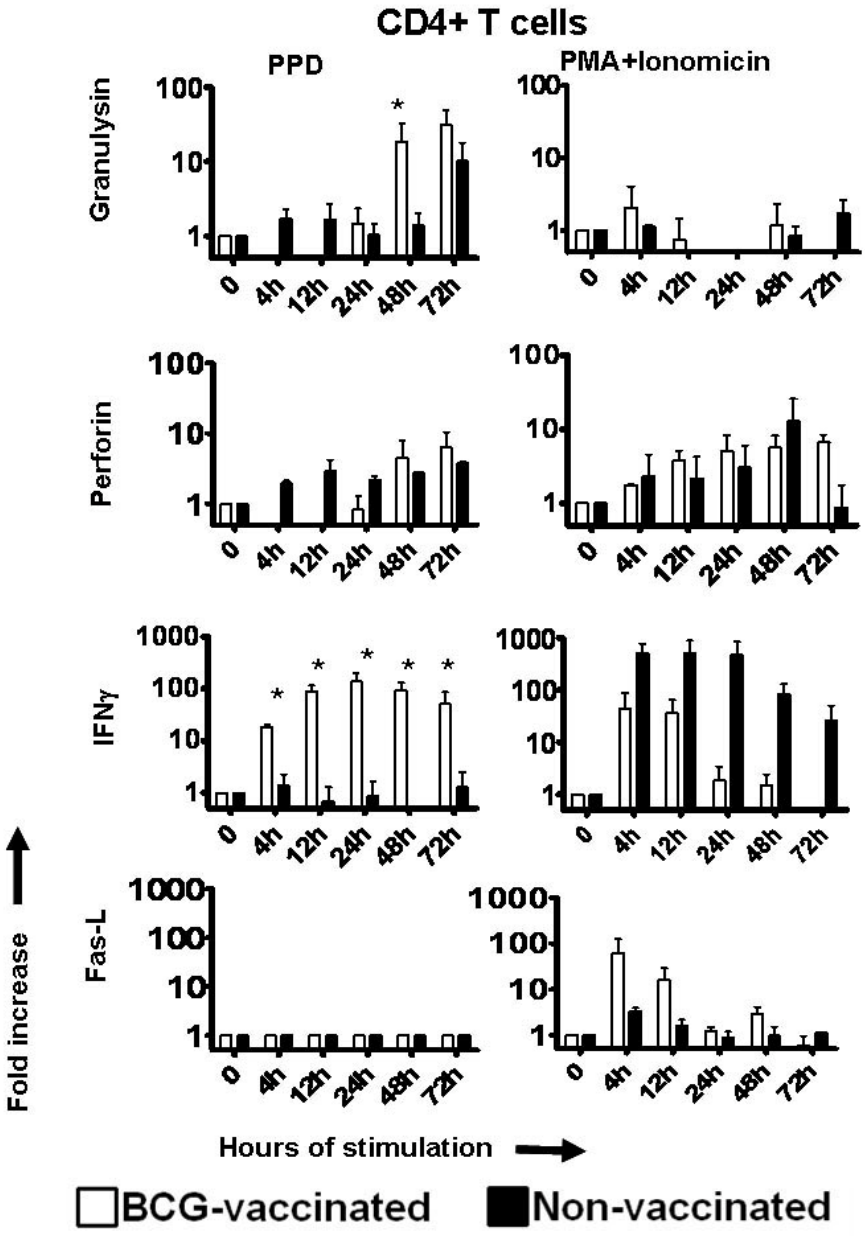

FIGURE 2A. Fold mRNA induction of granulysin, Perforin, IFNγ, and Fas-L measured by semi-quantitative real time PCR in $CD4^+$ T cells following 0, 4, 12, 24, 48 and 72 h of stimulation with 5μg/ml of boPPD or 10ng/ml of phorbol myristic acetate and 1 μg/ml of ionomycin, in both BCG-vaccinated (n=4) and non-vaccinated animals (n=4). Gene expression fold induction was calculated using the $2^{-\Delta\Delta Ct}$ method and non-stimulated cells were used as calibrator. Data were tested in duplicates and are presented as the means ±S.E.M. *P< 0.05, level of statistical difference between vaccinates and non-vaccinates.

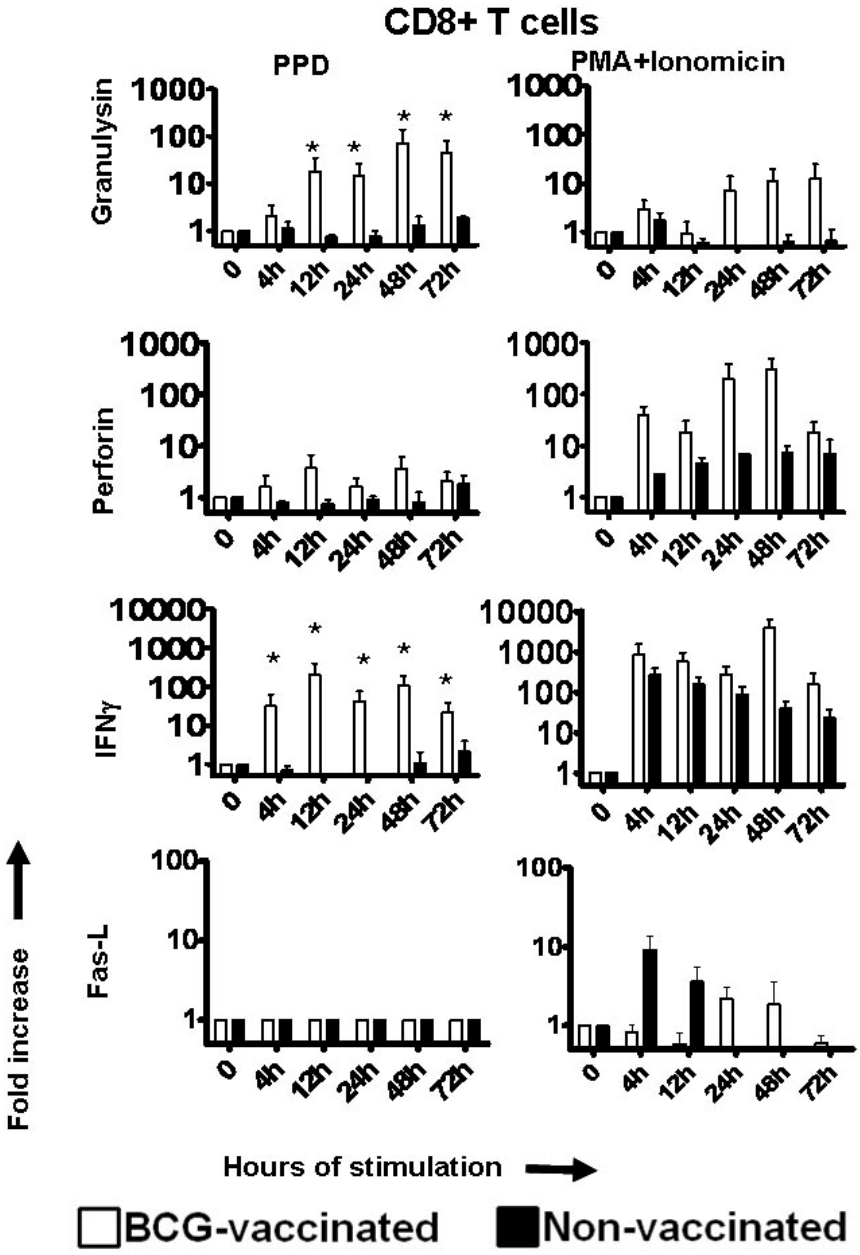

FIGURE 2B. Fold mRNA induction of granulysin, Perforin, IFNγ, and Fas-L measured by semi-quantitative real time PCR in $CD8^+$ T cells following 0, 4, 12, 24, 48 and 72 h of stimulation with 5μg/ml of boPPD or 10ng/ml of phorbol myristic acetate and 1 μg/ml of ionomycin, in both BCG-vaccinated (n=4) and non-vaccinated animals (n=4). Gene expression fold induction was calculated using the $2^{-\Delta\Delta Ct}$ method and non-stimulated cells were used as calibrator. Data were tested in duplicates and are presented as the means ±S.E.M. *P< 0.05, level of statistical difference between vaccinates and non-vaccinates.

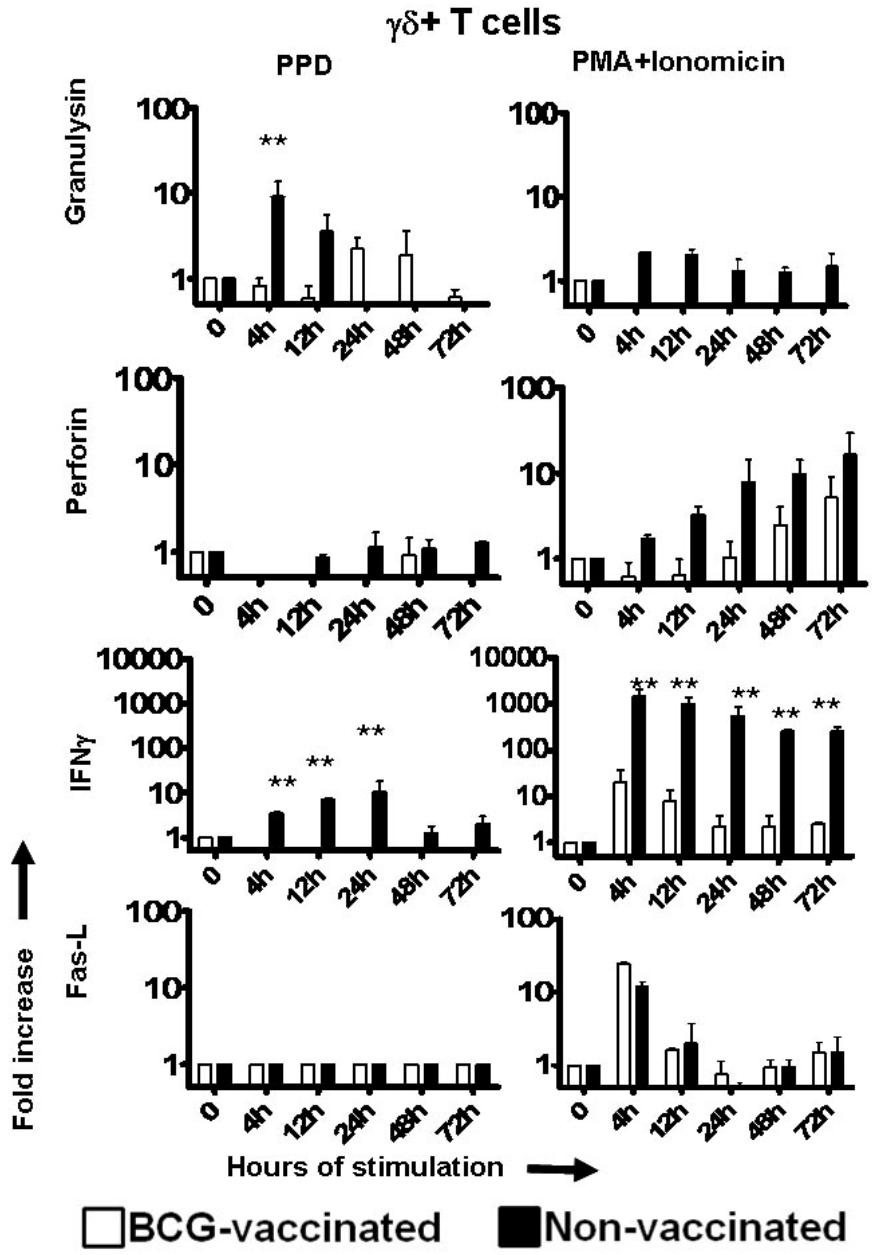

FIGURE 2C. Fold mRNA induction of granulysin, Perforin, IFNγ, and Fas-L measured by semi-quantitative real time PCR in $\gamma\delta^+$ T cells following 0, 4, 12, 24, 48 and 72 h of stimulation with 5μg/ml of boPPD or 10ng/ml of phorbol myristic acetate and 1 μg/ml of ionomycin, in both BCG-vaccinated (n=4) and non-vaccinated animals (n=4). Gene expression fold induction was calculated using the $2^{-\Delta\Delta Ct}$ method and non-stimulated cells were used as calibrator. Data were tested in duplicates and are presented as the means ±S.E.M. **P< 0.001, level of statistical difference between vaccinates and non-vaccinates.

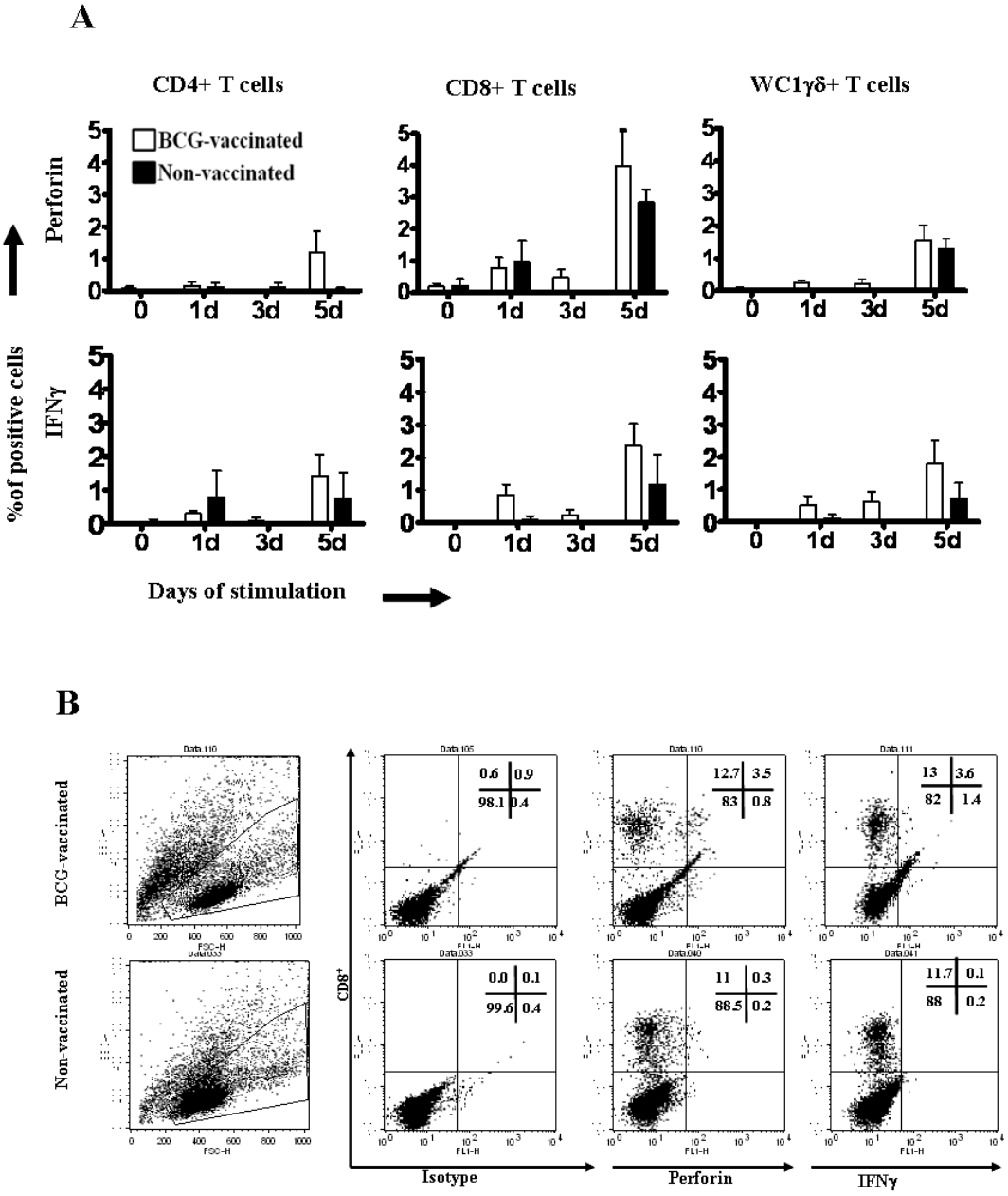

FIGURE 3. Flow cytometry detection of IFNγ and Perforin by intracellular staining, in $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T cells following PBMCs stimulation for 0, 1, 3 and 5 days with 20μg/ml of boPPD, in both BCG-vaccinated and non-vaccinated animals. *A*. Percentage of double positive gated T cells was calculated for each treatment group by subtracting a non-stimulated control. Data are presented as means ±S.E.M. of 2 independent experiments. *B*. FACS results for perforin and IFNγ in $CD8^+$ T cells in one animal for each treatment group after 5 days of stimulation.

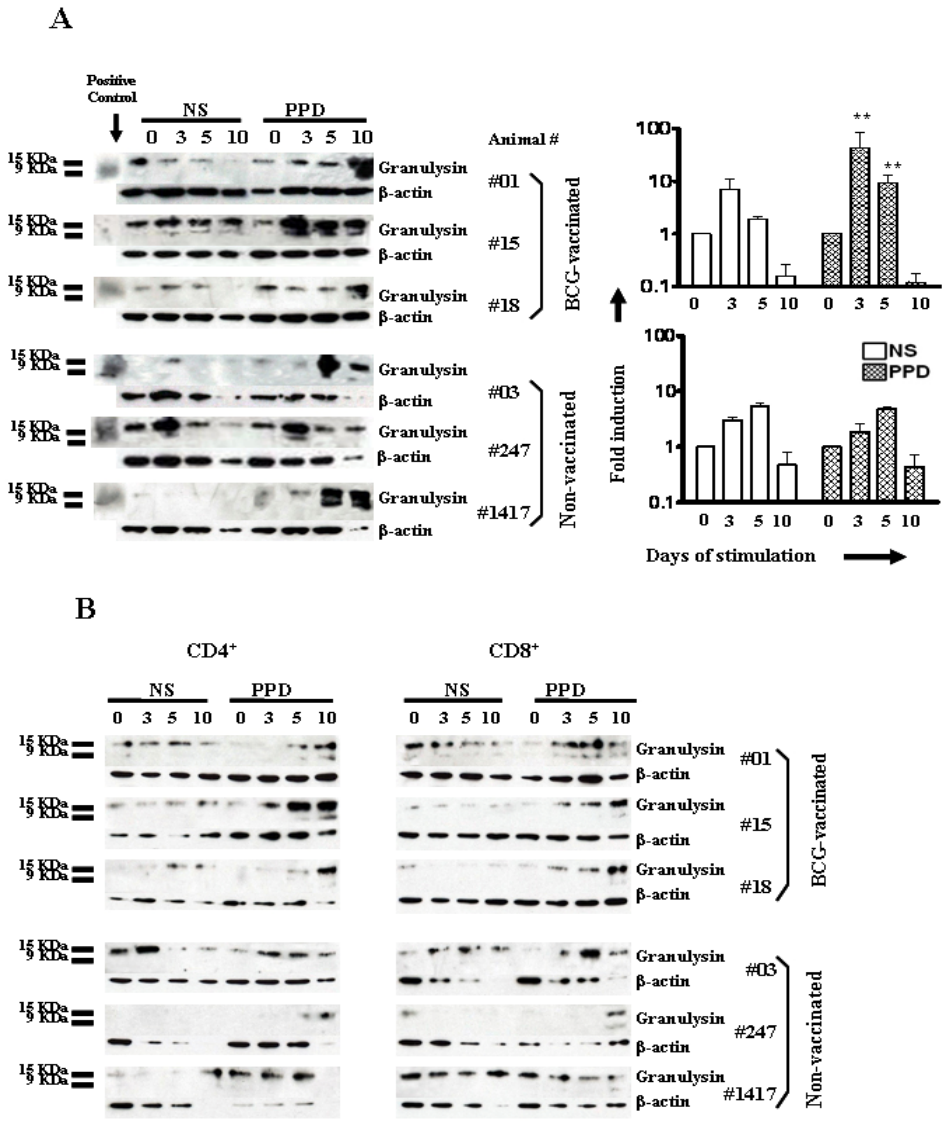

FIGURE 4. Granulysin detection by immunoblot. PBMCs (A) or purified $CD4^+$ and $CD8^+$ T cells (B) from both BCG- and non-vaccinates were stimulated with 20 μg/ml of boPPD or left unstimulated. Cells were harvested at 0, 3, 5 and 10 days either in RNA later for further RNA extraction and Quantitative Real-time PCR (PBMC only-right top panel) or were frozen at -80° C for immunoblot performed with specific bovine anti-granulysin antibody and an anti-mouse-IgM-HRP conjugated antibody. Fold induction mRNA was measured by real-time PCR calculated using the $2^{-\Delta\Delta Ct}$ method and non-stimulated cells were used as calibrator. Gene expression fold induction are presented as the means ±S.E.M. of 2 repetitions where ** P< 0.001. Protein concentration was equalized before loading into the gel and β–actin was used as loading control in the western blots.

Several other studies have demonstrated increased levels of this cytokine associated with disease persistence [63, 136, 220, 224]. The use of the bovine neonate as a model presents other advantages for use as a screening tool for vaccine efficacy including similarities in pathology, clinical presentation, transmission and host immune responses and effector pathways including granulysin [220, 225]. It also has been shown that a Th1 response is considered a good correlate of protection upon vaccination in both humans and bovine. Studies in cattle have shown that the balance of the host Th1/Th2 pathways are more similar to humans than to mice [226]. Granulysin was initially identified in effector T cells by subtractive hybridization [125]. The mechanism of action of granulysin is under active investigation but is thought to involve membrane disruption based on positively charged amino acids that interact with negative charges of the membrane of the microorganism [98, 106, 131, 202]. Granulysin is expressed in various effector populations including $CD4^+$, $CD8^+$, $WC1^+\gamma\delta$ T cells and NK cells [125, 129]. The molecular structure of granulysin indicates that it is a member of the saposin-like protein family [130] and is characterized by having a five helix bundle as demonstrated for other proteins of the same family [131]. Much of the lytic activities of granulysin were demonstrated based on the use of peptides derived from the granulysin sequence [126, 127, 132]. These peptides were demonstrated to have high cytotoxic/broad spectrum antimicrobial activity against both gram positive and gram negative bacteria [97, 106, 133]. Homologues of granulysin have been characterized for several species, including pigs, horses and cattle [79, 95, 134]. The gene for granulysin has not been identified in any rodent species to date, restricting the use of knock out mice for *in vivo*

studies. The bovine homologue of granulysin, based on nucleotide and amino acid sequences, exhibits a relatively high degree of conservation of functional domains with the other granulysin homologues, including the 5 helix bundle and antimicrobial activity against several types of bacteria such as *E.coli* and *M. bovis* BCG [79].

The expression of granulysin by the different T cell populations and the capacity to kill intracellular *Mycobacterium*, suggest that it may be a relevant biomarker for tracking the immune response against TB both pre- and post-exposure and could be used as a potential correlate of protection after vaccination against TB. Previous studies have demonstrated that elevated plasma levels of granulysin and reduced IFNγ levels correlate with curative responses against TB in human adults [227]. Similar results were observed following chemotherapy against TB, where elevated granulysin and reduced IFNγ levels correlate with curative responses [228].

Here we investigated the expression of granulysin in highly enriched effector T cell populations ($CD4^+$, $CD8^+$ and $WC1^+\gamma\delta$ T cells) in animals that were vaccinated with BCG or untreated. We demonstrated that granulysin is expressed early post exposure and is upregulated upon stimulation with bovine PPD. In a previous study, granulysin expression was not observed when pure $CD4^+$ T cells were stimulated with PMA and ionomycin but cytokines such as IFNγ and IL-21 and perforin levels were also elevated [216] (Appendix 1). This result is in accordance with our observations, suggesting that granulysin is upregulated in response to antigen-specific stimulation, and that its expression is dependent on the presence of an antigen presenting cells or other soluble

factors such as IL-2. Treatment with IL-2 alone was sufficient to induce granulysin protein at 5 days of stimulation in pure human $CD4^+$ T cells, but granulysin was not observed with the addition of α-CD3 to the culture [195] . This same study demonstrated that addition of IL-2 alone was able to induce granulysin dependent killing of *Cryptococcus neoformans* by $CD4^+$ T cells. To our knowledge, so far only one study has addressed signal transduction pathways involved in transcriptional regulation of the granulysin gene [229]. It is clear from our studies that signaling mediated via PMA and ionomycin does not activate transcription of granulysin as observed following antigen-specific stimulation. It is established that there is extensive alternative splicing in the granulysin gene where non-coding RNAs may function to stabilize or post-transcriptionally regulate granulysin gene expression [222].

To our knowledge, this is the first comprehensive study demonstrating and comparing granulysin gene expression and protein synthesis in T cell populations after BCG vaccination. We showed that both granulysin isoforms (15 KDa and 9 KDa) were present in T cell effector populations of BCG immunized animals and were increased upon a recall response using PPD loaded macrophages. Differences in our finding versus those reported in human studies are related to the different stimulation conditions used by us and the potential differences on T cell populations, mainly NK cells, that can constitutively express granulysin [81]. The presence of NK cells in our experiments was ruled out by sorting purified $CD4^+$ and $CD8^+$ T cells. When human $CD4^+$ and $CD8^+$ T cells were stimulated with *Listeria sp.* antigens, granulysin was observed after 6 to 12 days of stimulation, but LAK cells showed it as early as 4 days after stimulation [222].

Granulysin was detected by immunblot on purified human $CD4^+$ T cells stimulated with either IL-2 or anti-CD3 and IL-2 at 5 days of stimulation, increasing thereafter [195]. In the same work, increase in granulysin by $CD4^+$ T cells was accompanied by increased killing of *Cryptococcus neoformans* that was independent of perforin. The same group had showed years earlier that $CD8^+$ T cell –mediated killing of the pathogen by granulysin was independent of perforin, but dependent of IL-15 stimulation [230]. In summary, granulysin expression seems to be variable according the cell type and depending on the type of stimulation to which these cells are submitted.

As observed in our study there was a high variability among animals and between the 2 treatment groups for granulysin protein isoforms, making it difficult to interpret data and draw conclusions. The pre-exposure to *M. avium* constitutes a potential problem upon vaccination with BCG or other TB vaccine candidates and also affects diagnosis [231] and may be responsible for the antigen specific response observed in one non-vaccinated animal. One interesting observation was the strong response observed by purified $CD8^+$ T cells upon stimulation with a soluble antigen (*M. bovis* PPD). As suggested by Harding et al., (1994) [232], and reviewed by Vyas et al. (2008) [104], exogenous antigens may escape phagosomes and be further presented via MHC-I. Our observation is in accordance with additional reports where $CD8^+$ T cells proliferated and secreted IFNγ upon stimulation with the same soluble antigen [76, 105]. Bovine purified $CD8^+$ T cells were shown to proliferate upon stimulation with different antigen such as live *M. bovis* and *M. bovis* PPD. Even though response was superior to live organisms, considerable response was observed when using *M. bovis* PPD [105]. In summary, our

results suggest that granulysin and perforin are expressed upon an antigen specific stimulation in BCG-vaccinated animals.

CHAPTER 4

POTENTIAL USE OF BO-LYSIN AS A CORRELATE OF PROTECTION FOLLOWING VACCINATION WITH *M. bovis BCG* AND *M. bovis ΔRD1* AND CHALLENGE WITH VIRULENT *M. bovis*

INTRODUCTION

To further evaluate the potential use of granulysin and perforin, we evaluated protection by vaccination by using a new candidate vaccine against TB called *M. bovis ΔRD1*, in parallel with *M. bovis* BCG. The sequencing of the *M. tuberculosis* genome sequencing in 1998 was a very important step for vaccine development against TB [11, 233], making possible the development and testing of knock-out mutant strains of both *M. tuberculosis* and *M. bovis*. Live knock-out mutants of *M. tuberculosis* and *M. bovis* constitute important vaccine candidates since they still have limited replication capacity, imunogenicity, but lack aspects of the pathology observed in the wild type. As discussed earlier, comparison of *M. tuberculosis* and *M. bovis* genome with *M. bovis* BCG, showed that this last one lacks a so called Region of Difference 1 (RD1), which encodes a complex of 2 important proteins, ESAT-6 and CFP-10 [12]. These proteins are very important inducers of IFNγ, and are also involved on several aspect of the pathogenesis of *Mycobacterium spp.* In addition, RD1 genes that were engineered back to BCG resulted in a strain capable of promoting disease similar to wild type *M. bovis* [15].

The use of auxotrophic strains lacking the RD1 region and other metabolic pathway genes may be potential vaccine candidates and may provide important tool to differentiate between vaccination and infection for both bovine and humans. Another potential use for such vaccine strains is the application on wildlife reservoirs of *M. bovis*. Initial testing of a *ΔpanCD* mutant of *M. tuberculosis*, lacking the panthothenate

metabolic pathway was tested in normal and SCID mice showing better efficacy than BCG and protection against challenge [184] (Appendix 2). To further evaluate this vaccine candidate, a deletion of the RD1 region was performed and the strain used in vaccine trials in non-human primates and bovines, but results showed lack of protection for the mutant in both animal models [63]. In our current work we tested a second candidate based on a single deletion of the RD1 region on a strain of *M. bovis (M. bovis* Ravanel). We compared our vaccine candidate (*M. bovis* ΔRD1) with the gold standard BCG and then measured our potential correlates of protection at different time points after vaccination/before challenge and after challenge.

RESULTS

Granulysin expression is increased after vaccination against TB and challenge with virulent *M. bovis*

Here we analyzed the potential for correlation of gene expression with vaccine efficacy parameters (gross pathology score, radiographic assessment of lesions, and *M. bovis* colonization) using samples obtained from three vaccine groups, non-, BCG-, and ΔRD1-vaccinates (Fig. 5, n = 4 / vaccine treatment) in a neonatal vaccine trial. All 3 parameters were significantly lower in BCG-vaccinates as compared to non-vaccinates

(*P<0.05*). There were no statistically significant differences between BCG- and ΔRD1-vaccinates and between control non-vaccinated and ΔRD1-vaccinates (Fig. 5).

To examine the possible use of granulysin as a correlate of protection after vaccination against TB, we sampled animals at different time points after vaccination/before challenge (8, 9 and 10 weeks after vaccination) and one time point after challenge (2 weeks after challenge) with virulent *M. bovis*. Based on relative fold induction, granulysin was higher in both vaccinated groups as compared to non-vaccinates at all time points (*P<0.05*) before and after challenge (Fig. 6). When granulysin expression was averaged at all time points and compared among the three groups, the expression was elevated in BCG vaccinates as compared to controls (*P=0.04*) and in ΔRD1-vaccinates as compared to controls (*P=0.03*). There was no difference between the two vaccinated groups (BCG and ΔRD1). No significant differences were observed for Fas-L. Perforin transcript levels were elevated in BCG- and ΔRD1-vaccinates as compared to controls after the challenge (*P<0.05*). Similar results were observed when perforin was evaluated by intracellular staining in $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T cells after the challenge (Fig. 6) (*P<0.01* for BCG- and ΔRD1-vaccinates as compared to controls in $CD4^+$ T cells; *P<0.01* for BCG-vaccinates as compared to controls, and *P<0.0001* for ΔRD1-vaccinates as compared to controls in $CD8^+$T cells; *P<0.01* for ΔRD1-vaccinates as compared to controls in $\gamma\delta^+$ T cells).

IFNγ correlates with pathology

In parallel with granulysin and perforin, we also evaluated IFNγ, a claimed surrogate marker of protection upon vaccination against TB. IFNγ gene expression was greater in BCG- and ΔRD1-vaccinates as compared to controls before and after the challenge (*P<0.05*) but intracellular staining showed that the percent of $IFN\gamma^+$ cells in vaccinates was equivalent or lower than the controls (Fig. 6). IFNγ measured by ELISA showed that IFNγ responses to *M. bovis* PPD by ΔRD1-vaccinates was statistically higher *(P < 0.05)* as compared to controls non-vaccinated prior to challenge (Fig. 8). After challenge, IFNγ responses by the control non-vaccinated group exceeded the response of both BCG- and ΔRD1-vaccinates at 30 days after challenge (*P<0.001* for BCG- and ΔRD1-vaccinates, as compared to control) and at 60 days (*P<0.05* for BCG- and ΔRD1-vaccinates, as compared to control). In summary, both granulysin and perforin expression were upregulated in BCG and ΔRD1 vaccine groups as compared to control.

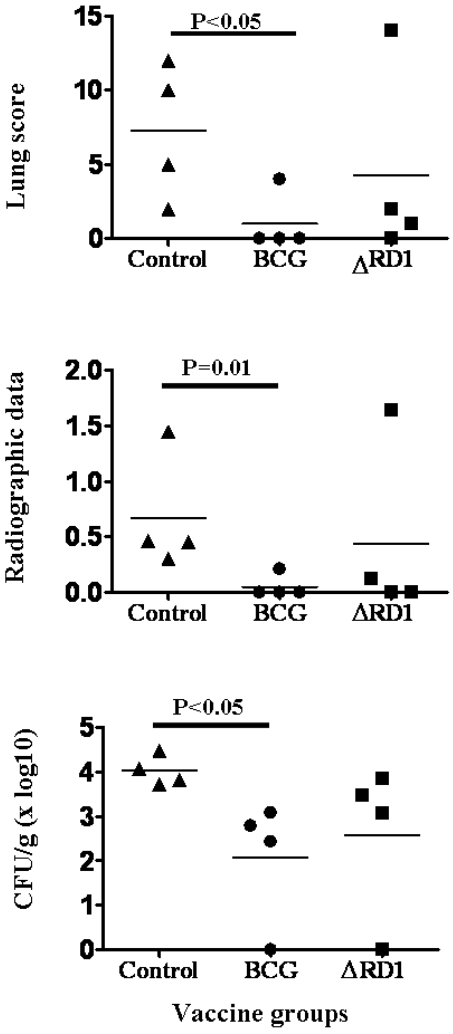

FIGURE 5. Comprehensive vaccine efficacy data. Lung gross pathology scores, radiographic data (percent lung affected), and CFU/g of tissue from 3 animal groups: control non-vaccinated (n=4), BCG vaccinated (n=4) and ΔRD1 (n=4) evaluated after necropsy. Lung score, radiographic data and Lung CFUs were measured as described by [63]. Values are presented as the means ±S.E.M.

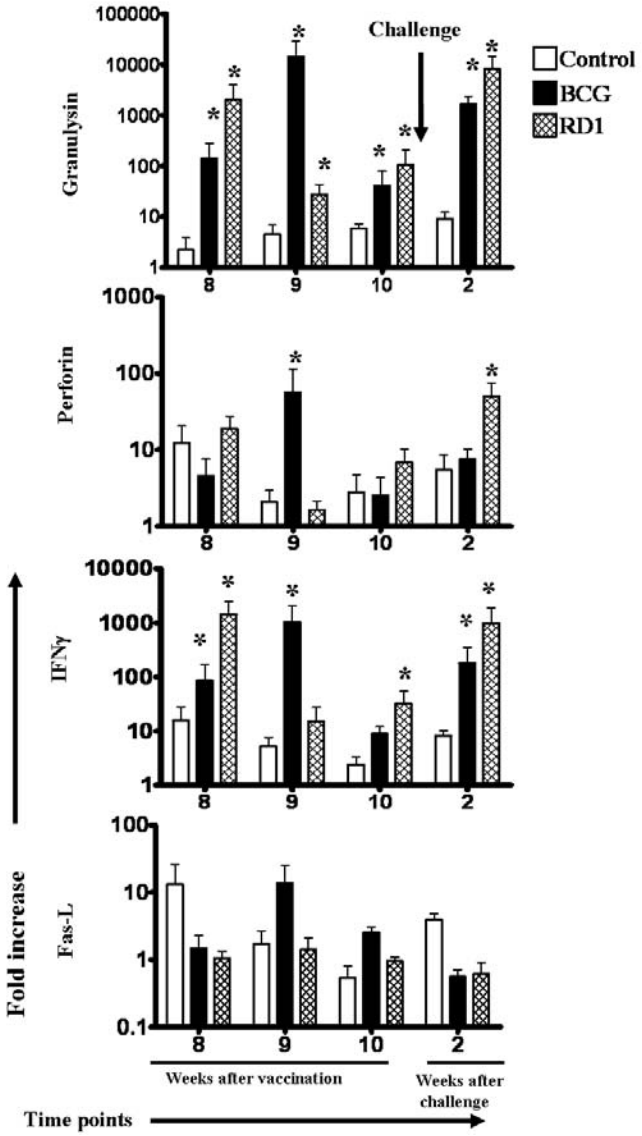

FIGURE 6. Fold mRNA induction of granulysin, Perforin, IFNγ, and Fas-L in 3 animal groups: control non-vaccinated (n=4), BCG vaccinated (n=4) and ΔRD1-vaccinated (n=4). PBMCs were stimulated for 5 days with 20μg/ml of boPPD or left unstimulated at 8, 9 and 10 weeks after vaccination and 2 weeks after challenge (14 weeks after vaccination). Cells were harvested and mRNA was measured by Quantitative Real-Time PCR. Gene expression fold induction was calculated using the $2^{-\Delta\Delta Ct}$ method and non-stimulated cells were used as calibrator. Data are presented as the means ±S.E.M. of 2 repetitions. * $P < 0.05$, level of statistical difference between vaccinates and non-vaccinates.

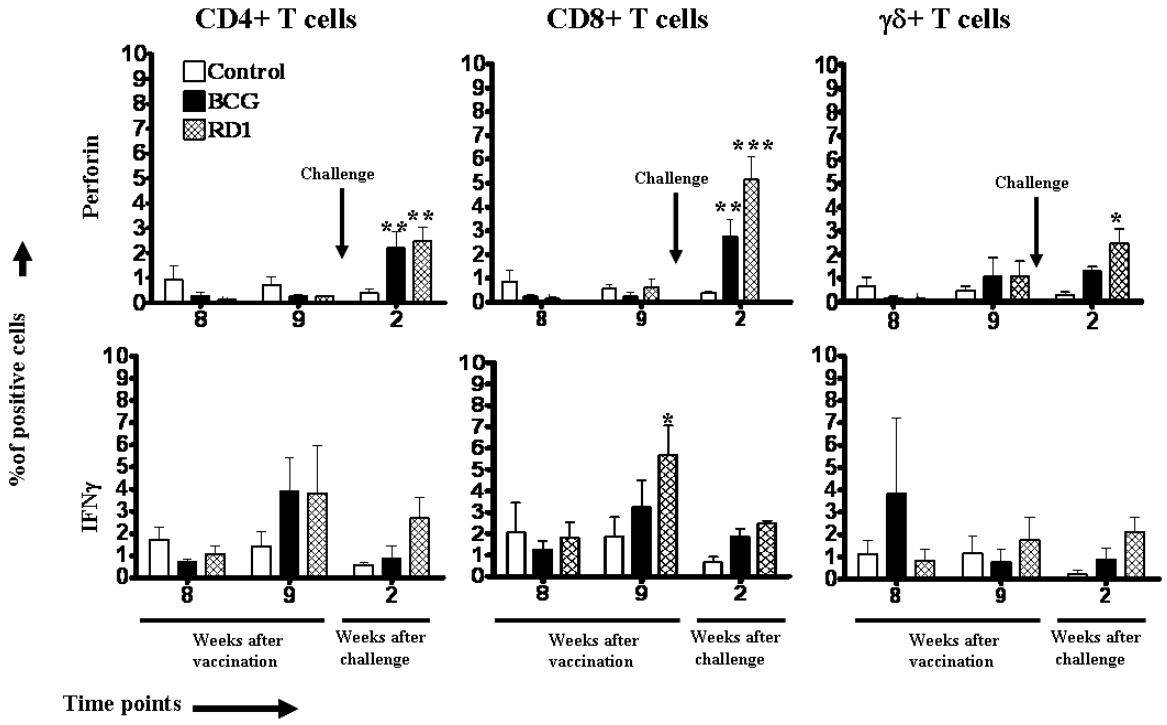

FIGURE 7. Flow cytometric analysis of intracellular staining of IFNγ and Perforin in $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T cells following PBMCs stimulation for 5 days with 20μg/ml of boPPD at 8, 9 and 10 weeks after vaccination and 2 weeks after challenge (14 weeks after vaccination) Treatment groups were: control non-vaccinated (n=4), BCG-vaccinated (n=4) and ΔRD1 -vaccinated (n=4) animals. Percentage of double positive gated T cells was calculated for each treatment group by subtracting non-stimulated control value for each individual animal. Data are presented as means ±S.E.M. * $P<0.05$, ** $P<0.01$, *** $P< 0.0001$, level of statistical difference between vaccinates and non-vaccinates.

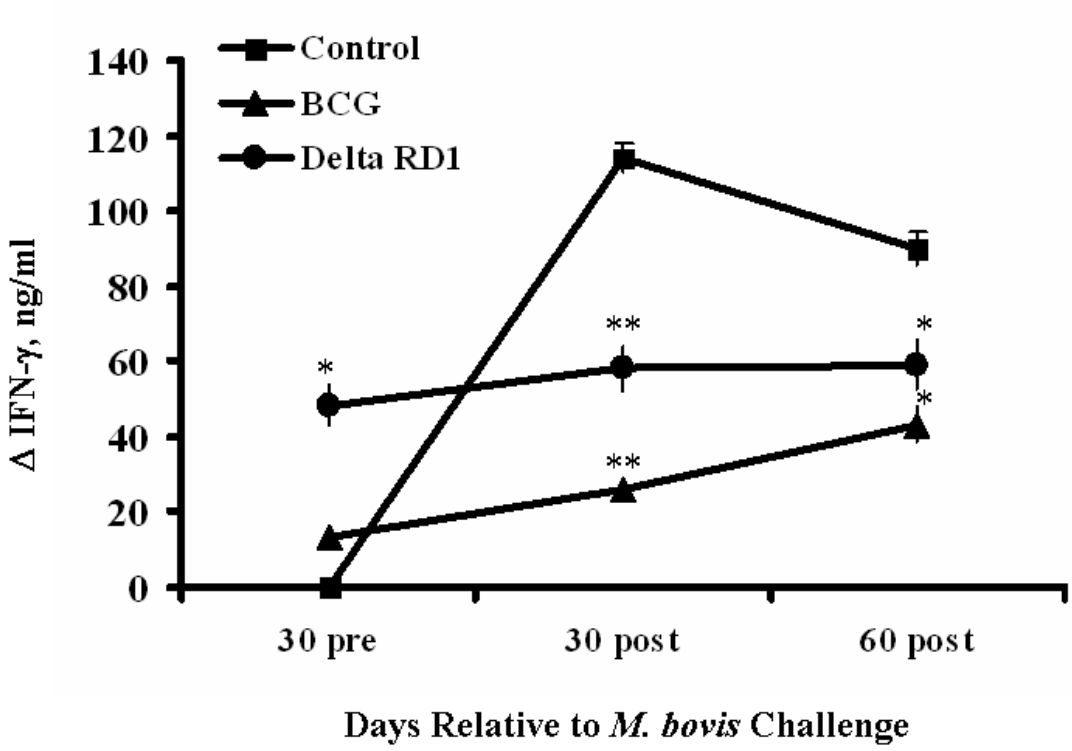

FIGURE. 8. Interferon-γ measured by ELISA. Blood mononuclear cells were isolated from each treatment group (n = 4, non-vaccinates; n = 4, *M. bovis* BCG-vaccinates; n = 4, *M. bovis* ΔRD1-vaccinates) 30 days prior challenge, 30 and 60 days after challenge and were cultured with 10 μg/ml *M. bovis* PPD or medium alone (no stimulation) for 48 hrs, when supernatants were harvested and IFN-γ concentrations determined by ELISA (Bovigam, Prionics, Ag). Values represent mean (± standard error) responses to antigen minus the response to media alone. * $P<0.05$ as compared to control group non-vaccinated, ** $P<0.001$ as compared to control group non-vaccinated.

DISCUSSION

Our results demonstrate the potential of granulysin and perforin as correlates of protective immunity to TB following vaccination and challenge of cattle. Even though our protection data did not show significant results to support ΔRD1 vaccine efficacy, comparable results were obtained with our gene expression analysis for both BCG and ΔRD1 vaccine groups. Both granulysin and perforin were upregulated at different time points before and after the challenge. Granulysin gene expression was significantly increased in both BCG and ΔRD1 vaccinates, and perforin was significantly increased in the ΔRD1 group as compared to control and BCG vaccinates after challenge. These results suggest that the ΔRD1 vaccine is able to induce an immune response comparable to BCG, and can be potentially used as a strain for future vaccine development, but further studies are needed in order to confirm our results. In regards to the bacterial burden, previous work from our group testing another vaccine candidate called $mc^2$6030, which showed protection only on the BCG vaccinated group as compared to control non-vaccinated [63], culture results were similar between control non-vaccinated , BCG- and $mc^2$6030 –vaccinated groups. This suggests that bacterial burden was not affected by vaccination, but in this particular case, other vaccine efficacy parameters, such as lung pathology, did show that BCG was protective as compared to the other 2 groups. Results from the current study and from our previous work suggest that bacterial burden has to be evaluated further in future vaccine trial and when developing new vaccine candidates. As

an overall conclusion we think that both granulysin and perforin do correlate with protection against pathology based on lung score and radiographic data.

Other studies in human TB patients evaluating plasma granulysin levels show that it correlate positively with TB recovery while high IFNγ levels corresponded to chronic TB [227]. In another study, there was a decrease in serum granulysin after successful chemotherapy treatment for TB in children [228]. The use of plasma granulysin as a way to evaluate protection may be influenced by potential degradation in the serum and by the time it takes from collection to testing of the plasma sample. Our results suggest that perforin should also be investigated further as a biomarker of protective immunity to human TB. Even though expression and intracellular detection of perforin were predominant after the challenge, they were considerable better than results observed for IFNγ in both vaccinated groups (BCG and ΔRD1) as compared to control group after the challenge. In addition to that, levels of IFNγ measured by ELISA were relatively higher in the Control non-vaccinated groups as compared to both BCG- and ΔRD1 vaccinates after the challenge, suggesting a correlation with pathology.

CHAPTER 5

EXPRESSION OF GRANULYSIN IN PARALLEL WITH IFNγ, PERFORIN AND FAS-L AT THE SITES OF INFECTION OF *M. bovis*.

INTRODUCTION

In order to provide additional information that could strengthen our evidence that both granulysin and Perforin could be used as potential correlates of protection, we sought to determine the gene expression profile on the mediastinal lymph node. Both lymphoid tissues and lung are foci of infection of *M. bovis*. Previously published data from our group showed that granulysin was successfully amplified from a bovine TB granuloma [79]. Analysis of tissue targets can provide strong insight on details of the type of immune response present in the tissue and to the cells involved on the process.

RESULTS

In order to establish a relationship of our PBMC data with *M. bovis* infection, we extracted RNA from portions of the mediastinal lymph node, adjacent to granulomas. Gene expression measured by quantitative real time PCR and analyzed as relative expression, showed that both granulysin and perforin had lower relative expression in both BCG-and ΔRD1 vaccinated groups as compared to control. IFNγ relative expression was lower in the Control and ΔRD1 vaccinated group as compared to BCG. Once sample concentrations are standardized, we can conclude that a lower relative expression of these molecules is correlated to higher gene expression fold induction (Fig. 9). No major differences were observed for Fas-L when comparing the 3 vaccine groups.

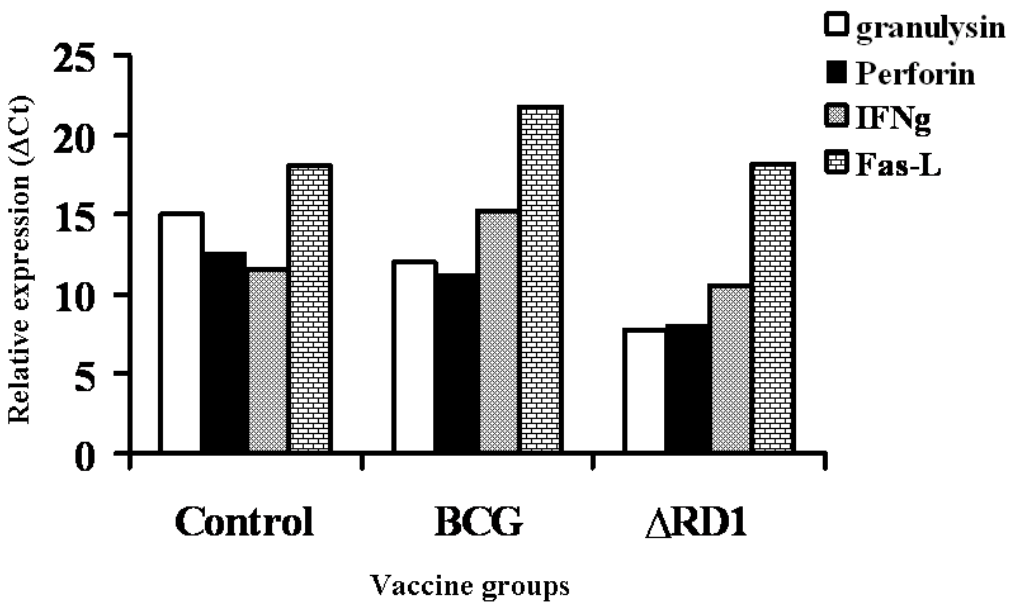

FIGURE 9- Relative gene expression of granulysin, perforin, IFNγ and Fas-L on Mediastinal lymph node. A total of 1g (gram) of tissue section from the lymph node from the 3 vaccine groups (Control n=4; BCG=4; ΔRD1 n=4), were homogenized in a microcentrifuge tube with a pestle and RNA was extracted by using manufactures protocol (Qiagen, Valencia, CA). Results are shown as relative expression (ΔCt) based on Ribosomal RNA.

DISCUSSION

Tuberculosis is predominantly a pulmonary disease. Infection is established via aerosol by a limited number of *M. tuberculosis* or *M. bovis* bacilli. The number of bacilli sufficient to establish infection is still a matter of controversy, but in the bovine numbers as low as 5 bacilli could be enough to result in infection [234]. Even though such a number is not established for human infections, less than 10 bacilli maybe enough, but could be influenced by factors such as droplet size [25]. Initial response starts with either macrophages in the lungs or DC lying on the trachea, which will engulf the pathogen. Further response is dependent on $\gamma\delta^{+}$, $CD4^{+}$ and $CD8^{+}$ T cells, either migrating to the site of infection without prior activation or following activation by DC and macrophages on the local draining lymph node, such as the mediastinal lymph node (MLN). Effector $CD4^{+}$ T cells responses were detected in the MLN as early as 10 days post-infection in mice [235]. In bovines that received intratonsillar inoculation of *M. bovis*, lesions at the MLN were observed at 28 days post infection, but were detected as early as 15 days after infection on the retropharyngeal lymph node [236].

In our studies we measured granulysin and perforin gene expression on MLN collected 100 days post challenge. Even though our measures are in relative expression and not calculated as fold induction, we observed that besides Fas-L and IFNγ, both granulysin and perforin were lower on the vaccine groups as compared to control, suggesting that MLN from both vaccinated groups had increased fold expression of cytotoxic granules. This observation is in accordance to the data observed on PPD

stimulated PBMC after challenge. As observed in our previous data, in which both gene expression and protein were measured, we can see that gene expression does not always indicate protein expression. However, gene expression profile can give us information related to the immune response against *M. bovis* infection, and as observed by Tacker et al. (2007) [237], it may suggest the type of response, either Th1 or Th2, predominant at the site of infection. In summary, results examining relative gene expression on tissues support and strengthen our evidence that both granulysin and perforin may be used as correlates of protection following vaccination against TB, together with IFNγ.

CHAPTER 6

SUMMARY, FINAL DISCUSSION/ CONCLUSIONS AND FUTURE DIRECTIONS

In our studies we have shown that (1) granulysin, perforin and IFNγ gene expression increase upon BCG vaccination; (2) both granulysin and perforin are upregulated after vaccination and challenge with *M. bovis* in both BCG and ΔRD1 vaccinated, as compared to control non-vaccinated group, (3) IFNγ correlated with pathology instead of protection, demonstrated by comparable positive cell numbers among 3 vaccine groups throughout the experiment, and by having secretion levels higher in the control group after challenge; (4) a comparable overall response was observed in both BCG and ΔRD1 vaccine groups demonstrating the potential use of the ΔRD1 strain in future vaccine trials.

Our results suggest that granulysin and perforin are potential correlates of protection upon vaccination, and can be used on future vaccine trials, together with IFNγ. The development of better diagnostic tools for granulysin, such as antibodies suitable for flow cytometric analysis and Confocal Microscopy, would help on providing more evidence in terms of the use of granulysin and perforin as correlates of protection on future vaccine trials. Other potential molecules that could be examined further would be FoxP3, a T regulatory cell marker. This molecule was shown to have higher expression after BCG vaccination and challenge, when compared to a control group non-vaccinated and a treatment group that received a *M. bovis ΔRD1ΔPanCD* vaccine candidate [63] (appendix 2). This vaccine conferred no protection upon challenge with virulent *M. bovis*. In addition to FoxP3 we have shown that IL-5 gene expression was relatively higher on the control as compared to the other vaccine groups. IL-5 is a Th2 cytokine

produced by mast cells, eosinophils, γδ T cells and NK cells, with relevant functions on the activation of eosinophils in asthma [238]. It is unclear what function IL-5 is playing, as of lack of protection, in TB.

Results observed for the ΔRD1 vaccine candidate are promising and require further evaluation as a potential vaccine candidate. Our results may have been masked by the number of animals used during the experiments and we have to consider increasing the number of animals in our next vaccine trials and evaluation of correlates of protection. The use of ΔRD1 may represent a potential choice as a vaccine candidate for both bovine and humans. As mentioned earlier, such vaccines can be combined with a test that differentiates vaccinated from infected animals (i.e. DIVA), based on using the protein complex ESAT-6 and CFP10.

APPENDIX 1

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Mycobacterium bovis BCG vaccination induces memory CD4+ T cells characterized by effector biomarker expression and anti-mycobacterial activity

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Abstract

The effector mechanisms used by CD4+ T cells to control mycobacteria differ between humans and rodent models of TB and should be investigated in additional animal models. In these studies, the bovine model was used to characterize the mycobactericidal CD4+ T cell response induced by vaccination with the attenuated *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). Antigenic stimulation of peripheral blood CD4+ T cells from BCG-vaccinated cattle enhanced expression of perforin and IFNγ in cells expressing a CD45RA−CD45RO+CD62L+ cell surface phenotype, enhanced transcription of granulysin, IFNγ, perforin, IL-4, IL-13, and IL-21, and enhanced anti-mycobacterial activity of CD4+ T cells against BCG-infected macrophages.

Keywords: Cytotoxicity; Tuberculosis; CD4+ T cells

1. Introduction

Tuberculosis (TB) is a global health emergency, affecting approximately one-third of the world's population [1,2]. Development of vaccines and therapeutics to curtail its spread requires improved understanding of the protective immune response to TB [3,4]. Immunity to TB is complex and includes components of both the innate and acquired immune systems. Cell mediated immune (CMI) responses are the most important correlate to protective immunity to TB in all animal models. The attenuated *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), currently in global use as a human TB vaccine and used experimentally in cattle, elicits strong CMI responses despite variable efficacy in vaccinated populations [4,5]. The widely accepted biomarkers of CMI responsiveness (IFNγ and lymphocyte proliferation) frequently fail to predict protective immunity to TB in adult populations [3]. Biomarkers of vaccine-induced protection against TB in small animal models are frequently not predictive of human immunity to TB [6–8]. Thus, there are currently no consistent immune correlates of vaccine-induced protection against TB [6–8]. These failures highlight the need to further characterize the cellular subsets and mechanisms of the CMI response, induced by BCG, that contribute to protective immunity to TB.

A substantial role for CD4+ T cells in protective immunity to TB is highlighted by the susceptibility of CD4-gene deleted mice and HIV-infected individuals [3]. A strong Type

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1 cytokine bias by $CD4^+$ T cells is thought to correlate with protective immunity against TB, with the degree of bias varying among model systems [9,10]. Enhanced microbicidal activity of infected macrophages (Mϕ) through inducible nitric oxide synthase (iNOS) is predominantly mediated by IFNγ produced by $CD4^+$ T cells and represents one of the most important protective mechanisms in the murine model of TB [3,8]. In human TB, the protective role of CD4-derived IFNγ is not fully understood and may be independent of iNOS mediated killing of intracellular mycobacteria [3]. Alternative mechanisms of Mϕ elimination of intracellular *M. tuberculosis* (*Mtb*) include activation through the $P2X_7$ purinergic receptor [11,12], acquisition of apoptotic neutrophil granules [13], and induction of cathelicidin by 1, 25-dihydroxy vitamin D3 [14]. $CD4^+$ T cells are the primary source of IL-21, a pleiotropic cytokine that enhances lytic activity of cytotoxic T cells and NK cells [15].

CTL activity by $CD4^+$, as well as $CD8^+$ and γδ T cells, may play an important role in human immunity to TB [16–19]. Results from *in vitro* experiments using *Mtb* specific T cell lines and peripheral blood mononuclear cells from *Mtb*-infected PPD^+ individuals demonstrate that cytotoxic activity of human $CD4^+$ T cells may also play an important role in immunity to TB [20,21]. Restriction of *Mtb* growth by human $CD4^+$ T cells is granule dependent and independent of Fas/FasL interactions [20]. Granulysin lyses intracellular *Mtb* in a perforin dependent manner, and correlates to inhibitory activity of human T cells isolated from peripheral blood of subjects reactive to PPD [18,22]. These preliminary studies in PPD^+ individuals and human cells lines suggest that granulysin and perforin may have important utility as biomarkers of TB immunity induced by vaccination or exposure to *Mtb*. The murine cell mediated immune response to mycobacteria appears to be less reliant on cytotoxic granule proteins in general, as perforin and granzyme B deficient mice are not more susceptible to infection with *Mtb* compared to wild type mice [23]. In cattle, bactericidal responses in peripheral blood T lymphocytes have been described [24–26]. Based on the incorporation of ^{3}H-Uracil by mycobacteria, these reports describe the mycobactericidal/mycobacteriostatic ability of total peripheral blood cells [24], $CD4^+$ [25] and $CD8^+$ T cells [25,26]. Importantly, it was shown the mycobactericidal/mycobacteriostatic activity of peripheral blood lymphocytes was independent of IFNγ [24].

Characterization of post-vaccination biomarkers in an animal model with clinical presentation similar to human TB, and similar expression of lytic granule molecules by T lymphocytes, represents an important avenue for investigation of protective immune responses to TB. Studies of the full complement of cytotoxic biomarkers in murine models are hindered by the lack of a granulysin gene homologue and a disproportionate reliance on IFNγ mediated Mϕ activation for containment of intracellular bacteria [3]. Cattle represent an important animal model of TB, with similar clinical presentation of disease and protective immune responses [5,27,28]. Studies of immunity to *M. bovis* infection in cattle and *Mtb* in humans demonstrate important similarities, including the relative importance and sources of IFNγ, the expression of memory markers by antigen specific T cells, the balance of Th1 and Th2 cytokines, and bacterial containment through T cell cytotoxicity [24–26,29–33]. The level of pathology in bovine TB (bTB) correlates to the amount of IFNγ produced by peripheral blood cells cultured *in vitro* with mycobacterial antigens [34]. Bovine homologues of granulysin and perforin are inducible upon T cell activation and are expressed in the granuloma cells of *M. bovis*-infected cattle [35]. Very recently, the bovine model of TB has been utilized to screen candidate neonatal TB vaccines prior to non-human primate studies [36].

The objective of the current work was to evaluate antigen specific recall of cytokine and lytic protein biomarkers in purified bovine $CD4^+$ T cells from peripheral blood following vaccination with BCG. Our results demonstrate that vaccination of cattle with BCG induces a subpopulation of mycobacteria specific $CD4^+$ T cells that are characterized by the expression of a cell-surface memory phenotype, enhanced expression of cytokines and mycobactericidal molecules, and anti-mycobacterial activity against intracellular BCG. These results, and similar studies in our laboratories characterizing $CD8^+$ T cell effector molecules, will allow for significant advancement in the use of the bovine model of TB to correlate *in vitro* immune biomarkers to protection from challenge in experimentally controlled vaccination programs.

2. Materials and methods

2.1. Animals

All animal experiments were carried out following local and National guidelines on the use of animals for experimentation and with the approval of local and National Ethics Committees. Peripheral blood samples used in these studies were obtained from BCG-vaccinated and non-vaccinated cattle housed at the Institute for Animal Health (Compton, UK) and the National Animal Disease Center (Ames, IA). All animals were vaccinated s.c. with 10^6 CFU of BCG. Animals were vaccinated with a second dose of 10^6 CFU of BCG, at 4 months following the primary dose, to boost T cell responses for analysis of effector activities.

2.2. Measurement of whole blood IFNγ

To measure production of IFNγ by peripheral blood cells, 200 μl blood/well was cultured in a round bottom 96-well plate with medium (negative control) or *M. bovis* PPD (PPD-B) (VLA, Surrey, UK) at 20 μg/ml final concentration and incubated at 37 °C in an atmosphere of 5% CO_2 and 95% humidity. After 24 h, plates containing blood cultures were centrifuged at 800 × *g* for 10 min and plasma harvested and stored at −20 °C until use. The concentration of IFNγ was

determined by standard capture ELISA as described previously [37].

2.3. Isolation and derivation of leukocyte populations

Monocytes from cattle vaccinated with attenuated *M. bovis* bacillus Calmette-Guérin (BCG) were isolated from peripheral blood mononuclear cells using human CD14$^+$ paramagnetic beads (Miltenyi) according to manufacturer recommendations. Mϕ were derived as previously described [38] using 0.2 U/ml recombinant bovine GM-CSF produced in Cos cells. Mϕ were infected with BCG (2.5×10^5 CFU), pulsed with PPD-B (20 μg/ml), or not treated. Autologous CD4$^+$ cells were isolated from peripheral blood mononuclear cells using 1 μg/ml per 10^6 cells mab CC8 (IgG2) (hybridoma supernatant) against bovine CD4 and paramagnetic beads conjugated to rat anti-mouse IgG2 (Miltenyi). Purity of the Macs sorted CD4$^+$ T cells from the individual donors varied from 92 to 98%, as assessed by flow cytometry. Purified CD4$^+$ T cells were exposed to non-infected, BCG-infected, or PPD-B pulsed Mϕ for 1, 3, and 6 days.

2.4. Flow cytometric analysis

CD4$^+$ T cells co-cultured with Mϕ were analyzed by three color flow cytometry for expression of CD4, CD45RA, CD45RO, or CD62L, using mabs CC8 (IgG2), CC76 (IgG1), IL-A116 (IgG3) and CC62 (IgG2b), respectively. Cells, 10^6 per sample, were stained with 25 μl of a 1 μg/ml dilution of each antibody, using titrated hybridoma supernatant. For intracellular staining of perforin or IFNγ, Brefeldin A (BD Sciences Pharmingen, San Diego, CA) was added to a final concentration of 10 μg/ml during the last 4 h of lymphocyte culture. The antibody to human perforin δG9 (IgG2b) (BD Sciences Pharmingen) has been shown to cross-react with bovine perforin [35] and was used according to manufacturer's instructions. The antibody to bovine IFNγ, CC330 (IgG1), was used at 1 μg/ml as affinity chromatography purified antibody. Cells were fixed and permeated using Becton Dickinson cytofix and FACS permeabilization buffer (BD Sciences Pharmingen) according to the protocol supplied by the manufacturer. Intracellular proteins were detected using PE or FITC conjugated mab to human perforin (BD Sciences Pharmingen), and PE or FITC conjugated antibody to bovine IFNγ (Serotec, Oxford, UK). Samples were fixed using 2% buffered paraformaldehyde prior to analysis by flow cytometry. Additional samples were stained with isotype matched, non-specific, surface (mouse immunoglobulin from clones AV20, AV37, AV29, IAH, Compton, UK) and intracellular antibodies (mouse IgG2b-PE or mouse IgG2b-FITC, BD Biosciences) to determine background levels of fluorescence. Samples were analyzed with a FACSCalibur instrument (BD Biosciences) by acquiring a minimum of 10,000 events within a live lymphocyte gate (forward and side scatter characteristics consistent with those of live lymphocytes. Memory subpopulations of purified CD4$^+$ T cells (CD45RO$^+$, CD45RO$^-$) cells were separated following activation with PPD-pulsed autologous Mϕ with a FACSAria instrument (BD Biosciences). Data were analyzed with FCS Express (De Novo software, Ontario, Canada).

2.5. Gene expression

RNA from mixed CD4$^+$ lymphocytes and Mϕ samples was harvested at 1, 3, and 6 days, following stimulation with medium, PPD-B, or BCG. RNA was harvested using RNEASY (Qiagen, Valencia, CA) and cDNA was synthesized using Superscript reverse transcriptase (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations. PCR was performed using bovine specific primers for perforin, IFNγ, Bo-lysin, IL-13, IL-21, and the housekeeping gene G3PDH using bovine specific primers as follows: perforin, forward 5′-GAT GCC AAC TTC GCC GCC CA-3′ and reverse, 5′-TGT CAG TCA CGT ACT TGC TC-3′; IFNγ, forward 5′-GCA AGT AGC CCA GAT GTA GC-3′ and reverse, 5′-GGT GAC AGG TCA TTC ATCAC-3′ Bo-lysin, forward 5′-CTG CTG CTC CAA GGA GAA GA-3′ and reverse, 5′-GCA GTG GAG GGA GTT TGG T-3′; G3PDH, forward 5′-GGA GAA ACC TGC CA-3′ and reverse, 5′-GTG TCG CGT GTT GA-3′; IL-13, forward 5′-CAC CAT GGC GCT CTT ATT G-3′ and reverse, 5′-TCA GTT GAA TCT TTC ATT GCG-3′; IL-4 forward 5′-GTC TCA CCT ACC AGC TGA TC-3′ and reverse, 5′-TCA GCG TAC TTG TGC TCG TC-3′, and IL-21, forward 5′-TAA GTC AAG CTC CCA AGG GCA AGA-3′ and reverse, 5′-CTT CTC CCT GTA TTT GTG GCA GGT-3′. Semi-quantitative real-time RT-PCR was performed to determine relative differences in gene expression as affected by antigen and days post-exposure to *in vitro* antigen. Differences in gene expression were determined using bovine specific primers and probes for IFNγ forward 5′-CAG AAA GCG GAA GAG AAG TCA GA-3′ reverse 5′-CAG GCA GGA GGA CCA TTA CG-3′ and probe TCT CTT TCG AGG CCG GAG AGC ATC A, IL-4 forward 5′-TTG AAC AAA TTC CTG GGC G-3′ reverse 5′-GGT CTT GCT TGC CAA GCT GT-3′ and probe CTT GAC AGG AAT CTC, IL-13 forward 5′-ACC AAG AGG ATG CTG AAT G-3′ reverse 5′-CTG CTT AGC TGA GGG CTT G-3′ and probe CAC TCT GTC CTC A, Bo-lysin (47), perforin, forward 5′-TTC GCC GCC CAG AAG AC-3′, reverse 5′-CAC TCC ACT AAG TCC ATG CTG AA-3′, and probe 5′-ACC AGG ACA ACT ACC G-3′, and IL-21, forward 5′-TTG CCT GAT GGT CAT CTT CTG TGG-3′, reverse 5′-TCT GGA GCT GGC AGA AAT TCA GGA-3′, and probe 5′-TCC CAA GGG CAA GAT CGC CTC TTT AT-3′ labeled with FAM (Applied Biosystems, Foster City, CA). VIC labeled 18s ribosomal control primers and probe (Applied Biosystems) were included in each reaction to standardize template. Effects of cytokine stimulation on relative gene expression were determined using an ABI PRISM 7000 sequence detector (Real-Time PCR Core Facility, UTMB, Galveston, TX).

2.6. Anti-mycobacterial activity

Mϕ were isolated from fresh peripheral blood, as described above, from each donor 1 day prior to determination of CD4+ T cell anti-mycobacterial activity. Mϕ were infected with BCG at a ratio of 10:1, 10 CFU of BCG to 1 Mϕ, and cultured for 16 h in universal tubes to prevent adherence. Following infection, Mϕ were harvested, counted using trypan blue, and washed three times in phosphate buffered saline at $300 \times g$ for 10 min as previously described. Using this protocol, approximately 40% of the Mϕ have intracellular mycobacteria, and >90% are viable, as determined by trypan blue exclusion. To determine the ability of CD4+ cells to contribute to the killing of mycobacteria, CD4+ cells were cultured with BCG-infected Mϕ at a ratio of 2:1 and a total cell concentration of 10^6 cells/ml. After 6 days, CD4+ cells were harvested and cultured with BCG-infected Mϕ at a ratio of 5:1 in 1 ml in 24-well plates at 10^6 cells/ml. After 24 h, the entire well was lysed with 1 ml PBS containing 1% Triton X-100/well. Colony forming units were determined by inoculating 7H10 agar plates with five-20 μl droplets of neat or serially diluted cell lysates. Plates were incubated for 3 weeks prior to determination of CFU.

2.7. Data analysis

Statistical analysis of the data was performed using Graphpad Prism® software (San Diego, CA). The probability of differences due to treatment effects was determined by one-way ANOVA. A Mann–Whitney *U*-test was used for individual comparisons to determine the level of significance of observed effects of antigen and CD4+ T cell subset on levels of intracellular effector molecules and CFU reduction. An un-paired *t*-test with Welch's correction was used to analyze one experiment where data were collected from only two animals per treatment. A value of $p < 0.05$ was accepted for significance. Real-time PCR results were evaluated using an internal control to equilibrate starting template in the analysis of relative changes in gene expression. Data was evaluated as the fold induction of equilibrated gene expression as compared to non-stimulated CD4+ T cells from the same animal, cultured under otherwise equivalent conditions.

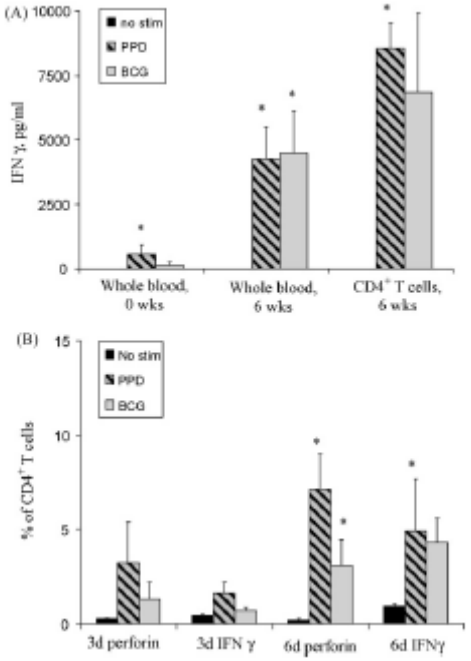

Fig. 1. BCG vaccination primes expression of effector molecules by CD4+ T cells. (A) Antigen specific secretion of IFNγ by peripheral blood leukocytes was determined at 0 and 6 weeks after vaccination with 10^6 CFU of BCG. IFNγ expression was determined by ELISA in supernatants from whole blood cultured with 20 μg/ml PPD, or 2.5×10^5 CFU BCG. IFNγ secretion by purified CD4+ T lymphocytes, 6 weeks following boost with 10^6 CFU of BCG, was determined following 6 days incubation with autologous Mϕ or autologous Mϕ pulsed with PPD or infected with BCG. (B) Intracellular expression of IFNγ and perforin by CD4+ T lymphocytes from BCG-vaccinated animals 6 weeks following boost with 10^6 CFU of BCG. Data are presented as the percentage of CD4+ T lymphocytes expressing intracellular IFNγ and perforin following 6 days co-culture with autologous Mϕ or autologous Mϕ pulsed with PPD or infected with BCG. Data are shown as average (±S.E.M.) of four, two, and three individual animals for whole blood IFNγ secretion, CD4+ T cell IFNγ secretion, and intracellular protein, respectively. *$p < 0.05$, level of statistical significance between *in vitro* stimulation and no stimulation (mean ± S.E.M.).

3. Results

3.1. Functional characterization of effector and memory CD4+ T cells following vaccination

A recall CMI response to mycobacterial antigens was measurable in a whole blood IFNγ assay (Fig. 1A) 6 weeks after vaccination with *M. bovis* BCG (BCG). It has been shown that CD4+ T cells are the main cell population secreting IFNγ in PBMC from mycobacteria-sensitized cattle following stimulation *in vitro* [39]. Accordingly, we isolated CD4+ T cells and sought to determine whether the response elicited following antigen presentation was influenced by the physical form of the antigen. Fig. 1B shows the expression of IFNγ and perforin by CD4+ T cells after 3 and 6 days stimulation with autologous Mϕ that have been pulsed with medium (negative control), PPD (soluble non-replicating antigen) or BCG (particulate replicating antigen). Secretion of IFNγ by, and accumulation of intracellular IFNγ in, purified CD4+ T cells from BCG-vaccinated cattle was increased following exposure to antigen-loaded Mϕ (Fig. 1A and B). Perforin expression also increased in CD4+ T cells from vaccinated animals after antigen exposure. The relative expression of perforin and IFNγ, however, was not affected

by the choice of antigen (PPD-B, BCG). Flow cytometric forward and side scatter (FSC/SSC) analysis of purified $CD4^+$ T lymphocytes, after 6 days *in vitro* incubation with PPD-pulsed or BCG-infected Mϕ showed an increase in size and granularity (data not shown). Analysis of the cultured cells showed that the majority of $perforin^+$ and $IFN\gamma^+$ cells were $CD4^+$, while a small population of $perforin^+$ and $IFN\gamma^+$ cells were also detected in a minor $CD4^-$ population (data not shown). Expression of intracellular perforin and IFNγ was not enhanced in $CD4^+$ T lymphocytes from non-vaccinated animals. The percentage of $perforin^+$ or $IFN\gamma^+$ $CD4^+$ T cells from two non-vaccinated animals was ≤1% and was not affected by exposure to PPD pulsed or BCG-infected autologous Mϕ (data not shown). Following antigen activation, $perforin^+$ and $IFN\gamma^+$ $CD4^+$ cells from vaccinated animals were characterized as having a memory phenotype ($CD45RA^-$, $CD45RO^+$, and $CD62L^+$) (Fig. 2). The mean fluorescent intensity (MFI) of CD62L expression by $CD4^+$ T cells was decreased following PPD or BCG activation (data not shown). The changes in expression of intracellular effector proteins and memory markers were determined by comparison to purified $CD4^+$ T cells from the same animals, cultured in the absence of antigen to account for potential artifacts induced by *in vitro* culture (Fig. 2). The expression of memory phenotype markers was not affected by the choice of *in vitro* antigen (PPD-B or BCG).

3.2. Kinetics of effector molecule expression by antigen-primed $CD4^+$ T cells

To determine expression of effector molecules and relevant cytokines, RT-PCR was performed on RNA from $CD4^+$ T cells exposed to PPD-B pulsed or BCG-infected Mϕ. Transcripts for IFNγ, perforin, and IL-21 were not detectable in RNA from non-stimulated $CD4^+$ T cells (Fig. 3). Increased expression of IFNγ was detected by 1 day post-exposure and enhancement of perforin and IL-21 expression was greatest at day 6. Bovine granulysin (bolysin) and IL-13 transcripts could be detected in RNA from non-activated CD4 T cells, but enhanced expression could be detected within 24 h of antigenic stimulation. Real-time Taqman PCR analysis of $CD4^+$ T cells from two donors exposed to PPD-B pulsed or BCG-infected Mϕ for 1, 3, and 6 days further demonstrated differences in the activation kinetics of effector molecules and cytokines (Fig. 4). Enhancement of IFNγ transcripts was greatest 3 days following stimulation with specific anti-

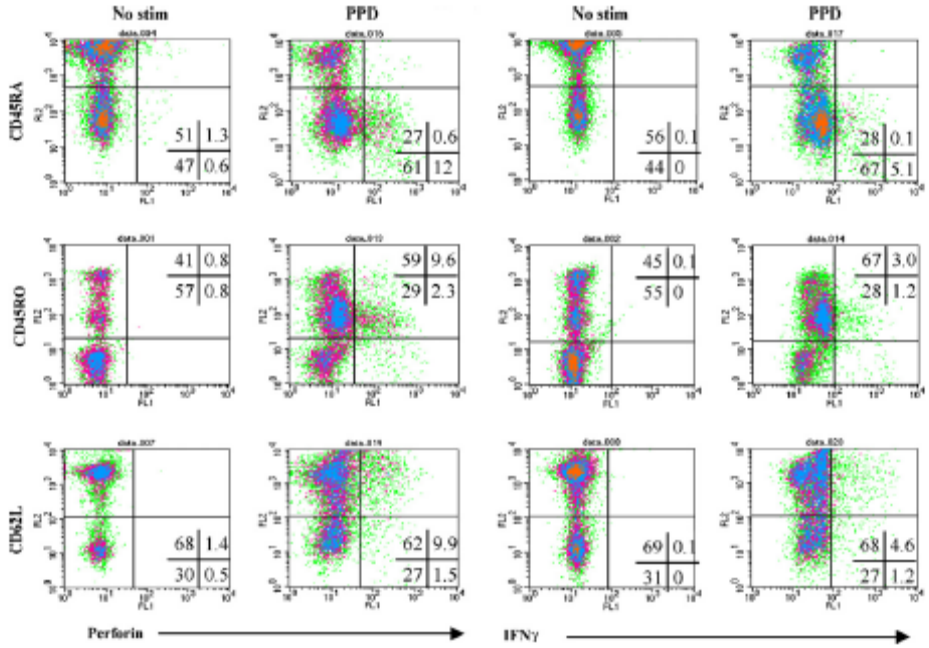

Fig. 2. Expression of effector/memory markers correlates with effector molecule protein expression following antigenic stimulation. Expression of intracellular perforin, IFNγ, and cell surface markers CD45RA, CD45RO, and CD62L, by purified $CD4^+$ T lymphocytes following 6 days *in vitro* exposure to non-stimulated autologous Mϕ or autologous Mϕ pulsed with PPD (20 μg/ml). $CD4^+$ T cells and Mϕ were isolated from peripheral blood of a BCG-vaccinated calf 6 weeks following boost with 10^6 CFU of BCG. Data shown are representative of results from four individual animals.

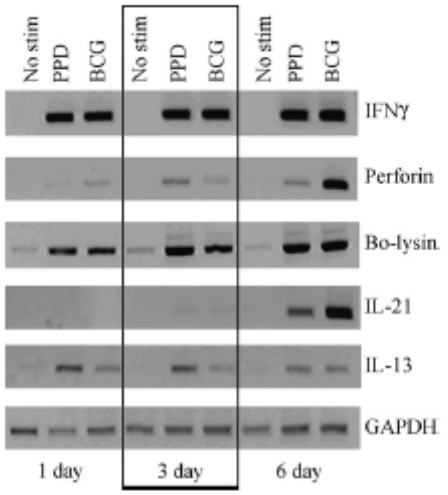

Fig. 3. Transcription of IFNγ, Bo-lysin, perforin, GAPDH, IL-21, and IL-13 from a BCG vaccinated animal after antigen stimulation. RNA was obtained from CD4+ T cells after 1, 3 or 6 days co-culture with non-stimulated, PPD pulsed or BCG-infected autologous Mϕ. PCR was performed with cDNA using bovine specific primers.

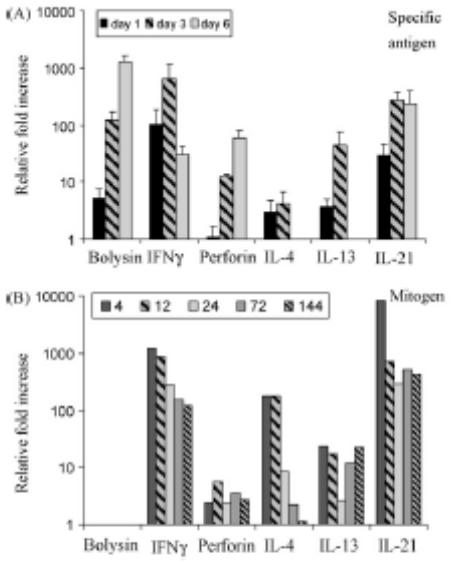

Fig. 4. Real-time PCR detection of effector molecule gene expression. (A) Antigen specific expression at 1, 3 and 6 days following stimulation of BCG specific CD4+ T cells with PPD pulsed autologous Mϕ. (B) Expression of effector molecules by CD4+ T cells after 4–144 h mitogen (PMA + ionomycin) stimulation. Results are presented as fold increase relative to non-stimulated CD4+ T lymphocytes from the same animal. Antigen specific data are shown as the average (±S.E.M.) of the fold increase from non-stimulated samples of three individual animals. Data shown for mitogen kinetics are representative of results from two individual animals.

gen, while transcription of bolysin and perforin was greatest at 6 days post-exposure (Fig. 4A). IL-4 and IL-13 expression was up-regulated at days 1 and 3, but was not different from non-activated sample levels by day 6. Expression of IL-21, however, was delayed compared to IL-4 and IL-13, with higher levels observed at days 3 and 6. *In vitro* exposure to antigen did not enhance relative expression of perforin and IFNγ in CD4+ T cells from non-vaccinated animals (data not shown). A non-specific increase in relative expression of bolysin was observed in three non-vaccinated donors (2–10-fold) by 3 days of *in vitro* exposure to PPD-B pulsed Mϕ (data not shown). The non-specific enhancement of bolysin gene expression by antigen was insignificant compared to the >100-fold increase in bolysin gene expression observed following antigen specific exposure (Fig. 4A).

To determine the potential of CD4+ T lymphocytes to express these genes, RNA was harvested from CD4+ T cells of a healthy non-vaccinated animal following 0, 4, 12, 24, 48, 72, and 144 h of mitogenic stimulation with phorbal myristate acetate and ionomycin (PMA-I) (Fig. 4B). As expected, IFNγ and IL-4 gene expression increased early and then decreased. Perforin expression increased moderately compared to the levels observed in the antigen specific response. Expression of IL-13 appeared to be a biphasic response. In contrast to the delayed response seen to specific antigen, IL-21 was up-regulated by 4 h mitogenic stimulation and remained elevated for at least the next 144 h. Real-time PCR data indicated a lack of antigen specific enhancement of IL-2, IL-6, IL-10, IL-15, TNFα, CD40L, while transcription of these genes was up-regulated in PMA-I stimulated CD4+ T cells (data not shown). Surprisingly, the robust enhancement of bolysin gene expression observed following antigenic stimulation in the vaccinated animals was not paralleled by enhanced gene expression following non-specific mitogenic stimulation with PMA-I. Transcripts for bolysin were detected following PMA-I stimulation, but did not differ from the non-stimulated samples. These results were repeated with additional BCG vaccinates to further characterize differences between antigenic and mitogenic enhancement of biomarker expression in the same pool of CD4+ T cells. As previously observed, increased bolysin expression was not detected after PMA-I stimulation of purified CD4+ T cells, while a 4–8-fold increase in perforin, and a 100–1000-fold increase in IFNγ transcripts were observed relative to non-stimulated samples (data not shown).

3.3. Activated CD4+ T cells reduce mycobacterial burden in Mϕ

To determine the relevance of expression of cytolytic machinery by antigen specific T cells, the ability of anti-

gen stimulated CD4+CD45RO+, and CD4+CD45RO− T cells from vaccinated animals to inhibit growth of intracellular BCG was determined. FACSARIA sorted populations of CD45RO+, and CD45RO− CD4+ T cells were 96 and 99% pure, respectively (Fig. 5A). FSC/SSC characteristics of the sorted populations indicated that CD45RO+ CD4 T cells are larger and more granular following antigen specific stimulation than CD45RO− CD4 T cells (Fig. 5A). The CD45RO+ population was also more numerous and may have greater anti-mycobacterial activity against BCG in infected autologous Mϕ (Fig. 5A and B) following stimulation with antigen. The CD45RO− CD4+ T cells, however, were also able to reduce growth as measured by CFU of BCG in infected autologous Mϕ (Fig. 5B). The anti-mycobacterial effects of purified CD4+ T cells stimulated with PPD pulsed autologous Mϕ for 5 days were also observed in two additional BCG vaccinates, although effects of individual memory populations on reduction of CFUs were not determined and statistical significance of anti-mycobacterial activity could not be evaluated (data not shown.) Despite variability among donors, CD4+ T cell inhibition of BCG CFU in infected Mϕ was detectable in all vaccinated donors. To a lesser extent, non-stimulated CD4+ T cells from vaccinated donors were able to reduce BCG CFU in infected autologous Mϕ (Fig. 5B).

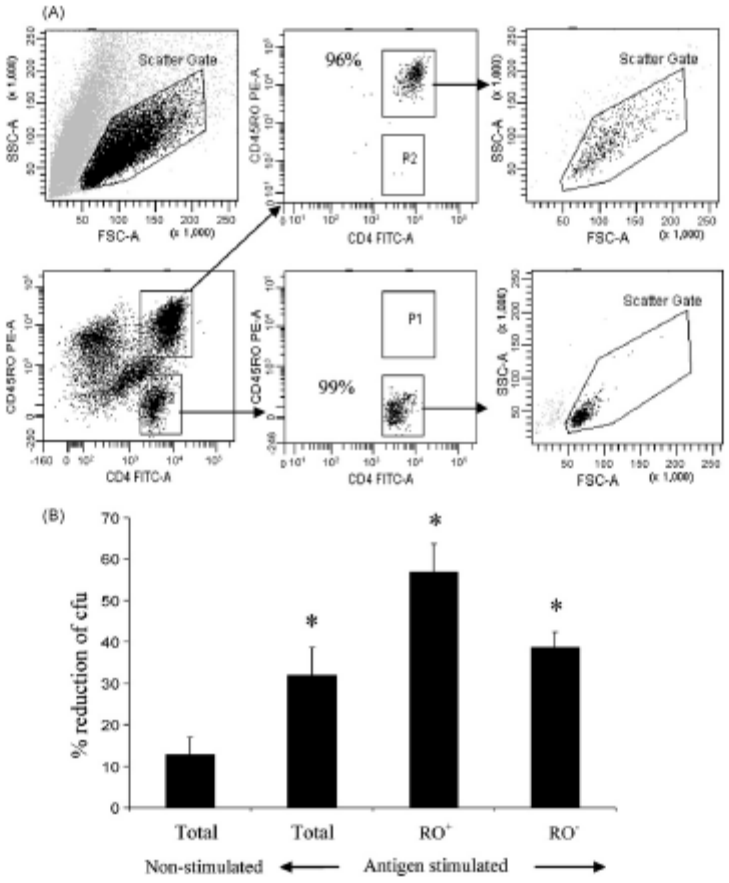

Fig. 5. Antigen specific CD4+ T cells reduce BCG CFU in infected Mϕ. (A) FSC/SSC characteristics and purity of CD4+ T cells after culture for 5 days with PPD-B pulsed autologous Mϕ and sorted by FACSARIA into CD45RO+ and CD45RO− populations. (B) BCG CFU inhibition in lysates from autologous, infected Mϕ after 24 h co-culture with non-activated total CD4+, or CD4+, CD4+CD45RO+ and CD4+CD45RO− T cells activated with PPD. Data are presented as the percent inhibition of CFU relative to BCG-infected Mϕ not exposed to CD4+ T lymphocytes. Data are shown as average (±S.E.M.) of four animals from two independent experiments. *$p < 0.05$, level of statistical significance between antigen stimulated and non-stimulated CD4+ T cell subsets.

4. Discussion

Major roadblocks to the design of vaccines that protect against TB are lack of surrogate markers of protection and lack of suitable animal models [4,40]. Understanding the mechanisms involved in the development of protective $CD4^+$ T cell memory responses is an important component of TB vaccine enhancement efforts. Thus, there is currently a need to characterize biomarkers of $CD4^+$ T cell mediated immunity in additional animal models of TB. In the present study, we used the bovine model of TB to determine the ability of BCG to elicit cytolytic-effector $CD4^+$ T cell responses upon re-exposure to vaccine antigen *in vitro*. Our results demonstrate that BCG vaccination induces mycobacteria specific $CD4^+$ T cells which: (A) express a memory/activated phenotype; (B) express cytolytic molecules and effector cytokines, and (C) contribute to reduction of BCG CFU in infected Mϕ.

Human and bovine TB have similar patterns of transmission, clinical presentation, and tissue pathology [5,27,28]. The human TB vaccine, *M. bovis* BCG, is an attenuated strain of the pathogen causing TB in cattle. BCG, as the only licensed vaccine available for TB, is our best tool to date to characterize the nature of the protective immune response against infection. Vaccine efficacy is variable in both species (0–80%) and is affected by environmental factors and immune status of the subject [4,5]. Cell mediated immune responses play predominant roles in containing mycobacterial pathogens in human and bovine TB. Type 1 T cell responses are thought to correlate with protection in human and bTB, and the balance of Type1/Type2 cytokines (IFNγ, IL-4) in cattle is more similar to human profiles than those seen in mice [41,42]. Activated bovine $CD4^+$ and $CD8^+$ T cell subsets also express homologues of perforin and granulysin, granule proteins with important roles in immunity to TB [35].

Effector activities of the CMI response include the production of cytokines and the cytolysis of infected cells. Cytotoxicity, as a general mechanism for pathogen control, can involve apoptosis of infected cells through Fas/FasL interaction or lysis/apoptosis of an infected cell resulting from release of cytotoxic granule proteins [43]. In TB, a disassociation between lysis of infected cells and reduction of *Mtb* CFU by $CD8^+$ T cells indicates functional separation of receptor and granule lytic mechanisms [20]. In the current trial, expression of FasL by purified $CD4^+$ T cells from BCG-vaccinated animals was not enhanced by exposure to antigen *in vitro* (not shown). Reports of *Mtb* specific expression of FasL by human $CD4^+$ and $CD8^+$ cells from PPD^+ individuals are conflicting. Reduction of *Mtb* in infected human Mϕ by $CD4^+$ and $CD8^+$ T cells, however, has been demonstrated to be granule dependent and FasL independent [20].

To date, the importance of granule proteins expressed by T cells in protective immunity to TB is not well characterized. Expression of granzymes A and B is enhanced following *Mtb* activation of peripheral blood from vaccinated individuals, although these molecules do not appear to have a protective role in human or murine TB [23,44]. The role of perforin in T cell mediated immunity to TB is controversial, as perforin knockout mice are not more susceptible to *Mtb* infection than wild type counterparts [23]. The role of perforin in granulysin-mediated reduction of intracellular *Mtb* indicates that perforin may play a more important role in human immunity to TB [45]. In the current studies, bovine perforin expression in $CD4^+$ T cells is primarily confined to *M. bovis* specific cells with a memory phenotype and does not represent a bystander activation event. Protein and mRNA analysis indicates that perforin is not constitutively expressed by $CD4^+$ T cells and is expressed late in response to specific antigen as compared to mitogen. Expression of perforin and IFNγ correlated with reduction of BCG CFU in infected Mϕ by the same pool of antigen specific $CD4^+$ T cells.

Antigen specific activation of $CD4^+$ T cells in the current trial resulted in a substantial increase in granulysin expression, as measured by real-time PCR. Granulysin has dramatic effects on *Mtb* viability, causing osmotic lysis through an electroporation effect on the organism's membrane [45]. Using recombinant proteins, Stenger and colleagues demonstrated that human granulysin alone could dramatically reduce extracellular *Mtb* and likely used perforin to access and kill intracellular *Mtb* [18]. Characterization of human granulysin expression and cytotoxicity to date has primarily been inferred from *in vitro* studies of *Mtb* expanded peripheral blood and CD1 restricted T cell lines specific for *Mtb* antigen [20,22,46]. Blocking studies of FasL, perforin, and granzyme substrates, in these cell lines indicated an important lytic role for granulysin in reduction of intracellular *Mtb* [20,47]. Clinical recovery of children from TB following chemotherapy correlates positively with expression of IFNγ and granulysin, and correlates negatively with proliferation of Vγ9δ2 gamma delta T cells in response to mycobacterial antigens [46]. Expression of granulysin, as compared to perforin, by $CD4^+$ T lymphocytes in contact with *M. leprae*-infected Mϕ is associated with containment of *M. leprae* [48]. A murine homologue to granulysin has not been identified to date, precluding the study of clinical effects of gene deletion in a tractable animal model of disease. We have previously demonstrated anti-mycobacterial activity of bovine granulysin peptides and demonstrated granulysin and perforin expression in the lymphocytic cuff of a forming granuloma in an animal infected with virulent *M. bovis* [35]. Analysis of granulysin transcripts by reverse transcriptase PCR and real-time PCR in the current trial showed that low-level expression is quickly induced relative to perforin, while significant induction of expression follows a pattern similar to perforin. Antigen specific recall of granulysin following controlled vaccination for any pathogen has not been previously demonstrated to the best of our knowledge. The current study is also the first demonstration of the kinetics of granulysin expression relative to perforin, IFNγ, and key T helper cytokines during an antigen specific response to a pathogen. It is surprising that expression of granulysin is observed early in the current study, as human studies indicate expression

is not measurable by RNase protection until 3–5 days and does not significantly amplify until at least 5–10 days post-stimulation [20,49]. Although these differences may indicate species differences between humans and cattle, it is more likely that the observed differences in granulysin expression are due to assay sensitivity, as RT-PCR, used in our studies, is significantly more sensitive than RNase protection.

In the current studies, lytic activity of antigen stimulated $CD4^+$ T cells correlated with expression of cell surface memory markers CD45RO and CD62L. Similar to previous studies, the level of CD62L expression by $CD4^+$ T cells decreased with activation [33,50,51]. In previous studies, expression of CD62L failed to correlate with antigen specific lympho-proliferative responses [33,51]. Expression of perforin and IFNγ in the current study, however, was shown to be primarily confined to the memory subpopulation expressing high levels of CD62L. Functional separation of antigen specific lymphoproliferation, and expression of effector molecules, by memory subsets of $CD4^+$ T cells is an important avenue for further investigation. Expression of granulysin RNA correlated to antigen activation conditions that resulted in enhanced expression of memory markers, lytic activity, and expression of cytotoxic/effector molecules, but could not be directly associated with memory phenotype. The precise mechanism of CFU reduction by $CD4^+$ T cells will be investigated in future studies using combinations of blocking antibodies and chemical inhibitors of granule exocytosis. Fas-mediated killing in mice has been shown not to result in reduction of CFU in co-culture [52]. Given the lack of gene deleted knockouts and the difficulty of siRNA or other loss of function studies in primary bovine T cells from vaccinated animals we cannot rule out the action of secreted IFNγ and Mϕ activation for at least partial reduction of CFU. The relative proportion of $CD4^+$ T cells with the potential for cytotoxicity (perforin positive) in the current study suggests this as a possible means of reduction of CFU. The $CD4^+$ $CD45RO^-$ cell population also reduced CFU following antigen specific activation. A low level of observed perforin and/or IFNγ in this population, as compared to the $CD4^+$ $CD45RO^+$ T cells, may account for these observed effects as well as the reduction of CFU by the total $CD4^+$ T cell population. An association of granulysin with memory T cell populations has not been demonstrated, to date, and may also account for the lytic activity of the $CD4^+$ $CD45RO^-$ and total $CD4^+$ T cell populations.

In the current experiments, mRNA expression of T helper cytokines IL-4, IL-13, and IL-21 was observed following antigen specific activation of CD4 T cells from vaccinated animals. The role of IL-13 in regulation of cellular immune responses in non-rodent species is significant particularly in cattle where IFN-γ dominates in response to intracellular pathogens and cell mediated damage can contribute to extensive immunopathology. IL-4 and IL-13 have both been shown to be elevated in TB patients relative to controls, and correlated with a less desirable disease outcome [53]. In cattle, it has not been demonstrated that IL-4 is expressed to the exclusion of IFN-γ [41,54]. Analysis of lymph node cell populations following challenge or vaccination/challenge of calves with virulent *M. bovis* demonstrated the potential importance of relative Type 1/Type 2 bias in immunity to TB [55]. A balanced IFNγ to IL-4 ratio correlated to protective immunity due to vaccination, while a more dominant IFNγ response correlated with increased pathology and bacterial load [55]. Expression of IL-21 by $CD4^+$ T cells from vaccinated animals in the current trial was enhanced following antigen specific exposure to BCG or PPD. IL-21 is a recently characterized Type 2 cytokine of the IL-2 family (IL-2, IL-7, IL-15), produced primarily by activated $CD4^+$ T cells [15]. IL-21 enhances the cytotoxicity of NK cells and $CD8^+$ CTLs [56,57], can suppress IFNγ production [57,58], and plays a regulatory role for DC maturation and B cell class switching [15]. In the current trial, antigen specific enhancement of IL-21 could be characterized as a late response, peaking subsequent to IFNγ, IL-4 and IL-13, but coincident with granulysin and perforin. This is the first characterization of antigen specific expression of IL-21 mRNA simultaneous with biomarkers following vaccination with mycobacteria. IL-21 may be an important regulator of molecules with delayed expression kinetics following antigen specific activation. Determining roles for IL-21 in regulating the balance of cytotoxic and effector molecules in the cell mediated immune response to TB will be an important focus of future studies.

The current work represents an important characterization of lytic and effector biomarkers that are enhanced in memory populations of post-vaccination $CD4^+$ CTL in bovine TB. Our results indicate that vaccination of cattle with BCG generates populations of $CD4^+$ T cells with enhanced anti-mycobacterial activity and further support the investigation of biomarkers with potentially important roles in human and bovine TB. The current studies further contribute to the general knowledge base of TB immunity by demonstrating an important association between surface markers of immune memory, anti-mycobacterial activity, and $CD4^+$ T cell derived lytic and effector molecules. The kinetic evaluation of the lytic molecules and effector cytokines in the current studies also provides insight into how the usage of cytolytic/effector mechanisms by $CD4^+$ T cells may alternate following mycobacterial exposure as compared to simultaneous expression. Future studies will define the important lytic role and relative predictive value of individual biomarkers of memory $CD4^+$ T cells in experimental vaccine/virulent challenge studies. These advances in the bovine model of TB will enable the investigation of important indicators of successful immune response to vaccine candidate alternatives to BCG in humans and cattle.

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APPENDIX 2

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Failure of a *Mycobacterium tuberculosis* ΔRD1 ΔpanCD double deletion mutant in a neonatal calf aerosol *M. bovis* challenge model: Comparisons to responses elicited by *M. bovis* bacille Calmette Guerin

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Abstract

An attenuated *Mycobacterium tuberculosis RD1* knockout and pantothenate auxotroph ($mc^2$6030) vaccine administered at 2 weeks of age failed to protect calves from low dose, aerosol *M. bovis* challenge at 2.5 months of age. In contrast, *M. bovis* bacille Calmette Guerin (BCG)-vaccinates had reduced tuberculosis-associated pathology as compared to non- and $mc^2$6030-vaccinates. Mycobacterial colonization was not impacted by vaccination. Positive prognostic indicators associated with reduced pathology in the BCG-vaccinated group were decreased antigen induced IFN-γ, iNOS, IL-4, and MIP1-α responses, increased antigen induced FoxP3 expression, and a diminished activation phenotype (i.e., ↓CD25+ and CD44+ cells and ↑CD62L+ cells) in mycobacterial-stimulated mononuclear cell cultures. The calf sensitization and challenge model provides an informative screen for candidate tuberculosis vaccines before their evaluation in costly non-human, primates.
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Keywords: Tuberculosis; Live bacterial vaccine; Auxotroph; BCG; Neonatal calf; Aerosol Inoculation; FoxP3

1. Introduction

Tuberculosis (TB) vaccines for eventual use in humans are typically screened for safety and efficacy using mouse (including immune deficient strains for safety assurance) and guinea pig models of infection [1]. The most promising candidates are evaluated further in non-human primates [2]. A key target population for TB vaccination is the newborn child, particularly in poverty stricken countries where nutritional, respiratory, and enteric diseases are common [3]. Neonatal cynomolgus macaques (*Macaca fascicularis*), while particularly relevant TB models, are costly to house in biosafety level-3 (BL-3) facilities and are limited in availability due to a monestrous reproductive cycle.

Another option for neonatal testing of candidate TB vaccines is the neonatal calf (*Bos taurus*) [4–6]. Large numbers of age-, sex- and breed-matched calves can be acquired throughout the year and calves are substantially less expensive to purchase and house than neonatal monkeys. Because cattle are out-bred, experimental variance is similar to that observed in non-human primates and humans. Additionally, the size of the newborn calf allows dose titration studies at a range relevant to studies in humans. Collection of large volumes of

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blood at frequent intervals is also an advantage of this model. Finally, the nutritional status, that can affect vaccine efficacy, can be manipulated in the neonatal calf to achieve degrees of protein-energy malnutrition [7] comparable to those experienced by human infants in developing countries.

Mycobacterium bovis, a member of the *M. tuberculosis* (*tb*) complex is the most frequently employed challenge strain in cattle studies. In addition to the serious economic and health concerns due to bovine TB, *M. bovis* is increasingly recognized as a major cause of disease in developing nations [8]. Recent TB outbreaks in several US cities are linked with ingestion of *M. bovis*-infected, non-pasteurized cheese from Mexico [9]. Thus, efficacy testing using newborn calves has relevance for evaluation of neonatal protective immunity, bovine TB control, duration of immunity studies, and as a screening tool for vaccines targeted for human testing. These attributes of the neonatal bovine model make it a useful model for screening and prioritizing vaccines before their evaluation in neonatal non-human primate models.

The region of difference 1 (*RD1*) deletion of *M. bovis* is the predominant attenuating defect in the vaccine strain developed by Calmette and Guerin in the early 1900's [i.e., *M. bovis* bacille Calmette Guerin (BCG)]. Deletion of *RD1* (i.e., Δ*RD1*) results in a loss of genes encoding early secretory antigenic target-6 kDa protein (ESAT-6) and culture filtrate protein 10 (CFP10) [10–13], as well as several other genes. Although vaccination of mice with *M. tuberculosis* Δ*RD1* provides similar protection as BCG, it has reduced safety in immune deficient mice [13]. Recent development of efficient tools for genetic manipulation of *Mycobacteria* spp. has enabled the targeted discovery of highly immunogenic attenuated live strains of *M. tuberculosis* and *M. bovis* (reviewed in [14]). Several *M. tuberculosis* auxotrophs induce significant levels of protection in mouse and guinea pig models of TB. Deletion of pantothenate biosynthesis genes from *M. tuberculosis* (i.e., *ΔpanCD*) results in an attenuated strain with limited capacity for in vivo replication while retaining similar efficacy to that of BCG in mice [15].

In the present study, a double deletion mutant of *M. tuberculosis* H37Rv strain (i.e., *ΔRD1ΔpanCD*, designated mc^{2}6030) was evaluated for efficacy in the neonatal calf model. The *RD1* deletion mimics the attenuation of BCG and the *panCD* deletion provides additional safeguards against prolonged in vivo replication, of particular importance with immune compromised hosts [13,14,16]. Responses and efficacy were compared to *M. bovis* BCG (Danish strain), the current standard for evaluation of TB vaccines.

2. Materials and methods

2.1. Animals, vaccination, and challenge procedures

Seventeen newborn Holstein bull calves were obtained from a TB-free herd in Newton, WI and housed in a biosafety level-3 (BSL-3) facility at the National Animal Disease Center, Ames, Iowa according to Institutional Biosafety and Animal Care and Use Committee guidelines. Vaccine treatment groups included: no vaccination ($n=7$), 1.8×10^5 cfu mc^{2}6030 vaccination ($n=6$), and 1.8×10^5 cfu BCG-Danish vaccination ($n=4$). Selection of mc^{2}6030 dosage (i.e., 1.8×10^5 cfu) was based on prior safety and efficacy studies using mice ([16] and unpublished observations). Vaccines were administered subcutaneously at 2 weeks of age. The BCG-Danish strain was kindly provided by M.J. Brennan, United States Food and Drug Administration, Bethesda, MD. The strain of *M. bovis* used for the challenge inoculum [95-1315, USDA, Animal Plant and Health Inspection Service (APHIS) designation] was originally isolated from a white-tailed deer in Michigan, USA [17]. The challenge inoculum of this strain was prepared as described previously [18].

Challenge inoculum of 1.6×10^3 cfu in 2 ml of phosphate-buffered saline solution (PBS, 0.01 M, pH 7.2) was administered at ~2.5 months of age by aerosol inoculation (Palmer et al., 2002). Briefly, inoculum was delivered to restrained calves by nebulization into a mask (Trudell Medical International, London, ON, Canada) covering the nostrils and mouth. Upon inspiration, inoculum was inhaled through a one-way valve into the mask and directly into the lungs via the nostrils. The process continued until the inoculum, a 1 ml PBS wash of the inoculum tube, and an additional 2 ml PBS were delivered, a process taking ~12 min. Strict BL-3 safety protocols were followed to protect personnel from exposure to *M. bovis*.

2.2. Vaccine efficacy evaluation: bacterial recovery, mean disease score, histopathology, and lung radiographic analysis

All calves were euthanized by intravenous administration of sodium pentobarbital approximately 4 months after challenge. Tissues collected and processed for the isolation of *M. bovis* and microscopic analysis included: palatine tonsil; lung; liver; spleen; and mandibular, parotid, medial retropharyngeal, mediastinal, hepatic, mesenteric, and superficial cervical lymph nodes. Lymph nodes were sectioned at 0.5 cm intervals and examined. Following radiography, each lung lobe was sectioned at 0.5–1.0 cm intervals and examined separately. Lungs and lymph nodes (mediastinal and tracheobronchial) were evaluated using a semi-quantitative gross pathology scoring system adapted from [19]. Lung lobes (left cranial, left caudal, right cranial, right caudal, middle and accessory) were individually scored based upon the following scoring system: 0 = no visible lesions; 1 = no external gross lesions, but lesions seen upon slicing; 2 = <5 gross lesions of <10 mm in diameter; 3 = >5 gross lesions of <10 mm in diameter; 4 = >1 distinct gross lesion of >10 mm in diameter; 5 = gross coalescing lesions. Cumulative mean scores were then calculated for each entire lung. Lymph node pathology was based on the following scoring system: 0 = no necrosis or visible lesions; 1 = small focus (1–2 mm in diameter); 2 = several small foci; 3 = extensive necrosis. Data are

presented as mean (±standard error) disease score for each tissue.

Tissues collected for microscopic analysis were fixed by immersion in 10% neutral buffered formalin. For microscopic examination, formalin-fixed tissues were processed by standard paraffin-embedment techniques, cut in 5 μm sections and stained with hematoxylin and eosin. Adjacent sections were cut from samples containing caseonecrotic granulomata suggestive of tuberculosis and stained by the Ziehl-Neelsen technique for identification of acid-fast bacteria. Microscopic tuberculous lesions were staged (I–IV) based on adaptations of the criteria described by [20] and [21]. Stage I (initial) granulomas are characterized by accumulations of epithelioid macrophages with low numbers of lymphocytes and neutrophils. Multinucleated giant cells may be present but necrosis is absent. Acid-fast bacilli are often absent or present in low numbers within macrophages or multinucleated giant cells. Stage II (solid) granulomas are characterized by accumulations of epithelioid macrophages surrounded by a thin connective tissue capsule. Infiltrates of neutrophils and lymphocytes may be present as well as multinucleated giant cells. Necrosis when present is minimal. Stage III (necrotic) granulomas are characterized by complete fibrous encapsulation. Necrotic cores are surrounded by a zone of epithelioid macrophages admixed with multinucleated giant cells and lymphocytes. Stage IV (necrotic and mineralized) granulomas are characterized by thick fibrous capsules, irregular multicentric granulomas with multiple necrotic cores. Necrotic cores contain foci of dystrophic mineralization. Epithelioid macrophages and multinucleated giant cells surround necrotic areas and there may be moderate to dense infiltratres of lymphocytes. Acid-fast bacilli are often present in moderate numbers primarily located within the caseum of the necrotic core. Data are presented as total and mean number of granulomas observed in each histologic lesion stage (i.e. I–IV) for lung and mediastinal lymph node sections.

For quantitative assessment of mycobacterial burden, tracheobronchial lymph nodes were removed, weighed, and homogenized in phenol red nutrient broth using a blender (Oster, Shelton, CT). Logarithmic dilutions (10^0 to 10^{-9}) of homogenates in PBS were plated in 100 μl aliquots on Middlebrook 7H11 selective agar plates (Becton Dickinson) or Middlebrook PAN (for mc^{2}6030, Teknova, Hollister, CA) and incubated for 8 weeks at 37 °C. Data are presented as mean (±standard error) cfu per gram of tissue.

To provide an additional measure of the extent of lung lesions, lung lobes were removed at necropsy and individually radiographed using a MinXray machine (Model HF-100, Diagnostic Imaging, Rapid City, SD) with 3M Asymetrix Detail Screens and Ultimate 2000 film (3M Animal Care Products, St. Paul, MN) as described [22]. Lesions were identified on digital images of scanned radiographs, outlined and measured using Image Pro Plus (Media Cybernetics, Silver Spring, MD) software (Fig. 1). Affected area was divided by total lung area then multiplied by 100 to determine percent affected lung. Using combined data from each lung lobe, results for individual animals are presented as the mean (±standard error) percentage of affected lung.

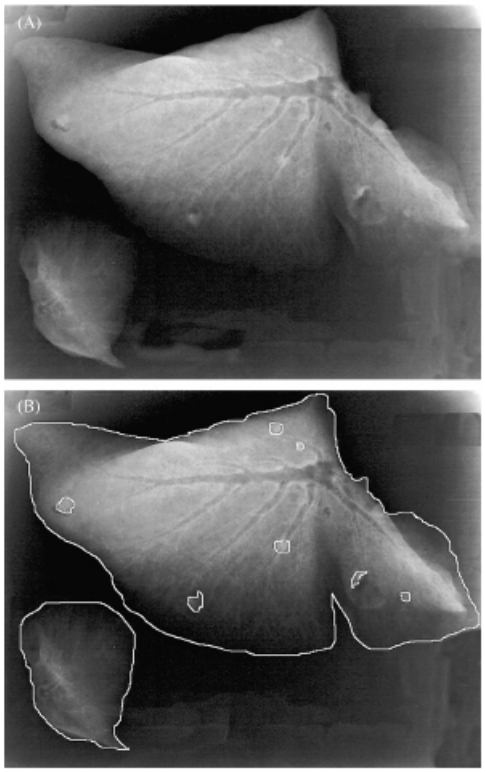

Fig. 1. Technique for identifying lung lobe margins and tuberculous lesions on radiographs for morphometry anlaysis. (A) Lung lobes were removed at necropsy and individually radiographed as described [22]. Right middle and caudal and accessory lung lobes are presented in this image. (B) Lung margins and lesions were outlined and measured using Image Pro Plus (Media Cybernetics) software.

2.3. *Interferon-γ analysis*

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation of peripheral blood buffy coat fractions collected into 2× acid citrate dextrose. Individual wells of 96-well round-bottom microtiter plates (Falcon, Becton-Dickinson; Lincoln Park, New Jersey) were seeded with 5×10^5 PBMC in a total volume of 200 μl per well. Medium was RPMI 1640 (GIBCO, Grand Island, New York) supplemented with 2 mM L-glutamine, 25 mM HEPES buffer, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 1% non-essential amino acids (Sigma), 2% essential amino acids (Sigma), 1% sodium pyruvate (Sigma), 50 mM 2-mercaptoethanol (Sigma), and 10% (v/v) fetal bovine sera (FBS). Wells contained medium plus 10 μg/ml

M. bovis PPD (Prionics AG, Schlieren, Switzerland), 1 μg/ml rESAT-6:CFP-10, 1 μg/ml pokeweed mitogen (PWM), or medium alone (no stimulation). After incubation for 48 h at 37 °C in 5% CO_2, culture supernatants were harvested and stored at −80 °C until thawed for analysis by an ELISA kit (Bovigam, Prionics AG). Concentrations of IFN-γ in test samples were determined by comparing absorbances of test samples with absorbances of standards within a linear curve fit. Mean IFN-γ concentrations (ng/ml) produced in 48-h cultures in response to antigen or mitogen minus concentrations in non-stimulated cultures (i.e., Δ IFN-γ) are presented.

2.4. Skin test procedures

Immediately prior to necropsy, calves received 0.1 ml (100 μg) of *M. bovis* PPD and 0.1 ml (40 μg) of *M. avium* PPD intradermally at separate clipped sites in the mid-cervical region according to guidelines described in USDA, APHIS circular 91-45-01 for the comparative cervical test. Skin thickness was measured with calipers prior to PPD administration and 72 h after injection. Balanced PPD's were obtained from the Brucella and Mycobacterial Reagents section of National Veterinary Services Laboratory, Ames, IA.

2.5. Real time rtPCR analysis

Mononuclear cell cultures were prepared as described for IFN-γ assay and incubated 16 h at 37 °C. Cells were harvested and lysed with 200 μl RLT buffer (Qiagen, Valencia, CA). RNA was isolated using an RNeasy Mini Kit (Qiagen) according to the manufacturer's directions and eluted from the column with 50 μl RNase Free water (Ambion, Austin, TX). Contaminating DNA was removed enzymatically by treating with DNA-free (Ambion) as directed by the manufacturer. Twenty microliters of RNA was reverse transcribed in a 50 μl reaction using Superscript II with 40 Units RNaseOUT and 0.25 μg Oligo$(dT)_{12–18}$ (Invitrogen, Calsbad, CA). Reverse-transcription was carried out at 42 °C for 60 min followed by 70 °C for 5 min. The resulting cDNA was stored at −80 °C until used in real-time PCR reactions. Real-time PCR was performed using SYBR Green Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's directions. Briefly, 2.5 μl of cDNA were added to a 25 μl reaction with 1 μM of each primer. Primers were designed with Primer3 software [23] using sequences from cattle (*Bos taurus*) and PCR products were sequenced to verify the primers. All reactions were performed in triplicate and data analyzed with the 2-ΔΔCt method as described [24]. β-Actin served as the internal control and the media only (no stimulation) sample from each animal was used as the calibrator. Validation of the use of β-actin as the internal control was performed as suggested by [24]. Data are presented as mean (±standard error) gene expression to antigen stimulation relative to that of no stimulation.

2.6. Flow cytometry

Isolated PBMC cultures were prepared as described for IFN-γ analysis and incubated for 6 days. Phenotype analysis of PBMC was performed as described previously [25]. Briefly, cells were harvested and incubated with 1 μg primary monoclonal antibodies/10^6cells (mAb's; CACT116A, CD25; BAT31A, CD44; BAQ92A, CD62L; GC50A1, CD4; BAQ111A, CD8; CACT61A, γδ TCR; all obtained from VMRD, Pullman WA) at room temperature for 15 min. Cells were then washed and stained with isotype appropriate goat anti-mouse phycoeythrin- (Southern Biotechnology Associates, Birmingham, AL), allophycocyanin- (Caltag Laboratories, Burlingame, CA), or Peridinin Chlorophyll Protein- (Becton Dickinson) conjugated secondary antibodies at room temperature for 15 min. Four-color flow cytometric analyses was performed using a Becton Dickinson LSR flow cytometer. Data were analyzed with FlowJo software (Tree Star Inc., San Carlos, CA) and presented as the mean (±standard error) percent positive cells or geometric mean fluorescence intensity (mfi) for each marker.

2.7. Evaluation of antibody responses to lipoarabinomannan (LAM)

LAM-specific antibody in serum obtained at vaccination (~14 days of age), before challenge (~2 months of age) and postchallenge at necropsy (~6 months of age) was quantified by ELISA. Aliquots of 50 μl LAM (3 μg/ml, kindly provided by J.T. Belisle, Department of Microbiology, Colorado State University, Fort Collins, CO) in carbonate buffer (pH 9.6) were added to wells of microtiter plates (Coming Inc., Corning, NY) and incubated at 37 °C for 1 h. Blocking was by addition of 3% bovine serum albumin (BSA, 200 μl per well) in Tris-buffered saline (TBS) at 37 °C for 1 h. After washing (3×), 50 μl calf sera diluted 1:50 in PBS was added to wells. Plates were incubated with sera for 1h at 37 °C, washed (3×), and incubated an additional 1 h at 37 °C with peroxidase-labeled goat anti-bovine IgM or IgG (10 μg/ml, 200 μl per well; Kirkegaard Perry Laboratories, Gaithersburg, MD). Plates were washed (5×) and the reaction developed by addition of 50 μl/well ABTS chromogen/substrate solution (Zymed Laboratories, Invitrogen, Carlsbad, CA). Washes were with TBS containing 0.05% Tween 20 (Sigma) in an ELISA plate washer (Skan washer, Molecular devices, Sunnyvale, CA) and absorbance was measured at 405 nm (Emax plate reader, Molecular devices, Sunnyvale, CA). Positive controls consisted of wells to which mAb 5c11 to LAM [26] were added. Negative controls consisted of wells prepared as described above but without the addition of LAM. Assays were performed in duplicate.

2.8. Statistics

Data were assessed for normality prior to statistical analysis and analyzed as a split-plot with repeated measures

ANOVA using Statview software (version 5.0, SAS Institute, Inc., Cary, NC). Fisher's protected-LSD test was applied when effects ($P < 0.05$) were detected. For antibody data, one-way ANOVA and pairwise Holm-Sidak test were used for normally distributed data and Kruskal-Wallis/Dunn's method non-normally distributed data using SigmaStat software (version 3.0, Systat Software Inc., San Jose, CA).

3. Results

3.1. Failure of mc²6030 to protect calves from aerosol challenge with virulent M. bovis

Variables used to evaluate vaccine efficacy included gross pathology (i.e., mean disease scores), radiographic morphometry, mycobacterial culture, and histopathology (Table 1 and Fig. 2). Mean disease scores for lungs and lung-associated lymph nodes did not differ between mc²6030-vaccinates and controls. Mean disease scores for lung-associated lymph nodes were lower ($P < 0.05$) for BCG-vaccinates than controls (Fig. 2 and Table 1). The percentage of affected lung area (i.e., radiographic morphometry data, Table 1) did not differ between mc²6030-vaccinates and controls; however, there was a trend for reduced disease (pathology) in BCG-vaccinates as compared to that of mc²6030-vaccinates ($P = 0.10$) and controls ($P = 0.12$).

Sections of mediastinal lymph node from non-vaccinated control calves and calves vaccinated with mc²6030 contained more granulomas and more granulomas of advanced stage (III–IV) than did calves vaccinated with BCG (Table 2). No microscopic granulomas were seen in sections of lung examined from BCG vaccinated cattle, while non-vaccinated cattle and cattle vaccinated with mc²6030 had lesions of all histologic stages (Table 3). Virulent *M. bovis* was cultured tracheobronchial lymph nodes (15 of 17), mediastinal lymph nodes (16 of 17), and one mesenteric lymph node from a control calf. Virulent *M. bovis* was cultured from lungs

Table 1
Comprehensive vaccine efficacy data

Treatment group	Gross pathology[a]	Radiographs[b]	Culture[c]
Control ($n = 7$)	2.43 (0.3)	1.93 (0.9)	3.04 (0.5)
BCG ($n = 4$)	0.50 (0)[†]	0.03 (0.02)[¶]	3.58 (0.3)
mc²6030 ($n = 6$)	1.67 (0.2)	2.08 (0.9)	4.05 (0.2)

[a] At necropsy, tracheobronchial lymph nodes were visually evaluated for lesions based upon a scoring system adapted from [19]. Values represent mean (±standard error) mean disease scores.

[b] Lung lobes were removed at necropsy and individually radiographed. Lesions were identified on digital images of scanned radiographs, outlined, and measured (Fig. 1). Affected area was divided by total lung area then multiplied by 100 to determine percent affected lung.

[c] Tracheobronchial lymph nodes were homogenized in phenol red nutrient broth for serial dilution plate counting on Middlebrook 7H11 selective agar plates (Becton Dickinson, 8-week culture). Data are presented as mean (±standard error) cfu per gram of tissue.

[†] $P < 0.05$ as compared to controls and mc²6030-vaccinates.

[¶] $P = 0.1$ as compared to mc²6030-vaccinates.

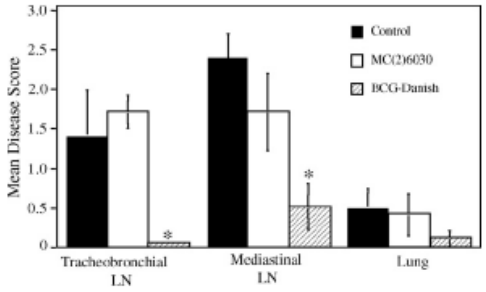

Fig. 2. Gross pathology. Lungs were evaluated based upon the following scoring system: 0 = no visible lesions; 1 = no external gross lesions, but lesions seen upon slicing; 2 = <5 gross lesions of <10 mm in diameter; 3 = >5 gross lesions of <10 mm in diameter; 4 = >1 distinct gross lesion of >10 mm in diameter; 5 = gross coalescing lesions. Scoring of lymph node pathology was based on the following system: 0 = no necrosis or visible lesions; 1 = small focus (1–2 mm in diameter); 2 = several small foci; 3 = extensive necrosis. Values represent mean (±standard error) pathology scores, ($n = 7$, controls; $n = 6$, mc²6030; $n = 4$, BCG). (*) Differs from controls, $P < 0.05$.

obtained from 3 of 7 controls, 2 of 6 mc²6030-vaccinates, and 1 of 4 BCG-vaccinates. Tracheobronchial lymph node colonization was not affected by vaccination (Table 1). The vaccine strains (BCG and mc²6030) were not isolated from tissues collected from any of the calves, including lymph nodes draining the site of vaccination. Overall assessment of vaccine efficacy utilizing gross pathology, radiographic mor-

Table 2
Histologic evaluation of lung-associated lymph node (i.e., mediastinal)

Treatment	Histologic stage[a]				
	I	II	III	IV	Total[b]
Controls ($n = 7$)	9.9	3.6	7.6	3.6	23.1 (5.3)
BCG ($n = 4$)	3.3	1.5	1.0	1.0	6.7 (3.5)[†]
mc²6030 ($n = 6$)	3.5	2.2	5.2	1.7	12.2 (4.4)

[a] Microscopic tuberculous lesions were staged (I–IV) based on adaptations of the criteria described by [20] and [21]. Disease severity progresses from stage I to IV. The number of granulomas in each histologic stage per section of mediastinal LN was enumerated.

[b] Mean (±standard error) number of granulomas (i.e., regardless of stage) detected on a section of mediastinal lymph node for each vaccine treatment.

[†] $P < 0.05$ as compared to controls.

Table 3
Histologic evaluation of lung

Treatment	Histologic stage[a]				
	I	II	III	IV	Total[b]
Controls ($n = 7$)	0.9	0.3	0.9	0.4	2.4 (0.9)
BCG ($n = 4$)	0	0	0	0	0 (0)[¶]
mc²6030 ($n = 6$)	0.3	0.2	0.5	0.2	1.2 (0.7)

[a] Microscopic tuberculous lesions were staged (I–IV) based on adaptations of the criteria described by [20] and [21]. Disease severity progresses from stage I to IV. The number of granulomas in each histologic stage per section of lung was enumerated.

[b] Mean number of granulomas (i.e., regardless of stage) detected on a section of lung for each vaccine treatment.

[¶] $P = 0.1$ as compared to controls.

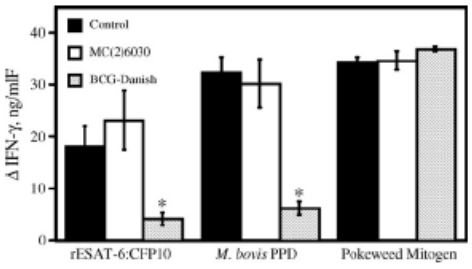

Fig. 3. Interferon-γ responses. Blood mononuclear cells were isolated from cattle ~3 months after challenge with virulent *M. bovis* (~5 months after vaccination) and cultured with 10 μg/ml *M. bovis* PPD (Prionics AG), 1 μg/ml rESAT-6:CFP-10, 1 μg/ml pokeweed mitogen (PWM), or medium alone (no stimulation) for 48 h. Interferon-γ concentrations in culture supernatants were quantified by ELISA. Values represent mean (±standard error) responses to stimulation (i.e., antigen or PWM) minus the response to media alone ($n=7$, controls; $n=6$, mc^{2}6030; $n=4$, BCG). Responses to PWM are presented to indicate a general responsiveness of the cell population to polyclonal stimulation (i.e., a positive control). (*) Differs from controls, $P<0.05$.

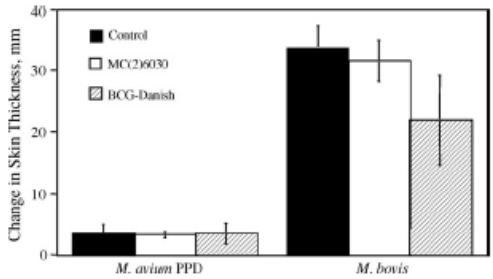

Fig. 4. Comparative cervical skin test. Immediatley prior to necropsy, animals received 0.1 ml (100 μg) of *M. bovis* PPD and 0.1 ml (40 μg) of *M. avium* PPD in the mid-cervical region according to guidelines described in USDA, APHIS circular 91-45-01. Responses are presented as mean (±standard error) skin thickness to purified protein derivative (PPD, mycobacterial origin indicated) at 72 h after injection minus the skin thickness prior to injection ($n=7$, controls; $n=6$, mc^{2}6030; $n=4$, BCG). Responses did not differ between vaccine treatment groups.

phometry, and mycobacterial culture indicated that neonatal vaccination with mc^{2}6030 failed to protect calves from low dose virulent *M. bovis* aerosol challenge (Table 1). Although BCG vaccination afforded protection from disease it did not reduce colonization of assayed tissues.

3.2. Reduced in vitro recall responses are positive prognostic indicators of vaccine efficacy

Three months after challenge and approximately 5 months after vaccination, antigen-specific IFN-γ, iNOS, IL-4, and MIP1-α responses of PBMC from BCG-vaccinates were lower than responses of cells from control and mc^{2}6030-vaccinates (Fig. 3 and Table 4). In contrast, cutaneous delayed type hypersensitivity (Fig. 4) and TNF-α (Table 4) responses evaluated concurrently were not affected by vaccination and FoxP3 responses were increased in BCG vaccinates (Table 4). IL-5 expression in antigen-stimulated cells from controls exceeded expression by cells from BCG- and mc^{2}6030-vaccinates (Table 4).

Three months after challenge, stimulation of PBMC with mycobacterial antigens resulted in increased percentages of CD4+ cell ($P<0.0001$) and decreased percentages of CD8+ cell ($P<0.05$) in PBMC cultures (Table 5). Percentages of CD4+ cells harvested from 6 d PBMC cultures differed ($P<0.05$) between each of the vaccine treatment groups [i.e., mc^{2}6030-vaccinates (10.2 ± 1.2) > controls

Table 5
Alterations in T cell subset composition upon in vitro antigen stimulation

Type of stimulation	CD4+[a]	CD8+	γδ TCR+
No stimulation ($n=17$)	3.8 (0.4)	11.6 (1.0)	17.5 (1.7)
rESAT-6:CFP10 ($n=17$)	10.2 (1.2)**	9.0 (0.9)*	15.4 (1.5)
M. bovis PPD ($n=17$)	10.0 (0.9)**	8.7 (0.5)*	18.3 (1.6)

[a] Blood mononuclear cells were isolated from cattle ~3 months after challenge with virulent *M. bovis* (~5 months after vaccination) and cultured with 10 μg/ml *M. bovis* PPD (Prionics AG), 1 μg/ml rESAT-6:CFP-10, or medium alone (no stimulation). After 6 days, cells were harvested and analyzed by flow cytometry for T cell subset composition. Data are presented as the mean (±standard error) percent positive cells in PBMC cultures according to in vitro stimulation, irrespective of vaccine treatment. Differs (*$P<0.05$, **$P<0.01$) from non-stimulated cultures (i.e., vertical comparisons).

Table 4
Gene expression by *M. bovis* PPD stimulated blood mononuclear cells

Treatment group [a]	iNOS	IL-4	IL-5	MIP1-α	TNF-α	FoxP3
Control ($n=7$)	7.8 (1.1)	5.9 (1.4)	264.0 (105.8)†	3.1 (1.0)	4.0 (0.8)	1.8 (0.9)
BCG ($n=4$)	4.1 (0.4)¶	2.1 (0.3)‡	4.3 (2.8)	1.4 (0.1)‡‡	2.5 (0.3)	48.3 (26.5)‡‡‡
mc^{2}6030 ($n=6$)	7.2 (0.8)	7.4 (2.0)	20.2 (7.5)	3.5 (0.9)	4.1 (0.7)	12.2 (10.2)

[a] Blood mononuclear cells were isolated from cattle ~3 months after challenge with virulent *M. bovis* (~5 months after vaccination) and cultured with either medium plus 10 μg/ml *M. bovis* PPD (Prionics AG) or medium alone (no stimulation). After 16 h of culture, cells were harvested, RNA isolated, and gene expression evaluated by real time RTPCR as described in the Methods. Data are presented as mean (±standard error) gene expression to *M. bovis* PPD stimulation relative to that of no stimulation. Similar responses were detected by PBMC in response to rESAT-6:CFP10 stimulation (data not shown).

¶ $P<0.05$ as compared to control and mc^{2}6030-vaccinates.

† $P<0.05$ as compared to BCG- and mc^{2}6030-vaccinates.

‡ $P<0.05$ as compared to mc^{2}6030-vaccinates and $P=0.07$ as compared to controls.

‡‡ $P<0.1$ as compared to mc^{2}6030-vaccinates.

‡‡‡ $P<0.05$ as compared to controls and $P=0.06$ as compared to BCG-vaccinates.

(7.8 ± 0.9) > BCG-vaccinates (5.0 ± 1.2)]. Percentages of γδ TCR+ cells were not affected by type of stimulation in vitro; however, increased percentages of γδ TCR+ cells were detected in cultures from BCG vaccinates (21.1 ± 2.1) as compared to control (15.0 ± 1.1) and mc^{2}6030-vaccinates (16.8 ± 1.5) ($P = 0.01$ and 0.07, respectively). As previously reported [25], stimulation with mycobacterial antigen resulted in increased percentages (and associated changes in mfi) of CD25+ and CD44+ cells and decreased percentages of CD62L+ cells in PBMC cultures ($P < 0.002$), irrespective of vaccine treatment (data not shown). Alterations in phenotype indicative of lymphocyte activation (i.e. increased CD25 and CD44 expression and reduced CD62L expression) were most prominent in PBMC cultures from mc^{2}6030-vaccinates and controls. Effects of vaccine treatment on antigen [i.e., rESAT-6:CFP10 (Fig. 5A) and *M. bovis* PPD (Fig. 5B)] induced responses are shown in Fig. 5. A diminished activation phenotype of fewer CD25+ and CD44+ cells and greater CD62L+ cells was evident in antigen-stimulated PBMC cultures from BCG-vaccinates as compared to control and mc^{2}6030-vaccinates.

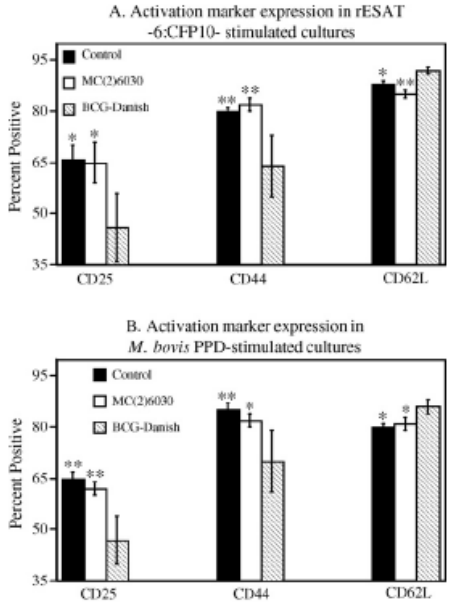

Fig. 5. Percent CD25+, CD44+, and CD62L+ cells in rESAT-6:CFP10-stimulated (A) and *M. bovis* PPD-stimulated (B) cultures. Mononuclear cells were isolated by density gradient centrifugation of buffy coat fractions and cultured with 1 μg/ml rESAT-6:CFP10 or 1 μg/ml *M. bovis* PPD. After 6 days of culture, cells were harvested for each individual animal according to treatment and stained with mAb's to cell surface markers for analysis by flow cytometry. Data are presented as mean (±standard error) percent expression of activation markers. Asterisks indicate differences (**$P < 0.1$, *$P < 0.05$) in PBMC cultures from control and mc^{2}6030-vaccinates as compared to BCG-vaccinates.

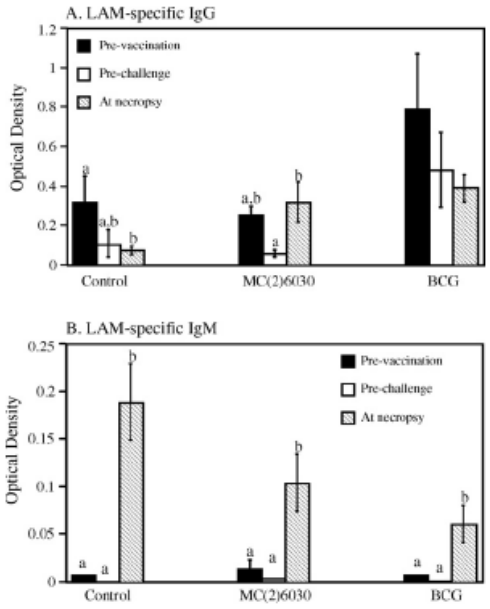

Fig. 6. Serum IgG (A) and IgM (B) to LAM. Serum was obtained from calves at 2 weeks of age (pre-vaccination), ~2 months of age (pre-challenge), and ~4 months after challenge (at necropsy) and evaluated for LAM-specific antibody by ELISA. Data are presented as mean (±standard error) absorbance. Bars with differing letter designation (i.e., a versus b) are different ($P < 0.05$) within vaccine treatment.

3.3. Antibody responses to lipoarabinomannan

With the exception of one calf from the unvaccinated group, LAM-specific IgG was detected in the serum from all calves prior to immunization (~2 weeks of age) (Fig. 6A), indicating colostral transfer of IgG reactive to LAM as demonstrated previously [6]. In general, LAM-specific IgG decreased throughout the study indicating decay in colostrum-derived antibody and blocking of an IgG response to both vaccination and subsequent infection. The exception was an increase ($P < 0.05$) in LAM-specific IgG in serum from mc^{2}6030-vaccinates after challenge with virulent *M. bovis* as compared to responses immediately pre-challenge (i.e., after vaccination with mc^{2}6030). In contrast to IgG, LAM-specific IgM was not detected prior to challenge and increased in all groups upon challenge (Fig. 6B).

4. Discussion

Vaccination with mc^{2}6030 failed to protect calves from virulent *M. bovis* aerosol challenge, despite a proven record of efficacy with immune-competent and immune-deficient strains of mice [16]. Similarly, vaccination trials with mc^{2}6030 failed to protect cynomolgus monkeys from virulent *M. tuberculosis* (Larsen et al., unpublished observations).

Experimental challenge with a strain of *M. tuberculosis* virulent to mice, monkeys, and humans did not elicit disease in cattle (Vordermeier and Hewinson, personal communication). For cattle, attenuated *M. tuberculosis* mutants may be less immunogenic as compared to those produced on a virulent *M. bovis* or BCG background strain. Thus, cattle may not be as useful as other models (e.g., monkeys) for the study of vaccine efficacy using *M. tuberculosis* mutants. Further studies are required to directly compare immunogenicity and virulence of *M. tuberculosis* versus *M. bovis* background mutants in cattle. Similar findings with cynomolgus monkey and neonatal calf trials, however, may be indicative of other, non-determined causes of vaccine failure such as inappropriate dose.

In contrast to mc^{2}6030, BCG-vaccinated cattle exhibited both limited disease dissemination and disease severity as evidenced by decreased lesion scores, decreased pulmonary involvement (radiographic morphometry) and fewer granulomas of advanced histologic stage in mediastinal lymph nodes and lungs. Development of fewer granulomas with less necrosis likely limits transmission, as it is later stage granulomas with extensive necrosis and large numbers of acid-fast bacilli that facilitate disease dissemination within individual hosts and between individuals. Colonization of tracehobronchial lymph nodes with virulent *M. bovis* was unaffected by vaccine treatment, suggesting that BCG vaccination reduced TB-associated pathology without limiting colonization.

Comparison of immune responses to mycobacterial antigen after virulent challenge demonstrated unique differences in the response associated with prior vaccine treatments. In general, BCG-vaccinates had a diminished activation profile as compared to mc^{2}6030-vaccinates and controls. BCG-vaccinates had reduced antigen-specific IFN-γ, iNOS, IL-4, and MIP1-α responses; reduced expansion of CD4+ cells in culture; and a diminished activation profile of decreased percentages of CD25+ and CD44+ cells and increased percentages of CD62L+ cells. Thus, a reduced immune stimulation profile was associated with administration of a disease limiting vaccine (i.e., BCG). These findings are in agreement with [19] and [27] demonstrating that a low response to ESAT-6 upon experimental challenge is a positive prognostic indicator for vaccine efficacy.

In cattle, T cell responses at the clonal level are biased to a Th1 response with few if any Th2 clones expressing IL-4 independent of IFN-γ [28,29]. The host response to mycobacterial infection relies, in part, on the production of IFN-γ by Th1 and CD8+ effector T cells in addition to cytolytic mechanisms for clearance of bacilli infected cells. This potentially harmful inflammatory response may be counter balanced by FoxP3 expressing CD4$^+$CD25$^+$T regulatory cells [30] or IL-13-/IL-4-expressing cells [31]. In the present study, high expression levels of FoxP3 and low expression levels of IL-4 and IL-5 were associated with a disease limiting vaccine (BCG) as compared to non-protected animals (i.e., non- and mc^{2}6030-vaccinates). In contrast, CD4$^+$CD25$^+$FoxP3$^+$ T regulatory cells increase in humans with active TB and suppress IFN-γ and IL-10 production, thereby, contributing to disease pathogenesis [30,32]. FoxP3 expression is increased in PBMC from humans with TB and correlates to increased numbers of circulating CD4$^+$CD25$^+$ T-regulatory cells [33]. Definitive characterization of the role of T regulatory cells in vaccine-induced immunity of cattle to TB will require further phenotypical and functional characterization of these populations.

LAM was chosen as the target antigen for evaluation of the antibody response because it is a major surface component of mycobacteria involved in the immunopathogenesis of tuberculosis including apoptosis, inhibition of phagosomal maturation, and macrophage interferon-γ signaling (reviewed in [34]). In the present study, LAM-specific IgG was detected in the serum from 2 weeks old calves. Although not statistically significant and of questionable relevance, LAM-specific IgG levels were numerically greater in BCG vaccinates as compared to other animals. IgG to mycobacterial antigens is transferred from the dam to the calf via colostrum with levels correlating to the immunologic experience of the dam ([6] and Waters/Nonnecke, unpublished observations). Exposure of dams to ubiquitous non-tuberculous, environmental mycobacteria elicits IgG reactive to LAM that is then transferred to the calf. In contrast, LAM-specific IgM was not detected prior to challenge (Fig. 6B), indicating a lack of maternal transfer of this isotype and no response to vaccination. As previously described [6], maternal LAM-specific IgG blocks production of antibody to LAM in the neonatal calf. Experimental challenge with *M. bovis*, on the other hand, induced significant levels of LAM-specific IgM. By this time (i.e., ~2.5 months of age), it is likely that the levels of IgG had waned to a level insufficient to block a response to mycobacterial infection; thus, as would be expected, an early IgM response to infection was elicited (Fig. 6B).

In summary, the calf sensitization and challenge model provides an informative screen for candidate tuberculosis vaccines before their evaluation in costly non-human, primates.

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APPENDIX 3

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An ESAT-6:CFP10 DNA vaccine administered in conjunction with *Mycobacterium bovis* BCG confers protection to cattle challenged with virulent *M. bovis*

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Abstract

The potency of genetic immunization observed in the mouse has demonstrated the utility of DNA vaccines to induce cell-mediated and humoral immune responses. However, it has been relatively difficult to generate comparable responses in non-rodent species. The use of molecular adjuvants may increase the magnitude of these suboptimal responses. In this study, we demonstrate that the co-administration of plasmid-encoded GM-CSF and CD80/CD86 with a novel ESAT-6:CFP10 DNA vaccine against bovine tuberculosis enhances antigen-specific cell-mediated immune responses. ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinated animals exhibited significant ($p < 0.01$) antigen-specific proliferative responses compared to other DNA vaccinates. Increased expression ($p \leq 0.05$) of CD25 on PBMC from ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinates was associated with increased proliferation, as compared to control DNA vaccinates. Significant ($p < 0.05$) numbers of ESAT-6:CFP10-specific IFN-γ producing cells were evident from all ESAT-6:CFP10 DNA vaccinated animals compared to control DNA vaccinates. However, the greatest increase in IFN-γ producing cells was from animals vaccinated with ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA. In a low-dose aerosol challenge trial, calves vaccinated as neonates with *Mycobacterium bovis* BCG and ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA exhibited decreased lesion severity in the lung and lung-associated lymph nodes following virulent *M. bovis* challenge compared to other vaccinated animals or non-vaccinated controls. These data suggest that a combined vaccine regimen of *M. bovis* BCG and a candidate ESAT-6:CFP10 DNA vaccine may offer greater protection against tuberculosis in cattle than vaccination with BCG alone.

Keywords: Adjuvant; Neonate; Tuberculosis

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1. Introduction

DNA vaccination of mice has demonstrated the utility of genetic immunization as a means of inducing robust cell-mediated and humoral responses to encoded antigens [1,2]. Development of successful, practical DNA vaccination in humans and other outbred non-rodent species has been hampered by several factors [3–5]. To induce significant immune responses, vaccinates are often subjected to several immunizations comprised of relatively large amounts of plasmid DNA [6–8]. Individuals vaccinated with DNA are relatively weak responders [6,7]. Molecular adjuvants such as cytokines, costimulatory molecules and immunostimulatory DNA sequences have been used to increase the potency of DNA vaccine-induced immune responses in non-rodent species [9–15].

Following intramuscular (IM) injection of a DNA vaccine, plasmid DNA is internalized by dendritic cells (DCs) and muscle cells. Myocytes express genes encoded on plasmids *in vitro* and *in vivo* [16,17]; however, these cells cannot initiate an immune response via naïve T cells, even when transfected with an appropriate array of costimulatory molecules or cytokines [18]. Effects of DNA vaccination are still apparent following ablation of tissue surrounding the injection site [19]. Plasmid DNA has been isolated directly from DCs within the draining lymph nodes and skin following IM or intradermal inoculation [20]. In addition, DCs can be directly transfected following IM plasmid immunization [21], suggesting that DCs are key mediators of DNA vaccine-induced immune responses. Strategies to manipulate DCs to increase the potency of DNA vaccination, include the use of growth factors such as granulocyte macrophage-colony stimulating factor (GM-CSF) [1,4,22] or *fms*-like tyrosine kinase-3 ligand (Flt-3L) [9,23,24]. In mice and humans, administration of these growth factors, used alone or in combination, increases numbers of DC's [25–29]. Bovine bone marrow cells cultured with GM-CSF alone, GM-CSF + IL-4 or GM-CSF + IL-4 + Flt-3L display morphological characteristics typical of DCs [30]. Experiments in cattle have shown that the co-administration of plasmid-encoded GM-CSF and Flt-3L enhances bovine $CD4^+$ T cell responses to an *Anaplasma marginale* major surface protein-1 (MSP-1) DNA vaccine [9]. The observed enhancement was attributed to an increase in DC recruitment at the site of immunization after growth factor treatment [9].

Early secretory antigenic target-6 kDa (ESAT-6) protein and culture filtrate protein 10 (CFP10) are potent immunogens encoded by region of difference-1 (RD1) in tuberculous mycobacteria [31] and are absent in *Mycobactrium bovis* bacille Calmette-Guerin (BCG) [32,33]. The loss of RD1 is associated with the attenuation of BCG [34,35]. Although no precise function has been attributed to these proteins, their expression correlates with an increased cytolytic ability of *M. tuberculosis* [36]. ESAT-6 is recognized by bovine T cells during the early phase of infection [37], resulting in the release of IFN-γ [37,38], making it an attractive candidate for vaccine development. ESAT-6 and CFP10 exist as a heterodimer in a 1:1 complex *in vivo* [39]. *In vitro*, the use of an ESAT-6:CFP10 fusion protein induces high levels of IFN-γ production [40] and robust proliferative responses by T cells from *M. bovis*-infected cattle.

In our previous study, we assessed the potential of CpG ODN, CD80, CD86 and CD154 to enhance the efficacy of a candidate bovine tuberculosis DNA vaccine directed against ESAT-6 [10]. Co-administration of CpG ODN with either CD80/CD86 or CD154, in a single prime/boost vaccination regimen, enhanced ESAT-6-specific IFN-γ responses as compared to non-vaccinated control animals [10]. However, following aerosol challenge with *M. bovis*, only animals vaccinated with CD80/CD86 demonstrated decreased lesion severity in the lungs and associated lymph nodes [10]. Collectively, this earlier study demonstrated that co-administration of CD80/CD86 is superior to CD154 in augmenting DNA vaccine-induced protection following aerosol challenge with *M. bovis* [10]. In the present study, we evaluated immune responses generated against a candidate ESAT-6:CFP10 DNA vaccine and assessed the ability of plasmid-encoded GM-CSF to further augment the adjuvant activity of plasmid-encoded CD80/CD86 in response to a novel ESAT-6:CFP10 DNA vaccine. In addition, we evaluated the use of a *M. bovis* BCG prime + ESAT-6:CFP10 DNA vaccine prime/boost strategy to induce protection against a low-dose aerosol challenge. Our results indicate that the co-administration of plasmids encoding GM-CSF and CD80/CD86 molecules enhances bovine immune responses to genetic immunization and that this ESAT-6:CFP10 DNA vaccine administered in conjunction with a *M. bovis* BCG prime confers protection against experimental bovine tuberculosis to a greater level than DNA vaccination or BCG vaccination alone.

2. Materials and methods

2.1. Plasmid DNA and recombinant protein production

Plasmids encoding bovine CD80 and bovine GM-CSF were kindly provided by Dr. Chris J. Howard (Institute of Animal Health, Compton, UK). The construction of bovine CD80 and GM-CSF plasmids was described previously [41,42]. For CD86 plasmid construction, total RNA was obtained from bovine PBMC using Trizol Reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed to cDNA using oligo dT 16 (Perkin-Elmer, Branchburg, NJ) according to the manufacturer's instructions. The cDNA was used to amplify an entire open reading frame of bovine CD86 by polymerase chain reaction (PCR) using primers FWD (5′-ACAGCAGAAATAACGAAAATGCG) and REV (5′-CATGGCGTTTACTCTTTAATTACA). PCR conditions were 94 °C (10 min), followed by 35 cycles of 94 °C (15 s), 60 °C (15 s) and 72 °C (2 min). PCR products were cloned into pCR3.1 using T4 ligase (Invitrogen). Sequence and

direction of the insert was confirmed by automated DNA sequencing.

The ESAT-6:CFP10 vaccine plasmid was constructed using the vector VR1020 (Vical Inc., San Diego, CA). The sequence encoding the protein fusion of ESAT-6:CFP10 was amplified from plasmid pISM2202 [40] using primers "ES-CFP-U-Not" (5′-AATGCGGCCGCATATGGGGGG) and "ES-CFP-L-EcoR1" (5′-GCTGAATTCCGAAGCCCATTTGC). The PCR product was gel purified, digested with *Not*I and *Eco*RI and ligated into, a derivative of plasmid VR1020, plasmid pISM2214. The resulting plasmid was designated plasmid pISM2215 (VR1020:6-His:ESAT-6:CFP10). The DNA sequence and frame orientation was confirmed by DNA sequence analysis. Plasmid DNA for immunization was chemically transformed into competent *Escherichia coli* TOP10 (Invitrogen). Plasmid DNA was purified using the Qiagen Plasmid Giga kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Purified recombinant protein was obtained following induction of TOP10 cells containing pISM2202 by metal chelate chromatography as described [40], dialyzed overnight at 4 °C in PBS and quantified by the Bradford assay.

2.2. Bacteria

M. bovis strain 1315 and attenuated *M. bovis* BCG strain Pasteur were grown in Middlebrook 7H9 media supplemented with 10% oleic acid–albumin–dextrose complex (Difco, Detroit, MI) plus 0.05% Tween 80 (Sigma, St. Louis, MO) as described previously [10]. *M. bovis* 1315 was originally isolated in 1995 from a naturally infected white-tailed deer [43]. Vaccine and challenge inocula consisted of mid-log growth phase mycobacteria. Bacilli were enumerated using serial dilution plate counting on Middlebrook 7H11 selective media (Becton Dickinson, Cockeysville, MD).

2.3. Animals and immunizations

All immunizations and challenge components of the study were conducted at the National Animal Disease Center, Ames, IA. Prior to experimentation, animal-related procedures were approved by the Institutional Animal Care and Use Committee of the National Animal Disease Center.

2.3.1. DNA vaccine trial #1

Twenty-five castrated, 4-month-old Holstein bull calves, were selected for their negative reactivity to purified protein derivative (PPD) from *M. avium* (PPDa) and *M. bovis* (PPDb), as well as rESAT-6:CFP10. Briefly, whole blood was incubated for 24 h *in vitro* in the presence of PPDa (10 μg/ml), PPDb (10 μg/ml) (Biocor Animal Health, Omaha, NE) or rESAT-6:CFP10 (10 μg/ml). Supernatants were harvested and antigen-specific IFN-γ production was determined using the Bovigam ELISA kit (Prionics Ag, Schlieren, Switzerland). Animals negative for previous exposure to mycobacterial antigens were then assigned randomly to the following experimental treatment groups: (1) control DNA (empty plasmid vector pcDNA3.1) ($n=5$); (2) ESAT-6:CFP10 DNA vaccination ($n=5$); (3) ESAT-6:CFP10 + GM-CSF DNA vaccination ($n=5$); (4) ESAT-6:CFP10 + CD80/CD86 DNA vaccination ($n=5$); (5) ESAT-6:CFP10 + GM-CSF + CD80/CD86 ($n=5$). DNA vaccinates were immunized intramuscularly in the right mid-cervical region with 2 mg of total plasmid DNA emulsified in 2 ml of incomplete Freund's adjuvant (IFA). All experimental vaccines contained 1 mg of ESAT-6:CFP10 encoding plasmid. The total amount of plasmid DNA was normalized to 2 mg by additional plasmids depending on the treatment. Cattle received an identical booster dose of vaccine 20 days following primary immunization.

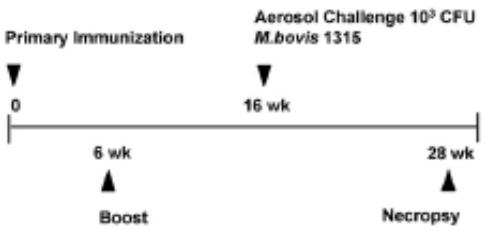

Fig. 1. Timeline detailing events during DNA vaccine trial #2. Calves receiving *Mycobacterium bovis* BCG were immunized with 1×10^6 CFU *M. bovis* BCG strain Pasteur at the time of primary immunization. Animals vaccinated with BCG + DNA or DNA alone received a primary dose of plasmids encoding ESAT-6:CFP10, GM-CSF, CD80 and CD86 in IFA, and an identical booster dose 6 weeks post-immunization. Sixteen weeks after primary immunization, cattle received 10^3 CFU of virulent *M. bovis* 1315 by aerosol and were euthanized 12 weeks after challenge.

2.3.2. DNA vaccine trial #2

Twenty-five Holstein calves less than 6 days of age were randomly assigned to the following experimental treatment groups: (1) non-vaccinated controls ($n=6$); (2) *M. bovis* BCG strain Pasteur vaccination ($n=5$); (3) ESAT-6:CFP10 + GM-CSF + CD80/CD68 DNA vaccination ($n=5$); (4) *M. bovis* BCG strain Pasteur + ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccination ($n=5$). At 1 week of age, DNA vaccinates were immunized subcutaneously with 2 mg of total plasmid DNA emulsified in 2 ml of IFA in the left mid-cervical region and received a booster dose at 6 weeks of age (Fig. 1). At the time of the first immunization, animals received 1.0×10^6 CFU *M. bovis* BCG strain Pasteur subcutaneously in the right mid-cervical region.

2.4. Aerosol challenge of vaccinated cattle

Low-dose aerosol challenge of cattle with 1.0×10^3 CFU *M. bovis* 1315 was conducted at the National Animal Disease Center as described previously [44].

2.5. Lymphocyte proliferation assays

PBMC were isolated via density centrifugation from buffy-coat fractions of peripheral blood collected in 2× acid–citrate–dextrose. Peripheral blood mononuclear cells (PBMC) (5×10^5) were cultured in triplicate wells of round-bottom 96-well plates (Falcon, Becton Dickinson, Lincoln Park, NJ) in a total volume of 200 μl. Medium was complete RPMI 1640 (cRPMI) supplemented with 2 mM L-glutamine, 25 mM HEPES buffer, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% non-essential amino acids (Sigma Chemical Co., St. Louis, MO), 2% essential amino acids (Sigma), 1% sodium pyruvate (Sigma), 50 μM 2-mercaptoethanol (Sigma), and 10% (v/v) fetal bovine sera (FBS). PBMC were stimulated *in vitro* with rESAT-6:CFP10 (5 μg/ml), PPDb (5 μg/ml), pokeweed mitogen (PWM) (1 μg/ml) or cRPMI alone. Cells were cultured for 6 days at 37 °C in 5% CO_2 humidified air with the addition of 0.5–1.0 μCi [^{3}H]-thymidine (specific activity, 6.7 Ci/mM, Amersham Life Science, Arlington Heights, IL) during the last 16–20 h of culture. Well contents were harvested onto glass fiber filters with a 96-well plate harvester (EG & G Wallac, Gaithersburg, MD), and the incorporated radioactivity measured by liquid scintillation counting. Treatments were run in triplicate and presented as mean cpm (±S.E.M.).

2.6. IFN-γ ELISA

Cells were cultured in round-bottom 96-well plates in a volume of 200 μl. PBMC (4×10^5) were cultured *in vitro* in the presence of rESAT-6:CFP10 (5 μg/ml), PPDb (5 μg/ml), pokeweed mitogen (PWM) (1 μg/ml) or cRPMI alone for 48 h at 37 °C in 5% CO_2 humidified air. Following incubation, supernatants were removed and stored at −80 °C until analysis. Supernatants were assayed for IFN-γ production using the Bovigam ELISA kit (Prionics) according to instructions provided by the manufacturer. Concentrations (ng/ml) in test samples were quantified by comparing the absorbance of duplicate test samples with the absorbance of standards within a linear curve fit.

2.7. IFN-γ ELISPOT assay

Antigen-specific IFN-γ production was assayed using an ELISPOT as described previously [10]. Anti-bovine IFN-γ monoclonal antibodies (MAb) CC302 and CC330 were kindly provided by Dr. Chris J. Howard. Briefly, 5×10^5 PBMC were added in 100 μl volumes containing either cRPMI alone, rESAT-6:CFP10 (5 μg/ml), PPDb (5 μg/ml) or PWM (1 μg/ml). Plates were incubated for 36 h at 37 °C in 5% CO_2 humidified air. IFN-γ spot-forming cells were enumerated using a standard dissection microscope or an ELISPOT Reader (CTL). For each animal, the mean number of spots of negative control (i.e. cRPMI alone) wells was subtracted from the number of spots in test wells to determine the mean number of antigen-specific IFN-γ spot-forming cells.

2.8. Detection of antigen-specific antibodies

Round-bottom 96-well plates (Falcon, Becton Dickinson) were coated with rESAT-6:CFP10 (1 μg/ml) in carbonate–bicarbonate coating buffer pH 9.6 overnight at 4 °C. Plates were washed three times with 1× phosphate buffered saline plus 0.05% Tween 20 (PBST) (Sigma) and then blocked for 1 h with milk diluent (Kirkegaard Perry Laboratories, Gaithersburg, MD) at 37 °C. Plates were then washed three times with PBST. Sera were diluted 1:100 in PBS and 100 μl added to wells in duplicate for antigen-coated and control wells (i.e. coating buffer alone) for 1 h incubation at 37 °C. Plates were washed three times with PBST before addition of 100 μl goat anti-bovine IgG (H + L) horseradish peroxidase-conjugated (KPL) secondary antibody diluted 1:10,000 in PBS + 0.1% fish gelatin. For isotype analysis, 100 μl of sheep anti-bovine IgG_1 (Serotec, Oxford, UK) or sheep anti-bovine IgG_2 (Serotec), diluted 1:5000 in PBS + 0.1% fish gelatin, was added as secondary antibody. Plates were incubated for 1 h at 37 °C and washed three times. Wells were developed with the addition of 100 μl TMB substrate (KPL) for 5 min at room temperature. The reaction was stopped by addition of 100 μl of 0.18 M sulfuric acid. A_{450} of individual wells was measured with an automated ELISA plate reader (Molecular Devices, Menlo Park, CA) Changes in optical density (ΔOD) readings were calculated by subtracting the mean OD readings for wells receiving coating buffer alone (two replicates) from the mean OD readings for antigen-coated wells (two replicates) receiving the same serum sample.

2.9. Flow cytometry

PBMC (4×10^5 per well) were cultured *in vitro* in the presence of rESAT-6:CFP10 (5 μg/ml) or cRPMI alone for 6 days at 37 °C in 5% CO_2 humidified air in a round-bottom 96-well plate. Following incubation, cells were subsequently pooled according to treatments. Approximately 4×10^5 pooled cells in 200 μl of culture medium were added to individual wells of round-bottom 96-well plates, centrifuged (2 min, 400 × *g*) and resuspended in 100 μl of primary antibody(s) (1 μg/ml in PBS containing 1% FBS and 0.1% sodium azide). Primary antibodies included anti-CD4 (GC50A1), anti-CD8α (BAQ111A), anti-γδ TCR (GB21A, specific for δ chain) and anti-CD25 (CACT116A) (VMRD, Pullman, WA). After 15 min incubation at room temperature, cells were centrifuged and stained with 100 μl of appropriate secondary antibody [fluorescein isothiocyanate (FITC, 1 μg/well)-conjugated goat anti-mouse IgG_2b, IgM (Southern Biotechnology Associates, Birmingham, AL), phycoerythrin (PE, 1 μg/well)-conjugated goat anti-mouse IgG_1]. Cells were incubated for 15 min at room temperature, centrifuged, washed and resuspended in PBS containing 0.04% sodium azide for acquisition (10,000 live gated events) using a FACScan (Becton Dickinson, San Jose, CA; 488 nm laser, two color) flow cytometer. Data analysis was performed

with commercially available software (FlowJo, Tree Star Inc., San Carlos, CA). Data are presented as the mean (±S.E.M.) percentage of cells expressing a marker.

2.10. Gross and histologic analysis

Lungs, mediastinal and tracheobronchial lymph nodes were subjected to a semi-quantitative gross lesion scoring system adapted from Vordermeier et al. [45]. Lung lobes (left cranial, left caudal, right cranial, right caudal, middle and accessory) were scored as follows: (0) no visible lesions; (1) no external gross lesions, but lesions seen upon slicing; (2) <5 gross lesions of <10 mm in diameter; (3) >5 gross lesions of <10 mm in diameter; (4) >1 distinct gross lesion of >10 mm in diameter; (5) gross coalescing lesions. Lymph node lesion severity was scored as follows: (0) no necrosis or visible lesions; (1) small focus (1–2 mm in diameter); (2) several small foci; (3) extensive necrosis.

Tissues collected for microscopic analysis were fixed by immersion in 10% neutral buffered formalin. For microscopic examination, formalin-fixed tissues were processed by standard paraffin-embedment techniques, cut into 5 μm sections and stained with hematoxylin and eosin. Adjacent sections were cut from samples containing caseonecrotic granulomata suggestive of tuberculosis and stained by the Ziehl–Neelsen method for visualization of acid-fast bacilli. Microscopic tuberculous lesions were staged (I–IV) based on adaptations of criteria described by Rhoades et al. [46] and Wangoo et al. [47]. One microscopic section from each sample collected was evaluated microscopically. The pathologist (M.V. Palmer) was blinded to treatment groups during analysis of tissues for gross and histologic lesion scoring. Data are presented as the mean (±S.E.M.) number of granulomas observed for lung and lung-associated lymph node sections.

2.11. Radiographic lesion morphometry

All lung lobes were radiographed after necropsy to substantiate pathology findings. Radiography was performed using a MinXray machine (Model HF-100, Diagnostic Imaging, Rapid City, SD) with 3M Asymetrix Detail Screens and Ultimate 2000 film (3M Animal Care Products, St. Paul, MN). Developed radiographs were scanned to create digital images. Radiographic lesions were then identified, outlined and measured using Image Pro Plus (Media Cybernetics, Silver Spring, MD) software. Affected area was divided by total lung area then multiplied by 100 to determine percent affected lung. Results for individual animals are presented as the mean percent affected lung for all lung lobes.

2.12. Statistical analysis

Data were analyzed by one-way ANOVA using commercially available software (Statview 5.0, SAS Institute Inc., Cary, NC). Pairwise comparisons between experimental groups were performed using Fisher's protected least significant difference and Student's *t*-test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Genetic immunization with plasmid DNA encoding ESAT-6:CFP10 + GM-CSF + CD80/CD86 enhances proliferative recall responses

To determine whether encoded GM-CSF enhanced DNA vaccine responses to ESAT-6:CFP10, the ability of bovine PBMC to proliferate following antigenic restimulation was compared among all experimental treatments. Animals immunized with ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA exhibited significant ($p < 0.01$) responses to rESAT-6:CFP10 stimulation as compared to all other groups at two (Fig. 2A) and 4 weeks post-boost (data not shown). Stimulation with PPDb did not result in significant ($p > 0.05$) proliferative responses following vaccination with plasmid-encoded ESAT-6:CFP10 compared to control vaccinated animals (Fig. 2B). Significant ($p < 0.05$) proliferative responses to rESAT-6:CFP10 were evident only in animals vaccinated with co-administered GM-CSF and CD80/CD86. These data suggest that the co-administration of GM-CSF and CD80/CD86 plasmids in addition to an ESAT-6:CFP10 DNA vaccine results in an antigen-specific recall response.

3.2. The frequency of ESAT-6:CFP10-specific IFN-γ-secreting cells increases following vaccination with ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA

To assess the adjuvant effects of plasmid-encoded GM-CSF, administered alone or administered with CD80/CD86 plasmids, we compared the ability of PBMC from different vaccinates to secrete IFN-γ in response to antigenic stimulation. IFN-γ ELISPOT assays were performed at 2 weeks post-boost to evaluate the frequencies of ESAT-6:CFP10-specific IFN-γ-producing cells (Fig. 3). ESAT-6:CFP10 DNA vaccination resulted in increased ($p < 0.05$) frequencies of IFN-γ-producing PBMC compared to control vaccination, as detected by ESAT-6:CFP10-specific IFN-γ ELISPOT. ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinates exhibited greater mean numbers of IFN-γ spot-forming cells (SFC) compared to control vaccinates ($p < 0.0001$), ESAT-6:CFP10 + CD80/CD86 DNA vaccinates ($p < 0.05$) and ESAT-6:CFP10 + GM-CSF DNA vaccinates ($p < 0.05$) (Fig. 3). Similarly, although not statistically significant ($p > 0.05$), ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinates produced the highest mean amount of IFN-γ, which was approximately 12-fold higher than control vaccinated cattle (data not shown). As expected, *in vitro* PPDb stimulation did not elicit IFN-γ production by ESAT-6:CFP10 DNA or control vaccinates (data not shown). Differences between ELISPOT responses of ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinates

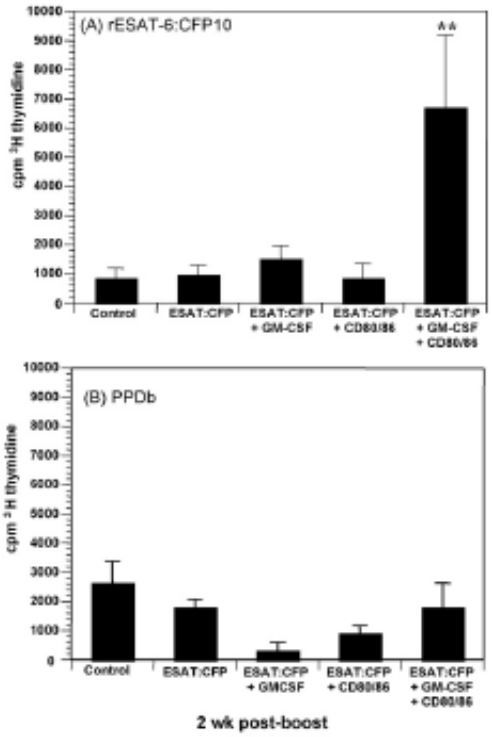

Fig. 2. Proliferative responses from ESAT-6:CFP10+GM-CSF+CD80/CD86 DNA vaccinates ($n=5$) are enhanced compared to all other DNA vaccinates following antigenic stimulation at 2 weeks post-boost. PBMC (5×10^5) were cultured *in vitro* for 7 days in the presence of (A) rESAT-6:CFP10 (5 μg/ml), (B) PPDb (5 μg/ml) or media alone. [^{3}H]-thymidine was added to wells the last 18–20 h of culture. **Indicates a significant difference ($p<0.01$) compared to all other groups. Data represent mean cpm from antigen stimulation cultures minus media alone cpm (±S.E.M.). Similar responses were detected at 4 weeks post-boost. Data were analyzed using ANOVA followed by Fisher's PLSD for post hoc analysis.

and cattle immunized with ESAT-6:CFP10 DNA alone were not significant ($0.05<p<0.1$) potentially due to donor variability. Therefore, vaccination with ESAT-6:CFP10 DNA vaccines results in the generation of increased frequencies of antigen-specific IFN-γ-producing cells; however, an additional trend towards greater numbers of SFC following co-administration of plasmid-encoded GM-CSF and CD80/CD86 was also observed.

3.3. $CD4^+$ cells from ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinates display increased expression of CD25 following antigenic restimulation

To evaluate whether increased expression of activation molecules was associated with enhanced cell-mediated responses, flow cytometric analysis of CD25 was conducted. Following 6-day culture, $CD4^+$ cells from ESAT-6:CFP10+GM-CSF+CD80/CD86 DNA vaccinates exhibited increased ($p<0.05$) expression of CD25 in response to rESAT-6:CFP10 stimulation when com-

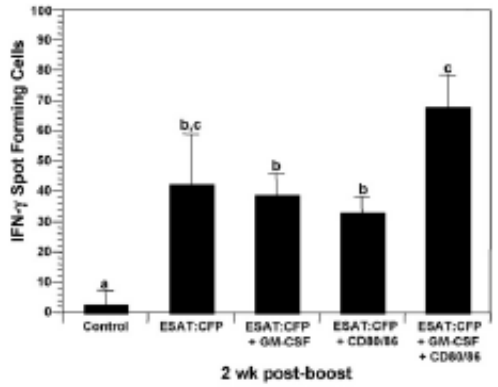

Fig. 3. Genetic immunization with ESAT-6:CFP10 DNA generates increased frequencies of antigen-specific IFN-γ producing cells. Two weeks post-boost, 5×10^5 PBMC were stimulated 36 h in the presence of rESAT-6:CFP10 (5 μg/ml) for the determination of the number of cells producing IFN-γ in response to rESAT:CFP. Data represent the mean (±S.E.M.) number of spot-forming cells from stimulated cultures minus control cultures. PBMC were cultured in triplicate. Letters (a–c) indicate that the treatment means for a specific type of stimulation (i.e. horizontal comparisons) differ ($p<0.05$). The same letter indicates that responses were not significantly different ($p>0.05$). Data were analyzed using ANOVA followed by Fisher's PLSD for post hoc analysis.

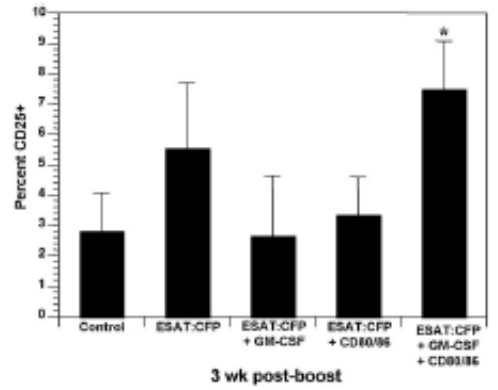

Fig. 4. ESAT-6+GM-CSF+CD80/CD86 DNA vaccinates ($n=5$) exhibit increased $CD4^+$ T cell expression of CD25 in response to antigen. Three weeks post-boost, PBMC from vaccinated animals were stimulated for 6 days in the presence of rESAT-6:CFP10 (5 μg/ml) or media alone. Cells were subsequently stained for expression of CD25, indicative of an activated phenotype. Data represent the mean (±S.E.M.) percent expression of CD25 minus control values (i.e. background) from animals in each treatment group.

Table 1
Serum antibody responses to ESAT-6:CFP10 genetic immunization[a]

Vaccination group	ΔOD at A_{450}					
	Total IgG day 0	Total IgG 2 weeks PB	IgG_1 day 0	IgG_1 2 weeks PB	IgG_2 day 0	IgG_2 2 weeks PB
Control ($n=5$)	0.111 (0.035)	0.176 (0.034)	0.016 (0.016)	0.178 (0.072)	0.005 (0.005)	0.138 (0.022)
ESAT-6:CFP10 ($n=20$)	0.140 (0.024)	0.281* (0.029)	0.027 (0.009)	0.370** (0.044)	0.006 (0.002)	0.196 (0.023)
ESAT-6:CFP10 alone ($n=5$)	0.057 (0.021)	0.231 (0.043)	0.000 (0.000)	0.350 (0.094)	0.010 (0.003)	0.214 (0.048)
ESAT-6:CFP10 + GM-CSF ($n=5$)	0.182 (0.066)	0.300** (0.036)	0.052 (0.028)	0.384 (0.089)	0.000 (0.000)	0.233 (0.051)
ESAT-6:CFP10 + CD80/CD86 ($n=5$)	0.151 (0.051)	0.297 (0.093)	0.025 (0.019)	0.402* (0.080)	0.005 (0.003)	0.174 (0.034)
ESAT-6:CFP10 + GM-CSF + CD80/CD86 ($n=5$)	0.170 (0.039)	0.296 (0.061)	0.030 (0.016)	0.347 (0.116)	0.009 (0.004)	0.164 (0.055)

[a] Serum antibody responses to ESAT-6:CFP10 were assessed prior to vaccination (day 0) and at 2 weeks post-boost (PB). Sera were diluted 1:100 and added to ESAT-6:CFP10-coated wells (1 μg/ml) in duplicate. The changes in optical density (ΔOD) readings were calculated by subtracting the mean OD readings for wells receiving coating buffer alone (two replicates) from the mean OD readings for antigen-coated wells (two replicates) receiving the same serum sample. Data are presented as group mean (±S.E.M.). Differs from respective response by controls, $^*p<0.1$, $^{**}p\leq0.05$.

pared to cattle vaccinated with control DNA at 3 weeks post-boost (Fig. 4). Differences in the expression of CD25 on $CD8^+$ and $\gamma\delta$ TCR^+ cells were not significant ($p>0.05$) at this timepoint (data not shown). Thus, increased proliferative responses observed from PBMC of ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinated subjects were associated with increased expression of CD25 on CD4-bearing cells.

3.4. Evaluation of antigen-specific humoral responses following ESAT-6:CFP10 DNA vaccination

Antigen-specific serologic responses from animals were evaluated to determine the effects of genetic immunization on antibody responses. Responses (total IgG, IgG_1 and IgG_2) directed toward ESAT-6:CFP were negligible at day 0 of the vaccine trial (Table 1). Analysis of antibody responses 2 weeks post-boost, revealed that increased antibody production was more likely attributable to ESAT-6:CFP10 DNA vaccination rather than the type of molecular adjuvant administered, although a significant ($p\leq0.05$) increase in total IgG against rESAT-6:CFP10 was seen in animals receiving ESAT-6:CFP10 + GM-CSF-encoding plasmids and a trend ($0.05<p<0.1$) toward increased IgG_1 production observed in cattle vaccinated with ESAT-6:CFP10 + CD80/CD86 DNA. The effect of immunizing with ESAT-6:CFP10 DNA vaccines was more telling when responses from all animals receiving ESAT-6:CFP10 DNA vaccine were pooled. Two weeks following vaccine boost, a trend ($0.05<p<0.1$) toward increased total IgG against rESAT-6:CFP10 was evident in all ESAT-6:CFP10 DNA vaccinates compared to control vaccinates (Table 1). Further analysis of antigen-specific antibody isotypes revealed a significant ($p\leq0.05$) IgG_1 antibody response against ESAT-6:CFP10 from all DNA vaccinates relative to responses of control animals (Table 1). These data suggest that DNA vaccination with ESAT-6:CFP10 results in the production of antigen-specific humoralresponses.

3.5. M. bovis

BCG + ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinates display reduced lesion severity in the lungs following aerosol challenge with virulent M. bovis

Results from DNA vaccine trial #1 indicated that ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccination elicited more robust immune responses to encoded antigen than all other DNA vaccinates. Therefore, we sought to evaluate whether or not this vaccine combination could confer protection in cattle aerosol challenged with virulent *M. bovis*, and to assess if the addition of BCG enhances plasmid-generated immunity. Previous experiments have determined the location of *M. bovis* after low-dose aerosol challenge of cattle (~120 days) to be primarily focused in the lungs and regional lymph nodes draining the lung. As shown in Table 2, BCG + ESAT-6:CFP10 DNA vaccinates possessed lower gross pathology scores as compared to control animals and other vaccinates. To reinforce these findings, we next employed radiographic lesion morphometry to quantify lung lesions. Non-vaccinated control animals exhibited the highest mean number of radiographic lesions per lung, followed by animals vaccinated with BCG alone and then DNA vaccinates (Table 2). BCG + ESAT-6:CFP10 DNA vaccinates possessed the fewest mean number of lesions compared to all other experimental treatment groups. Similarly, cattle vaccinated with BCG + ESAT-6:CFP10 DNA also displayed the lowest mean percent affected lung following aerosol challenge. Animals vaccinated with ESAT-6:CFP10 possessed comparable levels of percent affected lung and numbers of granulomas upon histologic analysis to BCG + ESAT-6:CFP10 DNA vaccinates, although exhibited a higher mean pathology score and a greater frequency of radiographic lesions in the lung. Numbers of granulomas of the various stages did not vary significantly between treatment groups (data not shown). These results suggest that BCG + ESAT-6:CFP10 DNA vaccination, and to some degree ESAT-6:CFP10 DNA vaccination, achieves reduced pathology of the lung

Table 2
Summary of lung pathology after challenge with virulent *Mycobacterium bovis*

Vaccination group[a]	Gross pathology[b]	Number of radiographic lesions	Animals with radiographic lesions	% Affected lung[c]	Histologic evaluation[d]
Control	1.32 ± 0.70	16.0 ± 9.36	5/5	1.29 ± 1.20	2.4 ± 1.3
BCG	1.44 ± 0.74	8.80 ± 5.15	3/5	0.58 ± 0.30	3.4 ± 2.3
ESAT-6:CFP10	1.20 ± 0.44	8.00 ± 2.72	4/4	0.16 ± 0.03	0.8 ± 0.4
BCG + ESAT-6:CFP10	0.48 ± 0.27	3.60 ± 2.01	3/5	0.12 ± 0.10	0.6 ± 0.4

Data are presented as group mean (±S.E.M.).
[a] Vaccine treatment groups were: non-vaccinated controls ($n = 5$), *M. bovis* BCG strain Pasteur vaccination ($n = 5$), ESAT-6:CFP10 + GM-CSF + CD80/CD68 DNA vaccination ($n = 4$), and *M. bovis* BCG strain Pasteur + ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccination ($n = 5$).
[b] Mean disease score: lungs were subjected to a semi-quantitative pathology scoring system described previously [45].
[c] Lung lobes were removed at necropsy and individually radiographed. Lesions were identified on digital images of scanned radiographs, outlined, and measured. Affected area was divided by total lung area then multiplied by 100 to determine percent affected lung.
[d] Mean number of granulomas detected per microscopic section upon histologic evaluation of lung tissue sections.

compared to control animals following virulent *M. bovis* challenge.

3.6. BCG + ESAT-6:CFP10 DNA vaccinates exhibit reduced pathology of the lung-associated lymph nodes

Following aerosol challenge, tracheobronchial and mediastinal lymph nodes were subjected to a previously described pathology scoring system [45]. Results are summarized in Table 3. BCG + ESAT-6:CFP10 DNA vaccinates possessed significantly reduced gross pathology scores of mediastinal and tracheobronchial lymph nodes compared to all other animals (Table 3). Additionally, the mean weights and number of granulomas detected upon histologic evaluation of the lung-associated lymph nodes were the lowest in animals receiving BCG + ESAT-6:CFP10 DNA. Numbers of granulomas of the various stages did not vary significantly between treatment groups (data not shown). These data suggest that vaccination with BCG + ESAT-6:CFP10 DNA results in reduced pathology of the lung-draining lymph nodes following aerosol challenge with virulent *M. bovis*.

4. Discussion

The goal of vaccination is to generate immunologic memory that will provide rapid protection against exposure to a specific pathogen. Following antigenic priming, $CD4^+$ and $CD8^+$ T cells undergo programmed division, resulting in the generation of effector and memory T cell populations [48]. This division occurs independently of further antigenic stimulation [48]. However, factors such as the strength and duration of antigenic stimulation, and level of costimulation affect T cell differentiation and their functional qualities [48]. In this study, we sought to generate improved recall responses to a candidate DNA vaccine (ESAT-6:CFP10) against tuberculosis by addition of plasmid-encoded costimulatory molecules (CD80/CD86) and encoded GM-CSF. In addition, we aimed to evaluate a vaccination regimen in which calves were primed with *M. bovis* BCG and prime/boosted with an ESAT-6:CFP10 DNA vaccine containing plasmid-encoded costimulatory molecules.

Following a single prime/boost regimen, animals immunized with ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccine displayed increased recall responses to antigen

Table 3
Summary of pathology in the lung-associated lymph nodes after challenge with virulent *M. bovis*

Vaccination group[a]	Gross pathology[b]	Lymph node weight (g)	Histologic evaluation[c]
Mediastinal lymph node			
Control	2.70 ± 0.21	29.62 ± 8.68	20.6 ± 7.9
BCG	2.00 ± 0.45	25.12 ± 3.54	23.4 ± 6.1
ESAT-6:CFP10	2.00 ± 0.32	21.42 ± 2.48	24.0 ± 8.6
BCG + ESAT-6:CFP10	0.60 ± 0.40	14.66 ± 1.84	6.8 ± 4.9
Tracheobronchial lymph node			
Control	2.20 ± 0.54	10.36 ± 1.62	24.2 ± 5.9
BCG	1.80 ± 0.58	11.22 ± 2.35	25.2 ± 8.3
ESAT-6:CFP10	2.00 ± 0.58	10.12 ± 1.05	20.2 ± 3.8
BCG + ESAT-6:CFP10	0.60 ± 0.40	7.46 ± 1.22	5.6 ± 3.9

[a] Data are presented as group mean (±S.E.M.), $n = 5$/group. Vaccine treatment groups were: non-vaccinated controls ($n = 5$), *M. bovis* BCG strain Pasteur vaccination ($n = 5$), ESAT-6:CFP10 + GM-CSF + CD80/CD68 DNA vaccination ($n = 4$), and *M. bovis* BCG strain Pasteur + ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccination ($n = 5$).
[b] Mean disease score: mediastinal and tracheobronchial lymph nodes were subjected to a semi-quantitative pathology scoring system described previously [45].
[c] Mean number of granulomas detected per microscopic section upon histologic evaluation of lymph node tissue sections.

as compared to control vaccinates. Lymphocytes from ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinated animals proliferated and increased CD25 expression on $CD4^+$ T cells in response to specific antigen. ESAT-6:CFP10 DNA vaccinates possessed increased frequencies of antigen-specific IFN-γ producing cells compared to control vaccinated animals, irrespective of co-administered molecular adjuvants, suggesting that ESAT-6:CFP10 DNA administered in a single prime/boost regimen is capable of inducing immune responses against encoded antigen. However, ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinates possessed statistically higher numbers of IFN-γ SFC compared to animals receiving ESAT-6:CFP10 + GM-CSF DNA or ESAT-6:CFP10 + CD80/CD86 DNA vaccines. Additionally, there appears to be an interaction between plasmid-encoded GM-CSF and CD80/CD86, as adjuvant effects on cellular responses were most potent when these molecules were co-administered. Serologic responses from DNA vaccinates revealed modest increases in ESAT-6:CFP10-specific total IgG ($0.05 < p < 0.1$) and IgG_1 ($p \leq 0.05$) production relative to responses of animals vaccinated with control DNA with few observed differences between DNA vaccination groups. These results suggest that co-administration of plasmid-encoded GM-CSF and CD80/CD86 induces enhanced cell-mediated, but not antibody responses to DNA vaccination.

DCs are a specialized cell population linking innate and adaptive immune responses. Adaptive immunity is highly dependent on DC responses, as this population of professional antigen presenting cells is the only subset capable of activating naïve T cells [49]. DCs also spontaneously secrete chemokines that selectively recruit/activate naïve and memory B cells [50]. However, for DCs to acquire these abilities, they must become activated and mature [49]. Several factors affect the maturation of immature DCs. Pathogen-derived signals (LPS, CpG, dsRNA), cytokines (GM-CSF and Flt-3L) and T cell signals (CD154), all induce the activation and maturation of DCs [49]. Activation of DCs results in the acquisition of a mature phenotype, characterized by morphologic changes, high expression of MHC II molecules, CD40 and costimulatory molecules (CD80 and CD86) [49], the latter being critical for providing the second signal needed for naïve T cell activation [51–53]. Therefore, in the present study we explored the ability of plasmid-encoded GM-CSF with or without plasmid-encoded CD80/CD86 to enhance immune responses to genetic immunization in cattle.

The effect of augmenting immune responses by GM-CSF appears to be mediated by providing DCs with a maturation signal so they become efficient antigen presenting cells. *In vitro* addition of GM-CSF to bovine bone marrow cells induces differentiation into DCs [30]. However, without addition of Flt-3L and IL-4, these cells lack potent capacity to stimulate T cells [30]. Further examination revealed that the ability to initiate a T cell response correlated with increased expression of CD80/CD86 and MHC II molecules that was dependent upon the presence of culture with Flt-3L [30]. Indeed, $CD4^+$ T cell responses to DNA vaccination are enhanced in cattle following co-administration of GM-CSF- and Flt-3L-expressing plasmids, explained, in part, by enhanced recruitment of DCs to the injection site [9].

Although direct comparisons cannot be made between our current and previous studies, it is noteworthy that adjuvant effects of CD80/CD86 plasmids were enhanced in the presence of a DC maturation signal (i.e. CpG oligodinucleotides (ODN) or GM-CSF).

In our previous study, we found that the co-administration of CD80/CD86 and CpG ODN to an ESAT-6 DNA vaccine, induced protection following *M. bovis* aerosol challenge [10]. The importance of a DC signal was not evident until the current study, which included controls that did not receive a DC maturation signal (i.e. no GM-CSF). Cell-mediated immune responses from animals vaccinated with ESAT-6:CFP10 + CD80/CD86 DNA were consistently lower than those of ESAT-6:CFP10 + GM-CSF + CD80/CD86 vaccinates. Enhanced immune responses observed in vaccinates, not administered CD80/CD86 and GM-CSF, may be attributable to DC maturation induced by tissue microinjury [2,54], caused by vaccination procedures. Therefore, in our system we speculate that a suboptimal DC maturation signal (GM-CSF alone) is compensated for by the addition of CD80/CD86-encoding plasmids. Based on this hypothesis, DNA vaccine trial #2 evaluated whether or not *M. bovis* BCG could further enhance DNA vaccine-induced protection following aerosol challenge with virulent *M. bovis*.

Following aerosol challenge with virulent *M. bovis*, calves vaccinated with *M. bovis* BCG and ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA exhibited marked reductions in lesion severity in the lung and lung-associated lymph nodes as compared to all other vaccinates. Radiographic analyses confirmed pathology findings in that *M. bovis* BCG + ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinates possessed the lowest mean percent affected lung and the lowest mean number of radiographic lung lesions compared to non-vaccinated control animals, cattle immunized with a single dose of BCG, or DNA vaccinates. DNA vaccinates possessed a higher level of lung lesion severity and a greater frequency of radiographic lesions, but a comparable level of mean percent affected lung when compared to *M. bovis* BCG + ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinates. Similarly, upon examination of lung-associated lymph nodes, *M. bovis* BCG + ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccinates possessed the lowest levels of mean lesion severity and a decreased lymph node weight compared to all other vaccinates. These data suggest that the co-administration of *M. bovis* BCG and ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA have additive effects on the reduction of lesion severity following virulent *M. bovis* challenge.

Combinatory approaches have been evaluated using BCG and DNA vaccines to improve vaccination against tuber-

culosis. In cattle, a DNA prime-BCG boost immunization regimen was shown to enhance protection following experimental challenge with *M. bovis* compared to non-vaccinated control animals and animals vaccinated with BCG alone [55]. In addition, Cai et al. recently demonstrated that a DNA vaccine prime followed by a BCG boost resulted in a 10–100-fold reduction of *M. bovis* in the lungs of cattle infected with virulent *M. bovis* compared to animals immunized with DNA alone or BCG alone [56]. In addition, several experiments in mice have been reported to induce enhanced protection using BCG and DNA vaccine strategies. The enhancement of immune responses seen with these combined approaches may be explained by several factors. The addition of DNA vaccines that encode proteins deleted in the attenuation of BCG, may provide important vaccine targets that are present in virulent mycobacteria (i.e. ESAT-6 and CFP10). The inclusion of BCG also presumably provides more T cell epitopes to which immune responses may be mounted. In addition, BCG provides a maturation signal to immature DCs through the involvement of toll-like receptors [57], which may explain our enhancement of DNA vaccine-generated immune responses in the presence of a molecular adjuvant. Further studies will be required to conclusively determine this effect.

To date, *M. bovis* BCG is the only approved vaccine against tuberculosis. However, it has been demonstrated that its efficacy in humans may range from 80% to 0% [58]. The efficacy of BCG vaccination in cattle has been similarly variable (reviewed in Refs. [59,60]). Recent progress regarding BCG vaccination of neonatal cattle and the use of combinatorial vaccines has been promising in finding alternative approaches to standard BCG vaccination. This may be important in controlling the spread of tuberculosis from wildlife reservoirs to cattle and thus, limiting the transmission of virulent mycobacteria to humans, particularly in developing countries or countries with endemic bovine tuberculosis. Natural transmission studies in the field will be needed to accurately determine the efficacy of our vaccine and/or vaccine regimen in preventing the spread of bovine tuberculosis.

In summary, our data demonstrate that ESAT-6:CFP10 DNA vaccination induces potent immune responses, and that co-administration of plasmid-encoded GM-CSF and CD80/CD86 as a single prime/boost regimen enhances immune responses of cattle to ESAT-6:CFP10 genetic immunization, particularly at the level of cell-mediated immunity. The combination of a DC maturation/activation signal and a T cell activation signal appear to be optimal for generating DNA vaccine-induced immune responses in cattle. Finally the combined *M. bovis* BCG and ESAT-6:CFP10 + GM-CSF + CD80/CD86 DNA vaccine prime/boost strategy afforded vaccinates the highest degree of protection against *M. bovis*-induced pathology when compared to BCG or a DNA vaccine administered separate of each other by possibly providing an adjuvant signal to potentiate DNA vaccine-induced responses.

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VITA

Charles Fernando Capinos Scherer was born on April 7, 1975 in Crissiumal, Rio Grande do Sul state, Brazil. His parents are Edmundo Scherer and Geni Maria Capinos Scherer. He attended Colégio Evangélico Augusto Pestana in Ijui, Brazil when he moved to Santa Maria to attend Universidade Federal de Santa Maria in 1993. There he received his Veterinary Medicine degree in 1998. Following his studies, Charles started Graduate School at the college of Veterinary Medicine at the same university (UFSM) where he got his Master's degree in Veterinary Virology, on December 2000. On March of 2000 Charles started teaching Veterinary Microbiology at UFSM until May 2002. In the meantime Charles continued doing research on the field of virology with Bovine Viral Diarrhea Virus (BVDV) and Bovine Herpesvirus (BHV). Following his interest in virology, on June 2002 Charles started as a Post-doctoral fellow at University of Missouri, Columbia, MO with Dr. D. Mark Estes but to work at Plum Island Animal Disease Center, Orient Point, NY, with Vesicular Stomatitis Virus pathogenesis and immune response. Following his interest in immunology, Charles started his PhD at the Graduate School of Biomedical Sciences, University of Texas Medical Branch (UTMB), on 2004. Charles joined the Microbiology and Immunology program in 2005.

As a graduate student at UTMB and in the Department of Microbiology and Immunology, Charles was co-chair of the International Student Organization from September 2006 to August 2007. He presented his work on several meeting, including the Conference of Research of Workers in Animal Diseases (CRWAD) in 2005 and at the American Association of Immunologist (AAI) Annual meeting in 2006 and 2007 and published 5 papers in peer –reviewed journals.

Charles is married to Juliana B. de Aguiar and has two lovely daughters Mariana and Carolina. He can be contacted through his in-laws at Dr. Barcelos, 775, Porto Alegre, RS, Brazil, 91910-251.

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