Bovine CD4 T cell memory subsets and immunological markers in *Mycobacterium bovis* infection and vaccination

by

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DEDICATION

To my mother, father, brother, sisters and husband. Meus amores, foi por vocês.

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CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

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 they were submitted. An overview and literature review, which comprises further details related to the dissertation, are included in Chapter 1. Chapter 2 has been published in *The Journal of Visualized Experiments*. Chapter 3 has been published in *Plos One*. Chapter 4 has been submitted for publication to *Frontiers in Immunology*. Chapter 5 encloses the general conclusions and future directions.

Overview

Mycobacterium bovis is the causative agent of tuberculosis in cattle. *M. bovis* is a member of the *Mycobacterium tuberculosis* complex (Mtbc) which also includes *M. tuberculosis*, *M. caprae*, *M. microti*, *M. africanum*, *M. canetti*, *M. pinnipedii*, *M. suricattae, M. mungi,* and *M. bovis Bacillus Calmette Guerin* (BCG) [1-3]. Despite what the nomenclature may imply, members of this group are not host species specific. The host range of *M. bovis* appears to be the broadest of the complex, causing disease in a number of species including humans [4]. Great strides have been made over the past century both toward the control of bovine tuberculosis (bTB) in cattle and also limiting the risk to human exposure (e.g., pasteurization of milk for dairy products); however, the disease still has great socioeconomic impact for livestock farmers. It is estimated that over 50 million cattle are currently infected worldwide, costing around $3 billion annually[5]. The WHO (World Health Organization), in conjunction

with FAO (Food and Agriculture Organization of the United Nations) and OIE (Office International des Épizooties), classify bTB as a neglected zoonosis [6,7].

A crucial component of the immune response to TB in humans, cattle and mice is the production of interferon (IFN)-γ by T helper 1 (Th1) CD4 T cells [8-12]. Immune deficiencies affecting CD4 T cells [e.g., human immunodeficiency virus (HIV) infection] and IL-12/IFN-γ /STAT1 signaling pathways result in more severe disease upon TB infection in humans [13,14]. In fact, IFN-γ release assays (IGRA) and delayed type hypersensitivity (i.e., skin test) responses are markers of infection in cattle and humans (reviewed respectively by Schiller *et al*. [15] and Walzl *et al*. [16]). Diagnostic IGRAs are measures of '*ex vivo*' immune responses relying on rapid production of IFN-γ in response to mycobacterial antigen stimulation in short-term (16–24 h) whole blood or peripheral blood mononuclear cell (PBMC) cultures. These *ex vivo* assays are generally considered a measure of T cell effector responses [12,17]. Most protective bTB vaccines elicit *ex vivo* IFN-γ responses; however, not all vaccines inducing this response are protective [5,18]. Cultured assays (i.e., culture of cells for 7-14 days before IFN-γ measurement) are assumed to quantify memory responses, primarily central memory T cells (Tcm) in humans [19-21]. In light of the idea that cultured ELISPOT measures Tcm responses, several studies have shown its association with protection against malaria, suppression of viral recrudescence in hepatitis B virus carriers, low virus levels in HIV infection, and favorable outcomes in human TB [22-26]. In cattle, responses measured by cultured IFN-γ ELISPOT following vaccination are the best-known positive correlate of protection in vaccine and challenge experiments [27-31].

Increasing interest has risen in order to characterize and assess the role of polyfunctionality in both protective and detrimental immune responses in TB. Polyfunctional T cells simultaneously produce two or more cytokines with IFN-γ, IL-2, and tumor necrosis

factor-α (TNF-α) being the most commonly measured Th-1 cytokines [32,33]. Association between protection and vaccination-induced polyfunctional T cells has been mainly studied in small animal models [34,35]. In humans, strong polyfunctional responses are detected in *M. tuberculosis*-infected individuals, high IL-2 production is associated with a positive clinical status (e.g., latent or treated disease), while a strong IFN-γ/TNF-α response is associated with a poor outcome (i.e., active TB) [36]. Human polyfunctional responses to vaccination both prior to TB exposure and in previously exposed individuals (i.e., latent infection) are extremely variable. In cattle, T cell polyfunctionality has only been measured upon *ex vivo* recall stimulation [26,37].These studies found no association between polyfunctional responses measured before challenge and vaccine success. Instead, polyfunctional responses to infection were associated with increased pathology and poor disease outcome [26]. Polyfunctional responses by long-term cultured cells for enrichment of Tcm responses have not been evaluated in spite of the fact that cultured IFN-γ ELISPOT is one of the most promising protection correlates in cattle [27-31].

The research included in this thesis had the objective to characterize memory cells phenotype and aspects of their functionality in cattle, specifically in response to bTB infection and vaccination. The discrimination of cell phenotype involved in cytokine production under both cultured and *ex vivo* conditions may be necessary to identify specific correlates of vaccine efficacy, useful for vaccine candidates selection for costly efficacy trials in biosafety level 3 (BSL-3) facilities.

Literature Review

CD4 T cell activation and function

Naïve CD4 T cells are primarily activated by mature antigen-loaded dendritic cells (DCs) in the T-cell areas of secondary lymphoid organs and the ensuing response is influenced by this initial interaction [38]. The clonal expansion that follows CD4 T-cell activation is accompanied by cell differentiation [39,40] both of which increase the precursor frequency in comparison to the naïve pool and results in greater ability of these cells to support CD8 and B cell responses. CD4 T cells are essential for B-cell germinal center formation, class switching and affinity maturation (such cells are defined as T follicular helper cells) [41]. CD4 T cells act via cytokine production and CD40L expression [41-43]. CD4 T cells also influence general immune responses, as their interactions with DCs are bi-directional, tailoring the activation of other cells[38]. Although CD8 T cells certainly can be activated in the absence of CD4 T cells, CD4 cells are essential for CD8 T cells long-term persistence and proliferative ability [42-45]. Fundamentally, CD4 cells play a major role for the optimal response of both CD8 T cells and B cells, and in their ability to establish memory.

Classically, antigen clearance is associated with the contraction phase, in which the majority of antigen-specific T cells are eliminated [46,47]. A small pool, however, become memory cells (Fig. 1). Considerable efforts have been made to identify markers that distinguish cells that will overcome the contraction phase to become memory from short-lived effector cells [40,45,48-62]. Memory development is associated with higher expression of survival signals [e.g., IL-7 receptor alpha chain (CD127) and B-cell lymphoma 2 protein (BCL-2) expression on activated CD4 T cells] and lower expression of inhibitory receptors, such as the killer cell

lectin-like receptor G1 (KLRG-1) and programmed cell death protein 1(PD-1) (reviewed by Mahnke *et al*. [47]).

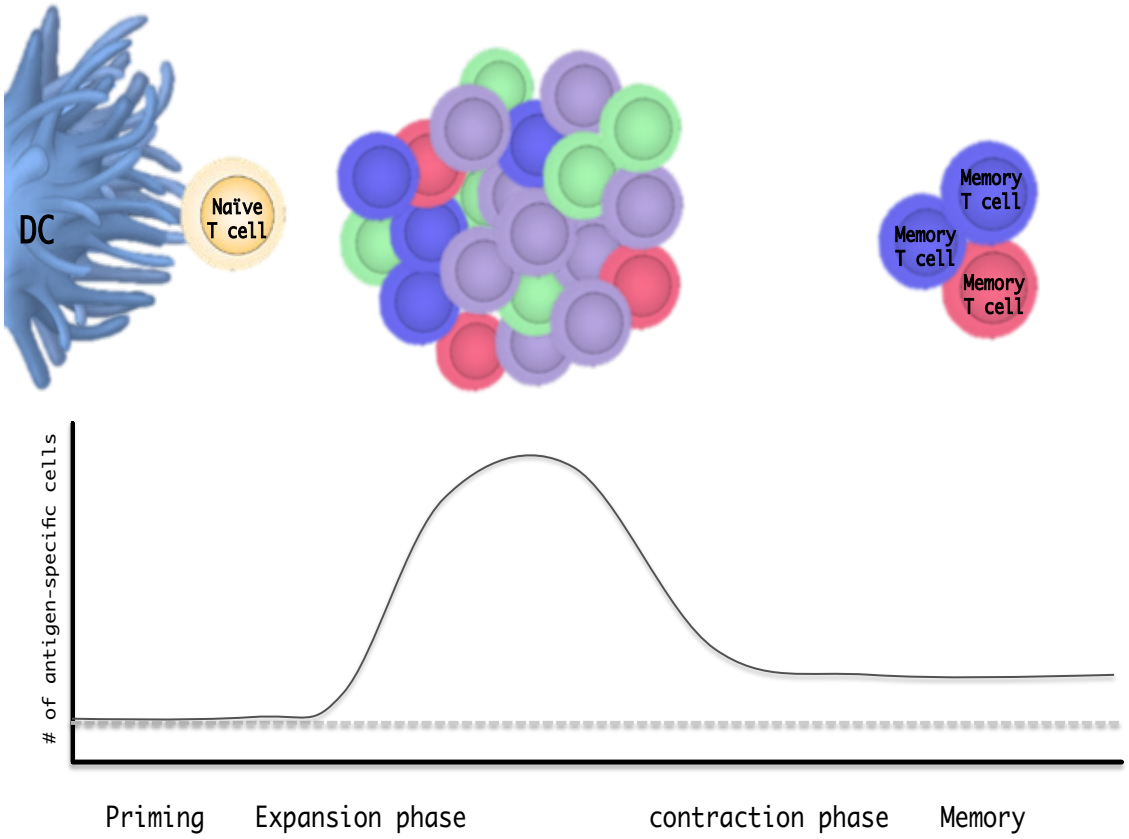

Figure 1. Kinetics of T cell primary responses. Upon antigen recognition, naïve T cells proliferate (during the expansion phase) and differentiate into effector cells. If infection is controlled, short-lived cells will be eliminated over the next several weeks (contraction phase). Only a small percentage of cells generated during the expansion phase (5–10%) survive and further develop into memory T cells. This process increases response fitness and yields higher precursor frequency (depicted as the gray dashed line) in comparison with primary immune response.

The strength of the signal through the T-cell receptor (TCR) has also been investigated and may influence CD4 T cell fate, with cells bearing higher affinity TCR outcompeting cells bearing lower affinity TCRs [48,49]. The role that self-peptide and MHC class II (pMHC), as

presented by antigen presenting cells (APCs), plays in memory T cell survival has also been the aim of extensive investigation. Experimentally, CD4 memory cells can survive in the absence of pMHC signaling, but whether this is also possible *in vivo* has been a matter of debate [52] .Jenkins *et al*. [40] demonstrated that TCR transgenic memory cells declined at a faster rate in a dose dependent fashion with transfer of increasing numbers of cells. Elevated numbers of cells would increase the competition for the same pMHC and other survival signals leading to speedy decline. Other factors, such as the signals present during T-cell priming, can also affect the long-term survival of memory cells. For example, immunization with antigen and Toll-like receptor 2 (TLR2) agonists generated memory cells that survived in greater numbers than those generated by immunization with the same antigen and a TLR4 agonist [53]. Cells with different fates may arise from the same progenitor cells, even though these cells bear TCR of the same affinity[54]. Harrington *et al.*[55] demonstrated that genetically marked IFN-γ producing CD4 T cells during a primary response were present in the subsequent memory pool. Relatedly, Lohning *et al.*[56] demonstrated that adoptively transferred cytokine-producing antigen-specific T cells are capable of becoming memory cells in recipient mice, indicating that not all effector cells are short lived and may become memory cells.

Certainly, antigen clearance appears to be crucial for memory formation under several circumstances. However, memory cells may arise soon after cell priming. Lauoar *et al.* [57] demonstrated the appearance of CD8 memory cells during the acute phase of a primary response to lymphocytic choriomeningitis virus (LCMV) as early as eight days after infection. Likewise, prime-boost vaccination with viral-vectored subunit malarial vaccine antigens induces both *ex vivo* and cultured IFN-γ ELISPOT responses by seven days [58]. Memory cells are also elicited during chronic infections, and although antigen clearance has crucial role in

response kinetics, pathogen clearance in some infections, such as TB (including *M. bovis* infection in calves), is not always naturally achievable.

Truly, the very factors governing effector / memory T cell differentiation remain largely unknown[59]. Whether memory cells arise (Figure 3): (**a**) as direct progenies of effector cells (linear differentiation model [60-62]), (**b**) via a linear process dictated by a progressive loss of T cell differentiation potential (i.e., from naïve → memory → effector → terminally differentiated cells) dependent on antigenic stimulation and signaling milieu (decreasing potential model [56,63]) or (**c**) as a separate lineage from naïve cells (divergent differentiation model [54,57,]) has long been debated. Still, the connection between naïve CD4 T cells and the various effector and memory fates these cells follow remains unclear, and a single unifying theory accounting for the diversity of CD4 T cell memory has not been determined. This suggests that CD4 T cell memory generation may be differentially engaged depending on various factors (e.g., level of inflammation, TCR affinity), and these models might not be mutually exclusive. Although many differences between memory and effector cell populations have been described, especially at the population level, technologies to drive memory T cell generation remain an elusive field.

Memory cells subsets: a growing family

The notion of immunity has been likely observed since ancient times through the observation of natural resistance and differences in disease severity presented by different individuals and animals. Thucydides, a Greek historian (460 BCE), gave perhaps the first documented evidence of immunological memory when he noticed that people who had

recovered from a new disease brought to Greece from Spartans during the Peloponnesian war would not “be attacked twice - never at least fatally” [65].

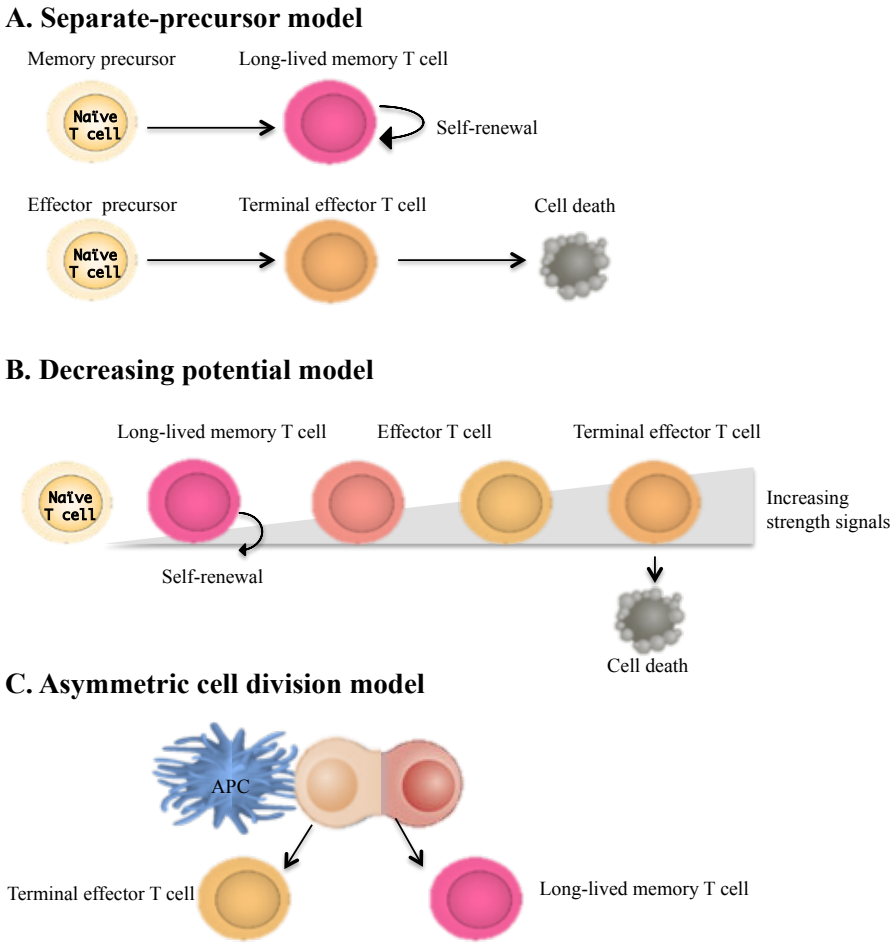

Figure 2. Models for T cell heterogeneity generation. A. Separate-precursor model, naive T cells are pre-programmed to follow a differentiation path; cell fate is determined during thymic development. **B.** Decreasing-potential model suggests that effector T cells adopt various differentiation states according to the cumulative history of signals that they encounter during infection. Repetitive stimulation with antigen and other signals, such as interleukin-2 (IL-2) and IL-12, drives greater effector cell proliferation and terminal differentiation. As the cells acquire terminally differentiated states they remain functional but lose memory cell properties, such as survival, plasticity and proliferative potential. The formation of heterogeneous effector cell populations is dependent on the overall 'strength' of the signals that are encountered early during T cell priming and throughout the infection. **C.** Asymmetric cell fate model states effector and memory T cell arise from a single precursor T cell through asymmetric cell division that occurs as early as the first cell division after antigen stimulation. Proximity with APCs may play a role in determining the cell fate of the daughter cells. (Adapted from: Kaech and Cui [64]).

In the 18th century, Ludwig Panun, a Danish physician, perceived that the immunological memory to measles could persist for a lifetime. He drew this conclusion observing two measles outbreaks occurring 65 years apart in an isolated island in the North Sea; individuals who had the disease in the first outbreak were not susceptible to re-infection [66]. Interestingly, the very concept of immunity was described much earlier than the understanding of both the immune system itself and microorganisms. Still the mechanisms governing immunological memory, especially T cell memory, are vastly unknown.

The ability to remember antigenic encounters and provide protection against pathogens are the foundation and the main goal of vaccination. Development of memory is a complex process, and so are memory T cells. In fact, memory comprises many subsets with distinct homing potentials and effector functions. Sallusto and colleagues [67] studied the expression of two lymph node homing receptors on CD45RA negative memory T cells: CC chemokine receptor type 7 (CCR7) and CD62L. Lymph node endothelial cells express CCR7 ligands: chemokine (C-C motif) ligand (CCL)-19 and -21. Consequently, the expression of CCR7 expression allows cells to follow its ligands gradient, guiding these cells to the T cell zone within lymph nodes. Increased CD62L expression facilitates T cell entrance into lymph nodes as its ligand, glycam-1, is highly expressed on high endothelial veins (HEV). The CD45 antigen has a cytoplasmic domain that, in T cells, eliminates the inhibitory phosphate groups on the tyrosine kinase Lck, promoting cell activation. Alternative splicing of the CD45 gene produces as many as eight different proteins: CD45RA, CD45RB, CD45RC, CD45RAB, CD45RAC, CD45RBC, CD45RO, CD45R (ABC). The most well known isoforms are CD45RA and CD45RO and depending on the activation status, these two isoforms are differentially expressed in T cells [68-71]. Naïve T cells express the larger CD45RA isoform,

while lacking CD45RO expression. Upon antigenic recognition, T cells switch the expression of CD45RA to the expression of the CD45RO isoform. The CD45RO isoform is smaller, as it lacks the RA, RB, and RC exons, easing the cell activation [71].

In their experiments, Sallusto and colleagues[67] described two subsets of memory T cells with distinct functionality: Tcm and Tem cells. Cells from both subsets express CD45RO and lack CD45RA. Tcm cells express CCR7 and CD62L, and are mainly located in T-cell areas of secondary lymphoid organs. Tcm cells have limited effector function, but show low activation threshold and retain high IL-2 production and proliferative capacity. Tem cells lack CCR7 and display receptors for homing to peripheral or inflamed tissues. Effector cells do not express CCR7 and express little to no CD62L, but promptly produce effector cytokines, such as IFN-γ. These cell subsets have also been detected in mice[67] cattle [72] and non-human primates [73,74].

Due to their peripheral localization, tissue-resident memory T cells (Trm) were not identified until recently [75] .Trm cells are confined to peripheral non-lymphoid tissues and they may be present long after the initial infection has been cleared, providing an immediate frontline adaptive response if a similar pathogen is reencountered at the entry site. In general, these cells express low levels of CCR7 and CD62L, and high levels of local non-lymphoid tissue-homing molecules such as CD103 and CD69. Trm cells seldom recirculate, explaining why studies investigating memory cells in the peripheral blood and secondary lymph organs (SLOs) failed to detect Trm cells [76,77].

Zhang and colleagues[78], studying graft-versus-host disease (GVHD) in mice, reported the existence of a subset of self-renewing CD8 T cells able to generate Tcm, Tem and effector T cells. The GVHD was observed upon transfer of these self-renewing cells to irradiated recipient mice, indicating their role in the disease. The phenotype of these cells was defined as

CD8 cells displaying low expression of the CD44 marker (an activation marker) and high expression CD62L and has been called stem-cell memory T cells (Tscm). This phenotype was later reported in humans and in non-human primates [79-82]. These cells maintain some naïve T cells characteristics, such as self-renewing capacity and plasticity. Although, human Tscm cells were first identified as a CD8 T [81] cell population, CD4 T cell were later shown to generate Tscm cells [82]. Tscm cells are not very numerous, only 2–3% of T cells from peripheral blood of healthy donors, and their identification relies on complex multiparameter flow cytometry cell analysis. The Tscm phenotype is described as $CD45RA^{+}CD45R0^{-}CCR7^{+}CD62L^{+}CD27^{+}CD28^{+}$ $CD95^{high}$. The intriguing expression of CD95 on Tscm cells raises the possibility of a new role for that receptor, indicating it may be important for the function and/or persistence of these cells [80,81]. Analysis of antigen-specific CD8 Tscm cells demonstrated that they retain self-renewal capacity while also exhibiting rapid cytokine production upon antigen re-exposure[82]. Transcriptional analysis of human CD4 Tscm cells indicates that Tscm cells are a transitional differentiation stage between naïve and Tcm cells [82]. In non-human primates, Tscm cells are distributed in peripheral blood, secondary lymphoid organs, and bone marrow [81]. After *in vitro* stimulation, Tscm cells often maintain their phenotype. When compared with Tcm cells, this self-renewing capability is twice as frequent in Tscm cells than in Tcm cells cultures[80]. Tscm cells exhibit high survival capability even in the absence of cognate antigens[81]. Not surprisingly, Tscm cells highly express pro-survival transcription factors (e.g., BCL2, Myeloid Cell Leukemia 1 protein -MCL1; and Lymphoid Enhancer-Binding Factor 1- LEF1) as compared to the other memory subsets and are able to differentiate into other memory subsets, including Tcm [81,82]. No other memory subset has been found to regenerate Tscm cells.

An obvious complication to the study of memory cells is the variability of markers and conditions under which their responses are assessed. The differential expression of a collection of molecules conferring lymphocyte functions is shown in Table 1. The multiple memory subsets are defined by differential function they exhibit due to the expression of these multiple molecules involved in migration, co-stimulation, cytotoxicity, and adhesion.

In cattle, memory responses have long been studied through the use of the antigen experienced splice variant marker CD45RO, activation markers (such as:CD25, CD69, MHC-II and CD26) and homing molecules (Table 2). Often, the measured responses were assumed to be due to memory cells by the timing of the assessment (the ensuing response upon an expected antigen clearance). Yet, Tcm and Tem subsets have only recently been characterized [76]. In cattle, Tcm have been shown to have a higher proliferative capacity than Tem and effector counterparts. Still, Trm and Tscm subsets have not been investigated in cattle.

Applied vaccinology has comprised the majority of immunological memory assessment in veterinary species. As most protective vaccine often rely on the neutralizing antibodies, serologic responses are frequently assessed immune parameter [83-86]. Cellular immune responses in cattle have mostly been studied through cytokine production by non-fractionated peripheral blood mononuclear cells (PBMC) and due to restrictions on reagent availability and research support, less sophisticated cell population analysis (i.e., flow cytometry) is often performed[86-90]. Frequently, memory responses are assessed through antigen clearance, typically achieved in vaccination or acute infection settings. These approaches are very useful, especially for vaccine development, but they provide limited advancement to the veterinary immunology field, and for the development of better tools to troubleshoot vaccine failure.

Table 1. Expression of functional molecules by circulating naïve and memory murine T-cell subsets according to differentiation stages

Category	Antigen	Function	Naïve	Tscm	Tcm	Tem
Costimulation/ survival	CD28	Costimulation	+	++	++	–
	CD27	Costimulation	++	+	+	–/+
	CD127	IL-7 signaling	++	+++	+++	–/+
	PD-1	Inhibition of effector function	–	–/+	+	+
	CD122	IL-2/IL-15 signaling	–	+	++	+++
	CD132	γc cytokine signaling	+	+	+	+
	KLRG-1	Inhibition of effector function	–	ND	–/+	++
Activation	HLA-DR	Peptide presentation	–	–	–/+	+
	CD38	Calcium flux/signal transduction	+	–/+	–	–
	CD69	Proliferation	–	–	–	–
	Ki-67	Proliferation	–	–	–/+	–/+
Adhesion	CD11a	Adhesion to APC/endothelium	+	++	++	+++
	CD58	Adhesion to APC	–	+	++	+++
	CD99	Transendothelial migration	–/+	+	++	++
Migration	CD62L	Secondary lymphoid tissues homing	+	+	+	–
	CD103	Gut homing / Residency	–	–	–	+
	CCR4	Chemokine response/Th2 associated	–/+	+	++	+++
	CCR5	Homing to inflamed tissues	–	–	+	+++
	CCR6	Chemokine response/Th17 associated	–	–	++	+++
	CCR9	Gut homing	–	ND	+	–
	CCR10	Skin homing	–	–	+	++
	CXCR3	Homing to inflamed tissues	–	–/+	+	+++
	CXCR4	Homing to bone marrow	+	++	+++	++
	CLA	Skin homing	–	ND	+	++
Cytolytic molecules	Granzyme A	Cleavage of cellular proteins	–	–	–	–/+
	Granzyme B	Cleavage of cellular proteins	–	–	–	–/+
	Perforin	Pore forming	–	–	–	–/+
Miscellaneous	CD161	Regulation of proliferation/cytotoxicity	–	–	–/+	+++
	IL-18Ra	Response to IL-18	–	–/+	+	+++
	c-Kit	Response to SCF	–	–	–	+++
	CD130	Response to IL-6	++	+	–/+	–

Table 2. Studies evaluating memory T cell in cattle and parameters analyzed.

Disease / model	Method	Cell marker	Cell function	Reference
Whole or cell-free colostrum fed calves, impact in their responses to vaccines	Flow cytometry	CD45RO CD62L CD4	Phenotype, memory	89
Oenothein B stimulation	Flow cytometry	CD45RO	IFN-γ , GM-CSF production	90
M. bovis, M. avium Paratuberculosis		CD45RO, CD25, CD26	Cell activation	91
Foot and mouth disease		CD45RO CD25 gd T cells		92
Vaccines against Contagious Bovine Pleuropneumonia	Flow cytometry, IFN-γ ELISPOT, ELISA	CD45R, CD45RO, CD62L	IFN-γ, proliferation	93
Impact of aging	Flow cytometry, IFN-γ ELISPOT, ELISA	CD4, CD45RO, CD62L, IFN-γ	Memory (as assessed by Cultured ELISPOT), IFN-γ production	94
M. bovis infection	Flow cytometry, IFN-γ ELISPOT	CD4, CD44, CD62L, CD45RO, CCR7, IFN-γ	IFN-γ production, cell proliferation	72
M. bovis BCG vaccination and challenge	Flow cytometry, ELISA, flow cytometry	CD4, IFNγ, TNFα, IL-2	Cytokine production	24
M. bovis BCG	Flow cytometry, cultured IFN-γ ELISPOT, ELISA	CD4, IFN-γ	Memory response (as assessed by Cultured ELISPOT), IFN-γ production	30
M. bovis DeltaRD1 and challenge	Cultured IFN-γ ELISPOT, IFN-γ ELISA rtPCR (IFN-γ and IL-17)	None	Memory response (as assessed by Cultured ELISPOT), IFN-γ and IL-17 expression.	27
M. bovis BCG followed by Viral booster and challenge	Flow cytometry, cultured IFN-γ ELISPOT, ELISA	CD4, CD8; CD25, γδ TCR, MHC class II	Cell activation, memory (as assessed by Cultured ELISPOT), proliferation, cytokine production	28
M. bovis	Flow cytometry	CD8, CD45RO, gammadelta TCR, proliferation, IFN-γ	Proliferation, cyottoxicity	95
M. bovis-BCG	Flow cytometry, rtPCR (IL-4, IL13, IL-21)	CD4, CD45RO, CD62L, IFN-γ	Cytokine production	96
M. bovis		CD4, CD8, gammadelta TCR CD45RO, CD26, CD25, proliferation	Cell activation, proliferation	97
Babesia bovis	Flow cytometry, Northern Blotting, ELISA (IL-2, IL-4, IFN-γ)	CD2, CD4, CD8, CD45R, CD45RO, CD62L	Cell activation, cytokine production	98
Theileria parva	Flow cytometry, cell sorting, rnaPCR (IL-2, IL-4, I, CD45), ELISA (IFN-γ)	CD2, CD4, CD8, gammadelta TCR,WC1, WC3,CD45RO+	Cytoknine production, proliferation, cell activation	99
Bovine viral diarrhea virus	Flow cytometry	CD4, CD8, CD25, CD45RO, CD62L	Cell expansion and populational size in BAL	100

Fortunately, advancements are being made in the field of veterinary immunology through increased reagent availability, more accessible technologies, and due to the revived appreciation for One Health approaches. These developments will likely prove to be useful for human research as well, especially for zoonotic pathogens, and to diseases to which laboratory models poorly recapitulate aspects of the disorder seen in humans and other primary host species.

Phenotype and function

The paradigm of segregating T cells into Tcm and Tem on the basis of their expression of CC-chemokine receptor 7 (CCR7) or CD62L, along with CD45RA[67], not only provided a conceptual advance in our understanding of T-cell dynamics and compartmentalization, it also enabled the identification of distinct patterns of cytokines (that is, IFN-γ and IL-2) associated with these specific subsets of memory T cells. In fact, both phenotype and function of these specific subsets provide two independent facets of the immune response and combined are more informative whether such responses in regards to protection predition. Although Tcm cells are prone to express IL-2, whereas Tem cells produce more IFN-γ, that dichotomy is not unconditional. In HIV-infected adults receiving chemotherapy, up to 80% of the IL-2-producing $CD4^+$ T cells specific for antigens from CMV, influenza virus, EBV, HSV or the HIV gag protein are Tem cells [101-103]. Similarly, in mice infected with *Leishmania major* [104] or humans infected with vaccinia virus [105-107] the majority of IL-2 was produced by multifunctional $CD4^+$ Tem cells. In cattle, natural *M. bovis* infection elicits strong polyfunctional T cell responses almost exclusively by $CD62L^{lo}$ CD4 cells [37]. Also, Tcm and Tem cells may produce all combinations of IFN-γ, IL-2, and TNF-α under long-term culture

condition. Several factors that influence the fate outcome, such as the relative frequency of Tem versus Tcm cells, the amount and duration of the antigen persistence, innate immunity, clonal competition and the characteristic of the pathogen. These factors will impact CD4 T cell memory, influence the predominant source of IL-2 and of other cytokines, contributing to cell functionality and infection outcome. In summary, evaluating the quality of a T cell response in combination with analysis of their phenotype increases the understanding of T cell memory and effector differentiation by defining the T cell functional capacity, strength and trafficking. Hence, a combined phenotypic and functional analysis of T cells should allow greater insight into whether a response is protective than either parameter alone, still, analysis of these parameters should always be analyzed taking in consideration the overall response and the nature of the infection.

The case for TB

Mycobacterium tuberculosis remains one of the most detrimental human pathogens and has been challenging to combat due to the lack of an effective vaccine. *M. tuberculosis* has co-evolved with humans for more than 70,000 years, resulting in the earth's most successful bacterial pathogen capable of establishing itself within its host for decades as a latent infection. It is estimated that more than 2 billion people worldwide are infected with *M. tuberculosis* with greater than 8.6 million developing acute pulmonary tuberculosis (TB) and about 1.3 million dying from the disease every year. TB infection ranks second among infectious diseases causing human deaths, only after HIV. Moreover, the emergence of multidrug-resistant (MDR), extensively drug resistant (XDR), and total drug-resistant (TDR) *M. tuberculosis* strains have increased the need for an effective vaccine [36]. Bovine tuberculosis (bTB) is a

chronic bacterial infection that affects animals and humans. *Mycobacterium bovis*, the main agent causing bTB, is a member of the *Mycobacterium tuberculosis* complex[1-4]. Zoonotic transmission of *M. bovis* occurs primarily via ingestion of unpasteurized dairy products or contact with infected cattle[5]. Before pasteurization became a common practice, infection with *M. bovis* resulted in up to 25% of TB cases in humans. The disease also has significant socioeconomic implications for livestock farmers with estimates of >50 million cattle infected worldwide, costing $3 billion annually[5]. The WHO (World Health Organization), in conjunction with FAO (Food and Agriculture Organization of the United Nations) and OIE (Office International des Épizooties), recently classified bTB as a neglected zoonosis [6,7]. Early discoveries in the field of TB indicate that the success of disease control in human populations would depend on the success of control measures in animals, and vice versa. Recognizing the zoonotic importance of a cattle-derived pathogen, eradication / control programs were initiated in many developed countries in the late 1800s. While costly and laborious, bTB control programs have generally been successful with a few notable exceptions (e.g., UK and Ireland). The resulting near disappearance of zoonotic TB from the human population exemplifies one of the largest One Health successes in medical history[5].

Numerous advances in TB diagnosis, vaccinology, molecular epidemiology and immunopathogenetic studies have been made within the disciplinary divides of human and animal health research. More recently, the discovery of similarities in the interactions between the natural hosts and the causative agents of TB, as well as similarities in the resulting disease, have led to a renewed appraisal of the benefits of collaborative approaches. For example, as a control measure, the currently approved vaccine against TB, BCG, is an attenuated strain from *M. bovis*. As with BCG, typical vaccination approaches aim to mimic the natural immune

response to infection using an attenuated pathogen expressing antigens broadly overlapping the antigen profile of the pathogenic agent. This approach has, in fact, resulted in the development of several highly successful vaccines against acute infections, to which natural immune response to infection is sufficient to protect against future infections, enabling eradication of pathogens, such as smallpox and rinderpest [108]. While BCG has been a valuable tool to prevent severe disease in children, it has failed to protect adults against TB infection and prevent active disease, especially in countries where the disease is endemic. Currently, there is a significant effort toward the development of a new TB vaccine [36]. Even though BCG reduces disease incidence in children, neither BCG nor prior infections promote an immune response sufficient to prevent disease reactivation or reinfection; the lack of protection emphases the challenge of developing a vaccine against an infection that requires cellular immunity to contain an established infection.

Untiringly, the majority of *M. tuberculosis* infected individuals will contain the infection and never develop clinical disease, supporting that sterile immunity can be usually achieved, which is supported by the observations of healed TB lesions at autopsies, from which no cultivable bacteria is isolated, suggesting that *M. tuberculosis* clearance occurred [109]. Nevertheless, several reports suggest that the absence of clinical TB is not a clear sign of sterile immunity. These reports include: (i) the fact that infected individuals maintain cellular responses to TB antigens for long periods of time, (ii) the finding of clinical isolates from the same from the same individuals taken years apart displaying identical genotype[110], and (iii) the isolation of *M. tuberculosis* DNA in fatal transit victims with no TB signs or history [111]; suggesting that sterile immunity may be less common than originally assumed. Moreover, reactivation of latent infection is frequently the cause of recurrent TB, especially under low-

endemic settings [112,113]. Even in high-incidence regions, only ~9% of recurrent TB cases in immune competent individuals is caused by re-infection. In this scenario, a vaccine able to stimulate long-term containment of infection preventing contagious lung disease rather than infection, would still undoubtedly represent an advance and impact disease control and global health[36].

BCG attenuation was a 13-year process, taking over 230 serial passages [114]. The genomic deletions responsible for BCG attenuation resulted in 16 genomic regions of differentiation (RD1–RD16, plus nRD18) as compared to the *M. tuberculosis* genome. All BCG strains lack RD1, a 9.5 kb DNA segment that encodes immunodominant T cell antigens including ESAT-6, CFP-10, Rv3873, and PPE protein. The attenuation gave rise to different BCG strains [115]. It was distributed worldwide as a TB vaccine for humans: BCG Russia (ATCC 35740), BCG Moreau/Rio de Janeiro, BCG Tokyo, BCG Sweden, BCG Birkhaug (ATCC 35731), BCG Denmark 1331 (ATCC 35733), BCG China, BCG Prague, BCG Glaxo (ATCC 35741), BCG Tice (ATCC 35743), BCG Frappier (ATCC 35735), BCG Connaught, BCG Phipps (ATCC 35744), and BCG Pasteur 1173. The search for better vaccines has included recombinant BCG (rBCG) and prime boost strategies using viral vector or subunits strategies investigation. rBCG strategy has mainly focused on the construction of: (a) Overexpression of immunodominant antigens already expressed by BCG, such as α-crystallin: HspX, and antigen 85 (Ag85) complex proteins (Ag85A, Ag85B, and Ag85C); (b) insertion of immunodominant antigens not expressed by BCG, such as RD1, RD2, RD3, RD14, RD15, RD16, and nRD18 encoded antigens; (c) both, overexpression and insertion of genes lost throughout attenuation; (d) BCG modification targeting cytokine production and immunomodulation[5]. The numerous BCG sub-strains with different attenuation levels and

antigenic repertoires, as well as the recombination process used may have profound influence on rBCG vaccine efficacy. Interestingly, pre-clinical animal (including cattle) and human studies have not supported that BCG strains provide different protection levels [29-31], in spite of eliciting different cytokine levels.

A major complication for developing a successful vaccine is the current lack of a gold standard of a fully protective immunological response in TB[5,36]. The use of better animal models, such as cattle, to more precisely reflect pathogenesis and ensuing immune responses in humans would be helpful for the understanding of the disease and perhaps its control. To achieve disease control in humans and animals, it will be paramount to exceed the prevalent focus on Th-1 cytokines, especially IFN-γ, and assess a wide range of immune parameters. This will allow signatures to be identified for correlation to vaccine success or failure. In the meantime, the search for better and safer vaccines should be continued. A future vaccine strategy able to hamper transmission (in human or cattle) and/or the establishment of infection would represent not only an improvement compared to BCG, but one of the biggest achievements in modern vaccinology.

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CHAPTER 2: APPLICATION OF LONG-TERM CULTURED INTERFERON- γ ENZYME-LINKED IMMUNOSPOT ASSAY FOR ASSESSING EFFECTOR AND MEMORY T CELL RESPONSES IN CATTLE

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Footnote: The video component of this article can be found at http://www.jove.com/video/52833/

Abstract

Effector and memory T cells are generated through developmental programing of naïve cells following antigen recognition. If the infection is controlled up to 95 % of the T cells generated during the expansion phase are eliminated (i.e., contraction phase) and memory T cells remain, sometimes for a lifetime. In humans, two functionally distinct subsets of memory T cells have been described based on the expression of lymph node homing receptors. Central memory T cells express C-C chemokine receptor 7 and CD45RO and are mainly located in T-cell areas of secondary lymphoid organs. Effector memory T cells express CD45RO, lack CCR7 and display receptors associated with lymphocyte homing to peripheral or inflamed tissues. Effector T cells do not express either CCR7 or CD45RO but upon encounter with antigen produce effector cytokines, such as interferon-γ. Interferon-γ release assays are used for the diagnosis of bovine and human tuberculosis and detect primarily effector and effector memory T cell responses. Central memory T cell responses by CD4+ T cells to vaccination, on the other hand, may be used to predict vaccine efficacy, as demonstrated with simian immunodeficiency virus infection of non-human primates, tuberculosis in mice, and malaria in humans. Several studies with mice and humans as well as unpublished data on cattle, have demonstrated that interferon-γ ELISPOT assays measure central memory T cell responses. With this assay, peripheral blood mononuclear cells are cultured in decreasing concentration of antigen for 10 to 14 days (long-term culture), allowing effector responses to peak and wane; facilitating central memory T cells to differentiate and expand within the culture.

Introduction

Effector and memory T cells are generated through developmental programing of naïve CD4+ T cells after antigen recognition. Differentiation of naïve CD4+ T cells into cytokine producing cells requires pathogen recognition by innate immune cells, antigen presentation to T cells, co- stimulation and transcriptional changes resulting in polarized cytokine production. For example: antigen presenting cells produce interleukin (IL)-12 in response to intracellular pathogens, which, together with antigen recognition, promotes differentiation of T cells into T helper 1 (Th1) cells[1,2] by signaling through signal transducer and activator of transcription 4 (STAT4) and T-box expressed in T cells (T-bet), leading to cell activation, IL-2 production, clonal expansion and interferon (IFN)-γ production[3,4]. If the infection is controlled, up to 95 % of the T cells generated during the expansion phase are eliminated (i.e., contraction phase) and memory T cells remain, sometimes for a lifetime[5]. Sallusto *et al*.[6], revealed two functionally distinct subsets of memory T cells in humans based on the expression of lymph node homing receptors. Central memory T cells (Tcm) express C-C chemokine receptor (CCR)-7 and CD45RO and are mainly located in T-cell areas of secondary lymphoid organs. Tcm have limited effector function, a low activation threshold and retain high IL-2 production and proliferative capacity. Effector memory T cells (Tem) express CD45RO, lack CCR7 and display receptors for homing to peripheral or inflamed tissues. Effector cells do not express either CCR7 or CD45RO but promptly produce effector cytokines, such as IFN-γ, upon antigen recognition.

IFN-γ release assays (IGRA) are used for the diagnosis of bovine and human tuberculosis[7]. *Mycobacterium bovis* is the principal agent of bovine tuberculosis (bTB) while

human cases of tuberculosis are caused mainly by *Mycobacterium tuberculosis*. With these tests, whole blood or peripheral blood mononuclear cells (PBMC) are stimulated with mycobacterial antigens for 16 to 24 hr and IFN-γ production within the supernatant is measured by ELISA or through detection of cells producing IFN-γ using ELISPOT techniques. As a result of the brief stimulation period (i.e., 16 to 24 h) and rapid cytokine production, ex vivo assays detect primarily effector and Tem responses. This has been confirmed by flow cytometric analysis of cell populations in these cultures[8-10].

Ex vivo IFN-γ responses are routinely included within the immune response panel evaluation of tuberculosis vaccines, including those used to evaluate responses by cattle. Most effective bovine tuberculosis vaccines elicit specific IFN-γ responses, but not all vaccines that induce IFN-γ responses are protective. Also, levels of IFN-γ elicited by vaccination, as measured before infection, do not necessarily correlate with protection. For instance, different BCG strains may have different capacities to induce ex vivo IFN-γ response, in spite of similar protection levels[11]. Thus, *ex vivo* IGRAs are valuable for tuberculosis diagnosis and for accessing vaccine immunogenicity; however, their use as predictors of vaccine efficacy is limited. Tcm responses to vaccination, on the other hand, may be used to predict vaccine efficacy, as demonstrated with simian immunodeficiency virus (SIV) infection in non-human primates[12,13], tuberculosis in mice14 and malaria in humans[15,16].

After an effective immune response resulting in pathogen clearance, Tcm are maintained and provide protection to an eventual second infection by the same agent. A notable exception to this scenario is *M. tuberculosis* infection of humans in which patients receiving curative anti-mycobacterial therapy are susceptible to re-infection[17,18]. Additionally, events governing immunological memory during chronic infections, wherein the antigenic stimulation persists,

are not well understood[19]. During chronic infections, such as with human immunodeficiency virus (HIV) and tuberculosis, a significant Tcm response is associated with a favorable outcome (e.g., latency with tuberculosis and subclinical disease with HIV)[20,21]. Several studies with mice and humans have demonstrated that long-term cultured IFN-γ ELISPOT assays measure Tcm responses[16,20-22]. With this assay, PBMCs are cultured in decreasing concentration of antigen for 10 to 14 days, allowing effector responses to peak and wane; facilitating Tcm to differentiate and expand within the culture.

Long-term cultured IFN-γ ELISPOT assays have also been used in veterinary research, yet the phenotype of responding cells has been difficult to assess due to a lack of critical reagents, especially an antibody to CCR7. Effective bovine tuberculosis vaccines [e.g. using a single dose of *M. bovis* Bacille Calmette Guerin (BCG), BCG followed by viral-vectored Antigen 85A subunit vaccine, or attenuated *M. bovis*ΔRD1] elicit long- term cultured IFN-γ ELISPOT responses following vaccination that correlate with protection (*i.e.*, lower mycobacterial burden and decreased TB- associated pathology) against subsequent challenge with virulent *M. bovis*[23,24]. Furthermore, the numbers of antigen-specific IFN-γ-secreting cells within long-term PBMC cultures are higher at 12 months but decrease at 24 months after BCG vaccination of neonatal calves, correlating with the degree of protection detectable post *M. bovis* challenge[25]. In this scenario, the cultured IFN-γ ELISPOT is an important tool for predicting vaccine efficacy, providing a means to prioritize vaccine candidates for high cost efficacy trials. Additionally, cultured ELISPOT techniques can be adapted for various hosts, pathogens and cytokines by altering antigens or antibodies for a variety of purposes in different research fields.

Protocol

1. Prepare the following solutions:

1.1. Prepare complete RPMI (cRMPI) by adding fetal bovine serum (FBS) to a concentration of 10 % (volume / volume), glutamine (2 μM), sodium pyruvate (1 μM), non-essential amino acids (0.1 μM), penicillin–streptomycin (100 units / ml penicillin and 0.1 mg / ml streptomycin) and 2-mercaptoethanol (50 mM) into RPMI 1640.

1.2. Prepare 2 X acid citrate dextrose, by mixing sodium citrate (77 μM), citric acid (38 μM), and dextrose (122 μM), in distilled water.

1.3. Prepare Ca^{2+} Mg^{2+} free phosphate buffered saline (PBS) pH 7.2: NaCl (137 mM), KCl (2.7 mM), Na_2HPO_4 (dibasic, 10 mM), KH_2PO_4 (monobasic, 2 mM) in distilled water; adjust the pH to 7.2.

1.4. Prepare PBS 1% (PBS 1 % BSA) by adding bovine serum albumin into PBS (volume / weight, 1g / 100mL of PBS).

1.5. Prepare PBS + 0.05 % tween 20 (PBST) by adding 0.05 % tween 20 into PBS (volume / volume, 500 l of tween 20 / l L of PBS).

1.6. Prepare 100 mM Tris HCL pH 8.2 buffer by mixing Tris (100 mM), HCl (0.50 mM) in distilled water.

1.7. Prepare 35 % ethanol solution by diluting ethyl alcohol (purity ≥99.5%) in distilled water (volume / volume, 35 ml of ethyl alcohol / 100 ml of distilled water).

1.8. Filter sterilize cRPMI, 2 x acid citrate dextrose, PBS, PBS 1 % BSA using 0.22 m filters.

2. Long-term cultured cells (14 day protocol)

(Day one)

2.1 Prepare antigen solutions at twice the final concentration in cRPMI. Antigen choice is critical and should be tailored to the purpose of the study.

2.2 Dilute recombinant *M. tuberculosis* antigens TB10.4 and antigen Ag85A to 2 g / ml (of each antigen; the final concentration is 1 g / ml).

2.3 Dilute *M. bovis* purified protein derivative (PPD-B, Prionics Ag) to 10 g / ml (the final concentration is 5 g / ml).

2.4 Dilute the recombinant early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) fusion protein (rESAT-6:CFP10) to 2 g / ml (final concentration of 1 g / ml).

NOTE: These antigens are immunodominant IFN-γ inducing antigens of tuberculous mycobacteria and may be included to stimulate PBMC from *M. bovis* infected animals. BCG or *M. bovis* RD1 vaccinated animals do not respond to rESAT-6:CFP10, as the region encoding these antigens (i.e., RD1) is deleted in these strains. rESAT-6:CFP10 used in these study was a kind gift from Dr. C. Minion, Iowa State University. TB10.4 and antigen Ag85A are immunodominant antigens present in virulent and vaccine *M. bovis* strains [37]. PPDB, as a purified protein derivative, is a complex of several antigens including antigens shared with non-tuberculous mycobacteria. Infected animals will potentially respond to all antigens (namely: TB10.4, Ag 85A, rESAT-6:CFP10 and PPDb), while vaccinated animals should not respond to rESAT-6:CFP10 [11].

2.5 Plate 500 l / well of antigen solution in quadruplicates for each animal in a 24 well plate. For this protocol, use antigens as a pool; thus use all wells contain all the antigens and no controls (such as, null or mitogen) this step. Incubate plate at 39 °C / 5 % CO_2. The normal temperature of cattle is 39 °C; thus, some investigators utilize 39 °C for culture of bovine cells. Also, in certain instances with human cells, cell culture at 39 °C provides added benefit[26,30].

2.6 Pre-load syringes coupled with 16 or 18 gauge hypodermic needles with 6 ml of 2 X acid-citrate-dextrose; and collect 60 ml of bovine blood by jugular venipuncture.

2.7 Isolate PBMC by standard density gradient centrifugation of the peripheral blood buffy coat fractions adjusting cell concentration to 4 X 10^6 cells / ml as described by Maue *et al*. [27]

2.8 Add cells (500 l / well) into antigen preloaded 24 well plate, in quadruplicate for each animal (step 2.5). Incubate plate at 39 °C / 5 % CO_2.

(Days three and seven)

2.9 Using sterile technique, carefully remove 500 l of the supernatant from each well without disturbing the cell layer.

2.10 Replenish well volume by adding 500 l / well of cRPMI containing IL-2 (30 U / l, *i.e.*, 15 U / well; recombinant human IL-2 Sigma I7908). Incubate plate at 39 °C / 5 % CO_2.

(Days 10 and 12)

2.11 Using sterile technique, remove 750 l of supernatant from each well. Replenish the volume with 750 l / well of cRPMI (**without** IL-2). Incubate plate at 39 °C / 5 % CO_2.

3. ELISPOT assay

Day one / 12th day of Long-term culture protocol

3.1 Label the ELISPOT plate properly for each set of samples, to avoid mistakes when plating cells: long-term cells plus antigen presenting cells (APCs), long-term cells without APCs and *ex vivo* / short-term cells (Figure 1). Replicates for each treatment (i.e. antigenic stimulation) should be done at least in duplicate.

3.2 Prepare capture antibody solution by diluting mouse anti-bovine IFN-γ antibody (MCA2112, clone CC330) in PBS (8 g / ml).

3.3 Using a multichannel pipettor pre-wet the wells of the ELISPOT plate with 15 l / well of 35 % ethanol for 1 min. To prevent membrane damage, do not touch the bottom of wells with pipette tips at any point during the assay.

3.4 Wash plate six times with 300 l / well of PBS. Wash fluid should be discarded by plate inversion. Washes should be done rapidly, not allowing plate wells to dry.

3.5 Pipette 100 l / well of capture anti-IFN-γ antibody (from step 1 above). Incubate at 4 °C overnight (keep the plate inside a zippered storage bag).

Day two / 13th day of the long-term culture protocol

3.6 Prepare antigen solutions at twice the final concentration. Treatments are: no stimulation (cRPMI), PPD-B (20g / ml, final concentration: 10g / ml), protein cocktail of TB10.4 and Ag85A (2 g / ml of each protein, final concentration: g / ml of each protein), rESAT-

6:CFP10 (for *M. bovis* infection, 2 μg / ml, final concentration: 1 g / ml), and a positive control, such as pokeweed (20 g / ml, final concentration: 10 g / ml). Other mitogen may be used instead of PWM (e.g., Concanavalin A).

NOTE: for this step antigens compose four different treatments and are not used as a pool (as in step 2.5). The four treatments are: NS, PPDb, protein cocktail (TB10.4 and Ag85A constitute one treatment) and PWM.

4. Short-term cell culture and adherent cell (APCs) isolation

4.1 Collect 60 ml blood from the same animals bled on day 1 (under: long-term culture, 14 day protocol) and isolate PBMCs as before.

4.2 Adjust cell concentration to 2 X 10^6 cells / ml. Label tubes clearly to prevent misallocation when plating cells. These cells will be used for APCs isolation and also as short-term cell culture (step 6.5). Label tubes with both the animal number and the type of culture (in this case: *ex vivo*, short-term or fresh cells).

4.3 Remove excess capture antibody from the ELISPOT by plate inversion. Wash plates 6 times with 300l PBST.

4.4 Remove as much PBST as possible. Pipette 50 l of cell suspensions to the wells labeled as long-term cells plus APCs. Block other wells with 200 l / well of cRPMI. Incubate 90 min at or 39 °C. Pre-warm cRPMI to or 39 °C (necessary for subsequent steps). Fresh PBMCs

not used for adherent cell (APCs) isolation will be needed in subsequent steps and should be stored.

5. Cultured cells

5.1 During the 90 min incubation (step 4.4, short-term culture and adherent cell isolation step), harvest long-term cultured cells.

5.2 Using 5 or 10 l pipettes, pipette media with cells up and down to detach cells, combine the quadruplicate replicates from each animal into a single 15 ml tube. Centrifuge the tubes for 5 min at 400 g.

5.3 After centrifugation, discard the supernatant by tube inversion. Dislodge cell pellet. Add 5 ml of PBS, gently re-suspend pellet, if necessary. Repeat the centrifugation. Repeat the washing step twice. Discard supernatant by tube inversion and gently re-suspend cells in 1ml cRPMI. Adjust cell concentration to 2 X 10^5 cells / ml.

6. Plating fresh and cultured PBMCs

6.1 After the 90 min incubation, shake plates on plate shaker for 30 seconds. Remove non-adherent cells by plate inversion. Pipette 150 l / well of warm cRPMI (from step 4, under: Short-term cells and adherent cell (APCs) isolation step).

6.2 Shake plates and discard wash fluid. Repeat wash 3 times. Add 100 l / well of each antigen into appropriate wells (pre-labeled).

6.3 To plate cells, pipette 100 l / well of long-term cultured cell suspension (2 x 10^5 cells / ml) into the wells labeled as long-term cells plus APCs. Ensure that the long-term cells added in this step are from the same animal as the APC's already in the well. Do not mix APC's and long-term cultured cells from different animals.

6.4 Pipette100 l / well of the long-term cultured cell suspension (2 x 10^5 cells / ml) into wells **without** APCs for assessment of APC requirement to the long-term culture response.

6.5 Add 100 l / well of short-term cells (freshly isolated and adjusted to 2 x 10^6 cells / ml) for *ex vivo* response assessment. Incubate plates overnight at 39 °C / 5 % CO_2 incubator. Ensure that plates are lying flat and do not stack plates.

NOTE: If analysis of cell phenotype is desired, flow cytometry can be performed as an ancillary assay. Cells are plated in 96 well U bottom plates (instead of ELISPOT plates) as described for ELISPOT assay. Capture antibody adsorption (steps 3.4 to 3.5) should be skipped. Cells are then incubated overnight at 39 °C / 5 % CO2 and a standard cell staining protocol performed. Cell staining reagents are included in the reagents table.

Day three (14th day of the Long-term culture protocol)

6.6 Dilute detection antibody (mouse anti-bovine IFN-γ, clone CC302) to 5 g / ml in PBS 1 % BSA. Discard fluids from wells and wash plates: six times with PBST (300 l / well),

placing on shaker each time for 10 sec before and in-between washes, once with dH_2O (300 l / well), an additional 6 times with PBST (300 l / well) and two times with PBS (300 l / well). After cells are removed from the plates, and if no biosafety concerns apply, procedures may be performed outside biological safety cabinets.

6.7 Discard wash fluid. Remove as much wash fluid as possible by tapping plate on paper towels. Add detection antibody (100 l / well).

6.8 Incubate plates for 120 min at or 39 °C. During this incubation step, prepare alkaline phosphatase solution following the manufacturer's instructions. Thirty min before use (i.e., 90 min after addition of detection antibody to wells) dilute the reagent in PBST. Invert the tube gently to mix reagents and incubated at room temperature.

6.9 After the 120 min incubation, discard excess detection antibody by plate inversion. Wash plate 6 times with PBST (300 l / well). Remove as much wash fluid as possible by tapping plate on paper towels.

6.10 Add 100 l / well of alkaline phosphatase solution (from step 3 above). Incubate plates for 45 min at room temperature. Discard fluid and wash each plate 6 times with PBST (300 l / well).

6.11 Prepare the substrate solution following the manufacturers' instructions (Vector Blue AP substrate III): Dilute reagents in 100 mM Tris HCL pH 8.2 buffer. Pipette 50 l / well of the substrate solution.

6.12 Incubate at room temperature until blue color starts to develop (approximately 30 min). Discard fluid and wash plates with copious amounts of dH_2O. Remove bottom plate to expose membrane, wash back of wells and allow plate to air dry.

6.13 Read plates on immunospot image analyzer or ELISPOT reader (Table 1), or manually, using a stereomicroscope. Alternatively, keep plates in dark at room temperature until reading. Because of the high stability of the reaction substrate, quality is preserved for several years.

NOTE: Cells and antigen concentrations were optimized to avoid wells with uncountable spots [23,24,27,37]. It is possible that uncountable spots formation occur in different settings or due to biological variation. If that is the case, cell concentration should be optimized so a readable spot counting is obtained.

7. Plates reading and data analysis

7.1 Perform analysis as per the Cellular Technology Limited (CTL) ELISPOT plate reader guide (Table 1). After the spots counts are obtained for each of the wells, calculate the average between duplicates. The specific immune response to each antigenic stimulus is calculated by

subtracting the average number of spots in non-stimulated wells from that of antigen-stimulated wells.

Table 1 AutoImmun Diagnostika ELISPOT reader image acquisition and cell counting procedure.

Acquiring image of plates	
1.	Turn on machine
2.	Open "Immuno Capture" Version 6.3 software.
3.	Step 1: select plate type.
4.	Step 2: load plate.
5.	Step 3: select scanning options.
6.	Step 4: start scanning.
7.	Obtain overview image of plate.
8.	Eject plate.
9.	Quit "Immuno Capture" software.
Counting the spot forming units	
1.	Open "Immuno Spot Capture" Version 5.0 software.
2.	Select object type: normal.
3.	Select counting module: smart count.
4.	Step 1: load plate.
5.	Step 2: define counting parameters: test accuracy of spot recognition on wells with different spots.
6.	Start auto count.
Quality control	
1.	Open "Immunospot Capture" Version 5.0 software.
2.	Select counting module: quality control.
3.	Step 1: load plate.
4.	Step 2: Analyze highlighted wells individually.
5.	Finish quality control.

Representative Results

Approximately one-month post-aerosol infection with *M. bovis* (10^4 colony-forming units), PBMCs from infected (n=8) and control animals (n=8) were cultured in the presence of antigens and IL-2 for 13 days. Development of Tcm responses after infection was determined using IFN- ELISPOT assay. Representative Tcm (in the presence or absence of APCs) and *ex*

vivo IFN-γ ELISPOT responses from infected animals (Three animals) and a non-infected animal (one animal) are shown in Figure 1. A successful long-term IFN- γ ELISPOT assay results in cells producing IFN-γ (Spot-Forming Cells, SFC) under stimulated conditions and near absence of a response from non-infected animals and under non-stimulated conditions. Also, a strong T cell response should occur in response to PWM. Specific responses to *M. bovis* were assessed by PPD-B or rESAT-6:CFP10 antigenic stimulation. As shown in Figure 1, robust Tcm and *ex vivo* responses to PPD-B and rESAT-6:CFP10 were detected from all three *M. bovis*-infected animals. Minimal to no Tcm and *ex vivo* responses were detected from the control animal.

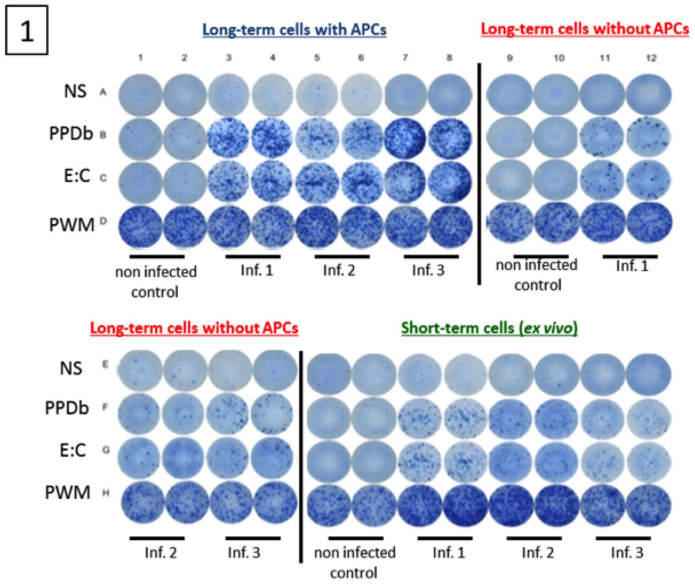

Figure 1 Image of wells from a long-term cultured IFN-γ ELISPOT assay.
Cultured IFN-γ ELISPOT assay was performed approximatelyone month after challenge with virulent *M. bovis* (three animals). Non-infected animals were included as controls (one animal). Long-term cell lines were generated by stimulating PBMC with a cocktail of recombinant Ag85A (1 µg / ml), TB10.4 (1 µg / ml), rESAT-6:CFP10 (1 µg / ml) and PPD-B (5 µg / ml) for 13 days followed by transfer to ELISPOT plates in the presence or absence of autologous APC. Short-term cells consisted of PBMC isolated on day 13 and plated directly into ELISPOT plates. Long- term and short-term cells were stimulated with PPD-B (10 µg / ml), rESAT-6:CFP10 (1 µg / ml), medium alone or pokeweed mitogen (5 µg / ml) for 24 h.

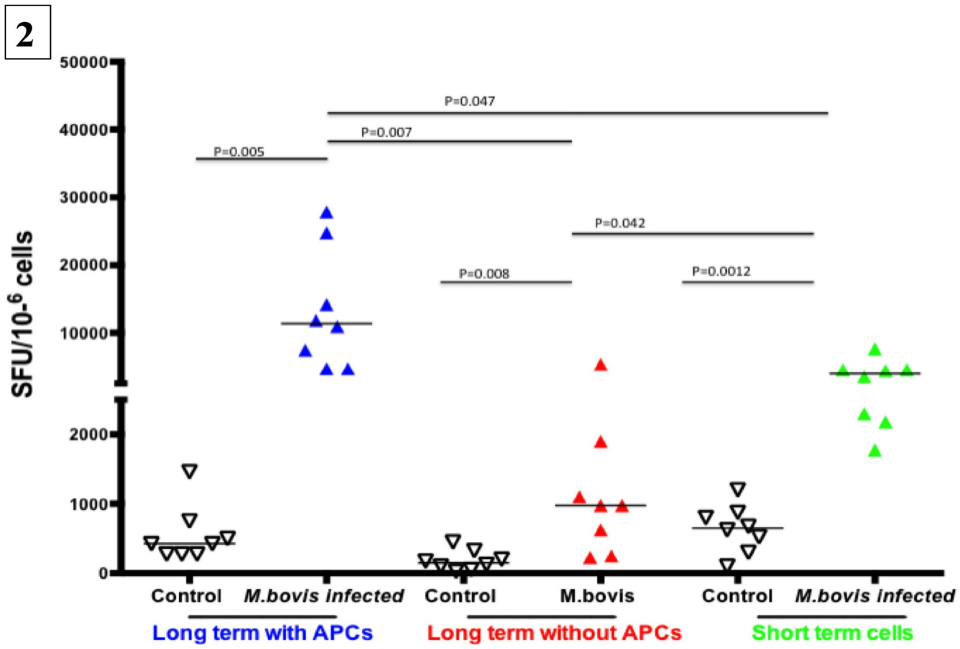

Figure 2 Representative results from a long-term cultured IFN-γ ELISPOT response to *M. bovis* purified protein derivative (PPD-B). Cultured IFN-γELISPOT assay was performed approximately one month after challenge with virulent*M. bovis* (eight animals). Non-infected animals were included as controls (eight animals Long-term cells were generated by stimulating PBMC with a cocktail of recombinant Ag85A (1 µg / ml), TB10.4 (1 µg / ml), rESAT-6:CFP10 (1 µg / ml) and PPD-B (5 µg / ml) for 13 days followed by transfer to ELISPOT plates in the presence or absence of autologous APCs. Short-term cells consisted of PBMC isolated on day 13 and plated directly into the ELISPOT plate. Long-term and short-term cells were stimulated with PPD-B (10 µg / ml) or medium alone for 24 h. Specific responses from each animal (SFC / 106 cells) are presented as response to PPD-B (average of duplicate samples) minus the response to media alone.

Discussion

Assessment of Tcm responses is also feasible by sorting these cells from PBMC. Direct enrichment of Tcm from PBMC, however, requires expensive devices, highly trained personal and is difficult as these cells are not numerous in the blood stream. Long-term culture of

PBMC provides enrichment of Tcm over Tem and effector cells without expensive devices; however, because these T cell populations are expanded *in vitro* they may be less representative of *in vivo* memory responses. It is possible to access IFN- memory responses (using the long-term culture or Tcm sorting strategies) by assays other than the ELISPOT such as: cytokine ELISAs[34], cytokine bead arrays (CBA), intracellular staining (ICS), or cytokine protein arrays (CPA). These methods; however, are generally less sensitive than ELISPOT assays[35].

An advantage of ELISPOT is its ability to detect the immediate capture of the cytokine shortly after its release preventing dilution in the supernatant and degradation by enzymatic cleavage or cytokine uptake by other cells. ELISPOT assays detect single cells producing cytokines providing precise results even in low signal to noise scenarios (i.e. low specific responses)[35]. Also, with ICS, detection of cytokines prior to release may result in false identification of cells producing cytokines (e.g., due to post-translational modulation before or during the secretory process)[34]. The transport inhibitors employed with ICS assays to minimize cytokine secretion during antigen stimulation (known as Golgi stop proteins) limit the duration of cell stimulation due to the cell toxicity of these proteins ultimately impacting cytokine production[35].

With that said - CBA, CPA and ICS are useful techniques for measuring multiple cytokines simultaneously and / or for determining cell surface marker expression (i.e., with ICS). Therefore, these methods may be used in combination with the IFN-γ ELISPOT assay[36]. While previous bovine TB vaccine efficacy studies measured Tcm responses via ELISPOT assay, the detection of Tcm responses by other techniques will likely yield comparable results. Importantly, the ELISPOT assay is less expensive and simpler to perform than CBA, CPA and

ICS analysis techniques[34]. Manual counting of the SFC is an alternative to automated counting, and ELISPOT plates can be stored at room temperature for long period of time with minimal quality loss, before or after spot counting[37]

The long-term cultured IFN-γ ELISPOT response has been applied to assess memory responses by human, and cattle when evaluating tuberculosis vaccine responses[14-16,31-32,33]. Tcm responses are also assumed to play significant roles in host responses to several other infectious agents of cattle, such as: *Mycoplasma mycoides* subsp. *mycoides*[42], *Anaplasma marginale*[38] and bovine respiratory syncytial virus[39]. Potentially, the long-term ELISPOT assay might be adapted for other animal species, cytokines and infections. These broader applications will be especially useful for veterinary immunology applications due to current limited availability of specific reagents.

Tcm responses are crucial for protection against several infections, but measuring them early after infection / immunization (for disease outcome prediction or vaccine efficacy) may be cumbersome. Analysis of the immune response under *ex vivo* and short antigenic stimulation conditions will more often represent effector responses, due to the overlap of memory and effector responses, especially during the beginning of immunological memory formation. In the context of chronic diseases in which the antigen load is continual, *ex vivo* responses will assess effector cell responses or a combination of memory and effector cell responses. The long-term cultured IFN- ELISPOT assay, described here, likely enables the measurement of Tcm responses rather than effector T cell or combined $CD4^+$ T cell responses. In summary, the long-term cultured IFN- ELISPOT assay provides a valuable approach for estimating T cell memory responses and has been employed successfully to evaluate memory responses of several species to a variety of infections agents[12-16, 20-25,29,31,33,34,38,39].

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Disclusure

The authors declare that they have no competing financial interests.

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CHAPTER 3: CHARACTERIZATION OF EFFECTOR AND MEMORY T CELL SUBSETS IN THE IMMUNE RESPONSE TO BOVINE TUBERCULOSIS IN CATTLE

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Abstract

Cultured IFN-γ ELISPOT assays are primarily a measure of central memory T cell (Tcm) responses with humans; however, this important subset of lymphocytes is poorly characterized in cattle. Vaccine-elicited cultured IFN-γ ELISPOT responses correlate with protection against bovine tuberculosis in cattle. However, whether this assay measures cattle Tcm responses or not is uncertain. The objective of the present study was to characterize the

relative contribution of Tcm ($CCR7^+$, $CD62L^{hi}$, $CD45RO^+$), T effector memory (Tem, defined as: $CCR7^-$, $CD62L^{low/int}$, $CD45RO^+$), and T effector cells ($CCR7^-$, $CD62L^{-/low}$, $CD45RO^-$), in the immune response to *Mycobacterium bovis*. Peripheral blood mononuclear cells (PBMC) from infected cattle were stimulated with a cocktail of *M. bovis* purified protein derivative, rTb10.4 and rAg85A for 13 days with periodic addition of fresh media and rIL-2. On day 13, cultured PBMC were re-stimulated with medium alone, rESAT-6:CFP10 or PPDb with fresh autologous adherent cells for antigen presentation. Cultured cells (13 days) or fresh PBMCs (*ex vivo* response) from the same calves were analyzed for IFN- γ production, proliferation, and CD4, CD45RO, CD62L, CD44, and CCR7 expression via flow cytometry after overnight stimulation. In response to mycobacterial antigens, ~75% of $CD4^+$ IFN-γ^+ cells in long-term cultures expressed a Tcm phenotype while less than 10% of the *ex vivo* response consisted of Tcm cells. Upon re-exposure to antigen, long-term cultured cells were highly proliferative, a distinctive characteristic of Tcm, and the predominant phenotype within the long-term cultures switched from Tcm to Tem. These finding suggest that proliferative responses of Tcm cells to some extent occurs simultaneously with reversion to effector phenotypes (mostly Tem). The present study characterizes Tcm cells of cattle and their participation in the response to *M. bovis* infection.

Introduction

Bovine tuberculosis (bTB) is a chronic bacterial disease of animals that may also infect humans. *Mycobacterium bovis*, the main agent causing bTB, is a member of the *Mycobacterium tuberculosis* complex, which also comprises: *M. tuberculosis*, *M. canettii*, *M.*

africanum, *M. pinnipedii*, *M. microti*, *M. caprae* and *M. mungi* [1, 2]. This genetically related group of bacteria causes TB with comparable pathology in a wide variety of hosts [3, 4]. Great strides have been made over the past century in the control of bTB in cattle and to limit the risk to humans (e.g., pasteurization of milk for dairy products); however, the disease persists as a significant socioeconomic hardship for livestock farmers with estimates of >50 million cattle infected worldwide, costing $3 billion annually. The WHO (World Health Organization), in conjunction with FAO (Food and Agriculture Organization of the United Nations) and OIE (Office International des Épizooties), recently classified bTB as a neglected zoonosis.

An essential component of the immune response to TB in humans, cattle and mice is the production of IFN-γ by T helper 1 (Th1) CD4 T cells [5-10]. Immune deficiencies affecting CD4 T cells (e.g., HIV infection) and IL-12/IFN-γ /STAT1 signaling pathways result in more severe disease upon TB infection in humans [11,12]. Given the importance of Th1 cells in the immune response to TB, it is not surprising that IFN-γ release assays (IGRA) and delayed type hypersensitivity (i.e., skin test) responses are useful correlates of infection (reviewed by Schiller *et al*. [13] for cattle and Walzl *et al*. [14] for humans). Widely utilized in IGRAs, early secretory antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) are potent inducers of Th-1 cytokines [15]. ESAT-6 and CFP-10 are co-secreted proteins encoded by the RD-1 region of the genome of *M. tb* complex mycobacteria. Such genes are absent in all *M. bovis* bacillus Calmette Guerin (BCG) strains and most other non-tuberculous mycobacteria species [16-19]. Diagnostic IGRA's are measures of '*ex vivo'* immune responses relying on rapid production of IFN-γ in response to mycobacterial antigen stimulation in short-term (16 – 24 hrs) whole blood or peripheral blood mononuclear cell (PBMC) cultures. *Ex vivo* assays for use in bTB diagnosis are generally considered a measure of T cell effector responses and are

frequently used to measure immune responses to bTB vaccines prior to and after challenge with virulent *M. bovis* [9,20]. While most protective bTB vaccines elicit *ex vivo* IFN-γ responses, not all vaccines that induce this response provide protection [21]. Additionally, levels of IFN-γ elicited by vaccination do not necessarily correlate with the level of protection afforded by the vaccine [22]. Thus, the identification of correlates of protection is needed to prioritize vaccine candidates for evaluation in costly BL-3 vaccination/challenge efficacy trials.

Recent vaccine efficacy studies in cattle have demonstrated that long-term cultured IFN-γ ELISPOT (so called, cultured IFN-γ ELISPOT) responses are positive predictors of vaccine efficacy [23-25] and duration of immunity [26]. Protection provided by vaccination is partial and protected animals have reduced mycobacterial burden and associated pathology following experimental infection. In this assay, PBMCs are stimulated with antigens for 10-13 days and maintained by fresh media exchange and exogenous IL-2. After this initial culture period, cells are re-stimulated for an additional 20 hrs in the presence of autologous antigen presenting cells (APC) in anti-IFN-γ coated ELISPOT plates. Studies with samples from humans have demonstrated that cultured ELISPOT responses are primarily a measure of T cell memory (Tcm) cells [27-29].

Sallusto *et al*. [30] identified two functionally distinct subsets of memory T cells (i.e., $CD45RA^{-}/CD45RO^{+}$) in mice and humans based on expression of the lymphoid homing receptors CD62L and CCR7. These two subsets are: (1) Tcm cells which express CD62L and CCR7 and are preferentially located in lymphoid tissues and (2) effector memory T (Tem) cells which lack CD62L and CCR7 expression and are preferentially located in peripheral tissues or remain blood associated, either circulating or contained within splenic red pulp or hepatic sinusoids [31]. Tem cells show immediate effector functions, maintaining preformed cytotoxic

granules for rapid cytolysis of infected host cells [32]. Tcm cells show elevated proliferation and IL-2 production capabilities, being able to generate Tem and effector cells [33] whereas Tem cells undergo relatively little proliferation and secrete minimal IL-2 upon restimulation [28,31,33]. While Tcm and Tem have different roles in the immune response, both subsets are thought to be important for protection against pathogens. Still, due to the high proliferative capacity and long life span of Tcm, the eliciting of Tcm is believed to provide long-term protection.

The cultured ELISPOT assay measures memory responses, primarily Tcm in humans [27-29]. Godkin *et al*. [27] tracked hepatitis C virus HLA-DR11-restricted epitopes in the course of the long-term culture, demonstrating that cultured ELISPOT IFN-γ production was due to long-lived $CD4^+$ Tcm expressing CCR7. The fundamental role of $CCR7^+$ CD4 cells was also reported by Todryk *et al*. [34]; these authors assessed the effect of depletion of CCR7 expressing cells on *ex vivo* or cultured ELISPOT responses to either influenza antigenic peptides or *M. tb* purified protein derivative (PPD). The depletion of $CCR7^+$ cells dramatically reduced cultured ELISPOT responses, yet had only a minimal effect on *ex vivo* responses. Supportive of the idea that the cultured ELISPOT response is a measure of Tcm responses, several studies have shown the association of responses measured by this assay with protection against malaria, suppression of viral recrudescence in hepatitis B virus carriers, low viremia in human immunodeficiency virus (HIV) infection, and favorable outcomes in human TB [28,35-38].

While responses measured by cultured IFN-γ ELISPOT following vaccination correlate to protection with bTB; the phenotype of the responding cells within the long-term cultures has not been determined for cattle in response to neither vaccination nor infection. A better

understanding of the cattle immune system may enable the development of improved vaccine strategies and consequently, greater protection against this zoonotic disease of cattle. In the present study, we characterize effector and memory T cell subsets in the immune response to *M. bovis* infection of cattle.

Materials and methods

Animal Use Ethics

All studies were approved by the National Animal Disease Center Animal Care and Use (Protocol #'s ACUP-2508 and ACUP-2688) / Institutional Biosafety (Permit #'s IBC-0285A and IBC-0004RA) committees and performed under appropriate project licenses within the conditions of the Animal Welfare Act originally signed into law in 1966 and in accordance with the most recent amendments. All animals were housed in appropriate biological containment facilities at the National Animal Disease Center. Animals did not develop clinical signs of bTB (such as: cough, dyspnea, anorexia and weight loss); however, one animal (from the non-infected control group) was euthanized by intravenous administration of sodium pentobarbital due to an umbilical infection.

Mycobacterium bovis aerosol challenge procedures

Two field strains of *M. bovis* were used for challenge inoculum: 95-1315 (Michigan white-tailed deer isolate) and 10-7428 (Colorado dairy isolate). Low passage (≤ 3) cultures of both strains were 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). Holstein steers (~ 6 months of age) were obtained from a bTB-free herd in Sioux Center, IA and housed in a biosafety level-3 (BSL-3) facility at NADC in separate rooms based upon treatment group. For the first experiment, treatment groups consisted of non-infected steers (n = 7) and animals receiving 10^4 colony-forming units (cfu) of *M. bovis* 95-1315 (n = 8), or *M. bovis* 10-7428 (n = 8). For the second study, a single group of steers (n=8) received 10^4 cfu *M. bovis* 10-7428. For both studies, *M. bovis* challenge inoculum was delivered to restrained calves (~9 months of age) by aerosol as described by Palmer *et al*. [39]. 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 mycobacteria to the lungs via the nostrils. The process continued until the inoculum, a 1 ml PBS wash of the inoculum tube, and an additional 2 ml PBS were delivered - a process taking ~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.

Mycobacterial isolation and assessment of lesions

All calves were euthanized ~4 months after challenge by intravenous administration of sodium pentobarbital. Tissues were examined for gross lesions and processed for microscopic analysis and isolation of *M. bovis*. Tissues collected included: lung; liver; mandibular, parotid, medial retropharyngeal, mediastinal, tracheobronchial, hepatic, and mesenteric lymph nodes. Lymph nodes were sectioned at 0.5 cm intervals and examined. Each lung lobe was sectioned at 0.5 – 1.0 cm intervals and examined separately. Lungs and lymph nodes (mediastinal and

tracheobronchial) were evaluated using a semi-quantitative gross pathology scoring system adapted from Vordermeier *et al.*[9]. Tissues collected for microscopic analysis were fixed by immersion in 10% neutral buffered formalin. For microscopic examination, formalin-fixed tissues were processed by standard paraffin-embedment techniques, cut in 5 μm sections and stained with hematoxylin and eosin. Adjacent sections from samples containing caseonecrotic granulomata suggestive of bTB were stained by the Ziehl-Neelsen technique for identification of acid-fast bacteria. Microscopic tuberculous lesions were staged (I-IV) based on a scoring system developed by Wangoo *et al*. [40].

***Ex vivo* and long-term cell culture**

PBMC were isolated from buffy coat fractions of blood collected in 2× acid-citrate-dextrose solution. Complete RPMI medium for PBMC cell culture was RPMI 1640 (GIBCO, Grand Island) supplemented with 2 mM L-glutamine, 25 mM HEPES buffer, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 1% non-essential amino acids (Sigma, St. Louis, MO), 2% essential amino acids (Sigma), 1% sodium pyruvate (Sigma), 50 mM 2-mercaptoethanol (Sigma), and 10% (v/v) fetal bovine sera (FBS). Long-term cell cultures were generated by stimulating 2×10^6/ml PBMC with a cocktail of *M. bovis* PPD (PPDb, 5 μg/ml, Prionics Ag, Sclieren, Switzerland) rAg85A (1 μg/ml, LIONEX Diagnostics and Therapeutics GmbH, Braunschweig, Germany), rTB10.4 (1 μg/ml, LIONEX Diagnostics and Therapeutics GmbH), and rESAT-6/CFP-10 (1 μg/ml, kind gift from Chris Minion, Iowa State University) in complete RPMI medium. Cells were cultured (2×10^6cells/well, 1ml/well) in 24 well flat-bottom microtiter plates (Nunc, Thermo Fisher, Waltham, MA). Media containing human rIL-2 (Sigma, 10 U/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. For ELISPOT assays, at day 13, cultured cells were added (2×10^4 of cultured PBMC/well) to anti-bovine IFN-γ capture-mAb (Serotec, Oxford, UK) coated 96-well ELISPOT plates (Millipore, Watford, UK) and incubated in the presence of autologous APCs and either PPDb (5 μg/ml), rESAT-6:CFP10 (1 μg/ml), pokeweed mitogen (PWM, Sigma) (1μg/ml) or medium alone. Autologous APCs were isolated by adherence incubating 1×10^5 freshly isolated PBMC in complete medium at 39°C/5% CO_2 for 90 min in ELISPOT plates. Non-adherent cells were discarded and the adherent cells (APCs) washed four times with warm RPMI 1640 media. Fresh complete media containing antigen and long-term cultured cells were then incubated 20h at 39°C/5% CO_2. For flow cytometric analysis, cultured cells were added (2×10^4 of cultured PBMC/well) to round-bottom well plates and incubated in the presence of APCs and either PPDb (5 μg/ml), rESAT-6:CFP10 (1 μg/ml), PWM (1μg/ml) or medium alone. Autologous APCs were prepared as described for the ELISPOT assay. Fresh complete media containing antigen and long-term cultured cells were then incubated 16h at 39°C/5% CO_2 with Brefeldin A (Sigma, 10μg/ml) added at 4h of culture.

IFN-γ ELISPOT

The *IFN-γ* ELISPOT assay was performed as described by Vordermeier *et al*. [41] and Whelan *et al*., [25]. Briefly, polyvinyldifluoride 96-well ELISPOT plates (Millipore) were coated at 4°C overnight with an anti-bovine IFN-γ capture mAb (Serotec), followed by a blocking step (10% fetal bovine serum (FBS) in RPMI 1640 media, 2h, 39°C/5% CO_2). Autologous APCs were isolated by adherence on coated and blocked ELISPOT plates. Long-term cultured cells (2×10^4 of cultured PBMC/well) were added to ELISPOT plates (Millipore,

Watford, UK) and incubated with either PPDb (5 μg/ml), rESAT-6:CFP10 (1 μg/ml), PWM (1μg/ml) or medium alone for 20h at 39°C/5% CO_2. Spot forming cells (SFC) were detected following the VECTASTAIN® ABC-AP Kit (Vector Laboratories, Burlingame, CA) standard procedures.

Flow Cytometry

Following the appropriate culture duration, cells were pooled from individual animals according to *in vitro* treatments (i.e., stimulation). Cells were stained as described by Maue *et al.,*[42] with the primary antibodies and appropriate secondary antibodies listed on the Table 1. CD4 T cells were analyzed as a separate panel, while CD8 and γδ T cells staining was performed together to enable the analysis of CD8 expressing γδ T cells. Intracellular staining was performed following BD Perm/Wash instructions (BD Biosciences, San Jose, CA).

Table 1 Primary and secondary monoclonal antibodies and proliferation staining reagents

Reagent or antibody	Specificity, Source	Secondary antibodies, Source
ILA11	Bovine CD4, Washington State University	Alexa-fluor 350, Life Technologies
ILA116	Bovine CD45RO, Washington State University	FITC, SouthernBiotech or Allophycocyanin-Cy7, Life Technologies
7D12	Human CCR7, BD Pharmingen	Allophycocyanin, SouthernBiotech
BAT31A	Bovine CD44, Washington State University	PE-Cy7, SouthernBiotech or Pacific blue, Life Technologies
BAQ92A	Bovine CD62L, Washington State University	Percp, SouthernBiotech
MCA1783-PE	Bovine IFN-γ, AbD Serotec	Not applicable
GB21A	Bovine TCR1 δ chain, Washington State University	PE-Cy7, SouthernBiotech
BAQ111A	Bovine CD8, Washington State University	PE, SouthernBiotech
CellTrace Violet	Not applicable, Life Technologies	Not applicable

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For cell-trace labeling, cells were labeled with CellTrace Violet (Invitrogen, Carlsbad, CA) following kit instructions. Briefly, either freshly isolated or long-term cells (cultured for 13 days) were resuspended at 1×10^7 cells in PBS containing 10 μM/ml of the cellTrace dye

with immediate vortexing to ensure rapid homogenous staining of cells. Staining was performed at 20°C and cell were incubated for 5 min. Cells were washed three times with PBS containing 10% FBS and cultured for additional six days in the presence of APCs and antigens in round-bottom 96-well plates before cell staining with primary and secondary antibodies. For the long-term cultured cells the culture length was 19 days. Flow cytometric analysis was performed with a BD LSR flow cytometer (BD Biosciences). Data were analyzed using FlowJo (Tree Star Inc., San Carlos, CA).

Statistical analysis

Data were analyzed using Analysis of Variance followed by Tukey's or Šídák's multiple comparisons test or Student's *t* test using GraphPAD Prism 6.0 (GraphPAD Software Inc., La Jolla, CA).

Results

Aerosol M. bovis infection of cattle elicits long-term cultured IFN-γ ELISPOT responses

Aerosol inoculation to cattle with *M. bovis* 95-1315 or *M. bovis* 10-7428 resulted in a similar distribution and severity of gross and microscopic tuberculous lesions as well as mycobacterial colonization, primarily affecting the lungs and lung-associated lymph nodes [43]. Specific cell-mediated and antibody responses, including kinetics of the response as well as antigen recognition profiles, were also comparable between the two treatment groups [43,44].

With bTB vaccine efficacy studies, long-term cultured (i.e., 14 days) IFN-γ ELISPOT

responses to vaccination (i.e., BCG, *M. bovis* ΔRD1, and viral-vectored Ag85) negatively correlates with mycobacterial burden and TB-associated pathology and positively correlates with vaccine-induced protection [23-25]. As with vaccination, *M. bovis* infection also elicited long-term cultured IFN-γ ELISPOT responses in cattle (Fig. 1). Three weeks after infection, long-term cultured IFN-γ ELISPOT responses by PBMCs from infected cattle to rESAT-6:CFP10 and PPDb exceeded ($P < 0.05$) respective responses by PBMCs from non-infected calves (Fig. 1A). Similar results were detected with *ex vivo* (i.e., short-term) responses (Fig. 1B). Also, Tcm and *ex vivo* responses did not differ ($P > 0.05$) between *M. bovis* 95-1315- and 10-7428-infected groups (Fig. S1). The weak response detected to PPDb by non-infected cattle in both *ex vivo* and long-term cultures was likely due to prior exposure to non-tuberculous mycobacteria (NTM, a common occurrence in US dairy cattle) as pre-infection whole blood (18 hr stimulation) IFN-γ responses to *M. avium* PPD (PPDa) exceeded ($P < 0.05$) respective responses to PPDb (Fig. S2). Using intracellular cytokine staining, IFN-γ responses to PPDb and to rESAT-6:CFP10 were also detected in long-term PBMC cultures at 3, 6, 8, and 12 wks after aerosol infection (Fig. 1C). Responses increased ($P < 0.05$) from 3 and 6 to 12 wks after infection. These findings demonstrate that infection of cattle with virulent *M. bovis* elicits long-term cultured IFN-γ ELISPOT responses, which are considered a surrogate of Tcm responses in humans [34,38,45].

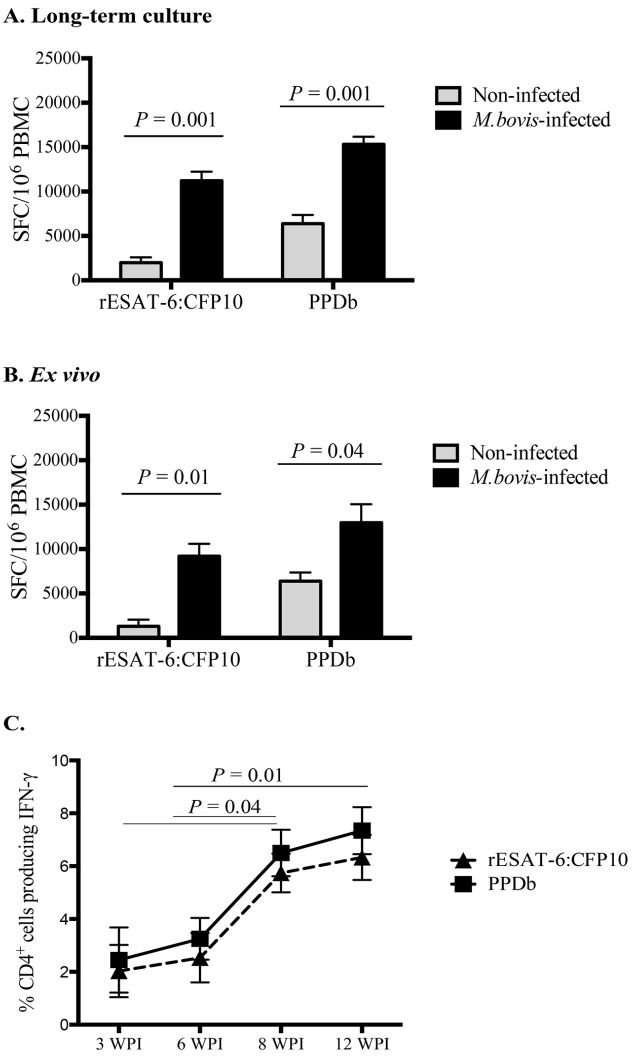

Figure 1 Long-term cultured and *ex vivo* IFN- γ responses by cattle after *M. bovis* aerosol challenge. Cultured ELISPOT analysis was performed ~3 weeks after challenge with virulent *M. bovis*. Long-term cultured cells were generated by stimulating PBMC with a cocktail of rAg85A (1 μg/ml), rTB10.4 (1 μg/ml), and rESAT-6:CFP10 (1 μg/ml) antigens as well as PPDb (5 μg/ml) for 13 days followed by transfer to ELISPOT plates with APCs and addition of either rESAT-6:CFP10, PPDb or medium alone. For the *ex vivo* response, freshly isolated PBMCs were stimulated with rESAT-6:CFP10, PPDb or medium alone for 16h. Medium control responses were subtracted from antigen-stimulated responses and results are presented as mean spot forming cells (SFC)/million cells (± SEM, n = 8) for **(A)** long-term culture or **(B)** *ex vivo* conditions. **(C)** The kinetics of the response is shown as the percent of $CD4^+$ cells producing IFN-γ in long-term cultures at 3, 6, 8, and 12 weeks post infection (WPI n = 6). Two-way ANOVA (Šídák's multiple comparison post-test).

Analysis of IFN-γ production in long-term cultures reveals a dominant contribution by Tcm

The expression of CD45RO, CD4, CCR7 and intracellular expression of IFN-γ by PBMC cells was evaluated following long-term or *ex vivo* culture (Fig. 2). CD4 T cells producing IFN-γ following long-term culture predominantly co-expressed CD45RO and CCR7 surface antigens (Fig. 3), consistent with the Tcm phenotype described for humans and mice (Fig. S3) [26],[46]. The phenotype of cells responding to PPDb ($CD4^+$ IFN-γ^+) was compared under *ex vivo* versus long-term culture conditions (Fig. 3A and S4A). The predominant cell phenotype responding to antigenic stimulation in long-term cultures was that of Tcm cells, whereas few Tcm were present under *ex vivo* conditions ($P < 0.01$, % Tcm in long-term versus *ex vivo* cultures). In contrast, effector cells contributed to *ex vivo* IFN-γ production, but only minimally to the long-term culture response ($P < 0.05$). Tem cells contributed to IFN-γ production in both *ex vivo* (~50%) and long-term cultures (~25%). The respective overall effector/memory CD4 T cells (i.e. $CD4^+$ IFN-$\gamma^{+/-}$ cells) proportions under both long- and short-term conditions are shown in Fig. S4B. The relative contribution of Tcm, Tem and effector $CD4^+$ T cells in the response to PPDb (Fig. 3B) and to rESAT-6:CFP10 (Fig. 3C) remained the same over the course of infection (i.e., at 6, 8, and 12 wks after challenge). In general a greater number of responding cells (IFN-γ^+) were observed in the long-term cultured assay as compared to the *ex vivo* assay (Fig. 3), perhaps due to the greater percentage of CD4 cells within long-term cultures (Fig. S5).

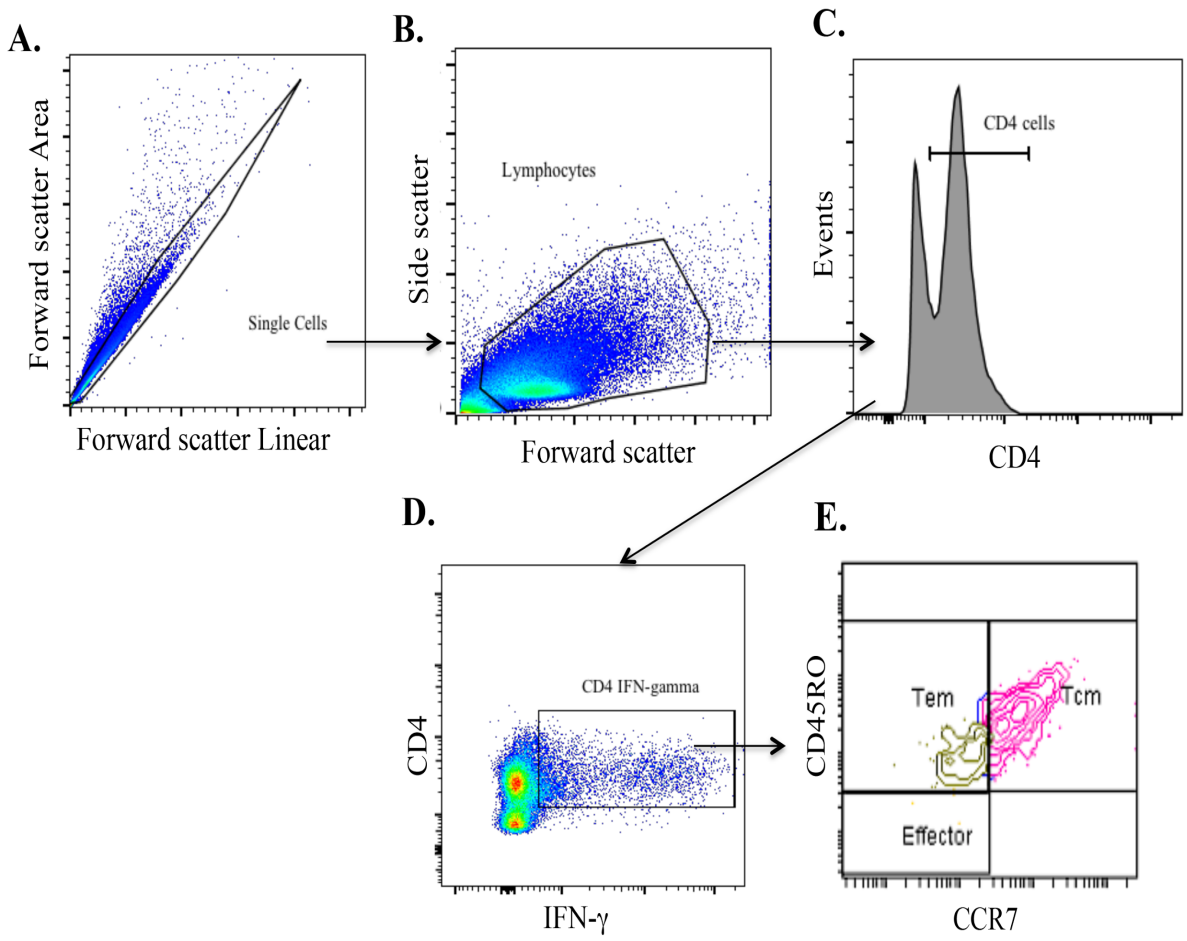

Figure 2 Representative gating strategy for evaluation of CD45RO and CCR7 expression on CD4 T cells producing IFN-γ. Approximately 8 weeks after aerosol challenge with *M. bovis*, long-term cultures were generated by stimulating PBMC with a cocktail of rAg85A (1 µg/ml), rTB10.4 (1 µg/ml) and rESAT-6:CFP10 (1 µg/ml) as well as PPDb (5 µg/ml) for 13 days followed by transfer of cells to ELISPOT plates with APCs and restimulation with PPDb. Gating hierarchy (gating sequence as depicted by the arrows): **(A)** Single cells (within the oblong gate), **(B)** Lymphocytes (within the polygon gate), **(C)** $CD4^+$ cells, **(D)** $CD4^+$ cells producing IFN-γ, and **(E)** CD45RO and CCR7 expression for determination of effector/memory phenotypes.

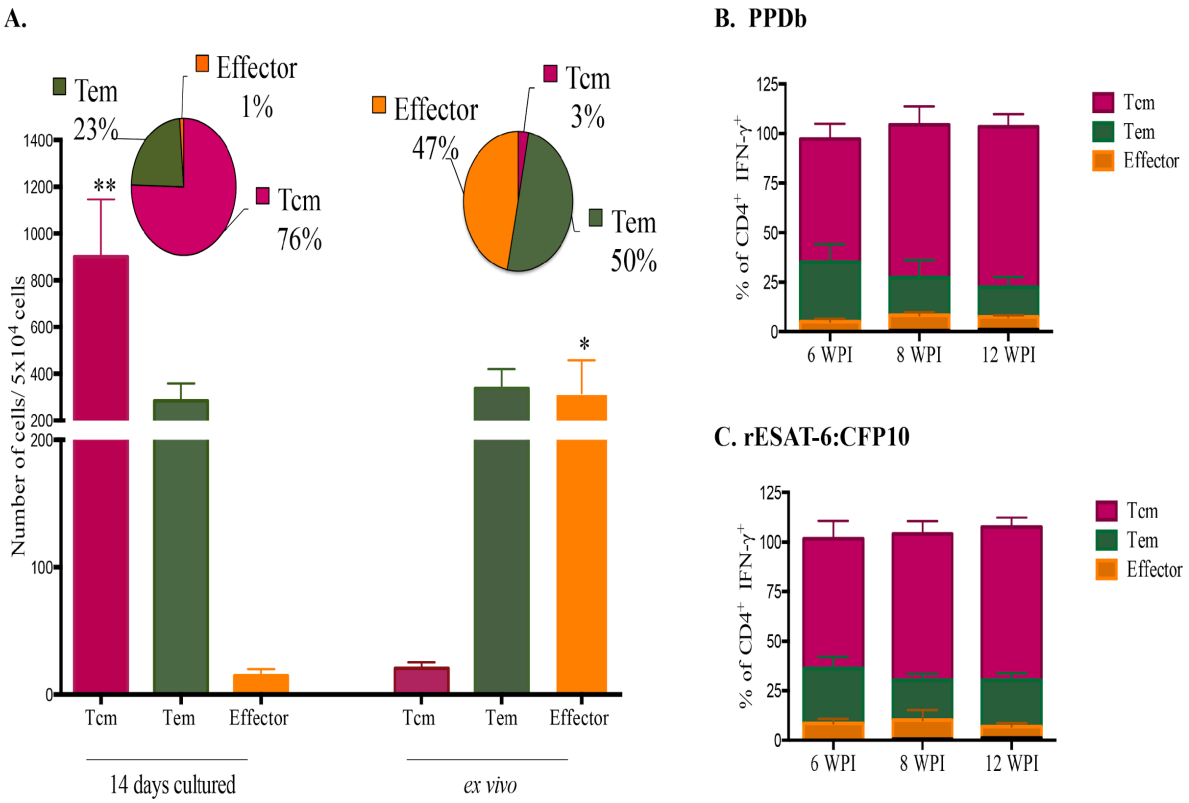

Figure 3 Frequencies of Tcm, Tem and effector cells producing IFN-γ in response to mycobacterial antigens in long-term and *ex vivo* assays. Peripheral blood mononuclear cells were isolated from calves ~ 8 weeks after challenge with virulent *M. bovis*. Cells were stimulated with a cocktail of rAg85A (1 µg/ml), rTB10.4 (1 µg/ml), and rESAT-6:CFP10 (1 µg/ml) as well as PPDb (5 µg/ml) for 13 days followed by transfer to 96 well round bottom plates with APCs and addition of media alone, PPDb or rESAT-6:CFP10 for an additional 16h. For *ex vivo* culture, PBMC were stimulated with media alone, PPDb or rESAT-6:CFP10 for 16h. **(A)** Relative contribution of Tcm, Tem, and T effector cells to IFN-γ production in response to PPDb by long-term (i.e., 14-day) (left) and *ex vivo* (i.e., 16 hr) (right) cultures, 8 weeks after *M. bovis* challenge. Data are presented in percentages (pies) and as mean (± SEM) number of cells producing IFN-γ / 10^4 cells (histograms) (n=16). Relative contribution of Tcm, Tem and T effector cells to IFN-γ production in response to PPDb **(B)** or to rESAT-6:CFP10 **(C)** in long-term cultures at three, six, eight or 12 weeks post-infection (WPI, n=6). Tcm, Tem and effector cell phenotypes were as defined in Fig. 2 and Fig. S3. Tcm and Effector T cell contribution to IFN-γ production differs (*$P < 0.05$; **$P < 0.01$, paired Student's t-tests) between short- and long-term cultures.

Tcm cells highly expressed ($P < 0.05$) CD62L and CD44 in response to either rESAT-6:CFP10 or PPDb stimulation (Fig. 4). Expression of CD62L was intermediate with Tem and

low to non-existent with effector cells (Fig. 4A). CD44 expression was low in both Tem and effector cells (Fig. 4B).

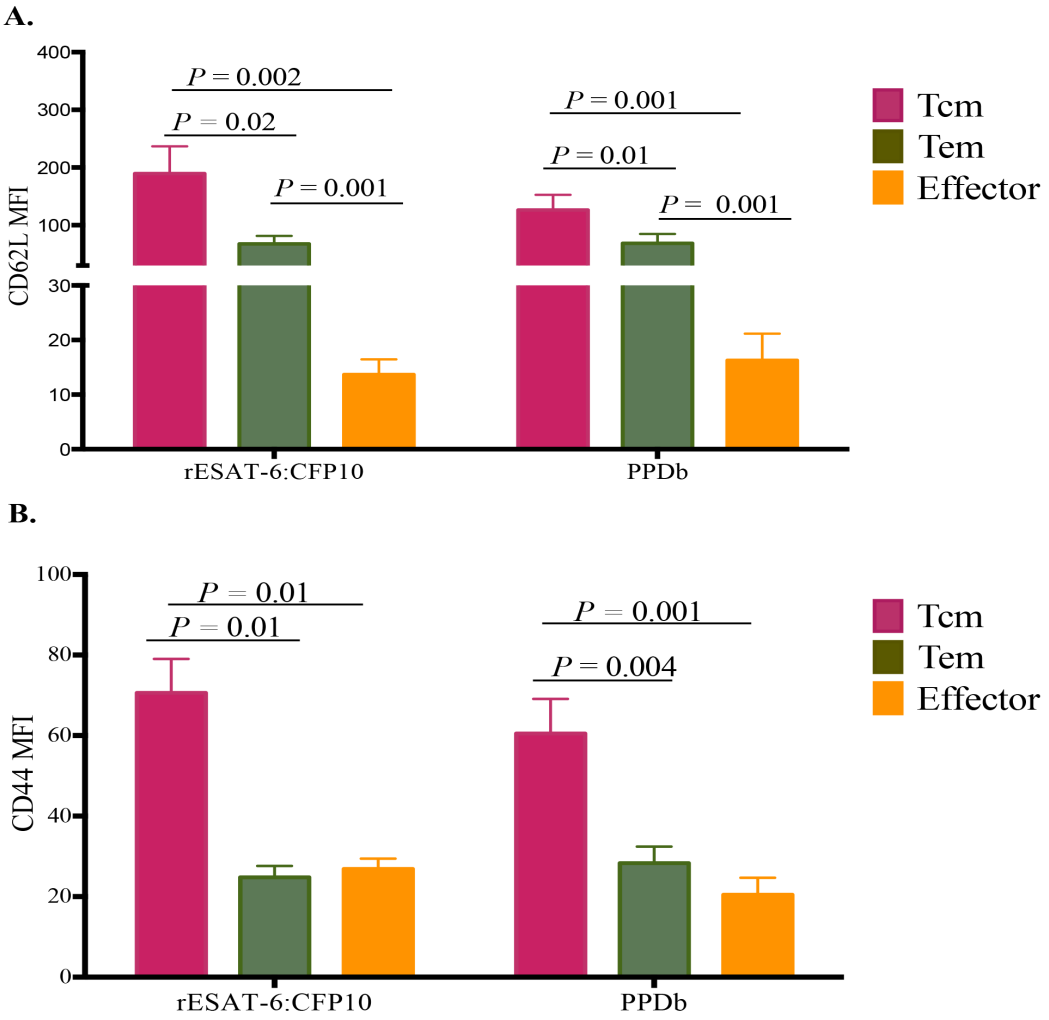

Figure 4 CD62L and CD44 expression by Tcm, Tem and effector CD4$^+$ cells in long-term (14 day) cultures. Analysis of long-term cultured PBMCs was performed ~ 8 weeks after aerosol challenge with virulent *M. bovis*. Cells were stimulated with a cocktail of rAg85A (1 µg/ml), rTB10.4 (1 µg/ml), and rESAT-6:CFP10 (1 µg/ml), as well as PPDb (5 µg/ml) for 13 days followed by transfer to 96 well round bottom plates with APCs and addition of PPDb or rESAT-6:CFP10. Data are presented as mean fluorescence intensity (MFI, y-axis, ± SEM) of CD62L or CD44 by CD4$^+$ cells of the various effector / memory subsets (x-axis). **(A)** CD62L expression by Tcm, Tem and effector cells in response to PPDb and rESAT-6:CFP10. **(B)** CD44 expression on Tcm, Tem and effector cells in response to PPDb and rESAT-6:CFP10. Tcm, Tem and effector cell phenotypes were as defined in Fig. 2 and Fig. S3. Paired Student's t-tests (n=8).

Tcm cells possess high proliferative capability

Human CD4 memory T cells, predominantly those exhibiting Tcm phenotype, proliferate in response to cytokine and antigenic stimulations, differentiating into Tem or effector T cells *in vitro* [30,47]. To assess the proliferative capacity of bovine Tcm cells following long-term culture, cells were harvested at day 13 and stained with CellTrace Violet. CellTrace Violet stained cells were re-stimulated with rESAT-6:CFP10 or PPDb for additional six days, without IL-2 (Fig. S5). For comparative purposes, freshly isolated PBMC were isolated and stained with CellTrace Violet and cultured for six days (short-term culture). Cells proliferated in response to antigenic stimulation under both long- and short-term conditions (Table 2). CD4 T cells were the most proliferative fraction ($P < 0.05$), followed by γδ, CD8 T cells, and CD8 expressing γδ T cells. In response to rESAT-6:CFP10 stimulation, the number of CD4 T cells proliferating in long-term cultures exceeded ($P < 0.05$) that of short-term cultures. Similarly, the CellTrace Violet mean fluorescence intensity (MFI) was significantly lower ($P = 0.003$), indicating greater cell proliferation in rESAT-6:CFP10-stimulated CD4 T cells in long- vs short-term cultures (Fig. 5 and Fig. 6). Greater percentages of CD4 T cells ($P < 0.05$) proliferated under long-term culture in response to either rESAT-6:CFP10 (Fig. 6A) or PPDb (Fig. 6B). These findings demonstrate that bovine Tcm cells are highly proliferative in response to repeated stimulation with recall antigen.

Table 2 Lymphocyte subset proliferative responses of *M. bovis*-infected cattle under short or long-term culture condition

Culture	Stimulation	Mean cell number (± SEM) by cell subset (50,000 cells)				
		Ungated	$CD4^+$	$CD8^+$	γδ TCR^+	γδ TCR^+ $CD8^+$
Long-term culture	rESAT-6:CFP-10	10,937 (±1476)	8,915 (±1,231) *	113 (±28) *	996 (±382)	51 (±9)
Short-term culture	rESAT-6:CFP-10	7,407 (±975)	4,076 (±567)	468 (±197)	942 (±17)	141 (±27)
Long-term culture	*M. bovis* PPD	15,061(±2187)	6,107 (±1343)	159 (±45) *	1354 (±68)	64 (±19)
Short-term culture	*M. bovis* PPD	13,146 (±2835)	4,841 (±1742)	496 (±12)	1612 (±427)	89 (±20)

Long-term cells consist of PBMC from *M. bovis*-infected cattle cultured in the presence of rAg85A, rTB10.4, rESAT-6:CFP10 and PPDb for 13 days. Subsequently, cells were stained with CellTrace violet dye and re-stimulated with either, PPDb, rESAT-6:CFP10 or medium in the presence of APCs for an additional six days. Short-term cells consist of CellTrace Violet-stained PBMC from *M. bovis*-infected cattle (n = 4) cultured for six days in the presence of either PPDb, rESAT-6:CFP10 or medium in the presence of APCs. Data are presented as the mean (±SEM) number of cells that had proliferated per 50,000 cells under short-term or long-term culture in response to antigen (PPDb or rESAT-6:CFP10) minus the response to media alone. *Responses under long-term culture differ ($P < 0.05$,two-way ANOVA, Šídák multiple comparison post-test) from the respective short-term culture (i.e., relative to lymphocyte subset and antigen stimulation).

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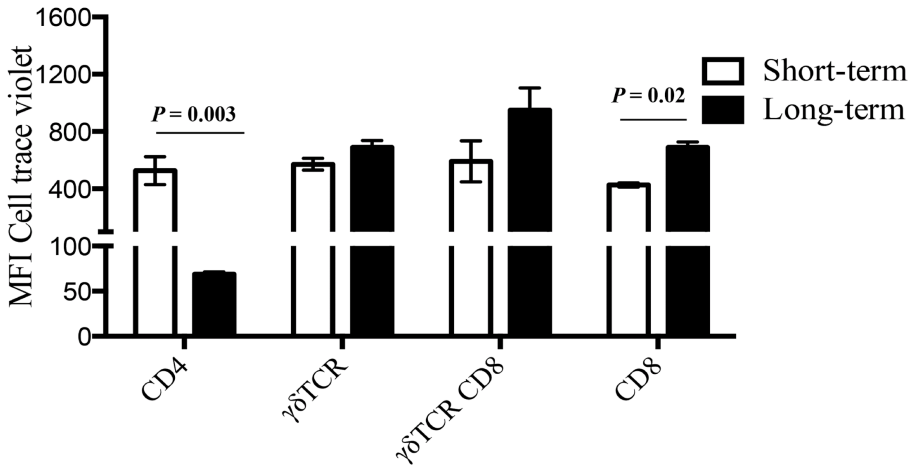

Figure 5 Proliferation of cell subsets in response to mycobacterial antigens. Approximately 7 weeks after *M. bovis* challenge, PBMCs from infected cattle were either long or short-term cultured (6 days). Long-term cells consisted of PBMCs cultured in the presence of rAg85A, rTB10.4, rESAT-6:CFP10 and PPDb for 13 days (n = 4). Subsequently, cells were stained with CellTrace violet dye and re-stimulated with either rESAT-6:CFP10 or medium in the presence of APCs for an additional six days. Short-term cells consist of CellTrace Violet-stained PBMC from *M. bovis*-infected cattle cultured for six days in the presence of either rESAT-6:CFP10 or medium (n=4). Proliferative responses of cells under both culture conditions were compared for each T cell subset. Data are presented as mean fluorescence intensity (MFI, y-axis) of CellTrace Violet for analysis of proliferation of T cell subsets (x-axis). Two-way ANOVA, n=6, Šídák's multiple comparison post-test.

Given the robust participation of CD4 T cells in the long-term proliferative response and the fact that Tcm cells are believed to generate effector cells, the phenotype of these cells (i.e., 14-day cultured cells re-stimulated with antigen for an additional 6 days) was further assessed. Again, PBMCs cultured under short-term culture conditions (6 days only) were used for comparison (Fig. 6). The distribution of CD4 T cells into Tcm and effector subsets was different between short- and long-term conditions following stimulation with rESAT-6:CFP10 (Fig. 6C) or PPDb (Fig. 6D). Greater ($P < 0.05$) numbers of Tcm cells were present in long-term (13 + 6 days) versus short-term (6 days) cultures; while more effector cells were present ($P < 0.05$) in short– versus long-term cultures. The numbers of Tem and naïve cells were similar between the two conditions. Tem cells were the main population in both cultures, whereas few naïve cells persisted under either culture condition (Fig. 6C and D). These findings indicate that highly proliferative Tcm cells partially revert to effector (predominantly Tem) phenotypes upon additional exposure to antigen for 6 days (Fig. 6C and D as compared to Fig. 3).

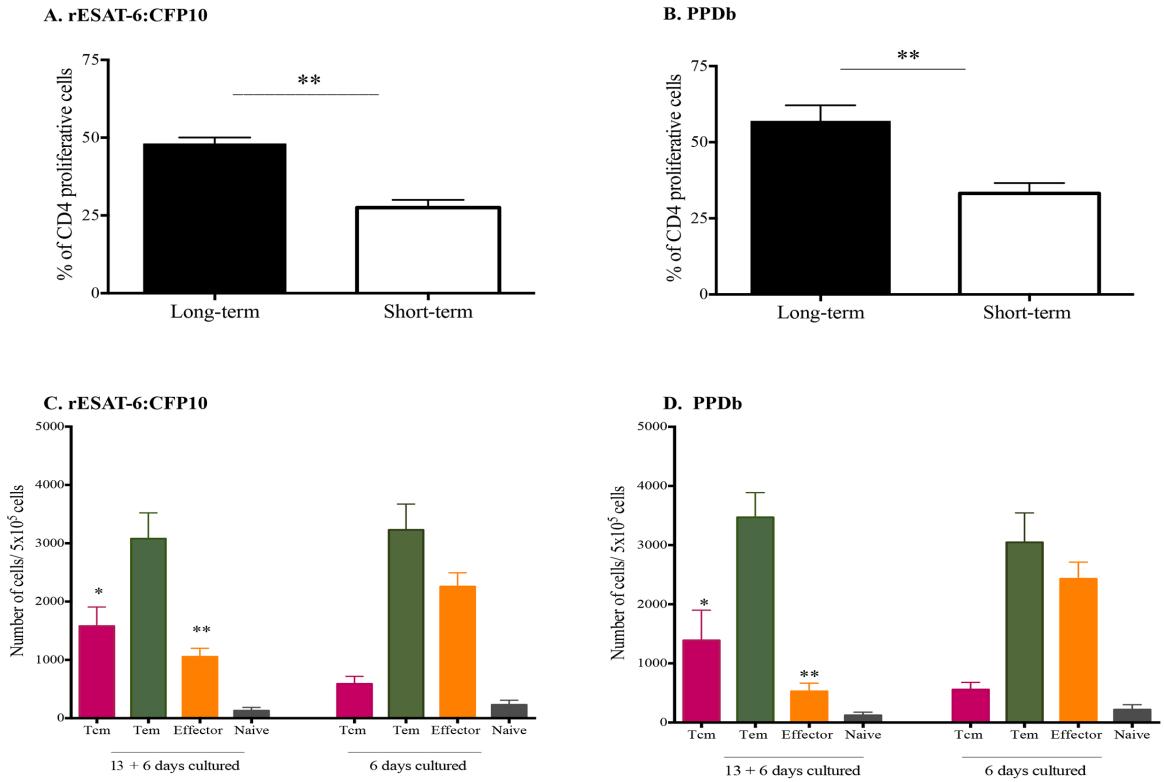

Figure 6 Long-term cultured cells have higher proliferative responses than short-term cells. Long-term and short-term cultured PBMCs were analyzed ~ 7 weeks after aerosol challenge with virulent *M. bovis*. Long-term cells consist of PBMC from *M. bovis* aerosol infected cattle cultured in the presence of rAg85A, rTB10.4, rESAT-6:CFP10 and PPDb for 13 days and then CellTrace violet-stained and re-stimulated with either rESAT-6:CFP10, PPDb or medium in the presence of fresh autologous adherent cells for an additional six days. Short-term cells consist of CellTrace violet-stained PBMC from *M. bovis* aerosol infected cattle cultured for six days in the presence of either rESAT-6:CFP10, PPDb or medium. The gating strategy was performed in accordance with procedures described in Figure 2A for single cells, and as shown in Figure S3 for lymphocytes, and $CD4^+$ cells. **(A)** Percentages of $CD4^+$ cells proliferating (low Celltrace dye MFI) in response to rESAT-6:CFP10 within long or short-term cultures. **(B)** Percentages of $CD4^+$ cells proliferating (low Celltrace dye MFI) in response to PPDb within long or short-term cultures. **(C)** Memory/effector phenotype of proliferating $CD4^+$cells within long-term or short-term cultures in response to rESAT-6:CFP10. **(D)** Memory/effector phenotype of proliferating $CD4^+$cells within long-term or short-term cultures in response to PPDb. For panels A and B, cell proliferation differs (**$P < 0.01$, n=6 paired Student's t-tests) between long and short-term cultures to either rESAT-6:CFP10 or PPDb. For panels C and D, Tcm and effector cell content differs (*$P < 0.05$; **$P < 0.01$, n=4 paired Student's t-tests) between short-term and long-term cultures to either rESAT-6:CFP10 or PPDb.

Discussion

This study characterizes Tcm cells in cattle and their participation in the immune response to *M. bovis* infection. As early as 6 weeks after *M. bovis* infection, $CD4^+$ Tcm cells ($CD45RO^+$, $CCR7^+$) were detected in long-term, antigen-stimulated PBMC cultures upon recall stimulation with specific (i.e., rESAT-6:CFP-10) or complex (i.e., PPDb) antigens of *M. bovis* (Fig. 3). Antigen-specific CD4 cells, as detected by IFN-γ production via either ELISPOT (Fig. 1A and B) or intracellular cytokine staining (Fig. 1C, 2 and 3), within long-term PBMC cultures were predominantly (~76%) Tcm cells ($CD45RO^+$ / $CCR7^+$), with the remainder being Tem cells ($CD45RO^+$ / $CCR7^-$, ~23%) (Fig. 3). Bovine Tcm were highly proliferative as antigen restimulation of cells within long-term cultures induced robust proliferation of $CD4^+$ cells that significantly exceeded that of short-term culture cells (Table 2, Fig. 5, 6A and S5). Further phenotypic analyses of repeat stimulated cultures indicated that a sub-population of bovine Tcm reverted to effector (both Tem and T effector) phenotypes upon repeat exposure to *M. bovis* antigens (Fig. 6B). The identification and characterization of CD4 Tcm and Tem subpopulations in cattle should prove useful for development of vaccines and the understanding of the immunopathogenesis for many infectious diseases of cattle.

Memory cells elicited either by vaccination [30,48] or during pathogen clearance [15,32] are thought to provide long-term protection due to their prolonged life-span, proliferation potential, and plasticity [7,33,48]. While events governing immunological memory during chronic infections (wherein the antigenic stimulation persists) are not well understood, a significant Tcm response is associated with a favorable outcome for chronic infections, such as HIV and TB (e.g., latency and self-healing with TB and subclinical disease

with HIV) [31,38]. Still, the relative importance of Tcm for protective immunity against TB is not fully established. Tcm and *ex vivo* responses are detected in *M. tb*-infected patients [33,37] and loss of Tcm responses (as measured by cultured IFN-γ ELISPOT) is associated with clinical disease progression [27,37]. Likewise, Tcm responses in the absence of *ex vivo* IFN-γ production indicates disease remission, either by self-healing [34,38] or anti-mycobacterial therapy, reinforcing the role that pathogen clearance has on Tcm function and/or maintenance [34,37]. Intriguingly, in spite of the presence of Tcm cells, patients receiving curative treatment are still susceptible to *M. tb* re-infection [28,31,49]. In the current set of experiments, each of the calves had mild progressive disease and were responsive to TB antigens in both cultured and *ex vivo* IFN-γ ELISPOT assays - similar to what occurs in humans with the mild active form of *M. tb* infection [38]. Flow cytometric analysis demonstrated that both Tem and Tcm cells were elicited relatively early after infection (3 weeks post-infection, Fig.1). It is uncertain if Tcm responses by the animals would decrease as the disease progresses, but it is frequently reported that animals in late stages of infection become anergic to measures of cell-mediated immunity, yielding false negative results upon skin test or *ex vivo* IFN-γ assays [50-52]. Prolonged infection trials with cattle are difficult due to biocontainment costs and ethical issues associated with extended duration of housing large animals within restrictive facilities. Thus, further studies to characterize the progression of Tcm responses to *M. bovis* infection in cattle may require sampling of field reactors with various clinical manifestations of the disease including: chronic progressive disease, *M. bovis* detected yet no visible lesions (i.e., analogous to latency in humans), and severe disseminated disease.

Although IFN-γ is key to a successful containment of mycobacterial infections, the interaction between pathogens and host is intricate. Several factors and cytokines may be

relevant to *M. bovis* infection outcome. Together with IFN-γ, tumor necrosis factor alpha (TNF-α) is crucial for the control of mycobacterial infections of humans and mice [53]. Also, T cells producing multiple cytokines have been recently identified and may play an important role in infection outcome. Polyfunctional T cells co-producing IFN-γ, TNF-α and IL-2 are associated with infection control in HIV, hepatitis C, leishmaniasis and malaria (reviewed in [54,55]). With TB, conflicting data indicate that polyfunctional T cell responses are associated with either clinical disease (i.e., as a biomarker of active TB) or infection control [55]. Polyfunctional T cells were also recently described in cattle in the response to *M. bovis* infection [56] and to anti-mycobacterial vaccines [57]. Studies on polyfunctional responses of bovine memory T cells under our system (long-term cultured cells) are currently in progress. Additionally, TNF-α and IL-2 have been evaluated in cattle for diagnostic purposes [58,59].

Upon *M. tb* infection, the primary immune response may take days to weeks to develop relying on exposure of naive T cells to antigens in secondary lymphoid organs, expansion of antigen-specific cells, and homing of effector cells to the site of infection [60]. With TB, this 2–3 week delay in the response at the primary site of infection seems to be advantageous for the bacteria, and is observed during infection in cattle, humans, and mice [61]. In theory, the 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. A more rapid homing of antigen-specific cells to the site of infection (e.g., as may occur in vaccinated animals) might be advantageous to the host, allowing more effective induction of immune responses (e.g., different activation status, cytokine profile) and circumvention of the pathogens regulation of host immunity [14,62,63]. These factors may partially explain why Tcm responses elicited by vaccination appear to be beneficial to the host, while similar Tcm

responses are detected in infected animals with progressive disease. The presence of a robust TB-specific Tcm population elicited by vaccination prior to infection may lead to pathogen clearance before establishment of immune evasion tactics by the pathogen. Present findings provide a basis for future studies to determine the relative role of these Tcm and Tem subsets in vaccine-elicited protection.

Lymphocyte homing and trafficking to sites of inflammation and lymphoid organs are mediated by the expression of numerous surface adhesion molecules, such as CCR7, CD62L and CD44. CD62L mediates cell adhesion to peripheral lymph node vascular addressins (e.g., GlyCAM-1 and MAdCAM-1) [64]. The expression of CD62L on naïve and memory T cells facilitates cell rolling on endothelium in secondary lymphatic organs, contributing to the compartmentalization of the immune response [64]. CD44 expression on T cells is up regulated upon activation, thereby promoting movement through the extracellular matrix via interactions with hyaluronic acid and fibronectin [65]. *In vitro* stimulation of antigen specific T cells is known to up-regulate CD44 while concurrently down regulating CD62L expression in humans [66], mice [67] and cattle [56,68]. As in humans [66], bovine T cells expressing CD44 upon *ex vivo* stimulation frequently co-express CD45RO, while down regulating CD62L [56,68]. In the present study, CD44 expression on CD4 cells did not differ between Tem and effector cells under *ex vivo* or long-term culture conditions (Fig. 4B). It is noteworthy that in the present study the expression of CD62L and CD44 was evaluated only among IFN-γ producing cells; thus, comparisons between resting/unstimulated versus responding/stimulated cells were not performed as previously described [68]. Also, the gating strategy employed (based on CCR7 and CD45RO expression) may also have contributed to the apparently discrepant findings. In the present work, the expression of CD62L was down regulated in effector cells, intermediate

on Tem cells, and high on Tcm cells (Fig. 4A). Although Tcm cells highly expressed CD62L, these cells maintained high CD44 expression. The relevance of the level of CD44 expression by memory T cells is controversial for both mice [15,69,70] and humans [66,69]. Although few authors investigated T cell memory responses in cattle [24,26,41,71-73], to our knowledge no other description of the expression of CD44 by bovine Tcm cells (as defined by CCR7 and CD45RO expression) has been published, making direct comparisons not possible. However, data from mice suggest that although the expression of CD44 is dispensable for early expansion, trafficking and cytokine production of Th1 cells; expression of CD44 is required for long-term cell survival and anamnestic responses to re-infection [70]. In humans and mice it is long known that, together with CD62L, CCR7 plays a major role for cell homing to secondary lymphoid organs (SLO). For cattle, CCR7 expression is required for CD4 T cell migration to SLO, while homing of γδ T cells to SLO is not mediated by CCR7 expression [74]. To our knowledge further cytometric analysis of CCR7 positive cells into Tcm and naïve subsets, as well the antigen specific memory response to infection has not been done in cattle. Totté *et al*. [73] reported a subset of CD4 cells with Tcm characteristics after *Mycoplasma mycoides* infection and pathogen clearance. Upon restimulation, cells expressing CD62L were highly proliferative, while being less prone to down regulate CCR7 transcription. Conversely, cells lacking CD62L showed greater IFN-γ production, lower proliferation and down regulation of CCR7 transcription. Our findings indicate that in response to antigenic stimulation, Tcm cells strongly proliferated (Fig. 5 and 6A) and were capable of switching to Tem and effector cells (CCR7 down regulation - Fig. 6B).

We also analyzed the contribution of γδ, CD8 and CD8-expressing γδ T cells to the proliferative response of long- and short-term cultures. In cattle, $CD8^+$ T-cells can bear either

α/β or γ/δ TCR, with profound impacts on antigen specificity and immunological memory, as classical antigen-specific immunological memory resides in α/β T-cells [75,76]. In BCG vaccinated animals, CD8 memory responses were elicited within the CD8 α/β TCR expressing cells, but not the CD8 expressing γδ T cells [76]. In the present study, $\gamma\delta^{-}$ $CD8^{+}$ T cells showed greater proliferation under short-term culture conditions, while the proliferative response of $\gamma\delta^{+}$ $CD8^{+}$ T cells did not differ based on culture length (i.e., short- vs long-term). However, $\gamma\delta^{+}CD8^{+}$ T cells constituted only a small population under both culture conditions (Table 1).

In summary, the present study demonstrates Tcm cells in the response to experimental *M. bovis* challenge. Our findings suggest that upon repeat *in vitro* stimulation Tcm cells proliferate into different effector cell types. The association of Tcm cells with vaccine-elicited protection, as well as in infection outcome, is still to be determined.

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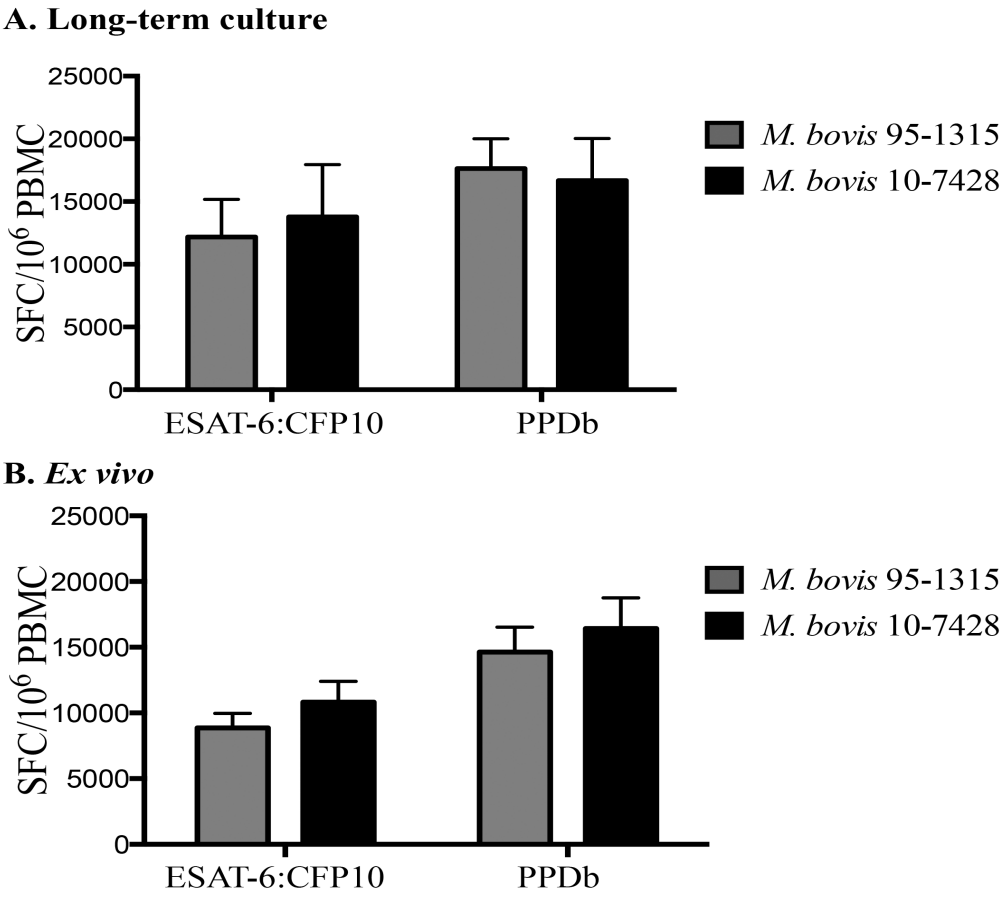
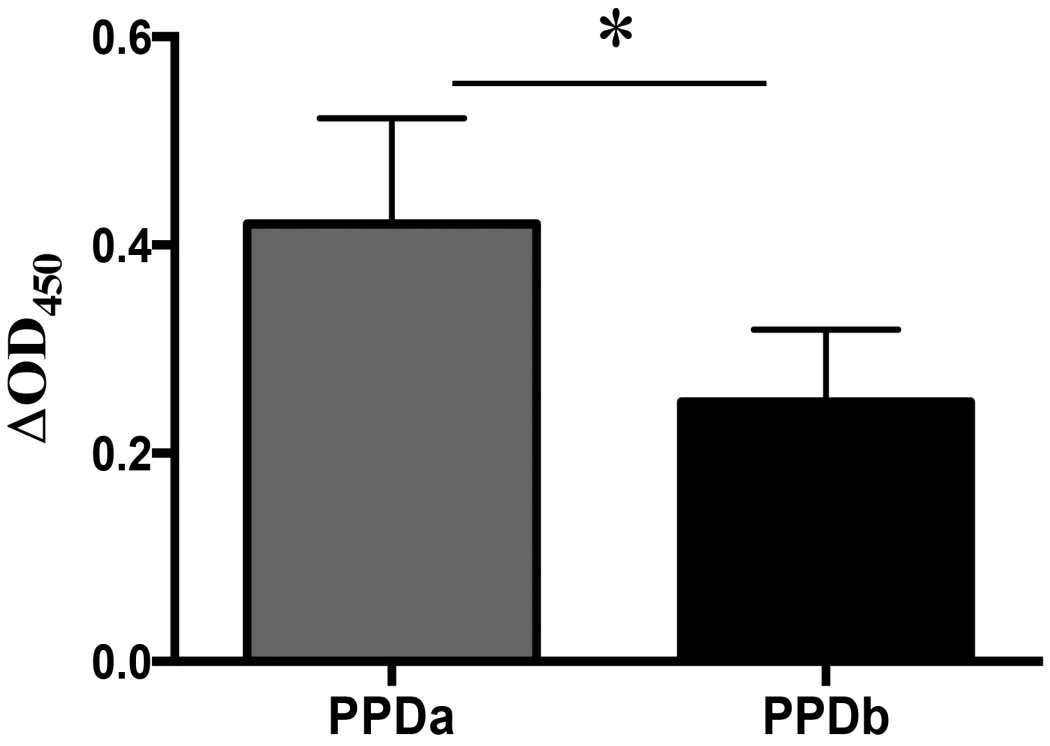
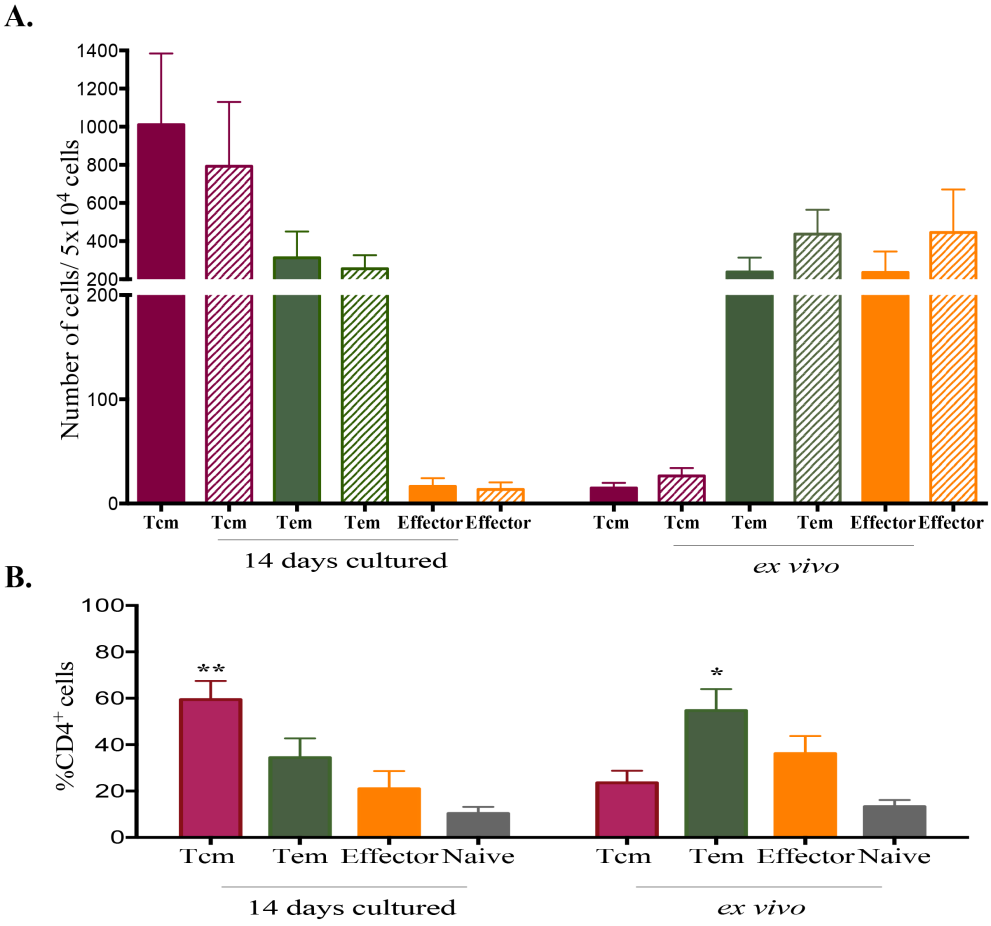
Supplemental information



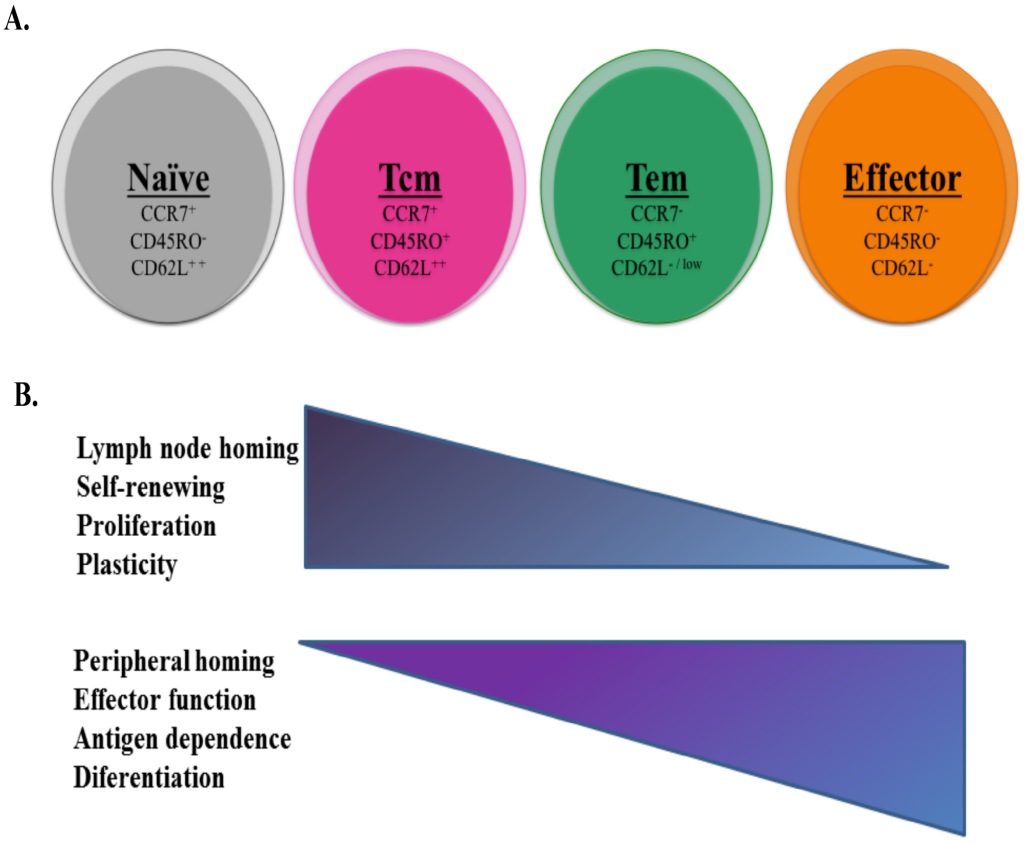
S1 Fig. Cattle infected with either *M. bovis* 95–1315 or *M. bovis* 10–7428 have similar long-term cultured and *ex vivo* IFN-γ responses after *M. bovis* aerosol challenge. Cultured ELISPOT analysis was performed ~3 weeks after challenge with virulent *M. bovis*. Long-term cultured cells were generated by stimulating PBMC with a cocktail of rAg85A (1 μg/ml), rTB10.4 (1 μg/ml), and rESAT-6:CFP10 (1 μg/ml) antigens as well as PPDb (5 μg/ml) for 13 days followed by transfer to ELISPOT plates with APCs and addition of either rESAT-6:CFP10, PPDb or medium alone. For the *ex vivo* response freshly isolated PBMCs were stimulated with rESAT-6:CFP10, PPDb or medium alone for 16h. Medium control responses were subtracted from antigen-stimulated responses and results are presented as mean spot forming cells (SFC)/million cells (± SEM, n = 8) for **(A)** long-term culture or **(B)** *ex vivo* conditions. Responses did not differ between *M. bovis* 95–1315 and *M. bovis* 10–7428 infection groups (Two-way ANOVA, followed by Tukey's multiple comparison).



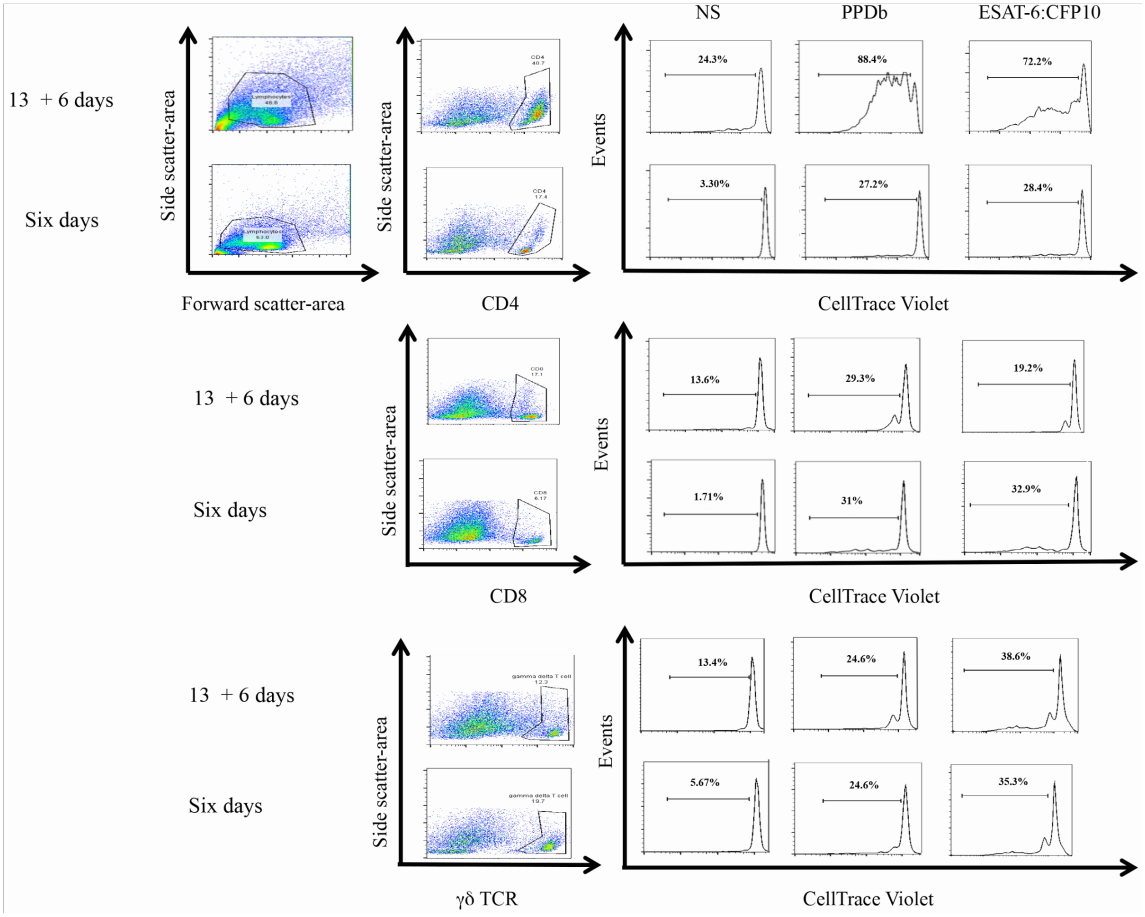
S2 Fig. Evaluation of IFN-γ responses to *M. avium*-derived PPD (PPDa) and *M. bovis*-derived PPD (PPDb) before challenge. Responses to PPDa and PPDb prior to challenge were examined using a commercial IFN-γ release assay (i.e., Bovigam, Prionics Ag, Schlieren, Switzerland) according to manufacturer instructions. Briefly, duplicate 250l heparinised whole blood aliquots were distributed in 96-well plates with PPDb (10g/ml, Prionics Ag), PPDa (10g/ml, Prionics Ag), or no antigen and incubated at 39°C/5% CO_2 for 20 hours. IFN-γ concentrations in stimulated plasma were determined using a commercial ELISA-based kit (Bovigam, Prionics Ag). Absorbencies of standards (recombinant bovine IFN-γ; Endogen, Rockford, IL) and test samples were read at 450 nm using an ELISA plate reader (Molecular Devices, Menlo Park, CA). Duplicate samples for individual treatments were analyzed and data presented as optical densities at 450 nm of the response to PPDb or PPDa minus the response to no-antigen (mean ± SEM). *Response to PPDa exceeded ($P < 0.05$, n=24, paired Student's t-test) the response to PPDb.



S3 Fig. Effector/memory phenotype of cells producing IFN-γ in response to PPDb under long-term and *ex vivo* conditions by infection group (A) and the respective efector/memory distribution of total CD4 cells, both IFN-γ$^+$ positive and IFN-γ$^-$ (B). Peripheral blood mononuclear cells were isolated from calves ~ 8 weeks after challenge with virulent *M. bovis* (n = 8). Cells were stimulated with a cocktail of rAg85A (1 μg/ml), rTB10.4 (1 μg/ml), and rESAT-6:CFP10 (1 μg/ml) as well as PPDb (5 μg/ml) for 13 days followed by transfer to 96 well round bottom plates with APCs and addition of media alone, PPDb or rESAT-6:CFP10 for an additional 16h. For *ex vivo* culture, PBMC were stimulated with media alone, PPDb or rESAT-6:CFP10 for 16h. (A) Relative contribution of Tcm, Tem, and T effector cells to IFN-γ production in response to PPDb by long-term (i.e., 14-day) (left) and *ex vivo* (i.e., 16 h) (right) cultures did not differ between *M. bovis* 95–1315 (solid) or *M. bovis* 10–7428 (dashed) infection groups for any of the phenotypes (Two-way ANOVA, Šídák's multiple comparison post-test). (B) Relative distribution of Tcm, Tem and T effector CD4$^+$ cells in response to PPDb. (mean ± SEM, *$P < 0.05$, **$P < 0.01$; n = 8, Two-way ANOVA, Šídák's multiple comparison post-test).



S4 Fig. **Cell function and expression of cell markers on different human T cell memory subsets.** (A) Identification of memory subsets in the human peripheral blood based on the expression of CD45R0, CCR7, CD62L in naïve, central memory T cells (Tcm), effector memory T cells (Tem) and effector T cells. (B) The differentiation of T cells occurs simultaneously with changes in cell functions. Adapted from Mahnkea *et al*. [46]



S5 Fig. Representative cytometric plots of the long-term and short-term cultured proliferative responses to mycobacterial antigens by CD4, CD8 and γδ T cells. Long-term and short-term cultured PBMCs were analyzed ~ 7 weeks after aerosol challenge with virulent *M. bovis*. Long-term cells consist of PBMC from *M. bovis* infected cattle cultured in the presence of rAg85A, rTB10.4, rESAT-6:CFP10 and PPDb for 13 days and then CellTrace violet-stained and re-stimulated with either rESAT-6:CFP10, PPDb or medium in the presence of fresh autologous adherent cells for an additional six days. Short-term cells consist of CellTrace violet-stained PBMC from *M. bovis* infected cattle cultured for six days in the presence of rESAT-6:CFP10, PPDb or medium.

CHAPTER 4: INCREASED TNF-α /IFN- γ /IL-2 AND DECREASED TNF-α /IFN- γ PRODUCTION BY CENTRAL MEMORY POLYFUNCTIONAL T CELLS ARE ASSOCIATED WITH PROTECTIVE RESPONSES AGAINST BOVINE TUBERCULOSIS FOLLOWING BCG VACCINATION

A manuscript submitted to *Frontiers in Immunology*

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Abstract

Central memory T cells (Tcm's) and polyfunctional CD4 T responses contribute to vaccine-elicited protection to both human and bovine tuberculosis (TB); however, their combined role in protective immunity to TB is unclear. To address this question, we evaluated polyfunctional cytokine responses by CD4 T cell effector / memory populations from bacille Calmette Guerin

(BCG) vaccinated and non-vaccinated calves prior to and after aerosol challenge with virulent *Mycobacterium bovis*. Polyfunctional cytokine expression patterns in the response by Tcm's, effector memory, and effector T cell subsets were similar between BCG-vaccinated and *M. bovis*-infected calves, only differing in magnitude (i.e., infected > vaccinated). BCG vaccination, however, did alter the kinetics of the ensuing response to virulent *M. bovis* infection. Early after challenge (3 weeks post infection), non-vaccinates had greater antigen-specific IFN-γ/TNF-α and lesser IFN-γ/TNF-α/IL-2 responses by Tcm's than did vaccinated animals. Importantly, these differences were also associated with mycobacterial burden upon necropsy. Polyfunctional responses to ESAT-6:CFP10 (antigens not synthesized by BCG strains) were detected in memory subsets, as well as effector cells, as early as three weeks after challenge. These findings suggest that cell fate divergence through asymmetric division occurs early after antigen priming in the response to bovine TB and that memory and effector T cells may expand concurrently during the initial phase of the immune response. In summary, robust IFN-γ/TNF-α responses by Tcm's are associated with greater mycobacterial burden while IFN-γ/TNF-α/IL-2 responses by Tcm's are indicative of a protective response to bovine TB.

Introduction

Bovine tuberculosis (TB) is a chronic bacterial infection affecting livestock, humans and wildlife [1]. *Mycobacterium bovis,* a member of the *Mycobacterium tuberculosis* complex, is the primary agent of TB in cattle, which causes substantial economic hardship for livestock producers. It is estimated that 50 million cattle are infected worldwide, costing approximately $3 billion annually to the livestock industry. In Great Britain alone, over 300,000 cattle were slaughtered from 2003 – 2013 in an attempt to control the disease at a cost to taxpayers of £500

million [2]. In addition to the socioeconomic ramifications of the disease, *M. bovis* poses a significant public health threat. The World Health Organization classifies bovine TB as one of the seven most neglected zoonotic diseases and as such, the disease is particularly devastating in resource poor settings due to limited regulatory control, consumption of non-pasteurized milk / non-inspected meat, and co-morbidities affecting host susceptibility and disease severity [3]. In addition to both animal and public health significance, *M. bovis* infection in cattle is an excellent model for human TB as adaptive immune responses and the ensuing immunopathogenesis are remarkably similar to that of *M. tuberculosis* infection in humans. Indeed, studies in cattle have been essential for the development of control strategies applicable to humans such as the tuberculin skin test, interferon-γ (IFN-γ) release assays (IGRA), bacille Calmette Guerin (BCG) vaccination, and approaches to differentiate infected from vaccinated individuals/animals (DIVA) (reviewed by [4]). The study of human TB, including mouse and non-human primate models, has also led to considerable progress in the understanding and control of bovine TB.

Immunological memory is a primary feature of adaptive immunity and an essential goal of vaccination [5].In naïve hosts, effector and memory T cells are generated through developmental programing of naïve cells following an infection or antigen exposure. If infection is controlled, the vast majority of T cells generated during the expansion phase are eliminated and memory T cells remain, sometimes for a lifetime [6]. Two major subsets of memory T cells (i.e., CD45RA-/CD45RO+) in humans and cattle are distinguishable based on expression of the lymphoid homing receptors CD62L and CCR7, reflecting important differences in function. Central memory T cells (Tcm) express CD62L and CCR7, are long-lived and home to lymphoid tissues while effector memory T cells (Tem) lack CCR7 and

express minimal to no CD62L. Tem's may remain blood associated or home to peripheral tissues, either circulating or remaining confined in splenic red pulp and hepatic sinusoids [6]. In humans, Tcm's exhibit elevated interleukin 2 (IL-2) production and proliferation potential, are long-lived, and able to generate heterogenic progeny capable of both terminal differentiation and self-renewal [7]. Tem's are higher producers of effector molecules, such as IFN-γ, but exhibit low proliferation capability [6, 8, 9]. In cattle, $CD45RO^{+}$ $CCR7^{+}$ memory cells in long-term cultures express high levels of CD62L (secondary lymphoid tissue homing receptor) and CD44 (homing cell adhesion molecule) and exhibit greater proliferation potential as compared to $CD45RO^{+}$ $CCR7^{-}$ memory cells [10]. While bovine $CD45RO^{+}$ $CCR7^{+}$ $CD62L^{hi}$ memory cells (Tcm's) are elicited by *M. bovis* infection, their role in the response to protective BCG vaccination has not been evaluated. However, long-term cultured IFN-γ ELISPOT assays (so called, cultured IFN-γ ELISPOT) may be used as a surrogate of Tcm responses [8, 10-13]. In cattle, cultured IFN-γ ELISPOT (i.e., 10-14 day culture followed by overnight recall stimulation) responses to BCG +/- subunit vaccines positively predict vaccine efficacy and duration of vaccine-induced protection [14-17]. As with BCG vaccination of humans, protection provided by BCG in cattle varies widely [18-19]. In humans, cultured IFN-γ ELISPOT responses are detected in spontaneously cured TB subjects in the absence of *ex vivo* responses (i.e., overnight recall stimulation) [20]. In contrast to the cultured IFN-γ ELISPOT, *ex vivo* assays detect primarily effector and Tem responses as a result of the brief stimulation period (i.e., 16 to 24 h) and rapid cytokine production [21-23].

Polyfunctional T cells simultaneously produce two or more cytokines with IFN-γ, IL-2, and tumor necrosis factor-α (TNF-α) being the most commonly measured [24, 25]. Associations between protection and vaccination-induced polyfunctional T cells have been mainly studied in

small animal models [26, 29]. In humans, strong polyfunctional responses are detected in *M. tuberculosis*-infected individuals and are generally a sign of disease progression. Still, high IL-2 production is associated with a positive clinical status (e.g., latent or treated disease), while a strong IFN-γ/TNF-α response is associated with a failed response (i.e., active TB) [30]. Human polyfunctional responses to vaccination both prior to TB exposure and in previously exposed individuals (i.e., latent infection) are extremely variable, much like BCG vaccination efficacy, and conflicting findings are reported[30]. Furthermore, the relative lack of efficacious TB vaccines for humans hinder the clear assessment of correlates of protection, including that of polyfunctional T cells [26, 27, 31-33]. In cattle, T cell polyfunctionality has only been measured upon *ex vivo* recall stimulation [21, 36]. These studies found no association between polyfunctional responses measured before challenge and vaccine success. However, polyfunctional responses to infection were associated with increased pathology and poor disease outcome [36]. Polyfunctional responses by long-term cultured cells for enrichment of Tcm responses have not been evaluated in spite of the fact that cultured IFN-γ ELISPOT is one of the most promising protection correlates in cattle [14-17]. Likewise, the discrimination of cell phenotype involved in cytokine production under both cultured and *ex vivo* conditions may be necessary to identify specific correlates of vaccine efficacy, useful for the prioritization of vaccine candidates for costly biosafety level 3 (BSL-3) efficacy trials.

Early secretory antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) are co-secreted proteins encoded by the RD-1 region of *M. tuberculosis* complex mycobacteria. Loss of the RD-1 region is the primary attenuating defect of BCG and this region is absent in non-tuberculous mycobacterial (NTM) species [37-40]. The differential expression of ESAT-6:CFP10 by tuberculous mycobacteria and their robust immunogenicity enables use of these

antigens as a tool to discriminate *M. bovis*-infected from NTM-exposed or BCG-vaccinated animals [41-45]. Antigen 85A (Ag85A) and protein TB10.4 are immunodominant antigens present in *M. tuberculosis* complex mycobacteria and BCG strains [1]. *M. bovis* purified protein derivative (PPD) is a complex antigenic formulation, including many antigens also found in NTM. In the present study, the use of these antigens permitted assessment of both broad (PPD) and specific (TB10.4, Ag 85A) responses to vaccination and infection, and exclusively to infection (ESAT-6:CFP10) [1].

Vaccine preparations included in the present study were BCG Danish (Statens Serum Institut, Copenhagen, Denmark, Strain 1331) and a cocktail of four BCG Danish (Strain 1331) deletion strains including BCG Δ*fdrA*, BCG Δ*leuCD* Δ*pks16*, BCG Δ*metA*, BCG Δ*mmaA4*. BCG Danish is a widely used human pediatric vaccine to reduce human tuberculosis. Likewise, BCG Danish has been shown to reduce bovine TB disease burden in both experimental and field studies and has a well-established safety profile in cattle [1, 46-47]. The BCG Danish deletion derivatives used in this study are more attenuated and safer than the parental BCG strain in immunocompromised mice [48,49] (and L. Berney-Meyer, M. Larsen, and W. R. Jacobs, unpublished data). In immunocompetent mice, the BCG deletions Δ*fdr8*, Δ*mmaA4*, and Δ*pks16* each result in enhanced mycobacterial immunogenicity through enhanced cross-presentation of mycobacterial antigens (Δ*fdr8*), cytokine modulation (Δ*mmaA4*), and biofilm formation (Δ*pks16*) as compared to the parental BCG [48] (and L. Berney-Meyer, M.H. Larsen, and W.R. Jacobs, unpublished data). BCG mutants, such as these, may also be used as vaccine vectors to promote epitope-specific responses (e.g., BCG Δ*pks12* for enhanced CD8 responses) [50]. In the present study and as presented elsewhere [51], BCG Danish and the cocktail of BCG Danish deletions (hereafter called BCG mutants) were equally protective and induced similar IFN-γ

and Th17-associated cytokine responses, as evaluated in *ex vivo* assays for diagnostic purposes.

A better understanding of the cattle immune system may be beneficial to the understanding of both human and bovine TB, and aid in the development of improved vaccine strategies in both species. Here, we investigated cytokine production (i.e., all combinations of IFN-γ, TNF-α, and IL-2) by flow cytometric analysis of memory T cells subsets in response to BCG vaccination and subsequent challenge with virulent *M. bovis* to determine how the elicited immune response correlated with infection outcome. Our findings indicate that early after infection, robust IFN-γ/TNF-α responses by Tcm's are associated with greater mycobacterial burden while IFN-γ/TNF-α/IL-2 responses by Tcm's are indicative of a protective response.

Materials and Methods

Animal Use Ethics and Biosafety

All studies were approved by the National Animal Disease Center Animal Care and Use and Institutional Biosafety committees and performed under appropriate project licenses within the conditions of the Animal Welfare Act. All animals were housed in appropriate biological containment facilities at the National Animal Disease Center. Given the low dose challenge and relatively short duration of the studies, animals did not develop clinical signs of bovine TB necessitating palliative therapy such as cough, dyspnea, anorexia or weight loss. 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 BSL-3 laboratory practices for handling *M. bovis* cultures and samples from *M. bovis*-infected animals.

Mycobacterium bovis vaccination and challenge procedures

Holstein steers were obtained from bovine TB-free herds in Iowa, USA and housed in a biosafety level-3 facility at the National Animal Disease Center, Ames, IA. For the first experiment (study 1), animals were experimentally infected with 10^4 colony-forming units (cfu) of *M. bovis* 10-7428 (n = 8) by aerosol inoculation as described by [52]. For the second study (study 2), calves were randomly assigned to the treatment groups: no vaccination (n = 10), vaccination with *M. bovis* BCG-Danish (Strain 1331; *n = 9*), vaccination with a cocktail of four BCG Danish deletion strains including BCG Δ*fdr8*, BCG Δ*leuCD* Δ*pks16,* BCG Δ*mmaA4* (Dao *et al.* 2008), and BCG Δ*metA* (Berney *et al.* 2015) *(n =10).* Vaccines (10^6 CFU, total dose) were administered subcutaneously at 2 weeks of age. Animals received *M. bovis* 10-7428 by aerosol (5 x 10^2 cfu) at 3.5 months of age (i.e., 3 months after vaccination) as described by [52].

Mycobacterial isolation and assessment of lesions

All calves were euthanized ~3.5 months after challenge with virulent *M. bovis* by intravenous administration of sodium pentobarbital. Tissues were examined for gross lesions and processed for microscopic analysis and isolation of *M. bovis*. Tissues collected included: lung; liver; mandibular, parotid, medial retropharyngeal, mediastinal, tracheobronchial, hepatic, and mesenteric lymph nodes. Lymph nodes were sectioned at 0.5 cm intervals and examined. Each lung lobe was sectioned at 0.5 – 1.0 cm intervals and examined separately. Lungs and lymph nodes (mediastinal and tracheobronchial) were evaluated using a semi-quantitative gross pathology scoring system adapted from Vordermeier *et al.* [53]. Tissues collected for microscopic analysis were fixed by immersion in 10% neutral buffered formalin. For microscopic

examination, formalin-fixed tissues were processed by standard paraffin-embedment techniques, cut in 5 mm sections and stained with hematoxylin and eosin. Adjacent sections from samples containing caseonecrotic granulomata suggestive of bovine TB were stained by the Ziehl-Neelsen technique for identification of acid-fast bacteria. Microscopic tuberculous lesions were staged (I-IV) based on a scoring system developed by Wangoo *et al*. [54].

Long-term and ex vivo cell culture

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coat fractions of blood collected in 2× acid-citrate-dextrose solution by density gradient centrifugation using ficoll-paque (Sigma, St. Louis, MO). Complete RPMI medium for PBMC cell culture was RPMI 1640 (GIBCO, Grand Island) supplemented with 2 mM L-glutamine, 25 mM HEPES buffer, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 1% non-essential amino acids (Sigma), 2% essential amino acids (Sigma), 1% sodium pyruvate (Sigma), 50 mM 2-mercaptoethanol (Sigma), and 10% (v/v) fetal bovine sera (FBS). Long-term cell cultures were generated by stimulating 2×10^6/ml PBMC with a cocktail of *M. bovis* PPD (PPD, 5 μg/ml, Prionics Ag, Sclieren, Switzerland) Antigen85A (Ag85A - 1 μg/ml, LIONEX Diagnostics and Therapeutics GmbH, Braunschweig, Germany), TB10.4 (1 μg/ml, LIONEX Diagnostics and Therapeutics GmbH), and/or ESAT-6/CFP-10 (1 μg/ml, kind gift from Chris Minion, Iowa State University) in complete RPMI medium. Cells were cultured (2×10^6cells/well, 1ml/well) in 24 well flat-bottom microtiter plates (Nunc, Thermo Fisher, Waltham, MA) at 39°C/5% CO_2 for 13 days. The normal body temperature of cattle (*Bos taurus*) is 39°C and incubation of human blood at 39°C, as compared to 37°C, augments cytokine responses [55]. Complete media containing

human rIL-2 (Sigma, 10 U/ml) was used to replace media from the PBMC cultures at days 3 and 7. Fresh complete media without IL-2 was used at days 10 and 12.

At day 13, cultured cells were plated (2×10^5 of cultured PBMC/well) into 96-well ELISPOT plates (Millipore, Watford, UK) and incubated in the presence of autologous APCs and either PPD (5 μg/ml), Ag85A/TB104 (1 μg/ml of each protein), ESAT-6:CFP10 (1 μg/ml), pokeweed mitogen (PWM, Sigma) (1μg/ml) or medium alone. Autologous APCs were isolated by adherence incubating 1×10^5 freshly isolated PBMC in complete medium at 39°C/5% CO_2 for 90 min in ELISPOT plates. Non-adherent cells were discarded and the adherent cells (APCs) washed four times with warm RPMI 1640 media. Fresh complete media containing antigen and long-term cultured cells were then incubated 20h at 39°C/5% CO_2. Long-term cultured cells were then incubated 16h at 39°C/5% CO_2 with Brefeldin A (Sigma, 10mg/ml) added at 4h of culture.

For *ex vivo* culture, fresh PBMC isolated from buffy coat fractions of blood collected in 2× acid-citrate-dextrose solution were plated into 96 well plates (2×10^5) and stimulated with either PPD (5 μg/ml), Ag85A/TB10[4] (1 μg/ml of each protein), ESAT-6:CFP10 (1 μg/ml), PWM (1μg/ml) or medium alone for 16h at 39°C/5% CO_2 with Brefeldin A (Sigma, 10mg/ml) added at 4h of culture [56].

Flow Cytometry

Following the appropriate culture duration, cells were pooled from individual animal replicates according to *in vitro* treatments (i.e., stimulation). Cells were stained as described by Whelan *et al.* [21] for assessment of bovine polyfunctional CD4 T cells and Maggioli *et al.* [10] for assessment of bovine memory/effector CD4 T cell subsets with antibodies listed in Table 1.

Intracellular staining was performed following BD Perm/Wash instructions (BD Biosciences, San Jose, CA). Flow cytometric analysis was performed with a BD LSR flow cytometer (BD Biosciences). Data were analyzed using FlowJo X (Tree Star Inc., San Carlos, CA). A representative plot of the gating strategy is depicted in Fig. S1.

Statistical analysis

Data were analyzed using Analysis of Variance followed by Sidak's or Tukey's multiple comparison test or Student's *t* test using GraphPAD Prism 6.0 (GraphPAD Software Inc., La Jolla, CA).

Table 1 Primary and secondary monoclonal antibodies and staining reagents

Reagent	Specificity, Source	Fluorophore, Source
ILA11	Bovine CD4, *Washington State University*	PE-Cy7, SouthernBiotech
ILA116	Bovine CD45RO, *Washington State University*	APC-Cy7, Life technologies
7D12	Human CCR7 (CD197), BD Pharmingen	AF 350, Life technologies or SpectralRed, SouthernBiotech
MCA1783-PE	Bovine IFN-γ, AbD Serotec	Not applicable
MCA2334-FITC	Bovine TNF-α, BD Pharmingen	Not applicable
AbD14386-DyLight649	Bovine IL-2, AbD Serotec	Not applicable
Live/Dead staining	PacificBlue, Life Technologies	Not applicable

Results

M. bovis infection of cattle elicits specific polyfunctional cytokine production by CD4 T cells in long-term and ex vivo cultures

Prior studies have demonstrated that *M. bovis* infection of cattle elicits polyfunctional CD4 T cell responses [21, 36]; however, the relative contribution of effector, Tem and Tcm subsets has not been evaluated. In the present study, individual cytokine (i.e., IFN-γ, TNF-α and IL-2) production by infected animals (study 1) did not differ between *ex vivo* or long-term cultured cells in response to either PPD or ESAT-6:CFP10 stimulation; however, the phenotype of responding cells greatly differed ($P < 0.05$) between culture conditions (Fig. 1). In response to either PPD (Fig. 1C) or ESAT-6:CFP10 (Fig. 1D), Tcm's ($CD4^+$ $CD45RO^+$ $CCR7^+$) comprised ~80% of cytokine producing cells in long-term cultures while Tem's ($CD4^+$ $CD45RO^+$ $CCR7^-$) comprised ~60% of cytokine producing cells in *ex vivo* cultures. Analysis of CD4 T cell polyfunctional profiles (i.e., all combinations of IFN-γ, TNF-α and IL-2) revealed frequent co-production of multiple cytokines under both *ex vivo* and long-term culture conditions in response to either PPD or ESAT-6:CFP10 by *M. bovis*-infected cattle (Table 2). IFN-γ/TNF-α and IFN-γ/TNF-α/IL-2 co-production were the predominant ($p < 0.05$) polyfunctional cytokine profile, regardless of culture condition. Also, long-term and *ex vivo* polyfunctional responses to either PPD or ESAT-6:CFP10 were comparable (i.e., comparison between the same profile under either *ex vivo* or long-term culture; $p > 0.05$) for all seven possible cytokine profiles (Table 2).

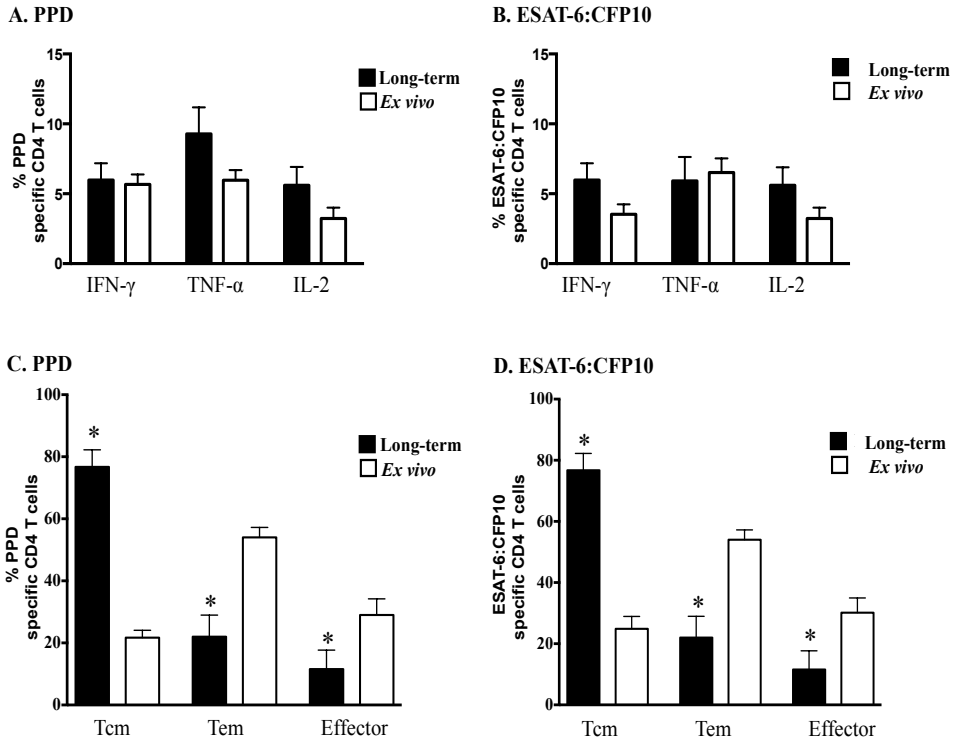

Figure 1. **Individual cytokines produced by different effector/memory phenotypes based upon culture conditions.** For long-term cultures, PBMCs were isolated and stimulated with a cocktail of Ag85A, TB10.4, and ESAT-6:CFP10 as well as PPD for 13 days followed by transfer to 96 well round bottom plates with APCs and addition of media alone, PPD or ESAT-6:CFP10 for an additional 16h. For *ex vivo* culture, PBMCs were stimulated with media alone, PPD or ESAT-6:CFP10 for 16 h. Results are presented as average and standard error for responses over the course of the study (n = 5 animals, 3 time points). Frequency long-term (closed bars) and *ex vivo* (open bars) cultured CD4 T cells producing IFN-γ, TNF-α or IL-2 in response to PPD (**A**) or ESAT-6:CFP10 (**B**). Phenotype of long-term (closed bars) and *ex vivo* (open bars) CD4 T cells producing any combination of IFN-γ, TNF-α or IL-2 in response to PPD (**C**) or ESAT-6:CFP10 (**D**) based on CD45RO and CCR7 expression (Fig. S1).
* Responses from PBMCs differ ($P < 0.05$, Sidak's) between long-term and *ex vivo* cultures.

Table 2 IFN-γ/TNF-α/IL-2 and IFN-γ/TNF-α are the predominant polyfunctional profile in response to *M. bovis* infection, as assessed by long-term and *ex vivo* assays.

	PPD		ESAT6:CFP10	
	Long-term	*ex vivo*	Long-term	*ex vivo*
Cytokine profile*	Average (SEM)	Average (SEM)	Average (SEM)	Average (SEM)
IFN-γ/TNF-α/IL-2	3.92 (0.55) [a]	4.03 (0.78) [a]	4.51 (0.64) [a]	3.79 (0.71) [a]
IFN-γ/TNF-α	4.44 (0.51) [a]	4.69 (0.59) [a]	5.37 (0.59) [a]	4.32 (0.70) [a]
IFN-γ/IL-2	1.31 (0.09) [b]	1.26 (0.22) [b]	1.01 (0.36) [b]	1.18 (0.24) [b]
TNF-α/IL-2	2.02 (0.24) [b]	2.09 (0.24) [b]	1.39 (0.14) [b]	2.06 (0.30) [b]
IFN-γ	1.15 (0.12) [b]	1.27 (0.35) [b]	0.86 (0.13) [b]	1.28 (0.41) [b]
TNF-α	0.31 (0.05) [b]	1.08 (0.23) [b]	0.20 (0.06) [b]	1.15 (0.28) [b]
IL-2	0.98 (0.36) [b]	1.83 (0.29) [b]	1.48 (0.35) [b]	1.79 (0.34) [b]

* Data are presented as frequencies (%) of cytokine producing cells within long-term or ex vivo cultures.
abc; Different letters represent differences ($P < 0.05$) in the frequency of profiles within each culture condition (long-term or *ex vivo)*. Differences in polyfunctional profile frequencies between long-term and *ex vivo* were not detected ($P > 0.05$, Tukey's).

As with the analysis of individual cytokine production, Tcm's were the main phenotype contributing to polyfunctional responses in long-term cultures whereas Tem's were the main subset producing cytokines in *ex vivo* cultures (Fig. 2).

Vaccine efficacy.

Three and half months after vaccination (study 2), animals received virulent *M. bovis* by aerosol. Vaccine efficacy was assessed upon necropsy at 4.5 months after challenge. BCG mutants and BCG-vaccinates had reduced bacterial load (Fig. 3A, $P < 0.05$) and gross

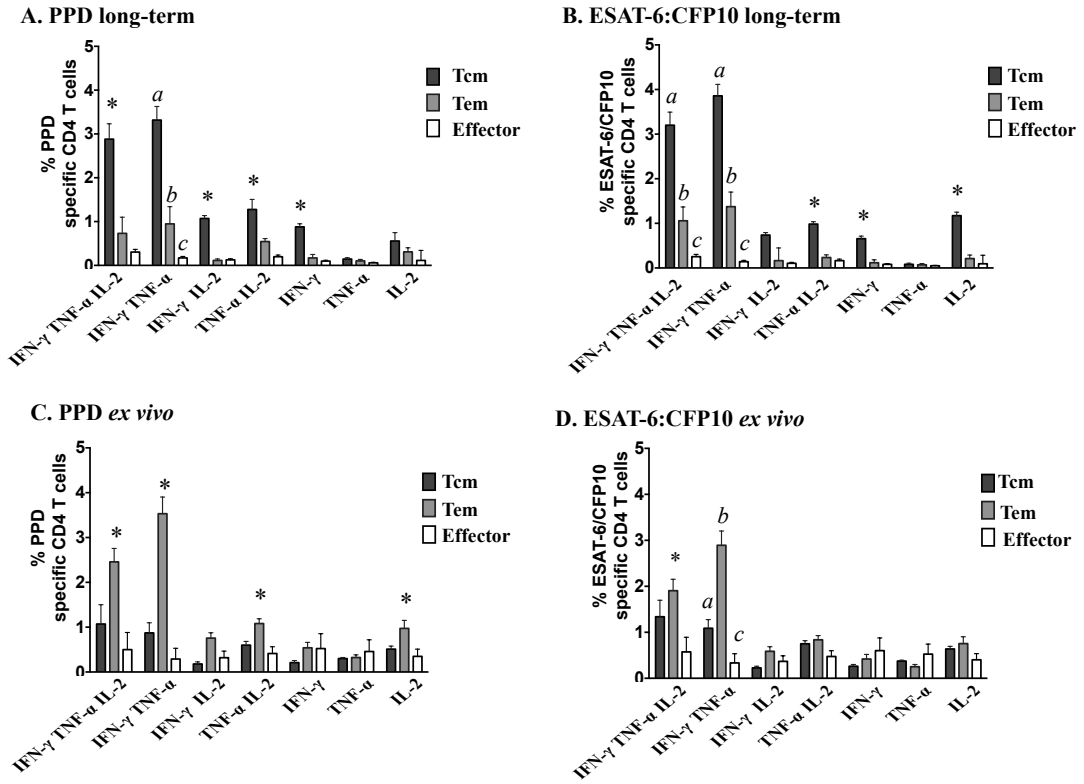

Figure 2. Polyfunctional profiles by effector/memory phenotypes differ based on culture conditions. For long-term culture, PBMCs were isolated and stimulated with a cocktail of Ag85A, TB10.4, and ESAT-6:CFP10 as well as PPD for 13 days followed by transfer to 96 well round bottom plates with APCs and addition of media alone, PPD or ESAT-6:CFP10 for an additional 16h. For *ex vivo* culture, PBMC were stimulated with media alone, PPD or ESAT-6:CFP10 for 16h. Results are presented as average and standard error for responses over the course of the study (n = 30, 5 animals x 3 time points). Percentages of polyfunctional profiles in response to long-term stimulation with PPD (**A**) or ESAT-6:CFP10 (**B**) or *ex vivo* stimulation with PPD (**C**) or ESAT-6:CFP10 (**D**) split by effector/memory phenotype based on CD45RO and CCR7 expression (Fig. S1).
* Responses differ ($P < 0.05$, Tukey's) from the other two phenotypes.
^{abc} Different letters represent differences ($P < 0.05$, Tukey's) in effector/memory phenotypes within a particular polyfunctional subset.

pathology as compared to non-vaccinates (Fig. 3B, $P < 0.05$). Mean disease scores and *M. bovis* colonization did not differ between BCG mutants and BCG-vaccinates. Lesion staging by histology corroborated gross lesion results (Fig. 3C). Noteworthy, only non-vaccinated calves developed stage IV granulomas that generally contain large numbers of acid-fast bacilli [57],

likely associated with increased transmission. In summary, both vaccines were exquisitely protective, decreasing pathology and bacterial burden.

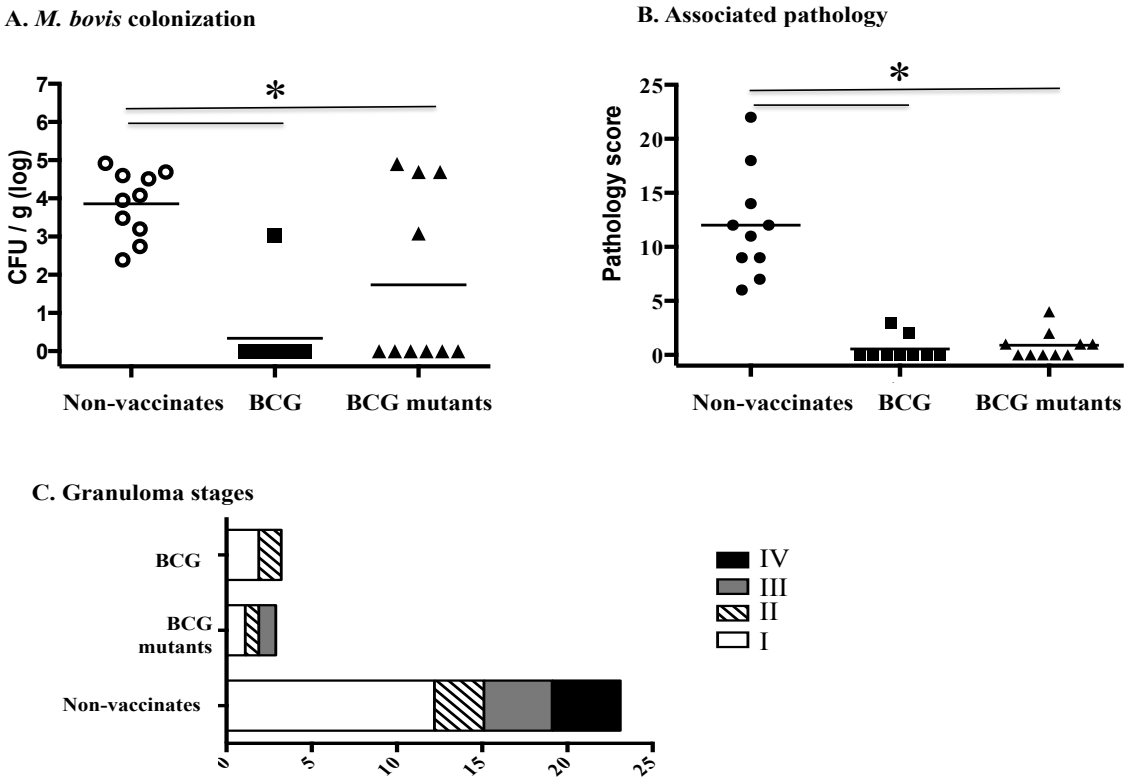

Figure 3. BCG mutants and BCG vaccination confers protection against virulent *M. bovis* challenge. (**A**) Quantitative assessment of mycobacterial burden in tracheobronchial lymph nodes. (**B**) Total gross pathology scores from lung, tracheobronchial and mediastinal lymph nodes. (**C**) Data are presented as average number of stage I - IV microscopic lesions observed in mediastinal lymph nodes, tracheobronchial lymph nodes and lung based on the scoring system developed by Wangoo *et al.* 2005.
* Parameters differ ($P < 0.05$, Tukey's) between non-vaccinates (n=10) and BCG mutants (n=10) or BCG vaccinates (n=9).

Cytokine responses elicited by BCG vaccination.

Vaccination of calves with either BCG or BCG mutants (study 2) elicited specific cytokine production at 6 weeks post-vaccination (WPV) in response to Ag85A/TB10.4 (Fig. 4) and PPD (Fig. S2). As expected for this time point prior to *M. bovis* infection, non-

vaccinated calves showed minimal or no responses to mycobacterial antigens. Long-term culture responses by vaccinated calves, as measured by IFN-γ, TNF-α and IL-2, were generally higher ($P < 0.05$) than the respective *ex vivo* responses (Fig. 4).

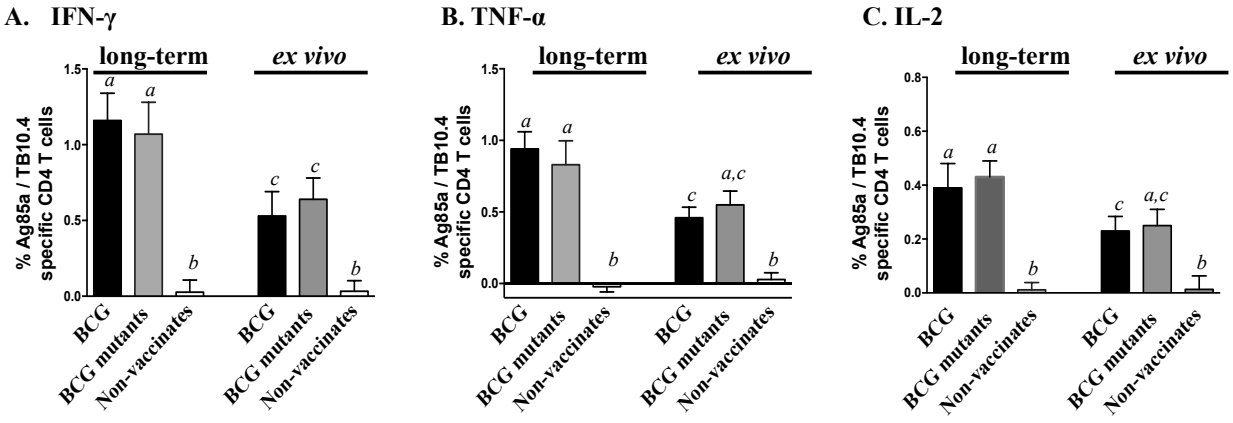

Figure 4. Long-term and *ex vivo* cytokine responses to vaccination. For long-term culture, PBMCs were isolated at 6 WPV and stimulated with a cocktail of Ag85A, TB10.4, and PPD for 13 days followed by transfer to 96 well round bottom plates with APCs and addition of media alone or Ag85A/TB10.4 for an additional 16h. Results are presented as average and standard error. Frequency of long-term (left) and *ex vivo* (right) cultured CD4 T cells producing IFN-γ (**A**) TNF-α (**B**) and IL-2 (**C**).
* *abc*; Different letters represent significant differences ($P < 0.05$) in cytokine production in long-term and *ex vivo* culture condition (Tukey's).

As observed in infected calves (study 1), the proportion of Tcm, Tem and effector cells contributing to cytokine production differed between long-term and *ex vivo* cultures (Fig. 5, $P < 0.05$). Responding cells in long-term cultures most frequently exhibited a Tcm phenotype (Fig. 5A, $P < 0.05$), while *ex vivo* cytokine production was mainly due to Tem's (Fig. 5B, $P < 0.05$). Effector cells contributed to cytokine production by vaccinated animals under both culture conditions, but were more frequent in the *ex vivo* response (Fig. 5C, $P < 0.05$). The contribution of naïve cells to the cytokine response was minimal, and did not differ based on vaccination or culture conditions (Fig. 5D, $P > 0.05$). Cell phenotype (Tcm, Tem, and

effector) and polyfunctional profiles within long-term or *ex vivo* cultures did not differ between BCG mutants and BCG vaccinates to Ag85A/TB10.4 (Fig. S3) nor PPD (Fig. S4) at 6 WPV.

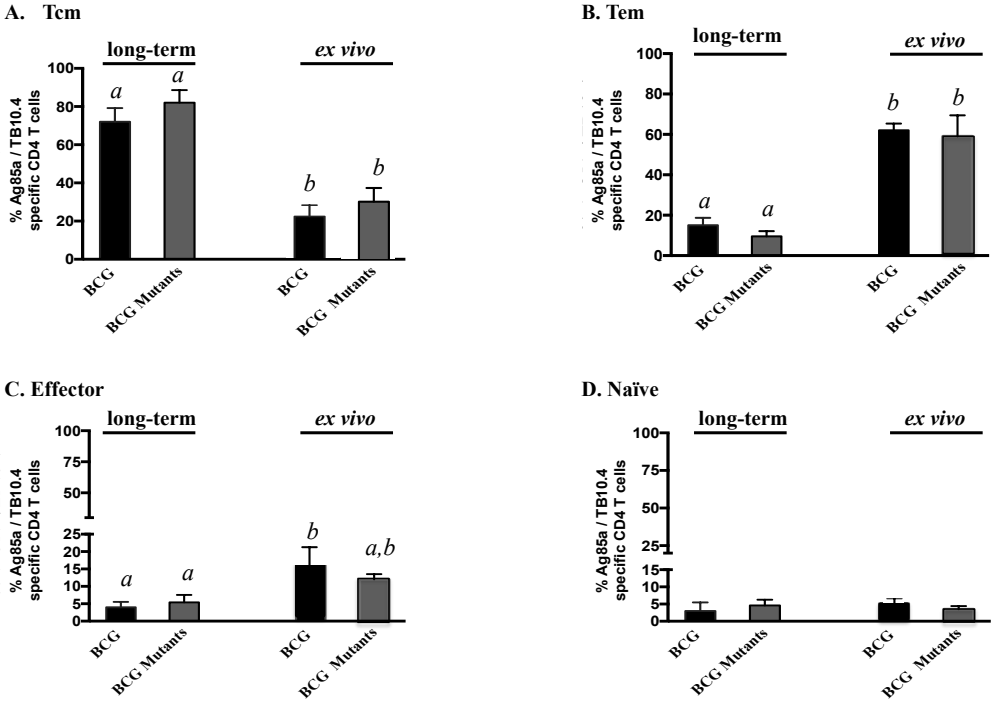

Figure 5. Vaccine elicited cytokine production is due to different effector/memory phenotypes based upon culture conditions. For long-term culture PBMCs were isolated at 6 WPV and stimulated with a cocktail of Ag85A, TB10.4, and PPD for 13 days followed by transfer to 96 well round bottom plates with APCs and addition of media alone or Ag85A/TB10.4 for an additional 16h. Results are presented as average and standard error. Frequency of cytokine producing cells under long-term or *ex vivo* culture exhibiting Tcm (**A**), Tem (**B**), Effector (**C**) or Naïve (**D**) phenotype using the gating strategy described in Fig. S1. [abc] Different letters represent differences ($P < 0.05$) in cytokine production within memory subsets (i.e., Tcm, Tem, Effector or Naïve) in both long-term and *ex vivo* cultures (Tukey's).

Vaccine-elicited polyfunctional responses were detected to PPD and Ag85A/TB10.4, but not to ESAT-6:CFP10 (Fig. 6). Thus, BCG vaccination elicits a specific polyfunctional CD4 T cell response and the culture duration (long-term vs *ex vivo*) dictates whether the response is primarily within Tcm or Tem subsets, respectively. These findings also confirm

that long-term cultured ELISPOT responses associated with protection to subsequent *M. bovis* infection in cattle are primarily a measure of CD4 Tcm responses[14, 58].

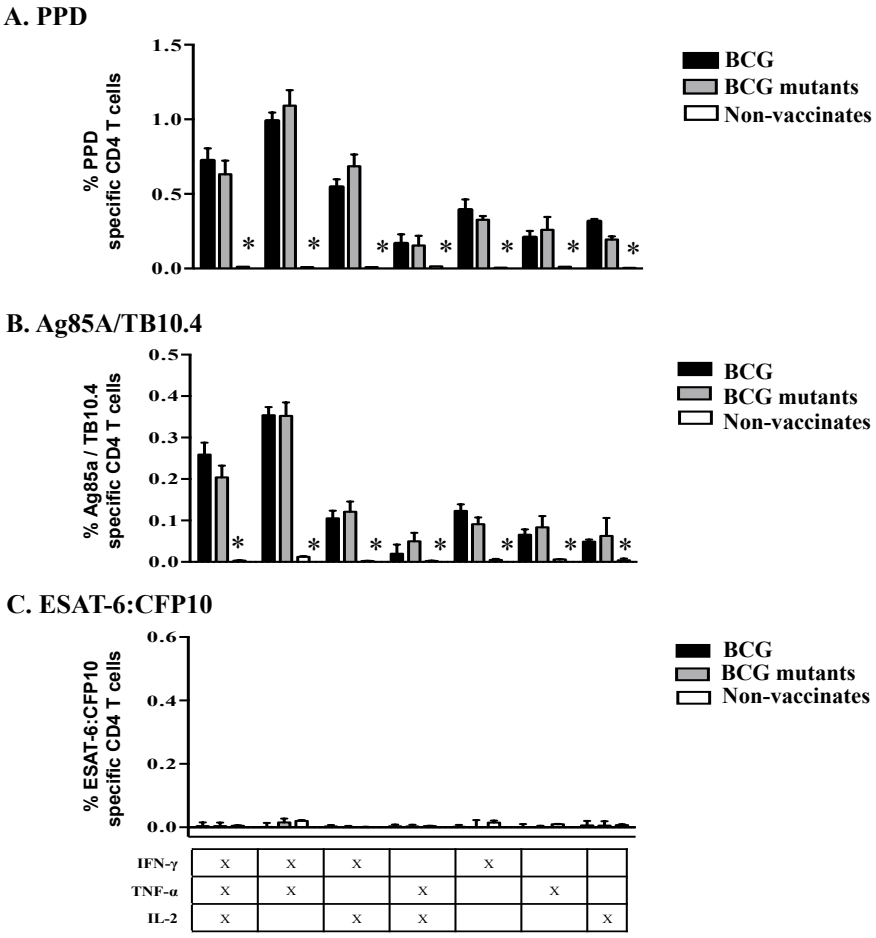

Figure 6. **Specific Tcm responses to vaccination at 6weeks post vaccination.** For long-term culture, PBMCs were isolated at 6 WPV and stimulated with a cocktail of Ag85A, TB10.4, and PPD for 13 days followed by transfer to 96 well round bottom plates with APCs and addition of media alone, PPD, Ag85A/TB10.4 or ESAT-6:CFP10 for an additional 16h. Results are presented as average and standard error. Polyfunctional responses by Tcm's (gated on $CD45RO^{+}/CCR7^{+}/CD4^{+}$ cells) were detected upon recall stimulation with PPD (**A**), Ag85A/TB10.4 (**B**), but not to ESAT-6:CFP10 (**C**).
* Different ($P < 0.05$, Tukey's) responses between non-vaccinates and both vaccinated groups.

Lower IFN-γ/TNF-α and higher IFN-γ/TNF-α/IL-2 co-production by Tcms early after infection by vaccinates as compared to non-vaccinates

Early after challenge with virulent *M. bovis* (3 weeks post-infection - WPI), non-vaccinates had greater ($P < 0.05$) IFN-γ/TNF-α responses to PPD, Ag85A/TB10.4 and ESAT-6:CFP10 by Tcm's than did vaccinated animals (Fig. 7). This difference was surprising given that vaccination elicited IFN-γ/TNF-α responses to PPD and Ag85A/TB10.4 and one might anticipate that these responses would be boosted after challenge with virulent *M. bovis*. In contrasted, the IFN-γ/TNF-α/IL-2 response by vaccinates exceeded ($P < 0.05$) the respective response by non-vaccinates early after challenge (Fig. 7). As with post vaccination responses, responses by BCG mutants and BCG vaccinates did not differ at 3 WPI (Fig. S5) and 8 WPI (data not shown). Also, polyfunctional responses by Tcm's at 8 WPI were similar among vaccinates and non-vaccinates (Fig. 8). To investigate how vaccination and infection elicited responses contrast, we compared the polyfunctional cytokine response by vaccinates at 6 WPV to that of non-vaccinates at 3 WPI and 8 WPI. At 8 WPI, long-term and *ex vivo* responses to infection exceeded ($P < 0.05$) respective responses to vaccination for most polyfunctional profiles to both Ag85A/TB10.4 and PPD (Fig. S6). Importantly, both IFN-γ/TNF-α/IL-2 and IFN-γ/TNF-α responses to infection at 3 WPI (data not shown) and 8 WPI (Fig. S6) exceeded ($P < 0.05$) respective responses to vaccination upon recall stimulation of long-term cultures with Ag85A/TB10.4. Thus, both attenuated and virulent strains of *M. bovis* elicit polyfunctional T cell responses; however, these responses differ in magnitude.

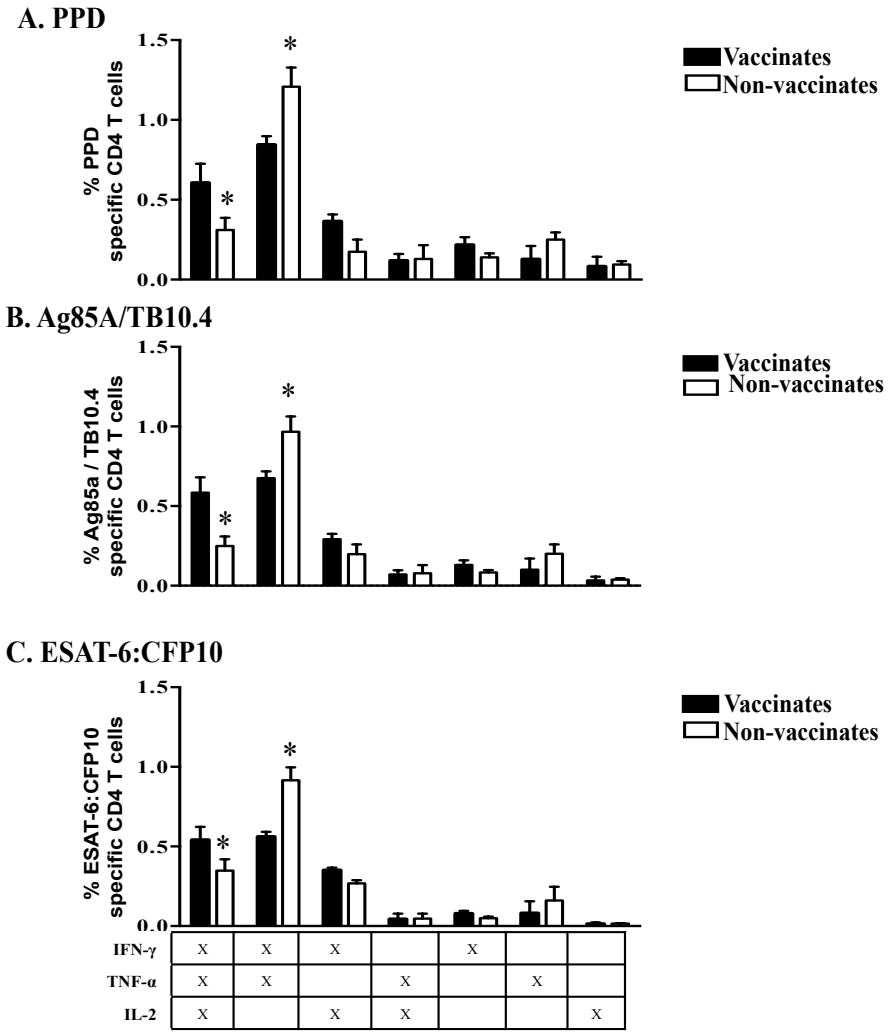

Figure 7. Higher IFN-γ/TNF-α and lower IFN-γ/TNF-α/IL-2 Tcm responses by non-vaccinates versus vaccinates at 3 weeks post challenge. For long-term culture, PBMCs were isolated at 3 WPI and stimulated with a cocktail of Ag85A, TB10.4, and PPD for 13 days followed by transfer to 96 well round bottom plates with APCs and addition of media alone, PPD, Ag85A/TB10.4 or ESAT-6:CFP10 for an additional 16h. Results are presented as average and standard error. Polyfunctional responses by Tcm's (gated on $CD45RO^{+}/CCR7^{+}/CD4^{+}$ cells) were detected upon recall stimulation with PPD (**A**), Ag85A/TB10.4 (**B**) or ESAT-6:CFP10 (**C**).
***** Parameters differ ($P < 0.05$, Tukey's) in cytokine production by vaccinates versus non-vaccinates.

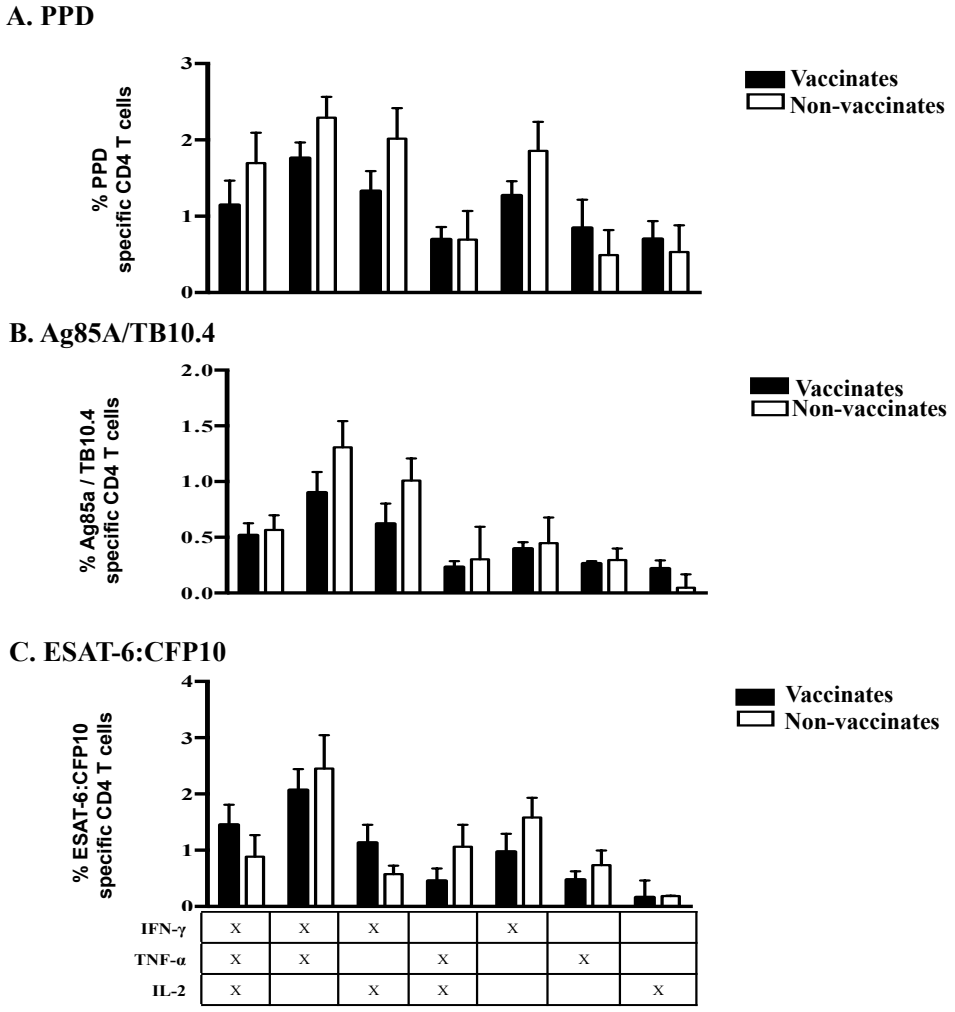

Figure 8. By 8 weeks post *M. bovis* challenge, polyfunctional responses by Tcm's did not differ between vaccinates and non-vaccinates. For long-term culture, PBMCs were isolated at 8 WPI and stimulated with a cocktail of Ag85A, TB10.4, and PPD for 13 days followed by transfer to 96 well round bottom plates with APCs and addition of media alone, PPD, Ag85A/TB10.4 or ESAT-6:CFP10 for an additional 16h. Results are presented as average and standard error. Polyfunctional responses by Tcm's (gated on $CD45RO^+/CCR7^+/CD4^+$ cells) upon recall stimulation with PPD (**A**), Ag85A/TB10.4 (**B**) or ESAT-6:CFP10 (**C**).

The most striking difference between vaccinates and non-vaccinates was the relative contribution of IFN-γ/TNF-α/IL-2 and TNF-α/IFN-γ production by Tcm's early after challenge (3 WPI). The kinetics of long-term culture Tcm's expressing these polyfunctional profiles is depicted in Fig. 9. Vaccination elicited IFN-γ/TNF-α/IL-2 and IFN-γ/TNF-α polyfunctional

Tcm responses to Ag85A/TB10.4, but not to ESAT-6:CFP10. Three weeks after challenge, IFN-γ/TNF-α responses by Tcm's were greater in non-vaccinates as compared to vaccinates (Fig. 9, *P < 0.05*), while vaccinates maintained higher percentages of IFN-γ/TNF-α/IL-2 producing Tcm's in comparison to non-vaccinates (Fig. 9, *P < 0.05)*. At eight weeks post challenge, TNF-α/IFN-γ/IL-2 and TNF-α/IFN-γ responses were similar between vaccinates and non-vaccinates (Fig. 9). Thus, BCG vaccination alters the ensuing polyfunctional profile upon infection with virulent *M. bovis,* favoring early IFN-γ/TNF-α/IL-2 production by $CD4^+$ Tcm's and dampening TNF-α/IFN-γ elicited by infection.

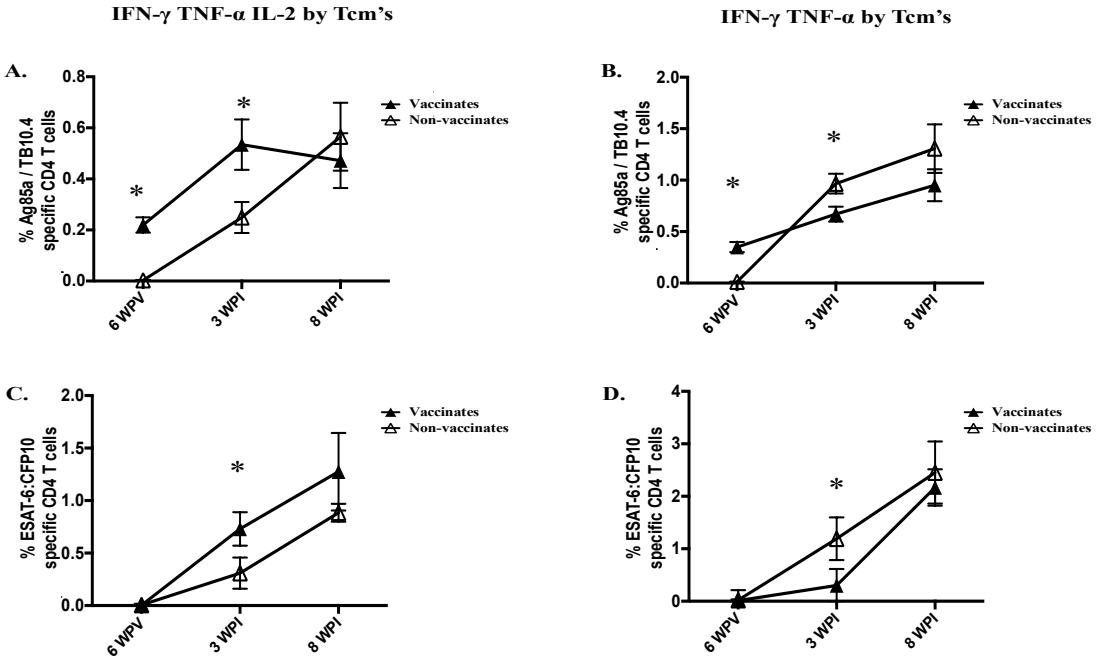

Figure 9. **Kinetics of IFN-γ TNF-α IL-2 and IFN-γ TNF-α responses by Tcm.**
For long-term culture, PBMCs isolated at 6 weeks post vaccination (6 WPV), 3 weeks post infection (3 WPI) and 8 WPI were stimulated with a cocktail of Ag85A, TB10.4, and PPD for 13 days followed by transfer to 96 well round bottom plates with APCs and addition of media alone, Ag85A/TB10.4 or ESAT-6:CFP10 for an additional 16h. Results are presented as average and standard error. Percentages of long-term cultured Tcm's (gated on $CD45RO^+/CCR7^+/CD4^+$ cells) producing IFN-γ/TNF-α/IL-2 or IFN-γ/TNF-α upon recall stimulation with Ag85A/TB10.4 (**A** and **B**) or ESAT-6:CFP10 (**C** and **D**).
* Differences (P < *0.05*, Tukey's) in the cytokine production by non-vaccinates and vaccinates.

Vaccination elicited protection is associated with reduced IFN-γ/TNF-α responses by Tcm's early after challenge.

After *M. bovis* challenge, Th-1 responses to mycobacterial antigens generally correlate with TB-associated pathology and poor vaccine efficacy [15, 53, 58,59]. In our study, both vaccines provided a high level of protection. *M. bovis* was isolated from only five vaccinated animals (4 BCG mutants and 1 BCG vaccinate). Retrospective analysis comparing responses to early infection (3 WPI) by non-vaccinates versus culture negative vaccinates (n=14) or culture positive vaccinates (n=5) revealed differential IFN-γ/TNF-α responses by Tcm's to ESAT-6:CFP10 among the groups (Fig. 10A, $P < 0.05$). IFN-γ/TNF-α responses by Tcm's from non-vaccinates were higher than that of both *M. bovis* culture negative and positive vaccinates (Fig. 10A, $P > 0.05$). *M. bovis* culture positive vaccinates produced intermediate cytokine levels, while culture negative animals had the lowest IFN-γ/TNF-α responses (Fig. 10A, $P < 0.05$). Conversely, IFN-γ/TNF-α/IL-2 production by Tcm's was higher in culture negative vaccinates as compared to culture positive non-vaccinates (Fig. 10B, $P > 0.05$). IFN-γ/TNF-α/IL-2 responses by Tcm's from culture positive vaccinates were intermediate; not differing from either culture negative vaccinates or non-vaccinates (Fig. 10B, $P < 0.05$). IFN-γ/TNF-α/IL-2 and IFN-γ/TNF-α production by Tem's in *ex vivo* assays did not differ among culture negative and culture positive animals (Fig. 10C and 10D, $P > 0.05$). Thus, these results indicate that strong IFN-γ/TNF-α responses by Tcm's are associated with greater mycobacterial burden while IFN-γ/TNF-α/IL-2 responses by Tcm's are indicative of a protective response provided by vaccination.

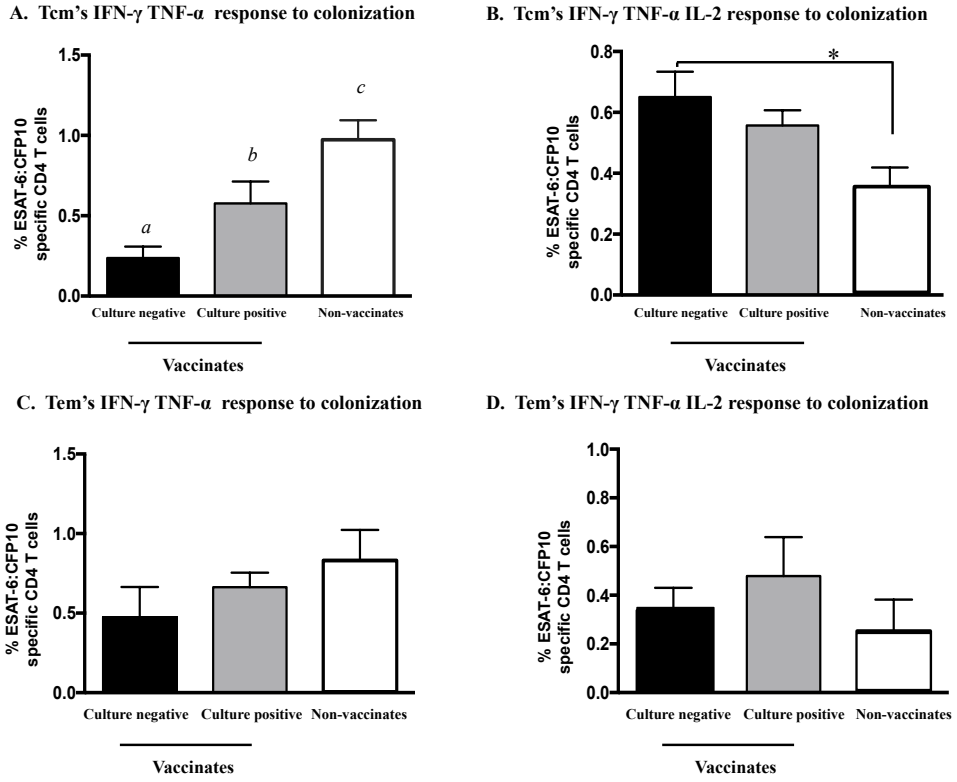

Figure 10. IFN-γ/TNF-α/IL-2 and IFN-γ/TNF-α polyfunctional reponses at 3 weeks post infection by Tcm's, but not by Tem's, differ based upon *M. bovis* colonization and vaccination status. Calves were grouped accordingly with both vaccine status and bacterial burden results (culture positive or negative) for assessment of of IFN-γ/TNF-α/IL-2 and IFN-γ/TNF-α Tcm response at 3 WPI. Long-term culture consisted of PBMCs isolated at 3 WPI and stimulated with a cocktail of Ag85A, TB10.4, and PPD for 13 days followed by transfer to 96 well round bottom plates with APCs and addition of media alone or ESAT-6:CFP10 for an additional 16h. Results are presented as average and standard error. IFN-γ/TNF-α and IFN-γ/TNF-α/IL-2 polyfunctional responses by Tcm's (gated on $CD45RO^+/CCR7^+/CD4^+$ cells) from vaccinates (either culture positive or negative) or non-vaccinates upon recall stimulation with ESAT-6:CFP10 (**A** and **B**). IFN-γ/TNF-α and IFN-γ/TNF-α/IL-2 polyfunctional responses by Tem's (gated on $CD45RO^+/CCR7^-/CD4^+$ cells) vaccinates (either culture positive or negative) or non-vaccinates upon recall stimulation with ESAT-6:CFP10 (**A** and **B**).

^{abc} Different letters differences ($P < 0.05$, Tukey's) in cytokine production among vaccinates (culture positive and negative) and non-vaccinates.

* Differences ($P < 0.05$, Tukey's) in the cytokine production between culture negative vaccinates and non-vaccinates.

Discussion

TB in cattle shares many features with TB in humans and studies with neonatal calves are particularly pertinent to human TB as children are the primary target population for vaccination and exposure to TB often occurs at a very young age [1]. Also, in contrast to laboratory mice, cattle are out-bred; thus, experimental variance is more similar to that of humans and of relevance for evaluation of safety and efficacy of vaccines, adjuvants, or other administered biologics. This can have significant advantages for a translational understanding of the mechanisms of pathogenesis and, more importantly, immunity. While correlates of vaccine-induced protection against TB are not entirely understood, Tcm's are presumed to be critical for protection in cattle and humans[1, 13, 18, 20, 27, 30]. Likewise, the role of polyfunctional CD4 T cells in the immune response to TB is not well established. Here we have shown that two protective BCG vaccine formulations elicited similar polyfunctional responses in vaccinated calves, and that early upon challenge with virulent *M. bovis,* responses by protected animals exhibited a different profile of response by Tcm's as compared to that of unprotected animals. Protected vaccinates had a lower frequency of ESAT-6:CFP10-specific Tcm's co-producing IFN-γ and TNF-α, suggesting that the incitement of strong Tcm IFN-γ/TNF-α responses early upon infection is related to a higher bacterial burden and inability of the host to control mycobacterial growth. On the other hand, higher frequencies of Tcm's producing IFN-γ/TNF-α/IL-2 early after infection were associated with vaccine elicited-protection and bacterial arrest by the host.

Polyfunctional cytokine responses were detected upon antigenic stimulation of freshly isolated and long-term cultured PBMCs with no difference in the percentages of cytokine producing cells based on culture condition. Still, the phenotype of responding cells greatly

differed, with enriched numbers of responding Tcm's in long-term cultured cells and Tem's in *ex vivo* cultures. This finding is consistent with previous research evaluating the phenotype of antigen specific cytokine producing cells in long-term cultures in humans [8, 11, 12] and cattle (Maggioli *et al.* 2014). As previously detected in naturally-infected cattle, experimental infection elicited polyfunctional responses biased toward IFN-γ/TNF-α and IFN-γ/TNF-α/IL-2 profiles [21]. In response to vaccination, long-term cultured PBMC responses were generally higher than that of *ex vivo* stimulated PBMCs. Long-term culture has been used as a method to improve the sensitivity of TB diagnostic tests for humans [60, 61], so it is reasonable to speculate that long-term culture enriches low frequency antigen-specific cells, as with vaccination, consequently aiding in response detection.

As early as 3 WPI, IFN-γ/TNF-α responses by both Tcm's and Tem's (i.e., in long-term cultured and *ex vivo* assays, respectively) from non-vaccinates exceeded that of vaccinates. Yet, whether detection of Tcm responses in *in vitro* cultures so early upon infection was a consequence of *in vitro* conditions or whether antigen-specific cells, or their progeny, maintained their true phenotype throughout the *in vitro* culture requires further investigation. Studies have shown that the antigen specificity of responding cells in *ex vivo* and cultured ELISPOT assays do not necessary correlate to each other with HIV [62] (Calarota *et al.* 2008), EBV [63], hepatitis C virus [11], or malaria infection [64, 65]; demonstrating that the nature of the response by cytokine producing cells may differ based on culture conditions. Likewise, it could be argued that the stimulation protocol might have induced *in vitro* priming of naïve T cells. *In vitro* priming, however, is unlikely due to the relatively short duration of the assay, absence of peptide antigen stimulation during the initial 13 day culture phase, no addition of cytokines apart from IL-2, and no enrichment of dendritic cells - all of which are required for *in vitro*

priming [66]. The absence of response to vaccine antigens by non-vaccinates prior to challenge further supports the notion that priming of naïve cells occurs *in vivo*, and not as a consequence of long-term culture. Moreover, IFN-γ responses to vaccination in long-term cultures are associated with protection upon *M. bovis* challenge, indicating that the assessed *in vitro* response does hold a connection to the *in vivo* outcome.

Polyfunctional responses to ESAT-6:CFP10 (antigens not synthesized by BCG strains) were detected in effector, Tem and Tcm populations from BCG vaccinates early after challenge (3 WPI). This finding is consistent with the early asymmetric division model of maturation, indicating memory and effector T cells expand concomitantly during the effector phase of the response and relatively early upon infection [67-72]. In the mouse model, BCG elicited Tcm's are detected as early as 20 days after vaccination and correlate with improved T cell recruitment to the lung. Also, the adoptive transfer of Tcm's, but not Tems, is sufficient to confer protection [5]. Laouar *et al.* [68] demonstrated the appearance of CD8 memory cells during the acute phase of a primary response to lymphocytic choriomeningitis virus as early as eight days after infection. Likewise, prime-boost vaccination with viral-vectored vaccines expressing malarial antigens induce both *ex vivo* and cultured IFN-γ ELISPOT responses by seven days after boost [65]. It is important to note that antigen clearance appears to have a crucial role on the kinetics of the response, and pathogen clearance in chronic infections such as TB (including *M. bovis* infection in calves), is not always achieved. Moreover, the very factors governing effector / memory T cell differentiation remain largely unknown. Whether memory cells arise (1) as direct progenies of effector cells (linear differentiation model; [73-75], (2) via a linear process dictated by a progressive loss of T cell differentiation potential (i.e., from naïve → effector → memory → terminally differentiated cells) dependent on antigenic stimulation and

signaling milieu (decreasing potential model; [76, 77], or (3) as a separate lineage from naïve cells (divergent differentiation model, [67, 69] has long been debated. Still, the connection between naïve CD4 T cells and the various effector and memory fates these cells follow remains elusive, and a single unifying theory accounting for the diversity of CD4 T cell memory has not been discovered [78, 79]. This fact suggests that various mechanisms of CD4 T cell memory generation may be differentially engaged depending on various factors (e.g., level of inflammation), and that these models are not necessarily mutually exclusive. Still, cell fate divergence (i.e., asymmetric division) early after priming has been reported by several authors [5, 65, 67-69], and is supported by the present findings, suggesting that memory generation begins prior to antigen clearance.

Early upon infection, vaccinates exhibited higher numbers of antigen-specific IFN-γ/TNF-α/IL-2 Tcm's than did non-vaccinates, and such dissimilar responses between vaccinates and non-vaccinates early after challenge is associated with disease outcome. Polyfunctionality is often associated with less differentiated T cells (Tcm and Tem, as opposed to terminally differentiated T cells expressing killer cell lectin-like receptor 1 - KLRG1), and better quality of responses [27, 76, 79]. Persistent or prolonged antigen stimulation is related to a progressive loss of the pool of long-lived central memory T cells, resulting in poorly fit immune responses by terminally differentiated, short-lived cells that produce single cytokine or loss of effector function all together [80, 27]. The higher numbers of IFN-γ/TNF-α/IL-2 Tcm cells may indicate that vaccinated calves were able to control antigen load, retaining greater levels of cell functionality, which was beneficial in the long run. In future studies, we will evaluate KLRG1 expression on CD4 T cell effector / memory populations with the various

polyfunctional profiles over a longer course of *M. bovis* infection to determine KLRG1 association with the expression of effector function by T cells in cattle.

As both vaccine treatments were proven to be exceptionally protective, we investigated whether vaccinated animals with no detectable *M. bovis* by quantitative culture responded to infection differently than did vaccinated animals with detectable *M. bovis* and/or non-vaccinates (all of which had detectable *M. bovis* by quantitative culture). Surprisingly, IFN-γ/TNF-α responses by Tcm's differed among all three groups, suggesting colonization, and perhaps antigen load, could be associated with the number of IFN-γ/TNF-α antigen-specific cells. Intriguingly, similar numbers of Tcm's producing IFN-γ/TNF-α/IL-2 were detected in vaccinates, regardless of their culture status. Still, IFN-γ/TNF-α/IL-2 responses were greater in vaccinates as compared to non-vaccinates, indicating that vaccine-induced IFN-γ/TNF-α/IL-2 responses may be associated with protection. Also, low levels of TB associated pathology was present in a few vaccinates with no detectable *M. bovis* by quantitative culture. It is known that few bacilli are required to cause TB associated pathology, and the vaccine may have reduced the bacterial burden below the limit of *M. bovis* detection. Interestingly, three vaccinates with detectable *M. bovis* by quantitative culture showed no TB associated pathology (lung and lung-associated lymph nodes). It is possible that TB associated pathology was only delayed by vaccination and these animals would still progress to clinical disease. It also indicates that even though vaccinates with detectable *M. bovis* had higher numbers of Tcm producing IFN-γ/TNF-α as compared to vaccinates with no *M. bovis*, the moderate levels of IFN-γ/TNF-α/IL-2 (or a combination of factors) would have conferred, if not a sterilizing protection, a significant decrease/delay in TB associated pathology even in the presence of the pathogenic bacilli. This scenario would still be beneficial to the host.

Tcm's expressing IL-2 are associated with latent TB or effective anti-mycobacterial therapy in humans, whereas patients with active TB predominately exhibit effector cells that express IFN-γ/TNF-α [32, 81,82]. This suggests that CD4 T cell functional capacity is driven toward terminally differentiated T cells by bacterial load and continuous antigen exposure [32].While the occurrence of latency is not well established in cattle, non-vaccinates and non-protected vaccinates exhibited higher IFN-γ/TNF-α responses by Tcm's than did protected calves early after challenge, similar to what occurs with active disease in humans. Additionally, protected vaccinates exhibited higher numbers of IFN-γ/TNF-α/IL-2 producing Tcm's. It is plausible that the amount of initial antigen exposure controls the extent of differentiation, and that vaccinated animals were more capable of containing the infection initially, preventing the loss of CD4 polyfunctional capacity. Moreover, single IL-2 producers did not differ among vaccinates and non-vaccinates or vary dependent upon *M. bovis* culture status at necropsy, which may indicate that the polyfunctional capacity, and not IL-2 production per se, was the most relevant protective response assessed. Such responses could be either a surrogate of other protective mechanisms or a just an indication of immune response fitness (e.g., the presence of Tcm exhibiting plasticity, more prone to long-lasting, or producing other cytokines). Protection to TB may require different levels of Tcm and Tem memory responses and also different ratios of Tcm's to Tem's in the different phases of this chronic infection [83]. It is also possible that the presence of Tcm's producing IFN-γ/TNF-α/IL-2 was a consequence of lower bacterial burden, rather than its cause. Importantly, Sakai *et al.* [84] demonstrated in the mouse model that differentiated T cells expressing KLGR1 elicited by BCG-vaccination are retained within lung blood vasculature, lacking the ability to migrate into the lung parenchyma. Although these cells may produce high levels of IFN-γ, they are still

functionally impaired and unable to confer protection in the lung. Kaushal *et al.* [85] showed that aerosol immunization of macaques with an MtbΔsigH mutant was highly protective against an otherwise lethal *M. tb* aerosol challenge. MtbΔsigH vaccinated macaques exhibited higher numbers of Tcm's in response to vaccination (as compared with BCG or non-vaccinated controls) and higher percentages of IFN-γ/TNF-α/IL-2 in their lungs after challenge. The assessment of T cell survival, senescence and peripheral migration markers [(e.g., B-cell lymphoma 2 protein (BCL-2), KLRG-1 and programmed cell death protein 1(PD-1)], local responses in the lungs, as well the measure of a broader panel of cytokines would further the understanding of our findings, but such analysis were not achievable in the present experiments.

Predicting vaccine efficacy is difficult, in part due to the lack of consistent immunological correlates of protection, especially one that would be appropriate throughout the different stages of chronic infections. Effective responses are likely not static, with different subsets displaying a variety of functions and migration capabilities necessary for disease prevention, bacterial arrest and infection resolution. Recently Ziraldo *et al.* [83] proposed a computational model to explore Tem and Tcm responses to a variety of mycobacterial antigens, generating a tool to predict vaccine formulations that would provide protective ratios of memory cells with desirable functionalities. If this approach proves to be successful, it would offer further insights into protection correlates and vaccination strategies to accomplish protection. In the mean time, present findings indicate that IFN-γ/TNF-α and IFN-γ/TNF-α/IL-2 responses by Tcm's early after *M. bovis* infection in cattle are associated with detrimental and protective outcomes, respectively.

In summary, determining the T cell responses by measuring their functionality in combination with cell surface phenotype is likely to increase the fundamental understanding of T cell memory and effector differentiation, as these parameters define the T cell functional capacity, life-span potentials, antigen exposure history and trafficking capabilities. Thus, phenotypic and functional analysis of T cells offers an assessment of the quality of the immune response, allowing a clearer evaluation of whether and how a response is protective, than either of these parameters measurement alone.

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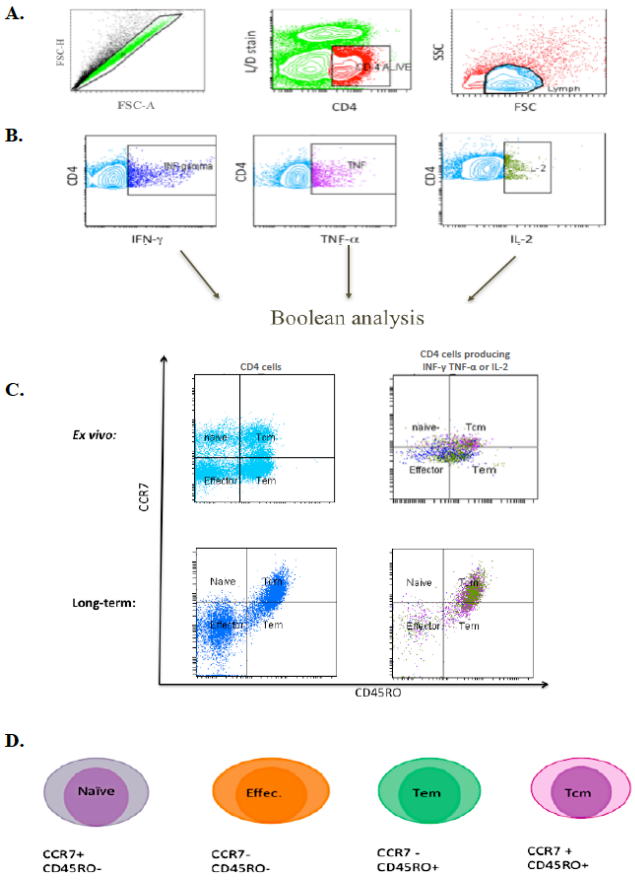
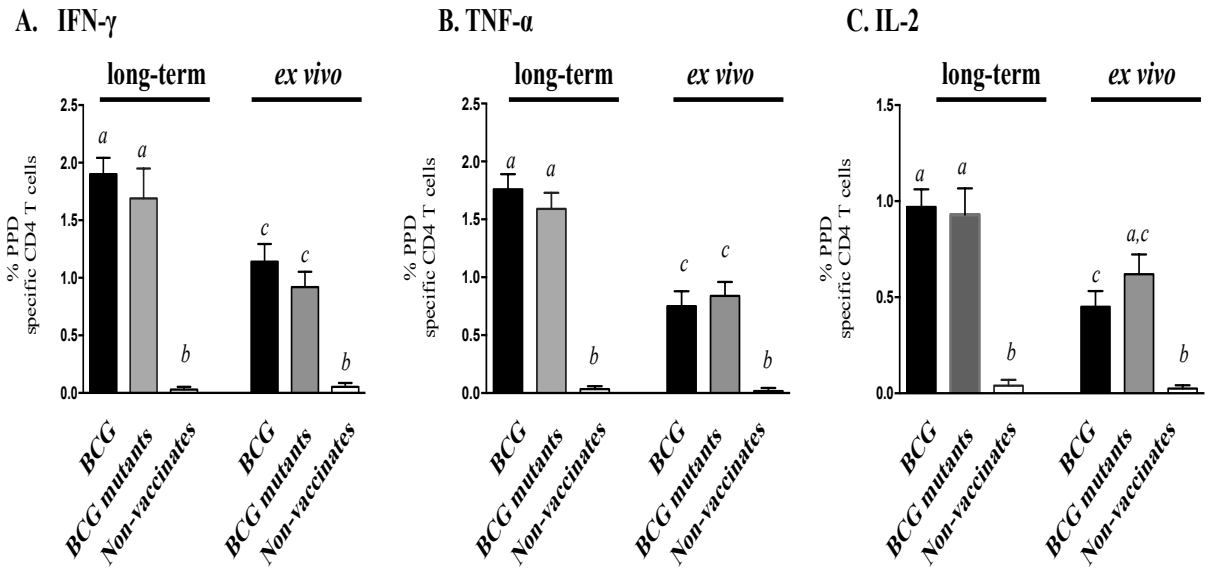
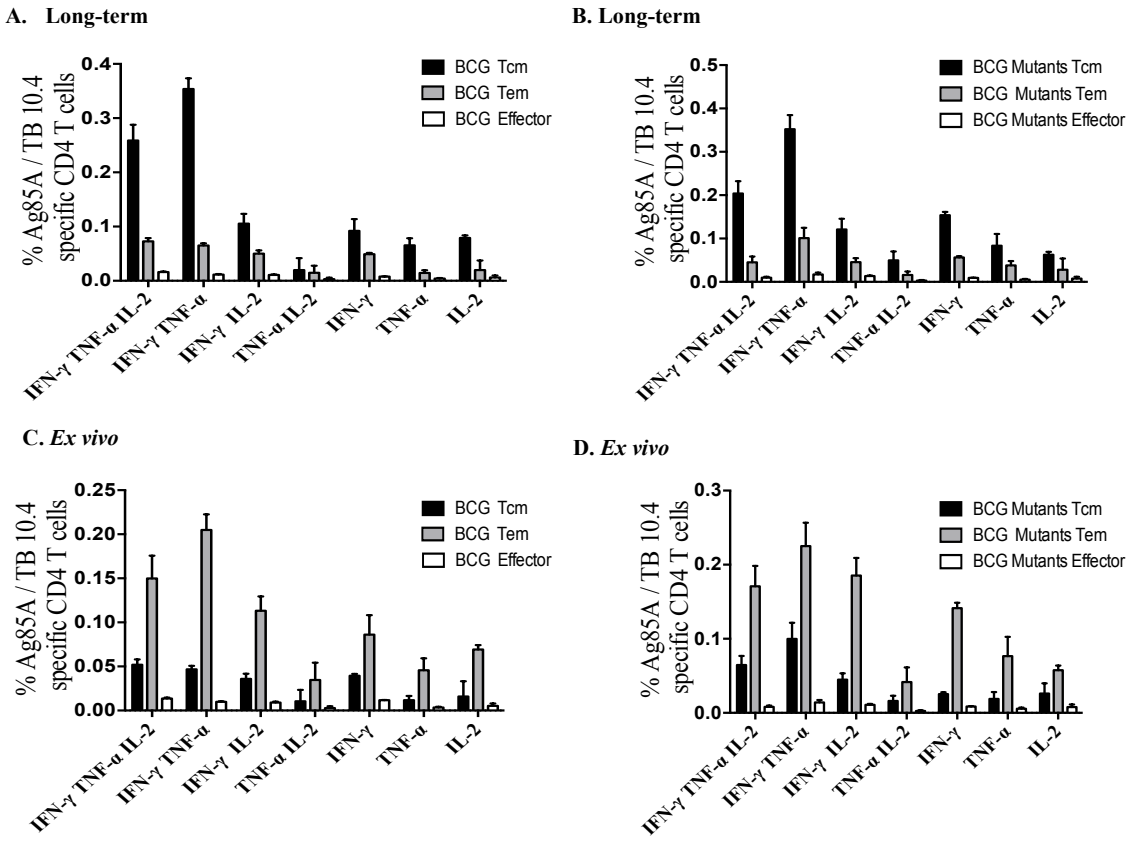
Supplemental Information



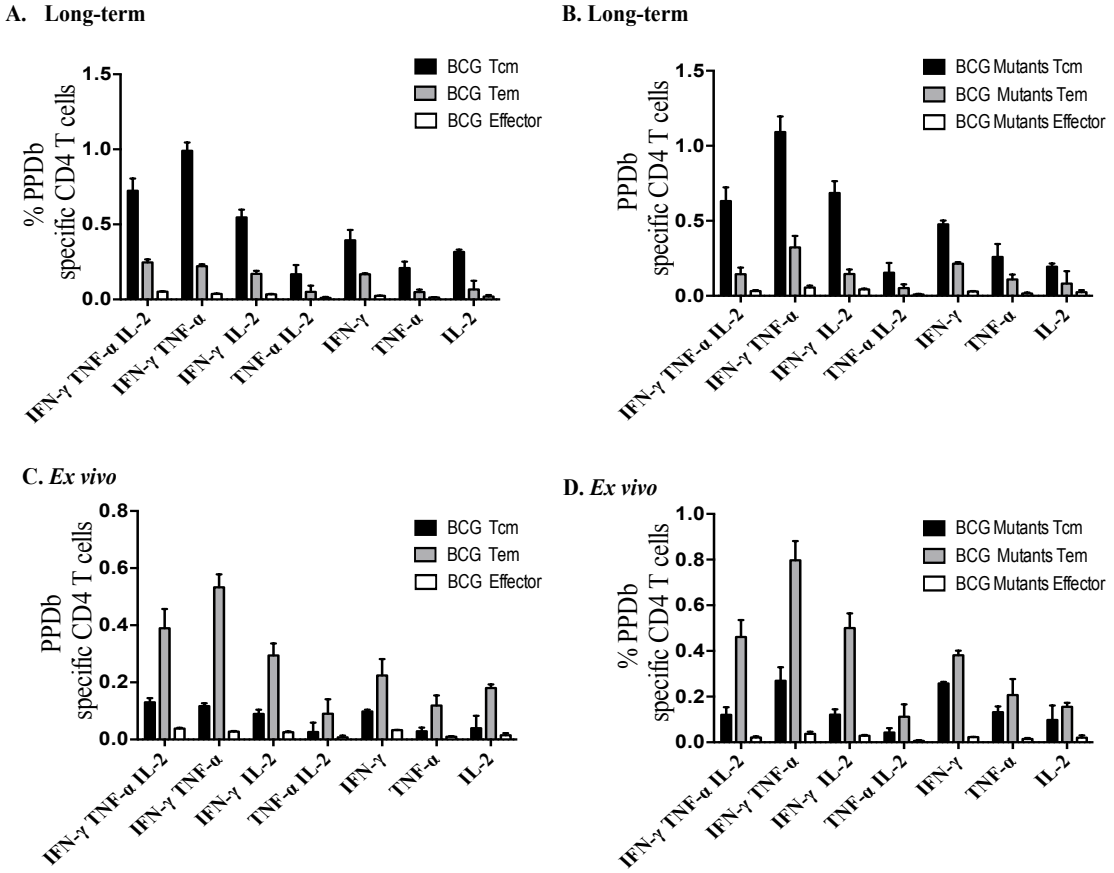
S1. Gating strategy for assessment of polyfunctional cytokine responses by effector/memory phenotypes in long-term and *ex vivo* culture conditions. A representative polyfunctional response from an infected animal is presented to demonstrate the gating strategy. (**A**) Selection of populations containing live $CD4^+$ singlets within the lymphocyte gate (based on FSC and SSC properties). Each subsequent panel shows only the population of interest that has been selected from the gate on the previous plot. (**B**) CD4 cells producing IFN-γ, IL-2 or TNF-α in response to PPD, followed by combinatory Boolean analysis for polyfunctional cytokine profiles. (**C**) CD4 T cells producing any cytokines of interest were then analyzed for CD45RO/CCR7 expression to further discriminate the polyfunctional CD4 T cell response by effector/memory phenotype in *ex vivo* (light blue) and long-term cultures (dark blue). (**D**) Expression of cell markers on different T cell memory/effector subsets.



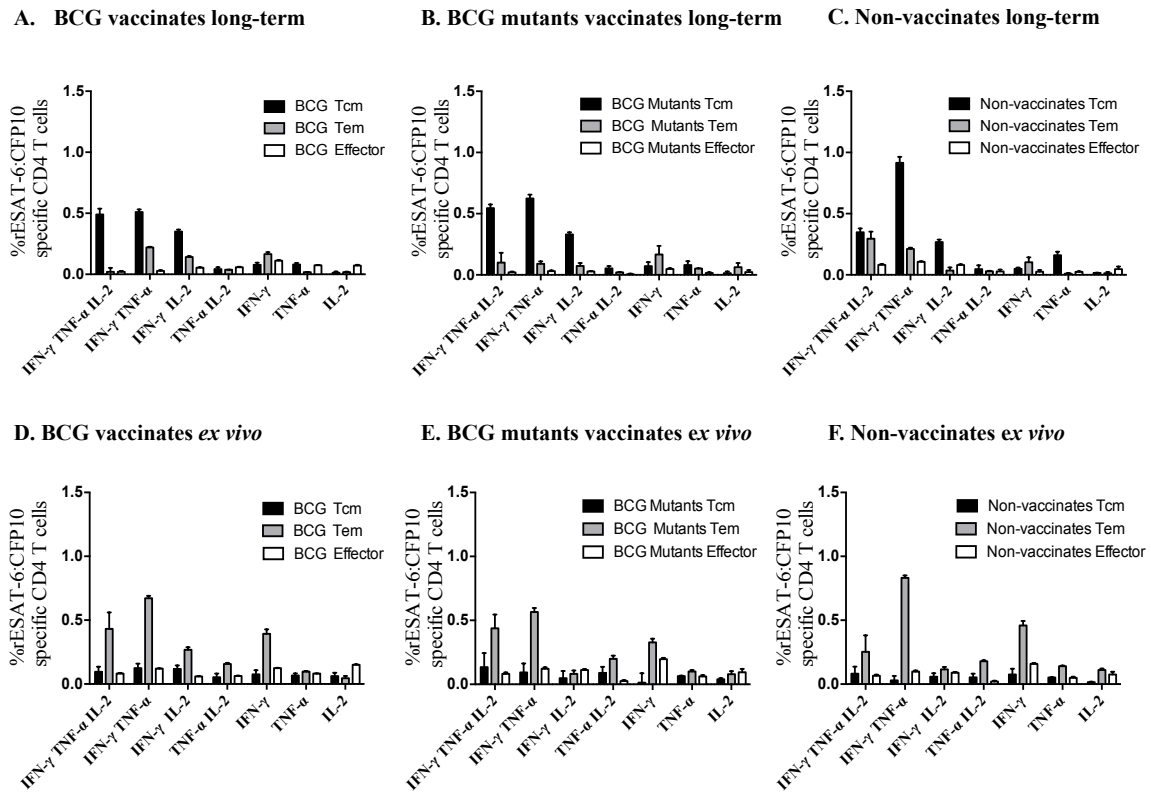
S2 . Cytokine production by CD4 T cells under long-term or *ex vivo* culture conditions in response to vaccination. For long-term culture, PBMCs were isolated and stimulated with a cocktail of Ag85A, TB10.4, and PPD for 13 days followed by transfer to 96 well round bottom plates with APCs and addition of media alone or PPD for an additional 16h. For *ex vivo* cultures, PBMCs were isolated and stimulated with media alone or PPD (5 μg/ml) for 16h. Results are presented as average and standard error. Frequency of long-term and *ex vivo* cultured CD4 T cells producing IFN-γ (**A**), TNF-α (**B**), or IL-2 (**C**).
[abc]Different letters represent differences ($p < 0.05$) in cytokine production in long-term and *ex vivo* culture conditions for each individual cytokine (ANOVA followed by Tukey's multiple comparisons test).



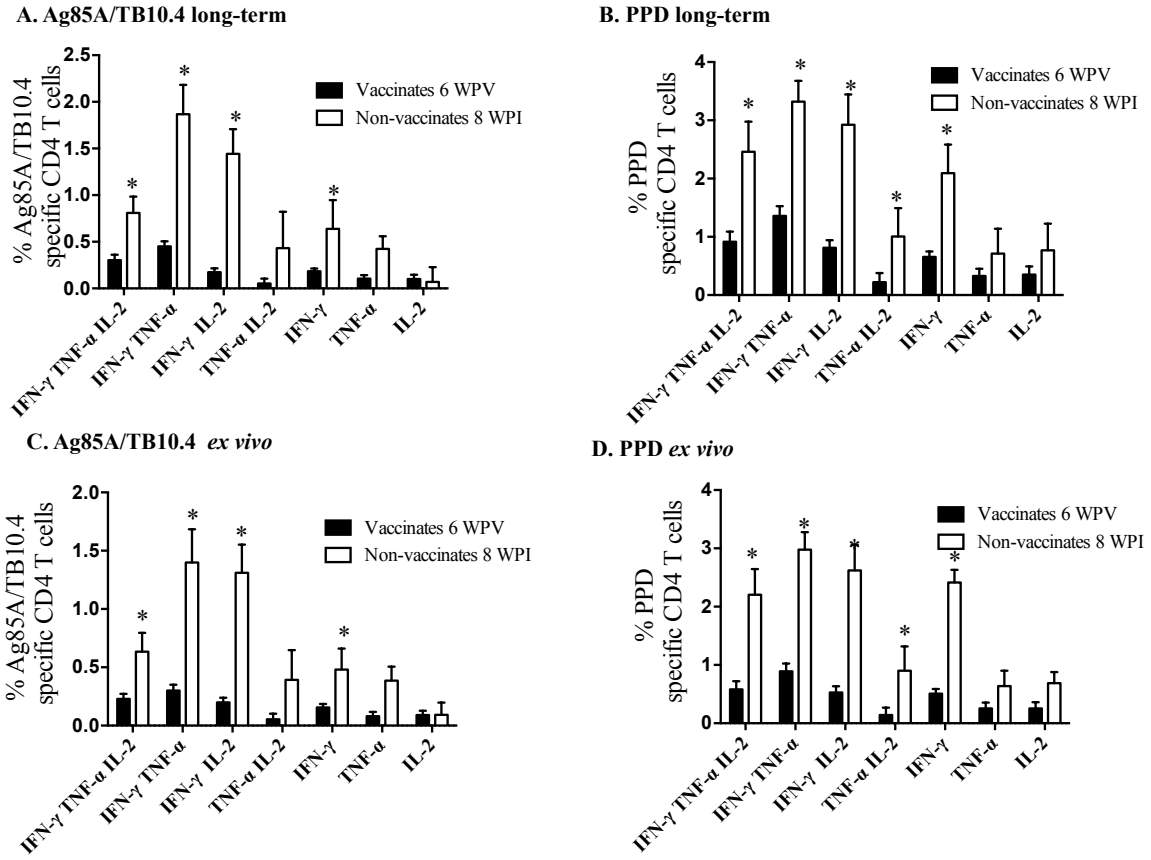
S3. Polyfunctional cytokine production to Ag85A/TB10.4 by PBMCs from BCG or BCG mutants under long-term or *ex vivo* cultures at 6 weeks post vaccination. For long-term culture, PBMCs were isolated and stimulated with a cocktail of Ag85A, TB10.4, and PPD for 13 days followed by transfer to 96 well round bottom plates with APCs and addition of media alone or Ag85A/TB10.4 for an additional 16h. For *ex vivo* culture, PBMCs were stimulated with media alone or a cocktail of Ag85A/TB10.4 for 16h. Results are presented as average and standard error. Percentage of cytokine production profiles among memory subsets in response to recall stimulation of long-term cultures with Ag85A/TB10.4 by BCG-vaccinates (**A**) or BCG mutants-vaccinates (**B**). Percentage of the cytokine production profiles among memory subsets in response to *ex vivo* stimulation with Ag85A/TB10.4 by BCG-vaccinates (**C**) or BCG mutants-vaccinates (**D**).



S4. Polyfunctional cytokine production to PPD by PBMCs from BCG mutants- or BCG-vaccinates under long-term or *ex vivo* cultures at 6 weeks post vaccination. For long-term culture, PBMCs were isolated and stimulated with a cocktail of Ag85A, TB10.4, and PPD for 13 days followed by transfer to 96 well round bottom plates with APCs and addition of media alone or PPD for an additional 16h. For *ex vivo* culture, PBMCs were stimulated with media alone or PPD for 16 h. Results are presented as average and standard error. Percentage of the cytokine production profiles among memory subsets in response to recall stimulation of long-term cultures with PPD by BCG-vaccinates (**A**) or BCG mutants-vaccinates (**B**). Percentage of the cytokine production profiles among memory subsets in response to *ex vivo* stimulation with PPD by BCG-vaccinates (**C**) or BCG mutants-vaccinates (**D**).



S5. Polyfunctional cytokine production by PBMCs from BCG mutants or BCG vaccinates under long-term or *ex vivo* culture conditions. For long-term culture, PBMCs were isolated at 3 weeks post infection and stimulated with a cocktail of rAg85A, rTB10.4, and PPD for 13 days followed by transfer to 96 well round bottom plates with APCs and addition of media alone or ESAT-6:CFP10 for an additional 16h. For *ex vivo* culture, PBMCs were stimulated with media alone or ESAT-6:CFP10 for 16 h. Results are presented as average and standard error. Percentage of the cytokine production profiles among memory subsets in response to recall stimulation of long-term cultures with ESAT-6:CFP10 by BCG-vaccinates (**A**), BCG mutants-vaccinates (**B**) or non-vaccinates (**C**). Percentage of the cytokine production profiles among memory subsets in response to *ex vivo* stimulation with ESAT-6:CFP10 by BCG-vaccinates (**D**), BCG mutants-vaccinates (**E**) or non-vaccinates (**F**).



S6. Cytokine production elicited by *M. bovis* infection exceeded respective responses to vaccination. For long-term culture, PBMCs were isolated at 8 weeks post infection and stimulated with a cocktail of rAg85A, rTB10.4, and PPD for 13 days followed by transfer to 96 well round bottom plates with APCs and addition of media alone or ESAT-6:CFP10 for an additional 16h. For *ex vivo* culture, PBMCs were stimulated with media alone or ESAT-6:CFP10 for 16 h. Results are presented as average and standard error. Percentage of the cells exhibiting polyfunctional profile to vaccination (6 WPV) and infection (non-vaccinates; 8WPI), in response to recall stimulation of long-term cultures with Ag85A/TB10.4 (**A**) or PPD (**B**). Percentage of the cells exhibiting polufunctional profile to vaccination (6 WPV) and infection (non-vaccinates; 8WPI), in response *ex vivo* recall stimulation with Ag85A/TB10.4 (**C**) or PPD (**D**).
* Differences ($P < 0.05$, Tukey's) in the cytokine production between by vaccinates (at 6WPV) and non-vaccinates at either 3 or 8 WPI (ANOVA followed by Tukey's multiple comparisons test).

CHAPTER 5: SUMMARY AND CONCLUSIONS

The research included in this thesis had the objective to characterize the phenotype of memory cells and aspects of their functionality in cattle, specifically in response to bTB infection and vaccination. The first manuscript describes long-term culture of PBMCs as a tool to enrich Tcm cells in cattle and an IFN-γ ELISPOT for cytokine production evaluation. The second manuscript describes for the first time, fundamental characteristics of Tcm (enriched by long-term cultures) in the response to bTB in cattle, as compared to Tem and effector counterparts. Bovine Tcm cells, as for many species, have greater proliferative capabilities and highly express SLO homing molecules. The third manuscript furthers the knowledge in memory responses to BCG vaccination by measuring IL-2 or TNF-α along with IFN-γ production, and also their association with protection or disease progression. Polyfunctional Tcm producing IFN-γ/TNF-α were associated with *M. bovis* culture, and these responses were significantly higher with culture positive calves in comparison with that of culture negative calves. Lack of cultivable *M. bovis* was associated with increased percentage of Tcm cells producing IFN-γ/TNF-α/IL-2. This study (presented in the third paper) provides relevant insights into memory formation and possible influences of bacterial load in the memory response in the context of bTB, representing a significant advancement to the veterinary immunology field. The discrimination of cell phenotype involved in cytokine production under both cultured and *ex vivo* conditions have proven to be helpful in identifying specific correlates of vaccine efficacy, which is a useful for selection of vaccine candidates for costly efficacy trials in BSL-3 facilities.

In the last few years it has became clear that memory T cells are extremely heterogeneous and complex. While improvements have been achieved to identify different components of immunological memory, little has been successfully applied to produce more effective vaccines against HIV and TB, or therapeutic approaches to infectious diseases. Also, as the classification of effector/memory subsets become more complex, concerns are raised regarding past studies. The use of fewer (or different) markers has possibly led to the wrong classification of memory and effector cells and consequently to incomplete conclusions. Also, studies have systematically excluded assessment of memory responses in peripheral tissue [1-3], which may constitute a relevant source of protection, and understanding of how these cells are established, and how to manipulate their formation may be necessary to improve vaccines strategies. Future studies should focus on analyzing resident memory cells, and also expand the markers and cytokines analyzed in both circulating and resident T cells.

Determining the T cell response by measuring their functionality in combination with cell surface phenotype expands the fundamental understanding of T cell memory and effector differentiation, by defining their functional capacity, life-span potential, antigen exposure history and trafficking characteristics. Thus, phenotypic and functional analysis of T cells offers an assessment of the quality of the immune response, allowing a clearer evaluation of how response are protective; than either of these measurements alone. Such knowledge is likely to yield more protective and safer vaccines, which would benefit livestock farmers, diminishing the socioeconomic impact of infectious diseases (including TB) to farm animals, while also improving food safety and reducing zoonotic transmissions to humans.

Several devastating human diseases and conditions (such as cancer, HIV, autoimmunity and organ transplantation rejections) remain relatively poorly treatable and not curable. Preventive and therapeutic immunization and cell treatments for such health problems are challenges to be overcome by immunologists in the years to come [4,5].

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