Characterization of bovine unconventional memory-like responses induced by *Mycobacterium bovis* infection and vaccination

by

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D.V.M., National Autonomous University of Mexico, 2012

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

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Abstract

Tuberculosis (TB) remains a leading cause of death from infectious diseases worldwide. Mycobacterium bovis is the causative agent of bovine tuberculosis (bTB) and zoonotic TB infection. CD4 T cells are necessary for host defense against *M. bovis*; however, CD4 T cell-mediated immunity is not sufficient to prevent or eliminate the infection and additional components of the immune system are required for optimum host protection. The innate immune system has long been considered primitive and nonspecific, thought to lack the capacity to mount immunological memory. However, evidence suggests that the innate immune system can improve with pathogen exposure and adopt memory-like traits, potentially conferring resistance to TB, as well as other unrelated diseases. The focus of this thesis was to characterize two types of nonconventional memory responses in calves responding to *M. bovis* vaccination or virulent infection.

Gamma Delta (GD) T cells are unconventional T cells that play a major role in defense against pathogens, especially at mucosal sites such as the lower respiratory tract. GD T cells participate in the response to *M. bovis* vaccination and infection. In the second chapter, we demonstrate that in cattle, virulent *M. bovis* infection elicits memory-like responses in circulating mycobacteria-specific GD T cells, characteristic of central memory (TCM) cells which have been reported in humans. We further show that bovine *M. bovis*-specific circulating GD T cells upregulate the surface expression of lung-homing receptors CXCR3 and CCR5, suggesting that GD have the capacity to home to sites of infection and participate in control of TB infection. In the third chapter, we expand upon our previous studies through a comparative assessment of the phenotype and function of *M. bovis*-specific GD T cells in the systemic and mucosal compartment following *M. bovis* Bacille Calmette Guerin (BCG) vaccination. We show that aerosol BCG immunization induces both systemic and mucosal *M. bovis*-specific γδ T cells with a surface phenotype associated with highly differentiated TCM and effector memory (TEM) cells, respectively.

Evidence from epidemiological studies in humans suggests that previous exposure to *M. bovis* BCG, a live attenuated vaccine, reduces the risk of childhood mortality due to prevention of sepsis, diarrhea and respiratory infections. It has been suggested that the nonspecific disease resistance induced by BCG is mediated by an enhanced memory-like response of the innate immune system known as ‘trained’ immunity. In the fourth chapter, we demonstrate that in vitro and in vivo infection with *M. bovis* BCG elicits long-term memory-like traits in bovine circulating innate cells, but not lung resident populations, during secondary in vitro Toll-like receptor (TLR) challenge. Consistent with reports in other species, this cellular hyperresponsiveness correlated with a metabolic shift towards glycolysis.

Together, our research suggests that effective control of *M. bovis* infection requires not only properly activated adaptive immune responses but may also benefit from nonconventional memory-like responses from populations such as ‘trained’ innate monocytes and differentiated GD T cells. Our results have important implications towards the development of novel intervention strategies for use against TB and related respiratory infections in humans and animals alike.

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Other work published but not included in this thesis.

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2. McGill JL, **Guerra-Maupome M**, Schneider S. Prophylactic digoxin treatment reduces IL-17 production in vivo in the neonatal calf and moderates RSV-associated disease. *PLoS One*. (2019);14(3): e0214407. doi.org/10.1371/journal.pone.0214407
3. **Guerra-Maupome, M.,** Palmer, M.V., McGill, J.L., Sacco, R.E. Neonatal calves as a model of human RSV infection. *Vaccines*. (2019) 20197;(1), 7. doi.org/10.3390/vaccines7010007.

Other work under review but not included in this thesis

1. McGill JL, Kelly MS, **Guerra-Maupome M**, Winkley E., Henningson J, Narasimhan B, Sacco RE. Vitamin A deficiency impairs the immune response to intranasal vaccination and RSV infection in neonatal calves. *Journal of Immunology.* (2019)
2. **Guerra-Maupome M,** McGill JL. Gamma delta T cell function in ruminants. *Veterinary Clinics of North America: Food Animal Practice*. (2019)

Chapter 1 - GENERAL INTRODUCTION AND BACKGROUND

DISSERTATION ORGANIZATION

This dissertation follows the organizational format in which each chapter is an independent manuscript that has been accepted or submitted. These chapters follow the submission guidelines for the specific journals to which they were submitted. An overview and literature review, which comprises further details related to the dissertation, are included in *Chapter 1*, part of which has will be published in Veterinary Clinics of North America: Food Animal Practice. *Chapter 2* has been submitted for publication in *Immunohorizons. Chapter 3* has been submitted for publication in *Scientific Reports. Chapter 4* has been published in *Plos One*. Chapter 5 encloses the general conclusions and future directions.

LITERATURE REVIEW

Mycobacteria are intracellular, slow-growing, aerobic, gram positive, rod-shaped bacteria. *Mycobacterium bovis (M. bovis)* is one of eight members of the *Mycobacterium tuberculosis* complex (Mtbc) *(1).* Members of the Mtbc are characterized by high genetic homology, with 99.9% similarity at the nucleotide level and identical 16S rRNA sequences; however, they differ in their hosts, phenotypes, and pathogenicity (2). The mycobacterial cell wall is complex, consisting of peptidoglycolipids, cord factors and sulpholipids. Unlike other gram-positive bacteria, members of the Mtbc do not retain the gram stain due to the structure of the cell wall. Mycobacteria are therefore identified as being acid-fast and are detected using the Ziehl-Nielsen stain.

M. bovis is the causative agent of bovine TB in cattle (bTB) and zoonotic TB infection in humans (3). BTB is a chronic infectious disease, characterized by granulomatous lesions localized primarily in the lower and upper respiratory tract, of cattle and a wide range of wildlife species. The disease is a cause of significant economic losses for the livestock industry, through the loss of animals to culling and the costs associated with disease testing and control (4). BTB is also a major public health risk to populations in developing countries and is of increasing concern to communities living at the human–animal interface (4-6). Thus, effective control of bTB may significantly impact human health. However, despite improvements in disease control strategies, many countries' efforts to eradicate are complicated by the presence of *M. bovis* infected wildlife species, which act as reservoirs of infection (4).

Disease pathogenesis and the development of innate and adaptive immune response in cattle infected with *M. bovis* reflect many of the characteristics of *M. tuberculosis* infection in humans. The discovery of these similarities has led to an appraisal of the benefits of collaborative

approaches between human and animal research (4, 7-9). One significant outcome of these collaborative approaches was the current vaccine that is used against TB in humans, the attenuated *M. bovis* strain Bacille Calmette-Guerin (BCG) (4). While the efficacy of the BCG vaccine in both cattle and humans is variable [reviewed in (4, 10)], neonatal vaccination in both species induces a significant degree of protective immunity (7, 11). Moreover, BCG expresses antigens broadly overlapping with virulent *M. bovis*; thus, knowledge gained by the use of BCG in experimental studies is vital for developing novel vaccines strategies (12).

Immune response to *M. bovis*

A range of complex factors such as animal genetics, age, nutritional status, the presence of other infections and the virulence of the particular strain is known to influence the host immune response to *M. bovis*. Innate immune responses are important for recruiting immune cells and establishing early lesion formation; however, in many cases, these responses do little to limit infection and it is the adaptive cell-mediated immune (CMI) response that is essential for controlling the disease [reviewed (8, 13-16)].

Innate immune response

Pulmonary bTB infection is primarily transmitted through inhalation of aerosol droplets containing *M. bovis* (17). Alveolar macrophages (AMs) are considered the major host cell for mycobacteria *in vivo*. AMs have important effector functions that aid in the control and destruction of the invading bacteria. Entry of *M. bovis* into macrophages is mediated by binding to macrophage surface receptors such as Toll-like receptors (TLR), complement receptors, C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain-containing protein 2 (NOD2), and mannose receptors (18). Once the bacteria are internalized, they are contained within

intracellular phagosomes. Several strategies have been described in relation with mycobacterial survival within a host [reviewed in (19)]. For example, mycobacteria inhibit lysosome fusion by the intercalation of mycobacterial membrane lipids with endosomal membranes, leading to the phagosome arrest in maturation and preventing the vesicle from acidifying (20). Infected macrophages are also impaired in their ability to produce Nitric Oxide (NO), TNFα, and IL-12 all of which are necessary for activation of the subsequent immune response.

Once macrophage infection is established, other immune cells are recruited to the site of infection to confine the bacilli and prevent further spread by forming granulomas. Granulomas are organized structures of immune cells that contain epithelioid or foamy macrophages, Langerhans-type multinucleated giant cells, lymphocytes, fibroblasts, and neutrophils (21). Host-pathogen interactions over the course of infection lead to changes in the immune cells recruited to the granuloma and the levels of immune mediators that are produced. Granulomas form in the early stages of disease and are primarily comprised of macrophages, neutrophils and other innate-like cells such as NK cells and γδ T cells. The accumulation of macrophages and dendritic cells (DC) ultimately initiates the adaptive immune response, resulting in later recruitment of adaptive immune cells such as CD4 and CD8 T cells and B cells.

Dendritic cells (DC) are important phagocytes that can ingest and harbor mycobacteria. Recognition of mycobacterial components by DCs occurs by interaction with CLRs, Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), and TLR receptors (22). Murine studies have shown that effective anti-mycobacterial T cell responses are mediated by antigen transport to draining lymph nodes by migratory DC (23). In particular, the most important role of DCs and macrophages is to initiate adaptive immune responses through the production of a wide array of cytokines in such as: TNFα, IL-1β, IL-6, IL-10, IL-12, IFNγ, and

TGF-β (24, 25). However, mycobacteria are capable of altering normal DC function, thus inhibiting these cells from inducing an effective subsequent adaptive immune response (26).

Neutrophils are the most abundant white blood cell population in the peripheral blood and are among the first inflammatory cells to localize at the periphery of the granuloma (21, 27). Although neutrophils have been shown to have mycobacteriocidal activity in some hosts, this appears to depend on several host factors and the stage of infection [reviewed (28)]. However, neutrophils clearly participate in immunity to TB in other capacities. Defective neutrophil functions or depletion of neutrophils exacerbates mycobacterial growth in different organs and reduces IFNγ levels and NO synthase activity (29), suggesting a protective role for neutrophils in host defense. Activated neutrophils also influence innate and adaptive immune responses by acting like antigen presenting cells (APC), producing antimicrobial molecules, pro-inflammatory cytokines and chemokines such as TNFα and CXCL10 (30, 31). However, a neutrophil-driven, interferon (IFN)-inducible gene profile in human patients with TB has been shown to correlate with clinical severity, suggesting that neutrophils may directly contribute to disease pathogenesis (32).

Other innate immune cells, such as natural killer (NK) cells, also play a role in the innate response to *M. bovis* infection. In calves, NK cells are present in higher numbers and are more functionally active compared to adults, and this appears to be important in the context of neonatal BCG vaccination (33). NK cells recognize endogenous host molecules with altered expression due to cellular stress through a combination of stimulatory and inhibitory receptors. *In vitro*, *M. tuberculosis* infected antigen presenting cells (APC) recruit NK cells and augment both their cytolytic activity and IFNγ release (23). *In vitro*, bovine NK cells have been shown to serve as an early source IFNγ, which reduces BCG proliferation in infected macrophages (34-36). Bovine NK

cells are also a source of perforin and granulysin that aids in the killing of infected cells, although these cells are not efficient at directly killing bacteria (8, 24).

Adaptive immune responses

Although innate immune responses are clearly essential for defense against TB infection, they are insufficient for the long-term control and prevention of the disease. Protective immunity against *Mycobacteria* is ultimately dependent upon the initiation of the adaptive immune response.

B lymphocytes are involved in TB granuloma maintenance in some species; however, only a small percentage of cattle infected with TB exhibit a measurable antibody response (37, 38). In *in vitro* models of BCG infection, mycobacteria-specific antibodies have been shown to increase uptake and killing of BCG bacteria by neutrophils and macrophages (39). In mice depletion of B cells results in exacerbated immunopathology and excessive neutrophil accumulation, which coincides with increased bacterial burden (39, 40). However, humoral immunity and antibody mediated immunity is generally regarded as nonprotective against TB in all species (41, 42).

Protective immunity against TB is dependent upon the interaction of T cells with infected macrophages. Initiation of the mycobacteria–specific T cell response is delayed 12-15 days after aerosol infection (43). Such delay allows the pathogen an opportunity to establish a niche and to direct the ensuing immune response in favor of bacterial persistence and chronic immune stimulation (61). It has been speculated that the delayed initiation of the acquired immune response by mycobacteria is a result of its slow growth and the relatively immunoprivileged state of the site of infection [reviewed (22)].

CD8 T cells, are often referred to as cytotoxic T lymphocytes (CTL). Upon mycobacterial infection, CTLs produce IFNγ, granulysin, and perforin, and are capable of lysing infected macrophages and mycobacteria (44, 45). In mice, CD8 T cells contribute to protection against

rapidly progressive *M. tuberculosis* infection; infected CD8 T cell–deficient mice succumb earlier than wild-type controls but later than CD4 T cell–deficient mice (46). In calves, *in vivo* depletion of CD8 T cells in the early stages of *M. bovis* infection results in reduced IFNγ production and reduced pathology scores in the head draining lymph nodes compared to nondepleted calves, suggesting that CD8 cells play a role in bTB immunopathology(47).

Control of TB infection requires CD4 T cells and is thought to be primarily mediated by IFNγ and TNFα, which activates macrophages and promotes their anti-microbial activities (48). Immune deficiencies affecting CD4 T cells (e.g., HIV infection) and IL-12/IFNγ /STAT1 signaling pathways result in more severe disease upon TB infection in humans (49, 50). Furthermore, IFNγ production is depressed in whole-blood cultures from advanced TB patients (51). However, despite the requirement for IFNγ in immunity to TB, its secretion by CD4 T cells is a poor correlate of protection (52, 53). This is further demonstrated by studies in which, despite increasing the frequency of IFNγ producing CD4 T cells, protective immunity was not increased (54). In some recent studies, it has been shown that polyfunctional T cells, in particular antigen-specific CD4 T cells producing a combination of IFNγ, IL-2, and/or TNFα, show greater association with protective immunity to TB than do IFNγ secreting monofunctional T cells (55). Consistent with these observations, protection against *M. bovis* infection in cattle has been strongly correlated with a high frequency of polyfunctional CD4 T cells (55). In humans, however, despite the expansion of polyfunctional T cells, the results obtained from a recent phase 2b vaccine trial in infants indicated no enhancement in protection (56). Thus, while CD4 T cells, and the cytokines they produce, are an essential component of the response to infection, their participation alone is not a biomarker indicative of protection against TB, further underscoring the need to engage other

components of the host immune system in protective responses against *Mycobacterium* infection (7, 8).

γδ T cells

Gamma delta T cells have been identified in all vertebrate species examined thus far (57). Since they were first identified in 1980's, a considerable effort has been made to understand their function and importance in human and animal health. In mice and adult humans, γδ T cells exist as a relatively small proportion of the peripheral blood T cells (~1 - 5%), yet in epithelial tissues γδ T cells can represent nearly 40-50% of T cells (58). In ruminants, γδ T cells constitute a major lymphocyte population in peripheral blood, epithelial tissues and sites of inflammation (59). The high frequency (20-60%) of γδ T cells in ruminants, particularly in young animals, suggests an important role in host defense.

Contrary to αβ T cells, γδ T cells can recognize a broad range of protein and non-protein ligands antigens [reviewed in (60)] without the presence of major histocompatibility complex (MHC). Upon antigen recognition, γδ T are primed for rapid effector function that correlates with their defined tissue distribution, T cell receptor (TCR) gene expression and phenotype. Identified γδ T cell functional responses include proinflammatory cytokine production (61-65), cytotoxicity (62, 66-68), antigen (Ag) presentation (63, 69), and a regulation of inflammatory processes (66, 70, 71).

Major differences between αβ and γδ T cells concern the specific anatomical sites where they localize, and the Ag recognized by the respective TCR molecules. γδ T cells are capable of rearranging a diverse set of germline-encoded variable (V), diversity (D), and joining (J) gene segments during development to generate functional Ag receptors. The overall repertoire of γ or

δ TCR gene segments available for recombination is much smaller than that seen for α or β TCR chains. Diversity in the γδ TCR is increased by the potential to join several D-gene segments in multiple reading frames within a single δ-chain, in addition to N- and P-nucleotide addition during recombination (72). Theoretically this increases the range of molecules that can be recognized by generating a particularly diverse set of CDR3 sequences. They also show canonical pairing of chains that incorporate the product of a particular Vγ gene segment with chains incorporating the product of a particular Vδ gene segment. As a result, the resident population of γδ T cells within an organ, such as the skin or gut, can be nearly homogeneous with regard to TCR expression (58).

Ruminant γδ T cell subsets

A large proportion of ruminant γδ T cells express WC1, a transmembrane glycoprotein and member of the scavenger receptor cysteine rich (SRCR). WC1 is thought to act as pathogen recognition receptor (PRR) and/or co-stimulatory molecule (73, 74). Although genes have been identified in humans and mice which share some homology with WC1, the receptor is not expressed in species other than ruminants and pigs (75). In cattle, the WC1 molecule is encoded by at least 13 genes. Bovine $WC1^+$ γδ T cells are $CD2^-$ $CD4^-$ $CD8^-$ and are a predominant subset in peripheral blood. In contrast, the $WC1^{neg}$ γδ T cell population is most numerous in the spleen, uterus, intestinal mucosa and mesenteric lymph nodes (LNs), and the majority express CD2 and CD8 (76, 77). $WC1^+$ γδ T cells in cattle can be further divided into three defined populations, differentiated by the WC1 genes they express and the pathogens to which they respond: $WC1.1^+$, $WC1.2^+$ and $WC1.3^+$ (78, 79). $WC1.3^+$ cells are contained within the $WC1.2^+$ subset. The bovine $WC1^+$ and $WC1^{neg}$ γδ T cell populations use different families of TCR genes, home to different tissue locations and thus, have distinct immunological roles in the ruminant immune system [(77, 79) and reviewed in (Guerra-Maupome & McGill, manuscript under review)].

γδ T cells and the immune response to *M. bovis*

Early after mycobacterial infection, γδ T cells undergo dynamic changes in frequency and activation status in the peripheral blood and accumulate in the lungs and the regional lymph nodes, suggesting an important role in the early stages of infection (9-14). Infection with *M. bovis* elicits vigorous *in vitro* proliferative responses, and robust IFNγ production by γδ T cells in response to protein and nonprotein Ags from *Mycobacterium* (22, 80-83), indicating that γδ T cells have the capacity to respond specifically and directly to mycobacteria (15-20). BCG vaccination and virulent *M. bovis* infection also induce increases in circulating $WC1^+$ γδ T cells, and these cells have with the capacity to lyse autologous *M. bovis*-infected monocytes (68, 84).

As mentioned previously, natural *M. bovis* infection occurs as a result of pathogen invasion from airborne transmission. Therefore, recruiting immune cells to the lungs is required for controlling the infection. One of the remarkable immune features of mycobacterial-specific γδ T cells is their capacity to traffic to lungs and mucosal surfaces in the early stages of disease. γδ T cells are amongst the first T cells to infiltrate early *M. bovis* granuloma lesions, arriving prior to CD4 T cells (85). $WC1.1^+$. $WC1.2^+$ and $WC1^{neg}$ γδ T cells can be found accumulating in the lymphoid mantle surrounding *M. bovis* granulomas in the lungs (82). In the developing granuloma, γδ T cells can serve as a major source of the pro-inflammatory cytokine, IL-17, which indirectly mediates neutrophil recruitment (86, 87). Depletion of γδ T cells prior to virulent *M. bovis* infection does not significantly alter disease pathology, but does result in increased production of antigen-specific IL-4 and impaired lymphocyte proliferative responses, suggesting alterations in the Th1 bias of the immune response following infection (27).

Memory-like responses of γδ T cells to Mycobacterial infection

While generally considered cells of the innate immune system, adaptive and memory-like features have been described for γδ T cells in the context of mycobacterial infection [reviewed in (88)]. In humans, BCG vaccination induces a population of γδ T cells that proliferate in response to *in vitro* stimulation with mycobacterial components (89). Interestingly, a clear memory-type response of Vγ2Vδ2 T cells can be detected soon after BCG reinfection and the magnitude of this expansion is 2–9-fold greater than that seen during primary BCG infection (89). In non-human primates, Vγ9Vδ2 T cells expand >25-fold in response to primary TB vaccination and display an accelerated recall response after a secondary *M. tb* challenge, which correlates with protection against fatal TB (90). Primary and recall expansions of γδ T cells are also seen following *M. bovis* and *M. tb* aerosol challenges (27, 90-93). Importantly, the capacity to rapidly expand Vγ9Vδ2 upon challenge coincides with reduced *M. tb* burdens and protection against fatal tuberculosis in BCG-vaccinated macaques (94). Administration of phosphoantigens and IL-2 induces marked expansion and pulmonary trafficking/accumulation of phosphoantigen-specific Vγ2Vδ2 T cells, resulting in significantly reduced bacterial burdens and decreased lung pathology upon subsequent *M. tb* challenge (95). These results suggest that γδ T cells, like αβ T cells, can acquire functional memory-like traits and participate in protection against mycobacterial infections.

Phenotypic analyses have shown that human γδ T cells can be differentiated into effector or memory phenotypes based on their expression of the surface molecules CD45RA and CD27 (96, 97). Effector/memory T cells are $CD45RA^{neg}$ and participate in recall responses to antigen (98-101). Bovine CD45RA and CD45RB isoforms are not yet defined; thus, CD45R is used as a marker for naïve lymphocytes in cattle (101). CD27 is a member of the tumor necrosis factor (TNF) family, known to be modulated in human, mice and pig CD4 and γδ T cells in response to

antigenic stimulation (96, 102, 103). CD27 expression has also been identified in bovine T cells, with an almost identical cellular distribution to that reported in humans (104).

Naïve CD45RA$^+$ CD27$^+$ γδ T cells represent ~10-20% of the circulating population in healthy adult humans. CD45RA$^-$ CD27$^+$ central memory (T_{CM}) cells are more plentiful in the blood and exhibit robust proliferative capacity, but limited effector functions. CD45RA$^-$ CD27$^-$ effector memory (T_{EM}) and CD45RA$^+$ CD27$^-$ (T_{EMRA}) γδ T cells are generally recognized to be fully differentiated subsets. Both T_{EM} and T_{EMRA} cells have low proliferative capacities but exhibit robust effector functions. Consistent with their differential homing capacity, certain chemokine receptors are also useful for classifying functional γδ T cell subsets (105). The expression of the homing receptors CXCR3, CCR5 and CD62L have been used to differentiate effector and memory subsets (106). The T_{EM} pool expresses high levels of CXCR3 and CCR5 and loses expression of the lymph node homing receptors CD62L and CCR7. T_{CM} cells may express CXCR3 and CCR5 but retain expression of CD62L (96).

Interestingly, serious TB disease results in reduced γδ T cell effector functions in the periphery (96). Consistent with this observation, there is a progressive loss of CD27^{neg} T_{EM} and T_{EMRA} γδ T cell subsets from the peripheral blood of patients with active TB (97, 107). The implications of these changes on control of the disease is not well defined, and there are limitations for assessing the biological significance of γδ T cells in the response to TB in humans. However, cattle have abundant γδ T cells in the periphery and, as a physiologic model of TB infection, can be applied to questions of γδ T cell biology during *Mycobacterium* infection (4, 7, 12). To date there are few published reports investigating γδ T cell differentiation in ruminants, or the impact of bTB infection on this process. Improved characterization of γδ T cell effector and memory-like

responses induced during mycobacterial infection will contribute to better strategies for harnessing the γδ T cell response in protection against TB for both humans and animals.

Innate immune memory

While dogma states that the innate immune system responds nonspecifically, and cannot improve following pathogen exposure, recent evidence has challenged that concept. It has long been known that that previous exposure to live vaccines such as *M. bovis* BCG, measles and yellow fever, as well as to some microbial components of pathogens (33, 108-113), reduces the risk of childhood mortality due to prevention of unrelated illnesses, such as sepsis, diarrhea and respiratory infections [(26, 114-116) and reviewed (117, 118)]. This nonspecific disease resistance has been shown to be independent of the adaptive response, and is instead mediated by enhanced memory-like responses of the innate immune system, a hypothesis that has been termed 'trained' innate immunity (119, 120).

In humans, BCG vaccination leads to an enhanced production of the monocyte-derived proinflammatory cytokines, TNFα and IL-6, in response to unrelated bacterial and fungal pathogens (*C. albicans, Staphylococcus aureus*) (112); and superior NK cell activity (111). Moreover, in neonates, intradermal BCG vaccination at birth influences the responses to common vaccine antigens at the systemic level, which appears to be mediated through differential activation of APCs, leading to enhanced activation of T lymphocytes (121). Similarly, compared with unvaccinated babies, cord blood monocytes from BCG vaccinated infants show increased expression of granulysin and perforin upon stimulation (122).

Evidence that trained immunity mediates the nonspecific protective effects seen after BCG vaccination came from proof-of-principle experimental studies in rodents. In these studies, BCG

vaccination of severe combined immunodeficiency (SCID) or recombination-activating gene 1 (rag1)-deficient mice, strains which are devoid of an adaptive immune system, induced protection against subsequent lethal *Candida albicans* (*C. albicans*) infection via a mechanism that was shown to require macrophages and proinflammatory cytokine production, both prototypical innate immune components (112, 123, 124). Mice vaccinated with BCG also show increased resistance to malaria infection, which is associated with increased transcription of antimicrobial proteins compared with nonvaccinated mice (125).

Trained immunity differs from adaptive memory for many aspects, including the lack of gene rearrangements, the involvement of epigenetic reprogramming, the type of cells involved (innate cells vs. T and B lymphocytes), and the receptors engaged in pathogen/antigen recognition [germline-encoded pattern-recognition receptors (PRR) vs. antigen-specific T cell and B cell receptors product of somatic recombination at the V(D)J locus] (33, 108-113, 119, 120, 124, 126).

Epidemiological studies in children have shown that BCG vaccination induces nonspecific protection for at least the duration of early childhood (127). Considering the short half-life of circulating monocytes, this suggests that the epigenetic reprogramming occurs in the bone marrow progenitors (113, 128). In an early study, the enhanced functional status of 'trained monocytes' was NOD2 dependent, and the increased gene expression of key proinflammatory cytokines was due to increased H3K4 trimethylation (112). Further mechanistic studies have since confirmed that trained immunity can be mediated by epigenetic modifications, including histone acetylation and methylation and modulation of miRNAs, which can be shaped by environmentally induced metabolic changes (112, 124, 126). Innate memory in rodents seems to be at least in part dependent on cellular metabolism, as the phenotype is correlated with a shift toward aerobic glycolysis and chemical inhibition of the AKT/mammalian target of rapamycin (mTOR)/hypoxia induced factor

1α (HIF1α) pathway blocks training (129, 130). Subsequent studies in human monocytes have identified a similar metabolic switch after BCG priming that involves distinct histone methylations at the H3K4 mark, with chromatin remodeling at a subset of cytokine promoters that lead to transcriptional programs associated with genes of the mTOR pathway (131). These metabolic changes result in differential gene expression and cell physiology leading to increased innate immune cells' capacity to respond to stimulation (112, 124, 126).

The nonspecific protection afforded by parenteral administration of BCG is increasingly appreciated as an aspect of innate immunity that can be exploited to enhance resistance against TB, as well as other pathogens of consequence [reviewed (132)]. In the context of TB, a 'trained' innate immune system may provide an immunological advantage in the extended time period before the adaptive immune response is generated, thus promoting early containment of the bacilli. Experiments aimed at understanding the mechanisms of innate immune training and identifying approaches for promoting enhanced innate resistance to TB and other respiratory infections are expected to significantly advance our ability to prevent and control infections in humans and animals.

Concluding remarks

There is a knowledge gap in our understanding of what constitutes a protective immune response to TB infection or vaccination. It is of pivotal importance to look beyond assumptions that Th1 cells and their cytokine signatures as cornerstones for protection against TB. The evidence presented in this literature review highlights the importance of analyzing alternative cells and immune components to better define the mechanisms of host defense against TB. These determinants may include mechanisms of innate 'training' that are IFNγ independent or may

emanate from unconventional T cell memory, such as that involving γδ T cells (**Fig. 1**). Study of the natural immune response to *M. bovis* in the bovine model is a useful strategy for uncovering the correlates of protection that will be essential for the development of improved intervention strategies for TB in humans and animals alike.

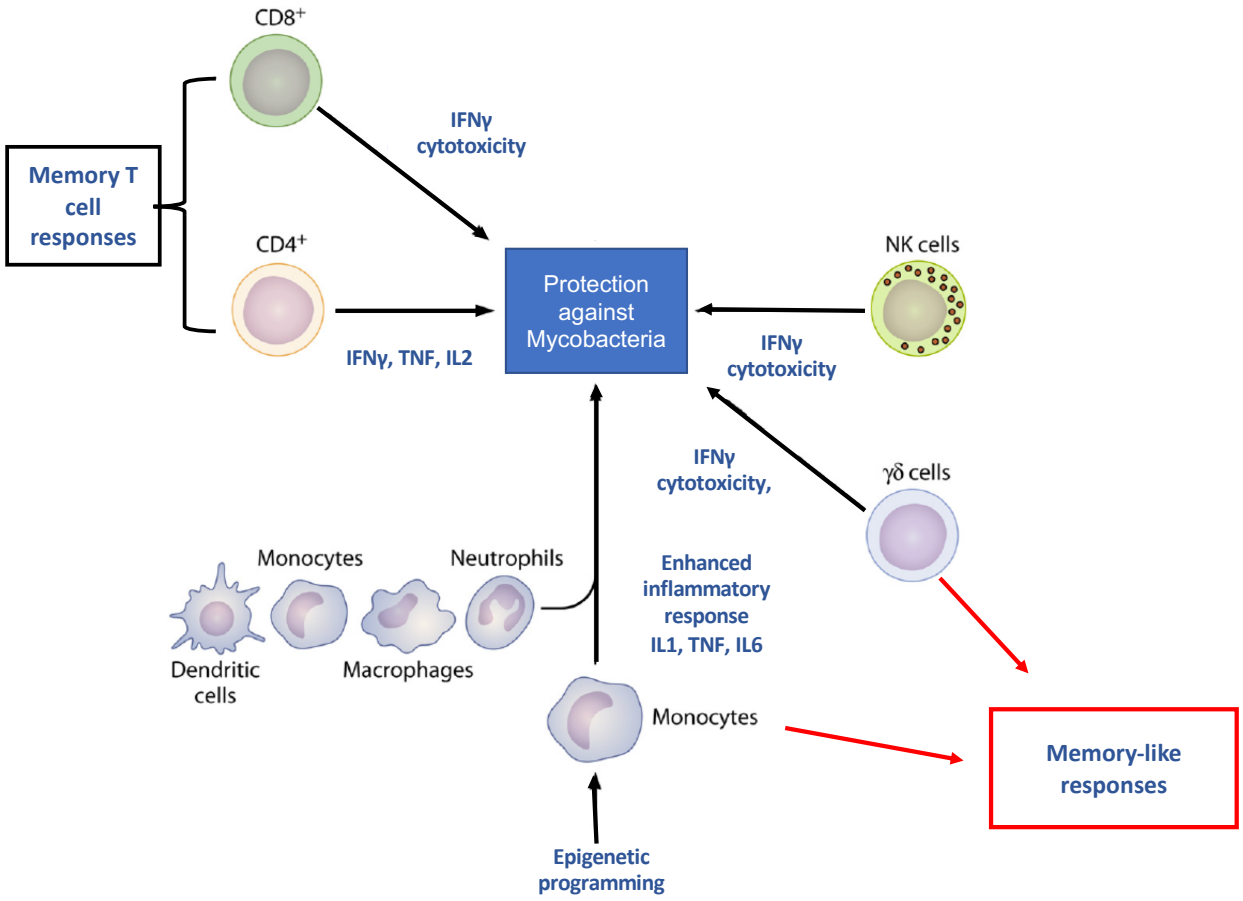

Figure 1. Determinants of protection against Mycobacteria

Protective immunity is dependent on the interactions between innate and adaptive compartments of the immune system. CD4 and CD8 T cells exhibit effector and memory function via secretion of cytokines and/or cytolysis of infected cells. Other immune cells, like NK cells, Neutrophils and DC (not pictured), are also reported to play a role in protective immunity via secretion of inflammatory cytokines, and cytotoxic T cell function. γδ T cells exhibit effector/memory-like function via secretion of IFNγ, cytotoxicity, etc. "Trained immunity", a key feature of the innate immune system is mediated through epigenetic programming, may also play a role in imprinting the innate cells towards an enhanced response to secondary antigenic challenge and thereby conferring in them a protective role, independent of the adaptive immune compartment. Image adapted from **(132).**

REFERENCES

1. Smith NH, Hewinson RG, Kremer K, Brosch R, Gordon SV. Myths and misconceptions: the origin and evolution of Mycobacterium tuberculosis. Nat Rev Microbiol. 2009;7(7):537-44.

2. Brosch R, Gordon SV, Marmiesse M, Brodin P, Buchrieser C, Eiglmeier K, et al. A new evolutionary scenario for the Mycobacterium tuberculosis complex. Proc Natl Acad Sci U S A. 2002;99(6):3684-9.

3. Organization WH. Global tuberculosis report 2018. 2018.

4. Waters WR, Palmer MV, Buddle BM, Vordermeier HM. Bovine tuberculosis vaccine research: historical perspectives and recent advances. Vaccine. 2012;30(16):2611-22.

5. Muller B, Durr S, Alonso S, Hattendorf J, Laisse CJ, Parsons SD, et al. Zoonotic Mycobacterium bovis-induced tuberculosis in humans. Emerg Infect Dis. 2013;19(6):899-908.

6. Schiller I, Oesch B, Vordermeier HM, Palmer MV, Harris BN, Orloski KA, et al. Bovine tuberculosis: a review of current and emerging diagnostic techniques in view of their relevance for disease control and eradication. Transbound Emerg Dis. 2010;57(4):205-20.

7. Endsley JJ, Waters WR, Palmer MV, Nonnecke BJ, Thacker TC, Jacobs WR, Jr., et al. The calf model of immunity for development of a vaccine against tuberculosis. Vet Immunol Immunopathol. 2009;128(1-3):199-204.

8. Waters WR, Palmer MV, Thacker TC, Davis WC, Sreevatsan S, Coussens P, et al. Tuberculosis immunity: opportunities from studies with cattle. Clin Dev Immunol. 2011;2011:768542.

9. Buddle BM, Skinner MA, Wedlock DN, de Lisle GW, Vordermeier HM, Glyn Hewinson R. Cattle as a model for development of vaccines against human tuberculosis. Tuberculosis (Edinb). 2005;85(1-2):19-24.

10. Buddle BM, Wedlock DN, Denis M, Vordermeier HM, Hewinson RG. Update on vaccination of cattle and wildlife populations against tuberculosis. Vet Microbiol. 2011;151(1-2):14-22.

11. Lopez-Valencia G, Renteria-Evangelista T, Williams Jde J, Licea-Navarro A, Mora-Valle Ade L, Medina-Basulto G. Field evaluation of the protective efficacy of Mycobacterium bovis BCG vaccine against bovine tuberculosis. Res Vet Sci. 2010;88(1):44-9.

12. Buddle BM, Pollock JM, Skinner MA, Wedlock DN. Development of vaccines to control bovine tuberculosis in cattle and relationship to vaccine development for other intracellular pathogens. Int J Parasitol. 2003;33(5-6):555-66.

13. McNair J, Welsh MD, Pollock JM. The immunology of bovine tuberculosis and progression toward improved disease control strategies. Vaccine. 2007;25(30):5504-11.

14. Pollock JM, Welsh MD, McNair J. Immune responses in bovine tuberculosis: towards new strategies for the diagnosis and control of disease. Vet Immunol Immunopathol. 2005;108(1-2):37-43.

15. Pollock JM, McNair J, Welsh MD, Girvin RM, Kennedy HE, Mackie DP, et al. Immune responses in bovine tuberculosis. Tuberculosis (Edinb). 2001;81(1-2):103-7.

16. Siddiqui N, Price S, Hope J. BCG vaccination of neonatal calves: potential roles for innate immune cells in the induction of protective immunity. Comp Immunol Microbiol Infect Dis. 2012;35(3):219-26.

17. Neill SD, Pollock JM, Bryson DB, Hanna J. Pathogenesis of Mycobacterium bovis infection in cattle. Vet Microbiol. 1994;40(1-2):41-52.

18. Glatzel A, Wesch D, Schiemann F, Brandt E, Janssen O, Kabelitz D. Patterns of chemokine receptor expression on peripheral blood gamma delta T lymphocytes: strong expression of CCR5 is a selective feature of V delta 2/V gamma 9 gamma delta T cells. J Immunol. 2002;168(10):4920-9.

19. Philips JA, Ernst JD. Tuberculosis pathogenesis and immunity. Annu Rev Pathol. 2012;7:353-84.

20. Russell DG. Mycobacterium tuberculosis and the intimate discourse of a chronic infection. Immunol Rev. 2011;240(1):252-68.

21. Palmer MV, Waters WR, Thacker TC. Lesion development and immunohistochemical changes in granulomas from cattle experimentally infected with Mycobacterium bovis. Vet Pathol. 2007;44(6):863-74.

22. Maue AC, Waters WR, Davis WC, Palmer MV, Minion FC, Estes DM. Analysis of immune responses directed toward a recombinant early secretory antigenic target six-kilodalton protein-culture filtrate protein 10 fusion protein in Mycobacterium bovis-infected cattle. Infect Immun. 2005;73(10):6659-67.

23. Marino S, Pawar S, Fuller CL, Reinhart TA, Flynn JL, Kirschner DE. Dendritic cell trafficking and antigen presentation in the human immune response to Mycobacterium tuberculosis. J Immunol. 2004;173(1):494-506.

24. Fenton MJ, Vermeulen MW. Immunopathology of tuberculosis: roles of macrophages and monocytes. Infect Immun. 1996;64(3):683-90.

25. Arts RJ, Blok BA, van Crevel R, Joosten LA, Aaby P, Benn CS, et al. Vitamin A induces inhibitory histone methylation modifications and down-regulates trained immunity in human monocytes. J Leukoc Biol. 2015;98(1):129-36.

26. Hollm-Delgado MG, Stuart EA, Black RE. Acute lower respiratory infection among Bacille Calmette-Guerin (BCG)-vaccinated children. Pediatrics. 2014;133(1):e73-81.

27. Kennedy HE, Welsh MD, Bryson DG, Cassidy JP, Forster FI, Howard CJ, et al. Modulation of immune responses to Mycobacterium bovis in cattle depleted of WC1(+) gamma delta T cells. Infect Immun. 2002;70(3):1488-500.

28. Lowe DM, Redford PS, Wilkinson RJ, O'Garra A, Martineau AR. Neutrophils in tuberculosis: friend or foe? Trends Immunol. 2012;33(1):14-25.

29. Pedrosa J, Saunders BM, Appelberg R, Orme IM, Silva MT, Cooper AM. Neutrophils play a protective nonphagocytic role in systemic Mycobacterium tuberculosis infection of mice. Infect Immun. 2000;68(2):577-83.

30. Wang J, Zhou X, Pan B, Yang L, Yin X, Xu B, et al. Investigation of the effect of Mycobacterium bovis infection on bovine neutrophils functions. Tuberculosis (Edinb). 2013;93(6):675-87.

31. Seiler P, Aichele P, Bandermann S, Hauser AE, Lu B, Gerard NP, et al. Early granuloma formation after aerosol Mycobacterium tuberculosis infection is regulated by neutrophils via CXCR3-signaling chemokines. Eur J Immunol. 2003;33(10):2676-86.

32. Berry MP, Graham CM, McNab FW, Xu Z, Bloch SA, Oni T, et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature. 2010;466(7309):973-7.

33. Sun JC, Beilke JN, Lanier LL. Adaptive immune features of natural killer cells. Nature. 2009;457(7229):557-61.

34. Portevin D, Young D. Natural killer cell cytokine response to M. bovis BCG Is associated with inhibited proliferation, increased apoptosis and ultimate depletion of NKp44(+)CD56(bright) cells. PLoS One. 2013;8(7):e68864.

35. Denis M, Keen DL, Parlane NA, Storset AK, Buddle BM. Bovine natural killer cells restrict the replication of Mycobacterium bovis in bovine macrophages and enhance IL-12 release by infected macrophages. Tuberculosis (Edinb). 2007;87(1):53-62.

36. Alvarez AJ, Endsley JJ, Werling D, Mark Estes D. WC1(+) gammadelta T cells indirectly regulate chemokine production during mycobacterium bovis infection in SCID-bo mice. Transbound Emerg Dis. 2009;56(6-7):275-84.

37. Johnson JE, Gonzales RA, Olson SJ, Wright PF, Graham BS. The histopathology of fatal untreated human respiratory syncytial virus infection. Mod Pathol. 2007;20(1):108-19.

38. Tsai MC, Chakravarty S, Zhu G, Xu J, Tanaka K, Koch C, et al. Characterization of the tuberculous granuloma in murine and human lungs: cellular composition and relative tissue oxygen tension. Cell Microbiol. 2006;8(2):218-32.

39. de Valliere S, Abate G, Blazevic A, Heuertz RM, Hoft DF. Enhancement of innate and cell-mediated immunity by antimycobacterial antibodies. Infect Immun. 2005;73(10):6711-20.

40. Taylor G, Wyld S, Valarcher JF, Guzman E, Thom M, Widdison S, et al. Recombinant bovine respiratory syncytial virus with deletion of the SH gene induces increased apoptosis and pro-inflammatory cytokines in vitro, and is attenuated and induces protective immunity in calves. J Gen Virol. 2014;95(Pt 6):1244-54.

41. Thoen CO, Kaplan B, Thoen TC, Gilsdorf MJ, Shere JA. Zoonotic tuberculosis. A comprehensive ONE HEALTH approach. Medicina (B Aires). 2016;76(3):159-65.

42. Pollock JM, Neill SD. Mycobacterium bovis infection and tuberculosis in cattle. Vet J. 2002;163(2):115-27.

43. Chackerian AA, Alt JM, Perera TV, Dascher CC, Behar SM. Dissemination of Mycobacterium tuberculosis is influenced by host factors and precedes the initiation of T-cell immunity. Infect Immun. 2002;70(8):4501-9.

44. Skinner MA, Yuan S, Prestidge R, Chuk D, Watson JD, Tan PL. Immunization with heat-killed Mycobacterium vaccae stimulates CD8+ cytotoxic T cells specific for macrophages infected with Mycobacterium tuberculosis. Infect Immun. 1997;65(11):4525-30.

45. Buddle BM, Skinner MA, Wedlock DN, Collins DM, de Lisle GW. New generation vaccines and delivery systems for control of bovine tuberculosis in cattle and wildlife. Vet Immunol Immunopathol. 2002;87(3-4):177-85.

46. Mogues T, Goodrich ME, Ryan L, LaCourse R, North RJ. The relative importance of T cell subsets in immunity and immunopathology of airborne Mycobacterium tuberculosis infection in mice. J Exp Med. 2001;193(3):271-80.

47. Villarreal-Ramos B, McAulay M, Chance V, Martin M, Morgan J, Howard CJ. Investigation of the role of CD8+ T cells in bovine tuberculosis in vivo. Infect Immun. 2003;71(8):4297-303.

48. Cooper AM. Cell-mediated immune responses in tuberculosis. Annu Rev Immunol. 2009;27:393-422.

49. Diedrich CR, Flynn JL. HIV-1/mycobacterium tuberculosis coinfection immunology: how does HIV-1 exacerbate tuberculosis? Infect Immun. 2011;79(4):1407-17.

50. Cooper AM, Solache A, Khader SA. Interleukin-12 and tuberculosis: an old story revisited. Curr Opin Immunol. 2007;19(4):441-7.

51. Hirsch CS, Toossi Z, Othieno C, Johnson JL, Schwander SK, Robertson S, et al. Depressed T-cell interferon-gamma responses in pulmonary tuberculosis: analysis of underlying mechanisms and modulation with therapy. J Infect Dis. 1999;180(6):2069-73.

52. Mittrucker HW, Steinhoff U, Kohler A, Krause M, Lazar D, Mex P, et al. Poor correlation between BCG vaccination-induced T cell responses and protection against tuberculosis. Proc Natl Acad Sci U S A. 2007;104(30):12434-9.

53. Majlessi L, Simsova M, Jarvis Z, Brodin P, Rojas MJ, Bauche C, et al. An increase in antimycobacterial Th1-cell responses by prime-boost protocols of immunization does not enhance protection against tuberculosis. Infect Immun. 2006;74(4):2128-37.

54. Skinner MA, Ramsay AJ, Buchan GS, Keen DL, Ranasinghe C, Slobbe L, et al. A DNA prime-live vaccine boost strategy in mice can augment IFN-gamma responses to mycobacterial antigens but does not increase the protective efficacy of two attenuated strains of Mycobacterium bovis against bovine tuberculosis. Immunology. 2003;108(4):548-55.

55. Maggioli MF, Palmer MV, Thacker TC, Vordermeier HM, McGill JL, Whelan AO, et al. Increased TNF-alpha/IFN-gamma/IL-2 and Decreased TNF-alpha/IFN-gamma Production by Central Memory T Cells Are Associated with Protective Responses against Bovine Tuberculosis Following BCG Vaccination. Front Immunol. 2016;7:421.

56. Tameris MD, Hatherill M, Landry BS, Scriba TJ, Snowden MA, Lockhart S, et al. Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. Lancet. 2013;381(9871):1021-8.

57. Hayday AC. [gamma][delta] cells: a right time and a right place for a conserved third way of protection. Annu Rev Immunol. 2000;18:975-1026.

58. Nielsen MM, Witherden DA, Havran WL. gammadelta T cells in homeostasis and host defence of epithelial barrier tissues. Nat Rev Immunol. 2017;17(12):733-45.

59. Hein WR, Mackay CR. Prominence of gamma delta T cells in the ruminant immune system. Immunol Today. 1991;12(1):30-4.

60. Born WK, Kemal Aydintug M, O'Brien RL. Diversity of gammadelta T-cell antigens. Cell Mol Immunol. 2013;10(1):13-20.

61. Bonneville M, O'Brien RL, Born WK. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. Nat Rev Immunol. 2010;10(7):467-78.

62. Brown WC, Davis WC, Choi SH, Dobbelaere DA, Splitter GA. Functional and phenotypic characterization of WC1+ gamma/delta T cells isolated from Babesia bovis-stimulated T cell lines. Cell Immunol. 1994;153(1):9-27.

63. Collins RA, Sopp P, Gelder KI, Morrison WI, Howard CJ. Bovine gamma/delta TcR+ T lymphocytes are stimulated to proliferate by autologous Theileria annulata-infected cells in the presence of interleukin-2. Scand J Immunol. 1996;44(5):444-52.

64. Jutila MA, Holderness J, Graff JC, Hedges JF. Antigen-independent priming: a transitional response of bovine gammadelta T-cells to infection. Anim Health Res Rev. 2008;9(1):47-57.

65. McGill JL, Nonnecke BJ, Lippolis JD, Reinhardt TA, Sacco RE. Differential chemokine and cytokine production by neonatal bovine gammadelta T-cell subsets in response to viral toll-

like receptor agonists and in vivo respiratory syncytial virus infection. Immunology. 2013;139(2):227-44.

66. Chiodini RJ, Davis WC. The cellular immunology of bovine paratuberculosis: the predominant response is mediated by cytotoxic gamma/delta T lymphocytes which prevent CD4+ activity. Microb Pathog. 1992;13(6):447-63.

67. Daubenberger CA, Taracha EL, Gaidulis L, Davis WC, McKeever DJ. Bovine gammadelta T-cell responses to the intracellular protozoan parasite Theileria parva. Infect Immun. 1999;67(5):2241-9.

68. Skinner MA, Parlane N, McCarthy A, Buddle BM. Cytotoxic T-cell responses to Mycobacterium bovis during experimental infection of cattle with bovine tuberculosis. Immunology. 2003;110(2):234-41.

69. Toka FN, Kenney MA, Golde WT. Rapid and transient activation of gammadelta T cells to IFN-gamma production, NK cell-like killing, and antigen processing during acute virus infection. J Immunol. 2011;186(8):4853-61.

70. Hoek A, Rutten VP, Kool J, Arkesteijn GJ, Bouwstra RJ, Van Rhijn I, et al. Subpopulations of bovine WC1(+) gammadelta T cells rather than CD4(+)CD25(high) Foxp3(+) T cells act as immune regulatory cells ex vivo. Vet Res. 2009;40(1):6.

71. Rhodes SG, Hewinson RG, Vordermeier HM. Antigen recognition and immunomodulation by gamma delta T cells in bovine tuberculosis. J Immunol. 2001;166(9):5604-10.

72. Clayton NS, Emery NJ. What do jays know about other minds and other times? In: Berthoz A, Christen Y, editors. Neurobiology of Umwelt: How Living Beings Perceive the World. Research and Perspectives in Neurosciences. Berlin: Springer-Verlag Berlin; 2009. p. 109-23.

73. Hsu H, Chen C, Nenninger A, Holz L, Baldwin CL, Telfer JC. WC1 is a hybrid gammadelta TCR coreceptor and pattern recognition receptor for pathogenic bacteria. J Immunol. 2015;194(5):2280-8.

74. Wang F, Herzig CT, Chen C, Hsu H, Baldwin CL, Telfer JC. Scavenger receptor WC1 contributes to the gammadelta T cell response to Leptospira. Mol Immunol. 2011;48(6-7):801-9.

75. Crocker G, Sopp P, Parsons K, Davis WC, Howard CJ. Analysis of the gamma/delta T cell restricted antigen WC1. Vet Immunol Immunopathol. 1993;39(1-3):137-44.

76. Baldwin CL, Sathiyaseelan T, Rocchi M, McKeever D. Rapid changes occur in the percentage of circulating bovine WC1(+)gamma delta Th1 cells. Res Vet Sci. 2000;69(2):175-80.

77. Machugh ND, Mburu JK, Carol MJ, Wyatt CR, Orden JA, Davis WC. Identification of two distinct subsets of bovine gamma delta T cells with unique cell surface phenotype and tissue distribution. Immunology. 1997;92(3):340-5.

78. Chen C, Herzig CT, Telfer JC, Baldwin CL. Antigenic basis of diversity in the gammadelta T cell co-receptor WC1 family. Mol Immunol. 2009;46(13):2565-75.

79. Rogers AN, VanBuren DG, Hedblom E, Tilahun ME, Telfer JC, Baldwin CL. Function of ruminant gammadelta T cells is defined by WC1.1 or WC1.2 isoform expression. Vet Immunol Immunopathol. 2005;108(1-2):211-7.

80. Welsh MD, Kennedy HE, Smyth AJ, Girvin RM, Andersen P, Pollock JM. Responses of bovine WC1(+) gammadelta T cells to protein and nonprotein antigens of Mycobacterium bovis. Infect Immun. 2002;70(11):6114-20.

81. Smyth AJ, Welsh MD, Girvin RM, Pollock JM. In vitro responsiveness of gammadelta T cells from Mycobacterium bovis-infected cattle to mycobacterial antigens: predominant involvement of WC1(+) cells. Infection and immunity. 2001;69(1):89-96.

82. McGill JL, Sacco RE, Baldwin CL, Telfer JC, Palmer MV, Waters WR. Specific recognition of mycobacterial protein and peptide antigens by gammadelta T cell subsets following infection with virulent Mycobacterium bovis. J Immunol. 2014;192(6):2756-69.

83. Rhodes SG, Hewinson RG, Vordermeier HM. Antigen recognition and immunomodulation by gamma delta T cells in bovine tuberculosis. Journal of immunology. 2001;166(9):5604-10.

84. Olin MR, Hwa Choi K, Lee J, Molitor TW. Gammadelta T-lymphocyte cytotoxic activity against Mycobacterium bovis analyzed by flow cytometry. J Immunol Methods. 2005;297(1-2):1-11.

85. Plattner BL, Huffman EL, Hostetter JM. Gamma-delta T-cell responses during subcutaneous Mycobacterium avium subspecies paratuberculosis challenge in sensitized or naive calves using matrix biopolymers. Vet Pathol. 2013;50(4):630-7.

86. Umemura M, Yahagi A, Hamada S, Begum MD, Watanabe H, Kawakami K, et al. IL-17-mediated regulation of innate and acquired immune response against pulmonary Mycobacterium bovis bacille Calmette-Guerin infection. J Immunol. 2007;178(6):3786-96.

87. Lockhart E, Green AM, Flynn JL. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. J Immunol. 2006;177(7):4662-9.

88. Chen ZW, Letvin NL. Vgamma2Vdelta2+ T cells and anti-microbial immune responses. Microbes Infect. 2003;5(6):491-8.

89. Hoft DF, Brown RM, Roodman ST. Bacille Calmette-Guerin vaccination enhances human gamma delta T cell responsiveness to mycobacteria suggestive of a memory-like phenotype. J Immunol. 1998;161(2):1045-54.

90. Shen Y, Zhou D, Qiu L, Lai X, Simon M, Shen L, et al. Adaptive immune response of Vgamma2Vdelta2+ T cells during mycobacterial infections. Science. 2002;295(5563):2255-8.

91. Koets A, Rutten V, Hoek A, van Mil F, Muller K, Bakker D, et al. Progressive bovine paratuberculosis is associated with local loss of CD4(+) T cells, increased frequency of gamma delta T cells, and related changes in T-cell function. Infect Immun. 2002;70(7):3856-64.

92. Pollock JM, Pollock DA, Campbell DG, Girvin RM, Crockard AD, Neill SD, et al. Dynamic changes in circulating and antigen-responsive T-cell subpopulations post-Mycobacterium bovis infection in cattle. Immunology. 1996;87(2):236-41.

93. Price S, Davies M, Villarreal-Ramos B, Hope J. Differential distribution of WC1(+) gammadelta TCR(+) T lymphocyte subsets within lymphoid tissues of the head and respiratory tract and effects of intranasal M. bovis BCG vaccination. Vet Immunol Immunopathol. 2010;136(1-2):133-7.

94. Chen ZW. Protective immune responses of major Vgamma2Vdelta2 T-cell subset in M. tuberculosis infection. Curr Opin Immunol. 2016;42:105-12.

95. Chen ZW, Letvin NL. Adaptive immune response of Vgamma2Vdelta2 T cells: a new paradigm. Trends Immunol. 2003;24(4):213-9.

96. Dieli F, Poccia F, Lipp M, Sireci G, Caccamo N, Di Sano C, et al. Differentiation of effector/memory Vdelta2 T cells and migratory routes in lymph nodes or inflammatory sites. J Exp Med. 2003;198(3):391-7.

97. Gioia C, Agrati C, Casetti R, Cairo C, Borsellino G, Battistini L, et al. Lack of CD27-CD45RA-V gamma 9V delta 2+ T cell effectors in immunocompromised hosts and during active pulmonary tuberculosis. J Immunol. 2002;168(3):1484-9.

98. Bembridge GP, MacHugh ND, McKeever D, Awino E, Sopp P, Collins RA, et al. CD45RO expression on bovine T cells: relation to biological function. Immunology. 1995;86(4):537-44.

99. Waters WR, Rahner TE, Palmer MV, Cheng D, Nonnecke BJ, Whipple DL. Expression of L-Selectin (CD62L), CD44, and CD25 on activated bovine T cells. Infect Immun. 2003;71(1):317-26.

100. Maggioli MF, Palmer MV, Thacker TC, Vordermeier HM, Waters WR. Characterization of effector and memory T cell subsets in the immune response to bovine tuberculosis in cattle. PLoS One. 2015;10(4):e0122571.

101. Howard CJ, Sopp P, Parsons KR, McKeever DJ, Taracha EL, Jones BV, et al. Distinction of naive and memory BoCD4 lymphocytes in calves with a monoclonal antibody, CC76, to a

restricted determinant of the bovine leukocyte-common antigen, CD45. Eur J Immunol. 1991;21(9):2219-26.

102. Hintzen RQ, de Jong R, Lens SM, Brouwer M, Baars P, van Lier RA. Regulation of CD27 expression on subsets of mature T-lymphocytes. J Immunol. 1993;151(5):2426-35.

103. Pepperberg IM. Avian cognition and social interaction: Fifty years of advances. Interact Stud. 2011;12(2):195-207.

104. Sopp P, Howard CJ. Cross-reactivity of monoclonal antibodies to defined human leucocyte differentiation antigens with bovine cells. Vet Immunol Immunopathol. 1997;56(1-2):11-25.

105. Geginat J, Sallusto F, Lanzavecchia A. Cytokine-driven proliferation and differentiation of human naive, central memory and effector memory CD4+ T cells. Pathol Biol (Paris). 2003;51(2):64-6.

106. Qin S, Rottman JB, Myers P, Kassam N, Weinblatt M, Loetscher M, et al. The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. J Clin Invest. 1998;101(4):746-54.

107. Meraviglia S, El Daker S, Dieli F, Martini F, Martino A. gammadelta T cells cross-link innate and adaptive immunity in Mycobacterium tuberculosis infection. Clin Dev Immunol. 2011;2011:587315.

108. Di Luzio NR, Williams DL. Protective effect of glucan against systemic Staphylococcus aureus septicemia in normal and leukemic mice. Infect Immun. 1978;20(3):804-10.

109. Marakalala MJ, Williams DL, Hoving JC, Engstad R, Netea MG, Brown GD. Dectin-1 plays a redundant role in the immunomodulatory activities of beta-glucan-rich ligands in vivo. Microbes Infect. 2013;15(6-7):511-5.

110. Munoz N, Van Maele L, Marques JM, Rial A, Sirard JC, Chabalgoity JA. Mucosal administration of flagellin protects mice from Streptococcus pneumoniae lung infection. Infect Immun. 2010;78(10):4226-33.

111. Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Jacobs C, Xavier RJ, et al. BCG-induced trained immunity in NK cells: Role for non-specific protection to infection. Clin Immunol. 2014;155(2):213-9.

112. Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Ifrim DC, Saeed S, et al. Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. Proc Natl Acad Sci U S A. 2012;109(43):17537-42.

113. Kaufmann E, Sanz J, Dunn JL, Khan N, Mendonca LE, Pacis A, et al. BCG Educates Hematopoietic Stem Cells to Generate Protective Innate Immunity against Tuberculosis. Cell. 2018;172(1-2):176-90 e19.

114. Aaby P, Roth A, Ravn H, Napirna BM, Rodrigues A, Lisse IM, et al. Randomized trial of BCG vaccination at birth to low-birth-weight children: beneficial nonspecific effects in the neonatal period? J Infect Dis. 2011;204(2):245-52.

115. Biering-Sorensen S, Aaby P, Napirna BM, Roth A, Ravn H, Rodrigues A, et al. Small randomized trial among low-birth-weight children receiving bacillus Calmette-Guerin vaccination at first health center contact. Pediatr Infect Dis J. 2012;31(3):306-8.

116. Levy O, Levy O. Ready to benefit from training: heterologous effects of early life immunization. Trans R Soc Trop Med Hyg. 2015;109(1):3-4.

117. Blok BA, Arts RJ, van Crevel R, Benn CS, Netea MG. Trained innate immunity as underlying mechanism for the long-term, nonspecific effects of vaccines. J Leukoc Biol. 2015;98(3):347-56.

118. Netea MG, van Crevel R. BCG-induced protection: effects on innate immune memory. Semin Immunol. 2014;26(6):512-7.

119. Netea MG, Quintin J, van der Meer JW. Trained immunity: a memory for innate host defense. Cell Host Microbe. 2011;9(5):355-61.

120. Netea MG, Joosten LA, Latz E, Mills KH, Natoli G, Stunnenberg HG, et al. Trained immunity: A program of innate immune memory in health and disease. Science. 2016;352(6284):aaf1098.

121. Ota MO, Vekemans J, Schlegel-Haueter SE, Fielding K, Sanneh M, Kidd M, et al. Influence of Mycobacterium bovis bacillus Calmette-Guerin on antibody and cytokine responses to human neonatal vaccination. J Immunol. 2002;168(2):919-25.

122. Semple PL, Watkins M, Davids V, Krensky AM, Hanekom WA, Kaplan G, et al. Induction of granulysin and perforin cytolytic mediator expression in 10-week-old infants vaccinated with BCG at birth. Clin Dev Immunol. 2011;2011:438463.

123. van 't Wout JW, Poell R, van Furth R. The role of BCG/PPD-activated macrophages in resistance against systemic candidiasis in mice. Scand J Immunol. 1992;36(5):713-9.

124. Quintin J, Saeed S, Martens JHA, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, et al. Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. Cell Host Microbe. 2012;12(2):223-32.

125. Parra M, Liu X, Derrick SC, Yang A, Tian J, Kolibab K, et al. Molecular analysis of non-specific protection against murine malaria induced by BCG vaccination. PLoS One. 2013;8(7):e66115.

126. Saeed S, Quintin J, Kerstens HH, Rao NA, Aghajanirefah A, Matarese F, et al. Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. Science. 2014;345(6204):1251086.

127. Stensballe LG, Nante E, Jensen IP, Kofoed PE, Poulsen A, Jensen H, et al. Acute lower respiratory tract infections and respiratory syncytial virus in infants in Guinea-Bissau: a beneficial effect of BCG vaccination for girls community based case-control study. Vaccine. 2005;23(10):1251-7.

128. Mitroulis I, Ruppova K, Wang B, Chen LS, Grzybek M, Grinenko T, et al. Modulation of Myelopoiesis Progenitors Is an Integral Component of Trained Immunity. Cell. 2018;172(1-2):147-61 e12.

129. Rodriguez-Prados JC, Traves PG, Cuenca J, Rico D, Aragones J, Martin-Sanz P, et al. Substrate fate in activated macrophages: a comparison between innate, classic, and alternative activation. J Immunol. 2010;185(1):605-14.

130. Cheng SC, Quintin J, Cramer RA, Shepardson KM, Saeed S, Kumar V, et al. mTOR- and HIF-1alpha-mediated aerobic glycolysis as metabolic basis for trained immunity. Science. 2014;345(6204):1250684.

131. Arts RJ, Joosten LA, Netea MG. Immunometabolic circuits in trained immunity. Semin Immunol. 2016;28(5):425-30.

132. Bhatt K, Verma S, Ellner JJ, Salgame P. Quest for correlates of protection against tuberculosis. Clin Vaccine Immunol. 2015;22(3):258-66.

Chapter 2 - Characterization of γδ T cell effector/memory subsets based on CD27 and CD45R expression in response to *Mycobacterium bovis* infection

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ABSTRACT

Tuberculosis (TB) remains a leading cause of death from infectious diseases worldwide. *Mycobacterium bovis* is the causative agent of bovine tuberculosis and zoonotic TB infection. γδ T cells are known to participate in the immune control of mycobacterial infections. Data in human and non-human primates suggest that mycobacterial infection regulates memory/effector phenotype and adaptive immune functions of γδ T cells. To date, the impact of *M. bovis* infection on bovine γδ T cells and their effector and memory differentiation remains unknown. In this study, we show for the first time that circulating γδ T cells from *M. bovis* infected cattle can be differentiated based on the expression of CD27, which is indicative of their capacity to respond to virulent *M. bovis* infection: $CD27^+$ γδ T cells proliferated in response to *M. bovis* antigen and thus, may compose the adaptive γδ T cell compartment in cattle. We further show that bovine *M. bovis*-specific γδ T cells express surface markers characteristic of T_{CM} cells (CD45R- CD27+$CD62L^{hi}$) and that *M. bovis*-specific CD4 and γδ T cells both upregulate the expression of the tissue-homing receptors CXCR3 and CCR5 during infection. Our studies contribute significantly to our understanding of γδ T cell differentiation during TB infection and provide important insights into

the link between phenotypic and functional subsets in the bovine. Accurate characterization of γδ T cell effector and memory-like responses induced during mycobacterial infection will contribute to improved strategies for harnessing the γδ T cell response in protection against TB for both humans and animals.

INTRODUCTION

Tuberculosis (TB) is a leading cause of deaths related to an infectious disease worldwide (1). *Mycobacterium bovis* is a member of the *M. tb* complex and is the causative agent of bovine tuberculosis (bTB) and zoonotic TB infection. Despite continuous efforts to control the disease, bTB is a significant cause of economic loss to the livestock industry, and a major public health risk to populations in developing countries (2-4). Furthermore, bTB parallels human TB in several aspects of disease pathogenesis and the development of innate and adaptive immune responses (2, 5, 6). Thus, experimental studies of bTB are an excellent model to understand the immune response to *M. tb* infection in humans.

Cell-mediated Th-1 immune responses are essential for controlling TB (7, 8). Importantly, however, γδ T cells been shown to contribute to the immune response against TB. *M. bovis* infection elicits a marked *in vitro* expansion and robust production of IFNγ by γδ T cells suggesting that bovine γδ T cells can mount memory-like responses upon restimulation (9-14). Expression of cell surface memory markers CD45RA and CD27 is linked to functional segregation of γδ T cell memory subsets in non-human primates and humans (15, 16). Naïve $CD45RA^{+}CD27^{+}$ γδ T cells represent ~10-20% of the circulating γδ T cell population in healthy human adults. $CD45RA^{-}CD27^{+}$ central memory (T_{CM}) γδ T cells are plentiful in peripheral blood and exhibit robust proliferative capacity, but limited effector functions. T_{CM} cells may express CXCR3 and CCR5 but retain expression of CD62L (15). $CD45RA^{-}CD27^{-}$ effector memory (T_{EM}) and $CD45RA^{+}CD27^{-}$ effector memory RA (T_{EMRA}) γδ T cells are generally recognized to be fully differentiated subsets. They are infrequent in the blood, but abundant in tissues and sites of inflammation. Both T_{EM} and T_{EMRA} cells have low proliferative capacity, but robust effector

functions. Consistent with their differential homing capacity, the T_{EM} pool expresses high levels of CXCR3 and CCR5 and loses expression of the lymph node homing receptors CD62L and CCR7.

In non-human primates, T_{EM} Vγ9Vδ2 T cell expansion after a secondary *M. tb* challenge correlates with protection against fatal TB (17-19). Importantly, however, serious TB disease results in a progressive loss of T_{EM} and T_{EMRA} γδ T cell subsets from the peripheral blood of humans with active TB (16, 20, 21). The implications of these changes on control of the disease is not well defined, and there are limitations for assessing the biological significance of γδ T cells in the response to TB in humans. Cattle have abundant γδ T cells and, as a physiologic model of TB infection, can be used to investigate γδ T cell biology in the context of *Mycobacterium* infection (2, 5, 22). Currently, little is known about bovine γδ T cell differentiation during TB infection. Characterization of γδ T cell effector and memory-like responses induced by mycobacterial infection will contribute to our understanding of their role in protection against TB for both humans and animals. To this end, cattle were infected via aerosol with virulent *M. bovis* and the effector and memory phenotypes of *M. bovis*-specific γδ T cells were examined by flow cytometry. We hypothesized that infection with virulent *M. bovis* would result in the development of circulating memory-like γδ T cell populations. *M. bovis*-specific γδ T cell effector and memory subsets were identified by their expression of CD27, CD45R and CD62L, and their expression of the activation and homing molecules CXCR3 and CCR5. Our results show, that CD27 and CD45R can differentiate functional (i.e. proliferating), *M. bovis*-specific γδ T cell subsets in bovine peripheral blood and suggest that bovine γδ T cells differentiate into effector and memory T cell subsets that are comparable to those populations which have been defined in humans with active TB infection.

MATERIALS AND METHODS

ANIMAL USE ETHICS

All animal studies were conducted according to federal and institutional guidelines and approved by the National Animal Disease Center Animal Care and Use committee and performed under appropriate project licenses. A total of 16 Holstein steers (~3 months of age) were used in the following experiments. Animals were housed in temperature- and humidity-controlled biosafety label-3 (BSL-3) containment rooms based upon treatment group, at the National Animal Disease Center in Ames, Iowa. Animals were acquired from a *M. bovis*-free herd in Sioux Center, IA.

Mycobacterium bovis

M. bovis strain 10-7428 isolated from a dairy farm in Colorado, was used for challenge inoculum. Low passage (≤ 3) cultures were prepared using standard techniques in Middlebrook 7H9 liquid media (Becton Dickinson, Franklin Lakes, NJ) supplemented with 10% oleic acid-albumin-dextrose 67 complex (OADC) plus 0.05% Tween 80 (Sigma, St. Louis, Missouri).

AEROSOL CHALLENGE PROCEDURES

Treatment groups consisted of non-infected steers (n =6) and animals receiving 10^4 colony-forming units (CFU) of *M. bovis* 10-7428 (n = 10). *M. bovis* challenge inoculum was delivered to restrained calves by aerosol as described by Palmer *et al.* (23). Briefly, inoculum was nebulized into a mask (Trudell Medical International, London, ON, Canada) covering the nostrils and mouth, allowing regular breathing and delivery of the bacterial inoculum to the upper and lower

respiratory tract 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 ~10 min. Strict biosafety protocols were followed to protect personnel from exposure to *M. bovis* throughout the study, including BSL-3 containment upon initiation of *M. bovis* challenge in animal rooms and standard laboratory practices for handling *M. bovis* cultures and samples from *M. bovis*-infected animals.

PBMC ISOLATION

Peripheral blood was drawn from the jugular vein into 2 × acid-citrate-dextrose solution. For peripheral blood mononuclear cell (PBMC) isolation, blood was diluted 1:1 in phosphate-buffered saline (PBS) and cells were isolated from buffy coat fractions, and PBMCs were isolated by density centrifugation on Histopaque (Sigma). PBMCs were collected and washed twice in PBS to remove platelets. Residual red blood cells (RBC) were removed by adding RBC lysis buffer. Finally, PBMCs were resuspended in complete Roswell Park Memorial Institute (RPMI)-1640 medium (GIBCO, Grand Island) supplemented with 2 mM L-glutamine, 25 mM HEPES buffer, 1% antibiotic-antimycotic (Sigma, St. Louis, MO), 1% non-essential amino acids (Sigma), 2% essential amino acids (Sigma), 1% sodium pyruvate (Sigma), 50 μM 2-mercaptoethanol (Sigma), and 10% (v/v) heat-inactivated fetal bovine sera (FBS).

PROLIFERATION ASSAY

To asses proliferation, PBMC were labeled using CellTrace Violet (Invitrogen, Carlsbad, CA) prior to cell culture following manufacturer's instructions. Briefly, freshy isolated cells were resuspended at 1×10^7 cells/mL in PBS containing 10 μM/ml of the CellTrace dye. After gently

mixing, PBMCs were incubated for 20 min at 37°C in a water-bath. Labeling was quenched by using an equal volume of FBS, and cells were washed three times with RPMI medium. Subsequently, cells ($5x10^5$ /well) were plated in round-bottom 96-well plate in duplicates, cultured for 6-days at 37°C, in the presence of 5% CO_2, in the presence of *M. bovis* PPD (PPD-b, 200 IU/mL, Prionics Ag, Schlieren, Switzerland), and recombinant ESAT-6/CFP-10 fusion protein (2 µg/ml, LIONEX Diagnostics and Therapeutics GmbH). Pokeweed mitogen (PWM, 1 µg/ml, Sigma) or complete RPMI medium were used as positive and negative control, respectively. After six days, cells were surface stained (see Flow cytometry section) and analyzed for proliferation and surface marker expression by flow cytometry.

FLOW CYTOMETRY

Following the appropriate culture duration, cells were stained with primary and secondary monoclonal antibodies (mAbs) listed on **Table I**. All incubation steps for staining were performed in FACS buffer (PBS with 10% FBS and 0.02% NA-azide) and incubated for 25 min at 4°C. Cells were washed and fixed with BD FACS lysis buffer (BD Biosciences, Mountain View, CA) for 10 min at room temperature, washed and resuspended in FACS buffer until analysis. Samples were acquired using a BD LSR Fortessa flow cytometer (BD Biosciences). Data were analyzed using Flowjo software (Tree Star Inc., San Carlos, CA). Electronic gates were set using Fluorescence minus one (FMO) controls.

Table 1. Reagents Flow cytometry

Reagent or antibody clone	Specificity, Source	Secondary antibodies, Source
ILA11	Bovine CD4, Washington State University	Allophycocyanin
GB21A	Bovine TCR1 delta chain, Washington State University	APC-Cy7, SouthernBiotech
ILA116	Bovine CD45RO, Washington State University	Alexa-fluor 488, Life Technologies
BAQ92A	Bovine CD62L, Washington State University	Allophycocyanin, Life technologies
GC6A	Bovine CD45R, Washington State University	PercpCy5.5, Life technologies
M-T271	Human CD27, Biolegend	Allophycocyanin or Pe-Cy7, Life technologies
G025H7	Human CXCR3 FITC, Biolegend	Not applicable
HM-CCR5	Human CCR5 PerCP/Cy5.5, Biolegend	Not applicable
Live Dead Aqua	Dead cells, Invitrogen	Not applicable
CellTrace Violet	Not applicable, Life technologies	Not applicable

STATISTICAL ANALYSIS

Results are expressed as mean ± standard errors of the mean. Statistical significance was determined by one-way Analysis of Variance (ANOVA) followed by Bonferroni test, or Student's t test using Prism software (GraphPad, La Jolla, CA).

RESULTS

Virulent *M. bovis* infection induces antigen-specific γδ T cell responses

We and others have previously demonstrated that γδ T cells from virulent *M. bovis*-infected cattle respond to both the complex mycobacterial antigen, PPD-b, and to the specific protein antigen, ESAT-6/CFP-10 (10, 11, 24). To corroborate our prior studies, animals were infected via aerosol inoculation with virulent *M. bovis* strain 10-7428 (n=10) or non-infected group (n=6). Following infection, PBMC were labeled with CellTrace dye and cultured for 6 days in the presence or absence of PPD-b or recombinant ESAT-6/CFP-10. On day 6, cells were analyzed by flow cytometry for the frequency of γδ and CD4 T cells that divided in response to the mycobacterial antigens. Representative proliferative responses of γδ and CD4 T cells following mycobacterial stimulation *in vitro* are depicted (**Fig 2A and B**). Robust proliferative responses to PPD-b and ESAT-6/CFP-10 were observed by γδ T cells at 4 weeks post infection and persisted until at least 12-weeks post infection **(Fig. 2C).** Consistent with prior reports, the mean CD4 T cell proliferative response to PPD-b and ESAT-6/CFP-10 peaked by 8 weeks post infection and persisted for at least 12 weeks post infection (**Fig. 2D**) (25). Recall responses from both γδ and CD4 T cell populations were *M. bovis*-specific, as neither CD4 nor γδ T cells from non-infected cattle responded to the specific or complex mycobacterial antigens.

Virulent *M. bovis* infection results in phenotypic changes on *M. bovis*-specific γδ and CD4 T cells

Similar to humans, bovine naïve and memory CD4 T cells can be distinguished based on the expression of CD45 isoforms. Naïve T cells express the high CD45RA isoform and upon antigenic recognition, T cells switch to the expression of the low CD45RO isoform (26, 27). In

the bovine CD45RA and CD45RB isoforms are not yet defined; thus, CD45R is used as a marker for naïve lymphocytes in cattle (26). To evaluate if CD45 isoform expression correlated with the bovine γδ T cell effector/memory population that responded to mycobacteria, we analyzed the CD45 phenotype of expanding *M. bovis*-specific γδ and CD4 T cells by flow cytometry following a 6-day *in vitro* restimulation with PPD-b (**Fig. 3A**). In accordance with previous data (25, 28), the mycobacterial-driven proliferative responses to PPD-b were within the $CD4^+$ $CD45RO^+$ T cell subset, and the antigen-responsive cells downregulated CD45R expression compared to non-responding cells (**Fig. 3B**). In contrast to CD4 T cells, γδ T cells from *M. bovis*-infected cattle showed no significant changes in cell surface expression of CD45RO, between antigen-specific (proliferating) and non-responding γδ T cell populations. However, antigen-responsive γδ T cells from infected cattle downregulated CD45R expression in response to PPD-b stimulation (**Fig. 3C**). Changes in expression of either CD45R or CD45RO were not detected in γδ T cells from noninfected animals after 6 days of culture. From these results it can be concluded that $CD45R^{neg}$ γδ and CD4 T cell subset participates in the *in vitro* recall responses against *M. bovis*.

Peripheral γδ T cells from *M. bovis* infected cattle possess an activated/memory phenotype based on CD27 expression

CD27, a costimulatory molecule, has been commonly used to identify stages of T cell differentiation (29). In humans, two subsets of γδ T cells develop in response to antigen stimulation and are identified based upon their expression of CD27. These subsets exhibit unique functions during mycobacterial infection that parallel the functions attributed to differentiated subsets of CD8 and CD4 T cells (15, 29). Moreover, flow-cytometric analysis of CD27 expression on circulating MTB-specific T cells can help to discriminate disease progression (16, 30).

CD27 expression has been reported in bovine PBMCs, however, its expression on bovine γδ and CD4 T cells has not been determined (31). Therefore, we performed *ex vivo* staining on PBMC from healthy cattle and observed that ~70% of CD4 T cells, and ~50% of γδ T cells express CD27 as measured by flow cytometry. Following mitogen stimulation, both CD4 and γδ T cells $CD27^{+}$ display significant higher clonogenic potential compared to $CD27^{neg}$ cells (**Fig. 4**). Thus, CD27 expression was abundantly expressed on bovine peripheral T cells, and can be used to identify CD4 and γδ T cell subsets based on their capacity to proliferate (16, 32).

CD27 expression on bovine γδ T cells identifies effector/memory subsets following virulent *M. bovis* infection

To determine whether mycobacteria-specific T cells could be defined by the expression of CD27, we analyzed the expression on *M. bovis*-specific γδ and CD4 T cells following *in vitro* restimulation with PPD-b and ESAT-6/CFP-10 (**Fig. 5A and B**). After 6 days in culture with PPD-b or ESAT-6/CFP-10, the majority of antigen-specific γδ T cells from *M. bovis* infected animals proliferated and upregulated expression of CD27 compared to non-responding γδ T cells in the same culture (**Fig. 5A**). Similarly, CD27 expression was significantly greater on the antigen-specific CD4 T cell population from *M. bovis*-infected animals compared to non-specific CD4 T cells in the same culture (**Fig. 5B**). Both γδ and CD4 T cells from infected animals exhibited significant increases in CD27 expression compared cells from noninfected control calves. Thus, from our results, increased expression of CD27 correlates with the capacity for bovine γδ T cells to proliferate in response to mycobacterial antigens, suggesting that bovine γδ T cell effector/memory phenotypes are consistent with those reported for human γδ T cells (16).

***M. bovis*-specific γδ T cells exhibit a T_{CM} phenotype based on CD27 and CD45R expression**

Expression of CD45RA and CD27 defines four subsets of memory γδ T cells in humans (15). Here, we sought to determine if the combination of both markers could be used to identify subsets of antigen-specific γδ T cells in cattle. PBMCs from *M. bovis*-infected animals were stimulated *in vitro* with PPD-b for 6 days. As shown in **Fig. 6A**, *M. bovis*-specific γδ T cells can be divided in four subsets: naïve ($CD27^+CD45R^+$), T_{CM} ($CD27^+CD45R^-$), T_{EM} ($CD27^-CD45R^-$), T_{EMRA} ($CD27^-CD45R^+$) following *in vitro* stimulation with PPD-b. $CD27^+CD45R^-$ T_{CM} γδ T cells exhibited the highest proliferative capacity following in vitro restimulation with mycobacterial antigens, followed by the $CD27^-CD45R^-$ T_{EM} γδ T cell subset (**Fig. 6B**). Previous reports have shown that antigen-specific bovine CD4 T cells can also be divided by their expression of CD27 and CD45R (25). To confirm these previous results and to validate our results of our γδ T cell analysis, we examined parallel cultures to determine the distribution of effector/memory CD4 subsets responding to *M. bovis* infection. As seen in Figure 5C, after 6 days, the CD4 T_{CM} subset exhibited the highest proliferative capacity following in vitro restimulation with mycobacterial antigens compared to non-responding CD4 T cells. Our results suggest that the T_{CM} subset comprises the majority of the *in vitro* proliferative response to mycobacterium antigens for both γδ and CD4 T cells.

Increased expression of CD27 on bovine peripheral γδ T cells correlates with memory-type responses

T_{CM} cells are highly proliferative, abundant in lymph nodes, and retain their expression of the homing receptors CCR7 and CD62L (33). The results described in **Fig. 6** suggest that peripheral *M. bovis*-specific γδ T cells share functional similarities (i.e. high proliferative capacity)

with central memory T cells (16). To address this hypothesis, we evaluated the expression of CD62L on peripheral *M. bovis*-specific γδ T cells. As seen in Fig 6, following a 6-day stimulation with PPD-b, $CD27^+$ *M. bovis*-responsive γδ T cells co-expressed CD62L compared to γδ T cells that did not proliferate to *M. bovis* antigens. In contrast, *M. bovis*-specific $CD27^+$ CD4 T cells were low for CD62L expression compared to non-*M. bovis*-specific CD4 cells. These data are consistent with previous descriptions identifying the majority of *M. bovis*-specific CD4 T cells in the peripheral blood as T_{EM} cells (**Fig. 7**) (25). Therefore, similar to human γδ T cells, *M. bovis*-responsive bovine γδ T cells display phenotypic and functional properties of T_{CM} memory cells (25, 33).

Bovine *M. bovis*-specific γδ T cells upregulate expression of the tissue-associated chemokine receptors CXCR3 and CCR5

Chemokine receptor expression is modulated during differentiation of both CD4 and γδ T cells (15, 33, 34). We hypothesized that antigen responsive γδ T cells would modify the expression of certain homing receptors that would allow cells to migrate to tissues and sites of inflammation. PBMCs from infected cattle were stimulated with PPD-b, as described above. As seen in **Fig. 8A** and **8B**, *M. bovis*-responsive bovine γδ T cells express higher surface levels of both CCR5 (RANTES/macrophage inflammatory protein-1α/-1β receptor) and CXCR3 compared to non-responding γδ T cells. As seen in **Fig. 8C**, antigen-specific CD4 T cells also express higher levels of CCR5 and CXCR3 compared to nonresponsive CD4 T cells. The increased expression of CCR5 and CXCR3 on *M. bovis*-responsive CD4 and γδ T cells is expected to enable these populations to migrate into the inflamed tissue.

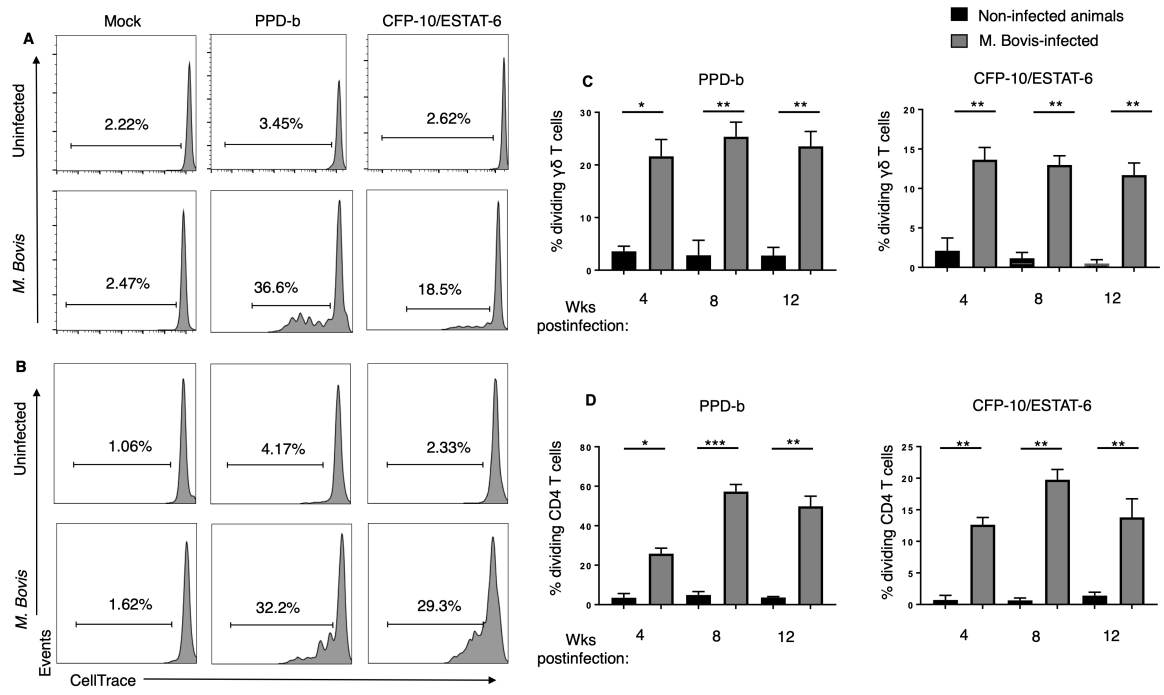

Figure 2 Proliferative responses in response to in vitro stimulation with mycobacterial antigens.

PBMCs from uninfected (n= 6) or virulent *M. bovis*–infected animals (n= 10) were labeled with CellTrace, and 5 × 10^6 cells/ml were cultured for 6 days in the presence or absence of 200 UI/ml PPD-b or 2 μg/ml ESAT-6/CFP-10. Cells were labeled with anti-bovine γδ TCR or CD4 and analyzed by flow cytometry for CellTrace dilution. Representative histograms of proliferative responses from a *M. bovis*-infected and control animal, gated on total live cells, lymphocytes (SSC-A vs FSC-A), and cells expressing the γδ TCR (**A**) or CD4 (**B**) Percentage of γδ T cells (**C**) and CD4 T cells (**D**) from *M. bovis* infected and uninfected animals that proliferated in response to mycobacterial antigens, as measured by CellTrace dilution, at 4-, 8- or 12-weeks post aerosol challenge. Analysis was performed with Flowjo software. Background proliferation was subtracted, and results represent change over mock. Data are mean ± SEM.

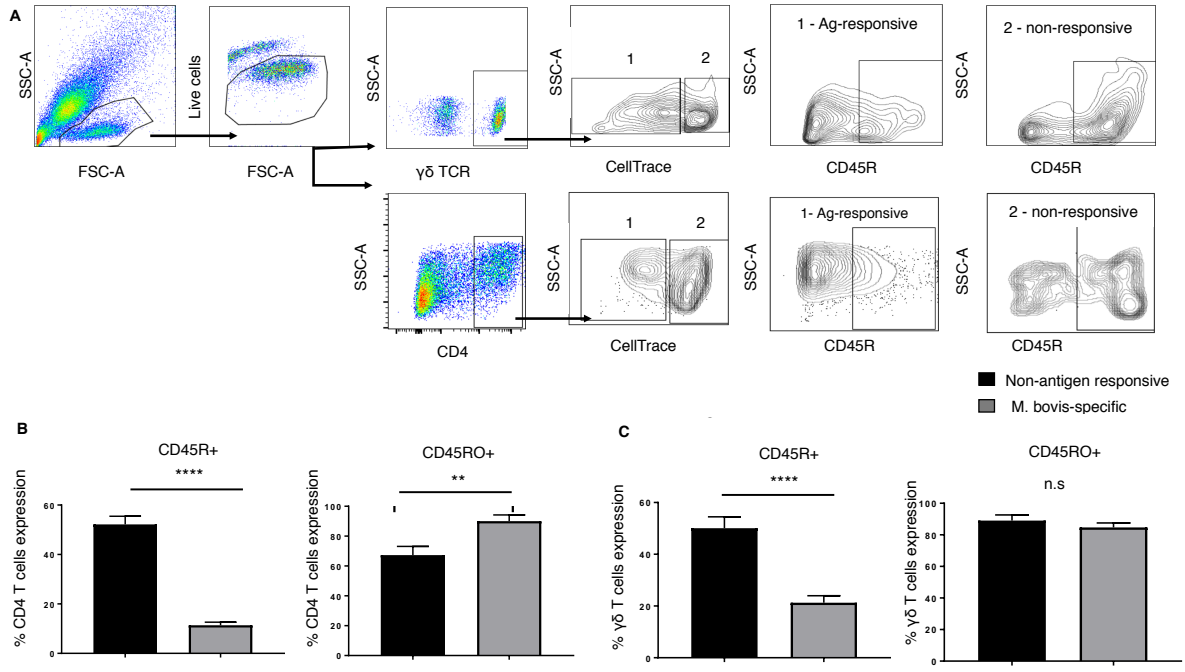

Figure 3. Evaluation of CD45RO and CD45R expression on *M. bovis*-specific γδ and CD4 T cells.

Approximately 8 weeks after aerosol challenge with *M. bovis*, PBMCs from *M. bovis*–infected animals (n= 10) were labeled with CellTrace, and 5×10^6 cells/ml were cultured for 6 days in the presence or absence of PPD-b. Cells were labeled with anti-bovine CD4, or γδ TCR; and CD45RO or CD45R expression by flow cytometry within proliferative subsets as determined by CellTrace dilution. **(A)** Gating hierarchy (gating sequence as depicted by the arrows): lymphocytes (gate 1), live cells (gate 2), γδ or CD4 T cells (gate 3), proliferating (1) and non-proliferating cells (2) and CD45RO or CD45R expression (gate 5). **(B and C)** The expression of CD45RO or CD45R was determined for individual T cell subsets CD4 T cells (**B**) or γδ T cells (**C**) or based on CellTrace staining intensity (i.e., bright or dim). Data are presented as means (± SEM). p values for differences from responding and non-responding fractions for the respective T cell subset (i.e., comparisons between black and grey bars for each graph) were as follows: *, $p < 0.05$; **, $p < 0.01$, p ***<0.001, p****<0.0001 as determined by Student's t test.

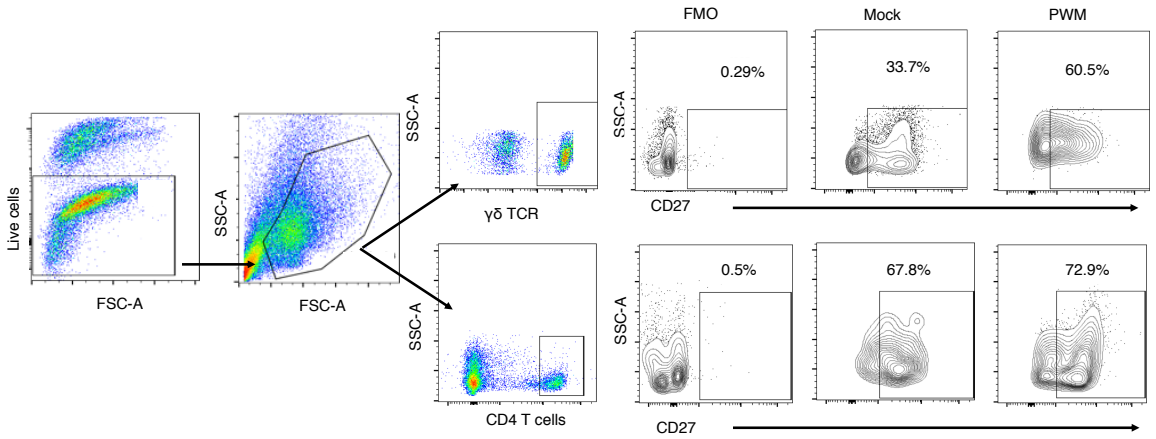

Figure 4. CD27 expression on bovine γδ and CD4 T cells ex vivo and in response to mitogen. Using flow cytometry, bovine PBMCs from healthy cows were analyzed for CD4 or γδ TCR expression. Gating hierarchy (gating sequence as depicted by the arrows): live cells (gate 1), lymphocytes (gate 2), γδ or CD4 T cells (gate 3). Each lymphocyte population was further analyzed for CD27 expression following mock or PWM stimulation for 6 days (dot-plots). Data are representative of 6 independent experiments.

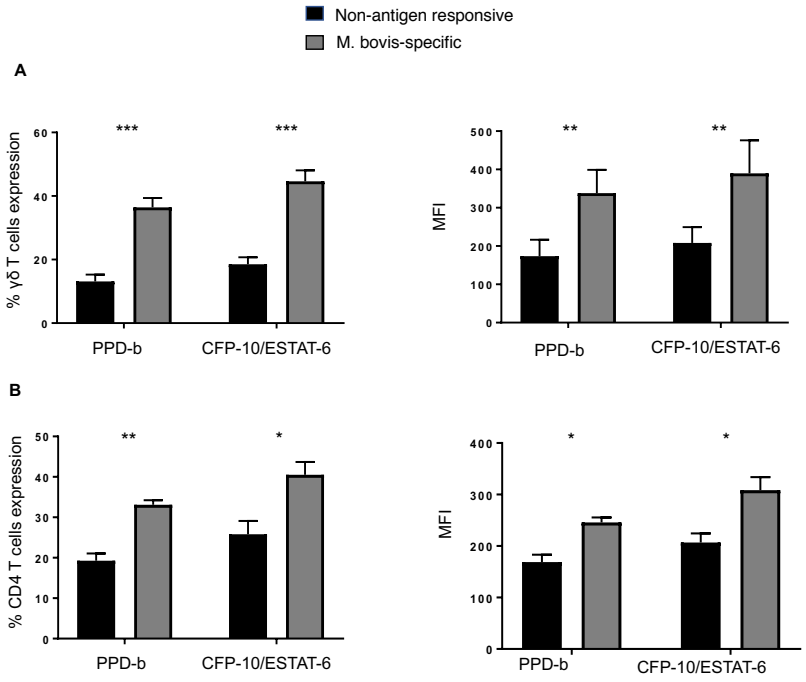

Figure 5. CD27+ expression correlates with robust antigen-specific responses to *M. bovis*.

Approximately 8 weeks after virulent *M. bovis* infection, PBMCs (5×10^6 cells/ml) were labeled with CellTrace violet and restimulated *in vitro* for 6 days in the presence or absence of PPD-b, ESAT-6/CFP-10 or left unstimulated. Cells were then labeled with anti-bovine CD4 or γδ TCR, and CD27. CellTrace dilution (proliferative responses) and CD27 cell surface expression was determined for (**A**) γδ T cells and (**B**) CD4 T cells. Data are presented as means ± SEM; n = 10. p values indicate differences between CellTrace dim and positive fractions for the respective T cell subset (i.e., comparisons between black and grey bars for each graph) as follows: *, $p < 0.05$; **, $p < 0.01$, p ***<0.001, p****<0.0001 as determined by Student's t test.

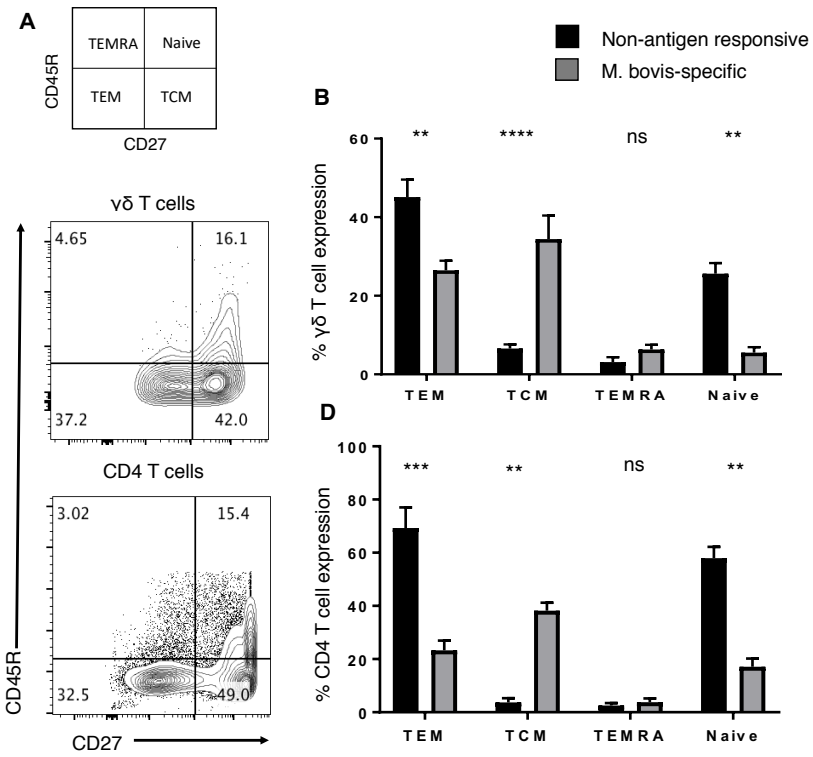

Figure 6. Phenotype of TCM, TEM and effector γδ and CD4 T cells proliferating in response to *M. bovis*.

PBMCs were isolated from calves ~ 8 weeks after challenge with virulent *M. bovis.* Cells were stained with CellTrace dye and incubated with *M. bovis* PPD-b for 6 days. A flow cytometric-based proliferation assay was used to study CD45R and CD27 expression on *M. bovis*-specific CD4 and γδ T cells. **(A)** Stimulated PBMCs were analyzed for proliferative responses. Live CD4 and γδ T cells were gated based on response to *M. bovis* (i.e., proliferating cells) and analyzed for CD27 versus CD45R expression within CellTrace bright (i.e., non-proliferative fraction; black bars) or CellTrace dim (i.e., proliferative fraction; grey bars) fractions. **(B and C)** Relative contribution of γδ and CD4 T cells to proliferative response to PPD-b **within** CD45R/CD27 defined cell populations (CD45R+CD27+; CD45R-CD27+; CD45R-CD27-; CD45R+CD27-). Data are presented as mean (± SEM) (n = 10). *, $p < 0.05$; **, $p < 0.01$, p ***<0.001, p****<0.0001 as determined by Student's t test.

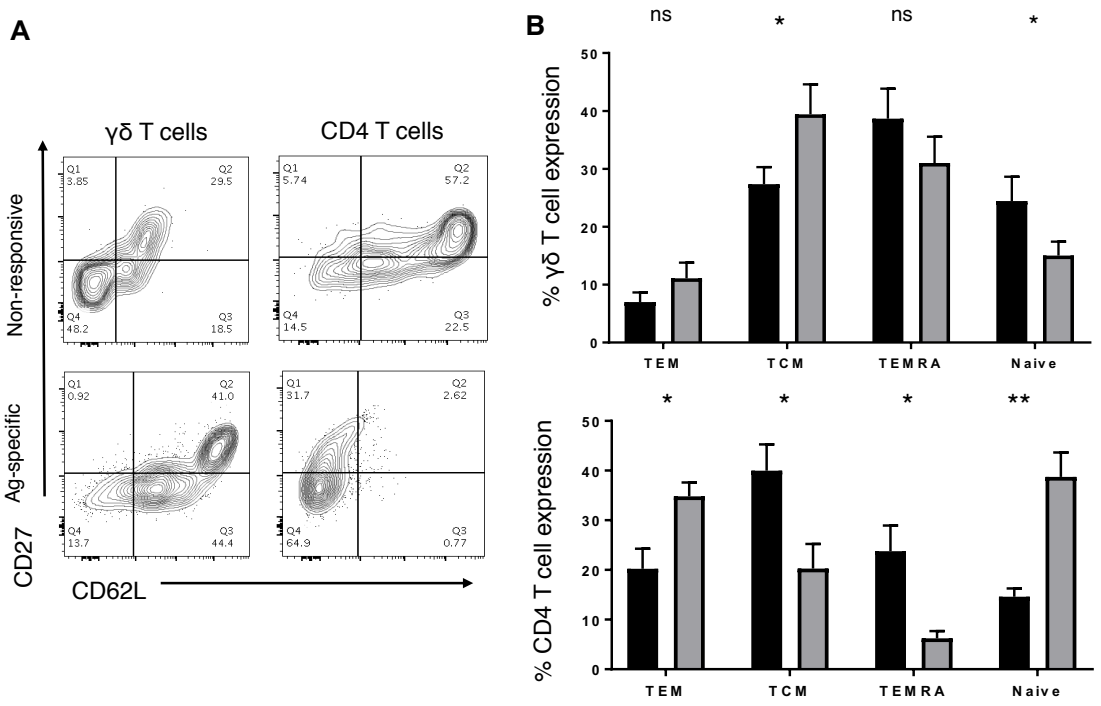

Figure 7. Memory marker expression of CD27 and CD62L T cell subsets of *M. bovis*-infected cattle.

PBMCs were isolated from calves ~ 8 weeks after challenge with virulent *M. bovis*. Cells were stained with CellTrace dye and incubated with *M. bovis* PPD-b for 6 days. Flow cytometry was used to study both CD27 and CD62L expression on *M. bovis*-specific T cells. Live lymphocytes, CD4 or γδ T cells were gated based on responsiveness to *M. bovis* (i.e., proliferating cells) and analyzed for CD27 versus CD62L expression. (**A**) Representative dot plots from an *M. bovis* infected animal, gated on dividing and nondividing (non-specific) CD4 or γδ T cells. Data are presented as CD27 versus CD62L expression. Cumulative data depicting the frequencies of CD27 and CD62L expressing γδ T cell subsets **(B)** and CD4 T cell subsets **(C)** by dividing (dark bars) and nondividing (grey) cells are shown for all infected animals. Data are presented as mean (± SEM) (n = 10). *, $p < 0.05$; **, $p < 0.01$, p ***<0.001, p****<0.0001 as determined by ANOVA.

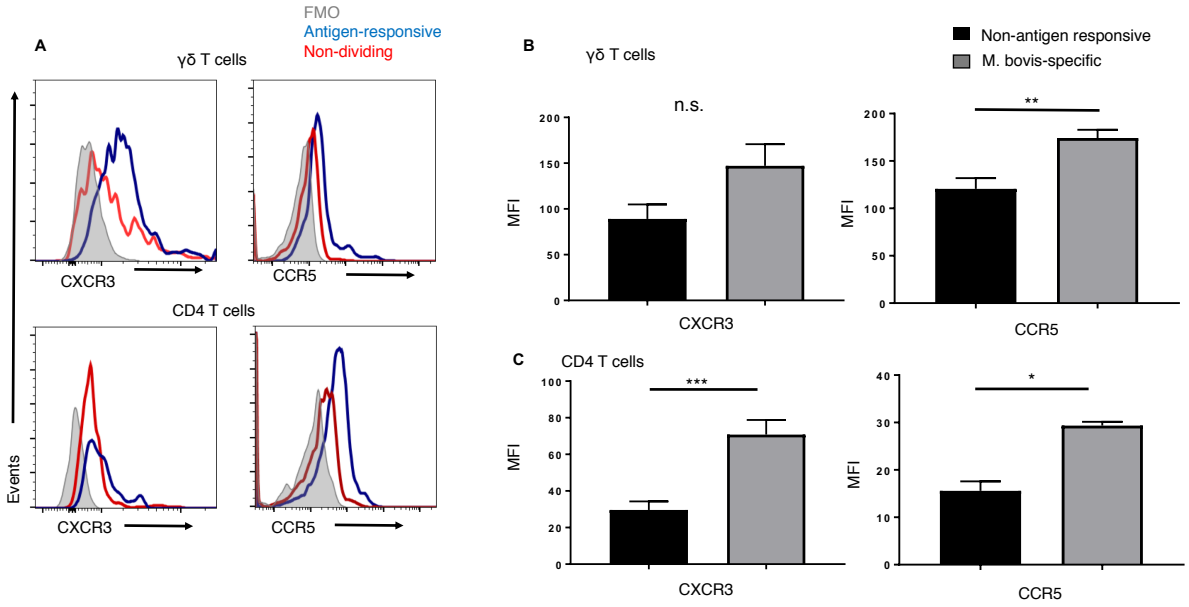

Figure 8. Surface expression of chemokine receptors on *M. bovis*-specific peripheral γδ and CD4 T cells.

PBMCs were isolated from calves ~ 8 weeks after challenge with virulent *M. bovis*. Cells were stained with CellTrace dye and incubated with PPD-b for 6 days. Flow cytometry was used to study CXCR3 and CCR5 expression on *M. bovis*-specific CD4 and γδ T cells. Cells were surface stained and then analyzed by flow cytometry for CXCR3 and CCR5 expression. **(A)** Representative histograms of CXCR3 and CCR5 expression on antigen-responsive (blue solid line) and non-responsive CD4 and γδ T cells (red solid line). Cumulative results from live γδ **(B)** and CD4 T cells **(C)**, gated based on response to *M. bovis* (i.e., proliferating cells) and analyzed for CXCR3 or CRR5 expression. The FMO control is shown in grey shaded histograms. Data are presented as mean ± SEM MFI expression of cell surface markers. *, $p < 0.05$; **, $p < 0.01$, p ***<0.001 indicates a significant difference ($p< 0.05$) from antigen-responsive cells compared to non-responsive cells as determined by Students *t*-test.

DISCUSSION

Following *M. bovis* infection, a marked in vitro expansion suggests that bovine γδ T cells can mount memory-like responses upon restimulation with mycobacterial antigen (**Fig. 1**) and [reviewed (35)]. However, to date, a combination of surface markers that effectively identify effector/memory-like subsets of bovine antigen-specific γδ T cells have not been reported. Here, we show that compared to CD4 T cells, proliferating *M. bovis*-specific γδ T cells do not significantly alter CD45RO expression after restimulation with mycobacterial antigens *in vitro*. Our results agree with reports showing that γδ T cells from human TB patients do not modulate CD45RO expression compared to γδ T cells from non-infected subjects (16, 17). Thus, γδ T cells may acquire a 'pre-activated' state early in their development and thus, CD45RO is not useful to identify antigen-experienced γδ T cells. In contrast to CD45RO expression, we found that proliferating γδ T cells from *M. bovis* infected cattle, downregulated the expression of CD45R following antigen restimulation *in vitro* compared to non-responsive γδ T cells (**Fig. 2**) (28, 36). These observations indicate that mycobacteria-specific γδ T cells that can be identified using CD45R.

In humans, two subsets of γδ T cells develop in response to antigen stimulation and are identified based upon their expression of CD27 (22). These subsets exhibit unique functions during mycobacterial infection that parallel the functions attributed to differentiated subsets of CD8 and CD4 T cells (15, 29). Moreover, flow-cytometric analysis of CD27 expression on circulating MTB-specific T cells can help to discriminate disease progression (30). Thus, assessing the changes in γδ T cell effector/memory phenotype based on CD27 might be helpful to monitor changes related to TB disease progress and vaccine-induce protection as previously reported (16, 18). Although the expression of CD27 in bovine cells has been reported before, its

expression on bovine γδ and CD4 T cells has not been determined (31). We observed that in healthy adult cattle ~70% of CD4 T cells, and ~50% of γδ T cells express CD27. Importantly, we observed that a subpopulation of γδ $^{+}$CD27^{+} effector/memory-like T cells from *M. bovis* infected cattle develop in response to Ag stimulation and exhibit high proliferative capacity after restimulation with either PPD-b or the *M. bovis* protein antigen, ESAT-6/CFP-10. Our observations are consistent with findings in humans, where patients with acute pulmonary TB, and BCG vaccinated individuals, demonstrate increases in circulating CD27^{+} Vγ9Vδ2^{+} T cells, and these cells exhibit enhanced proliferative activity upon stimulation with mycobacterial antigens (16, 37). Thus, our results suggest that CD27 expression may be potentially used to monitor γδ T cell responses to *M. bovis* infection in cattle.

Coexpression of cell surface markers CD45RA and CD27 has been used to functional segregate subsets of human and non-human primate γδ T cells (15, 16). In non-human primates, TEM (CD45RA- CD27+) Vγ9Vδ2 T cells expansion after a secondary *M. tb* challenge correlates with protection against fatal TB (17-19). Interestingly, there is a significant reduction in effector γδ cells (CD27^{-} T_{EM} and T_{EMRA} cells) in patients with active infection, but the population rebounds after successful clinical treatment (38). Our data show that *M. bovis*-specific γδ T cells can be divided into four distinct subsets: naïve (CD27^{+}CD45R^{+}), T_{CM} (CD27^{+}CD45R^{-}), T_{EM} (CD27^{-} CD45R^{-}), T_{EMRA} (CD27^{-}CD45R^{+}). Consistent with reports from humans, our data show that virulent *M. bovis* infection is associated with an expansion in primarily the γδ T_{CM} subset in the blood (**Fig. 5**) (39). Although effector functions were not assessed in this study, it has been shown that γδ T effectors develop functional defects and progressively lose the ability to produce IFNγ compared with γδ T cells from healthy tuberculin positive adults (40). Whether the loss of γδ T cell effectors impacts the ability to acquire memory T cell properties is not known. Together, our

observations, coupled with reports from human TB patients, demonstrate a negative correlation between γδ T effector ($CD27^-$ $CD45R^-$) phenotype and disease. Further studies should address the functional implications of these phenotypic changes following *M. bovis* experimental challenge and vaccine efficacy studies in the bovine.

Lastly, we show that *M. bovis*-specific bovine γδ T cells exhibited higher CCR5 and CXCR3 compared to non-responding γδ T cells. Similarly, a higher expression of CXCR3 and CCR5 is preferentially found on circulating γδ T cells from TB patients (38, 41). The expression of CCR5 and CXCR3 on *M. bovis*-specific γδ and CD4 T cells suggests that these cell populations are memory/effector T cells (15, 38, 41). Similar to our results, Blumerman *et al.* showed that bovine γδ T cells participating in recall responses against *Leptospira* antigens exhibited increased transcription of CCR5 and CXCR3 (42). γδ T cells are amongst the first cells to localize to the granuloma following *M. bovis* infection (43). The increased expression of CCR5 and CXCR3 on *M. bovis*-responsive γδ T cells presumably enables this population to migrate into the inflamed peripheral tissue. We did not investigate the functionality of CCR5 and CXCR3 on this population, however studies in human γδ T cells have confirmed their capacity to migrate towards the inflammatory chemokines RANTES and IP-10 (38). Importantly, the chemokine receptor profile of peripheral γδ T cells may differ from that of γδ T cells at the site of infection; thus, further studies will address the functionality of CXCR3 and CCR5 in bovine γδ T cells, and the expression of tissue homing receptors such as CXCR3 and CCR5 on cells within granuloma lesions of *M. bovis*-infected cattle.

In conclusion, our results show that, like humans, γδ T cells from *M. bovis*-infected cattle can be divided into phenotypic and functional subsets based upon their surface expression of CD27, CD45R, and the homing molecules, CXCR3 and CCR5. Further elucidation of the pathway

of γδ T cell differentiation and the functional implications will contribute significantly to our ability to engage the γδ T cell response in effective immune responses to mycobacterial diseases of both humans and animals.

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REFERENCES

1. Organization WH. Global tuberculosis report 2018. 2018.

2. Waters WR, Palmer MV, Buddle BM, Vordermeier HM. Bovine tuberculosis vaccine research: historical perspectives and recent advances. Vaccine. 2012;30(16):2611-22.

3. Muller B, Durr S, Alonso S, Hattendorf J, Laisse CJ, Parsons SD, et al. Zoonotic Mycobacterium bovis-induced tuberculosis in humans. Emerg Infect Dis. 2013;19(6):899-908.

4. Schiller I, Oesch B, Vordermeier HM, Palmer MV, Harris BN, Orloski KA, et al. Bovine tuberculosis: a review of current and emerging diagnostic techniques in view of their relevance for disease control and eradication. Transbound Emerg Dis. 2010;57(4):205-20.

5. Endsley JJ, Waters WR, Palmer MV, Nonnecke BJ, Thacker TC, Jacobs WR, Jr., et al. The calf model of immunity for development of a vaccine against tuberculosis. Vet Immunol Immunopathol. 2009;128(1-3):199-204.

6. Waters WR, Palmer MV, Thacker TC, Davis WC, Sreevatsan S, Coussens P, et al. Tuberculosis immunity: opportunities from studies with cattle. Clin Dev Immunol. 2011;2011:768542.

7. Diedrich CR, Flynn JL. HIV-1/mycobacterium tuberculosis coinfection immunology: how does HIV-1 exacerbate tuberculosis? Infect Immun. 2011;79(4):1407-17.

8. Cooper AM, Solache A, Khader SA. Interleukin-12 and tuberculosis: an old story revisited. Curr Opin Immunol. 2007;19(4):441-7.

9. Rhodes SG, Hewinson RG, Vordermeier HM. Antigen recognition and immunomodulation by gamma delta T cells in bovine tuberculosis. J Immunol. 2001;166(9):5604-10.

10. Smyth AJ, Welsh MD, Girvin RM, Pollock JM. In vitro responsiveness of gammadelta T cells from Mycobacterium bovis-infected cattle to mycobacterial antigens: predominant involvement of WC1(+) cells. Infect Immun. 2001;69(1):89-96.

11. McGill JL, Sacco RE, Baldwin CL, Telfer JC, Palmer MV, Waters WR. Specific recognition of mycobacterial protein and peptide antigens by gammadelta T cell subsets following infection with virulent Mycobacterium bovis. J Immunol. 2014;192(6):2756-69.

12. Price S, Davies M, Villarreal-Ramos B, Hope J. Differential distribution of WC1(+) gammadelta TCR(+) T lymphocyte subsets within lymphoid tissues of the head and respiratory tract and effects of intranasal M. bovis BCG vaccination. Vet Immunol Immunopathol. 2010;136(1-2):133-7.

13. Vesosky B, Turner OC, Turner J, Orme IM. Gamma interferon production by bovine gamma delta T cells following stimulation with mycobacterial mycolylarabinogalactan peptidoglycan. Infect Immun. 2004;72(8):4612-8.

14. Kabelitz D, Bender A, Prospero T, Wesselborg S, Janssen O, Pechhold K. The primary response of human gamma/delta + T cells to Mycobacterium tuberculosis is restricted to V gamma 9-bearing cells. J Exp Med. 1991;173(6):1331-8.

15. Dieli F, Poccia F, Lipp M, Sireci G, Caccamo N, Di Sano C, et al. Differentiation of effector/memory Vdelta2 T cells and migratory routes in lymph nodes or inflammatory sites. J Exp Med. 2003;198(3):391-7.

16. Gioia C, Agrati C, Casetti R, Cairo C, Borsellino G, Battistini L, et al. Lack of CD27-CD45RA-V gamma 9V delta 2+ T cell effectors in immunocompromised hosts and during active pulmonary tuberculosis. J Immunol. 2002;168(3):1484-9.

17. Shen Y, Zhou D, Qiu L, Lai X, Simon M, Shen L, et al. Adaptive immune response of Vgamma2Vdelta2+ T cells during mycobacterial infections. Science. 2002;295(5563):2255-8.

18. Shen L, Frencher J, Huang D, Wang W, Yang E, Chen CY, et al. Immunization of Vgamma2Vdelta2 T cells programs sustained effector memory responses that control tuberculosis in nonhuman primates. Proc Natl Acad Sci U S A. 2019;116(13):6371-8.

19. Chen CY, Yao S, Huang D, Wei H, Sicard H, Zeng G, et al. Phosphoantigen/IL2 expansion and differentiation of Vgamma2Vdelta2 T cells increase resistance to tuberculosis in nonhuman primates. PLoS Pathog. 2013;9(8):e1003501.

20. Meraviglia S, El Daker S, Dieli F, Martini F, Martino A. gammadelta T cells cross-link innate and adaptive immunity in Mycobacterium tuberculosis infection. Clin Dev Immunol. 2011;2011:587315.

21. Dieli F, Ivanyi J, Marsh P, Williams A, Naylor I, Sireci G, et al. Characterization of lung gamma delta T cells following intranasal infection with Mycobacterium bovis bacillus Calmette-Guerin. J Immunol. 2003;170(1):463-9.

22. Buddle BM, Pollock JM, Skinner MA, Wedlock DN. Development of vaccines to control bovine tuberculosis in cattle and relationship to vaccine development for other intracellular pathogens. Int J Parasitol. 2003;33(5-6):555-66.

23. Palmer MV, Waters WR, Whipple DL. Aerosol delivery of virulent Mycobacterium bovis to cattle. Tuberculosis (Edinb). 2002;82(6):275-82.

24. Waters WR, Rahner TE, Palmer MV, Cheng D, Nonnecke BJ, Whipple DL. Expression of L-Selectin (CD62L), CD44, and CD25 on activated bovine T cells. Infect Immun. 2003;71(1):317-26.

25. Maggioli MF, Palmer MV, Thacker TC, Vordermeier HM, Waters WR. Characterization of effector and memory T cell subsets in the immune response to bovine tuberculosis in cattle. PLoS One. 2015;10(4):e0122571.

26. Michie CA, McLean A, Alcock C, Beverley PC. Lifespan of human lymphocyte subsets defined by CD45 isoforms. Nature. 1992;360(6401):264-5.

27. Bembridge GP, MacHugh ND, McKeever D, Awino E, Sopp P, Collins RA, et al. CD45RO expression on bovine T cells: relation to biological function. Immunology. 1995;86(4):537-44.

28. Maue AC, Waters WR, Davis WC, Palmer MV, Minion FC, Estes DM. Analysis of immune responses directed toward a recombinant early secretory antigenic target six-kilodalton protein-culture filtrate protein 10 fusion protein in Mycobacterium bovis-infected cattle. Infect Immun. 2005;73(10):6659-67.

29. Hintzen RQ, de Jong R, Lens SM, Brouwer M, Baars P, van Lier RA. Regulation of CD27 expression on subsets of mature T-lymphocytes. J Immunol. 1993;151(5):2426-35.

30. Streitz M, Tesfa L, Yildirim V, Yahyazadeh A, Ulrichs T, Lenkei R, et al. Loss of receptor on tuberculin-reactive T-cells marks active pulmonary tuberculosis. PLoS One. 2007;2(8):e735.

31. Sopp P, Kwong LS, Howard CJ. Cross-reactivity with bovine cells of monoclonal antibodies submitted to the 6th International Workshop on Human Leukocyte Differentiation Antigens. Vet Immunol Immunopathol. 2001;78(2):197-206.

32. DeBarros A, Chaves-Ferreira M, d'Orey F, Ribot JC, Silva-Santos B. CD70-CD27 interactions provide survival and proliferative signals that regulate T cell receptor-driven activation of human gammadelta peripheral blood lymphocytes. Eur J Immunol. 2011;41(1):195-201.

33. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature. 1999;401(6754):708-12.

34. Caccamo N, Meraviglia S, Ferlazzo V, Angelini D, Borsellino G, Poccia F, et al. Differential requirements for antigen or homeostatic cytokines for proliferation and differentiation of human Vgamma9Vdelta2 naive, memory and effector T cell subsets. Eur J Immunol. 2005;35(6):1764-72.

35. McGill JL, Sacco RE, Baldwin CL, Telfer JC, Palmer MV, Waters WR. The role of gamma delta T cells in immunity to Mycobacterium bovis infection in cattle. Vet Immunol Immunopathol. 2014;159(3-4):133-43.

36. Howard CJ, Sopp P, Parsons KR, McKeever DJ, Taracha EL, Jones BV, et al. Distinction of naive and memory BoCD4 lymphocytes in calves with a monoclonal antibody, CC76, to a restricted determinant of the bovine leukocyte-common antigen, CD45. Eur J Immunol. 1991;21(9):2219-26.

37. Hoft DF, Brown RM, Roodman ST. Bacille Calmette-Guerin vaccination enhances human gamma delta T cell responsiveness to mycobacteria suggestive of a memory-like phenotype. J Immunol. 1998;161(2):1045-54.

38. Glatzel A, Wesch D, Schiemann F, Brandt E, Janssen O, Kabelitz D. Patterns of chemokine receptor expression on peripheral blood gamma delta T lymphocytes: strong expression of CCR5 is a selective feature of V delta 2/V gamma 9 gamma delta T cells. J Immunol. 2002;168(10):4920-9.

39. Martino A, Casetti R, Sacchi A, Poccia F. Central memory Vgamma9Vdelta2 T lymphocytes primed and expanded by bacillus Calmette-Guerin-infected dendritic cells kill mycobacterial-infected monocytes. J Immunol. 2007;179(5):3057-64.

40. Swaminathan S, Gong J, Zhang M, Samten B, Hanna LE, Narayanan PR, et al. Cytokine production in children with tuberculous infection and disease. Clin Infect Dis. 1999;28(6):1290-3.

41. Eberl M, Engel R, Beck E, Jomaa H. Differentiation of human gamma-delta T cells towards distinct memory phenotypes. Cell Immunol. 2002;218(1-2):1-6.

42. Blumerman SL, Wang F, Herzig CT, Baldwin CL. Molecular cloning of bovine chemokine receptors and expression by WC1+ gammadelta T cells. Dev Comp Immunol. 2007;31(1):87-102.

43. Pollock JM, Pollock DA, Campbell DG, Girvin RM, Crockard AD, Neill SD, et al. Dynamic changes in circulating and antigen-responsive T-cell subpopulations post-Mycobacterium bovis infection in cattle. Immunology. 1996;87(2):236-41.

Chapter 3 - Characterization of mucosal and circulating bovine γδ T cell responses to respiratory BCG vaccination

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ABSTRACT

The *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) vaccine is administered parenterally to infants and young children to prevent tuberculosis (TB) infection. However, the protection induced by BCG is highly variable and the vaccine does not prevent pulmonary TB, the most common form of the illness. Until improved TB vaccines are available, it is crucial to use BCG in a manner which ensures optimal vaccine performance. Immunization directly to the respiratory mucosa has been shown to promote greater protection from TB in animal models. γδ T cells play a major role in host defense at mucosal sites and are known to respond robustly to mycobacterial infection. Their positioning in the respiratory mucosa ensures their engagement in the response to aerosolized TB vaccination. However, our understanding of the effect of respiratory BCG vaccination on γδ T cell responses in the lung is unknown. In this study, we used a calf model to investigate the immunogenicity, and phenotypic profile of peripheral and mucosal γδ T cells responding to aerosol BCG vaccination. We observed robust mucosal and systemic *M. bovis*-specific IFN-γ and IL-17 production by both γδ and CD4 T cells. Importantly, BCG vaccination induced effector and memory cell differentiation of γδ T cells in both the airways and peripheral blood, with accumulation of a large proportion of effector memory γδ T cells in both compartments. Our results demonstrate the potential of the neonatal calf model to evaluate the

safety and immunogenicity of TB vaccine candidates that are to be administered via the respiratory tract and suggest that aerosol immunization is a promising strategy for engaging γδ T cells in vaccine-induced immunity against TB.

INTRODUCTION

Mycobacterium bovis is a member of the *M. tb* complex and is the causative agent of bovine TB (bTB) and zoonotic TB infection (1). The attenuated *M. bovis* vaccine strain, Bacille Calmette-Guerin (BCG), is the only vaccine that is currently available to prevent TB infection in humans. It is approved for intradermal use and is commonly administered at birth to infants in TB endemic areas. The BCG vaccine has been tested experimentally in cattle, and like humans, the protection induced by parenteral BCG vaccination is transient and highly variable [reviewed (2)]. Although parenteral BCG vaccination is not efficacious against pulmonary TB, no other vaccine has shown improved efficacy over BCG, and it remains the 'gold-standard' by which all other TB vaccines are compared in both humans and cattle. Furthermore, BCG has well-recognized health benefits in human infants and is likely to be continued to be administered to populations in developing countries [reviewed in (3)]. Therefore, until improved TB vaccines are available, it is crucial to use BCG in a manner which ensures optimal vaccine performance.

Immunization directly to the respiratory mucosa with BCG, attenuated *M. tb* and vectored vaccines has been shown to promote greater protection from TB in rodents and non-human primates (4-10). A single intranasal vaccination with a recombinant human type 5 adenovirus expressing antigen 85A (AdHu5Ag85A) offered superior protection to subcutaneous BCG vaccination alone (11). In BCG-vaccinated cattle, boosting via the respiratory mucosa with AdAg85A induced local mucosal and systemic responses that were similar in magnitude to intradermal boosting (12). The vaccine-induced protection seen after immunization via the respiratory tract is believed to be associated with the preferential recruitment of antigen-specific CD4 T cells to the lung airways (5, 13-18), which allows for an immediate response upon pathogen exposure, preventing bacilli from establishing infection (19-21). While it is clear that IFNγ-

secreting CD4 T cells are an essential component for protection against TB, IFNγ secretion is a poor correlate of protection (22, 23). The activity of other lymphocyte populations, notably γδ T cells, may represent another major essential component for protection or resistance to TB [reviewed (24)].

γδ T cells play a major role in defense against pathogens, especially at mucosal sites such as the lower respiratory tract (25-28). Although γδ T cells fall into the innate-like category, adaptive features have also been reported in multiple studies [reviewed (29, 30)]. Humans vaccinated with BCG have a population of γδ T cells that expands robustly in response to *in vitro* restimulation with mycobacteria antigens (31). In non-human primates, administration of phosphoantigens/IL-2 induced a marked expansion and pulmonary accumulation of phosphoantigen-specific Vγ2Vδ2 T cells, significantly reducing *M. tb* burdens and associated lung pathology (9, 32).

Like CD4 T cells, γδ T cells have the capacity to differentiate into subsets that differ in their migratory and functional properties. In humans, γδ T cell subsets are divided according to the surface expression of CD45RA and CD27. Naïve $CD45RA^+$ $CD27^+$ cells represent ~10-20% of the γδ T cells circulating population in healthy adults. Central memory (T_{CM}) cells $CD45RA^-$ $CD27^+$ are more plentiful in the blood and exhibit robust proliferative capacity, but limited effector functions (33). Effector memory (T_{EM}) and $CD45RA^+$ $CD27^-$ (T_{EMRA}) γδ T cells are generally recognized to be fully differentiated subsets and express receptors for homing to inflamed tissues, display immediate effector functions and are highly prevalent in sites of inflammation (34). Consistent with their differential homing capacity, certain chemokine receptors are also useful for classifying functional γδ T cell subsets (35). The expression of the homing receptors CXCR3, CCR5 and CD62L have been used to differentiate effector and memory γδ T cells subsets (36, 37).

Effector T cells expand during active disease, whereas memory cells correlate with reduced mycobacterial burden and associated pathology following experimental infection (38, 39). Interestingly, serious TB disease results in reduced γδ T cell effector functions in the periphery (33, 34). Consistent with this observation, there is a progressive loss of $CD27^{neg}$ T_{EM} and T_{EMRA} γδ T cell subsets from the peripheral blood of patients with active TB (34, 40). We have recently shown that virulent *M. bovis* infection results in differentiation of circulating bovine γδ T cells to a T_{CM} phenotype similar to that described in humans (Guerra-Maupome *et al.,* manuscript under review). However, little is known regarding the response by lung-associated γδ T cells during *Mycobacterial* infection and vaccination (41, 42), and there are limitations for assessing the biological significance of γδ T cells in the response to TB in humans.

As a natural host of TB infection, cattle represent a highly relevant animal model to investigate the immune response of γδ T cells to *Mycobacterium* vaccination and infection (2, 43, 44). Furthermore, respiratory BCG vaccination is an established, well-characterized experimental system that is particularly useful for studying the development of TB-specific immune responses in the lungs. To this end, calves were vaccinated with BCG via the respiratory tract, and vaccine immunogenicity, and the differentiation of responding *M. bovis*-specific γδ T cells was examined in the peripheral blood and lower respiratory tract. We hypothesized that vaccination with BCG via the respiratory tract would result in the development of robust immune responses and phenotypic changes in mucosal and circulating γδ T cell populations. Improved characterization of γδ T cell phenotype and function during mycobacterial infection and vaccination will contribute to the development of improved strategies for harnessing their response in protection against TB.

MATERIALS AND METHODS

Animal Use Ethics

All animal procedures were conducted in strict accordance with federal and institutional guidelines and were approved by the Kansas State University Institutional Animal Care and Use Committee (Protocol Number: 27–2956). A total of 28 Holstein steers (~6-8-week-old) were used in the following experiments. Animals were housed in outdoor pens at the College of Veterinary Medicine, Kansas State University in Manhattan, KS. Animals had ab libitum access to hay, water, and concentrate. Steps were taken to avoid prolonged restraint and discomfort during all handling procedures. Body temperature was assessed if animal demonstrated signs of clinical illness and antibiotics and analgesics were administered as needed if animals presented with clinical disease independent of the experimental protocol. At the end of the study animals were humanely euthanized by barbiturate overdose. The calves showed no clinical signs following administration of the vaccine, and we observed no pathological changes in the lungs or tissues of the upper respiratory tract.

Mycobacterium bovis BCG aerosol vaccine procedures

M. bovis BCG Danish strain was a gift from Dr. Ray Waters at the National Animal Disease Center, USDA. BCG was prepared using standard techniques in Middlebrook 7H9 liquid media (Becton Dickinson, Franklin Lakes, NJ) supplemented with 10% oleic acid-albumin-dextrose complex (OADC) plus 0.05% Tween 80 (Sigma, St. Louis, Missouri). For the first experiment, treatment groups consisted of non-infected steers (n = 3) and animals receiving 1 x 10^8 colony-forming units (CFU) of *M. bovis* BCG Danish strain (n = 11). For the second study, treatment

groups consisted of non-infected calves (n = 7) and animals receiving 1 x 10^8 CFU of *M. bovis* BCG Danish strain (n = 7). For both studies, BCG inoculum was delivered to restrained calves by aerosol as described by Palmer *et al.* (45). Briefly, inoculum was nebulized into a mask (Trudell Medical International, London, ON, Canada) covering the nostrils and mouth, allowing regular breathing and delivery of the bacterial inoculum to the upper and lower respiratory tract via the nostrils. Clinical signs, including cough, dyspnea, and loss of appetite were monitored daily throughout the study. No clinical signs or pathology associated with BCG immunization were observed.

PBMC Isolation

Peripheral blood was drawn from the jugular vein into 2 × acid-citrate-dextrose solution. For peripheral blood mononuclear cells (PBMC) isolation, blood was diluted 1:1 in phosphate-buffered saline (PBS), cells were collected from buffy coat fractions, and PBMCs were isolated by density centrifugation on Histopaque (Sigma). Contaminating red blood cells were removed using RBC lysis buffer. Finally, cells were washed three times, counted in an hemocytometer and resuspended in complete RPMI (cRPMI) composed of RPMI-1640 (Gibco, Carlsbad, CA) supplemented with 2 mM L-glutamine, 25 mM HEPES buffer, 1% antibiotic-antimycotic solution, 1% non-essential amino acids 2% essential amino acids, 1% sodium pyruvate, 50 μM 2-mercaptoethanol (all from Sigma, St. Louis, MO), and 10% (v/v) heat-inactivated fetal bovine sera (FBS).

Bronchoalveolar Lavage Fluid Collection and cell Isolation

Broncheoalveolar lavage (BAL) samples were collected using a modified stallion urinary catheter (JorVet, Jorgensen Laboratories). The catheter was blindly passed through the nose and advanced through the trachea until lodging in the bronchus. A total of 180 mL of sterile saline was divided into three aliquots. An aliquot was first introduced to the lower respiratory tract, followed by immediate suction to obtain lower airway washes. The procedure was repeated twice more. All three aliquots were pooled at the end of the procedure. BAL samples were kept on ice, filtered over sterile gauze, and centrifuged at 200 x g for 10 minutes at 4°C. Contaminating red blood cells were removed using RBC lysis buffer. Cells were washed, resuspended in cRPMI, counted and 1 x 10^6 cells/well were resuspended in cRPMI and plated in 96 well plates. BAL cells were stimulated as described above. Plates were incubated for 72 hours for cytokine measurement at 37° C, 5% CO_2 incubator.

Tissue collection and cell suspension

All calves were euthanized 16 weeks after vaccination by intravenous administration of barbiturate overdose. Tissues were examined for gross lesions. Mediastinal and tracheobronchial lymph node tissues were collected and placed in cold cRPMI. Cells from lymph nodes were gained by sieving small pieces of tissue through steel meshes. Contaminating red blood cells were removed using RBC lysis buffer. Finally, cells were washed three times, counted in a hemocytometer and resuspended in cRPMI. Subsequently, cells (5 x 10^5 /well) were plated in round-bottom 96-well plate in duplicates, cultured for 6-days at 37°C, in the presence of 5% CO_2, and stimulated with *M. bovis* PPD (PPD-b, 200 IU/mL, Prionics Ag, Sclieren, Switzerland), recombinant (r)Ag85A (1 µg/ml, LIONEX Diagnostics and Therapeutics GmbH, Braunschweig, Germany) and rTB10.4 (1 µg/ml, LIONEX Diagnostics and Therapeutics GmbH). Concanavalin

A mitogen (ConA, 5 μg/ml, Sigma) or cRPMI medium were used as positive and negative control, respectively.

Long-term cell culture

Following PBMC isolation, long-term cell culture was performed as described by Maggioli *et al. (39, 46).* Briefly, PBMC were cultured (2×10^6 cells/well) in 24 well flat-bottom microtiter plates (Nunc, Thermo Fisher, Waltham, MA) and stimulated with a cocktail of *M. bovis* PPD (PPD-b, 200 IU/ml, Prionics Ag, Sclieren, Switzerland), rTB10.4 and rAg85A (1 μg/ml each) in cRPMI medium for 12 days 37°C, 5% CO_2. Media containing human rIL-2 (Sigma, 10 IU/ml) was used to replace media from the PBMC cultures at days 3 and 7. Fresh media without IL-2 was used at days 10 and 12. At day 13, autologous APCs were isolated by adherence incubating 2×10^5/well of freshly isolated PBMC in complete medium at 37°C, 5% CO_2 for 90 min in 96-well ELISPOT plates (Millipore, Watford, UK) previously coated overnight with anti-bovine IFNγ capture-mAb (Kingfisher) and blocked in cRPMI, for 2 h at 37°C, 5% CO_2. Non-adherent cells were discarded, and the adherent cells washed twice times with warm cRPMI. Long-term cell cultures and antigen were added (2×10^4 /well) to the ELISPOT plate and incubated for 20 h at 37°C, 5% CO_2 in the presence of autologous APC.

Ex vivo and Long-term ELISPOT

The IFNγ and IL-17A ELISPOT assay was performed as described by Maggioli *et al.* (39, 46). Briefly, 96-well ELISPOT plates (Millipore) were coated at 4°C overnight with an anti-bovine IFNγ capture mAb or IL-17A capture mAb (both from Kingfisher Biotech, Inc., St. Paul, MN), followed by a blocking step in cRPMI, for 2 h at 37°C, 5% CO_2. Fresh isolated PBMC or

long-term cultured cells (2×10^4/well) were added to ELISPOT plates and stimulated with either PPD-b (5 μg/ml), rTB10.4 and rAg85A (1 μg/ml each), ConA (5 μg/ml) or medium alone. Plates were incubated 20 h (IFNγ) or 48 h (IL-17A) at 37°C, 5% CO_2. Spot forming cells (SFC) were detected following the Vectastain ABC-AP Kit (Vector Laboratories, Burlingame, CA) standard procedures.

Proliferation

PBMC were labeled using CellTrace Violet (Invitrogen, Carlsbad, CA) prior to cell culture following manufacturer's instructions. Briefly, freshy isolated PBMCs were resuspended at 1×10^7 cells/mL in PBS containing 10 μM/ml of the CellTrace dye. After gently mixing, PBMCs were incubated for 20 min at 37°C in a water-bath. Labeling was quenched by using an equal volume of FBS, and cells were washed three times with RPMI medium. Subsequently, cells (5×10^5 /well) were plated in round-bottom 96-well plate in duplicates, cultured for 6-days at 37°C, 5% CO_2, in the presence of *M. bovis* PPD (PPD-b, 200 IU/mL). ConA (5 μg/ml) or cRPMI medium were used as positive and negative control, respectively. After six days, cells were surface stained (see Flow cytometry section) and analyzed for proliferation and surface marker expression by flow cytometry.

Flow Cytometry

Following the appropriate culture duration, cells were stained with primary and secondary monoclonal antibodies (mAbs) listed on **Table 2**. All incubation steps for staining were performed in FACS buffer (PBS with 10% FBS and 0.02% NA-azide) and incubated for 25 min at 4°C. Cells

were washed and fixed with BD FACS lysis buffer (BD Biosciences, Mountain View, CA) for 10 min at room temperature, washed and resuspended in FACS buffer until analysis.

For intracellular staining, 1 x 10^6 cells were incubated in cRPMI containing antigen for 16 h at 37°C, 5% CO_2 with Brefeldin A (GolgiPlug; BD Pharmingen, 10 μg/ml) added at 4 h of culture. After staining cell-surface markers, cells were permeabilized for 30 min using BD CytoFix/CytoPerm solution (BD Biosciences) and incubated with IFNγ (**Table 2**) for 45 min. Non-stimulated samples served as negative controls. Samples were acquired using a BD LSRII Fortessa flow cytometer (BD Biosciences). Data were analyzed using Flowjo software (Tree Star Inc., San Carlos, CA). Lymphocytes were identified in PBMC and BAL samples as shown in Supplemental Figs and were further subdivided by their CD4 and γδ T cell expression patterns.

Table 2 Flow cytometry reagents

Reagent or antibody clone	Secondary Antibody/ Source	Specificity/ Source
ILA11a	APC, Life Technologies	Bovine CD4, Washington State University
GB21a	APC-Cy7, Southern Biotech	Bovine TCR1 delta chain, Washington State University
GC6a	PE-Cy7, Life Technologies	Bovine CD45R, Washington State University
M-T271	AF488, Life Technologies	Human CD27, Biolegend
G025H7	Not applicable	Human CXCR3 FITC, Biolegend
HM-CCR5	Not applicable	Human CCR5 Percpcy5.5, Biolegend
CC302	Not applicable	Bovine IFN-γ PE, Serotec
Cell Trace Violet	Not applicable	Not applicable, Life Technologies

ELISA

PBMC and BAL cells were stimulated *in vitro* as described above. Plates were incubated for 6 days (PBMC) or 72 hours (BAL) at 37° C, 5% CO_2 incubator. Cell culture supernatants were stored at -80°C until ELISA analysis. The concentration of cytokines in cell culture supernatants was determined using bovine commercial ELISA kits for IFNγ, and IL-17A (all from Kingfisher) according to manufacturer's instructions. Each sample assayed was measured in duplicate.

Statistical Analyses

Results are expressed as averages ± standard errors of the mean (SEM). Statistical significance was determined by one-way Analysis of Variance (ANOVA) followed by Bonferroni test, or Student's t test using Prism software (GraphPad, La Jolla, CA).

RESULTS

Results

Aerosol BCG vaccination induced robust mucosal and systemic cellular immune responses

One of the primary objectives of this study was to determine if a vaccine delivered by aerosol to the respiratory tract of calves was safe and immunogenic. To this end, calves were vaccinated via aerosol inoculation with BCG. Control calves remained unvaccinated. PBMC were isolated at 4, 8, 12- and 16-weeks post vaccination (**Fig. 9A**). Cells were restimulated *in vitro* as described in Materials & Methods. IFNγ (**upper panels**) and IL-17A (**lower panels)** concentrations were analyzed in cell culture supernatants by sandwich ELISA. Calves were monitored twice daily, and we observed no clinical signs in the calves following administration of the vaccine, suggesting that this is a safe vaccination approach in immunocompetent individuals. Aerosol immunization was also highly immunogenic and elicited potent *M. bovis*-specific immune responses in peripheral blood, as indicate by the robust IL-17A and IFNγ secretion by Ag-stimulated PBMC. The response was significantly increased over PBMC from unvaccinated controls throughout the 16-week study (**Fig. 9A**).

Vaccine-induced responses in the respiratory mucosa were measured from BAL mononuclear cells at 4-, 12- and 16-weeks post vaccination (**Fig. 9B**). BAL cells from BCG vaccinated calves produced significant quantities of IL-17A and IFNγ in recall response to mycobacterial antigens, while BAL cells from unvaccinated animals did not (**Fig. 9B**).

Vaccine-induced cellular immune responses were also evaluated in lung-draining lymph nodes during necropsy, 16 weeks after vaccination (**Fig. 10**). Single cell suspensions were prepared from the mediastinal and tracheobronchial LN, and *in vitro* antigen stimulation assays were performed as described in Materials & Methods. Cell culture supernatants were analyzed by

sandwich ELISA for IFNγ (**upper panels**) and IL-17A (**lower panels**). Similar to the results from the BAL, cells isolated from the tracheobronchial (**Fig. 10A**) and mediastinal LNs (**Fig. 10B**) of BCG-vaccinated calves secreted IFNγ and IL-17A in specific response to *in vitro* PPD-b restimulation and the response was significantly increased over samples from unvaccinated controls at 16 week's post vaccination.

Mucosal BCG vaccination elicits effector and central memory T cell responses

Ex vivo assays are considered a measure of effector and effector memory T cell responses. While most protective TB vaccines elicit *ex vivo* IFNγ responses, not all vaccines that induce this response provide protection (2). Long-term cultured IFNγ ELISPOT responses to TB vaccination negatively correlate with mycobacterial burden and TB-associated pathology and positively correlate with vaccine-induced protection (47-50). In cattle and humans, the long-term cultured IFNγ ELISPOT assay evaluates central memory T cell responses following TB infection and vaccination (38, 39). Here, we determined if aerosol BCG vaccination was capable of eliciting both *ex vivo* and long-term cultured responses, as has been previously reported for other protective TB vaccines (39, 47, 48).

PBMC were isolated and short term stimulations were performed to quantify the numbers of *ex vivo* effector IFNγ and IL-17A-secreting cells (spot-forming cells, SFC) by ELISPOT assays (39, 46). Aerosol BCG vaccination elicited significant numbers of effector PPD-b and rTb10.4/Ag85a-specific T cells in the peripheral blood (**Fig. 11A)**. Long-term cultures were performed to quantify the numbers of *M. bovis*-specific central memory T cells in peripheral blood. As seen in **Fig. 11B**, aerosol BCG vaccination elicited significant numbers of IFNγ-producing central memory T cells. Antigen-specific responses to rTb10.4/Ag85a and PPD-b both exceeded

the long-term ELISPOT responses measured from non-vaccinates (**Fig. 11B**). Thus, aerosol BCG vaccination of elicits strong recall effector and central memory responses in calves (51, 52).

Aerosol BCG vaccination induces a robust systemic and mucosal antigen-specific γδ T cell responses

Having demonstrated that BCG vaccination via the respiratory tract elicited effector and memory T cell responses in the calf, we next investigated the differentiation status of the responding γδ T cell populations. Using flow cytometry, we assessed mycobacterial-specific γδ T cell responses in the peripheral and mucosal compartment. It is well established that aerosol BCG vaccination induces effector and memory CD4 T cell differentiation (53); therefore, where relevant, we made comparisons to the *M. bovis*-specific CD4 T cell populations in the same animals.

Following 4-, 8-, 12- and 16-weeks post vaccination, PBMC were isolated, labeled with CellTrace dye and cultured for 6 days in the presence or absence of PPD-b. On day 6 of culture, cells were analyzed by flow cytometry for T cells that divided in response to mycobacterial antigen restimulation. Representative γδ and CD4 T cell flow plots from a vaccinated and control animal are depicted in **Fig. 12A**. Consistent with the results in Fig. 1-3, aerosol BCG vaccination induced long-term, robust systemic cellular responses, and γδ T cells appear to participate in this response, as evidenced by the robust proliferative responses that persisted in vaccinated calves through at least 16-weeks post vaccination **(Fig. 12C)**. The observed γδ T cell recall responses were *M. bovis*-specific, as cells from non-vaccinated cattle did not respond to the complex or protein mycobacterial antigens. Similarly, only CD4 T cells from BCG-vaccinated animals, and not

control animals, participated in the recall response to mycobacterial antigen restimulation *in vitro* (**Fig. 12B and D).**

The frequency of vaccine-elicited IFNγ-producing γδ T cells was determined by intracellular cytokine staining on PBMC and BAL cells after ~8 weeks post vaccination. Aerosol BCG vaccination induced *M. bovis*-specific, IFNγ-producing γδ T cells in both the peripheral and mucosal compartments (**Fig. 13A**). The frequency of γδ T cells producing IFNγ in response to PPD-b stimulation was significantly higher in animals that were vaccinated with BCG compared to unvaccinated controls. Similarly, aerosol vaccination induced IFN-γ-producing antigen-specific CD4 T cells in the peripheral and mucosal compartment (**Fig. 13B**). Thus, aerosol BCG vaccination induces functional, *M. bovis*-specific γδ T cells in both the mucosal and systemic compartment.

***M. bovis*-specific γδ T cells from BCG vaccinated calves adopt a memory phenotype based on CD27 and CD45R expression**

We have recently shown that virulent *M. bovis* infection in cattle promotes the differentiation of circulating γδ T cells into subsets that share characteristics with human T_{CM} γδ T cells (CD45R- CD27+ $CD62L^{hi}$) (M. Guerra-Maupome, M. V. Palmer, W. R. Waters, J. L. McGill, manuscript under review). Here, we investigated the phenotype of both systemic and lung-associated γδ T cells to determine the impact of aerosol BCG vaccination on γδ T cell differentiation.

In the circulating compartment, aerosol BCG vaccination induced a significant increase in γδ T cells with a T_{CM} ($CD45R^{-}$ $CD27^{+}$) phenotype, and a moderate increase in γδ T cells with a T_{EM} ($CD45R^{-}$ $CD27^{-}$) phenotype (**Fig. 14A**). The cumulative proportions of effector/memory γδ

T cells from all calves are shown in **Fig**. **14B**. Our results suggest that BCG vaccination, similar to virulent *M. bovis* infection, elicits a γδ T_{CM} phenotype in the circulation that comprises the majority of the *M. bovis*-specific proliferative response. Interestingly, however, in contrast to our previous studies with virulent *M. bovis* infection, BCG vaccination also induced the expansion of circulating γδ T cells with a T_{EM} ($CD45R^{-}$ $CD27^{+}$) phenotype.

In the mucosal compartment, we observed that aerosol BCG vaccination preferentially induced the differentiation of γδ T_{EM} ($CD45R^{-}$ $CD27^{+}$) cells, with low proportions of T_{CM} cells compared to the circulating population (**Fig. 15A)**. The relative contribution of mucosal γδ T cell subsets in the response to PPD-b are shown in **Fig. 15B**.

Thus, BCG vaccination via the respiratory route induces the differentiation of antigen-specific γδ T cells with a T_{CM} phenotype in the periphery, and a T_{EM} phenotype in the mucosal compartment.

M. bovis-specific γδ T cells modulate expression of the tissue-associated chemokine receptors CXCR3 and CCR5

Expression of the chemokine receptors CXCR3 and CCR5 has been used to differentiate effector and memory T cell subsets (35). We have previously shown that circulating γδ T cells modulate their expression of CXCR3 and CCR5 during virulent *M. bovis* infection (M. Guerra-Maupome, M. V. Palmer, W. R. Waters, J. L. McGill, manuscript under review). The increased expression of CCR5 and CXCR3 on *M. bovis*-responsive T cells is expected to enable these populations to migrate into the inflamed tissue. Therefore, we next investigated the effect of aerosol BCG vaccination on the acquisition of lung homing receptors by *M. bovis* responsive γδ T cell in peripheral blood and BAL cells.

We observed marked differences in the patterns of chemokine receptor expression by *M. bovis*-responsive γδ T cells isolated from mucosal versus peripheral compartments. The majority of mucosal *M. bovis*-specific γδ T cells recovered from the BAL expressed lower surface levels of CXCR3 compared to non-responding γδ T cells (**Fig. 16A)**. As seen in **Fig. 16B**, mucosal antigen-specific CD4 T cells also expressed lower levels of CXCR3 compared to nonresponsive CD4 T cells. Comparison of chemokine receptor expression by mucosal γδ T cells compared with circulating *M. bovis*-specific γδ T cells, revealed significantly higher expression of CXCR3 by circulating antigen-specific populations (**Fig. 17C).** The expression of CCR5 on both CD4 and γδ T cells followed a similar trend, with increased expression by circulating, *M. bovis*-specific cells and reduced by *M. bovis*-responsive T cells recovered from the airways; however, this change was not statistically significant (Supp. Fig 6). Together our data show that aerosol BCG vaccination induces up-regulation of CXCR3 by circulating γδ T cells, which may confer lung homing properties under inflammatory conditions and correlate with the observed effector memory phenotype (36). Conversely, analysis of airway, *M. bovis*-responsive γδ T cells indicates a tendency towards downregulation of chemokine receptor expression, perhaps indicating that T cells localized to the lung no longer require these receptors.

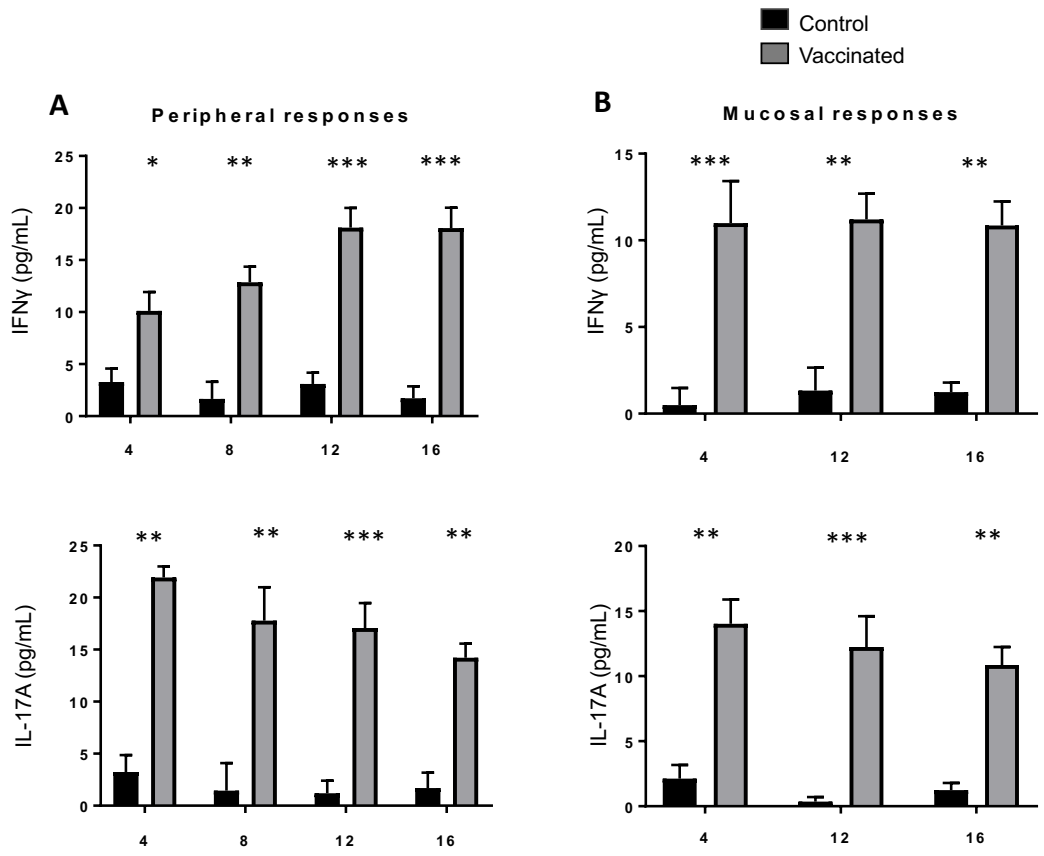

Figure 9. Cellular immune responses in the airways and peripheral blood of BCG-vaccinated calves.

(**A**) Peripheral blood was collected 4-, 8-, 12- and 16-weeks after vaccination. PBMC were isolated and stimulated for 6 days with PPD-b. Control wells remained unstimulated. (**B**) BAL were collected at 4-, 12- and 16-weeks after vaccination. BAL cells were stimulated for 72 hours *in vitro* with PPD-b. Control wells remained unstimulated. Cell culture supernatants were collected from BAL and PBMC cultures and analyzed by commercial ELISA kit for IFNγ (upper panel) and IL-17 (lower panel). Data represent means ± SEM. *$p<0.05$, **$p<0.01$ ***$p<0.001$ as determined by Student's t test.

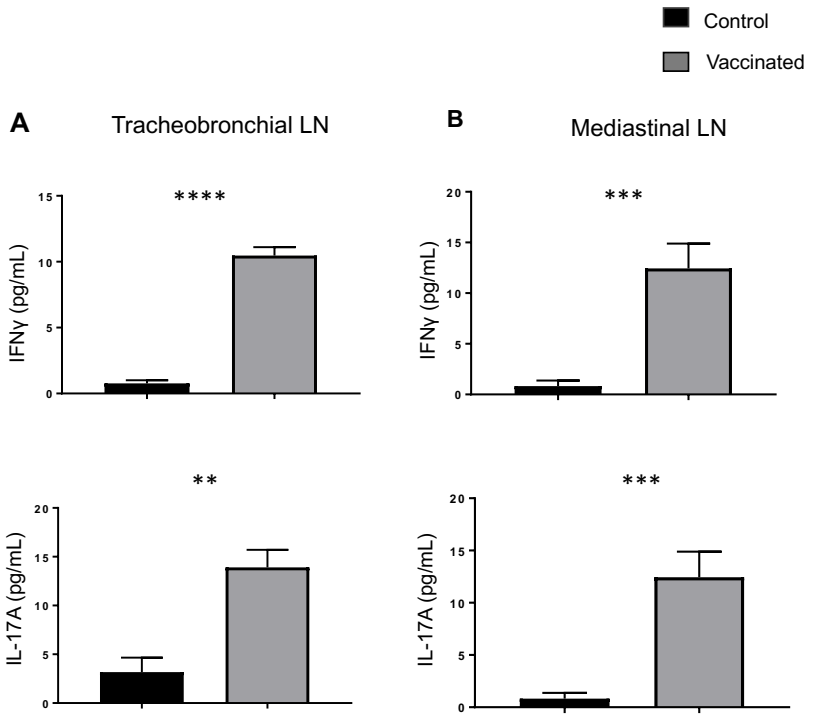

Figure 10. Cellular immune responses in lung-draining lymph nodes of BCG-vaccinated calves.

Lung-draining tracheobronchial (**A**) and mediastinal lymph nodes (**B**), were collected from control (n=7) or BCG–vaccinated animals (n= 7) during necropsy at 16 weeks after vaccination. Cells were isolated as described in Materials & Methods and restimulated for 6 days with PPD-b. Control wells remained unstimulated. Cell culture supernatants were collected from cultures and analyzed by commercial ELISA kit for IFNγ (**upper panel**) and IL-17 (**lower panel**). Data represent means ± SEM. **p<0.01 ***p<0.001 ****p<0.001 as determined by Student's t test.

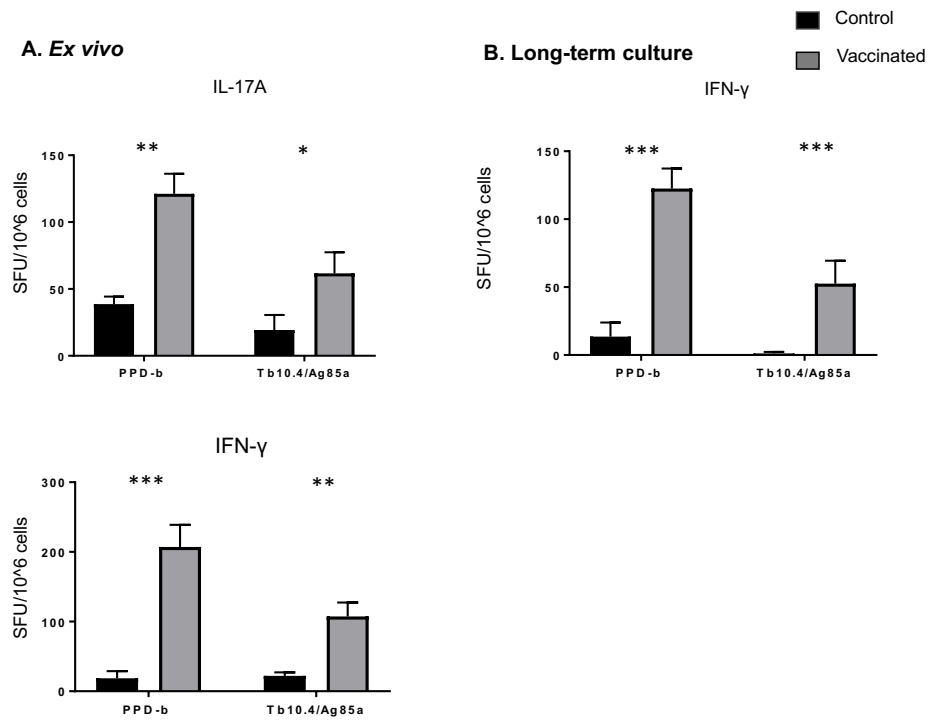

Figure 11. Long-term cultured and ex vivo IFNγ responses by cattle after *M. bovis* aerosol vaccination.

Cultured ELISPOT analysis was performed ~8 weeks after BCG aerosol vaccination. (**A**) For the *ex vivo* response, freshly isolated PBMCs were stimulated with rTb10.4/Ag85a, PPD-b or medium alone for 20 h (IFNγ) or for 48 hours (IL-17A). (B) Long-term cultured cells were generated by stimulating PBMC with a cocktail of rTB10.4 and rAg85A (1 µg/ml each), and PPD-b (200 IU/ml) for 13 days followed by transfer to ELISPOT plates with APCs and the addition of either rTb10.4/Ag85a, PPD-b or medium alone. Medium control responses were subtracted from antigen-stimulated responses and results are presented as mean spot forming cells (SFC)/10^6 cells (± SEM) for (**A**) *ex vivo* conditions or (**B)** long-term culture *$p<0.05$ **$p<0.01$ ***$p<0.001$ as determined by Student's t test.

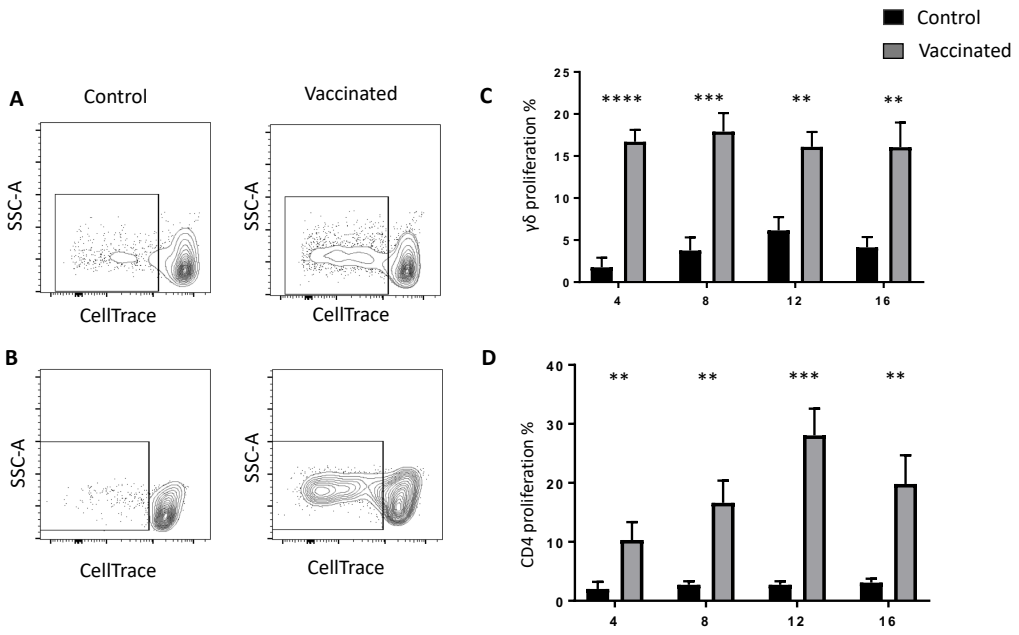

Figure 12. Systemic *M. bovis*-specific responses in response to in vitro restimulation with mycobacterial antigens.

PBMCs from control (n= 10) or BCG–vaccinated animals (n= 18) were labeled with CellTrace, and 5 × 10^6 cells/ml were cultured for 6 days in the presence or absence of PPD-b. Cells were labeled with anti-bovine γδ TCR or CD4 (**see Table 1**) and analyzed by flow cytometry for CellTrace dilution. (**A and B**) Representative contour plots of proliferative responses to *in vitro* PPD-b stimulation from a control and BCG-vaccinated animal. Gating hierarchy (gating sequence as depicted by the arrows): Single cells (SSC-A vs SSC-H), lymphocytes (SSC-A vs FSC-A), γδ T cells (**A**) or CD4 (**B**) and CellTrace dilution as shown in Fig. S1 (**C and D**) Cumulative percentage of γδ T cells (**C**) and CD4 T cells (**D**) from vaccinated and control animals that proliferated in response to mycobacterial antigens, as measured by CellTrace dilution, at 4-, 8- 12- or 16-weeks post vaccination. Analysis was performed with Flowjo software. Background proliferation was subtracted, and results represent change over mock. Data represent means ± SEM. **p<0.01 ***p<0.001 ****p<0.001 as determined by Student's t test.

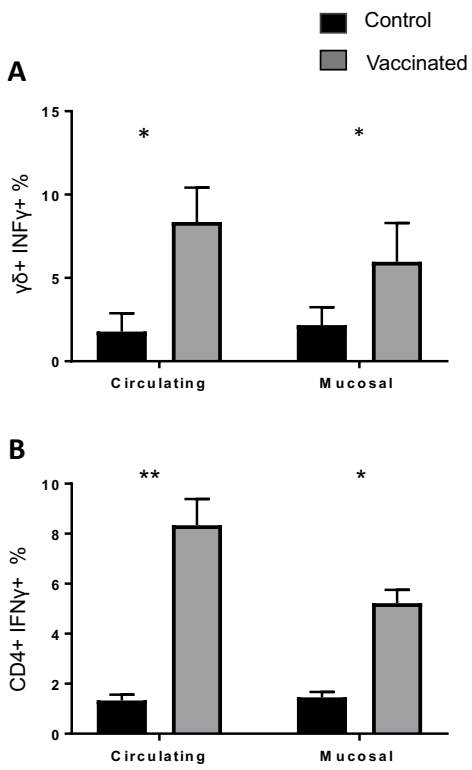

Figure 13. Circulating and Mucosal *M. bovis*-specific IFNγ expression in response to in vitro restimulation with mycobacterial antigens.

Approximately ~8 weeks after aerosol vaccination, IFNγ expression was analyzed in circulating (PBMC) and in mucosal (BAL) compartment from control (n=7) or BCG–vaccinated animals (n= 7). 1 x 10^6 cells/well were stimulated *in vitro* with PPD-b (200 IU/ml) for 16 hours. Cells were then stained for intracellular IFNγ expression and analyzed by flow cytometry. Gating hierarchy (gating sequence as depicted by the arrows): Single cells (SSC-A vs SSC-H), lymphocytes (SSC-A vs FSC-A), γδ or CD4 T cells and IFNγ expression as shown in Fig. S2 and Fig. S3 (**A and B**) The proportions of circulating and mucosal (**A**) $\gamma\delta^+$ $IFN\gamma^+$ and (**B**) $CD4^+$ $IFN\gamma^+$ conditions are shown. Analysis was performed with Flowjo software. Data represent means ± SEM. *$p<0.05$, **$p<0.01$ as determined by Student's t test.

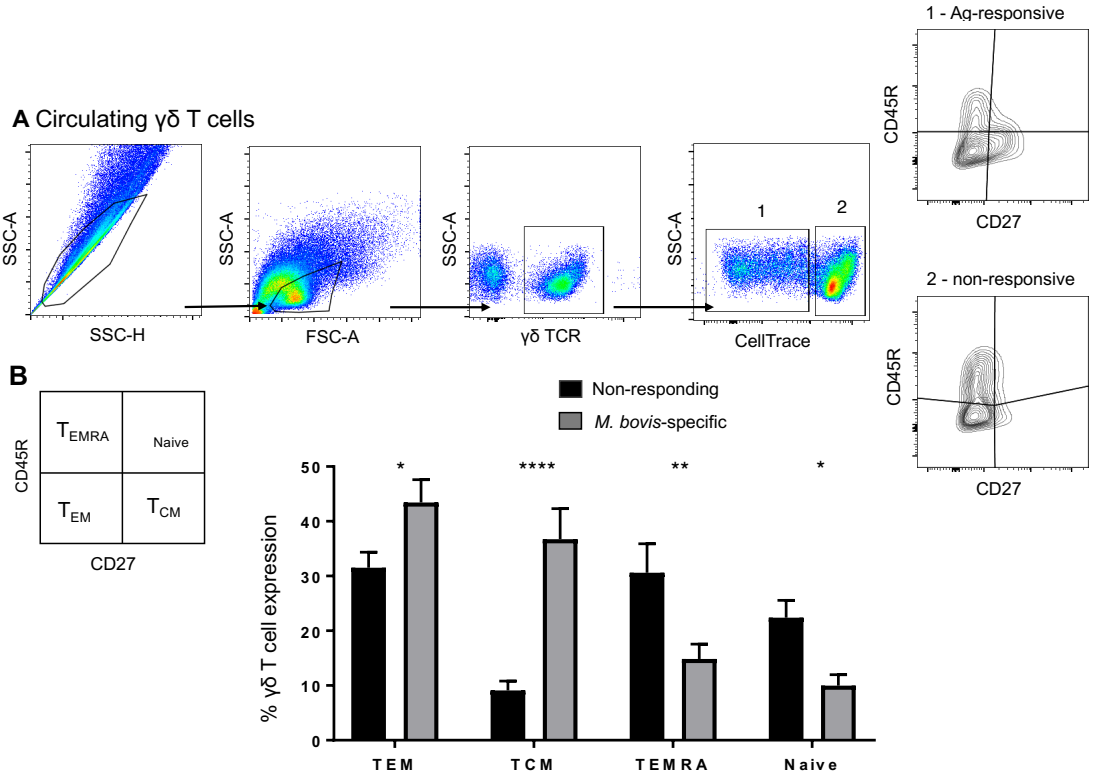

Figure 14. Phenotype of circulating antigen-specific γδ T cells.

PBMCs were isolated from calves ~ 8 weeks after BCG vaccination. Cells were stained with CellTrace dye and incubated with *M. bovis* PPD-b for 6 days. Cells were analyzed by flow cytometry and the expression of CD45R and CD27 was evaluated on *M. bovis*-specific (proliferating) γδ T cells. (**A**) Gating hierarchy (gating sequence as depicted by the arrows): Single cells (SSC-A vs SSC-H), lymphocytes (SSC-A vs FSC-A), γδ T cells, proliferating (1) and non-proliferating cells (2) in response to PPD-b and analyzed for CD27 versus CD45R expression. (**B**) Proportion of *M. bovis*-specific (CellTrace dim; grey bars) and nonspecific (CellTrace bright; black bars) γδ T cells in CD45R/CD27 defined subsets (Naïve: $CD45R^{+}CD27^{+}$; T_{CM}: $CD45R^{-}CD27^{+}$; T_{EM}: $CD45R^{-}CD27^{-}$; T_{EMRA}: $CD45R^{+}CD27^{-}$). Data are presented as ± SEM. *$p < 0.05$; **$p < 0.01$, p****<0.0001 indicates a significant difference from antigen-responsive cells compared to non-responsive cells as determined by Student's t-test.

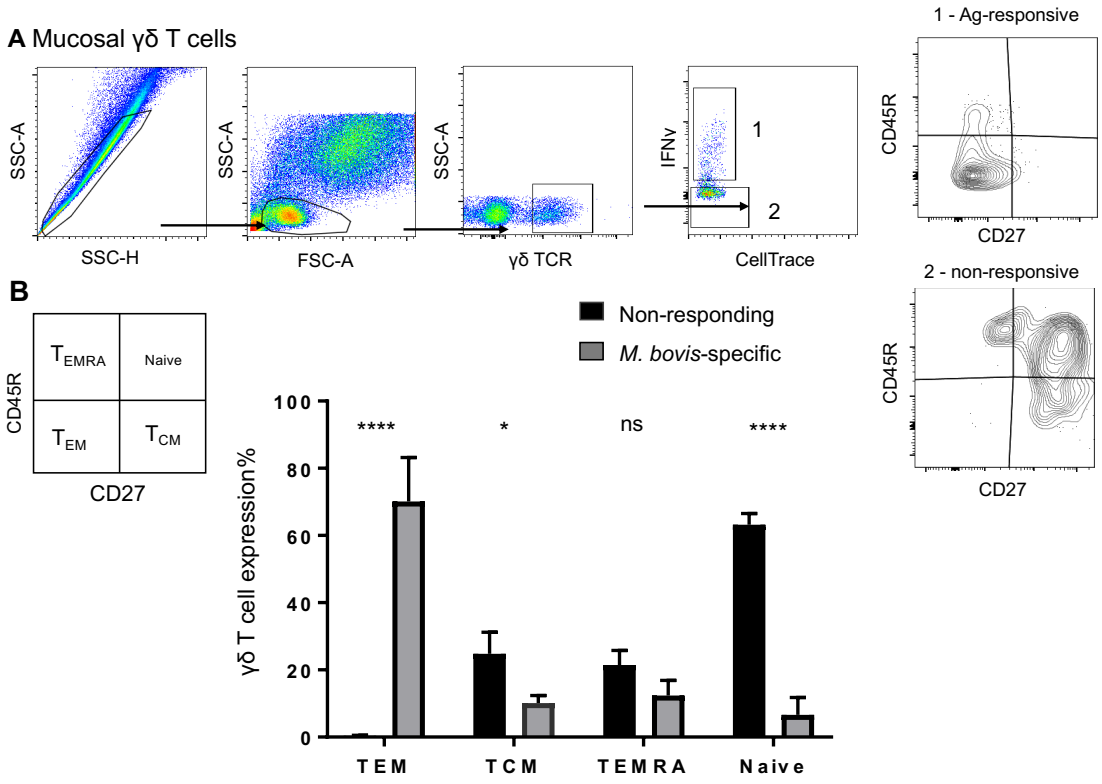

Figure 15. Phenotype of mucosal antigen-specific γδ T cells.

BAL cells were isolated from calves ~8 weeks after vaccination. Cells were stimulated with PPD-b *in vitro* for 16 hours. Cells were surface stained and stained for intracellular IFNγ, and then analyzed by flow cytometry to evaluate CD45R and CD27 expression on *M. bovis*-specific γδ T cells. (**A**) Gating hierarchy (gating sequence as depicted by the arrows): Single cells (SSC-A vs SSC-H), lymphocytes (SSC-A vs FSC-A), γδ T cells, IFNγ^{+} (grey bars) and IFNγ^{neg} (black bars) cells and CD27 versus CD45R expression. (**B**) Proportion of *M. bovis*-specific (IFNγ^{+}; grey bars) and nonspecific (IFNγ^{neg}; black bars) γδ T cells in CD45R/CD27 defined subsets (Naïve: CD45R^{+}CD27^{+}; T_{CM}: CD45R^{-}CD27^{+}; T_{EM}: CD45R^{-}CD27^{-}; T_{EMRA}: CD45R^{+}CD27^{-}). Data are presented as ± SEM. *p < 0.05, p****<0.0001 indicates a significant difference from antigen-responsive cells compared to non-responsive cells as determined by Student's t-test.

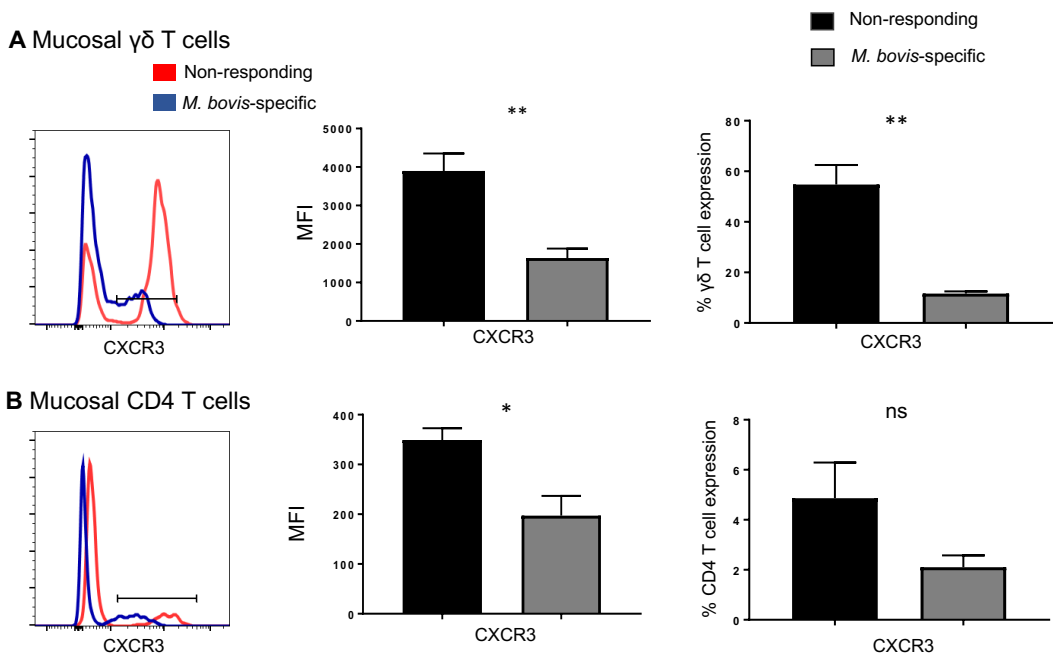

Figure 16. Surface expression of chemokine receptors on mucosal *M. bovis*-specific circulating γδ and CD4 T cells.

BAL cells were isolated from calves ~8 weeks after vaccination. Cells were stimulated with PPD-b *in vitro* for 16 hours. Cells were surface stained, stained for intracellular IFNγ expression, and the analyzed by flow cytometry for expression of CXCR3. Gating hierarchy (gating sequence as depicted by the arrows): Lymphocytes (SSC-A vs FSC-A), γδ T cells or CD4 cells, $IFN\gamma^{+}$ and $IFN\gamma^{neg}$ cells and CXCR3 as shown in Fig. S4. The left panels depict representative histogram plots of CXCR3 expression on *M. bovis*-specific γδ (**A**) and CD4 T cells (**B**). Cumulative results from mucosal γδ T cells (**A)** and CD4 T cells (**B**) are depicted: relative expression (MFI) (middle panels) and the frequency of $CXCR3^{+}$ cells (right panels) on *M. bovis*-specific (grey bars) or *M. bovis* nonresponsive (black bars). Data are presented as mean ± SEM. Not significant (ns), * $p < 0.05$, **$p < 0.01$ indicates a significant difference from antigen-responsive cells compared to non-responsive cells as determined by Student's t-test.

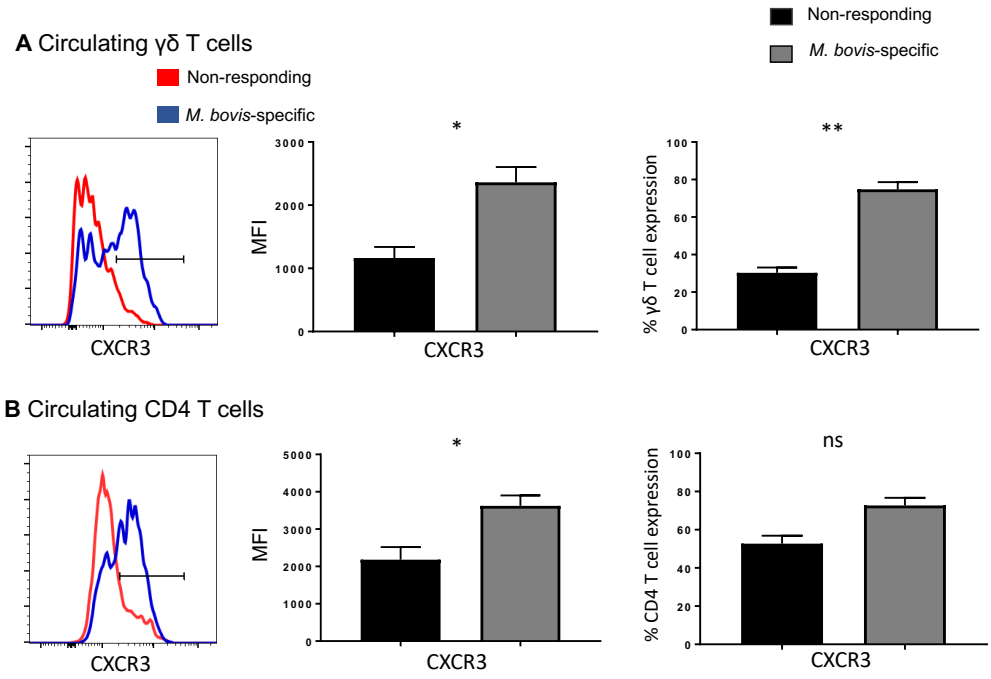

Figure 17. Surface expression of chemokine receptors on circulating *M. bovis*-specific γδ and CD4 T cells.

(A and B) PBMCs were isolated from calves ~8 weeks after vaccination. Cells were stained with CellTrace dye and incubated with PPD-b for 6 days. Cells were surface stained and then analyzed by flow cytometry to study CXCR3 and CCR5 expression on circulating *M. bovis*-specific γδ (**A**) and CD4 T cells (**B**). Gating hierarchy (gating sequence as depicted by the arrows): Lymphocytes (SSC-A vs FSC-A), γδ T cells or CD4 cells, proliferating and non-proliferating cells (CellTrace dilution) and CXCR3 or CCR5 expression as shown in Fig. S5. The left panels depict representative histogram plots of CXCR3 expression on *M. bovis*-specific γδ (**A**) and CD4 T cells (**B**). Cumulative results from blood γδ T cells (**A)** and CD4 T cells (**B**) are depicted: relative expression (MFI) (middle panels) and the frequency of $CXCR3^{+}$ cells (right panels) on *M. bovis*-specific (grey bars) or *M. bovis* nonresponsive (black bars). Data are presented as mean ± SEM. Not significant (ns), * $p < 0.05$, **$p < 0.01$ indicates a significant difference from antigen-responsive cells compared to non-responsive cells as determined by Student's t-test.

DISCUSSION

Current vaccine regimens against TB are ineffective; thus, development of improved intervention strategies and a better understanding of the nature of protective immunity remain important goals for TB research. Vaccination via the respiratory tract has been demonstrated to induce significant protection against virulent mycobacterial challenge (4-9). Although Th1 cells are of major importance for immunity to TB, other immune populations, notably γδ T cells [(9, 31, 54, 55) and reviewed (24)], may represent another essential component for the anti-mycobacterial immune response. The focus of this study was to investigate the immunogenicity, and generation of systemic and mucosal γδ T cell responses to BCG vaccination via the respiratory tract.

Infants are an important target population for TB vaccination. However, the infant immune system represents significant challenges when designing vaccines [reviewed (56)]. There are few suitable models suitable for studying infant immunology to TB, and most mucosal TB vaccines have been conducted in adult animals. Although we often attempt to extrapolate our knowledge of host defense and vaccine-induces immunity from adults to infants, we currently know little about neonatal immune response to mucosal vaccination. Furthermore, despite the promise of a mucosal vaccine strategy, there are safety concerns associated to the administration of a live replicating pathogen directly to the respiratory mucosa of immunosuppressed individuals. Thus, a reliable animal model that reflects the immune response to this route of vaccination in human infants is urgently required. Here, we observed that aerosol BCG immunization was safe in young calves, with no associated lung pathology or clinical symptoms. Furthermore, we show that aerosol BCG immunization of young calves is highly immunogenic and induces antigen-specific immune responses in both the systemic and mucosal compartment (**Figs. 1 and 2**). Our results are consistent with those reported by Hoft *et al*., showing that oral delivery of BCG to humans induced

both systemic and mucosa-associated immune responses in the airways (57). The capacity of mucosal BCG vaccination to induce responses in both the systemic and mucosal immune compartments in cattle is in contrast to the observations made in mice, where vaccine responses are compartmentalized following mucosal vaccination (58). In non-human primates, responses are less compartmentalized, but the magnitude of response correlates with the route of vaccination, with strong systemic responses after systemic vaccination and strong responses in the respiratory tract in animals immunized by aerosol delivery (5). Given the limitations of other animal models, our results are encouraging and demonstrate the potential of the calf model to evaluate the safety and immunogenicity of other TB vaccine candidates through this route of immunization.

In parallel with efforts to evaluate the safety and immunogenicity of the aerosol route of delivery, the identification of immunological correlates of protection is essential to reduce costs of challenge experiments and to prioritize vaccine candidates. Assessing the magnitude and frequency of IFNγ-producing CD4 T cells has remained the standard measurement of vaccine induced T cell memory for many years. However, it has become clear recently that this ex *vivo* assay cannot consistently predict vaccine success (59). Alternatively, long-term cultured IFNγ ELISPOT responses to TB vaccination have been shown to negatively correlate with mycobacterial burden and TB-associated pathology, and to positively correlate with vaccine success (46-49, 60) . In humans and cattle, the cultured ELISPOT assay is a surrogate of T_{CM} responses (39, 47, 48). Using this assay and the conventional ELISPOT, we evaluated if aerosol BCG vaccination was capable of eliciting both effector and T_{CM} responses. Consistent with the results of previous bovine TB vaccine trials examining parenteral BCG vaccination (47, 49), we demonstrate here that aerosol BCG vaccination elicits significant long-term cultured IFNγ ELISPOT responses to both PPD-b and the protein antigens Tb10.4 and Ag85a (**Fig. 3**). Thus

aerosol BCG vaccination elicits effector and central memory T cell (T_{CM}) responses in both humans and cattle (39, 49, 51, 52).

In humans, serious TB infection induces expansion of phenotypically immature, T_{CM} Vγ9Vδ2 T cells, and a reduction in the pool of Vγ9Vδ2 T cells with immediate effector functions (T_{EM} and T_{EMRA} cells), suggesting a functional impairment of γδ T cells perhaps, due to a persistent stimulation (61). Similarly, we have recently reported that virulent *M. bovis* infection in cattle can significantly alter the differentiation of circulating *M. bovis*-specific γδ T cells towards a T_{CM} (CD45R- CD27+CD62L^{hi}) phenotype (M. Guerra-Maupome, M. V. Palmer, W. R. Waters, J. L. McGill, manuscript under review). In this study, phenotypic analyses of circulating γδ T cells showed that *M. bovis*-specific γδ T cells are strongly affected by BCG vaccination; however, in contrast to virulent infection, the vaccine strain induced the expansion of both T_{CM} and T_{EM} γδ T cells in the blood (**Fig. 6**). As particular memory subsets of CD4 T cells have been shown to correlate with reduced mycobacterial burden, or to indicate disease progression, we speculate that changes in the differentiation status of memory γδ T cell subsets may also be indicative of particular disease states or serve as a correlate of immune status.

Previous reports in humans have shown that T_{EM} γδ T cells are highly represented in sites of inflammation and display immediate effector functions (33). Consistent with these findings, BCG vaccination via the respiratory tract was associated with an increase in mucosal IFNγ^{+} γδ T cells (**Fig. 5**) which exhibited a T_{EM} phenotype (**Fig. 7**). In non-human primates, accumulation of phosphoantigen-specific Vγ2Vδ2 T cells in the lungs significantly reduces *M. tb* burden and associated lung pathology (9, 28). Similarly, in mice, *M. bovis* BCG intranasal infection elicits increased levels of IFNγ^{+} γδ T cells with cytotoxic activity against infected macrophages (41). Thus, accumulation of T_{EM} *M. bovis*-responsive γδ T cells in the airway mucosa may enable an

immediate response upon pathogen re-exposure. Identifying strategies to elicit memory γδ T cell differentiation with a high frequency of γδ T cell effectors in the lung, as well as peripheral mycobacteria-specific γδ T cells that have the capacity to rapidly traffic into the lung to enhance and sustain protection, may be expected to confer optimal vaccine-induced protection (38, 39)

Few studies have examined γδ T cell chemokine expression (33); however, mycobacterial antigen stimulation appears to alter γδ T cell chemokine receptor expression (37). In our previous studies, we have observed increased expression of both CXCR3 and CCR5 on circulating γδ T cells from cattle infected with virulent *M. bovis* (M. Guerra-Maupome, M. V. Palmer, W. R. Waters, J. L. McGill manuscript under review). Similarly, we show here that aerosol BCG vaccination is associated with an increase in surface expression of CXCR3 in circulating *M. bovis*-specific γδ T cells compared to non-responding cells (**Fig. 9**). CXCL10, the ligand of CXCR3, is involved in trafficking of Th1 lymphocytes to areas of inflammation. It has been shown that peripheral human Vγ2Vδ2 CXCR3$^+$ T cells are recruited efficiently by CXCL10 (37, 62). Moreover, CXCL10 is found *in vivo* in lymph nodes and TB granulomas (63), suggesting that CXCR3 expression in circulating γδ T cells may enable rapid migration to the lungs upon *M. bovis* challenge (64, 65). Interestingly, comparison of CXCR3 expression on circulating T cell populations with *M. bovis*-specific T cells recovered from the BAL on the same day, revealed significantly reduced surface expression by both γδ and CD4 T cells in the airways (**Fig. 9A and B**). These findings are intriguing, as Poggi *et al.* showed that the homeostatic chemokine CCL21 was more efficient at inducing transendothelial migration of lung-resident Vγ2Vδ1 CXCR3$^+$ T cell populations than inflammatory CXCL10, suggesting that lung-associated γδ T cells preferentially respond to homeostatic chemokines, while circulating Vγ2Vδ2 CXCR3$^+$ T cells are more sensitive to inflammatory chemokines and thus might not require high expression of CXCR3 (62). It may

also be possible that CXCR3 is internalized or shed by cells which have migrated to the lung, thus explaining the reduced surface expression. Functional studies to assess CXCR3-dependent lymphocyte trafficking were not performed in this study, thus its importance in facilitating γδ T cell recruitment during *M. bovis* infection in the bovine remains to be fully determined.

BCG remains the most efficacious, 'gold-standard' TB vaccine available to prevent TB in humans and animals. Therefore, until improved vaccines are available, it is crucial to identify the most effective strategies for using BCG. As observed here, mucosal vaccination in juvenile calves is safe and immunogenic, priming robust effector and memory *Mycobacterium*-specific T cell responses in both peripheral and lung compartment. Based on our observations, there is merit in evaluating this route of immunization using other vaccine candidates, and in employing the infant calf model, as it can provide critical insights into mucosal TB-specific immune responses in infant humans. Through a comparative assessment of the phenotype and functions of *M. bovis*-specific γδ T cells in the systemic and mucosal compartment, we have shown that a single BCG immunization via the respiratory tract induces differentiation of T_{CM} and T_{EM} *M. bovis*-responsive γδ T cells, with a predominance of T_{EM} γδ T cells in the lungs, where they would be expected to mediate immediate effector functions and rapid protection against TB invasion. γδ T cells are known to promote resistance to *Mycobacterium* infection (9, 10, 32). Thus, engaging γδ T cells through novel, aerosol vaccination strategies may be particularly beneficial for promoting local protection in the respiratory tract.

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REFRENCES

1. Organization WH. Global tuberculosis report 2018. 2018.

2. Waters WR, Palmer MV, Buddle BM, Vordermeier HM. Bovine tuberculosis vaccine research: historical perspectives and recent advances. Vaccine. 2012;30(16):2611-22.

3. Butkeviciute E, Jones CE, Smith SG. Heterologous effects of infant BCG vaccination: potential mechanisms of immunity. Future Microbiol. 2018;13:1193-208.

4. Kaushal D, Foreman TW, Gautam US, Alvarez X, Adekambi T, Rangel-Moreno J, et al. Mucosal vaccination with attenuated Mycobacterium tuberculosis induces strong central memory responses and protects against tuberculosis. Nat Commun. 2015;6:8533.

5. White AD, Sibley L, Dennis MJ, Gooch K, Betts G, Edwards N, et al. Evaluation of the safety and immunogenicity of a candidate tuberculosis vaccine, MVA85A, delivered by aerosol to the lungs of macaques. Clin Vaccine Immunol. 2013;20(5):663-72.

6. Goonetilleke NP, McShane H, Hannan CM, Anderson RJ, Brookes RH, Hill AV. Enhanced immunogenicity and protective efficacy against Mycobacterium tuberculosis of bacille Calmette-Guerin vaccine using mucosal administration and boosting with a recombinant modified vaccinia virus Ankara. J Immunol. 2003;171(3):1602-9.

7. Giri PK, Verma I, Khuller GK. Protective efficacy of intranasal vaccination with Mycobacterium bovis BCG against airway Mycobacterium tuberculosis challenge in mice. J Infect. 2006;53(5):350-6.

8. Chen L, Wang J, Zganiacz A, Xing Z. Single intranasal mucosal Mycobacterium bovis BCG vaccination confers improved protection compared to subcutaneous vaccination against pulmonary tuberculosis. Infect Immun. 2004;72(1):238-46.

9. Shen Y, Zhou D, Qiu L, Lai X, Simon M, Shen L, et al. Adaptive immune response of Vgamma2Vdelta2+ T cells during mycobacterial infections. Science. 2002;295(5563):2255-8.

10. Shen L, Frencher J, Huang D, Wang W, Yang E, Chen CY, et al. Immunization of Vgamma2Vdelta2 T cells programs sustained effector memory responses that control tuberculosis in nonhuman primates. Proc Natl Acad Sci U S A. 2019;116(13):6371-8.

11. Wang J, Thorson L, Stokes RW, Santosuosso M, Huygen K, Zganiacz A, et al. Single mucosal, but not parenteral, immunization with recombinant adenoviral-based vaccine provides potent protection from pulmonary tuberculosis. J Immunol. 2004;173(10):6357-65.

12. Grode L, Seiler P, Baumann S, Hess J, Brinkmann V, Nasser Eddine A, et al. Increased vaccine efficacy against tuberculosis of recombinant Mycobacterium bovis bacille Calmette-Guerin mutants that secrete listeriolysin. J Clin Invest. 2005;115(9):2472-9.

13. Lai R, Afkhami S, Haddadi S, Jeyanathan M, Xing Z. Mucosal immunity and novel tuberculosis vaccine strategies: route of immunisation-determined T-cell homing to restricted lung mucosal compartments. Eur Respir Rev. 2015;24(136):356-60.

14. Li W, Deng G, Li M, Liu X, Wang Y. Roles of Mucosal Immunity against Mycobacterium tuberculosis Infection. Tuberc Res Treat. 2012;2012:791728.

15. Manjaly Thomas ZR, McShane H. Aerosol immunisation for TB: matching route of vaccination to route of infection. Trans R Soc Trop Med Hyg. 2015;109(3):175-81.

16. Santosuosso M, Zhang X, McCormick S, Wang J, Hitt M, Xing Z. Mechanisms of mucosal and parenteral tuberculosis vaccinations: adenoviral-based mucosal immunization preferentially elicits sustained accumulation of immune protective CD4 and CD8 T cells within the airway lumen. J Immunol. 2005;174(12):7986-94.

17. Horvath CN, Xing Z. Immunization strategies against pulmonary tuberculosis: considerations of T cell geography. Adv Exp Med Biol. 2013;783:267-78.

18. Satti I, Meyer J, Harris SA, Manjaly Thomas ZR, Griffiths K, Antrobus RD, et al. Safety and immunogenicity of a candidate tuberculosis vaccine MVA85A delivered by aerosol in BCG-vaccinated healthy adults: a phase 1, double-blind, randomised controlled trial. Lancet Infect Dis. 2014;14(10):939-46.

19. Horvath CN, Shaler CR, Jeyanathan M, Zganiacz A, Xing Z. Mechanisms of delayed anti-tuberculosis protection in the lung of parenteral BCG-vaccinated hosts: a critical role of airway luminal T cells. Mucosal Immunol. 2012;5(4):420-31.

20. Shaler CR, Horvath C, Lai R, Xing Z. Understanding delayed T-cell priming, lung recruitment, and airway luminal T-cell responses in host defense against pulmonary tuberculosis. Clin Dev Immunol. 2012;2012:628293.

21. Jeyanathan M, Heriazon A, Xing Z. Airway luminal T cells: a newcomer on the stage of TB vaccination strategies. Trends Immunol. 2010;31(7):247-52.

22. Mittrucker HW, Steinhoff U, Kohler A, Krause M, Lazar D, Mex P, et al. Poor correlation between BCG vaccination-induced T cell responses and protection against tuberculosis. Proc Natl Acad Sci U S A. 2007;104(30):12434-9.

23. Majlessi L, Simsova M, Jarvis Z, Brodin P, Rojas MJ, Bauche C, et al. An increase in antimycobacterial Th1-cell responses by prime-boost protocols of immunization does not enhance protection against tuberculosis. Infect Immun. 2006;74(4):2128-37.

24. Salerno A, Dieli F. Role of gamma delta T lymphocytes in immune response in humans and mice. Crit Rev Immunol. 1998;18(4):327-57.

25. Hayday AC. [gamma][delta] cells: a right time and a right place for a conserved third way of protection. Annu Rev Immunol. 2000;18:975-1026.

26. Hayday AC. Gammadelta T cells and the lymphoid stress-surveillance response. Immunity. 2009;31(2):184-96.

27. Siddiqui N, Price S, Hope J. BCG vaccination of neonatal calves: potential roles for innate immune cells in the induction of protective immunity. Comp Immunol Microbiol Infect Dis. 2012;35(3):219-26.

28. Qaqish A, Huang D, Chen CY, Zhang Z, Wang R, Li S, et al. Adoptive Transfer of Phosphoantigen-Specific gammadelta T Cell Subset Attenuates Mycobacterium tuberculosis Infection in Nonhuman Primates. J Immunol. 2017;198(12):4753-63.

29. Chen ZW, Letvin NL. Adaptive immune response of Vgamma2Vdelta2 T cells: a new paradigm. Trends Immunol. 2003;24(4):213-9.

30. McGill JL, Sacco RE, Baldwin CL, Telfer JC, Palmer MV, Waters WR. The role of gamma delta T cells in immunity to Mycobacterium bovis infection in cattle. Vet Immunol Immunopathol. 2014;159(3-4):133-43.

31. Hoft DF, Brown RM, Roodman ST. Bacille Calmette-Guerin vaccination enhances human gamma delta T cell responsiveness to mycobacteria suggestive of a memory-like phenotype. J Immunol. 1998;161(2):1045-54.

32. Chen CY, Yao S, Huang D, Wei H, Sicard H, Zeng G, et al. Phosphoantigen/IL2 expansion and differentiation of Vgamma2Vdelta2 T cells increase resistance to tuberculosis in nonhuman primates. PLoS Pathog. 2013;9(8):e1003501.

33. Dieli F, Poccia F, Lipp M, Sireci G, Caccamo N, Di Sano C, et al. Differentiation of effector/memory Vdelta2 T cells and migratory routes in lymph nodes or inflammatory sites. J Exp Med. 2003;198(3):391-7.

34. Gioia C, Agrati C, Casetti R, Cairo C, Borsellino G, Battistini L, et al. Lack of CD27-CD45RA-V gamma 9V delta 2+ T cell effectors in immunocompromised hosts and during active pulmonary tuberculosis. J Immunol. 2002;168(3):1484-9.

35. Geginat J, Sallusto F, Lanzavecchia A. Cytokine-driven proliferation and differentiation of human naive, central memory and effector memory CD4+ T cells. Pathol Biol (Paris). 2003;51(2):64-6.

36. Qin S, Rottman JB, Myers P, Kassam N, Weinblatt M, Loetscher M, et al. The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. J Clin Invest. 1998;101(4):746-54.

37. Glatzel A, Wesch D, Schiemann F, Brandt E, Janssen O, Kabelitz D. Patterns of chemokine receptor expression on peripheral blood gamma delta T lymphocytes: strong expression of CCR5 is a selective feature of V delta 2/V gamma 9 gamma delta T cells. J Immunol. 2002;168(10):4920-9.

38. Godkin AJ, Thomas HC, Openshaw PJ. Evolution of epitope-specific memory CD4(+) T cells after clearance of hepatitis C virus. J Immunol. 2002;169(4):2210-4.

39. Maggioli MF, Palmer MV, Thacker TC, Vordermeier HM, Waters WR. Characterization of effector and memory T cell subsets in the immune response to bovine tuberculosis in cattle. PLoS One. 2015;10(4):e0122571.

40. Meraviglia S, El Daker S, Dieli F, Martini F, Martino A. gammadelta T cells cross-link innate and adaptive immunity in Mycobacterium tuberculosis infection. Clin Dev Immunol. 2011;2011:587315.

41. Dieli F, Ivanyi J, Marsh P, Williams A, Naylor I, Sireci G, et al. Characterization of lung gamma delta T cells following intranasal infection with Mycobacterium bovis bacillus Calmette-Guerin. J Immunol. 2003;170(1):463-9.

42. de Bree C, Marijnissen RJ, Kel JM, Rosendahl Huber SK, Aaby P, Benn CS, et al. Bacillus Calmette-Guerin-Induced Trained Immunity Is Not Protective for Experimental Influenza A/Anhui/1/2013 (H7N9) Infection in Mice. Front Immunol. 2018;9:869.

43. Endsley JJ, Waters WR, Palmer MV, Nonnecke BJ, Thacker TC, Jacobs WR, Jr., et al. The calf model of immunity for development of a vaccine against tuberculosis. Vet Immunol Immunopathol. 2009;128(1-3):199-204.

44. Waters WR, Palmer MV, Thacker TC, Davis WC, Sreevatsan S, Coussens P, et al. Tuberculosis immunity: opportunities from studies with cattle. Clin Dev Immunol. 2011;2011:768542.

45. Palmer MV, Waters WR, Whipple DL. Aerosol delivery of virulent Mycobacterium bovis to cattle. Tuberculosis (Edinb). 2002;82(6):275-82.

46. Maggioli MF, Palmer MV, Vordermeier HM, Whelan AO, Fosse JM, Nonnecke BJ, et al. Application of Long-term cultured Interferon-gamma Enzyme-linked Immunospot Assay for Assessing Effector and Memory T Cell Responses in Cattle. J Vis Exp. 2015(101):e52833.

47. Waters WR, Palmer MV, Nonnecke BJ, Thacker TC, Scherer CF, Estes DM, et al. Efficacy and immunogenicity of Mycobacterium bovis DeltaRD1 against aerosol M. bovis infection in neonatal calves. Vaccine. 2009;27(8):1201-9.

48. Whelan AO, Wright DC, Chambers MA, Singh M, Hewinson RG, Vordermeier HM. Evidence for enhanced central memory priming by live Mycobacterium bovis BCG vaccine in comparison with killed BCG formulations. Vaccine. 2008;26(2):166-73.

49. Vordermeier HM, Villarreal-Ramos B, Cockle PJ, McAulay M, Rhodes SG, Thacker T, et al. Viral booster vaccines improve Mycobacterium bovis BCG-induced protection against bovine tuberculosis. Infect Immun. 2009;77(8):3364-73.

50. Hope JC, Thom ML, McAulay M, Mead E, Vordermeier HM, Clifford D, et al. Identification of surrogates and correlates of protection in protective immunity against Mycobacterium bovis infection induced in neonatal calves by vaccination with M. bovis BCG Pasteur and M. bovis BCG Danish. Clin Vaccine Immunol. 2011;18(3):373-9.

51. Keating SM, Bejon P, Berthoud T, Vuola JM, Todryk S, Webster DP, et al. Durable human memory T cells quantifiable by cultured enzyme-linked immunospot assays are induced by heterologous prime boost immunization and correlate with protection against malaria. J Immunol. 2005;175(9):5675-80.

52. Millington KA, Gooding S, Hinks TS, Reynolds DJ, Lalvani A. Mycobacterium tuberculosis-specific cellular immune profiles suggest bacillary persistence decades after spontaneous cure in untreated tuberculosis. J Infect Dis. 2010;202(11):1685-9.

53. Perdomo C, Zedler U, Kuhl AA, Lozza L, Saikali P, Sander LE, et al. Mucosal BCG Vaccination Induces Protective Lung-Resident Memory T Cell Populations against Tuberculosis. MBio. 2016;7(6).

54. Blumerman SL, Herzig CT, Baldwin CL. WC1+ gammadelta T cell memory population is induced by killed bacterial vaccine. Eur J Immunol. 2007;37(5):1204-16.

55. Worku S, Gorse GJ, Belshe RB, Hoft DF. Canarypox vaccines induce antigen-specific human gammadelta T cells capable of interferon-gamma production. J Infect Dis. 2001;184(5):525-32.

56. Ghazal P, Dickinson P, Smith CL. Early life response to infection. Curr Opin Infect Dis. 2013;26(3):213-8.

57. Hoft DF, Xia M, Zhang GL, Blazevic A, Tennant J, Kaplan C, et al. PO and ID BCG vaccination in humans induce distinct mucosal and systemic immune responses and CD4(+) T cell transcriptomal molecular signatures. Mucosal Immunol. 2018;11(2):486-95.

58. Belyakov IM, Moss B, Strober W, Berzofsky JA. Mucosal vaccination overcomes the barrier to recombinant vaccinia immunization caused by preexisting poxvirus immunity. Proc Natl Acad Sci U S A. 1999;96(8):4512-7.

59. Maue AC, Waters WR, Davis WC, Palmer MV, Minion FC, Estes DM. Analysis of immune responses directed toward a recombinant early secretory antigenic target six-kilodalton protein-culture filtrate protein 10 fusion protein in Mycobacterium bovis-infected cattle. Infect Immun. 2005;73(10):6659-67.

60. Wangoo A, Johnson L, Gough J, Ackbar R, Inglut S, Hicks D, et al. Advanced granulomatous lesions in Mycobacterium bovis-infected cattle are associated with increased expression of type I procollagen, gammadelta (WC1+) T cells and CD 68+ cells. J Comp Pathol. 2005;133(4):223-34.

61. Meraviglia S, Caccamo N, Salerno A, Sireci G, Dieli F. Partial and ineffective activation of V gamma 9V delta 2 T cells by Mycobacterium tuberculosis-infected dendritic cells. J Immunol. 2010;185(3):1770-6.

62. Poggi A, Carosio R, Fenoglio D, Brenci S, Murdaca G, Setti M, et al. Migration of V delta 1 and V delta 2 T cells in response to CXCR3 and CXCR4 ligands in healthy donors and HIV-1-infected patients: competition by HIV-1 Tat. Blood. 2004;103(6):2205-13.

63. Ferrero E, Biswas P, Vettoretto K, Ferrarini M, Uguccioni M, Piali L, et al. Macrophages exposed to Mycobacterium tuberculosis release chemokines able to recruit selected leucocyte subpopulations: focus on gammadelta cells. Immunology. 2003;108(3):365-74.

64. Fuller CL, Flynn JL, Reinhart TA. In situ study of abundant expression of proinflammatory chemokines and cytokines in pulmonary granulomas that develop in cynomolgus macaques experimentally infected with Mycobacterium tuberculosis. Infect Immun. 2003;71(12):7023-34.

65. Mikhak Z, Fleming CM, Medoff BD, Thomas SY, Tager AM, Campanella GS, et al. STAT1 in peripheral tissue differentially regulates homing of antigen-specific Th1 and Th2 cells. J Immunol. 2006;176(8):4959-67.

Chapter 4 - Aerosol vaccination with Bacille Calmette-Guerin induces a trained innate immune phenotype in calves

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ABSTRACT

Mycobacterium bovis Bacillus Calmette-Guérin (BCG) is a live attenuated vaccine for use against tuberculosis (TB); however, it is known to reduce childhood mortality from infections other than TB. The unspecific protection induced by BCG vaccination has been associated with the induction of memory-like traits of the innate immune system identified as 'trained' immunity. In humans and mouse models, *in vitro* and *in vivo* BCG training leads to enhanced production of monocyte-derived proinflammatory cytokines in response to secondary unrelated bacterial and fungal pathogens. While BCG has been studied extensively for its ability to induce innate training in humans and mouse models, BCG's nonspecific protective effects have not been defined in agricultural species. Here, we show that *in vitro* BCG training induces a functional change in bovine monocytes, characterized by increased transcription of proinflammatory cytokines upon restimulation with the toll-like receptor agonists. Importantly, *in vivo*, aerosol BCG vaccination in young calves also induced a 'trained' phenotype in circulating peripheral blood mononuclear cells (PBMCs), that lead to a significantly enhanced TLR-induced proinflammatory cytokine response compared to PBMCs from unvaccinated control calves. Our results show that BCG training not

only induces increased cytokine production but also identifies glycolysis as a fundamental molecular basis. Similar to the long-term training effects of BCG reported in humans, our results suggest that in young calves, the effects of BCG induced innate training can last for at least 3 months in circulating immune populations. Interestingly, however, aerosol BCG vaccination did not 'train' the innate immune response at the mucosal level, as alveolar macrophages from aerosol BCG vaccinated calves did not mount an enhanced inflammatory response to secondary stimulation, compared to cells isolated from control calves. Together, our results suggest that, like mice and humans, the innate immune system of calves can be 'trained'; and that BCG vaccination could be used as an immunomodulatory strategy to reduce disease burden in juvenile food animals before the adaptive immune system has fully matured.

INTRODUCTION

Evidence from epidemiological studies suggests that previous exposure to *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG), a live attenuated vaccine used to prevent tuberculosis, reduces the risk of childhood mortality due to prevention of sepsis, diarrhea and respiratory infections [(1-4) and reviewed (5, 6)]. It has been suggested that the nonspecific disease resistance induced by BCG is mediated by an enhanced memory-like response of the innate immune system known as 'trained' immunity (7, 8).

Trained immunity is induced primarily in myeloid cells (monocytes and macrophages) and natural killer (NK) cells (8-11), after previous exposure to some live vaccines like BCG, measles and yellow fever, as well as to some microbial components of pathogens (11-17), which results in superior cytokine expression and ultimately, enhanced capacity to prevent infection. Mechanistic studies have demonstrated that trained immunity is independent of adaptive immunity (18), and is caused by epigenetic reprogramming and alterations in basal intracellular metabolic pathways, which result in changes in gene expression and cell physiology leading to increased innate immune cells' capacity to respond to stimulation (9, 10, 16).

Evidence that trained immunity mediates the nonspecific protective effects seen after BCG vaccination came from proof-of-principle experimental studies. In these studies, BCG vaccination of severe combined immunodeficiency (SCID) or recombination-activating gene 1 (rag1)-deficient mice induced protection against subsequent lethal *Candida albicans* (*C. albicans*) infection via a mechanism that requires macrophages and proinflammatory cytokine production, both prototypical innate immune components (9, 16, 19). Mice vaccinated with BCG also show increased resistance to malaria infection, which is associated with increased transcription of antimicrobial proteins compared with nonvaccinated mice (20).

In humans, BCG vaccination leads to an enhanced production of the monocyte-derived proinflammatory cytokines, tumor necrosis alpha (TNFα) and interleukin 6 (IL-6), in response to unrelated bacterial and fungal pathogens (*C. albicans, Staphylococcus aureus*) (16); and superior NK cell activity (11). Moreover, in neonates, intradermal BCG vaccination at birth influences the responses to common vaccine antigens at the systemic level, which appeared to be mediated through activation of T lymphocytes by APCs (21). Similarly, compared with unvaccinated babies, cord blood monocytes from BCG vaccinated infants show increased expression of granulysin and perforin upon stimulation (22). Taken together, these observations suggest that BCG vaccination has the capacity to train the innate immune system to develop memory-like traits that result in enhanced disease resistance.

The nonspecific protection afforded by parenteral administration of some live vaccines is increasingly appreciated as an aspect of innate immunity that can be exploited for enhancing disease resistance in subjects at high risk from infections (23). Similar to young human infants, neonatal and juvenile agricultural species are most susceptible to respiratory diseases during the first weeks of life, due to waning maternal immunity and the still-developing neonatal adaptive immune system. In cattle, nearly a quarter of pre-weaned deaths and nearly half of postweaning calf deaths can be attributed to respiratory diseases, with calf pneumonia affecting as many as 20-30% of calves in some operations (24, 25). Therefore, methods to enhance disease resistance and limit pathogen colonization are highly needed to reduce the risk of respiratory disease in calves, which also serve as valuable animal model for juvenile human respiratory diseases.

The mucosal surface of the respiratory tract represents the principal portal of entry for most human and animal pathogens. It is believed that matching the route of infection with the route of vaccination will provide the best protection against disease (26). Thus, there are ongoing efforts

to evaluate mucosal routes of vaccination (i.e. intranasal or aerosol) for delivery of live respiratory pathogens (27). BCG vaccination of juvenile calves is a well-described model that has been used previously to explore the adaptive immune response to bovine tuberculosis; and to model the human infant immune response to TB infection (28-31). Aerosol vaccines have not only the immunological advantage of being able to replicate in the respiratory mucosa and prime the mucosal immune system, but also have practical and logistical advantages (32). However, few studies have looked at the ability of this route to induce a 'trained' immune phenotype at mucosal surfaces, at which there may be a more efficient route of inducing memory-like innate responses against mucosal diseases. Therefore, using the calf model of aerosol BCG vaccination, the objective of this study was to test the hypothesis that both *in vitro* and *in vivo* BCG exposure will 'train' bovine innate immune cells to develop memory-like characteristics against unrelated secondary stimuli, and thus can be harnessed as an approach to reduce disease burden in juvenile food animals.

MATERIALS AND METHODS

Animal Use Ethics

All animal procedures were conducted in strict accordance with federal and institutional guidelines and were approved by the Kansas State University Institutional Animal Care and Use Committee (Protocol Number: 27-2956). All the animals in this study, were housed in pens outside at Kansas State University in Manhattan, KS. Animals had *ad libitum* access to hay, water, and concentrate. The animal care protocol included avoidance of prolonged restraint, and methods to minimize pain and distress as determined by the discretion of the attending clinical veterinarian. No animals died or required euthanasia. For the *in vitro* studies, peripheral blood was collected from a total of six Holstein heifers (one-two years old).

For the vaccine study, fourteen, 6-8-week-old Holstein bull calves were randomly assigned to two groups (n = 7 animals/group): vaccinated and unvaccinated group. Clinical signs like cough, dyspnea, and appetite were monitored daily throughout the study. No adverse effects from immunization were observed. Peripheral blood and broncheoalveolar lavage samples were collected at 4 and 12 weeks postvaccination for evaluation of peripheral and mucosal immune responses, respectively.

Bacterial culture

M. bovis BCG Danish strain was a gift from Dr. Ray Waters at the National Animal Disease Center, USDA. BCG was prepared using standard techniques in Middlebrook 7H9 liquid media (Becton Dickinson, Franklin Lakes, NJ) supplemented with 10% oleic acid-albumin-dextrose complex (OADC) plus 0.05% Tween 80 (Sigma, St. Louis, Missouri). For *in vitro* and *in vivo* experiments, bacteria in log phase were centrifuged (3000 rpm for 15 minutes) and resuspended

in cRPMI without antibiotics for *in vitro* studies, or sterile PBS for *in vivo* vaccination studies (see below for additional details).

Peripheral blood mononuclear cell preparation, cryopreservation and monocyte isolation

Peripheral blood was collected into 2x acid citrate dextrose via the jugular vein. Peripheral blood monocular cells (PBMCs) were isolated from buffy coats by density centrifugation as previously described (33). Contaminating red blood cells were removed using hypotonic lysis. Cells were washed three times, counted and resuspended in complete RPMI (cRPMI) composed of RPMI-1640 (Gibco, Carlsbad, CA) supplemented with 2 mM L-glutamine, 25 mM HEPES buffer, 1% antibiotic-antimycotic solution, 1% non-essential amino acids 2% essential amino acids, 1% sodium pyruvate, 50 μM 2-mercaptoethanol (all from Sigma, St. Louis, MO), and 10% (v/v) fetal bovine sera (FBS).

PBMCs from the vaccine study where cryopreserved as follows: briefly, PBMCs were resuspended at 2 x10^7 cells/mL in 1 mL of precooled FBS containing 10% dimethyl sulfoxide (DMSO), and rapidly brought to −80°C in polystyrene containers, which ensured a slow drop in temperature. After 24 h, the cryovials were transferred to a liquid nitrogen tank where they remained until analysis.

Monocytes were isolated using Magnetic Activated Cell Sorting (MACS) according to manufacturer's instructions. Briefly, PBMCs were resuspended at 10^7 cells/mL in MACS buffer (0.5% BSA, 2mM EDTA in PBS) and labeled with 10 μg/mL mouse anti-bovine CD14 (Clone CAM36A) from Washington State Monoclonal Antibody center (Pullman, WA) for 20 min at 4°C. Cells were then washed and labeled with anti-mouse IgG1 magnetic beads (Miltenyi Biotech) and purified by positive selection.

Bronchoalveolar lavage fluid collection, cell isolation and cryopreservation

Broncheoalveolar lavage (BAL) samples were collected from the group of calves in the vaccine study (n = 7/ group) at 4 weeks post immunization. A modified stallion catheter was blindly passed through the nose and advanced through the trachea until lodging in the bronchus. A total of 180 mL of sterile saline was divided into three aliquots. An aliquot was introduced to the lower respiratory tract, followed by immediate suction to obtain lower airway washes. The procedure was repeated twice more. All three aliquots were pooled at the end of the procedure. BAL samples were kept on ice, filtered over sterile gauze, and centrifuged at 200 x g for 10 minutes. Contaminating red blood cells were removed using hypotonic lysis. Cells were washed, counted and cryopreserved as described above.

In vitro training model in bovine monocytes

In vitro immune training was performed as described previously (16, 34) (**Fig. 1A).** Briefly, isolated bovine monocytes were plated in a total volume of 200 µl/well of cRPMI, at a concentration of $1x10^5$ cells/well in flat-bottom, 96-well tissue-culture-treated plates (Falcon). Monocytes were incubated with culture medium only as a negative control. Cells were infected at a 5:1 multiplicity of infection (MOI) with BCG strain Danish for 24 hours at 37° C/5% CO_2, washed once with warm culture media, resuspended in cRPMI and incubated for 6 days. The media was replaced once, at day 3. After the incubation, the media was removed and cells were re- stimulated with cRPMI, E. coli lipopolysaccharide (LPS; serotype 055:B5; Sigma-Aldrich, 1 µg/mL), tripalmitoylated lipopeptide (Pam3CSK4; InvivoGen, 10 µg/mL) or polyinosinic:polycytidylic acid (Poly(I:C); InvivoGen, 10 µg/mL) for 4 hours (mRNA expression)

or 72 hours (protein expression). After the indicated times, cells were pelleted, supernatants collected for cytokine measurement and cells lysed with Trizol Reagent (Invitrogen, Life Technologies) for mRNA transcription assessment and stored at -80°C.

In vivo experiments

BCG vaccination

For the vaccine study, each calf in the vaccinated group (n = 7) received $1x10^8$ colony-forming units (CFU) of *Mycobacterium bovis* BCG strain Danish via aerosol inoculation as we have previously described (35). Briefly, the inoculum was nebulized into a mask (Trudell Medical International, London, ON, Canada) covering the nostrils and mouth, allowing regular breathing and delivery of the mycobacteria to the lungs via the nostrils.

Thawing and *ex vivo* stimulation

For thawing, PBMCs or alveolar macrophages were removed from the liquid nitrogen and thawed in a 37°C water bath for 2 minutes. Once thawed, the cells were rapidly transferred to 15-ml polystyrene tubes containing 8 ml of warm cRPMI to remove DMSO. Finally, the cells were washed twice with complete medium and counted. Cell viability was assessed using Trypan Blue method and found to be >85%. For stimulation experiments, PBMCs or BAL cells ($1x10^6$ cells/mL, 1 mL/well) were added to 24-well, tissue-culture-treated plates and rested overnight at 37°C, 5% CO_2. Cell cultures were then washed once with warm cRPMI, and the cells were stimulated with cRPMI (negative control), LPS, Pam3CSK and incubated for 4 hours (mRNA expression) or 72 hours (protein expression). After the indicated times, cells were pelleted, and

lysed with Trizol Reagent (Invitrogen, Life Technologies) or cell culture supernatants were collected, and stored at −80°C.

Real-time PCR

RNA isolation, cDNA preparation and qPCR were performed as described (33). The primer sequences have been published (36, 37) (**Table 1).** Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method, with RPS9 as the reference housekeeping gene (38). Reactions were performed on an Agilent MX3000P Real-Time PCR machine or Quantstudio 3 Real-Time PCR machine. The following amplification conditions were used: 2 min at 50°, 10 min at 95°, 40 cycles of 15 seconds at 95° and 1 min at 60°, and a dissociation step (15 seconds at 95°, 1 min at 60°, 15 seconds at 95°, 15 seconds at 60°).

ELISAs

The concentration of cytokines in cell culture supernatants was determined after 72 hours of incubation, using bovine commercial ELISA kits for interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), and IL-6 (all from Kingfisher Biotech, Inc., St. Paul, MN) according to manufacturer's instructions.

Flow cytometry

For flow cytometric analysis, PBMC were added to 96-well round bottom plates ($1X10^6$ cells/well). Staining was performed at 4°C. Cells were labeled for 25 minutes with Live/Dead Aqua (Thermo Fisher) and 10 μg/mL of the following primary antibodies: mouse anti-bovine CD14 (clone CAM36A) and CD11b (clone MM10A) from Washington State Monoclonal

Antibody center. Cells were washed once, and then incubated for 25 minutes with 0.5 ug/mL of the following secondary antibodies: PeCy7 (IgG1, Biolegend) and APC-Cy7 (IgG2b, Southern biotech). Samples were acquired using a BD LSR Fortessa flow cytometer (BD Biosciences). Data were analyzed using FlowJo (Tree Star Inc., San Carlos, CA).

Metabolite measurements

The concentration of glucose and lactate in cell culture supernatants was determined after 72 hours of incubation, using Glucose Colorimetric Assay Kit (Catalog # K06-100, Biovision) and Lactate Colorimetric Assay Kit (Catalog # K07-100, Biovision), respectively.

Statistics

$2^{-\Delta\Delta Ct}$ method was used in the statistical analysis of relative gene expression (shown as expression relative to uninfected or unstimulated control samples). Statistical comparisons were performed using One-way ANOVA followed by Tukey's multiple comparison test. p value below 0.05 was considered statistically significant. All data were analyzed using GraphPad Prism software version v6.0f (La Jolla, CA, USA). Data are shown as means ± standard errors of the means.

Table 3. Primers used for RT-PCR

Gene	Forward (5'-3')	Reverse (5'-3')	Accession number[1]
IL-6	CTGAAGCAAAAGATCGCAGATCTA	CTCGTTTGAAGACTGCATCTC	NM_173921.2
IL-1b	TTCTGTGTGACGCACCCGTGC	AGCACACATGGGCTAGCCAGC	X54796
TNFα	CGGGGTAATCGGCCCCCAGA	GGCAGCCTTGGCCCCTGAAG	NM_174088.1
RPS9	CGCCTCGACCAAGAGCTGAGG	CCTCCAGACCTCAGGTTTGTTCC	NM_001101152
TLR2	CGATGACTACCGCTGTGACTC	CCTTCCTGGGCTTCCTCTT	NM_174198.6
TLR4	TGCCTTCACTACAGGGACTTT	TGGGACACCACGACAATA AC	NM_174197.2

[1] Accession numbers from NCBI database http://www.ncbi.nih.gov.

RESULTS

In vitro BCG priming induces trained bovine monocytes, characterized by increased transcription of proinflammatory cytokines

In vitro BCG priming induces enhanced proinflammatory cytokine expression in response to unrelated antigens in murine and human monocytes (11, 16, 34, 39). To test if bovine monocytes have the capacity for innate training, we utilized a traditional *in vitro* protocol of trained immunity (**Fig. 18A**) (16, 34). Circulating bovine monocytes were isolated from peripheral blood and exposed to BCG *in vitro* for 24 hours, rested for 6 days and stimulated with unrelated microbe-associated molecular patterns (LPS, Poly(I:C) or Pam3CSK4). RT-PCR was used to analyze changes in gene expression of IL-6 and TNFα. As shown in **Fig. 18B**, bovine monocytes primed *in vitro* with BCG mounted an enhanced response to LPS or Pam3CSK4 compared to non- trained cells, characterized by higher expression of TNFα and IL-6. Interestingly, cells exposed to BCG did not mount an enhanced response following secondary exposure to Poly(I:C) (data not shown). These results suggest that, similar to observations in humans and mice models (16, 34, 39), *in vitro* BCG priming of bovine monocytes induces increased responsiveness to secondary unrelated stimuli, as measured by expression of the proinflammatory cytokines TNFα and IL-6.

BCG vaccination of young calves induces enhanced nonspecific production of monocyte-derived proinflammatory cytokines.

Previous studies have demonstrated that parenteral BCG immunization in healthy adult humans enhances the immune response of peripheral monocytes, macrophages and NK cells upon subsequent *ex vivo* peripheral blood mononuclear cell restimulation (11, 16, 40). Therefore, we next determined if BCG vaccination also had the capacity to train peripheral mononuclear cells in

calves. To explore the possible impact of mucosal immune training on the juvenile immune system, we employed an aerosol route of BCG vaccination for our studies. A total of 14 calves were divided into two groups (n = 7 animals/group). The vaccinated group received $1x10^8$ cfu BCG strain Danish via aerosol inoculation (**Fig. 19A**). The remaining calves served as unvaccinated controls. Four weeks after vaccination, PBMCs were isolated and restimulated *in vitro* with LPS or Pam3CSK4 for 4 hours (mRNA expression) or 72 hours (protein expression). As shown in **Fig. 19B** and **19C**, PBMC from calves receiving *in vivo* BCG vaccination demonstrated significantly enhanced expression of the monocyte-derived proinflammatory cytokines IL-6, Il-1β and TNFα, in response to *ex vivo* stimulation with LPS and Pam3CSK. Together, our results confirm previous reports in human and murine species, showing that BCG vaccination induces an hyperresponsive state in bovine circulating mononuclear cells, characterized by the enhanced production of monocyte-derived proinflammatory cytokines upon secondary stimulation with unrelated antigen. Our results also confirm the ability of BCG to induce memory-like innate immune traits in early life.

BCG vaccination increases glucose consumption and lactate production in bovine trained monocytes

Studies have suggested that trained immunity has a molecular basis as an underlying mechanism, which relies upon specific epigenetic reprogramming that affect immune signaling pathways and cellular metabolism (8-10, 43). Here we observed that when bovine PBMCs form aerosol BCG-vaccinated animals were stimulated *in vitro* with LPS or Pam3CSK4 for 72 hours the amount of glucose and lactate production was significantly higher than PBMCs from unvaccinated animals (**Fig. 20**). Our findings are consistent with human monocytes trained with

β-glucan where a metabolic switch from oxidative phosphorylation to glycolysis has been described (41). Thus, results observed here are compatible with the hypothesis that elevated glycolysis is the metabolic basis for trained immunity in BCG-vaccinated animals.

Aerosol BCG immunization does not alter the number of circulating bovine monocytes or activation marker expression

Compared to cells from non-vaccinated individuals, monocytes and NK cells from BCG-vaccinated human adults and infants display slightly increased expression of the surface markers CD14, CD11b and Toll-like receptor 4 (TLR4); but no changes in TLR2 or dectin-1 (11, 16). To further characterize the phenotype of trained bovine monocytes induced by BCG vaccination, we measured the expression of surface markers by flow cytometry. As seen in Fig. 3A, no differences were detected in the frequency of CD14+ cells or in the surface expression of CD11b+ between vaccinated and unvaccinated calves. Similarly, TLR4 and TLR2 mRNA expression did not differ between groups at 4 weeks after vaccination (**Fig. 20B-C**). In accordance with our results, blocking of TLR4 or TLR2 during preincubation with live BCG did not significantly influence the magnitude of trained immunity in mice (42).

BCG vaccination induces a long-term 'trained' phenotype in bovine PBMCs.

The effects of BCG vaccination on innate immune training have been shown to persist for at least 3 months to a year (6, 16, 40). As seen in **Fig. 21**, BCG vaccination also leads to long-term functional changes in bovine peripheral mononuclear cells. PBMC isolated 12 weeks after vaccination displayed enhanced production of proinflammatory cytokines IL-6, IL-1β and TNFα in response to LPS and Pam3CSK4 compared to the unvaccinated group **(Fig. 21B).**

Bovine alveolar macrophages do not adapt a 'trained' phenotype in response to aerosol BCG vaccination

Respiratory infections are a major cause of juvenile human and animal morbidity and mortality. In the lung, alveolar macrophages (AMs) are the most abundant immune-cell type present under homoeostatic conditions (43). Considering that BCG influences the functional state of circulating monocytes, and that AMs are critical in immunity to respiratory infections, we hypothesized that aerosol BCG vaccination could induce functional changes at the level of the respiratory mucosa, that could enhance the AMs immune properties. Therefore, we collected BAL samples at 4 weeks post aerosol BCG vaccination. Alveolar macrophages were plated at $1x10^5$ cells/well in 24-well plates and stimulated *in vitro* with LPS or Pam3CSK4 for 4 hours (mRNA expression) or 72 hours (protein expression). As shown in **Fig. 22B-C**, compared to the control group, aerosol exposure of BCG did not impact gene and protein expression of the proinflammatory cytokines, IL-6, Il-1β or TNFα, in response to *ex vivo* stimulation with LPS and Pam3CSK4. These results indicate that aerosol BCG vaccination does not 'train' alveolar macrophages from juvenile calves.

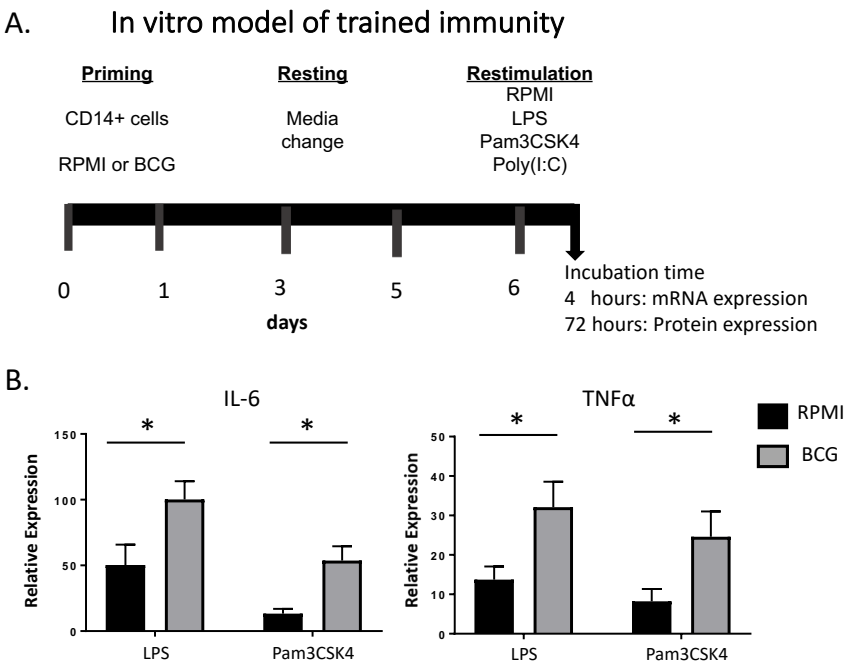

Figure 18. In vitro BCG infection of bovine monocytes enhances the expression of proinflammatory cytokines after stimulation with LPS or Pam3CSK4.

(A) Diagram of the i*n vitro* training model used in bovine isolated monocytes. **(B)** IL-6 and TNFα expression after restimulation for 4 hours. Monocytes isolated from peripheral blood of six healthy heifers were plated at $1x10^6$ cells/mL in a 96-well plate. Cells were infected *in vitro* with BCG at a MOI of 5:1 for 24 hours, washed once with warm cRPMI, incubated for 6 days, and stimulated with LPS, Poly(I:C) or Pam3CSK4 for 4 hours. Uninfected cells (cRPMI) served as negative controls. Changes in gene expression were assessed by RT-PCR. Data represent means ± SEM (n=6). *$p<0.05$.

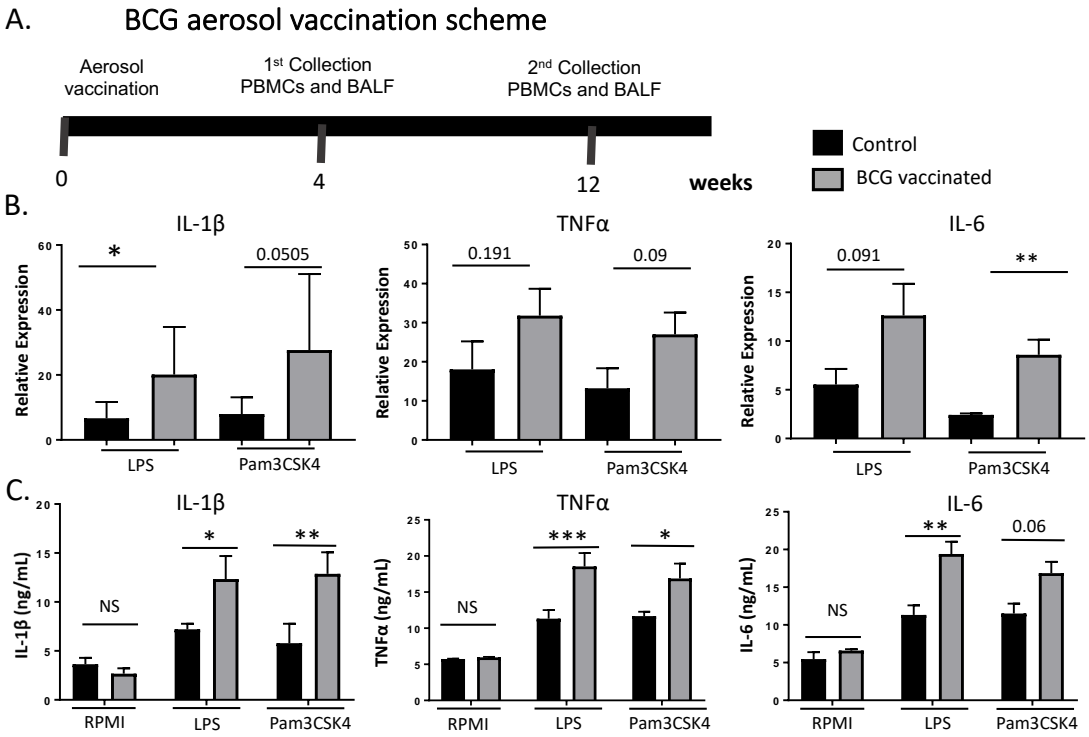

Figure 19. BCG vaccination increased nonspecific production of monocyte-derived proinflammatory cytokines.

(A) Diagram showing the BCG vaccination schedule in calves. Calves were vaccinated with 1 x 10^8 colony forming units (CFU) BCG Danish via aerosol. Peripheral blood was collected four weeks post vaccination from calves in both groups. **(B)** PBMCs isolated from calves after four weeks post vaccination, were stimulated *in vitro* with LPS or Pam3CSK4 for 4 or 72 hours to measure cytokine expression. Proinflammatory cytokine gene expression was assessed by RT-PCR (B), and protein expression was assessed by ELISA on the cell supernatants (C). Data represent means ± SEM. *p<0.05, **p<0.01.

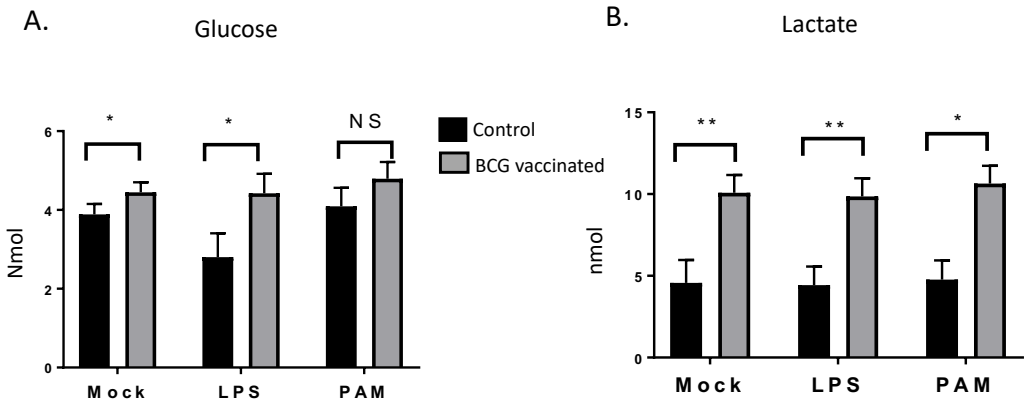

Figure 20. BCG vaccination increases glucose consumption and lactate production in bovine trained monocytes.

Peripheral blood was collected four weeks postvaccination from calves in both groups. PBMCs were stimulated *in vitro* with LPS or Pam3CSK4 for 72 hours to measure glucose and lactate concentration in cell culture media using Glucose Colorimetric Assay Kit (Catalog # K06-100, Biovision) and Lactate Colorimetric Assay Kit (Catalog # K07-100, Biovision), respectively. (**A**) Changes of glucose consumption and lactate production (**B**) of PBMCs from unvaccinated (grey bars) and BCG vaccinated animals (black bars). Data represent means ± SEM. No significant (NS), *p<0.05, **p<0.01, *** p<0.001.

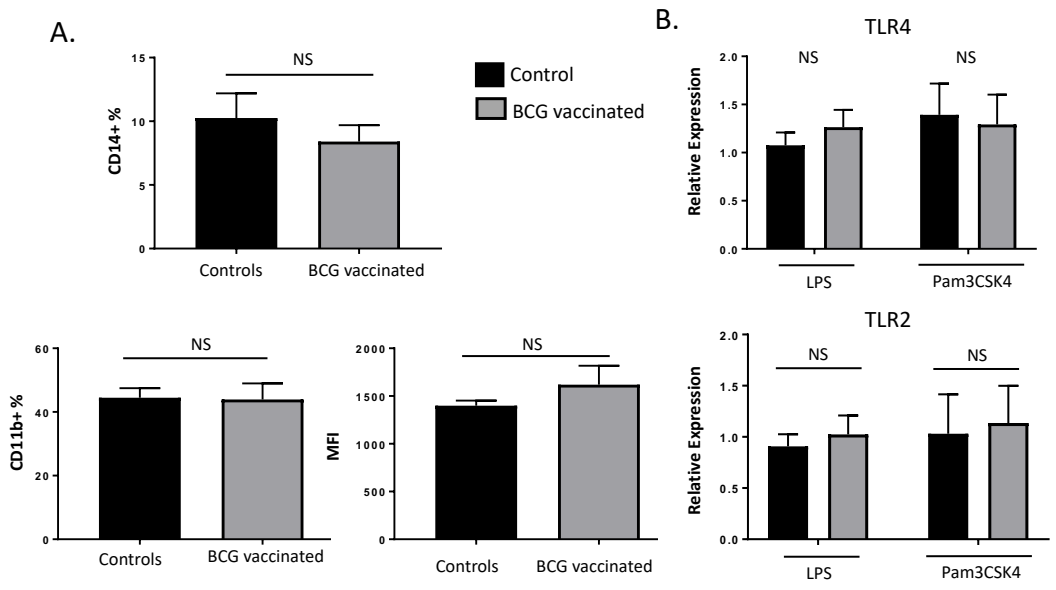

Figure 21. BCG aerosol immunization does not alter the frequency or surface marker expression of bovine circulating monocytes.

(A) Flow cytometric analysis of circulating CD14+ CD11b+ monocytes isolated 4 weeks after BCG immunization. Average frequencies of CD14+ CD11b+ cells isolated from both groups of calves (n = 7/group). **(B)** Monocytes were isolated as described in Materials and Methods section and analyzed by RT-PCR for the mRNA expression of TLR2 and TLR4, after 4 weeks of BCG vaccination. Data represent means ± SEM. No significant (NS) differences were observed between treatment groups.

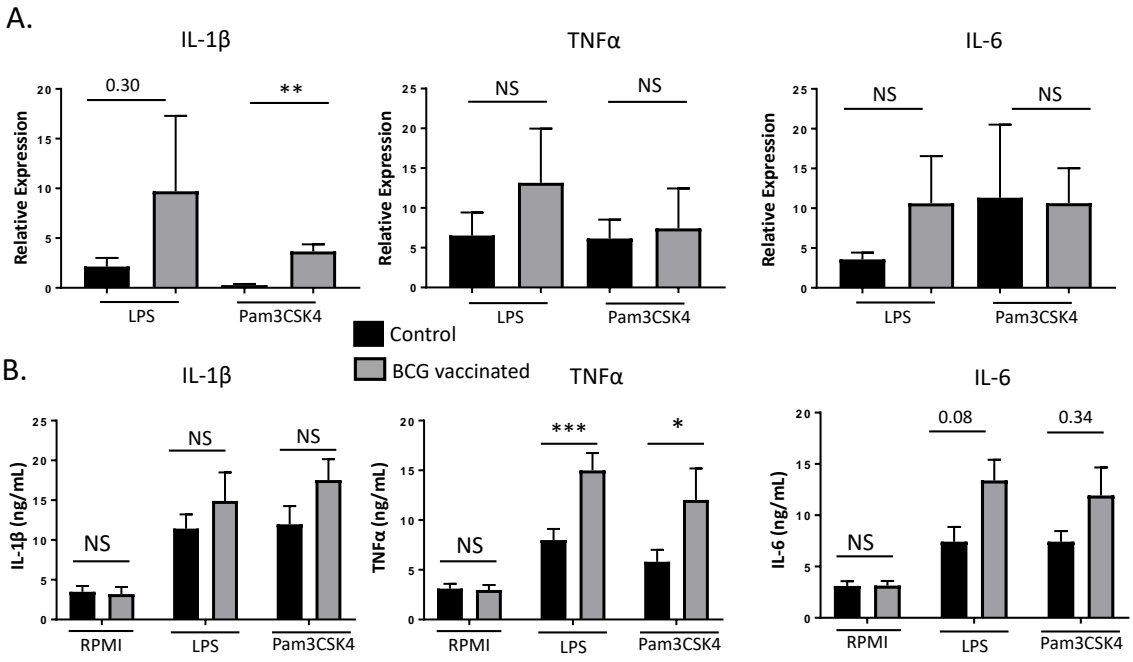

Figure 22. BCG vaccination induces long-term 'trained' phenotype in bovine PBMCs. PBMCs isolated from calves after twelve weeks postvaccination, were stimulated *in vitro* with LPS or Pam3CSK4 for 4 or 72 hours to measure cytokine expression. Proinflammatory cytokine gene expression was assessed by RT-PCR **(A)** and protein expression was assessed by ELISA in the supernatants **(B).** Data represent means ± SEM. No significant (NS), *p<0.05, **p<0.01, *** p<0.001.

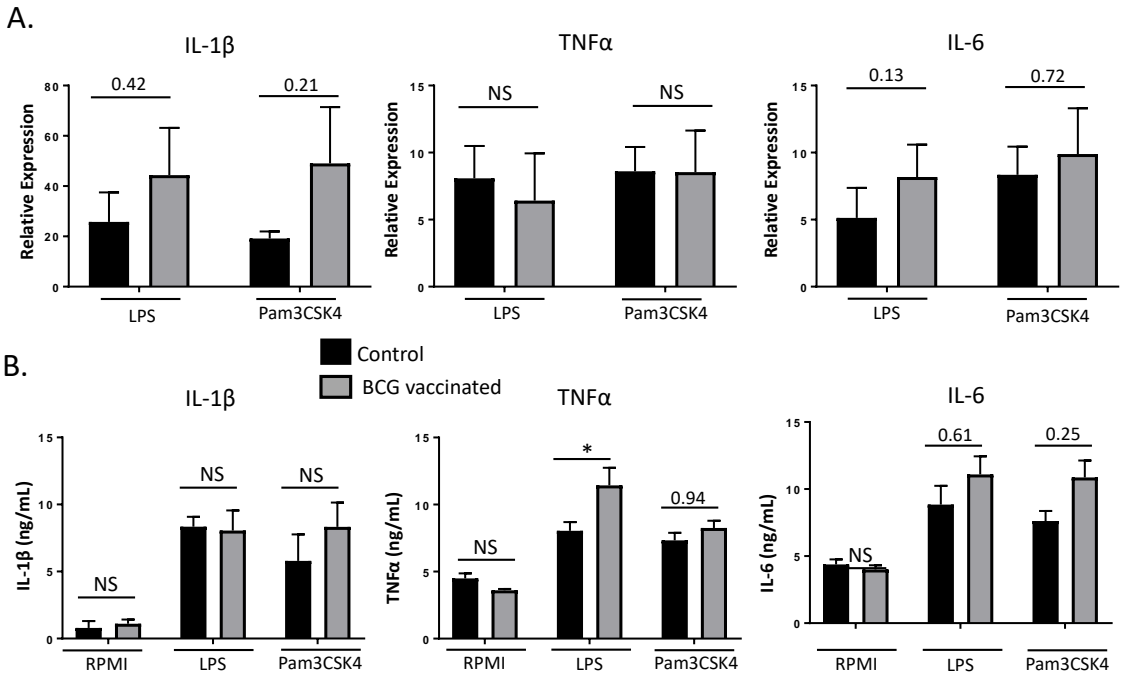

Figure 23.BCG aerosol vaccination does not alter the function of alveolar macrophages. Calves were vaccinated with $1x10^8$ colony forming units (CFU) BCG Danish via aerosol. Peripheral blood was collected four weeks post vaccination from calves in both groups. PBMCs isolated from calves after four weeks post vaccination, were stimulated *in vitro* with LPS or Pam3CSK4 for 4 or 72 hours to measure cytokine expression. Proinflammatory cytokine gene expression was assessed by RT-PCR **(A),** and protein expression was assessed by ELISA in the supernatants **(B).** Data represent means ± SEM. No significant (NS), *p<0.05.

DISCUSSION

BCG parenteral vaccination has long been known to have beneficial effects against childhood diseases other than TB, and it has recently become clear that these effects arise through a mechanism known as innate training or 'trained' immunity (7, 8, 16). However, the nonspecific effects of BCG have yet to be studied in agricultural species. Here, we demonstrate that, consistent with previous studies in human and mouse models (11, 16, 29, 32, 33), BCG vaccination has the capacity to 'train' bovine PBMCs, leading to a significantly enhanced TLR-induced proinflammatory cytokine response compared to PBMCs from control calves (**Fig. 2**). Thus, our observations have important clinical relevance, as they suggest that the use of BCG vaccination is likely to confer improved disease resistance, through at least 6 months of age, when the 'window of susceptibility' to infection with common diseases in juvenile animals is greatest.

In agreement with data obtained in plants and invertebrates, studies have suggested that trained immunity has a molecular basis as an underlying mechanism, which relies upon specific epigenetic reprogramming that affect immune signaling pathways and cellular metabolism (8-10, 44). In this study, we identify metabolic pathways induced in bovine PBMCs by aerosol BCG vaccination, demonstrating a metabolic switch toward aerobic glycolysis (**Fig. 5**). Similarly, earlier studies reported that cell activation and proliferation induced a switch toward aerobic glycolysis in macrophages (45). Subsequent studies in human monocytes, identified a similar metabolic switch after β-glucan priming that involve distinct histone methylations at H3K4 mark with chromatin remodeling at a subset of cytokine promoters that lead to transcriptional programs associated with genes of the mTOR pathway (10, 16, 41). To our knowledge our study is the first to identify the glycolytic process induced by BCG training *in vivo*. Our results show that BCG

training not only induces increased cytokine production but also identifies glycolysis as a fundamental molecular basis.

There is little information on the duration of the transcriptional changes provided by trained immunity. Regarding trained immunity in monocytes, epidemiological studies in children have shown that BCG vaccination induces nonspecific protection for at least the duration of early childhood (46). Considering the short half-life of circulating monocytes, this suggests that the epigenetic reprogramming occurs in the bone marrow progenitors (17, 47). Identification of core signatures of innate training is thus important, as it allows the better characterization of these functional states, and may have consequences in the selection of antigens, development of adjuvants and vaccine design.

In addition to epigenetic reprogramming, the functional modulation of monocytes, macrophages and NK cells during trained immunity has been linked to an slightly increased expression of PRRs and activation markers crucial for pathogen recognition such as CD14, CD11b, and Toll-like receptor 4 (TLR4), but no changes in TLR2 or dectin-1 (11, 16). In our study, no differences were detected in the frequencies of circulating monocytes (CD14+), or in the surface expression of CD11b+ between vaccinated and unvaccinated calves. Also, TLR4 and TLR2 mRNA expression did not differ between BCG-vaccinated and control calves 4 weeks after vaccination (**Fig. 3A-C**). In accordance with our results, blocking of TLR4 or TLR2 during preincubation with live BCG did not significantly influence the magnitude of trained immunity in mice (42), suggesting that the 'trained' phenotype is likely not simply due to increased expression of PRR or other surface activation markers. A limitation of our study is that we did not investigate the molecular mechanism by which trained immunity was induced in bovine cells. However, epigenetic alterations that facilitate gene transcription has been demonstrated to a mechanism that

influences the 'trained' immune response to secondary stimulation in other models (9-11, 16, 17). While this was beyond the aims of the present study, future experiments in cattle should aim to identify the epigenetic program induced after BCG priming, as it an important question that will allow better characterization of these functional states.

The mucosal surface of the respiratory tract represents one of the principal portals of entry for most human and animal pathogens. Juvenile agricultural species, similar to young human infants, are most susceptible to respiratory diseases during the first weeks of life, as the adaptive immune system develops. Alveolar macrophages (AMs) have an essential role in lung innate immunity and in protecting the host against respiratory diseases (43). AMs fulfil a variety of functions including, removal of cellular debris, immune surveillance, microbial clearance, responses to infection and the resolution of inflammation. Given that AMs are an important regulator of the local innate response against respiratory pathogens, it is logical to assume that enhanced function may be a more efficient at reducing the risk of respiratory infections and thus, reducing juvenile mortality. However, few studies have looked at the ability to induce an immune 'trained' phenotype in AMs. In the current study, we assessed if aerosol BCG vaccination induces functional changes at the level of the respiratory mucosa that could alter the functional properties of AMs. As shown in **Fig. 6B-C**, our data demonstrate that AM's from BCG-immunized calves do not adopt a 'trained' phenotype in response to *ex vivo* stimulation with LPS and Pam3CSK4. Similar to our results, a low dose of intranasal administration of BCG (TICE strain) in mice has been shown to result in decreased TNF-α mRNA expression and increased IL-10 mRNA expression in AMs (48). We speculate that the inability to induce a 'trained' phenotype in AM's after BCG vaccination is related to the inherent immunosuppressive properties of AM and the lung environment, as demonstrated by the upregulation of IL-10 in mice (43, 48). The type of

hyperresponsive innate immune state induced by innate training has beneficial effects during host defense, as showed in epidemiological studies, but it could also trigger enhanced tissue damage (49). It is worth to mentioning, however, that although AMs from mice in the study of Mukherjee *et. al*. exhibits an immunosuppressive phenotype, the animals were still protected against a lethal infection with mouse-adapted influenza virus A/Puerto Rico/8/34 (PR8) (H1N1) after intranasal BCG vaccination (48). Moreover, the nasal route of BCG immunization was more effective than the systemic route against the respiratory challenge. The authors hypothesize that this enhanced resistance is due to increased efferocytosis in animals receiving intranasal BCG vaccination. Thus, limiting immune responses to foreign antigens may be an essential form of adaptation that reduces tissue damage in sites where high pathogen burden occurs, and thus may be an aspect of lung tolerance and resistance to infection. Future studies should be aimed at comparing different routes of immune training (parenteral or mucosal), identifying possible therapies that induce mucosal innate training of AMs, and determining the potential of mucosal 'trained' immunity to prevent respiratory infection.

Epidemiological studies show that the nonspecific effects of BCG vaccination are most pronounced in the first year of life, suggesting that trained immunity is most strongly activated during this first year (1, 2). However, few immunological studies in human infants and neonates have been performed, due to ethical concerns and difficulty obtaining samples. Thus, the full spectrum of innate training and its capacity to promote disease resistance in children has not been fully explored. The calf represents an excellent translational model to investigate the effect of BCG vaccination on susceptibility to common respiratory diseases, as it is a tractable model of the infant immune system and allows for the longitudinal collection of large volumes of peripheral blood and BAL fluid that can be used for cytological, immunological or virological studies. Thus,

studies in the calf could benefit both human and animal health and provide information to develop novel vaccination strategies that would more effectively induce both classic adaptive immunity and innate trained immunity to reduce the incidence of common pathogens.

In conclusion, although the effects of BCG on trained immunity have been described before, we provide evidence for the first time that in the young calf, *in vivo* BCG training induces memory-like traits comparable to what has been described in adult human volunteers and mouse models. While BCG vaccination might not be an ideal agent to use for innate training in agricultural species due to regulatory concerns (i.e. skin-testing for monitoring *M. bovis* infection), our results warrant further research to identify the microbial components of BCG capable of combining effective induction of adaptive immune responses with elicitation of trained immunity, to enhance diseases resistance against infectious agents in both humans and agricultural species.

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REFERENCES

1. Aaby P, Roth A, Ravn H, Napirna BM, Rodrigues A, Lisse IM, et al. Randomized trial of BCG vaccination at birth to low-birth-weight children: beneficial nonspecific effects in the neonatal period? J Infect Dis. 2011;204(2):245-52.

2. Biering-Sorensen S, Aaby P, Napirna BM, Roth A, Ravn H, Rodrigues A, et al. Small randomized trial among low-birth-weight children receiving bacillus Calmette-Guerin vaccination at first health center contact. Pediatr Infect Dis J. 2012;31(3):306-8.

3. Levy O, Levy O. Ready to benefit from training: heterologous effects of early life immunization. Trans R Soc Trop Med Hyg. 2015;109(1):3-4.

4. Hollm-Delgado MG, Stuart EA, Black RE. Acute lower respiratory infection among Bacille Calmette-Guerin (BCG)-vaccinated children. Pediatrics. 2014;133(1):e73-81.

5. Blok BA, Arts RJ, van Crevel R, Benn CS, Netea MG. Trained innate immunity as underlying mechanism for the long-term, nonspecific effects of vaccines. J Leukoc Biol. 2015;98(3):347-56.

6. Netea MG, van Crevel R. BCG-induced protection: effects on innate immune memory. Semin Immunol. 2014;26(6):512-7.

7. Netea MG, Quintin J, van der Meer JW. Trained immunity: a memory for innate host defense. Cell Host Microbe. 2011;9(5):355-61.

8. Netea MG, Joosten LA, Latz E, Mills KH, Natoli G, Stunnenberg HG, et al. Trained immunity: A program of innate immune memory in health and disease. Science. 2016;352(6284):aaf1098.

9. Quintin J, Saeed S, Martens JHA, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, et al. Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. Cell Host Microbe. 2012;12(2):223-32.

10. Saeed S, Quintin J, Kerstens HH, Rao NA, Aghajanirefah A, Matarese F, et al. Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. Science. 2014;345(6204):1251086.

11. Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Jacobs C, Xavier RJ, et al. BCG-induced trained immunity in NK cells: Role for non-specific protection to infection. Clin Immunol. 2014;155(2):213-9.

12. Di Luzio NR, Williams DL. Protective effect of glucan against systemic Staphylococcus aureus septicemia in normal and leukemic mice. Infect Immun. 1978;20(3):804-10.

13. Marakalala MJ, Williams DL, Hoving JC, Engstad R, Netea MG, Brown GD. Dectin-1 plays a redundant role in the immunomodulatory activities of beta-glucan-rich ligands in vivo. Microbes Infect. 2013;15(6-7):511-5.

14. Munoz N, Van Maele L, Marques JM, Rial A, Sirard JC, Chabalgoity JA. Mucosal administration of flagellin protects mice from Streptococcus pneumoniae lung infection. Infect Immun. 2010;78(10):4226-33.

15. Sun JC, Beilke JN, Lanier LL. Adaptive immune features of natural killer cells. Nature. 2009;457(7229):557-61.

16. Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Ifrim DC, Saeed S, et al. Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. Proc Natl Acad Sci U S A. 2012;109(43):17537-42.

17. Kaufmann E, Sanz J, Dunn JL, Khan N, Mendonca LE, Pacis A, et al. BCG Educates Hematopoietic Stem Cells to Generate Protective Innate Immunity against Tuberculosis. Cell. 2018;172(1-2):176-90 e19.

18. Aaby P, Kollmann TR, Benn CS. Nonspecific effects of neonatal and infant vaccination: public-health, immunological and conceptual challenges. Nat Immunol. 2014;15(10):895-9.

19. van 't Wout JW, Poell R, van Furth R. The role of BCG/PPD-activated macrophages in resistance against systemic candidiasis in mice. Scand J Immunol. 1992;36(5):713-9.

20. Parra M, Liu X, Derrick SC, Yang A, Tian J, Kolibab K, et al. Molecular analysis of non-specific protection against murine malaria induced by BCG vaccination. PLoS One. 2013;8(7):e66115.

21. Ota MO, Vekemans J, Schlegel-Haueter SE, Fielding K, Sanneh M, Kidd M, et al. Influence of Mycobacterium bovis bacillus Calmette-Guerin on antibody and cytokine responses to human neonatal vaccination. J Immunol. 2002;168(2):919-25.

22. Semple PL, Watkins M, Davids V, Krensky AM, Hanekom WA, Kaplan G, et al. Induction of granulysin and perforin cytolytic mediator expression in 10-week-old infants vaccinated with BCG at birth. Clin Dev Immunol. 2011;2011:438463.

23. Benn CS, Netea MG, Selin LK, Aaby P. A small jab - a big effect: nonspecific immunomodulation by vaccines. Trends Immunol. 2013;34(9):431-9.

24. USDA. Part III. Health and Management and Biosecurity in U.S. Feedlots. 1999.

25. USDA. PartII. changes in the U.S. Dairy Cattle Industry. 2007.

26. Belyakov IM, Ahlers JD. What role does the route of immunization play in the generation of protective immunity against mucosal pathogens? J Immunol. 2009;183(11):6883-92.

27. Whittaker E, Goldblatt D, McIntyre P, Levy O. Neonatal Immunization: Rationale, Current State, and Future Prospects. Front Immunol. 2018;9:532.

28. Buddle BM, Wedlock DN, Parlane NA, Corner LA, De Lisle GW, Skinner MA. Revaccination of neonatal calves with Mycobacterium bovis BCG reduces the level of protection against bovine tuberculosis induced by a single vaccination. Infect Immun. 2003;71(11):6411-9.

29. Hope JC, Thom ML, Villarreal-Ramos B, Vordermeier HM, Hewinson RG, Howard CJ. Vaccination of neonatal calves with Mycobacterium bovis BCG induces protection against intranasal challenge with virulent M. bovis. Clin Exp Immunol. 2005;139(1):48-56.

30. Waters WR, Palmer MV, Nonnecke BJ, Thacker TC, Scherer CF, Estes DM, et al. Failure of a Mycobacterium tuberculosis DeltaRD1 DeltapanCD double deletion mutant in a neonatal calf aerosol M. bovis challenge model: comparisons to responses elicited by M. bovis bacille Calmette Guerin. Vaccine. 2007;25(45):7832-40.

31. Endsley JJ, Waters WR, Palmer MV, Nonnecke BJ, Thacker TC, Jacobs WR, Jr., et al. The calf model of immunity for development of a vaccine against tuberculosis. Vet Immunol Immunopathol. 2009;128(1-3):199-204.

32. Giudice EL, Campbell JD. Needle-free vaccine delivery. Adv Drug Deliv Rev. 2006;58(1):68-89.

33. McGill JL, Rusk RA, Guerra-Maupome M, Briggs RE, Sacco RE. Bovine Gamma Delta T Cells Contribute to Exacerbated IL-17 Production in Response to Co-Infection with Bovine RSV and Mannheimia haemolytica. PLoS One. 2016;11(3):e0151083.

34. Bekkering S, Blok BA, Joosten LA, Riksen NP, van Crevel R, Netea MG. In Vitro Experimental Model of Trained Innate Immunity in Human Primary Monocytes. Clin Vaccine Immunol. 2016;23(12):926-33.

35. Palmer MV, Waters WR, Whipple DL. Aerosol delivery of virulent Mycobacterium bovis to cattle. Tuberculosis (Edinb). 2002;82(6):275-82.

36. Sacco RE, Nonnecke BJ, Palmer MV, Waters WR, Lippolis JD, Reinhardt TA. Differential expression of cytokines in response to respiratory syncytial virus infection of calves with high or low circulating 25-hydroxyvitamin D3. PLoS One. 2012;7(3):e33074.

37. Fu Y, Liu B, Feng X, Liu Z, Liang D, Li F, et al. Lipopolysaccharide increases Toll-like receptor 4 and downstream Toll-like receptor signaling molecules expression in bovine endometrial epithelial cells. Vet Immunol Immunopathol. 2013;151(1-2):20-7.

38. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.

39. Arts RJ, Blok BA, van Crevel R, Joosten LA, Aaby P, Benn CS, et al. Vitamin A induces inhibitory histone methylation modifications and down-regulates trained immunity in human monocytes. J Leukoc Biol. 2015;98(1):129-36.

40. Kleinnijenhuis J, Quintin J, Preijers F, Benn CS, Joosten LA, Jacobs C, et al. Long-lasting effects of BCG vaccination on both heterologous Th1/Th17 responses and innate trained immunity. J Innate Immun. 2014;6(2):152-8.

41. Cheng SC, Quintin J, Cramer RA, Shepardson KM, Saeed S, Kumar V, et al. mTOR- and HIF-1alpha-mediated aerobic glycolysis as metabolic basis for trained immunity. Science. 2014;345(6204):1250684.

42. Arts RJ, Blok BA, Aaby P, Joosten LA, de Jong D, van der Meer JW, et al. Long-term in vitro and in vivo effects of gamma-irradiated BCG on innate and adaptive immunity. J Leukoc Biol. 2015;98(6):995-1001.

43. Allard B, Panariti A, Martin JG. Alveolar Macrophages in the Resolution of Inflammation, Tissue Repair, and Tolerance to Infection. Front Immunol. 2018;9:1777.

44. Kurtz J. Specific memory within innate immune systems. Trends Immunol. 2005;26(4):186-92.

45. Rodriguez-Prados JC, Traves PG, Cuenca J, Rico D, Aragones J, Martin-Sanz P, et al. Substrate fate in activated macrophages: a comparison between innate, classic, and alternative activation. J Immunol. 2010;185(1):605-14.

46. Stensballe LG, Nante E, Jensen IP, Kofoed PE, Poulsen A, Jensen H, et al. Acute lower respiratory tract infections and respiratory syncytial virus in infants in Guinea-Bissau: a beneficial effect of BCG vaccination for girls community based case-control study. Vaccine. 2005;23(10):1251-7.

47. Mitroulis I, Ruppova K, Wang B, Chen LS, Grzybek M, Grinenko T, et al. Modulation of Myelopoiesis Progenitors Is an Integral Component of Trained Immunity. Cell. 2018;172(1-2):147-61 e12.

48. Mukherjee S, Subramaniam R, Chen H, Smith A, Keshava S, Shams H. Boosting efferocytosis in alveolar space using BCG vaccine to protect host against influenza pneumonia. PLoS One. 2017;12(7):e0180143.

49. Arts RJW, Joosten LAB, Netea MG. The Potential Role of Trained Immunity in Autoimmune and Autoinflammatory Disorders. Front Immunol. 2018;9:298.

Chapter 5 - SUMMARY AND FUTURE DIRECTIONS

In the studies described here I describe a system in which infection with *M. bovis* generates memory-like responses in bovine immune cells. In the first part of my research, I characterized *bovine M. bovis*-specific γδ T cell effector/memory subsets by the development of a defined, surface marker expression panel, similar to previously reported in non-human primates and humans (1, 2). Following *M. bovis* infection, a marked *in vitro* expansion of γδ T cells suggests that bovine γδ T cells can mount memory-like responses upon restimulation with mycobacterial antigen. Further, I demonstrate that virulent *M. bovis* infection modulates peripheral γδ T cell responses, and elicits phenotypic characteristics consistent with that of TCM (CD45R- CD27+) cells (3, 4). Contrary to virulent infection, attenuated *M. bovis* BCG infection elicited both γδ TEM (CD45R- CD27-) and TCM (CD45R- CD27+) subsets in peripheral blood, suggesting that mycobacterial virulence may influence γδ T cell memory differentiation. In particular, the antigen-specific γδ T cell response in the mucosal compartment following BCG infection showed a unique modulation of surface markers characteristic of γδ TEM (CD45R- CD27-) cells when stimulated with antigen compared to that which occurred in the peripheral compartment. Together with the phenotypic expression of memory markers CD45R and CD27 our results indicate that in the bovine, antigen-specific γδ T cells can be characterized of TCM and TEM subsets based on the expression of those markers in CD4 T cells. Our observations on chemokine modulation, also provide some insight into the mechanism utilized by antigen-specific γδ T cells to rapidly localize to sites of inflammation at early stages of the granuloma development.

Our results appear to be consistent with observations in patients with active TB, were TEM γδ T cells are reduced in peripheral blood, and are successfully reestablished to basal levels after

drug therapy (4, 5). Moreover, expansion of TEM γδ T cells through vaccination or phosphoantigen administration correlates with protection against fatal TB in non-human primates (6-9). In recent work by Shen et al., they demonstrate that selective activation of Vγ2Vδ2 T cells *in vivo* with a single respiratory administration of phosphoantigen (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP)-producing attenuated *Listeria monocytogenes* (Lm *ΔactA prfA**), elicited robust Vγ2Vδ2 T cell expansion, Th1-like responses, and reduced TB pathology after moderate-dose TB challenge. In this study, analysis of antigen-specific Vγ2Vδ2 T cells revealed that protection correlated with rapid memory-like, Th1-like Vγ2Vδ2 T cell response, the presence of tissue-resident Vγ2Vδ2 T effectors coproducing IFNγ and perforin, inhibition of intracellular *M. t*b growth, and enhanced CD4 and CD8 T cell responses (8).

These observations together with those described here, suggest that the capacity to selectively activate bovine γδ TEM subsets in the peripheral and the mucosal compartment, may relate with protection or resistance to TB infection (10). However, the implication of these γδ T cell changes in the bovine following *M. bovis* infection, cannot be fully appreciated, since the role of each γδ T effector/memory subset was not assessed. Phenotypic analysis of human T cells has shown that memory CD27+ T cell subset have higher clonogenic potential and are able to secrete IL-2, IFNγ and TNFα. This subset does not exhibit cytolytic activity without prior *in vitro* stimulation (11). By comparison, CD27- γδ T cell subset can produce IFNγ and TNFα but do not secrete IL-2 and depend on IL-2 and IL-15 for proliferation (12). Interestingly, CD27− cells contain perforin and granzyme and have high cytolytic activity without in vitro stimulation (11). Previous reports in the bovine, have shown that γδ T cells are capable of producing large amounts of Th1 cytokines, and inhibiting intracellular mycobacterial growth by producing perforin and granzyme (13). Thus, further studies should identify the conditions for optimal activation of γδ

TEM subsets, and the functional role of each subset *in vitro,* using simultaneous staining with CD45R and CD27.

Effector T cell function is dependent not only on the expression of cytokines but also on the capacity of these cells to migrate to sites of antigen encounter. Examination of the lung-homing receptors, CXCR3 and CCR5, from circulating antigen-specific γδ T cell populations revealed that *M. bovis* infection resulted in upregulation of chemokine receptors when stimulated with antigen *in vitro*. These results suggest that there might be a preferential migration of antigen-specific γδ T cells to the lung from peripheral blood after *M. bovis* infection, as seen in granuloma lesions. Surprisingly, BAL fluid recovered antigen-specific γδ T cells downregulated CXCR3 expression when stimulated with antigen *in vitro*. Therefore, we cannot exclude the possibility that different populations of γδ T cells in the airways respond to infection. Thus, detailed characterization of *M. bovis*-specific γδ T cells using an *in vitro* migration assay might provide insight into the mechanism involved in γδ T cell tissue trafficking and homing and might explain differences between circulating and mucosal γδ T cells chemokine expression seen here. Understanding these changes may shed light into strategies to more efficiently engage the γδ T cell response and may provide insight into incorporating γδ T cell specific activation in future vaccine strategies may confer enhanced protection against TB (4, 8).

There is increasing evidence that appropriate activation of innate immune system is essential for protection against TB (14). Earlier reports have shown that BCG induces a long-term state of "trained immunity" in innate cells that confers increased non-specific protection (15, 16). Therefore, the second part of this work was to test if *M. bovis* BCG vaccination elicits memory-like traits in bovine innate cells, as described in humans and mice [reviewed (17)]. We demonstrate

that *M. bovis* BCG infection induces a 'trained' phenotype in bovine cells, characterized by increased production of proinflammatory cytokines, IL-1β, TNFα and IL-6, in response to secondary TLR challenge. We also demonstrate that this cellular hyperresponsiveness correlated with a metabolic shift towards glycolysis, previously implicated in innate immune memory (18). The possibility that BCG elicits strong boosting effects on innate immune responses can have important consequences for developing new TB vaccines that may act more efficiently if combining effects on adaptive and innate immunity. However, very little has been done to address this in the field of TB immunology. Further work is needed to define if part of the protection induced by BCG against TB is mediated by innate immune memory and to investigate the mechanisms responsible for these effects. Since the work described here utilized a live bacterium as 'priming' antigen, there are several possible components that may have elicited the response. Analysis of the mycobacterial components capable of stimulating this response in innate cells may provide insight into the mechanisms involved in 'training' and thus, provide better strategies to engage the innate immune system in the early response to mycobacteria.

In addition to the changes in innate immunity in peripheral blood seen here and reported by others (15), there may be differences in respiratory mucosal innate cells. Interestingly, a distinct response was observed here after BCG priming of in BAL fluid cells. We observed that aerosol BCG vaccination did not result in enhanced cytokine production following TLR stimulation in bovine AMs *in vitro*. Suggesting that a tissue-specific immune response may occur in the lung in response to BCG infection. However, this experiment was performed in only 7 vaccinated calves at one fixed time point after *ex vivo* stimulation. Given the strong variation in the outbred population, a larger number of animals is strongly warranted. Furthermore, in infection models with *M. tb* and *M. avium*, an overall shift of glucose metabolism from oxidative phosphorylation

to fermentation (the Warburg effect) has been reported, and this was linked to pro-inflammatory cytokine production and bacterial survival.

In recent work by Gleeson et al., they demonstrated a shift from oxidative phosphorylation to anaerobic glycolysis in primary human AMs infected with *M. tb in vitro*. Inhibition of this shift resulted in decreased levels of proinflammatory IL-1β and decreased, increased levels of anti-inflammatory IL-10, and increased intracellular bacillary survival. Although induction of glycolytic reprogramming by *M. tb* infection did not directly impact TNF-α production, IL-1β was shown to augment TNF-α signaling; thus, glycolysis may also affect this cytokine indirectly at later time points (19). Thus, an effective vaccine against TB might need to induce metabolic changes in AM that could facilitate early enhanced clearance of the bacillus. Additionally, drugs that manipulate host metabolism (i.e., metformin, meclizine) may be exploited as adjuvants for future therapeutic and vaccination strategies. Further studies in cattle, are needed in order to identify the optimal methods to enhance immunometabolism and training immunity in the future that may be specifically relevant to develop protective vaccination strategies against TB.

It is of pivotal importance to look beyond assumptions that Th1 cells and their cytokine signature are cornerstone for protection against TB. There is good evidence that effective control of *M. bovis* infection requires not only properly activated adaptive immune responses but may also benefit from nonconventional memory-like responses from populations such as 'trained' innate monocytes and differentiated γδ T cells. Our results are promising but a much better understanding of the memory-like responses is urgently needed. More investigation is needed to optimize strategies to prime/boost memory-like responses and may be gained from well-planned studies in the bovine model.

References

1. Dieli F, Ivanyi J, Marsh P, Williams A, Naylor I, Sireci G, et al. Characterization of lung gamma delta T cells following intranasal infection with Mycobacterium bovis bacillus Calmette-Guerin. J Immunol. 2003;170(1):463-9.

2. Martino A, Casetti R, Sacchi A, Poccia F. Central memory Vgamma9Vdelta2 T lymphocytes primed and expanded by bacillus Calmette-Guerin-infected dendritic cells kill mycobacterial-infected monocytes. J Immunol. 2007;179(5):3057-64.

3. Dieli F, Poccia F, Lipp M, Sireci G, Caccamo N, Di Sano C, et al. Differentiation of effector/memory Vdelta2 T cells and migratory routes in lymph nodes or inflammatory sites. J Exp Med. 2003;198(3):391-7.

4. Gioia C, Agrati C, Casetti R, Cairo C, Borsellino G, Battistini L, et al. Lack of CD27-CD45RA-V gamma 9V delta 2+ T cell effectors in immunocompromised hosts and during active pulmonary tuberculosis. J Immunol. 2002;168(3):1484-9.

5. Meraviglia S, El Daker S, Dieli F, Martini F, Martino A. gammadelta T cells cross-link innate and adaptive immunity in Mycobacterium tuberculosis infection. Clin Dev Immunol. 2011;2011:587315.

6. Shen Y, Zhou D, Qiu L, Lai X, Simon M, Shen L, et al. Adaptive immune response of Vgamma2Vdelta2+ T cells during mycobacterial infections. Science. 2002;295(5563):2255-8.

7. Qaqish A, Huang D, Chen CY, Zhang Z, Wang R, Li S, et al. Adoptive Transfer of Phosphoantigen-Specific gammadelta T Cell Subset Attenuates Mycobacterium tuberculosis Infection in Nonhuman Primates. J Immunol. 2017;198(12):4753-63.

8. Shen L, Frencher J, Huang D, Wang W, Yang E, Chen CY, et al. Immunization of Vgamma2Vdelta2 T cells programs sustained effector memory responses that control tuberculosis in nonhuman primates. Proc Natl Acad Sci U S A. 2019;116(13):6371-8.

9. Chen CY, Yao S, Huang D, Wei H, Sicard H, Zeng G, et al. Phosphoantigen/IL2 expansion and differentiation of Vgamma2Vdelta2 T cells increase resistance to tuberculosis in nonhuman primates. PLoS Pathog. 2013;9(8):e1003501.

10. Hoft DF, Brown RM, Roodman ST. Bacille Calmette-Guerin vaccination enhances human gamma delta T cell responsiveness to mycobacteria suggestive of a memory-like phenotype. J Immunol. 1998;161(2):1045-54.

11. Baars PA, Maurice MM, Rep M, Hooibrink B, van Lier RA. Heterogeneity of the circulating human CD4+ T cell population. Further evidence that the CD4+CD45RA-CD27- T cell subset contains specialized primed T cells. J Immunol. 1995;154(1):17-25.

12. Weninger W, Crowley MA, Manjunath N, von Andrian UH. Migratory properties of naive, effector, and memory CD8(+) T cells. J Exp Med. 2001;194(7):953-66.

13. Skinner MA, Parlane N, McCarthy A, Buddle BM. Cytotoxic T-cell responses to Mycobacterium bovis during experimental infection of cattle with bovine tuberculosis. Immunology. 2003;110(2):234-41.

14. Bhatt K, Verma S, Ellner JJ, Salgame P. Quest for correlates of protection against tuberculosis. Clin Vaccine Immunol. 2015;22(3):258-66.

15. Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Ifrim DC, Saeed S, et al. Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. Proc Natl Acad Sci U S A. 2012;109(43):17537-42.

16. Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Jacobs C, Xavier RJ, et al. BCG-induced trained immunity in NK cells: Role for non-specific protection to infection. Clin Immunol. 2014;155(2):213-9.

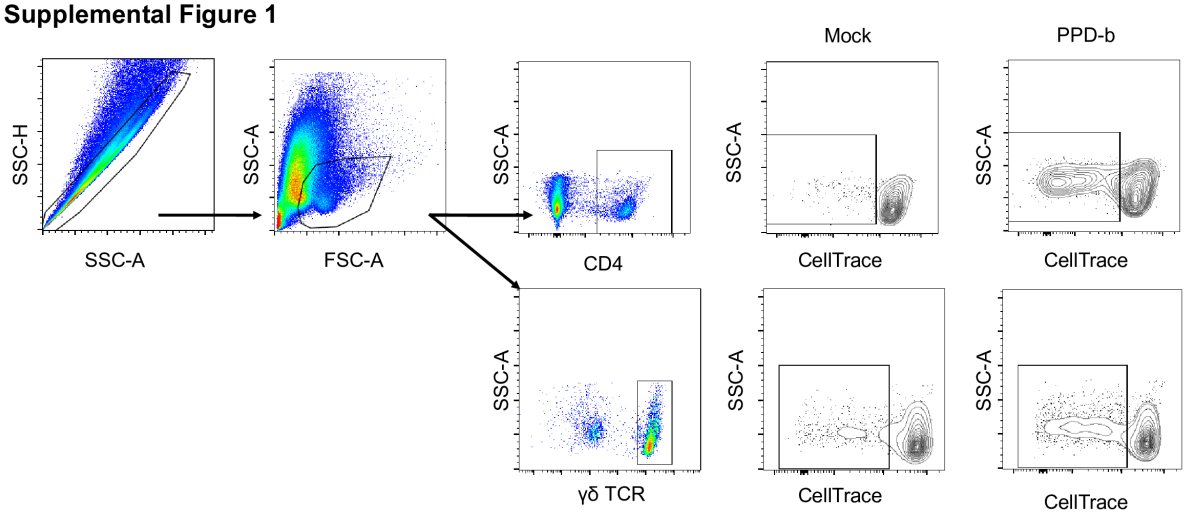
17. Netea MG, Quintin J, van der Meer JW. Trained immunity: a memory for innate host defense. Cell Host Microbe. 2011;9(5):355-61.

18. Arts RJ, Joosten LA, Netea MG. Immunometabolic circuits in trained immunity. Semin Immunol. 2016;28(5):425-30.

19. Gleeson LE, Sheedy FJ, Palsson-McDermott EM, Triglia D, O'Leary SM, O'Sullivan MP, et al. Cutting Edge: Mycobacterium tuberculosis Induces Aerobic Glycolysis in Human Alveolar Macrophages That Is Required for Control of Intracellular Bacillary Replication. J Immunol. 2016;196(6):2444-9.

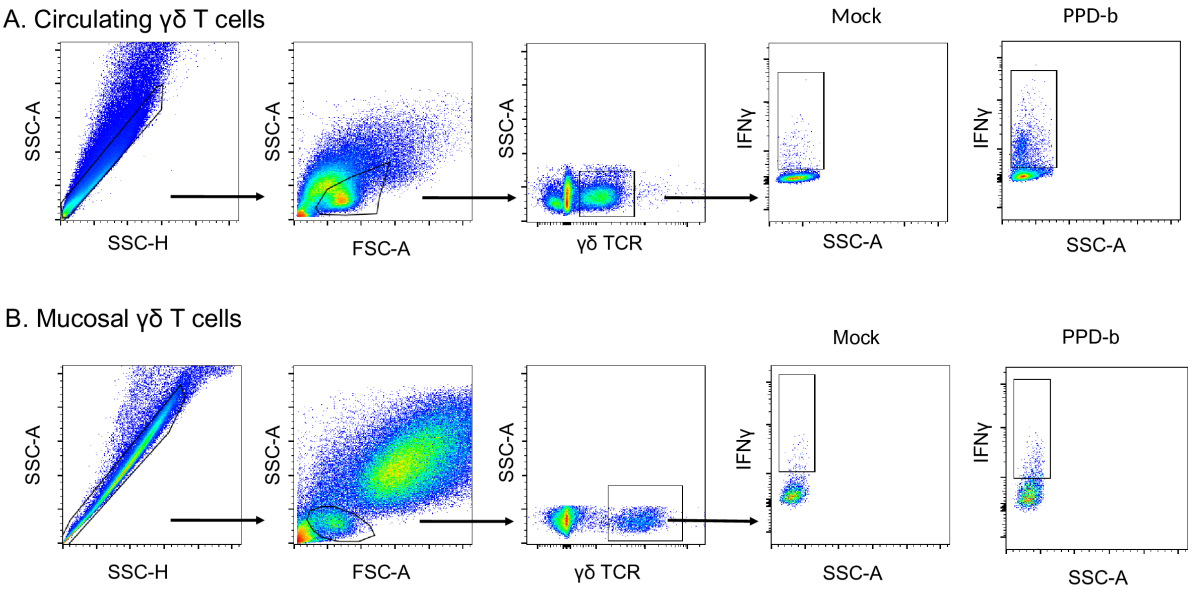
Supplemental information Chapter 3

Supplemental Figure 1



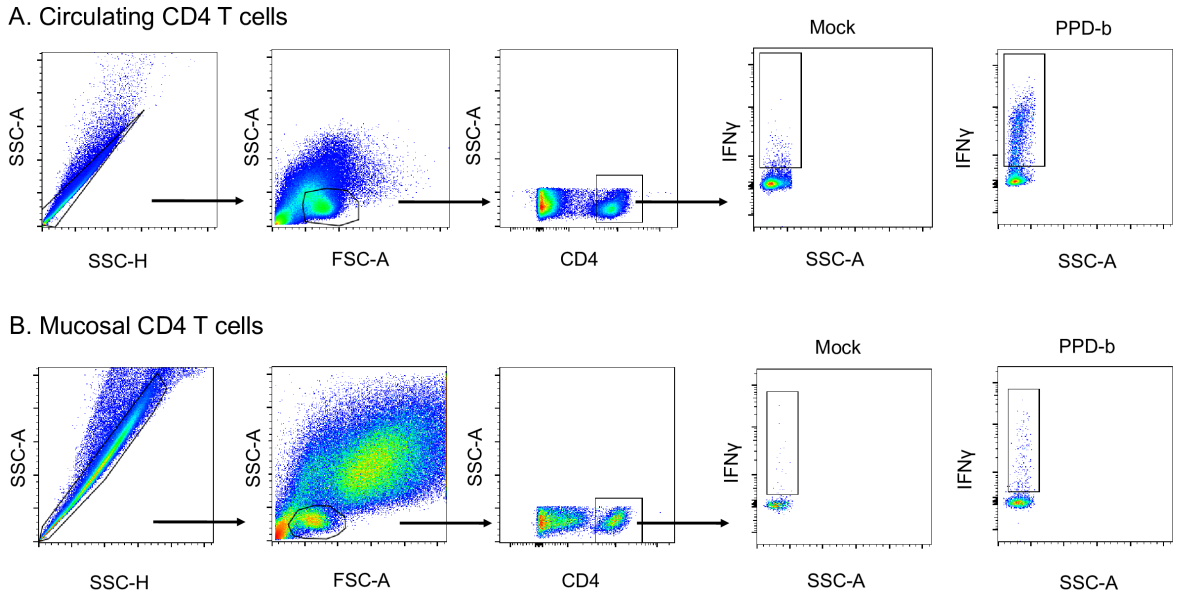
Supplementary Figure 1. PBMC gating strategy. PBMCs from control (n= 10) or BCG–vaccinated animals (n= 18) were labeled with CellTrace, and 5×10^6 cells/ml were cultured for 6 days in the presence or absence of PPD-b. Cells were labeled with anti-bovine γδ TCR or CD4 and analyzed by flow cytometry for CellTrace dilution. Gating hierarchy (gating sequence as depicted by the arrows): Single cells (SSC-A vs SSC-H), lymphocytes (SSC-A vs FSC-A), γδ T cells or CD4 and CellTrace dilution. Representative contour plots of proliferative responses to *in vitro* PPD-b stimulation. Analysis was performed with Flowjo software.

Supplemental Figure 2



Supplementary Figure 2. PBMC and BAL cells gating strategy. Approximately ~8 weeks after aerosol vaccination, IFNγ expression was analyzed in circulating **(A, PBMC)** and in mucosal **(B, BAL)** compartment from control (n=7) or BCG–vaccinated animals (n= 7). 1 x 10^6 cells/well were stimulated in vitro with PPD-b (200 IU/ml) for 16 hours. Cells were then stained for intracellular IFNγ expression and analyzed by flow cytometry. Gating hierarchy (gating sequence as depicted by the arrows): Single cells (SSC-A vs SSC-H), lymphocytes (SSC-A vs FSC-A), γδ T cells and IFNγ expression as shown. Analysis was performed with Flowjo software.

Supplemental Figure 3



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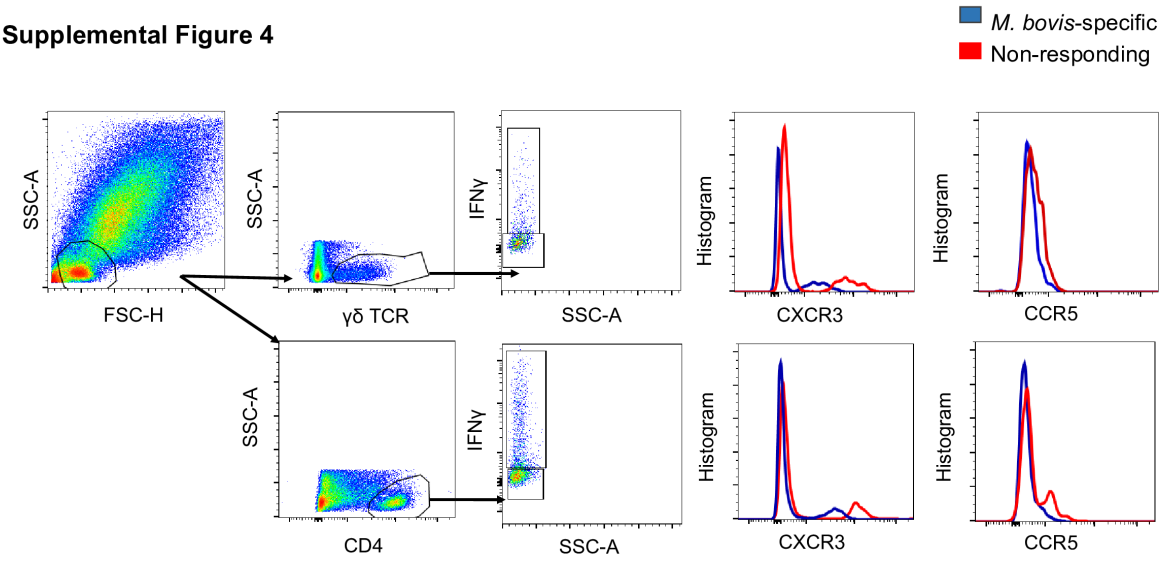
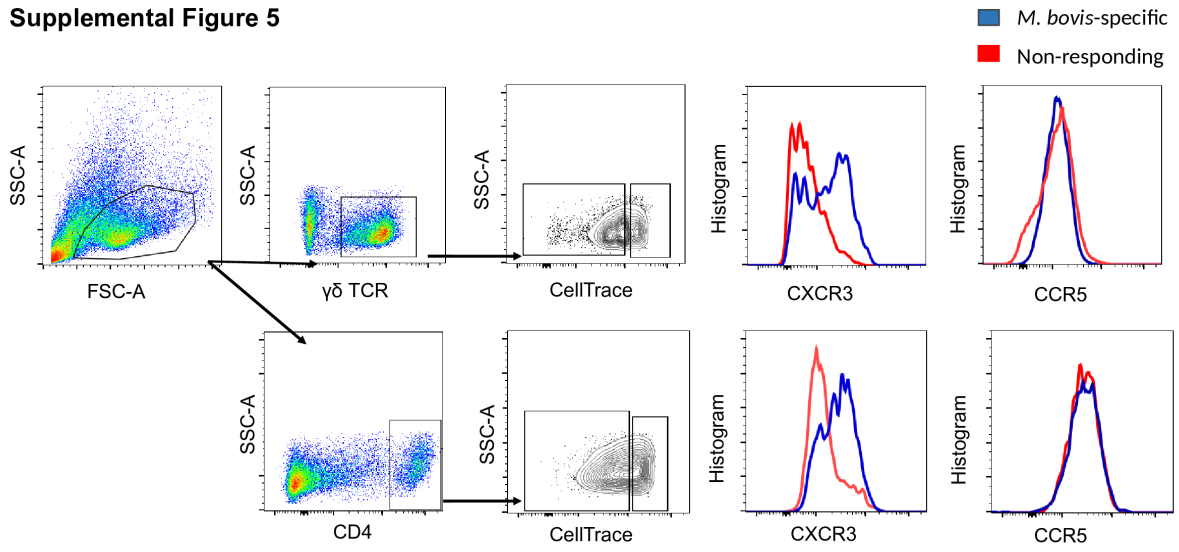
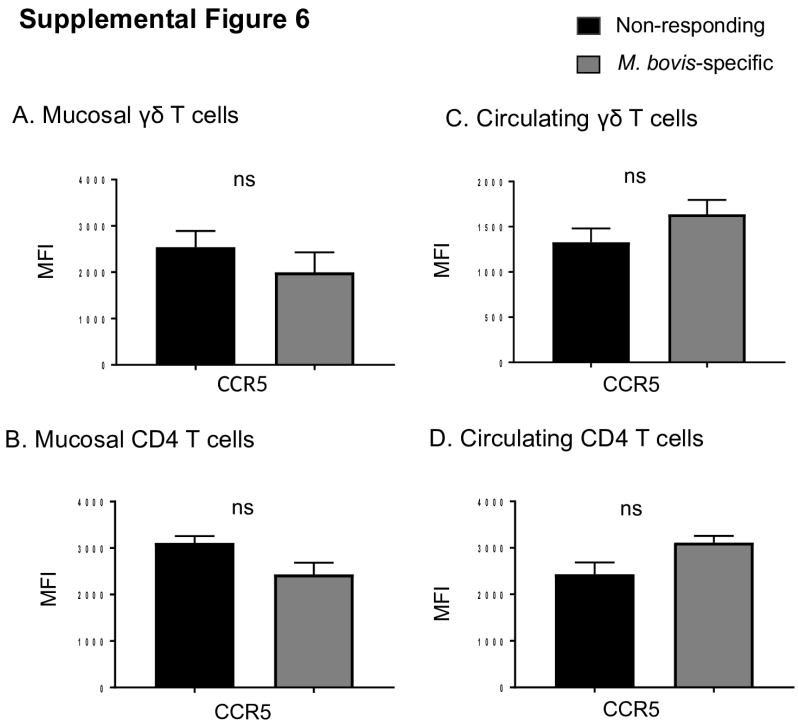
Supplemental Figure 4



Supplementary Figure 4. BAL cells gating strategy for expression of chemokine receptors on mucosal γδ and CD4 T cells. BAL cells were isolated from calves ~8 weeks after vaccination. Cells were stimulated with PPD-b in vitro for 16 hours. Cells were surface stained and then analyzed by flow cytometry to study CXCR3 and CCR5 expression on mucosal *M. bovis*-specific γδ and CD4 T cells. Gating hierarchy (gating sequence as depicted by the arrows): Lymphocytes (SSC-A vs FSC-A), γδ T cells or CD4 cells, IFNγ+ and IFNγ^{neg} cells and CXCR3 or CCR5 expression as shown in histogram. Analysis was performed with Flowjo software.



Supplementary Figure 5. PBMC cells gating strategy for expression of chemokine receptors on mucosal γδ and CD4 T cells. PBMCs were isolated from calves ~8 weeks after vaccination. Cells were stained with CellTrace dye and incubated with PPD-b for 6 days. Cells were surface stained and then analyzed by flow cytometry to study CXCR3 and CCR5 expression on circulating *M. bovis*-specific γδ and CD4 T cells. Gating hierarchy (gating sequence as depicted by the arrows): Lymphocytes (SSC-A vs FSC-A), γδ T cells or CD4 cells, proliferating and non-proliferating cells (CellTrace dilution) and CXCR3 or CCR5 expression as shown in histogram. Analysis was performed with Flowjo software.



Supplementary Figure 6. PBMC and BAL-recovered cells expression of chemokine receptors on mucosal γδ and CD4 T cells. Cells were isolated from calves ~8 weeks after vaccination. Cells were stimulated in vitro as indicated in Materials and Methods, surface stained and then analyzed by flow cytometry to study CXCR3 and CCR5 expression on γδ and CD4 T cells. Analysis was performed with Flowjo software. Not significant (ns) difference from antigen-responsive cells compared to non-responsive cells as determined by Student's t-test.

Supplemental information Chapter 4

Supplemental 1. CD11b expression