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Characterization of γδ T Cell Effector/Memory Subsets Based on CD27 and CD45R Expression in Response to Mycobacterium bovis Infection

Mariana Guerra-Maupome,* Mitchell V. Palmer,† W. Ray Waters,† and Jodi L. McGill*

*Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA 50010; and †Infectious Bacterial Diseases Research Unit, National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, IA 50010

ABSTRACT

Tuberculosis (TB) remains a leading cause of death from infectious diseases worldwide. Mycobacterium bovis is the causative agent of bovine TB and zoonotic TB infection. γδ T cells are known to participate in the immune control of mycobacterial infections. Data in human and nonhuman primates suggest that mycobacterial infection regulates memory/effector phenotype and adaptive immune functions of γδ T cells. To date, the impact of M. bovis infection on bovine γδ T cells and their effector and memory differentiation remains unknown. In this study, we show that circulating γδ T cells from M. bovis–infected cattle can be differentiated based on the expression of CD27, which is indicative of their capacity to respond to virulent M. bovis infection: CD27+ γδ T cells proliferated in response to M. bovis Ag and, thus, may comprise the adaptive γδ T cell compartment in cattle. We further show that bovine M. bovis–specific γδ T cells express surface markers characteristic of central memory T cells (CD45R+CD27+CD62Lhi) and that M. bovis–specific CD4 and γδ T cells both upregulate the expression of the tissue-homing receptors CXCR3 and CCR5 during infection. Our studies contribute significantly to our understanding of γδ T cell differentiation during TB infection and provide important insights into the link between phenotypic and functional subsets in the bovine. Accurate characterization of γδ T cell effector and memory-like responses induced during mycobacterial infection will contribute to improved strategies for harnessing the γδ T cell response in protection against TB for humans and animals. ImmunoHorizons, 2019, 3: 208–218.

INTRODUCTION

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

Cell-mediated Th1 immune responses are essential for controlling TB (7, 8). Importantly, however, γδ T cells have been shown to contribute to the immune response against TB. M. bovis infection elicits a marked in vitro expansion and robust production of IFN-γ by γδ T cells, suggesting that bovine γδ T cells can mount memory-like responses upon restimulation (9–14). Expression of cell surface memory markers CD45RA and CD27 is linked to functional

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Address correspondence and reprint requests to: Dr. Jodi L. McGill, Iowa State University, 1907 Christensen Drive, Veterinary Medical Research Institution Building 5, Ames, IA 50010. E-mail address: jlmcgill@iastate.edu

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Abbreviations used in this article: bTB, bovine TB; PPD-b, M. bovis purified protein derivate; TB, tuberculosis; TCM, central memory T; TEM, effector memory T; TEMRA, effector memory RA T.

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MATERIALS AND METHODS

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

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

Aerosol challenge procedures
Treatment groups consisted of noninfected steers (n = 6) and animals receiving 1 × 10^6 CFU of M. bovis 10-7428 (n = 10). M. bovis challenge inoculum was delivered to restrained calves by aerosol as described by Palmer et al. (23). Briefly, inoculum was nebulized into a mask (Trudell Medical International, London, ON, Canada) covering the nostrils and mouth, allowing regular breathing and delivery of the bacterial inoculum to the upper and lower respiratory tract via the nostrils. The process continued until the inoculum, a 1-ml PBS wash of the inoculum tube, and an additional 2 ml PBS were delivered, a process taking ~10 min. Strict biosafety protocols were followed to protect personnel from exposure to M. bovis throughout the study, including biosafety label 3 containment upon initiation of M. bovis challenge in animal rooms and standard laboratory practices for handling M. bovis cultures and samples from M. bovis–infected animals.

PBMC isolation
Peripheral blood was drawn from the jugular vein into 2× acid/citrate/dextrose solution. For PBMC isolation, blood was diluted 1:1 in PBS and cells were isolated from buffy coat fractions, and PBMCs were isolated by density centrifugation on Histopaque (Sigma-Aldrich). PBMCs were collected and washed twice in PBS to remove platelets. Residual RBC were removed by adding RBC lysis buffer. Finally, PBMCs were resuspended in complete RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 2 mM L-glutamine, 25 mM HEPES buffer, 1% antibiotic/antimycotic (Sigma-Aldrich), 1% nonessential amino acids (Sigma-Aldrich), 2% essential amino acids (Sigma-Aldrich), 1% sodium pyruvate (Sigma-Aldrich), 50 μM 2-ME (Sigma-Aldrich), and 10% (v/v) heat-inactivated FBS.

Proliferation assay
To assess proliferation, PBMC were labeled using CellTrace Violet (Invitrogen, Carlsbad, CA) prior to cell culture following the manufacturer’s instructions. Briefly, freshly isolated cells were resuspended at 1 × 10^7 cells/ml in PBS containing 10 μM/ml of the CellTrace dye. After gently mixing, PBMCs were incubated for 20 min at 37°C in a water bath. Labeling was quenched by using an equal volume of FBS, and cells were washed three times with RPMI medium. Subsequently, cells (5 × 10^6 cells/well) were plated in round-bottom, 96-well plate in duplicates, cultured for 6 d at 37°C, in the presence of 5% CO_2, in the presence of M. bovis.
purified protein derivate (PPD-b; 200 IU/ml; Prionics, Schlieren, Switzerland), and rESAT-6/CFP-10 fusion protein (2 μg/ml; LIONEX Diagnostics and Therapeutics). PWM (1 μg/ml; Sigma-Aldrich) or complete RPMI medium were used as positive and negative controls, respectively. After 6 d, cells were surface stained (see Flow cytometry section) and analyzed for proliferation and surface marker expression by flow cytometry.

Flow cytometry

Following the appropriate culture duration, cells were stained with primary and secondary mAbs listed in Table I. All incubation steps for staining were performed in FACS buffer (PBS with 10% FBS and 0.02% NA/azide) and incubated for 25 min at 4°C. Cells were washed and fixed with BD FACS lysis buffer (BD Biosciences, Mountain View, CA) for 10 min at room temperature.

FIGURE 1. Proliferative responses from M. bovis–infected and noninfected cattle in response to in vitro restimulation with mycobacterial Ags. PBMCs from uninfected (n = 6) or virulent M. bovis–infected animals (n = 10) were labeled with CellTrace, and 5 × 10^6 cells/ml were cultured for 6 d in the presence or absence of 200 UI/ml PPD-b or 2 μg/ml ESAT-6/CFP-10. Cells were labeled with anti-bovine γδ TCR or CD4 and analyzed by flow cytometry for CellTrace dilution (Supplemental Fig. 1). Representative histograms of proliferative responses from a M. bovis–infected and control animal are shown in panels A and B. Percentage of γδ T cells (C) and CD4 T cells (D) from M. bovis–infected and uninfected animals that proliferated in response to mycobacterial Ags, as measured by CellTrace dilution, at 4, 8 or 12 wk after aerosol challenge. Analysis was performed with FlowJo software. Background proliferation was subtracted, and results represent change over mock. Data are mean ± SEM and are representative of one independent experiment. The p values comparing responses between uninfected and M. bovis–infected animals were as follows: *p < 0.05, **p < 0.01, ***p < 0.001, as determined by Student t test.

### TABLE I. Primary and secondary Abs and staining reagents

<table>
<thead>
<tr>
<th>Reagent or Ab Clone</th>
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<th>Secondary Abs, Source</th>
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<tr>
<td>ILA11</td>
<td>Bovine CD4, Washington State University</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>G821A</td>
<td>Bovine TCR1 β chain, Washington State University</td>
<td>Allophycocyanin/Cy7, SouthernBiotech</td>
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<td>ILA16</td>
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<td>Allophycocyanin, Life Technologies</td>
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<td>GC6A</td>
<td>Bovine CD45R, Washington State University</td>
<td>PerCP/Cy5.5, Life Technologies</td>
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<td>Allophycocyanin or PE/Cy7, Life Technologies</td>
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</tr>
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<td>Dead cells, Invitrogen</td>
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</tr>
<tr>
<td>CellTrace Violet</td>
<td>Not applicable, Life Technologies</td>
<td>Not applicable</td>
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temperature, washed, and resuspended in FACS buffer until analysis. Samples were acquired using a BD LSRFortessa flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, San Carlos, CA). Electronic gates were set using fluorescence minus one controls.

Statistical analysis
Results are expressed as mean ± SE of the mean. Statistical significance was determined by one-way ANOVA, followed by Bonferroni test, or Student t test using Prism software (GraphPad, La Jolla, CA).

RESULTS
Virulent M. bovis infection induces Ag-specific γδ T cell responses
We and others have previously demonstrated that γδ T cells from virulent M. bovis–infected cattle respond to both the complex mycobacterial Ag, PPD-b, and to the specific protein Ag, ESAT-6/CFP-10 (10, 11, 24). To corroborate our prior studies, animals were infected via aerosol inoculation with virulent M. bovis strain 10-7428 (n = 10) or noninfected group (n = 6). Following infection, PBMC were labeled with CellTrace dye and cultured for 6 d in the presence or absence of PPD-b or rESAT-6/CFP-10. On day 6, cells were analyzed by flow cytometry for the frequency of γδ and CD4 T cells that divided in response to the mycobacterial Ags (Supplemental Fig. 1). The mAbs and reagents used in the flow cytometry experiments are described in Table I. Representative proliferative responses of γδ and CD4 T cells following mycobacterial stimulation in vitro are depicted (Fig. 1A, 1B). Robust proliferative responses to PPD-b and ESAT-6/CFP-10 were observed by γδ T cells at 4 wk postinfection and persisted until at least 12 wk postinfection (Fig. 1C). Consistent with prior reports, the mean CD4 T cell proliferative response to PPD-b and ESAT-6/CFP-10 peaked by 8 wk postinfection and persisted for at least 12 wk postinfection (Fig. 1D) (25). Recall responses from both γδ and CD4 T cell populations were M. bovis specific, as neither CD4 nor γδ

FIGURE 2. Evaluation of CD45RO and CD45R expression on M. bovis–specific γδ and CD4 T cells.
Approximately 8 wk after aerosol challenge with M. bovis, PBMCs from M. bovis–infected animals (n = 10) were labeled with CellTrace, and 5 × 10⁶ cells/ml were cultured for 6 d in the presence or absence of PPD-b. Cells were labeled with anti-bovine CD4 or γδ TCR and CD45RO or CD45R expression by flow cytometry within proliferative subsets as determined by CellTrace dilution. (A) Gating hierarchy (gating sequence as depicted by the arrows): lymphocytes (gate 1), live cells (gate 2), γδ or CD4 T cells (gate 3), proliferating (1) and nonproliferating cells (2), and CD45RO or CD45R expression (gate 5). (B) The expression of CD45RO or CD45R was determined for CD4 T cells or γδ T cells based on CellTrace staining intensity (i.e., bright or dim). Data are presented as means (±SEM) and are representative of one independent experiment. The p values for differences from responding and nonresponder fractions for the respective T cell subset (i.e., comparisons between black and gray bars for each graph) were as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, as determined by Student t test. SSC-A, side scatter A; SSC-H, side scatter H.

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T cells from noninfected cattle responded to the specific or complex mycobacterial Ags.

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

Similar to humans, bovine naive and memory CD4 T cells can be distinguished based on the expression of CD45 isoforms. Naive T cells express the high CD45RA isoform, and upon antigenic recognition, T cells switch to the expression of the low-CD45RO isoform (26, 27). In the bovine, CD45RA and CD45RB isoforms are not yet defined; thus, CD45R is used as a marker for naive lymphocytes in cattle (26). To evaluate if CD45 isoform expression correlated with the bovine γδ T cell effector/memory population that responded to mycobacteria, we analyzed the CD45 phenotype of expanding M. bovis–specific γδ and CD4 T cells by flow cytometry following a 6-d in vitro restimulation with PPD-b (Fig. 2A). In accordance with previous data (25, 28), the mycobacterial-driven proliferative responses to PPD-b were within the CD4+ CD45RO+ T cell subset, and the Ag-responsive cells downregulated CD45R expression compared with nonresponding cells (Fig. 2B). In contrast to CD4 T cells, γδ T cells from M. bovis–infected cattle showed no significant changes in cell surface expression of CD45RO between Ag-specific (proliferating) and nonresponding γδ T cell populations. However, Ag-responsive γδ T cells from infected cattle downregulated CD45R expression in response to PPD-b stimulation (Fig. 2C). Changes in expression of either CD45R or CD45RO were not detected in response to PPD-b stimulation (Fig. 2C). In accordance with previous data (25, 28), the mycobacterial-driven proliferative responses to PPD-b were within the CD4+ CD45RO+ T cell subset, and the Ag-responsive cells downregulated CD45R expression compared with nonresponding cells (Fig. 2B). In contrast to CD4 T cells, γδ T cells from M. bovis–infected cattle showed no significant changes in cell surface expression of CD45RO between Ag-specific (proliferating) and nonresponding γδ T cell populations. However, Ag-responsive γδ T cells from infected cattle downregulated CD45R expression in response to PPD-b stimulation (Fig. 2C). Changes in expression of either CD45R or CD45RO were not detected in γδ T cells from noninfected animals after 6 d of culture. From these results, it can be concluded that CD45Rneg γδ and CD4 T cell subset participates in the in vitro recall responses against M. bovis.

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

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

CD27 expression has been reported in bovine PBMCs; however, its expression on bovine γδ and CD4 T cells has not been determined (31). Therefore, we performed ex vivo staining on PBMC from healthy cattle and observed that ~70% of CD4 T cells and ~50% of γδ T cells express CD27, as measured by flow cytometry. Following mitogen stimulation, both CD4 and γδ T cells CD27+ display higher clonogenic potential (Fig. 3). Thus, CD27 expression was abundantly expressed on bovine peripheral T cells and can be used to identify CD4 and γδ T cell subsets based on their capacity to proliferate (16, 32).

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

To determine whether mycobacteria-specific T cells could be defined by the expression of CD27, we analyzed the expression on M. bovis–specific γδ and CD4 T cells following in vitro restimulation with PPD-b and ESAT-6/CFP-10 (Fig. 4A, 4B). After 6 d in culture with PPD-b or ESAT-6/CFP-10, the majority of Ag-specific γδ T cells from M. bovis–infected animals proliferated and upregulated expression of CD27 compared with nonresponding γδ T cells in

**FIGURE 3. CD27 expression on bovine γδ and CD4 T cells ex vivo and in response to mitogen.**

Using flow cytometry, bovine PBMCs from healthy cows were analyzed for CD4 or γδ TCR expression. Gating hierarchy (gating sequence as depicted by the arrows): live cells (gate 1), lymphocytes (gate 2), and γδ or CD4 T cells (gate 3). Each lymphocyte population was further analyzed for CD27 expression following mock or PWM stimulation for 6 d (dot plots). Data are representative of n = 12 animals from six independent experiments. FMO, fluorescence minus one; FSC-A, forward scatter A; SSC-A, side scatter A; SSC-H, side scatter H.

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the same culture (Fig. 4A, Supplemental Fig. 2). Similarly, CD27 expression was significantly greater on the Ag-specific CD4 T cell population from *M. bovis*–infected animals compared with nonspecific CD4 T cells in the same culture (Fig. 4B). Both γδ and CD4 T cells from infected animals exhibited significant increases in CD27 expression compared cells from noninfected control calves. Thus, from our results, increased expression of CD27 correlates with the capacity for bovine γδ T cells to proliferate in response to mycobacterial Ags, suggesting that bovine γδ T cell effector/memory phenotypes are consistent with those reported for human γδ T cells (16).

**M. bovis**–specific γδ T cells exhibit a T<sub>CM</sub> phenotype based on CD27 and CD45R expression

Expression of CD45RA and CD27 define four subsets of memory γδ T cells in humans (15). In this study, we sought to determine if the combination of both markers could be used to identify subsets of Ag-specific γδ T cells in cattle (Supplemental Fig. 3). PBMCs from *M. bovis*–infected animals were stimulated in vitro with PPD-b for 6 d. As shown in Fig. 5A, *M. bovis*–specific γδ T cells can be divided in four subsets: naïve (CD27<sup>−</sup>CD45R<sup>−</sup>), T<sub>CM</sub> (CD27<sup>+</sup>CD45R<sup>−</sup>), T<sub>EM</sub> (CD27<sup>−</sup>CD45R<sup>−</sup>), and T<sub>EMRA</sub> (CD27<sup>−</sup>CD45R<sup>+</sup>) cells following in vitro stimulation with PPD-b. CD27<sup>−</sup>CD45R<sup>−</sup> T<sub>CM</sub> γδ cells exhibited a change in distribution and in absolute numbers relative to the other subsets following in vitro restimulation with mycobacterial Ags, followed by the CD27<sup>−</sup>CD45R<sup>−</sup> T<sub>EM</sub> γδ cell subset (Fig. 5B, 5C). Previous reports have shown that Ag-specific bovine CD4 T cells can also be divided by their expression of CD27 and CD45R (25). To confirm these previous results, and to validate our results of our γδ T cell analysis, we examined parallel cultures to determine the distribution of effector/memory CD4 subsets responding to *M. bovis* infection. As seen in Fig. 5D and 5E, after 6 d, the CD4 T<sub>CM</sub> and T<sub>EM</sub> subset exhibited the highest change in distribution and in absolute numbers relative to the other subsets following in vitro restimulation with mycobacterial Ags compared with nonresponding CD4 T cells. Our results suggest that the T<sub>CM</sub> subset comprises the majority of the in vitro proliferative response to *Mycobacterium* Ags for γδ T cells.

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

T<sub>CM</sub> cells are highly proliferative, abundant in lymph nodes, and retain their expression of the homing receptors CCR7 and CD62L (33). The results described in Fig. 5 suggest that peripheral *M. bovis*–specific γδ T cells share functional similarities (i.e., high proliferative capacity) with central memory T cells (16). To address

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this hypothesis, we evaluated the expression of CD62L on peripheral M. bovis–specific γδ T cells. As seen in Fig. 6A, following a 6-d stimulation with PPD-b, CD27+ M. bovis–responsive γδ T cells coexpressed CD62L compared with γδ T cells that did not proliferate to M. bovis Ags. In contrast, M. bovis–specific CD27+ CD4 T cells were low for CD62L expression compared with non–M. bovis–specific CD4 cells. These data are consistent with previous descriptions identifying the majority of M. bovis–specific CD4 T cells in the peripheral blood as TEM cells (Fig. 6A, 6D, 6E) (25). Therefore, similar to human γδ T cells, M. bovis–responsive bovine γδ T cells display phenotypic and functional properties of TCM cells (Fig. 6B, 6C) (25, 33).

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

Chemokine receptor expression is modulated during differentiation of both CD4 and γδ T cells (15, 33, 34). We hypothesized that Ag-responsive γδ T cells would modify the expression of certain homing receptors that would allow cells to migrate to tissues and sites of inflammation. PBMCs from infected cattle were stimulated with PPD-b, as described above. As seen in Fig. 7A and 7B, M. bovis–responsive bovine γδ T cells express higher surface levels of both CCR5 (RANTES/macrophage inflammatory protein-1α/1β receptor) and CXCR3 compared with nonresponding γδ T cells. As seen in Fig. 7C, Ag-specific CD4 T cells also express higher levels of CCR5 and CXCR3 compared with nonresponsive CD4 T cells. The increased expression of CCR5 and CXCR3 on M. bovis–responsive CD4 and γδ T cells is expected to enable these populations to migrate into the inflamed tissue.

**DISCUSSION**

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

In humans, two subsets of γδ T cells develop in response to Ag stimulation and are identified based upon their expression of CD27 (22). These subsets exhibit unique functions during mycobacterial infection that parallel the functions attributed to differentiated subsets of CD8 and CD4 T cells (15, 29). Moreover, flow cytometric analysis of CD27 expression on circulating MTB–specific T cells can help to discriminate disease progression (30). Thus, assessing the changes in γδ T cell effector/memory phenotype based on CD27 might be helpful to monitor changes related to TB disease progress and vaccine-induced protection, as previously reported (16, 18). Although the expression of CD27 in bovine cells has been reported before, its expression on bovine γδ and CD4 T cells has not been determined (31). We observed that, in healthy adult cattle, ~70% of CD4 T cells and ~50% of γδ T cells express CD27. Importantly, we observed that a subpopulation of γδ CD27+ effector/memory-like T cells from M. bovis–infected cattle develop in response to Ag stimulation and exhibit high proliferative capacity after restimulation with either PPD-b or the M. bovis protein Ag, ESAT-6/CFP-10. Our observations are consistent with findings in humans, in which patients with acute pulmonary TB, and bacillus Calmette–Guérin-vaccinated individuals, demonstrate increases in circulating CD27+γδVγVδ2+ T cells, and these cells exhibit enhanced proliferative activity upon stimulation with mycobacterial Ags (16, 37). Thus, our results suggest that CD27 expression may be potentially used to monitor γδ T cell responses to M. bovis infection in cattle.

Coexpression of cell surface markers CD45RA and CD27 has been used to functionally segregate subsets of human and nonhuman primate γδ T cells (15, 16). In nonhuman primates, TEM (CD45RA–CD27+) VγVδ2 T cell expansion after a secondary M. tuberculosis challenge correlates with protection against fatal TB (17–19). Interestingly, there is a significant reduction in effector γδ cells (CD27+ TEM and TEMRA cells) in patients with active infection, but the population rebounds after successful clinical treatment (38). Our data show that M. bovis–specific γδ T cells can
be divided into four distinct subsets: naive (CD27+CD45R+), TCM (CD27+CD45R2), TEM (CD272CD45R2), and TEMRA (CD272CD45R+) cells. Consistent with reports from humans, our data show that virulent M. bovis infection is associated with an expansion in primarily gd TCM subset in the blood (Fig. 5) (39). Although effector functions were not assessed in this study, it has been shown that gd Teffectors develop functional defects and progressively lose the ability to produce IFN-γ compared with gd T cells from healthy, tuberculin-positive adults (40). Whether the loss of gd T effector cells impacts the ability to acquire memory T cell properties is not known. Together, our observations, coupled with reports from human TB patients, demonstrate a negative correlation between gd Teffector (CD272CD45R2) phenotype and disease. Further studies should address the functional implications of these phenotypic changes following M. bovis experimental challenge and vaccine efficacy studies in the bovine.

In conclusion, our results show that, like humans, gd T cells from M. bovis–infected cattle can be divided into phenotypic and functional subsets based upon their surface expression of CD27,
CD45R, and the homing molecules CXCR3 and CCR5. Further elucidation of the pathway of γδ T cell differentiation and the functional implications will contribute significantly to our ability to engage the γδ T cell response in effective immune responses to mycobacterial diseases of both humans and animals.

DISCLOSURES

The authors have no financial conflicts of interest.

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

M. Guerra-Maupome*, M. V. Palmer †, W. R. Waters† J. L. McGill*

*Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA, USA, †Infectious bacterial Diseases Research Unit, National Animal Disease Center, Agricultural Research Service, USDA, Ames, IA, USA.

Corresponding author: Jodi L. McGill

Email: jlmcgill@iastate.edu

Phone number: 515-294-2635
Supplemental Fig. 1. Gating strategy to identify proliferating γδ T cells from *M. bovis*-infected and non-infected cattle. PBMCs from uninfected (n= 6) or virulent *M. bovis*-infected animals (n= 10) were labeled with CellTrace, and 5 × 10^6 cells/ml were cultured for 6 days in the presence or absence of 200 UI/ml PPD-b or 2 µg/ml ESAT-6/CFP-10. Cells were labeled with anti-bovine γδ TCR or CD4 and analyzed by flow cytometry for CellTrace dilution. Arrows depict the gating strategy to identify dividing cells. Cells were gated on single cells (doublet exclusion), lymphocytes (FSC-A vs. SSC-A), CD4 or the γδ TCR, then cells which diluted CellTrace violet. Background proliferation (cRPMI stimulated cells) was subtracted, and results were depicted as change over mock.
Supplemental Figure 2. Gating strategy to identify CD27 cell surface expression. Approximately 8 weeks after virulent *M. bovis* infection, PBMCs (5×10⁶ cells/ml) were labeled with CellTrace violet and restimulated *in vitro* for 6 days in the presence or absence of PPD-b, ESAT-6/CFP-10 or left unstimulated. Cells were then labeled with anti-bovine CD4 or γδ TCR, and CD27. Depicted is the gating hierarchy for CellTrace dilution (proliferative responses) and CD27 cell surface expression on γδ T cells and CD4 T cells.
Supplemental Figure 3. Gating strategy for defining *M. bovis*-responsive and nonresponsive TEM and TCM subsets. PBMCs were isolated from calves ~ 8 weeks after challenge with virulent *M. bovis*. Cells were stained with CellTrace dye and incubated with *M. bovis* PPD-b for 6 days. Gating strategy is depicted for a representative animal. Live CD4 and γδ T cells were gated based on response to *M. bovis* (i.e., proliferating cells) and analyzed for CD27 versus CD45R expression within CellTrace bright (i.e., non-proliferative fraction; black bars) or CellTrace dim (i.e., proliferative fraction; grey bars) fractions.