Analysis of Immune Responses Directed toward a Recombinant Early Secretory Antigenic Target Six-Kilodalton Protein–Culture Filtrate Protein 10 Fusion Protein in *Mycobacterium bovis*-Infected Cattle

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Cell-mediated immune responses are critical for protective immunity to mycobacterial infections. Recent progress in defining mycobacterial antigens has determined that region of difference 1 (RD1) gene products induce strong T-cell responses, particularly the early secretory antigenic target 6-kDa (ESAT-6) protein and culture filtrate protein 10 (CFP10). However, comprehensive analysis of the immune response towards these antigens is incompletely characterized. To evaluate recall responses to ESAT-6 and CFP10, peripheral blood mononuclear cells from *M. bovis*-infected cattle were stimulated in vitro with a recombinant ESAT-6 (rESAT-6)–CFP10 fusion protein and compared to responses induced by *M. bovis*-derived purified protein derivative. Following antigenic stimulation, activation marker expression was evaluated. Significant proliferative responses ($P < 0.05$) were evident in CD4⁺, CD8⁺, immunoglobulin M-positive, and CD172a⁺ cell fractions after 6 days of culture. Expression of CD25 and CD26 was increased ($P < 0.05$) on CD4⁺, CD8⁺, and $\gamma\delta$ T-cell-receptor-positive cells. CD4⁺ and CD8⁺ cells also exhibited significant changes ($P < 0.05$) in expression of CD45 isoforms. Using a flow cytometry-based proliferation assay, it was determined that CD45R expression is downregulated ($P < 0.05$) and that CD45RO expression is upregulated ($P < 0.05$) on proliferating (i.e., activated) CD4⁺ cells. Collectively, data indicate that recall immune responses directed toward the rESAT-6–CFP10 fusion protein or purified protein derivative are comparable and that recall to mycobacterial antigens correlates with a CD45RO⁺ phenotype.

Comparative genomic analysis between *Mycobacterium bovis* bacille Calmette-Guerin (BCG) and *M. tuberculosis* has revealed that several deletions exist in the vaccine strain (4, 28). The cause for the attenuation of BCG was mapped to region of difference 1 (RD1) (21, 27, 34). RD1 is present in all virulent members of the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*) (4, 19). Given the reported genetic variability within strains of BCG, it is interesting that RD1 is the only region absent in all strains of BCG (4, 19). This region encodes several proteins that are expressed early during infection, including the early secretory antigenic 6-kDa (ESAT-6) protein and culture filtrate protein 10 (CFP10) (6). In vivo, ESAT-6 and CFP10 associate as a heterodimer in a tight 1:1 complex (36). Although no direct function has been attributed to these proteins, their expression correlates with an increased cytolytic ability of *M. tuberculosis* (21). These proteins are recognized by human, murine, and bovine T cells (9, 32), which respond by proliferating and secreting gamma interferon (IFN- γ) (1, 32, 39). Due to its relatively early expres-

sion and antigenicity, ESAT-6 has been used as a candidate subunit vaccine against tuberculosis. ESAT-6 DNA vaccination of rodents and cattle induces protection against challenge with tubercle bacilli (23, 29). The reintroduction of RD1 into BCG improves the efficacy of BCG as a vaccine (35). Mice and guinea pigs vaccinated with recombinant RD1-expressing BCG possess reduced bacterial burdens following challenge with *M. tuberculosis* (35). Thus, ESAT-6 and other proteins of RD1, alone or in combination with BCG, show promise in establishing new vaccine regimens against tuberculosis. However, further evaluation of the immune response directed toward RD1 proteins is needed.

Cell-mediated immune responses are critical in controlling mycobacterial infection. Protective roles have been attributed to CD4⁺, CD8⁺, and $\gamma\delta$ T-cell-receptor-positive (TCR⁺) T cells (8, 17). Mice possessing defective CD4⁺- and CD8⁺-T-cell responses are quite susceptible to infection with *M. tuberculosis* (3, 13, 17, 41). Both subsets contribute to IFN- γ production required for macrophage activation (17). IFN- γ is an indispensable component of the antimycobacterial response (14, 18). In the mouse, 1 week following infection with *M. tuberculosis*, the number of activated CD4⁺ and CD8⁺ T cells increases in the lung-associated lymph nodes (16, 17, 38). Following activation by mycobacteria, T cells upregulate mole-

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cules involved in homing to sites of infection while conversely decreasing the expression of molecules that restrict trafficking (16, 17, 38). Murine CD4⁺ cells homing to sites of *M. tuberculosis* infection possess an activated phenotype defined by the increased expression of CD25, CD44, and CD69 and decreased expression of CD62L (2). In response to purified protein derivative (PPD), T cells from *M. bovis*-infected cattle also up-regulate CD25 and CD44 while decreasing expression of CD62L (46). Subsequent to the increase of activated T cells in the lymph nodes, these T-cell populations migrate to the lung and display an effector/memory phenotype (CD44^{hi} CD45^{lo} CD62L⁻) (17). Long-term immunity in the mouse is likely mediated by memory CD4⁺ cells that express a naïve phenotype until restimulated by antigen (2). Human memory CD4⁺ T cells specific for tuberculin have been shown to possess a CD45RO⁺ phenotype (42). Naïve, activated, and memory T-cell subsets are relatively defined in humans and mice; however, few studies have been conducted in cattle, especially with regard to tuberculosis.

Recently, progress in defining mycobacterial antigens has advanced; however, a comprehensive analysis of immune responses directed toward these antigens is lacking. Aims were to further define effector/memory populations in cattle and to compare the activation profiles generated by recombinant ESAT-6 (rESAT-6)-CFP10 and PPD. Analysis of the activation phenotype of lymphocytes from *M. bovis*-infected cattle in response to a defined antigen (rESAT-6-CFP10) or a crude mycobacterial antigen (PPD) was determined using flow cytometry. In the present study, it is demonstrated that several cell subsets proliferate in response to the rESAT-6-CFP10 fusion protein, CD4⁺ T cells expressing CD45RO expand in response to rESAT-6-CFP10, and several additional activation molecules are upregulated following mycobacterium-induced activation.

MATERIALS AND METHODS

Animals, bacterial culture, and challenge procedures. Eight cross-bred cattle approximately 6 months old were selected based on negative reactivity to PPD derived from *M. bovis* using the Bovigam (Biorad Animal Health, Omaha, NE) assay for IFN- γ production. Infected cattle ($n = 4$) were housed in temperature- and humidity-controlled rooms within a biosafety level 3 confinement facility with negative airflow exiting the building through high-efficiency particulate air (HEPA) filters. Directional airflow ensured that air from animal pens was pulled toward a central corridor and passed through HEPA filters before exiting the building. Noninfected control animals ($n = 4$) were housed similarly in a separate building. Personnel in contact with *M. bovis*-infected animals wore full-face HEPA-filtered respirators. All animals were housed at the National Animal Disease Center, Ames, Iowa, according to institutional guidelines for animal care.

M. bovis strain 95-1315 was used for experimental infections. This strain was originally isolated from a white-tailed deer in Michigan in 1994 (37). Challenge inocula consisted of 10⁶ CFU of mid-log-phase *M. bovis* strain 95-1315 cultures grown in Middlebrook 7H9 medium supplemented with 10% oleic acid-albumin-dextrose complex (Difco, Detroit, MI) plus 0.05% Tween 80 (Sigma, St. Louis, MO) as described previously (7). Enumeration of *M. bovis* strain 1315 was done by serial dilution plate counting on Middlebrook 7H11 selective agar plates (Becton Dickinson, Cockeysville, MD). For intratracheal inoculation, cattle ($n = 4$) were sedated by intravenous administration of xylazine (0.025 mg/kg). Challenge inocula were instilled directly into the tonsillar crypts of sedated cattle as described previously for white-tailed deer (31). The effects of sedation were reversed by intravenous administration of tolazoline (4 mg/kg; Lloyd Laboratories, Shenandoah, IA). At the conclusion of the experiment, cattle were euthanized by intravenous administration of sodium pentobarbital (Sleepaway; Fort Dodge Laboratories, Fort Dodge, IA).

TABLE 1. Primary antibodies, isotypes, and specificities^a

MAb	Ig isotype	Specificity
ILA-11A	IgG _{2a}	CD4
CACT138A	IgG ₁	CD4
CACT187A	IgG ₁	CD4
7C2A	IgG _{2a}	CD8 α
CACT80C	IgG ₁	CD8 α
BAT82A	IgG ₁	CD8 β
CACT116A	IgG ₁	CD25
CACT114A	IgG _{2b}	CD26
GS5A	IgG ₁	CD45R
GC44A1	IgG ₃	CD45RO
DH59B	IgG ₁	CD172a
GB21A	IgG _{2b}	TCR1 δ chain
CACT61A	IgM	TCR1 δ chain
CACT148A	IgM	TCR1 δ chain
BAQ44A	IgM	B cell
CACT200A	IgG ₁	ACT1
CACT26A	IgG ₁	ACT2
LCTB6A	IgG ₁	ACT9
GB110A	IgM	ACT16
CACT195A	IgM	ACT27
CACT216A	IgM	ACT28
CACT225A	IgG ₁	ACT29
CACT185A	IgG ₁	ACT30
CACT152A	IgM	ACT31
CACT191A	IgM	ACT32

^a MAb, monoclonal antibody.

Cloning and expression of rESAT-6-CFP-10 fusion protein. The construction of pISM2202 expressing the ESAT-6-CFP10 fusion protein has been described previously (45). Purified recombinant protein was obtained by metal chelate chromatography as described previously (45), dialyzed overnight at 4°C in phosphate-buffered saline, and quantified by the Bradford assay.

Cell culture. At 12 months postinfection, peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat fractions of peripheral blood collected in 2 $\times$ acid citrate dextrose (12). Wells of 96-well round-bottom microtiter plates (Falcon; Becton-Dickinson, Lincoln Park, NJ) were seeded with 5×10^5 PBMCs in a total volume of 200 μ l per well. Medium used was RPMI 1640 supplemented with 2 mM L-glutamine, 25 mM HEPES buffer, 100 U/ml penicillin, 100 μ g/ml streptomycin, 1% nonessential amino acids (Sigma), 2% essential amino acids (Sigma), 1% sodium pyruvate (Sigma), 50 μ M 2-mercaptoethanol (Sigma), and 10% (vol/vol) fetal bovine serum. Wells contained medium plus rESAT-6-CFP10 (10 μ g/ml), *M. bovis* PPD (5 μ g/ml), or medium alone. Cells were cultured in vitro for 3 or 6 days at 37°C in 5% CO₂.

PKH67 proliferation assay. For four-color flow cytometric analysis, PBMCs were stained with PKH67 prior to cell culture in accordance with the manufacturer's instructions (Sigma) and as described previously (44, 46). Briefly, 2×10^7 PBMCs were centrifuged (10 min, 400 $\times$ g), supernatants were removed, and cells were resuspended in 1 ml of diluent provided in the PKH67 kit. Diluted cells were added to 1 ml of PKH67 green fluorescent dye (2 μ M; Sigma) and incubated for 5 min, followed by a 1-min incubation with 2 ml of fetal bovine serum to absorb excess dye. Cells were then washed (10 min, 400 $\times$ g) three times with RPMI 1640. Cells were counted and plated out at a density of 5×10^5 cells per well. PKH67 proliferation was analyzed using commercially available software (Modfit Proliferation Wizard; Verity Software House Inc., Topsham, ME). Proliferation data are presented as the mean ($\pm$ standard error of the mean [SEM]) numbers of cells that had proliferated per 10,000 PBMCs minus no-stimulation (medium alone) values.

Flow cytometry. Following the appropriate culture duration, cells were pooled from individual animals according to in vitro treatments (i.e., stimulation). Cells were then replated to ensure equal cell numbers. Cells were stained with primary antibodies (Table 1) at room temperature for 15 min. All primary antibodies were provided by the Washington State University Monoclonal Antibody Center VMRD (Pullman, WA). Antibodies were used at a concentration of 1 μ g/10⁶ cells. Primary antibodies have been described previously (25). Following incubation, cells were washed and stained with appropriate goat anti-mouse fluorescein isothiocyanate-, phycoerythrin-, allophycocyanin-, Cy5-, or peridinin chlorophyll protein-conjugated secondary antibodies at room temperature for 15

FIG. 1. Proliferation of PBMCs from *M. bovis*-infected cattle in response to rESAT-6-CFP10 and *M. bovis* PPD. Representative figures indicate PBMC gating strategy (A, B, C), and PKH67 histograms indicate proliferative responses of PBMCs from *M. bovis*-infected cattle in response to medium alone (D), rESAT-6-CFP10 fusion protein (10 μ g/ml) (E), or *M. bovis* PPD (5 μ g/ml) (F) following 6 days of culture. As cells divide, PKH67 fluorescence diminishes, resulting in subsequent daughter generations with similar intensities. Note that stimulation with mycobacterial antigen results in the formation of numerous daughter peaks. Analysis was performed with Modfit Proliferation Wizard (Verity Software House).

min. Three- and four-color flow cytometric analyses were performed with FAC-Scan and BD LSR flow cytometers (Becton Dickinson), respectively. Data were analyzed using commercially available software (FlowJo [Tree Star Inc., San Carlos, CA] and CellQuest [Becton Dickinson]). Data are presented as the mean ($\pm$ SEM) percentages of cells expressing a given marker minus control values (medium alone) or as percent expression.

Statistical analysis. Data were analyzed using commercially available software (InStat 2.00; GraphPAD Software, San Diego, CA). Pairwise comparisons between *M. bovis*-infected and control animals were performed using Student's *t* test. Statistical differences were considered significant at a *P* value of <0.05 .

RESULTS

PBMCs from *M. bovis*-infected cattle exhibit robust proliferative responses when stimulated with rESAT-6-CFP10 fusion protein. To evaluate recall responses of specific cell sub-

sets to rESAT-6-CFP10 from *M. bovis*-infected animals, cells were stained with the membrane-labeling dye PKH67 (Fig. 1.). The proliferation of total PBMCs from infected cattle in response to rESAT-6-CFP10 or *M. bovis* PPD exceeded that of control cultures ($P < 0.01$) after 6 days of culture (Table 2). Differences in proliferation between infected and control animals were not significant at 3 days after culture (data not shown). Previous studies using *M. bovis*-infected cattle have demonstrated that CD4⁺ and $\gamma\delta$ TCR⁺ cells are the predominant cell subsets responding to PPD stimulation (46). Stimulation with rESAT-6-CFP10 induced significant ($P < 0.05$) expansion of CD4⁺, CD8⁺, immunoglobulin M-positive (IgM⁺), and CD172a⁺ cell subsets in cultures from infected animals (Table 2). *Mycobacterium bovis* PPD stimulation resulted in a similar proliferation profile with the exception of greater CD4⁺ cell proliferation compared to that of rESAT-6-CFP10 stimulation. CD4⁺ cells from *M. bovis*-infected cattle stimulated with PPD proliferated at nearly fourfold greater levels than did CD4⁺ cells stimulated with rESAT-6-CFP10. $\gamma\delta$ TCR⁺ cells did not display significant ($0.05 < P < 0.1$) proliferative responses compared to control animals in this experiment due to variation within group responses and a low level of nonspecific proliferation by control animals; however, ESAT-6 is a major antigenic target of WC1⁺ $\gamma\delta$ TCR⁺ cells that induces their proliferation and secretion of IFN- γ (47).

CD4⁺ and CD8⁺ cells from *M. bovis*-infected cattle display an activated phenotype in response to rESAT-6-CFP10 stimulation. To determine if upregulation of CD25 expression correlated with an increase in proliferative responses, PBMCs were stained for the interleukin-2R (IL-2R) α chain (CD25). After 6 days in culture, PBMCs from infected animals had increased percentages of cells expressing CD25 after stimulation with either PPD or rESAT-6-CFP10. Notably, CD4⁺, CD8⁺, and $\gamma\delta$ TCR⁺ cell subsets all exhibited significant increases in CD25 expression compared to that of noninfected control cultures (Fig. 2A, B, and C). Upregulation of CD25 expression was also detected on CD4⁺ and $\gamma\delta$ TCR⁺ cells after 3 days of in vitro culture with rESAT-6-CFP10 or *M. bovis* PPD (data not shown). Additionally, there was a notable increase in the number of cells expressing CD26. The cellular upregulation of CD26, an ectoenzyme, has been associated with an activated phenotype in cattle (26). CD4⁺, CD8⁺, and $\gamma\delta$ TCR⁺ cells from *M. bovis*-infected cattle, but not from noninfected animals, upregulated their expression of CD26 at 6 days of culture with mycobacterial antigens (Fig. 2D, E, and

TABLE 2. Proliferation of cell subsets in response to defined (rESAT-6-CFP10) or complex (*M. bovis* PPD) mycobacterial antigens^a

Stimulation	Group (<i>n</i> = 4)	Mean cell no. ($\pm$ SEM) by cell subset					
		Ungated	CD4 ⁺	CD8 ⁺	$\gamma\delta$ TCR ⁺	IgM ⁺	CD172a ⁺
rESAT-6-CFP10	Infected	3,668 (544)**	333 (98)*	427 (85)*	853 (247) ^b	957 (207)**	1,081 (357)*
rESAT-6-CFP10	Control	503 (196)	84 (33)	74 (48)	247 (134)	58 (35)	40 (19)
<i>M. bovis</i> PPD	Infected	4,415 (780)**	1,224 (408)*	361 (101)*	699 (115)	1,051 (160)**	1,424 (659) ^b
<i>M. bovis</i> PPD	Control	613 (350)	91 (43)	51 (30)	325 (172)	173 (70)	59 (40)

^a PBMCs from *M. bovis*-infected cattle (*n* = 4) or control cattle (*n* = 4) were stained with PKH67; cultured with medium alone, rESAT-6-CFP10 (10 μ g/ml), or *M. bovis* PPD (5 μ g/ml) for 6 days; stained for cell surface markers for flow cytometry; and analyzed for proliferation. Data represent the mean ($\pm$ SEM) numbers of cells that had proliferated per 10,000 PBMCs minus values from nonstimulated (medium alone) cultures. A single asterisk indicates a significant difference ($P < 0.05$) from *M. bovis*-infected animals compared to noninfected animals. A double asterisk indicates a significant difference ($P < 0.01$) from *M. bovis*-infected animals compared to noninfected animals.

^b Difference ($P < 0.1$) from *M. bovis*-infected animals compared to noninfected animals.

FIG. 2. Increased expression of CD25 and CD26 on PBMCs from *M. bovis*-infected cattle in response to stimulation with rESAT-6-CFP10 or *M. bovis* PPD. Mean percentages of CD4⁺ (A, D), CD8⁺ (B, E), and γδ TCR⁺ (C, F) cells expressing CD25 and CD26 are shown. PBMCs from *M. bovis*-infected ($n = 4$) or noninfected ($n = 4$) animals were stimulated with either medium alone, rESAT-6-CFP10 (10 μg/ml), or *M. bovis* PPD (5 μg/ml) for 6 days. After cell culture, cells were stained for surface and activation markers and analyzed using flow cytometry. Data are presented as mean ($\pm$ SEM) percentages of cells expressing a given activation marker in response to antigen minus values from medium alone. A single asterisk indicates a significant difference ($P < 0.05$) from *M. bovis*-infected animals compared to noninfected animals. A double asterisk indicates a significant difference ($P < 0.01$) from *M. bovis*-infected animals compared to noninfected animals.

F). After 3 days of in vitro culture with rESAT-6-CFP10 or *M. bovis* PPD, CD26 expression was increased ($P < 0.05$) on CD4⁺ and γδ TCR⁺ cells from *M. bovis*-infected cattle (data not shown).

Nine additional activation molecules (ACT) were evaluated to further characterize the activation phenotype of PBMCs from *M. bovis*-infected cattle. These molecules are expressed on activated bovine lymphocytes (25). Stimulation with *M. bovis* PPD resulted in increased expression of all ACT molecules except for ACT29. When compared to control animals, greater percentages of CD4⁺ cells from *M. bovis*-infected cattle expressed ACT molecules ($P < 0.01$) following stimulation with PPD (Fig. 3A). rESAT-6-CFP10 induced a similar expression pattern; however, stimulation with this antigen did not result in significant increases in the expression of ACT1 or ACT16. Expression of other ACT molecules by PBMCs of infected animals restimulated with rESAT-6-CFP10 exceeded ($P < 0.05$) those of their control counterparts (Fig. 3A).

CD4⁺ cells from *M. bovis*-infected cattle cultured with PPD increase their expression of CD45RO and downregulate expression of CD45R upon restimulation in vitro. To examine if rESAT-6-CFP10 or *M. bovis* PPD induces the expansion of effector/memory T-cell populations, we analyzed the expression of CD45RO and CD45R on CD4⁺ and CD8⁺ cells. After 6 days of culture, PBMCs from infected animals treated with

medium alone possessed the highest and lowest mean percentages of CD4⁺ cells expressing CD45R and CD45RO, respectively (Fig. 4A and B). Stimulation with *M. bovis* PPD resulted in a significant expansion of a CD4⁺ CD45RO⁺ population compared to cultures with no antigen (Fig. 4B). The expression of CD45R followed an inverse trend and was found to significantly ($P < 0.05$) contract compared to control culture (Fig. 4A). Stimulation with rESAT-6-CFP10 resulted in a CD4⁺ CD45RO⁺ phenotype intermediate to that of PPD and control cultures (Fig. 4B). In contrast to CD4⁺ cells, CD8⁺ cells decreased ($P < 0.05$) expression of CD45R (Fig. 4C) and increased ($P < 0.05$) expression of CD45RO (Fig. 4D) in response to rESAT-6-CFP10 stimulation. Changes in expression of either CD45R or CD45RO were not detected in cultures from noninfected control animals (data not shown). Likewise, differences were not evident following 3 days of culture of CD4⁺ cells from infected or noninfected animals (data not shown).

CD4⁺ CD45RO⁺ cells from experimentally infected cattle possess an activated phenotype following antigenic stimulation. To determine if effector/memory cells (i.e., CD45RO⁺) that expanded following stimulation with rESAT-6-CFP10 or *M. bovis* PPD displayed an activated phenotype, CD4⁺ CD45RO⁺ cells were stained for activation molecules. Six days of culture with PPD increased expression of ACT1, ACT16,

FIG. 3. Expression of ACT molecules on CD4⁺ (A) or CD4⁺ CD45RO⁺ (B) subsets. PBMCs from *M. bovis*-infected ($n = 4$) or noninfected ($n = 4$) animals were stimulated with either medium alone, rESAT-6-CFP10 (10 μ g/ml), or *M. bovis* PPD (5 μ g/ml) for 6 days. After cell culture, cells were stained for CD4, CD45RO, and activation markers and then analyzed using flow cytometry. Data are presented as mean ($\pm$ SEM) percentages of cells expressing a given activation marker in response to antigen minus values from medium alone. A single asterisk indicates a significant difference ($P < 0.05$) from *M. bovis*-infected animals compared to noninfected animals for the identical stimulation. A double asterisk indicates a significant difference ($P < 0.01$) from *M. bovis*-infected animals compared to noninfected animals for the identical antigenic stimulation.

ACT30, ACT31, and ACT32 ($P < 0.05$) on CD4⁺ CD45RO⁺ cells from *M. bovis*-infected cattle compared to noninfected control cattle (Fig. 3B). Similarly, stimulation with rESAT-6-CFP10 induced a similar activation phenotype. Culture with rESAT-6-CFP10 increased the expression of ACT16, ACT30, and ACT31 ($P < 0.05$) on CD4⁺ CD45RO⁺ cells from *M. bovis*-infected cattle compared to their noninfected counterparts (Fig. 3B). Differences between groups and treatments were not apparent after 3 days of culture (data not shown). The expression of CD26 on CD4⁺ CD45RO⁺ and CD8⁺ CD45RO⁺ cells was not detected in this study (data not shown).

CD4⁺ cells proliferating in response to mycobacterial antigens exhibit increased expression of CD45RO and decreased expression of CD45R. By utilizing PKH67 staining, analysis of CD45 isoforms was conducted to evaluate changes in expression on proliferating and nonproliferating cell populations. Cells actively proliferating display decreased PKH67 fluorescence (i.e., PKH67^{lo}), whereas nonproliferative cells remain highly fluorescent (i.e., PKH67^{hi}). Total lymphocytes from *M. bovis*-infected cattle displayed increased ($P < 0.01$) expression of CD45RO in PKH67^{lo} fractions compared to nonproliferative fractions after 6 days of culture with either the rESAT-6-

CFP10 fusion protein or *M. bovis* PPD (Fig. 5A). CD4-gated cells in the proliferative cell populations of infected animals exhibited decreased ($P < 0.05$) expression of CD45R after 6 days of restimulation with mycobacterial antigens (Fig. 5B). Conversely, CD45RO was increased ($P < 0.01$) on CD4-gated proliferating cells compared to noncycling cells (Fig. 5C). A similar trend was observed with regard to CD8⁺ cells. CD8⁺ cells within the cycling population exhibited significant reductions ($P < 0.01$) in CD45R expression (Fig. 5D); however, they did not change expression of CD45RO between PKH67 fractions.

Increased expression of CD45RO correlates to CD4⁺ and CD8⁺ proliferative recall responses to mycobacterial antigens. To evaluate recall responses by CD45RO⁺ cell subsets to the rESAT-6-CFP10 fusion protein or *M. bovis* PPD, cells were stained with PKH67, CD4, CD8, and CD45RO. Proliferative responses by total CD45RO⁺ PBMCs from infected cattle in response to rESAT-6-CFP10 or *M. bovis* PPD exceeded that of control cultures ($P < 0.01$) after 6 days (Table 3). CD4⁺ CD45RO⁺ cells also significantly expanded ($P < 0.05$) following antigenic restimulation (Table 3). Although CD8⁺ cells did not increase their expression of CD45RO (Fig. 5E) between proliferating and nonproliferating cell populations, the subset

FIG. 4. Expression of CD45R (A, C) and CD45RO (B, D) on CD4⁺ and CD8⁺ cell populations. PBMCs from *M. bovis*-infected cattle ($n = 4$) were stimulated with either medium alone (NS), rESAT-6-CFP10 (10 μ g/ml), or *M. bovis* PPD (5 μ g/ml) for 6 days. Following cell culture, cells were stained for CD4, CD8, CD45R, and CD45RO. Flow cytometric analysis was utilized to determine the percentage of each cell subset expressing CD45 molecules. Data are presented as mean ($\pm$ SEM) percentages of cells expressing CD45R or CD45RO. A single asterisk indicates a significant difference ($P < 0.05$) from antigenic stimulation compared to no stimulation (medium alone). A double asterisk indicates a significant difference ($P < 0.01$) from antigenic stimulation compared to no stimulation (medium alone).

of CD8⁺ cells expressing CD45RO from *M. bovis*-infected animals did significantly ($P < 0.01$) expand in culture after 6 days (Table 3). Thus, it appears that expression of CD45RO correlates with recall responses to mycobacterial antigens.

DISCUSSION

A key feature of an immune response is the development of T-cell memory. Following activation by foreign antigen, a population of antigen-specific T cells expands. Subsequent contraction of the ensuing effector response results in the formation of a stable pool of memory T cells characterized by rapid kinetics following secondary antigen encounter (15). Relatively clear descriptions of memory phenotypes have been identified for mouse and human (15). However, few studies of effector or memory T-cell development in cattle have been performed. In the present study, we analyzed recall responses of *M. bovis*-infected cattle to a defined or complex antigen using flow cytometric analysis.

Several cell types exhibited robust proliferative responses following stimulation with mycobacterial antigens. CD4⁺, CD8⁺, $\gamma\delta$ TCR⁺, IgM⁺, and CD172a⁺ cell subsets in cultures from infected animals expanded severalfold following exposure to antigen. Expansion of IgM⁺ and CD172a⁺ cell subsets to rESAT-6-CFP10 has not been reported in cattle to date. The expansion of a CD172a⁺ population is particularly interesting. CD172a (MyD1, SIRP α) is a member of a designated group of signal-regulatory proteins (10, 24). CD172a expression is restricted to cells of myeloid lineage, specifically, monocytes, macrophages, and granulocytes (10). Monoclonal antibody-mediated blockade of CD172a inhibits proliferation of bovine CD4⁺ T cells in response to antigen, thus suggesting a role in costimulating T-cell responses (10). CD172a signaling

also modulates cells of the innate immune system (40). Cross-linking of CD172a inhibits the secretion of tumor necrosis factor alpha, but not the expression of tumor necrosis factor alpha mRNA, by human monocytes in response to stimulation with lipopolysaccharide, zymosan, or PPD (40). Although no functional analyses were conducted in this experiment, additional studies will need to be conducted to elucidate the precise role of CD172a-bearing cells in bovine tuberculosis.

CD4⁺, CD8⁺, and $\gamma\delta$ TCR⁺ cells displayed increased expression of CD25 and CD26 following stimulation with either the rESAT-6-CFP10 fusion protein or *M. bovis* PPD. The expression of CD25 correlates with an activated bovine T-cell population (46), as IL-2 is needed to drive a proliferative response. Membrane-expressed CD26 functions as a costimulatory signal in T cells (30). Ligation of CD26 increases the recruitment of CD26 to lipid rafts and facilitates association with CD45RO, thus enhancing the phosphorylation of downstream signaling molecules and enhancing IL-2 production (22). Differences in the expression of CD26 on CD4⁺ CD45RO⁺ or CD8⁺ CD45RO⁺ cells were not detected from proliferative cell populations in this study. An explanation for this observation is that CD26 may be internalized to lipid rafts following ligation, where it binds the cytoplasmic region of CD45RO (22). Additionally, CD4⁺ and CD4⁺ CD45RO⁺ cells upregulated expression of several ACT molecules. To date, these ACT molecules remain incompletely characterized, and data presented here will be beneficial as their identities become characterized (e.g., CD26 was formerly ACT3) (26). Regardless, CD4⁺, CD8⁺, and $\gamma\delta$ TCR⁺ cells from *M. bovis*-infected cattle exhibit an activated phenotype when restimulated with either an rESAT-6-CFP10 fusion protein or *M. bovis* PPD.

FIG. 5. Activated CD4⁺ cells display an effector/memory phenotype. PKH67-stained PBMCs from *M. bovis*-infected cattle ($n = 4$) were stimulated with either medium alone, rESAT-6-CFP10 (10 $\mu\text{g/ml}$), or *M. bovis* PPD (5 $\mu\text{g/ml}$) for 6 days. Following restimulation, PBMCs were harvested and stained for subset markers (CD4 and CD8) and CD45 (CD45R and CD45RO) for flow cytometry. Gating was set on PKH67^{lo} (proliferating) and PKH67^{hi} (nonproliferating) fractions. CD45RO within the PKH67 gates was analyzed on total PBMCs (A), CD4⁺ cells (C), and CD8⁺ cells (E). CD45R was similarly analyzed on CD4⁺ cells (B) and CD8⁺ cells (D). Data are presented as mean ($\pm$ SEM) percentages of cells expressing a given CD45 isoform in response to antigen minus values from medium alone. A single asterisk indicates a significant difference ($P < 0.05$) from antigen-stimulated PKH67^{lo} cell populations compared to antigen-stimulated PKH67^{hi} cell populations. A double asterisk indicates a significant difference ($P < 0.01$) from antigen-stimulated PKH67^{lo} cell populations compared to antigen-stimulated PKH67^{hi} cell populations.

CD45R is a marker for naïve lymphocytes in cattle (20), and bovine CD45RA and CD45RB isoforms are not yet defined. CD45RO expression on bovine CD4⁺ cells is indicative of antigenic priming and can be used to identify effector/memory cell populations (5). Following activation, CD4⁺ and CD8⁺ T cells from infected animals exhibited decreased expression of CD45R. Decreased expression of CD45R was most evident in CD4⁺ cells restimulated with PPD and in CD8⁺ cells restimulated with rESAT-6-CFP10. CD45RO expression followed an inverse pattern. Following antigenic stimulation, CD45RO expression generally increased on CD4⁺ and CD8⁺ cell subsets. The trends in CD45 isoform expression were more highly pronounced when proliferative cell populations were evaluated. Significant downregulation of CD45R expression was evident on proliferating CD4⁺ and CD8⁺ cells stimulated with either mycobacterial antigen. CD45RO expression was greatly enhanced on proliferating CD4⁺ cells but was unchanged on proliferating CD8⁺ cells. These data corroborate observations presented previously by Bembridge et al., in which proliferative responses of CD4⁺ cells were entirely within the CD45RO⁺ subset (5). Bembridge et al. also reported that CD8⁺ cells downregulate expression of CD45RO after activation (5). Initially, increases in CD45RO expression were observed for CD8⁺ cells; however, following analysis of proliferative (i.e.,

TABLE 3. Expansion of CD45RO⁺ subsets following antigenic restimulation^a

Stimulation	Group ($n = 4$)	Mean cell no. ($\pm$ SEM) by CD45RO ⁺ cell subset		
		Ungated	CD4 ⁺	CD8 ⁺
rESAT-6-CFP10	Infected	3,139 (484)**	484 (142)*	385 (59)**
	Control	560 (277)	101 (34)	26 (10)
<i>M. bovis</i> PPD	Infected	3,740 (512)**	1,426 (494)*	303 (55)**
	Control	257 (155)	49 (30)	12 (9)

^a PBMCs from *M. bovis*-infected cattle ($n = 4$) or control cattle ($n = 4$) were stained with PKH67; cultured with medium alone, rESAT-6-CFP10 (10 $\mu\text{g/ml}$), or *M. bovis* PPD (5 $\mu\text{g/ml}$) for 6 days; stained for CD4, CD8, and CD45RO; and analyzed for proliferation. Data represent the mean ($\pm$ SEM) numbers of cells that had proliferated per 10,000 PBMCs minus values from nonstimulated (medium alone) cultures. A single asterisk indicates a significant difference ($P < 0.05$) from *M. bovis*-infected animals compared to noninfected control animals. A double asterisk indicates a significant difference ($P < 0.01$) from *M. bovis*-infected animals compared to noninfected control animals.

activated) CD8⁺ cells, it was evident that activated CD8⁺ cells did not significantly alter CD45RO expression after restimulation with soluble mycobacterial antigens. As *M. bovis*-infected cattle were used as PBMC donors in this study, one cannot define all responsive CD45RO⁺ populations as memory cells, as they most likely represent an effector/memory subset of cells.

Immune responses from *M. bovis*-infected cattle directed towards rESAT-6-CFP10 fusion protein or *M. bovis* PPD were relatively comparable in most aspects except for the proliferation of CD4⁺ cells. The magnitude of CD4⁺ cell proliferation in response to PPD was approximately four times as large as what was observed for restimulation with rESAT-6-CFP10. A likely explanation for this discrepancy is that PPD is a complex antigen containing multiple antigens capable of stimulating CD4⁺ cells from tuberculous animals. The results presented here may be of importance for future vaccine design involving ESAT-6 and CFP10 in cattle, as functional CD4⁺ cells are an indispensable component of protective immunity against tuberculosis (17). Additionally, the decreased CD4⁺ proliferation may aid in explaining the increased time needed to develop delayed-type hypersensitivity reactions to ESAT-6 skin testing in cattle compared to administration of PPD (33). However, given the comparable level of activation relative to PPD, the data suggest that the rESAT-6-CFP10 fusion protein may be a promising candidate for diagnostic applications, as its use could allow for the differentiation of mycobacterial infections (11, 43, 45).

Findings presented in this study describe bovine recall responses to a defined mycobacterial antigen (rESAT-6-CFP10) and a complex antigen mixture (PPD). In conclusion, data reveal that bovine PBMCs, particularly CD4⁺, CD8⁺, and $\gamma\delta$ TCR⁺ cells, from *M. bovis*-infected cattle become highly activated following restimulation with an rESAT-6-CFP10 fusion protein. Additionally, novel findings describing the expansion of IgM⁺ and CD172a⁺ cell subsets of tuberculous cattle in response to rESAT-6-CFP10 were reported. Increased expression of CD25 and CD26 on CD4⁺, CD8⁺, and $\gamma\delta$ TCR⁺ cells corresponded with increased cellular proliferation. Generally, rESAT-6-CFP10- and PPD-induced immune responses were similar. Finally, data corroborate the finding that CD4⁺

CD45RO⁺ cells represent a subset of effector/memory cells, and we report that CD45RO⁺ cell subsets are responsible for proliferative recall responses to mycobacterial antigen and that effector/memory responses induced by rESAT-6-CFP10 stimulation are comparable to those induced by PPD.

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