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Increased TNF-α/IFN-γ/IL-2 and Decreased TNF-α/IFN-γ Production by Central Memory T Cells Are Associated with Protective Responses against Bovine Tuberculosis Following BCG Vaccination

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Central memory T cell (Tcm) and polyfunctional CD4 T cell responses contribute to vaccine-elicited protection with 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 by flow cytometry prior to and after aerosol challenge with virulent *Mycobacterium bovis*. Polyfunctional cytokine expression patterns in the response by Tcm, 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 interferon-γ (IFN-γ)/tumor necrosis factor-α (TNF-α) and lesser IFN-γ/TNF-α/IL-2 responses by Tcm cells 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 in effector cells, as early as 3 weeks after challenge. These findings suggest that cell fate divergence may occur 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 response by Tcm cells is associated with greater mycobacterial burden, while IFN-γ/TNF-α/IL-2 response by Tcm cells are indicative of a protective response to bovine TB.

Keywords: polyfunctional T cells, central memory T cells, bovine tuberculosis, calf model

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 to 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 Ref. (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 (6). In humans, Tcm cells 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). Tcm cells 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) 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- to 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., 16–24 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 (26–28). 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) (29). 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, 31). Furthermore, the relative lack of efficacious TB vaccines for humans hinders the clear assessment of correlates of protection, including that of polyfunctional T cells (26, 32–35). 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 region of differentiation (RD)-1 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 subsequent challenge, or 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*, and BCG Δ*mmaA4*. BCG Danish is a widely used human pediatric vaccine to reduce human TB. 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 Berney-Meyer et al., 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 Berney-Meyer et al., 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, adding 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 cell 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 are associated with greater mycobacterial burden, while IFN-γ/TNF-α/IL-2 responses by Tcm cells 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 IA, USA, and housed in a BSL-3 facility at the National Animal Disease Center, Ames, IA, USA. 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 following treatment groups: no vaccination ($n = 10$), vaccination with *M. bovis* BCG Danish (Strain 1331; $n = 9$), and vaccination with a cocktail of four BCG Danish deletion strains including BCG *Δfdr8*, BCG *ΔleuCD Δpks16*, BCG *ΔmmaA4* (48), and BCG *ΔmetA* (49) ($n = 10$). Vaccines (10^6 cfu, total dose) were administered subcutaneously at 2 weeks of age. Animals received *M. bovis* 10-7428 by aerosol (5×10^2 cfu) at 3.5 months of age (i.e., 3 months after vaccination) as described by Palmer et al. (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; and 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- to 1.0-cm intervals and examined separately. Lungs and lymph nodes (mediastinal and tracheobronchial) were evaluated using a semiquantitative 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-μm 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 (PBMCs) were isolated from buffy coat fractions of blood collected by jugular venipuncture in 2× acid citrate dextrose solution, using density gradient centrifugation with Ficoll-Paque (Sigma, St. Louis, MO, USA). Complete RPMI medium for PBMC cell culture was RPMI 1640 (GIBCO, Grand Island) supplemented with 2-mM L-glutamine, 25-mM

HEPES buffer, 100 U/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, 1 ml/well) in 24-well flat-bottom microtiter plates (Nunc, Thermo Fisher, Waltham, MA, USA) 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 (2×10^6 of cultured PBMC/well) into 96-well ELISPOT plates (Millipore, Watford, UK) and incubated in the presence of autologous antigen-presenting cells (APCs) and PPD (5 μg/ml), Ag85A/TB104 (1 μg/ml of each protein), ESAT-6:CFP10 (1 μg/ml), pokeweed mitogen (PWM, Sigma) (1 μg/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 96-well plates. Non-adherent cells were discarded, and the adherent cells (APCs) were washed four times with warm RPMI 1640 media. Fresh complete media containing antigen and long-term cultured cells were then incubated for 20 h at 39°C/5% CO_2. Long-term cultured cells were then incubated for 16 h at 39°C/5% CO_2 with Brefeldin A (Sigma, 10 μg/ml) added at 4 h 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 PPD (5 μg/ml), Ag85A/TB10[4] (1 μg/ml of each protein), ESAT-6:CFP10 (1 μg/ml), PWM (1 μg/ml), or medium alone for 16 h at 39°C/5% CO_2 with Brefeldin A (Sigma, 10 μg/ml) added at 4 h 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, USA). 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, USA). A representative plot of the gating strategy is depicted in Figure S1 in Supplementary Material. A representative unstimulated condition response is depicted in Figure S2 in Supplementary Material.

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 Spectral Red, 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	Pacific Blue, Life Technologies	Not applicable

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, USA).

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, **Figures 1A,B**) 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. In response to either PPD (**Figure 1C**) or ESAT-6:CFP10 (**Figure 1D**), Tcm cells ($CD4^+$ $CD45RO^+$ $CCR7^+$) comprised ~80% of cytokine-producing cells in long-term cultures, while Tem ($CD4^+$ $CD45RO^+$ $CCR7^-$) comprised ~60% of cytokine-producing cells in *ex vivo* cultures. Analysis of CD4 T cell cytokine profiles (i.e., all combinations of IFN-γ, TNF-α, and IL-2) revealed frequent coproduction 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**). As with the analysis of individual cytokine production, Tcm was the main phenotype contributing to polyfunctional responses in long-term cultures, whereas Tem was the main subset producing cytokines in *ex vivo* cultures (**Figure 2**).

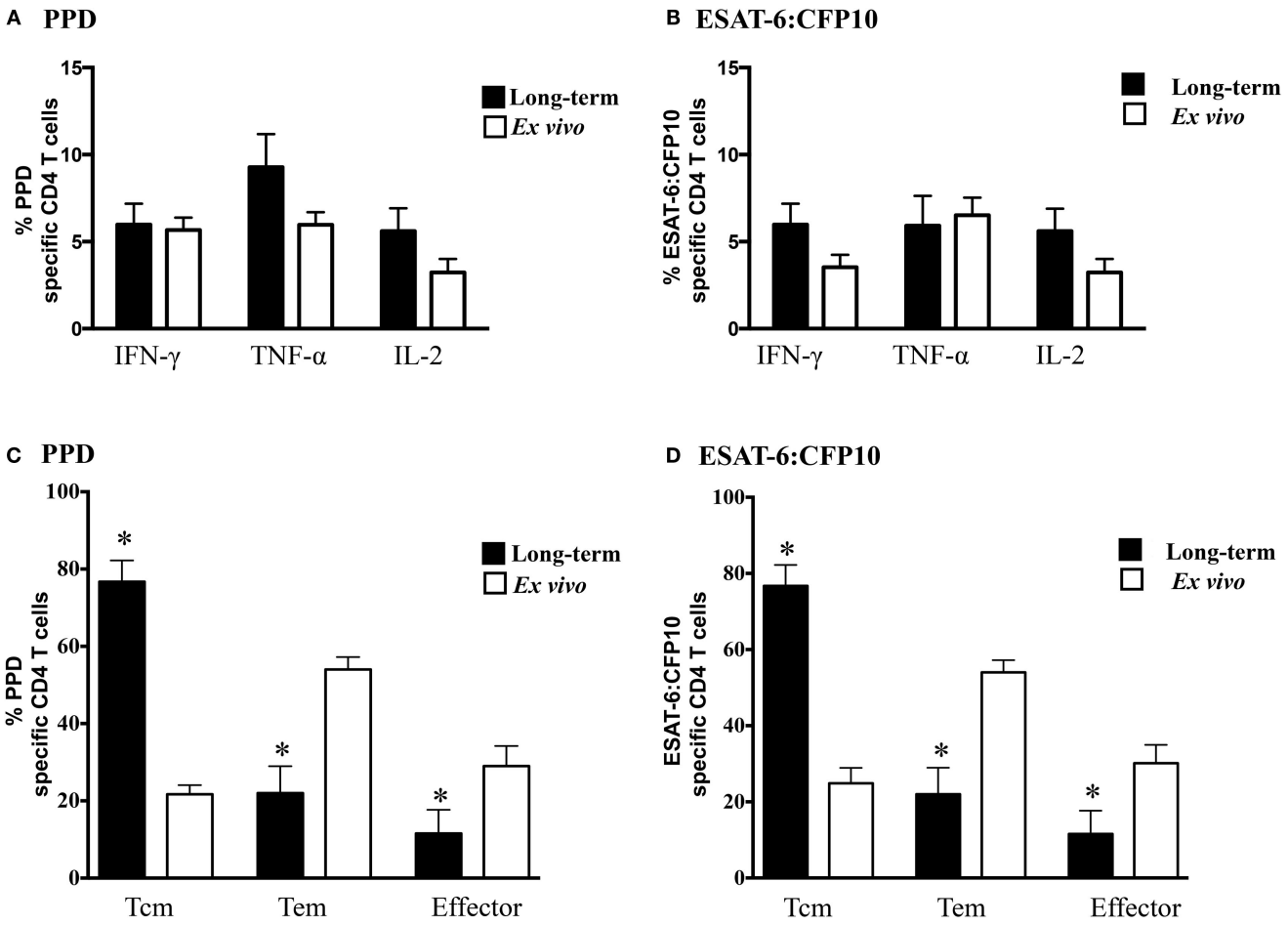

FIGURE 1 | Individual cytokines produced by different effector/memory phenotypes based upon culture conditions. For long-term cultures, PBMCs from infected calves were isolated and stimulated with a cocktail of Ag85A, TB10.4, and ESAT-6:CFP10 as well as PPD for 13 days followed by transfer to 96-well round bottom plates with APCs and addition of media alone, PPD, or ESAT-6:CFP10 for an additional 16 h. For *ex vivo* culture, PBMCs were stimulated with media alone, PPD or ESAT-6:CFP10 for 16 h. Cytokine production and effector/memory phenotype were determined by ICS flow cytometry. Results are presented as average and SE 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 (Figure S1 in Supplementary Material). *Responses from PBMCs differ ($P < 0.05$, Sidak's) between long-term and *ex vivo* cultures.

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 (**Figure 3A**, $P < 0.05$) and gross pathology as compared to non-vaccinates (**Figure 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 (**Figure 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.

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 (**Figure 4**) and PPD (Figure S3 in Supplementary Material). 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 (**Figure 4**). As observed in infected calves (study 1), the proportion of Tcm, Tem, and effector cells contributing to cytokine production in response to PPD and Ag85A/TB10.4 differed 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.

Cytokine profile[c]	PPD		ESAT6:CFP10	
	Long-term Average (SEM)	*Ex vivo* Average (SEM)	Long-term Average (SEM)	*Ex vivo* 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]

[a,b]Different letters indicate 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).

[c]Data are presented as frequencies (%) of cytokine-producing cells within long-term or ex vivo cultures.

(**Figure 5**, $P < 0.05$). Responding cells in long-term cultures most frequently exhibited a Tcm phenotype, while *ex vivo* cytokine production was mainly due to Tem cells. The contribution of naïve cells to the cytokine response was minimal and did not differ based on vaccination or culture conditions. 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 (Figure S4 in Supplementary Material) nor PPD (Figure S5 in Supplementary Material) at 6 WPV. Vaccine-elicited polyfunctional responses were detected to PPD and Ag85A/TB10.4 but not to ESAT-6:CFP10 (**Figure 6**). Thus, BCG vaccination elicits a specific polyfunctional CD4 T cell response and the culture duration (long-term versus *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).

Lower IFN-γ/TNF-α and Higher IFN-γ/TNF-α/IL-2 Coproduction 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 cells than did vaccinated animals (**Figure 7**). This difference was surprising given that vaccination elicited IFN-γ/TNF-α responses to PPD as well as Ag85A/TB10.4; and one might anticipate that these responses would be boosted after challenge with virulent *M. bovis*. In contrast, the IFN-γ/TNF-α/IL-2 response by vaccinates exceeded ($P < 0.05$) the respective response by non-vaccinates early after challenge (**Figure 7**). As with post-vaccination responses, responses by BCG mutants and BCG vaccinates did not differ at 3 WPI (Figure S5 in Supplementary Material) and 8 WPI (data not shown). Also, polyfunctional responses by Tcm cells at 8 WPI were similar among vaccinates and non-vaccinates (**Figure 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 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 (Figure S7 in Supplementary Material). 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 (Figure S7 in Supplementary Material) 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.

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 cells early after challenge (3 WPI). The kinetics of long-term culture Tcm cells expressing these polyfunctional profiles is depicted in **Figure 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 cells were greater in non-vaccinates as compared to vaccinates (**Figure 9**, $P < 0.05$), while vaccinates maintained higher percentages of IFN-γ/TNF-α/IL-2-producing Tcm cells in comparison to non-vaccinates (**Figure 9**, $P < 0.05$). At 8 weeks post-challenge, TNF-α/IFN-γ/IL-2 and TNF-α/IFN-γ responses were similar between vaccinates and non-vaccinates (**Figure 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 cells and dampening TNF-α/IFN-γ elicited by infection.

Vaccination-Elicited Protection Is Associated with Reduced IFN-γ/TNF-α Responses by Tcm Cells 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 (four BCG mutants and one 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 cells to ESAT-6:CFP10 among the groups (**Figure 10A**, $P < 0.05$). IFN-γ/TNF-α responses by Tcm cells from non-vaccinates were higher than that of both *M. bovis* culture-negative and -positive vaccinates (**Figure 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 (**Figure 10A**, $P < 0.05$). Conversely, IFN-γ/TNF-α/IL-2 production by Tcm cells was higher in culture-negative vaccinates as compared to culture-positive non-vaccinates (**Figure 10B**, $P > 0.05$). IFN-γ/TNF-α/IL-2 responses by Tcm cells from culture-positive

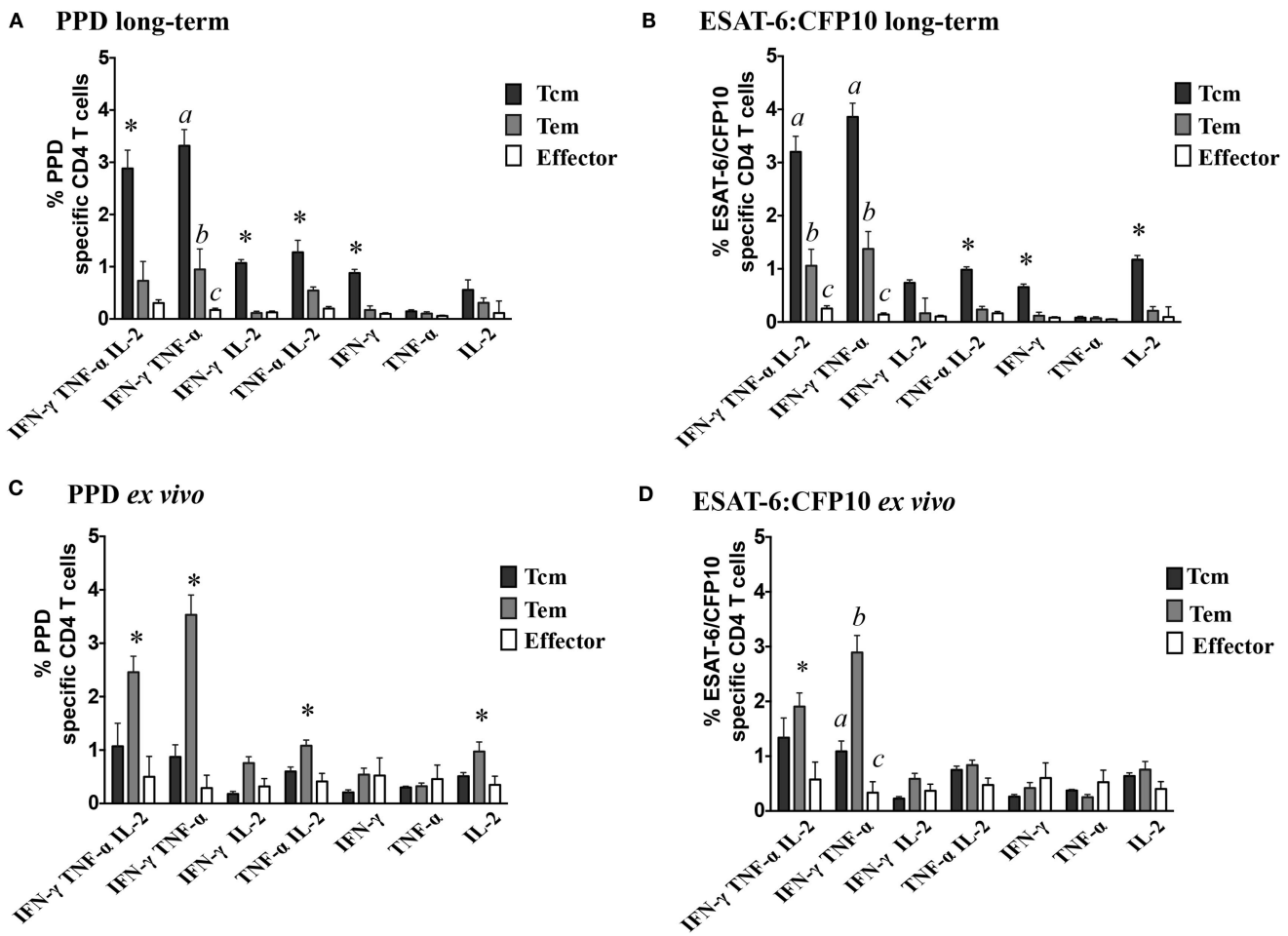

FIGURE 2 | Polyfunctional profiles by effector/memory phenotypes differ based on culture conditions. For long-term culture, PBMCs were isolated and stimulated with a cocktail of Ag85A, TB10.4, and ESAT-6:CFP10 as well as PPD for 13 days followed by transfer to 96-well round bottom plates with APCs and addition of media alone, PPD, or ESAT-6:CFP10 for an additional 16 h. For *ex vivo* culture, PBMC were stimulated with media alone, PPD, or ESAT-6:CFP10 for 16 h. Cytokine production and effector/memory phenotype were determined by ICS flow cytometry. Results are presented as average and SE for responses over the course of the study ($n = 30$, 5 animals × 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 (Figure S1 in Supplementary Material). *Responses differ ($P < 0.05$, Tukey's) from the other two phenotypes. [a,b,c]Different letters indicate differences ($P < 0.05$, Tukey's) in effector/memory phenotypes within a particular polyfunctional subset.

vaccinates were intermediate, not differing from either culture-negative vaccinates or non-vaccinates (**Figure 10B**, $P < 0.05$). IFN-γ/TNF-α/IL-2 and IFN-γ/TNF-α production by Tcm cells in *ex vivo* assays did not differ among culture-negative and culture-positive animals (**Figures 10C,D**, $P > 0.05$). Thus, these results indicate that strong IFN-γ/TNF-α responses by Tcm cells are associated with greater mycobacterial burden, while IFN-γ/TNF-α/IL-2 responses by Tcm cells are indicative of a protective response provided by vaccination.

DISCUSSION

Tuberculosis in cattle shares many features with TB in humans, and studies with neonatal calves are particularly pertinent to human TB, since children are the primary target population for vaccination with exposure to TB often occurring 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 cells are presumed to be critical for protection in cattle and humans (1, 13, 18, 20, 29, 32). 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 cells as compared to that of unprotected animals. Protected vaccinates had a lower frequency of

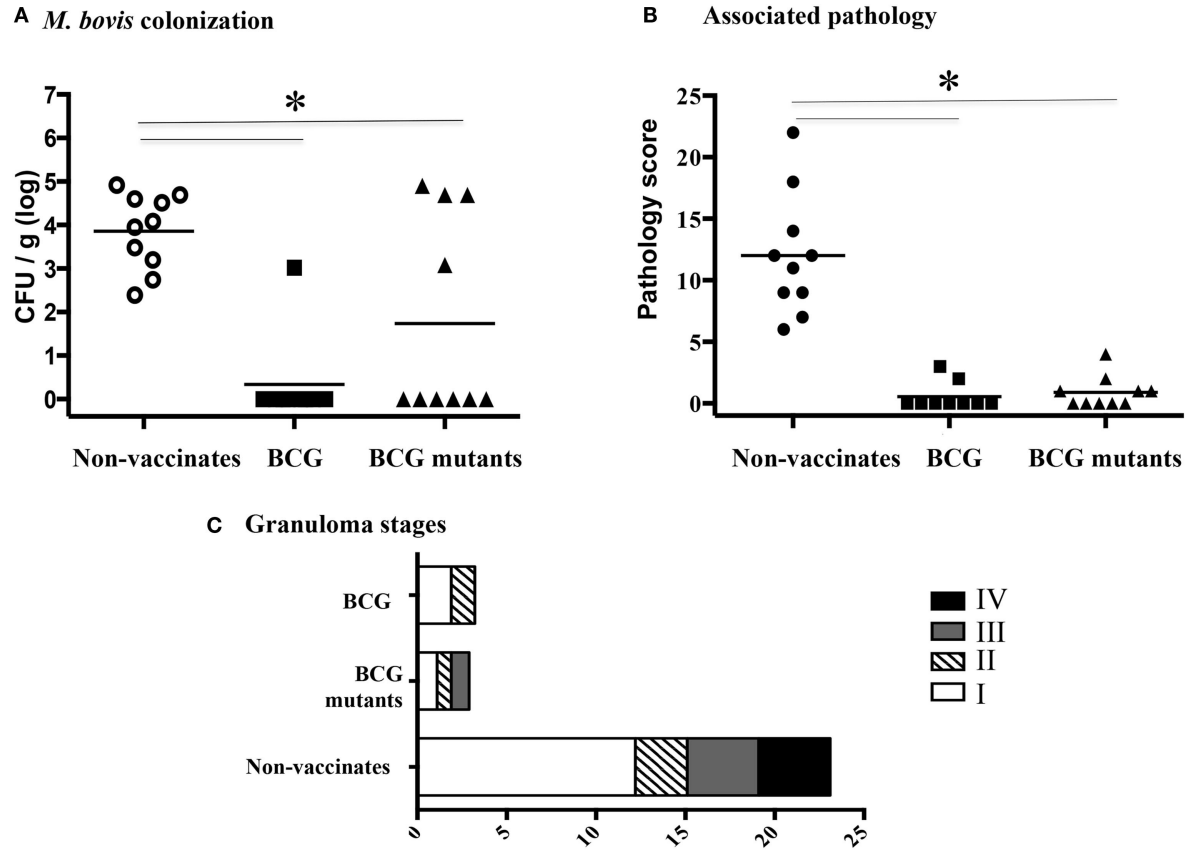

FIGURE 3 | BCG mutants and BCG vaccination confers protection against virulent *M. bovis* challenge. **(A)** Quantitative assessment of mycobacterial burden in tracheobronchial lymph nodes. **(B)** Total gross pathology scores from lung and tracheobronchial and mediastinal lymph nodes. **(C)** Data are presented as average number of stages I–IV microscopic lesions observed in mediastinal lymph nodes, tracheobronchial lymph nodes, and lung based on the scoring system developed by Wangoo et al. (54). *Parameters differ ($P < 0.05$, Tukey's) between non-vaccinates ($n = 10$) and BCG mutants ($n = 10$) or BCG vaccinates ($n = 9$).

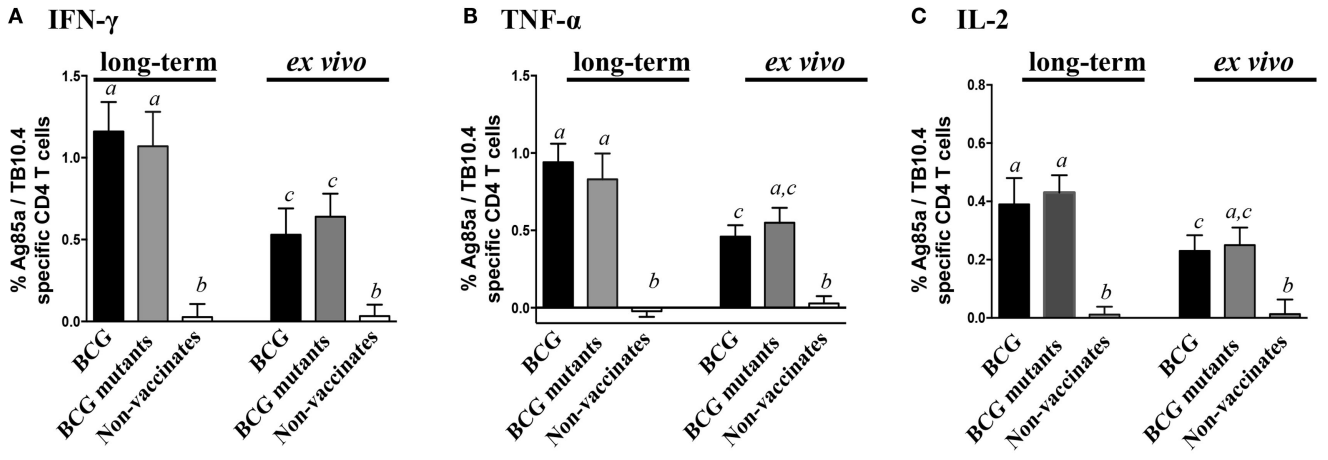

FIGURE 4 | Long-term and *ex vivo* cytokine responses to vaccination. For long-term culture, PBMCs were isolated at 6 WPV and stimulated with a cocktail of Ag85A, TB10.4, and PPD for 13 days followed by transfer to 96-well round bottom plates with APCs and addition of media alone or Ag85A/TB10.4 for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

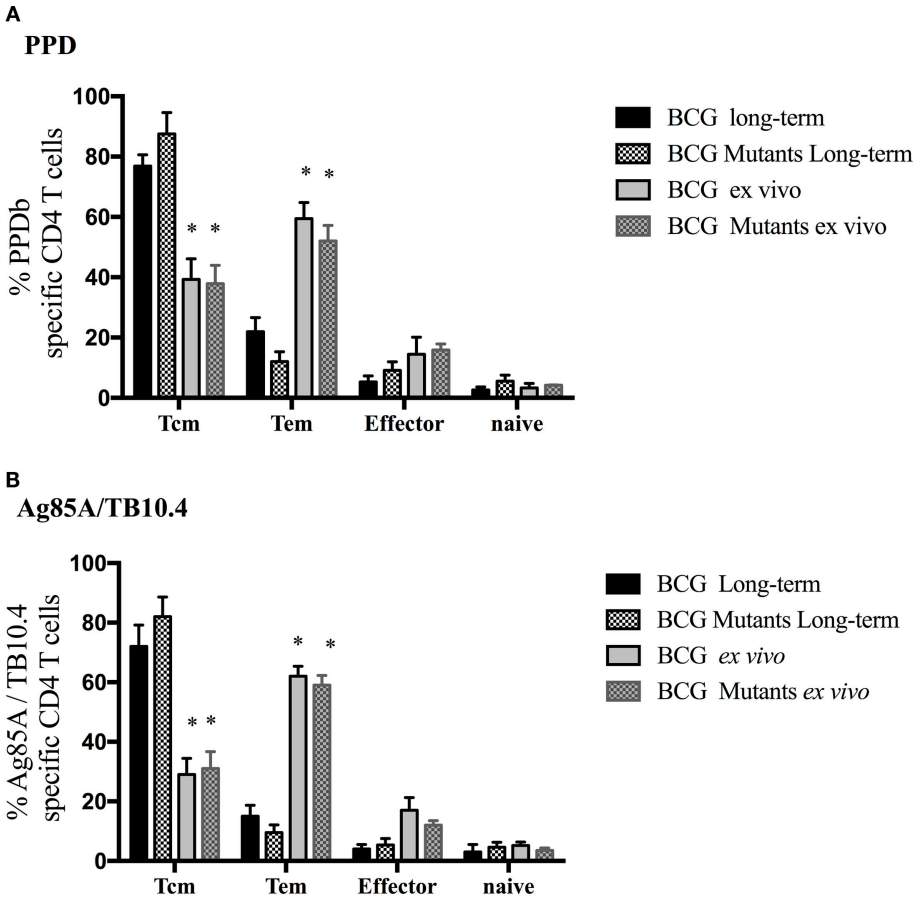

FIGURE 5 | Vaccine-elicited cytokine production is due to different effector/memory phenotypes based upon culture conditions. For long-term culture, PBMCs were isolated at 6 WPV and stimulated with a cocktail of Ag85A, TB10.4, and PPD for 13 days followed by transfer to 96-well round bottom plates with APCs and addition of media alone PPD **(A)** or Ag85A/TB10.4 **(B)** for an additional 16 h. Cytokine production and effector/memory phenotype were determined by ICS flow cytometry. Results are presented as average and SE. Frequency of cytokine-producing cells under long-term or *ex vivo* culture exhibiting Tcm, Tem, effector, or naïve phenotype using the gating strategy described in Figure S1 in Supplementary Material. *Differences ($P < 0.05$) in cytokine production within memory subsets (i.e., Tcm, Tem, effector, or naïve) in long-term as compared to *ex vivo* cultures (Tukey's).

ESAT-6:CFP10-specific Tcm cells coproducing 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 cells 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 cells in long-term culture and Tem cells 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 (10). 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 and Tem cells (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 necessarily correlate to each other with HIV (62), EBV (63), hepatitis C virus (11), or malaria infection (64, 65), demonstrating that the nature of the response by cytokine-producing cells

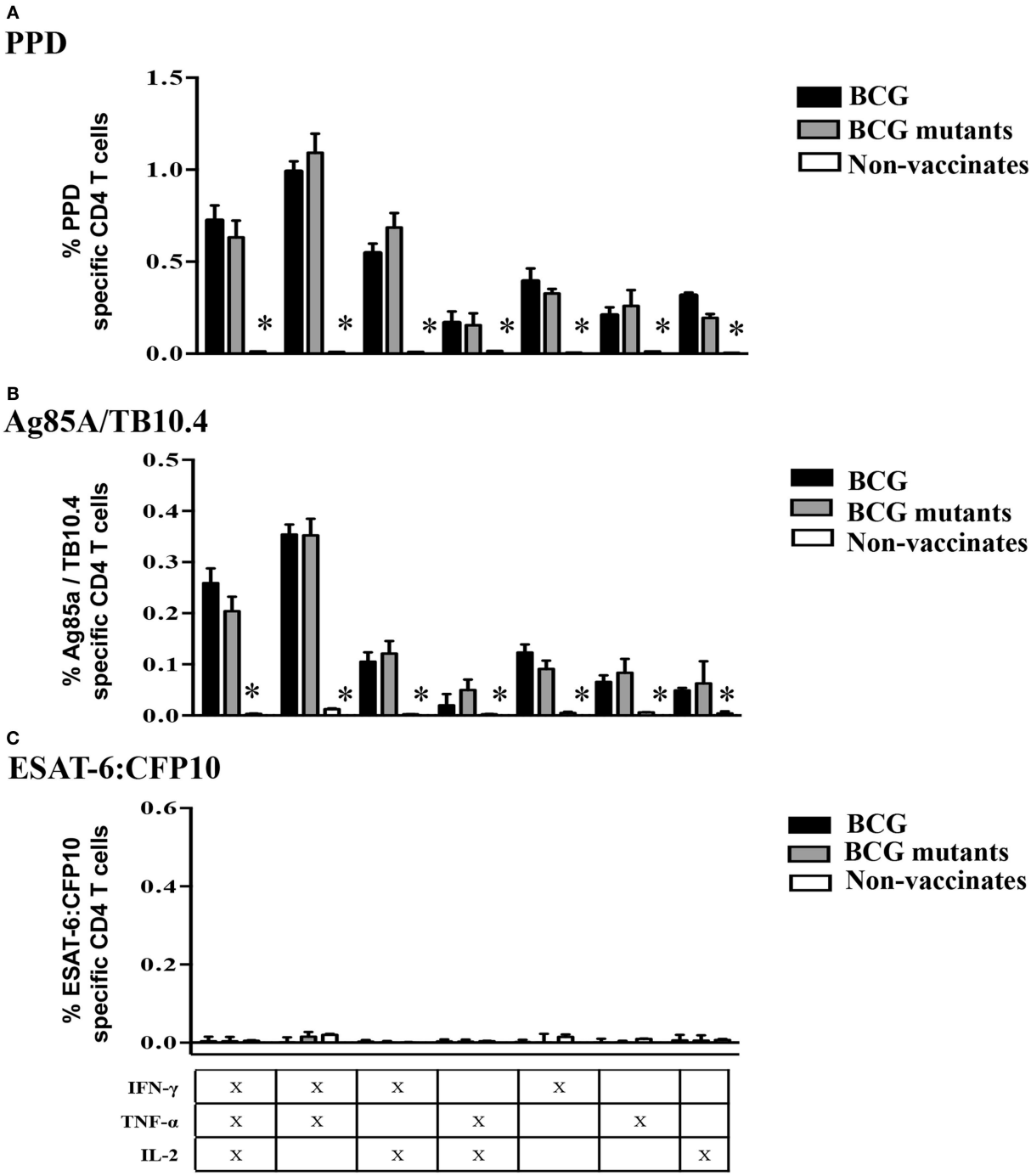

FIGURE 6 | Specific Tcm responses to vaccination at 6 weeks post-vaccination. For long-term culture, PBMCs were isolated at 6 WPV and stimulated with a cocktail of Ag85A, TB10.4, and PPD for 13 days followed by transfer to 96-well round bottom plates with APCs and addition of media alone, PPD, Ag85A/TB10.4, or ESAT-6:CFP10 for an additional 16 h. Cytokine production and effector/memory phenotype were determined by ICS flow cytometry. Results are presented as average and SE. Polyfunctional responses by Tcm cells (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.

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

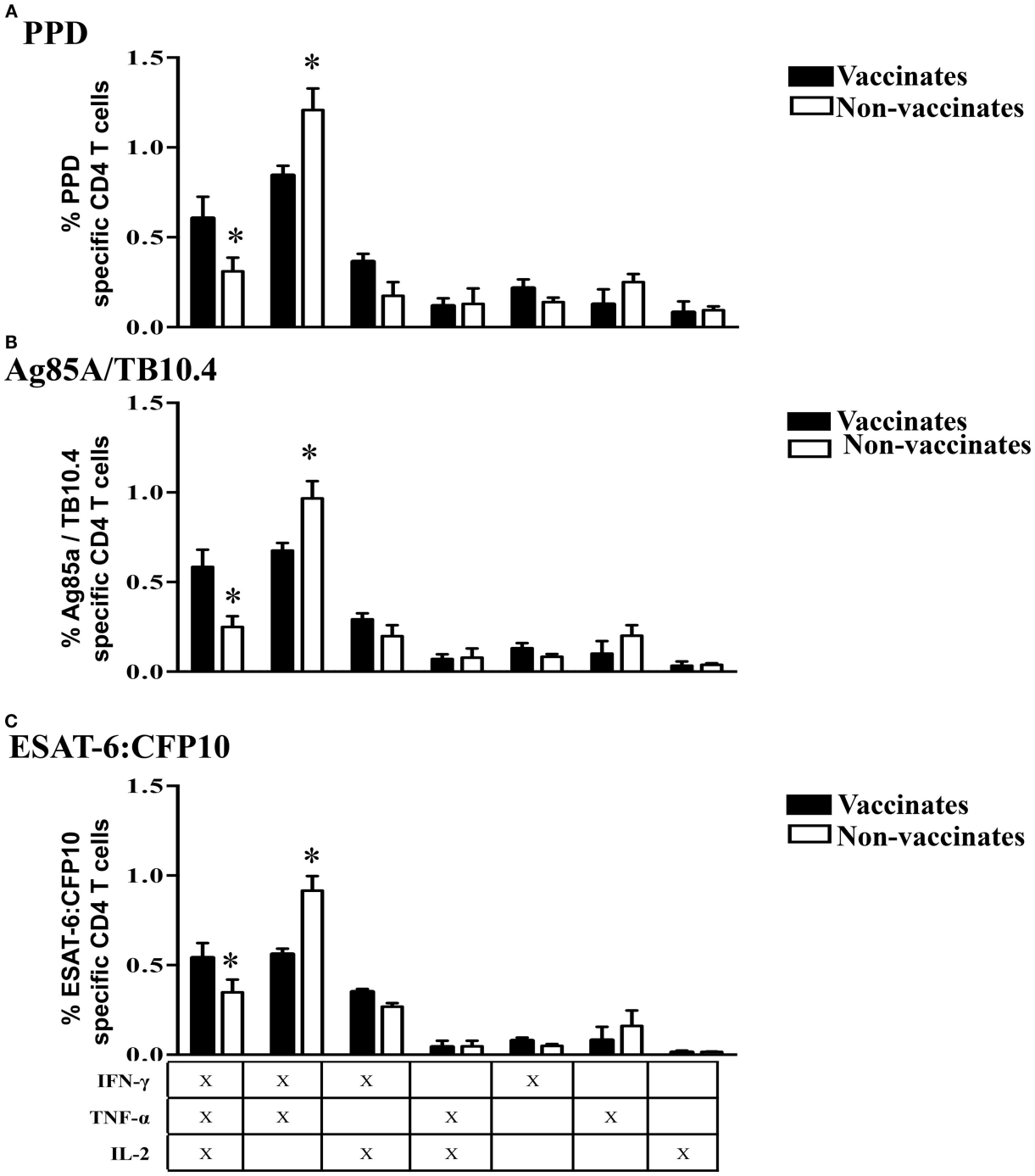

FIGURE 7 | Higher IFN-γ/TNF-α and lower IFN-γ/TNF-α/IL-2 Tcm responses by non-vaccinates versus vaccinates at 3 weeks post-challenge. For long-term culture, PBMCs were isolated at 3 WPI and stimulated with a cocktail of Ag85A, TB10.4, and PPD for 13 days followed by transfer to 96-well round bottom plates with APCs and addition of media alone, PPD, Ag85A/TB10.4, or ESAT-6:CFP10 for an additional 16 h. Cytokine production and effector/memory phenotype were determined by ICS flow cytometry. Results are presented as average and SE. Polyfunctional responses by Tcm cells (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.

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.

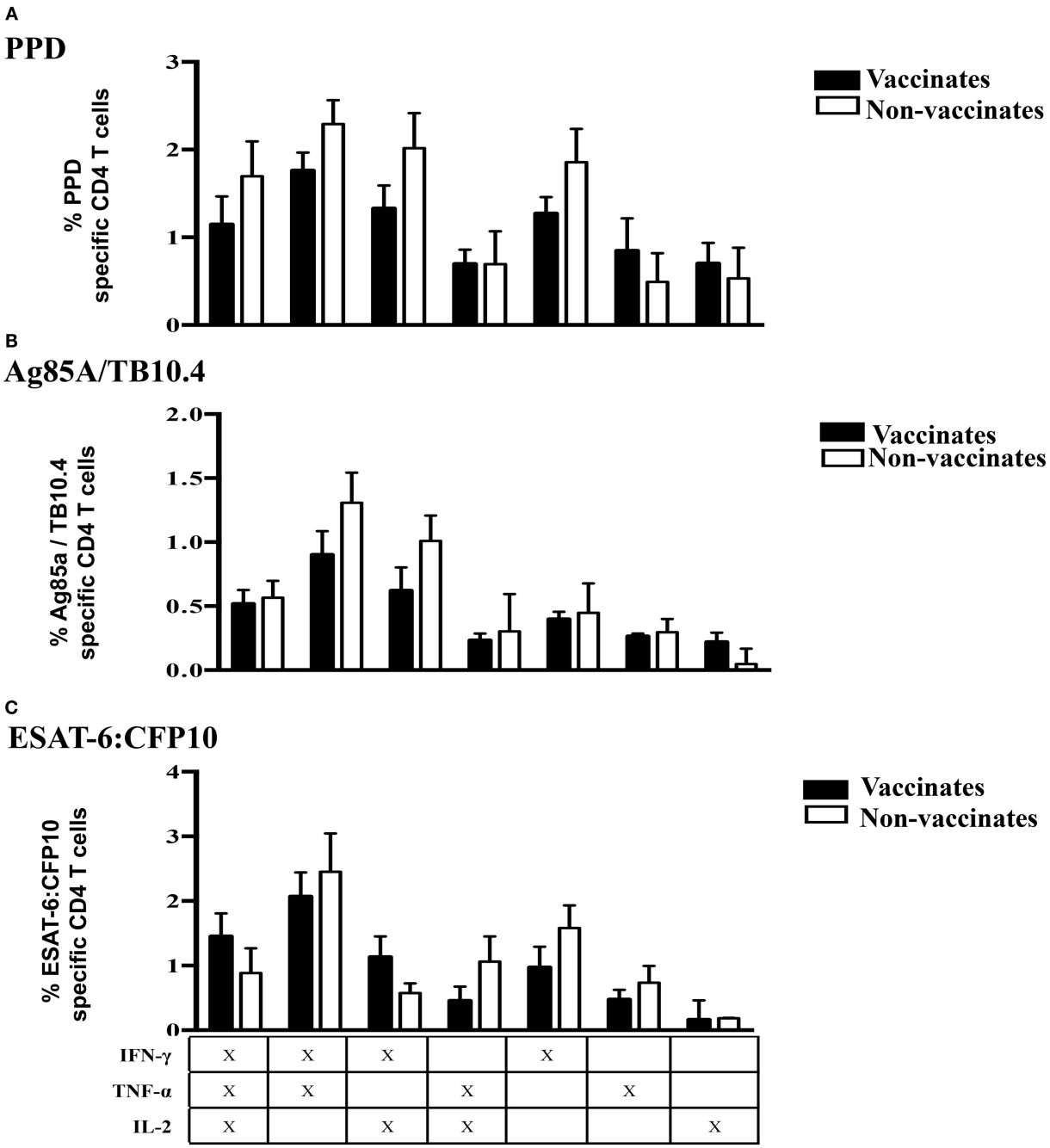

FIGURE 8 | By 8 weeks post-*M. bovis* challenge, polyfunctional responses by Tcm cells did not differ between vaccinates and non-vaccinates. For long-term culture, PBMCs were isolated at 8 WPI and stimulated with a cocktail of Ag85A, TB10.4, and PPD for 13 days followed by transfer to 96-well round bottom plates with APCs and addition of media alone, PPD, Ag85A/TB10.4, or ESAT-6:CFP10 for an additional 16 h. Cytokine production and effector/memory phenotype were determined by ICS flow cytometry. Results are presented as average and SE. Polyfunctional responses by Tcm cells (gated on $CD45RO^+/CCR7^+/CD4^+$ cells) upon recall stimulation with PPD **(A)**, Ag85A/TB10.4 **(B)**, or ESAT-6:CFP10 **(C)**. *Differences ($P < 0.05$, Tukey's) in cytokine production by vaccinates versus non-vaccinates.

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 cells 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, but not Tem cells, is sufficient to confer protection (5). Laouar et al. (68) demonstrated the appearance of CD8 memory cells during the acute phase of

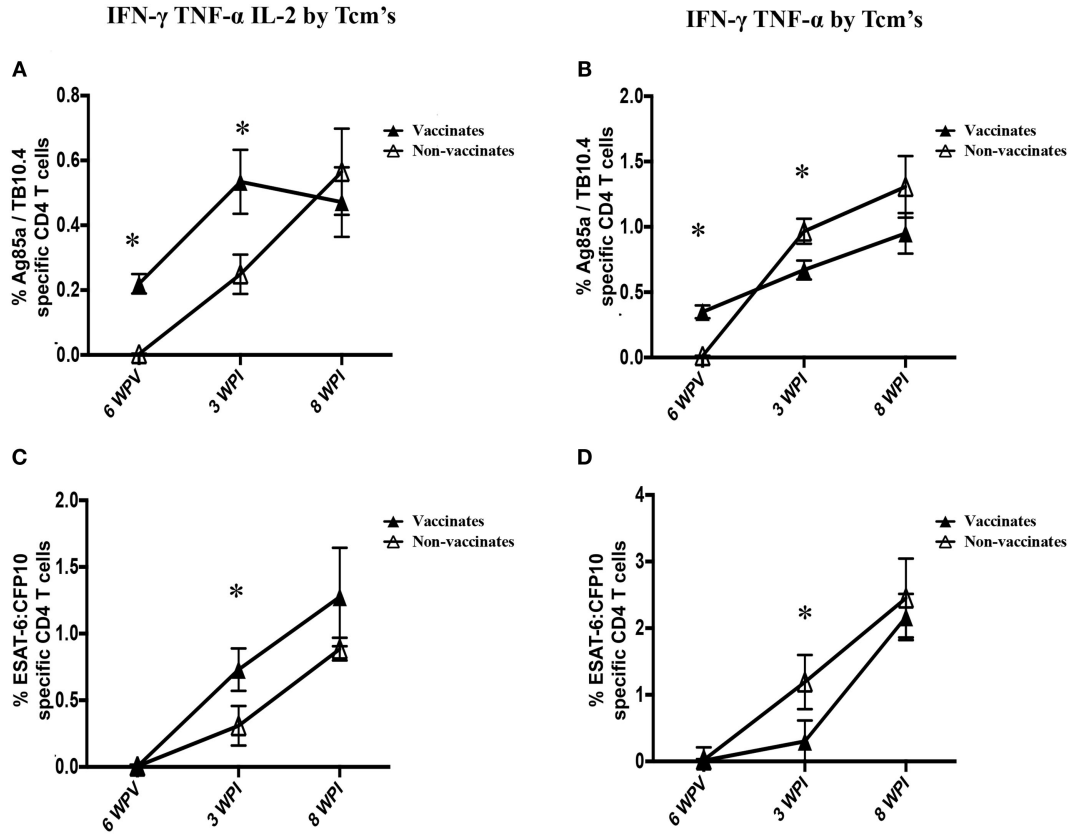

FIGURE 9 | Kinetics of IFN-γ TNF-α IL-2 and IFN-γ TNF-α responses by Tcm. For long-term culture, PBMCs isolated at 6 weeks post-vaccination (6 WPV), 3 weeks post-infection (3 WPI) and 8 WPI were stimulated with a cocktail of Ag85A, TB10.4, and PPD for 13 days followed by transfer to 96-well round bottom plates with APCs and addition of media alone, Ag85A/TB10.4 or ESAT-6:CFP10 for an additional 16 h. Cytokine production and effector/memory phenotype were determined by ICS flow cytometry. Results are presented as average and SE. Percentages of long-term cultured Tcm cells (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,B)** or ESAT-6:CFP10 **(C,D)**. *Differences ($P < 0.05$, Tukey's) in the cytokine production by non-vaccinates and vaccinates.

a primary response to lymphocytic choriomeningitis virus as early as 8 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 7 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 (a) as direct progenies of effector cells [linear differentiation model (73–75)], (b) *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 (c) 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 cells 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 (32, 76, 79). Persistent or prolonged antigen stimulation is related to a progressive loss of central memory T cell pool, resulting in unfit immune responses by terminally differentiated, short-lived cells that produce single cytokine or loss of effector function all together (32, 80). The higher numbers of IFN-γ/TNF-α/IL-2 Tcm cells may indicate that vaccinated calves were able to control antigen load, retaining

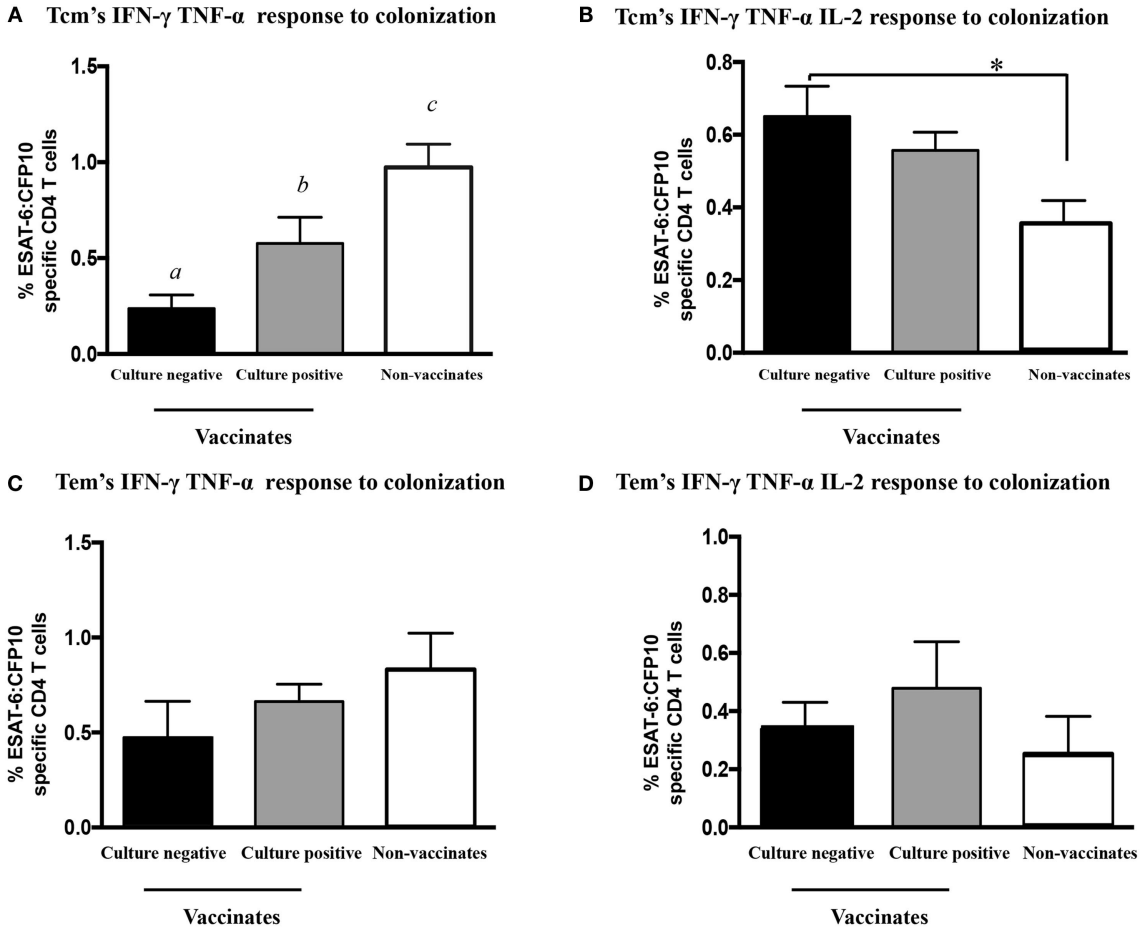

FIGURE 10 | IFN-γ/TNF-α/IL-2 and IFN-γ/TNF-α polyfunctional responses at 3 weeks post-infection by Tcm, but not by Tem cells, differ based upon *M. bovis* colonization and vaccination status. Calves were grouped accordingly with both vaccine status and bacterial burden results (culture positive or negative) for assessment of IFN-γ/TNF-α/IL-2 and IFN-γ/TNF-α Tcm response by flow cytometry 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 16 h. Results are presented as average and SE. IFN-γ/TNF-α and IFN-γ/TNF-α/IL-2 polyfunctional responses by Tcm cells (gated on $CD45RO^+/CCR7^+/CD4^+$ cells) from vaccinates (either culture positive or negative) or non-vaccinates upon recall stimulation with ESAT-6:CFP10 **(A,B)**. IFN-γ/TNF-α and IFN-γ/TNF-α/IL-2 polyfunctional responses by Tem cells (gated on $CD45RO^+/CCR7^-/CD4^+$ cells) vaccinates (either culture positive or negative) or non-vaccinates upon recall stimulation with ESAT-6:CFP10 **(C,D)**. ^{a,b,c}Different letters indicate 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.

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 cells 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 cells 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 level 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.

Central memory T cells 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 (34, 81, 82). This suggests that CD4 T cell functional capacity is driven toward terminally differentiated T cells by bacterial load and continuous antigen exposure (34). 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 cells 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 cells. 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-term survival, or producing other cytokines). Protection to TB may require different levels of Tcm and Tem memory responses and also different ratios of Tcm to Tem in the different phases of this chronic infection (83). It is also possible that the presence of Tcm cells 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 KLRG1 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 *Mtb*Δ*sigH* mutant was highly protective against an otherwise lethal *M. tuberculosis* aerosol challenge. *Mtb*Δ*sigH* vaccinated macaques exhibited higher numbers of Tcm cells 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 programed 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 analyses 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 meantime, present findings indicate that IFN-γ/TNF-α and IFN-γ/TNF-α/IL-2 responses by Tcm cells 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.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: MP, TT, JM, WW, and MM. Performed the experiments: MM, MP, TT, and WW. Analyzed the data: MM and WW. Contributed reagents/materials/analysis tools: AW, HV, ML, and WJ. Wrote the paper: MM and WW.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00421

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