Loss of Immunization-Induced Epitope-Specific CD4 T-Cell Response following Anaplasma marginale Infection Requires Presence of the T-Cell Epitope on the Pathogen and Is Not Associated with an Increase in Lymphocytes Expressing Known Regulatory Cell Phenotypes

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We have shown that in cattle previously immunized with outer membrane proteins, infection with Anaplasma marginale induces a functionally exhausted CD4 T-cell response to the A. marginale immunogen. Furthermore, T-cell responses following infection in nonimmunized cattle had a delayed onset and were sporadic and transient during persistent infection. The induction of an exhausted T-cell response following infection presumably facilitates pathogen persistence. In the current study, we hypothesized that the loss of epitope-specific T-cell responses requires the presence of the immunizing epitope on the pathogen, and T-cell dysfunction correlates with the appearance of regulatory T cells. In limited studies in cattle, regulatory T cells have been shown to belong to γδ T-cell subsets rather than be CD4 T cells expressing forkhead box protein P3 (FoxP3). Cattle expressing the DRB3*1101 haplotype were immunized with a truncated A. marginale major surface protein (MSP) 1a that contains a DRB3*1101-restricted CD4 T-cell epitope, F2-5B. Cattle either remained unchallenged or were challenged with A. marginale bacteria that express the epitope or with A. marginale subs. centrale that do not. Peripheral blood and spleen mononuclear cells were monitored for MSP1a epitope F2-5B-specific T-cell proliferative responses and were stained for γδ T-cell subsets or CD4⁺CD25⁺ FoxP3⁺ T cells before and during infection. As hypothesized, the induction of T-cell exhaustion occurred only following infection with A. marginale, which did not correlate with an increase in either CD4⁺CD25⁺FoxP3⁺ T cells or any γδ T-cell subset examined.

Anaplasma marginale is a tick-borne intraerythrocytic rickettsial pathogen found in most temperate and tropical regions of the world and causes significant anemia and a mortality rate of up to 30% in naive cattle. Cattle that survive acute disease remain persistently infected for life with cyclic, but microscopically undetectable, levels of bacteria that do not cause clinical disease (1). Of note, the antigen load in animals during acute and persistent infection is high, reaching 10⁹ bacteria per ml of blood during acute infection and 10⁷ bacteria per ml of blood in recurrent peaks during persistent infection (2). The mechanisms by which A. marginale is capable of persisting in the immunocompetent host have not been completely elucidated. A. marginale undergoes extensive antigenic variation in immunodominant and abundant major surface protein 2 (MSP2) and MSP3 by gene conversion of whole pseudogenes and segments of pseudogenes into a single expression site (3). Antigenic variation in MSP2, which is rich in T- and B-lymphocyte epitopes, allows the organism to escape specific adaptive immune responses and contributes to persistence (4–7). Our studies have shown that infection of A. marginale in cattle previously immunized with either native MSP2 or recombinant MSP1a resulted in a complete loss of functional CD4⁺ T-cell responses to the specific immunogen beginning near the peak of acute infection (7, 8). The T cells were unable to proliferate or produce gamma interferon (IFN-γ). The loss of MSP2-specific T-cell responses occurred for both conserved and antigenically variant epitopes, showing that the induction of T-cell anergy via altered peptide ligand antagonism was not the sole explanation (7). The similar loss of MSP1a-specific functional CD4⁺ T-cell responses in MSP1a vaccinates was paralleled by the rapid depletion of MSP1a-specific CD4⁺ T cells, monitored with major histocompatibility complex (MHC) class II tetramers, from the peripheral blood. Functional MSP1a-specific CD4⁺ T cells could not be recovered from lymph node, spleen, or liver samples, although...
significantly higher numbers of tetramer-positive cells were detected in some spleen and liver samples than in blood and lymph node samples (8). Additionally, responses of blood and splenic CD4 T cells primed by A. marginale infection were first detected at 5 to 7 weeks or 15 to 16 weeks postinfection but were transient and sporadic thereafter for up to 1 year (2). In contrast, Clostridium vaccine-induced CD4⁺ T-cell responses were unimpaired. This finding is consistent with the continual downregulation or deletion of newly primed antigen-specific T cells throughout recurrent cycles of bacteremia observed during persistent infection.

The residual tetramer-positive CD4⁺ T cells in the spleen and liver might represent exhausted cells on the pathway to destruction or regulatory T cells that fail to respond ex vivo to antigen stimulation, because they fail to produce sufficient interleukin-2 (IL-2) (9, 10). T-cell exhaustion is a progressive loss of effector T-cell functions, beginning with the production of IL-2, followed by tumor necrosis factor alpha (TNF-α) and IFN-γ, eventually leading to T-cell death (11). This has been shown to occur for both CD8 and CD4 T cells (12, 13), but the most widely studied examples show a loss of effector CD8 T-cell function during chronic viral infections characterized by a relatively high antigen load (11, 13–19). We recently characterized the exhausted phenotype in A. marginale-specific CD4 T cells following infection as a progressive loss of proliferation (presumably IL-2 driven) and IFN-γ and TNF-α secretion, which was reversible upon clearing the infection with tetracyclines (20). This result suggests there is active suppression of A. marginale-specific T cells during persistent infection, in addition to T-cell exhaustion and deletion.

Forkhead box protein P3 (FoxP3)⁺ regulatory T cells can play a role in exhaustion because they can suppress or kill functional antigen-specific CD4 T cells during persistent infection, preventing excessive inflammation and enabling the pathogen to persist (21–27). In cattle, although FoxP3⁺ T cells have been identified, they have not been shown to have suppressive activity, whereas subsets of γδ T cells have regulatory activity for bovine T cells ex vivo (28–30).

This study was designed to test two hypotheses. The first hypothesis is that the exhaustion of immunization-induced epitope-specific T cells following infection requires the presence of the same T-cell epitope on the infecting bacteria and is not a result of Anaplasma infection per se. The second hypothesis is that infection with Anaplasma is characterized by an increase in CD4⁺ CD25⁺ FoxP3⁺ T cells or a subset of γδ T cells during infection that might explain the loss of functional antigen-specific T cells. To test these hypotheses, CD4⁺ CD25⁺ FoxP3⁺ T cells and γδ T-cell subsets in blood and spleen samples were enumerated by flow cytometry before and during acute infection in cattle immunized with an MSP1a peptide containing a DRB3*1101-restricted T-cell epitope. Cattle were infected with A. marginale expressing the F2-5B T-cell epitope (31, 32) or with the A. marginale subsp. centrale vaccine strain that does not share the identical epitope sequence (33). Our results show that the loss of the MSP1a F2-5B-specific T-cell response occurs only after infection with bacteria that express this epitope, and the loss of responsiveness is not associated with an increase in cells with a regulatory T-cell phenotype.

### Table 1: DRB3 haplotypes and composition of experimental animal groups

| Animal and infection group | DRB3 | Allele |
|----------------------------|------|-------|
| **A. marginale**           |      |       |
| 31858                     | 22   | *1101 |
| 31882                     | 12   | *1701 |
| 31889                     | 22   | *1101 |
| 31917                     | 16   | *1501 |
|                           | 22   | *1101 |
| **A. marginale subsp. centrale** |     |       |
| 31879                     | 12   | *1701 |
| 31892                     | 22   | *1101 |
| 31898                     | 22   | *1101 |
| 31935                     | 11   | *0902 |
|                           | 22   | *1101 |
| **No infection**          |      |       |
| 31877                     | 16   | *1501 |
| 31878                     | 22   | *1101 |
| 31880                     | 22   | *1501 |
| 31896                     | 22   | *1101 |
|                           | 24   | *0101 |

### Materials and Methods

**Expression and purification of recombinant protein.** The recombinant MSP1a F2–F3 region was expressed from plasmid pTrcHis TOPO (Invitrogen), as previously described (34). This construct encodes two MSP1a repeats, J and B, and previously identified CD4 T-cell epitopes, including the F2-5B T-cell epitope from MSP1a presented by DRB3*1101 (32, 35). Protein was purified using denaturing conditions, according to the Protinex nickel purification kit instructions (Invitrogen). Protein concentration was determined with the Quick Start Bradford protein assay (Bio-Rad), and purity was assessed using 1 and 10 µg of sample/well separated by using Coomassie blue-stained precast sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 4 to 20% gradient gels (Bio-Rad).

**Cattle immunization.** Twelve age-matched Holstein steer calves were purchased from local dairies and shown to be serologically negative for A. marginale by competitive inhibition enzyme-linked immunosorbent assay (ELISA) for MSP-5 (VMRD). All cattle were immunized with Vision 7-killed Clostridium sp. vaccine (Merck Animal Health) before the start of the study. DRB3 haplotypes (Table 1) were determined by PCR-restriction fragment length polymorphism (PCR-RFLP), as described previously (36). Animals expressing one copy of the MHC class II DRB3*1101 allele were chosen for the study, because the DRB3*1101 molecule was shown to present to CD4 T cells an immunodominant F2-5B epitope within the F2 region of A. marginale major surface protein 1a (MSP1a) (8, 32, 37). All animals underwent a surgery to marsupialize the body and tail of the spleen, as previously described (38). The calves were immunized subcutaneously six times with 50 µg/ml recombinant MSP1a F2–F3 protein at 3-week intervals. Primary immunizations were given in complete Freund’s adjuvant, and booster immunizations were given in incomplete Freund’s adjuvant, and booster.
controls (8). Cells were cultured for 6 days at 37°C in 5% CO2, labeled with 1:1,000 in complete RPMI 1640 medium were also used as positive controls. A. marginale and A. centrale and percent drop in PCV between cattle infected with A. marginale was determined by an examination of Giemsa-stained blood smears, and 1 ml of blood diluted in phosphate-buffered saline (PBS) was administered with 10% fetal bovine serum (FBS), 100 μM-glutamine, 50 μM-2-mercaptoethanol (2-ME), 24 mM HEPES buffer, and 50 μM-2-ME cultured for 4 days with 2 × 107 irradiated autologous PBMC, as a source of antigen-presenting cells, per well in triplicate with 1 and 10 μg/ml peptide, and the cells were pulsed and harvested, as described previously (39). The results are presented as the stimulation index (SI), determined as the mean counts per minute (CPM) for replicate cultures stimulated with the test antigen divided by the mean CPM of replicate cultures stimulated with medium. For all assays, responses to antigen compared to those with medium were considered statistically significant at a P value of < 0.05 using a one-tailed Student’s t test with Bonferroni correction.

**Monoclonal antibodies and cells used for flow cytometric analysis.** A variety of bovine leukocyte differentiation determinant-specific monoclonal antibodies (MAb) were used to define cell phenotypes. They included CACT138A (CD4, IgG1), CACT108A (CD25, IgG2a), GB21A (y6 TcR1, IgG2b), CAM66A (CD14, IgM), CACTB32A (WC1.2, IgG1), and 7C2B (CD8e, IgG2a) obtained from the Washington State University (WSU) Monoclonal Antibody Center, Pullman, WA. The appropriate isotype control MAb Colis69A (IgG1), Colis89A (IgG1), Colis265D (IgG2a), and Colis169A (IgG2b) were used to stain cells in parallel for all phenotyping studies. Appropriate isotype secondary conjugates labeled with fluorescein isothiocyanate (FITC), phycoerythrin (PE) (Southern Biotechnology), Alexa Fluor 488, Alexa Fluor 647, PE-Cy5.5, or Cy5 (all Invitrogen) were used for multicolor analysis. In addition, primarily labeled FoxP3-specific MAb FKJ-16-5-PE-Cy5.5 and isotype control MAb eB2Ra-PE-Cy5.5 (eBioscience) were used, as per the manufacturer’s protocol. PBMC were obtained 0 to 3 days before infection, and spleen biopsy samples were obtained 0 to 10 days before infection for the preinfection samples. PBMC or spleen samples were obtained between 2 to 32 weeks postinfection.

**Flow cytometric analysis of Foxp3-expressing CD4 T cells and y6 T-cell subsets.** Four-color analysis of FoxP3-expressing CD4 T cells was performed by staining cells according to a modification of a previously described protocol (40). Briefly, PBMC or splenocytes were thawed, washed, and incubated with 15 mg/ml MAb CACT138A and CACT108A for 15 min. Cells were washed three times and incubated with anti-IgG1-FITC and anti-IgG2a-PE for 15 min on ice in the dark. Cells were then washed and incubated with the LIVE/DEAD fixable red dead cell stain kit (Invitrogen) to identify viable cells. Surface-labeled LIVE/DEAD-stained cells were then incubated in fixation/permeabilization buffer (eBioscience), washed, and stained for the presence of FoxP3. y6 T cells were examined using viable cells purified from PBMC or splenocytes on Histopaque-1077 gradients and washed twice in complete medium. Cells from each sample were stained for phenotypic markers with MAb GB21A, CAM66A, CACTB32A, and 7C2B and stained with appropriate secondary conjugates. Additional cells were stained with the corresponding isotype control MAb. Cells were evaluated on a FACSCalibur flow cytometer with CellQuest Pro, collecting 18,000 to 20,000 events. For FoxP3 T-cell analysis, live CD4 cells were gated and analyzed for CD25 versus FoxP3 expression using FCS Express (De Novo software). For y6 T-cell analysis, CD14+ cells were gated out, and the remaining lymphocyte population was analyzed for T-cell subset frequencies. Significant differences in the frequencies of CD4+ T cells, CD4+ CD25+ FoxP3+ cells, TcR1 y6 T cells, CD8+ y6 T cells, WC1.2 y6 T cells, and WC1.2 y6 T cells from PBMC or spleen cells in samples obtained before infection and at various time points after infection were determined using Student’s two-tailed paired t tests with a Bonferroni correction for multiple comparisons. To compare the lymphocyte subset frequencies between groups at individual time points, a two-tailed Student’s t test was used.

**TABLE 2** Proliferative response of T cells homoygous for DRB3*1101 to Am F2-5B and AcF2-5BL

| Antigen      | Proliferation (mean CPM ± SD) to antigen at*: |
|--------------|-----------------------------------------|
| AmF2-5BL     | 1,128 ± 286 (17.4)                      |
| AcF2-5BL     | 124 ± 50 (1.9)                          |

*Background proliferation to medium was 65 ± 28 CPM. The results in bold type are significantly greater than medium (P < 0.05), and the results in parentheses are stimulation indices.

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Freund’s adjuvant. Animal studies were conducted using an approved Institutional Animal Care and Use Center (Washington State University, Pullman, WA) protocol. An additional animal, 4848, which was homozygous for DRB3*0101 and previously immunized with A. marginale (St. Maries strain) outer membranes (39), was used initially to determine the cross-reactivity between peptides AmF2-5B and AcF2-5BL.

**Infection with A. marginale or A. marginale subsp. centrale.** Approximately 10 months after the primary immunization, four calves were infected intravenously with 1 × 108 erythrocytes infected with A. marginale St. Maries strain, and four calves were infected with 1 × 105 erythrocytes infected with A. marginale subsp. centrale. For infection, blood was obtained from a splenectomized calf undergoing acute infection, bacteremia was determined by an examination of Giemsa-stained blood smears, and 1 ml of blood diluted in phosphate-buffered saline (PBS) was administered intravenously to each calf. A third group of four immunized calves was maintained as uninfected controls. The health of each animal was monitored daily. Blood samples were monitored for packed erythrocyte cell volume (PCV) and bacteremia, determined by light microscopic examination of Giemsa-stained blood films. A Student two-tailed t test was used to determine statistically significant differences in maximum bacteremia and percent drop in PCV between cattle infected with A. marginale and A. marginale subsp. centrale. Spleen aspirates and peripheral blood samples were obtained at 2 weeks during ascending bacteremia, at 6 or 7 weeks near the peak of infection, and at 17 weeks (spleen samples) and 32 weeks (peripheral blood mononuclear cell [PBMC] samples), when bacteremia had been controlled. It was not possible to obtain spleen biopsy samples past 17 weeks. Mononuclear cells were purified from gently disrupted spleen aspirates or whole-blood samples by Histopaque gradient centrifugation, repetitively washed with Hank’s balanced salt solution (Gibco), and suspended in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), 100 mM-l-glutamine, 50 μM 2-mercaptoethanol (2-ME), 24 mM HEPES buffer, and 50 μg/ml gentamicin sulfate (complete medium), as described previously (2, 8). Lymphocytes were either used immediately in proliferation assays or cryopreserved in fetal bovine serum containing 10% dimethyl sulfoxide (DMSO).

**Lymphocyte proliferation assays.** Fresh or cryopreserved PBMC or spleen mononuclear cells obtained preinfection and at various time points after infection were assayed in triplicate using 2 × 105 viable cells/well in complete RPMI 1640 medium (2). Cells were stimulated in 96-well round-bottomed plates in a volume of 100 μl/well with antigen. The antigens assayed included 10 μg/ml A. marginale St. Maries outer membranes (OM), A. marginale peptide AmF2-5B (ARSVLETAGHVDALG) (8, 32, 37), and the orthologous A. marginale subsp. centrale peptide AcF2-5BL (GSGILDTSIGYNSWE) (33) (Table 2). Peptides were synthesized by Gerhardt Munske, Laboratory for Biotechnology and Bioanalysis, I. Washington State University, Pullman, WA. A negative-control antigen consisted of 10 μg/ml membranes prepared from uninfected red blood cells (URBC). Bovine T-cell growth factor (TCGF) diluted 1:10 and Vision 7-killed Clostridium sp. vaccine (Merck Animal Health) diluted 1:1000 in complete RPMI 1640 medium were also used as positive controls (8). Cells were cultured for 6 days at 37°C in 5% CO2, labeled with 0.25 μg/μl [3H]thymidine for 18 h, harvested using a Tomtec Harvester96, and quantified with a PerkinElmer 1450 TriLux MicroBeta liquid scintillation counter. For one experiment, a 2-week cell line from A. marginale OM-immunized animal 4848, which was homozygous for DRB3*1101 (39), was established by culturing PBMC for 1 week with 5 μg/ml MSP1a peptide F2-5 (GGVSYNGNASAIRSLTGTVLTHDVNLG) (37) that contains the F2-5B epitope and resting for 1 week. The 2-week cell line was then tested with the A. marginale and A. marginale subsp. centrale peptides AmF2-5B and AcF2-5BL, respectively, to ensure there was no cross-reactivity between the two peptides. For this assay, 3 × 107 T cells were cultured for 4 days with 2 × 107 irradiated autologous PBMC, as a source of antigen-presenting cells, per well in triplicate with 1 and 10 μg/ml peptide, and the cells were pulsed and harvested, as described previously (39). The results are presented as the stimulation index (SI), determined as the mean counts per minute (CPM) for replicate cultures stimulated with the test antigen divided by the mean CPM of replicate cultures stimulated with medium. For all assays, responses to antigen compared to those with medium were considered statistically significant at a P value of < 0.05 using a one-tailed Student’s t test with Bonferroni correction.
TABLE 3 Proliferative responses of PBMC to peptides AmF2-5B and AcF2-5BL over the course of infection

| Infection group and animal | Peptide AmF2-5B | Peptide AcF2-5BL |
|---------------------------|-----------------|-----------------|
|                           | Preinfection    | Wk 2            | Wk 6            | Wk 32           | Preinfection    | Wk 2            | Wk 6            | Wk 32           |
| A. marginale              |                 |                 |                 |                 |                 |                 |                 |                 |
| 31858                     | 31.5 (7.3)      | 48.1 (10.3)     | 0.5 (0.3)       | 1.0 (0.1)       | 3.1 (1.6)       | 4.3 (1.2)       | 0.3 (0.2)       | 2.5 (1.3)       |
| 31882                     | 28.5 (2.7)      | 694.9 (91.5)    | 2.5 (0.3)       | 1.1 (0.3)       | 1.9 (0.9)       | 5.7 (1.1)       | 0.5 (0.3)       | 0.9 (0.7)       |
| 31889                     | 17.0 (0.9)      | 97.7 (2.7)      | 1.3 (0.1)       | 8.8 (7.3)       | 1.7 (0.8)       | 0.1 (0)         | 0.6 (0.1)       | 9.7 (5.1)       |
| 31917                     | 249.5 (12.2)    | 290.3 (10.4)    | 4.2 (1.1)       | 18.8 (22.2)     | 2.1 (0.3)       | 0.4 (0.2)       | 4.8 (0.4)       | 2.0 (0.9)       |
| A. marginale subsp. centrale |               |                 |                 |                 |                 |                 |                 |                 |
| 31879                     | 271.3 (69.6)    | 302.1 (10.8)    | 146.4 (5.0)     | 203.1 (39.0)    | 2.3 (1.2)       | 5.5 (0.5)       | 41.0 (3.0)      | 9.1 (5.8)       |
| 31892                     | 156.8 (8.7)     | 162.6 (7.9)     | 379.5 (48.7)    | 8.3 (0.1)       | 12.5 (1.1)      | 6.6 (3.4)       | 7.0 (3.4)       | 3.8 (2.8)       |
| 31898                     | 75.7 (3.7)      | 43.2 (17.1)     | 35.1 (4.2)      | 90.8 (16.3)     | 3.7 (1.7)       | 0.4 (0.2)       | 0.7 (0.1)       | 1.3 (0.1)       |
| 31935                     | 372.5 (107.6)   | 379.3 (32.1)    | 741.3 (130.5)   | 214.4 (62.5)    | 5.8 (0.02)      | 6.2 (3.9)       | 5.6 (2.2)       | 7.9 (11.3)      |
| No infection              |                 |                 |                 |                 |                 |                 |                 |                 |
| 31877                     | 122.4 (29.8)    | 307.6 (68.9)    | 98.3 (8.3)      | 122.4 (29.8)    | 2.2 (1.7)       | 4.2 (2.3)       | 0.5 (0.3)       | 2.2 (1.7)       |
| 31878                     | 316.4 (45.2)    | 136.6 (28.4)    | 477.8 (7.3)     | 49.0 (1.5)      | 6.6 (8.5)       | 29.3 (2.4)      | 11.8 (9.2)      | 27.2 (6.8)      |
| 31880                     | 35.6 (3.8)      | 80.0 (27.7)     | 99.6 (30.4)     | 42.5 (2.8)      | 9.4 (4.7)       | 1.5 (0.9)       | 7.3 (2.5)       | 6.9 (0.4)       |
| 31896                     | 46.1 (7.8)      | 180.5 (53.2)    | 120.5 (8.1)     | 100.6 (28.0)    | 5.4 (0.7)       | 39.7 (16.8)     | 10.7 (10.1)     | 12.2 (6.2)      |

*Numbers in bold type represent responses significantly greater than medium calculated for each animal at each time point (P < 0.05, determined by the one-tailed Student t-test with Bonferroni correction for two comparisons).

**Proliferative response to peptide AmF2-5B is significantly greater than the response to peptide AcF2-5BL (P < 0.05, determined with a one-tailed Student t-test).**

**RESULTS**

**Clinical response to infection.** All animals became infected and experienced clinical signs. Cattle infected with *A. marginale* experienced a mean ± standard deviation peak bacteremia of 3.6% ± 4.6% (range, 0.83 to 10.5%), and cattle infected with *A. marginale* subsp. *centrale* experienced a mean peak bacteremia of 0.88% ± 0.26% (range, 0.86 to 1.2%), and these were not significantly different. In a comparison of PCVs, *A. marginale*-infected animals had a mean percent drop in PCV of 51.2% ± 63.2% (range, 45.4 to 63.2%), and *A. marginale* subsp. *centrale*-infected animals had a mean percent drop in PCV in 45.1% ± 7.3% (range, 38.7 to 55.6%), and these were not significantly different. The *A. marginale* bacteremia reached peak levels between 4 and 5 weeks postinfection, whereas *A. marginale* subsp. *centrale* bacteremia peaked between 6 and 7 weeks postinfection.

**Response of PBMC and spleen lymphocytes to peptides AmF2-5B and AcF2-5BL.** A short-term T-cell line from *A. marginale* outer membrane-immunized animal 4848, homologous for *DRB3*+*1101* (39), which responds to peptide F2-5B, was used to verify a lack of T-cell cross-reactivity between the *A. marginale* F2-5B peptide and the corresponding *A. marginale* subsp. *centrale* peptide, which shares only four of the 16 amino acids. The response to peptide Am-F2-5B was significant, whereas the response to peptide AcF2-5BL was not (Table 2), showing the epitope is not conserved between the two *Anaplasma* organisms.

To test our hypothesis that the exhaustion of immunization-induced epitope-specific T cells following infection requires the presence of the same T-cell epitope on the infecting bacteria, cattle expressing the *DRB3*+*1101* gene product, known to present the CD4 T-cell epitope MSP1a F2-5B from *A. marginale* (32), were immunized with an MSP1a protein construct (F2–F3 region) that expresses the F25-B epitope (34). The immunized cattle were challenged with either *A. marginale* ST Maries strain that expresses the F2-5B epitope (31) or with the *A. marginale* subsp. *centrale* vaccine strain that does not (33). Next, the response of PBMC and spleen cells to the *A. marginale* and *A. marginale* subsp. *centrale* peptides containing CD4 T-cell epitopes were determined after immunization, but prior to infection, and at several time points after infection with either *A. marginale* or *A. marginale* subsp. *centrale* (Tables 3 and 4). The time points correspond to ascending bacteremia (2 weeks), near the peak of infection (6 and 7 weeks), and during persistent infection (17 and 32 weeks). As we observed previously for cattle immunized with a DNA construct encoding the MSP1a F2-5B epitope (8, 41), the AmF2-5B peptide-specific PBMC response was lost in 75% of animals by 6 weeks following infection with *A. marginale*, just after bacteremia peaked at 4 to 5 weeks, and remained insignificant at 32 weeks (Table 3). In contrast, T-lymphocyte responses remained significant at all time points sampled in cattle infected with *A. marginale* subsp. *centrale* that does not express the AmF2-5B epitope and in uninfected controls. Similar results were found for spleen lymphocytes, although the responses by spleen cells were generally lower than those by PBMC (Table 4). For both PBMC and spleen cells, there were sporadic significant responses to the *A. marginale* subsp. *centrale* AcF2-5BL peptide that occurred in all three groups. However, the responses to the AcF2-5BL peptide were not consistent across all time points and were all significantly lower than the responses to the corresponding AmF2-5BL peptide. This result shows that the loss of epitope-specific CD4 T-cell response following infection is dependent on the expression of the epitope by the infecting bacteria and not due to *Anaplasma* infection or levels of bacteremia per se. This also confirms our previous finding that the loss of responding T cells from blood is not due to their trafficking to the spleen (8), where infected erythrocytes are eliminated (42).

**Responses of PBMC and spleen lymphocytes to purified *A. marginale* outer membranes.** The three groups of cattle immunized with recombinant MSP1a containing the F2-5B epitope...
were also monitored over the course of infection for T-cell proliferation to native antigen in the form of outer membranes. The results of testing PBMC and spleen lymphocytes are shown in Tables 5 and 6, respectively. The results are similar to what was observed for peptides in that the PBMC response in A. marginale-infected cattle was reduced or insignificant by 6 weeks, near the peak of bacteremia, and remained insignificant in two cattle at 32 weeks (Table 5). The responding animal 31917 had no response at 32 weeks, and it is likely that the response at 32 weeks represents a response to native antigen by T cells primed to other A. marginale proteins during infection, as we previously observed transient and sporadic responses during infection in naive cattle (2). For all cattle in the A. marginale subsp. centrale-infected and -uninfected groups, the response to outer membranes remained significant across all time periods. For all groups, there were occasional responses to negative-control uninfected erythrocyte membrane antigen, although these were always significantly less than the response to A. marginale. Similar results were shown for spleen lym-

### TABLE 4 Proliferative responses of splenocytes to AmF2-5B and AcF2-5BL over the course of infection

| Infection group and animal | Preinfection | Wk 2 | Wk 7 | Wk 17 | Preinfection | Wk 2 | Wk 7 | Wk 17 |
|----------------------------|--------------|------|------|-------|--------------|------|------|-------|
| A. marginale               |              |      |      |       |              |      |      |       |
| 31858                      | 5.8 (0.1)    | 27.9 (4.6) | 1.8 (0.6) | 1.0 (0.4) | 1.4 (1.0) | 4.8 (4.2) | 0.5 (0.3) | 0.4 (0.4) |
| 31882                      | 30.8 (6.6)   | 62.8 (11.7) | 9.0 (3.1) | 0.2 (0)   | 0.3 (0.1) | 5.4 (2.1) | 0.9 (0.6) | 1.3 (1.6) |
| 31889                      | 6.8 (1.7)    | 121.0 (16.0) | 1.2 (0.1) | 0.3 (0)   | 0.6 (0.1) | 4.1 (3.1) | 1.6 (0.4) | 0.9 (0.2) |
| 31917                      | 253.1 (10.1) | 94.0 (10.8) | 0.8 (0.3) | 0.3 (0.1) | 1.0 (0.1) | 2.6 (1.6) | 0.3 (0.1) | 0.6 (0.1) |

### TABLE 5 Proliferative responses of PBMC to A. marginale OM and uninfected erythrocyte membranes over the course of infection

| Infection group and animal | Preinfection | Wk 2 | Wk 6 | Wk 32 | Preinfection | Wk 2 | Wk 6 | Wk 32 |
|----------------------------|--------------|------|------|-------|--------------|------|------|-------|
| A. marginale               |              |      |      |       |              |      |      |       |
| 31858                      | 60.6 (10.8)  | 230.6 (26.4) | 6.2 (3.0) | 2.7 (0.7) | 1.6 (0.8) | 5.1 (1.4) | 1.1 (1.1) | 3.1 (0.6) |
| 31882                      | 20.6 (3.8)   | 128.9 (14.0) | 6.0 (0.4) | 1.9 (1.3) | 3.5 (0.7) | 1.5 (2.4) | 3.1 (2.5) | 0.8 (0.3) |
| 31889                      | 63.1 (12.5)  | 183.0 (1.7) | 7.4 (4.5) | 0.6 (0.2) | 0.3 (0.1) | 0.3 (0.3) | 14.7 (4.5) | 0.3 (0.1) |
| 31917                      | 148.0 (20.8) | 223.3 (16.4) | 0.8 (0.1) | 61.8 (6.9) | 8.1 (0.5) | 4.0 (0.2) | 4.0 (3.2) | 3.9 (2.1) |

### Notes
- Numbers in bold type represent responses significantly greater than medium calculated for each animal at each time point (P < 0.05, determined by the one-tailed Student t test with Bonferroni correction for two comparisons).
- Proliferative response to peptide AmF2-5B is significantly greater than the response to peptide AcF2-5BL (P < 0.001, determined with a one-tailed Student t test).
Phagocytes (Table 6), although in general, the proliferative responses by spleen cells were lower than those by PBMC.

**Response of PBMC and spleen lymphocytes to T-cell growth factor and *Clostridium* antigen.** To rule out generalized immunosuppression in cattle infected with *A. marginale* in which immune responses were lost, we also performed proliferation assays using bovine T-cell growth factor and *Clostridium* vaccine antigen (Tables 7 and 8). The PBMC of all cattle responded robustly to T-cell growth factor (Table 7). PBMC from all cattle except 31858 responded significantly to *Clostridium* vaccine antigen. Similarly, spleen lymphocytes from all cattle at all time points responded to T-cell growth factor, and most gave significant responses to *Clostridium* (Table 8).

**Frequencies of CD4⁺ CD25⁺ FoxP3⁺ T cells and γδ T-cell subsets before and after infection.** Although CD4⁺ CD25⁺ FoxP3⁺ T cells have a demonstrated regulatory function in mice and humans (43), recent studies show a lack of suppressive function of these cells in cattle (28, 30). In contrast, γδ T cells, and in

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**TABLE 6** Proliferative responses of splenocytes to *A. marginale* OM and uninfected erythrocyte membranes over the course of infection

| Infection group and animal | Preinfection | Wk 2 | Wk 7 | Wk 17 | Preinfection | Wk 2 | Wk 7 | Wk 17 |
|----------------------------|--------------|------|------|-------|--------------|------|------|-------|
| **A. marginale**           |              |      |      |       |              |      |      |       |
| 31858                      | 35.5 (7.1)   | 54.1 (3.7) | 6.2 (3.1) | 1.7 (1.4) | 1.1 (0.2) | 1.2 (0.5) | 1.8 (1.8) | 0.5 (0.2) |
| 31882                      | 23.2 (9.1)   | 94.9 (2.9) | 28.0 (15.4) | 0.4 (0.3) | 3.5 (4.7) | 3.9 (2.2) | 2.6 (0.9) | 0.2 (0.1) |
| 31889                      | 5.4 (0.6)    | 104.9 (8.2) | 18.7 (7.7) | 1.7 (0.3) | 0.5 (0.1) | 7.7 (1.3) | 0.8 (0.7) | 1.0 (0.9) |
| 31917                      | 656.7 (111.3) | 108.2 (6.6) | 2.7 (0.4) | 0.7 (0.9) | 1.5 (1.0) | 3.4 (1.4) | 2.9 (1.0) | 0.5 (0.1) |
| **A. marginale subsp. centrale** |              |      |      |       |              |      |      |       |
| 31879                      | 131.3 (20.8) | 25.0 (2.8) | 52.6 (4.8) | 7.5 (0.1) | 1.5 (0.5) | 1.9 (1.1) | 2.6 (2.7) | 2.1 (0.6) |
| 31892                      | 111.4 (7.6)  | 547.7 (52.5) | 56.2 (17.7) | 34.7 (3.5) | 0.3 (0) | 1.8 (1.0) | 2.3 (1.0) | 2.1 (2.3) |
| 31898                      | 326.4 (50.4) | 36.6 (12.5) | 6.6 (1.9) | 4.6 (0.3) | 1.2 (0.5) | 6.7 (0.5) | 5.3 (0.5) | 1.3 (0) |
| 31935                      | 5.8 (2.4)    | 88.2 (7.7) | 39.5 (19.7) | 10.8 (1.3) | 0.5 (0.1) | 6.6 (8.9) | 3.3 (2.5) | 5.9 (3.2) |
| **No infection**           |              |      |      |       |              |      |      |       |
| 31877                      | 19.6 (9.2)   | 151.9 (24.0) | 97.2 (37.2) | 92.9 (42.6) | 1.3 (0.5) | 3.6 (4.2) | 1.1 (0.3) | 3.6 (1.0) |
| 31878                      | 56.8 (22.2)  | 41.8 (17.4) | 4.4 (0.3) | 4.2 (0.1) | 1.2 (0.2) | 0.7 (0.2) | 0.8 (0.1) | 0.9 (0.2) |
| 31880                      | 3.4 (0.8)    | 8.7 (4.5) | 9.5 (0.5) | 157.4 (7.9) | 2.2 (0.8) | 2.6 (2.0) | 2.2 (0.6) | 0.7 (0.4) |
| 31896                      | 11.6 (2.6)   | 95.3 (2.3) | 17.9 (2.7) | 129.3 (31.6) | 0.4 (0.2) | 0.5 (0) | 1.2 (1.3) | 1.4 (0.4) |

* Numbers in bold type represent responses significantly greater than medium calculated for each animal at each time point (*P* < 0.05, determined by the one-tailed Student *t* test with Bonferroni correction for two comparisons).

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**TABLE 7** Proliferative responses of PBMC to T-cell growth factor and *Clostridium* vaccine antigen over the course of infection

| Infection group and animal | Preinfection | Wk 2 | Wk 6 | Wk 32 | Preinfection | Wk 2 | Wk 6 | Wk 32 |
|----------------------------|--------------|------|------|-------|--------------|------|------|-------|
| **T-cell growth factor**   |              |      |      |       |              |      |      |       |
| 31858                      | 145.7 (18.2) | 168.3 (11.2) | 14.5 (0.7) | 5.2 (1.1) | 4.4 (2.6) | 3.8 (0.3) | 8.2 (5.3) | 4.2 (1.6) |
| 31882                      | 72.8 (33.2)  | 123.1 (15.9) | 81.6 (18.7) | 10.9 (0.3) | 52.6 (19.0) | 62.4 (24.0) | 71.6 (24.9) | 17.9 (2.4) |
| 31889                      | 142.6 (22.1) | 45.1 (3.3) | 54.6 (5.5) | 63.8 (7.6) | 60.6 (14.0) | 5.2 (1.5) | 19.6 (2.9) | 54.9 (10.0) |
| 31917                      | 395.0 (27.0) | 263.1 (39.8) | 333.2 (17.1) | 75.6 (4.4) | 152.2 (7.0) | 269.0 (23.3) | 170.4 (9.5) | 233.3 (15.0) |
| **Clostridium vaccine antigen** |              |      |      |       |              |      |      |       |
| 31879                      | 113.4 (15.2) | 218.4 (34.3) | 287.9 (34.2) | 49.1 (7.3) | 103.4 (3.4) | 223.8 (20.5) | 282.4 (6.7) | 317.6 (7.7) |
| 31892                      | 31.1 (3.1)   | 179.2 (57.8) | 110.6 (28.6) | 39.2 (2.7) | 71.1 (16.2) | 69.7 (29.4) | 57.0 (125.7) | 97.6 (17.9) |
| 31898                      | 12.7 (2.9)   | 22.1 (2.7) | 44.0 (3.6) | 97.0 (16.7) | 27.3 (5.5) | 82.8 (11.2) | 109.9 (30.2) | 187.1 (21.7) |
| 31935                      | 669.9 (28.8) | 275.7 (44.7) | 305.8 (11.5) | 71.0 (6.5) | 61.4 (4.0) | 27.9 (19.6) | 32.2 (2.7) | 277.2 (64.9) |

* Numbers in bold type represent responses significantly greater than medium calculated for each animal at each time point (*P* < 0.05, determined by the one-tailed Student *t* test with Bonferroni correction for two comparisons).

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particular the WC1.2+ subset of γδ T cells, have been implicated in the suppression of bovine CD4 and CD8 T cells in vitro (28–30). To determine if regulatory T cells increased in cattle undergoing acute A. marginale infection, PBMC and spleen cells were analyzed by flow cytometry after staining cells with MAb to detect regulatory T-cell markers. Figure 1A shows an example of gated live CD4+ cells from PBMC that were used to enumerate CD4+ CD25+ FoxP3+ cells, which in this animal had a frequency of 1.7%. The average frequencies of CD4+ T cells and CD4- T cells that express FoxP3 or FoxP3 and CD25 are presented for each group in Table 9. There was a significant increase in CD4+ CD25+ FoxP3+ cells at week 3 postinfection in spleen cells in the A. marginale-infected group only. However, this increase was not sustained and was not significantly different from the frequency of CD4- CD25+ FoxP3+ cells at the 3-week postinfection time points in the other two groups. Furthermore, when the A. marginale-infected cattle were compared to either A. marginale subsp. centrale-infected cattle or -noninfected cattle at individual time points, there were no significant differences in the frequency of any T-cell subset at any time point. Figure 1B shows an example of the frequencies of γδ T cells, CD8+ γδ T cells, and WC1.2+ γδ T cells after CD14+ monocytes were gated out. In this animal, of the total PBMC, 7.9% were γδ T cells, 0.5% were CD8+ γδ T cells, 4.2% were WC1.2+ γδ T cells, and 3.7% were WC1.2- cells. The mean frequencies of these γδ T-cell subsets in PBMC and spleen are presented for each group in Table 10. There were never significant increases in the frequency of TcR1+ γδ T cells, CD8+ γδ T cells, WC1.2+ γδ T cells, or WC1.2- γδ T cells in any group postinfection in a comparison of the frequencies to preinfection levels. When the A. marginale-infected group was compared to the A. marginale subsp. centrale-infected group at individual time points, the only significant differences were fewer WC1.2+ γδ T cells (6.8% versus 10.4%) and fewer CD8+ γδ T cells (0.9% versus 2.1%) in the spleen at 5 weeks postinfection with A. marginale (P < 0.05).

**DISCUSSION**

Pathogens that cause a persistent infection or chronic disease have evolved mechanisms to evade both innate and adaptive immune responses (44, 45). A. marginale, an intracellular rickettsial pathogen, lacks lipopolysaccharide (LPS) and peptidoglycan (31, 46) and does not appear to evoke a pathological inflammatory response during acute infection. Through gene conversion, A. marginale undergoes extensive and continual antigenic variation in surface MSP2 and MSP3 throughout persistent infection (3), enabling it to avoid preexisting antibody response to the dominant MSP2 variant (5). Thus, antigenic variation is one way this pathogen avoids the adaptive immune response.

Unexpectedly, the induction of strong MSP2-specific T-cell and antibody responses following immunization with gel-purified native MSP2 did not provide any protection against infection with bacteria expressing the same major MSP2 variants. These MSP2 variants attained high levels after infection, suggesting that antigenic variation was not essential to escape a protective adaptive immune response (7). In that study and those following, we documented that infection with A. marginale resulted in a loss of immunization-induced CD4 T-cell proliferation and cytokine production (7, 8, 20). Using MHC class II tetramers, we demonstrated the loss of epitope-specific CD4 T cells from blood during the course of acute infection that could not be accounted for in lymph node, spleen, or liver tissues (8). This dysfunctional response is characteristic of an exhausted T-cell phenotype leading to an antigen-specific T-cell deletion observed during chronic infection with pathogens that cause high antigen load (11, 13–19). Furthermore, we showed that a nonfunctional A. marginale-specific T-cell response became functional once the infection was cleared, indicating the requirement for continual antigenic stimulation to maintain the nonresponsive phenotype (20).

This study was designed to test the hypothesis that epitope-specific T-cell exhaustion observed following infection requires the pres-
ence of the epitope on the infecting pathogen and is not a result of *Anaplasma* infection per se. Our results show that following immunization of cattle expressing DRB3*1101*, which presents the MSP1a peptide F2-5B epitope, with a recombinant protein expressing this epitope, only animals infected with *A. marginale* expressing this epitope failed to respond to antigen. Immunized but uninfected cattle or immunized cattle challenged with *A. marginale* subsp. *centrale*, which does not express the F2-5B epitope, maintained significant responses to both the F2-5B epitope and to native outer membrane antigen. Furthermore, nearly all cattle maintained significant responses to *Clostridium* vaccine antigen or T-cell growth factor, showing that the dysfunctional T-cell response is antigen specific. We therefore can accept our hypothesis, and these results further support the concept that T-cell exhaustion observed during infection with *A. marginale* results from high antigen load and antigen-specific T-cell exhaustion/deletion.

**FIG 1** Example of flow cytometric analysis of PBMC. Four-color analysis was performed. (A) Live CD4+ lymphocytes were gated versus side light scatter (SSC) and analyzed for CD25 and FoxP3 expression. As a control, cells were stained with an anti-CD25 and an isotype-matched irrelevant MAb. The percentage of FoxP3+/CD25+ cells is shown in the upper right quadrant. (B) Live PBMC obtained following Histopaque purification were stained with MAb to the γδ TcR (TcR1), CD8α, WC1.2, or CD14. CD14+ cells were gated out and lymphocytes analyzed for the expression of TcR1 and CD8α and of TcR1 and WC1.2. Isotype controls were included. The percentages of TcR1+CD8α or TcR1+WC1.2α cells are shown in the upper right quadrants.
TABLE 9 Average frequencies of CD4 T cells and CD4 T cells that express FoxP3 or FoxP3 and CD25 in PBMC and spleen lymphocytes in uninfected cattle and in cattle before and after infection with *Anaplasma*

| Infection group and lymphocyte subset | Frequency (%) (mean ± SD) at wk³ |
|--------------------------------------|----------------------------------|
|                                       | 0      | 3   | 5   | 7   |
| **A. marginale**                     |        |     |     |     |
| PBMC                                 |        |     |     |     |
| CD4⁺ cells                           | 4.3 (1.9) | 7.8 (3.8) | 2.4 (1.6) | 4.3 (1.2) |
| FoxP3⁺ cells (of CD4⁺)               | 3.4 (2.0) | 1.0 (0.3) | 1.5 (1.0) | 0.5 (0.5) |
| CD25⁺ FoxP3⁺ cells (of CD4⁺)         | 3.0 (1.6) | 0.9 (0.3) | 1.3 (1.9) | 0.3 (0.4) |
| Spleen                               |        |     |     |     |
| CD4⁺ cells                           | 5.1 (1.3) | 7.0 (2.9) | 1.7 (0.8) | 4.2 (0.9) |
| FoxP3⁺ cells (of CD4⁺)               | 2.2 (0.6) | 3.4 (1.0) | 1.4 (1.3) | 0.6 (0.5) |
| CD25⁺ FoxP3⁺ cells (of CD4⁺)         | 2.1 (0.6) | 3.0 (0.8) | 0.8 (0.6) | 0.3 (0.3) |
| **A. marginale subsp. centrale**     |        |     |     |     |
| PBMC                                 |        |     |     |     |
| CD4⁺ cells                           | 5.9 (3.2) | 7.3 (0.6) | 2.2 (0.3) | 5.4 (1.6) |
| FoxP3⁺ cells (of CD4⁺)               | 2.8 (1.8) | 0.9 (0.1) | 0.7 (0.8) | 0.3 (0.1) |
| CD25⁺ FoxP3⁺ cells (of CD4⁺)         | 2.3 (1.4) | 0.8 (0.1) | 0.6 (0.7) | 0.1 (0.1) |
| Spleen                               |        |     |     |     |
| CD4⁺ cells                           | 4.3 (1.2) | 5.1 (2.9) | 2.9 (1.0) | 4.3 (1.5) |
| FoxP3⁺ cells (of CD4⁺)               | 2.0 (1.1) | 2.7 (1.3) | 1.0 (0.6) | 0.8 (0.4) |
| CD25⁺ FoxP3⁺ cells (of CD4⁺)         | 1.9 (1.0) | 2.4 (1.2) | 0.4 (0.4) | 0.8 (1.0)³⁴ |
| **No infection**                     |        |     |     |     |
| PBMC                                 |        |     |     |     |
| CD4⁺ cells                           | 11.0 (7.6) | 5.5 (2.2) | 9.8 (5.4) | 7.9 (1.9) |
| FoxP3⁺ cells (of CD4⁺)               | 2.1 (1.3) | 2.5 (0.5) | 0.6 (0.3) | 0.3 (0.2) |
| CD25⁺ FoxP3⁺ cells (of CD4⁺)         | 1.8 (1.0) | 2.3 (0.4) | 0.4 (0.2) | 0.2 (0.1) |
| Spleen                               |        |     |     |     |
| CD4⁺ cells                           | 2.6 (0.6) | 3.9 (0.5) | 6.9 (8.6) | 5.2 (4.8) |
| FoxP3⁺ cells (of CD4⁺)               | 3.9 (1.4) | 3.1 (2.1) | 0.5 (0.4) | 1.0 (0.7) |
| CD25⁺ FoxP3⁺ cells (of CD4⁺)         | 3.7 (1.4) | 3.0 (1.0) | 0.2 (0.3) | 0.4 (0.4) |

⁴ Indicates the percentage of CD4⁺ T cells in PBMC and percentage of CD4⁺ T cells that express FoxP3 or CD25 and FoxP3 after subtracting any background staining obtained with the isotype control antibody.

There was a significantly lower percentage of cells obtained at week 0, just before infection (P < 0.05, using a 2-tailed Student t test for paired samples and Bonferroni correction for multiple comparisons).

The percentage of cells was significantly greater than that at week 0.

The mechanism leading to T-cell exhaustion during *A. marginale* infection has not been determined, but we previously found no evidence for the functional suppression by PBMC of cattle immunized with native MSP2 following infection and loss of antigen-specific proliferation and IFN-γ secretion (7). Specifically, mixing nonresponsive T cells with responding T cells at different cell ratios did not reveal any suppression of the responding cells (7). However, in that study, spleen cells were not examined, and we hypothesized that we might observe an increase in T cells with a regulatory phenotype during ascending bacteremia, especially in the spleen, where infected erythrocytes are removed, before the loss of response. To address this, PBMC and spleen cells were sampled before and at various time points after infection and analyzed for the frequencies of CD4 T cells expressing FoxP3 and CD25 or γδ T cells, including CD8⁺ γδ T cells and WC1.2⁺ γδ T cells. The results of the flow cytometric analysis did not reveal any consistent increase in either CD4⁺ CD25⁺ FoxP3⁺ cells or any γδ T-cell subset examined in either spleen or blood. These results are consistent with the lack of increased IL-10 or TGF-β production by PBMC cultured with antigen during acute rickettsiaemia following infection of MSP2-immunized cattle (7). Evidence suggests that in cattle, regulatory T cells are not CD4⁺ CD25⁺ Foxp3⁺ cells but can be γδ T cells that express the scavenger receptor WC1⁺. In cattle, WC1⁺ and WC1.2⁺ γδ T cells had suppressive function *in vitro* and expressed high levels of IL-10 (28), but this was not measured in the context of chronic infection. In calves infected with bovine respiratory syncytial virus (BRSV), the WC1.2⁻ subset of γδ T cells produced high levels of IL-10 and transforming growth factor beta (TGF-β) when stimulated with BRSV *ex vivo*, suggesting a regulatory function for these cells (29). More recently, in foot and mouth disease virus (FMDV)-vaccinated cattle, CD4⁺ CD25⁺ Foxp3⁺ T cells were not suppressive, whereas WC1⁻ and WC1.2⁺ γδ T cells made IL-10 and TGF-β when stimulated with antigen *ex vivo*. However, only the WC1.2⁺ cells suppressed FMDV-specific CD4 T cells *ex vivo* (30). Unfortunately, we did not have sufficient cells to perform similar cytokine assays on purified γδ T-cell subsets.

In summary, the results of our studies suggest that *A. marginale* infection, characterized by high antigen load, causes an antigen-specific, exhausted CD4 T-cell response leading to T-cell deletion that requires the presence of the epitope on the pathogen. T-cell exhaustion is not clearly associated with an increase in either CD4⁺ CD25⁺ Foxp3⁺ T cells that have a regulatory phenotype and can contribute to T-cell exhaustion in mice and humans (19, 47) or with WC1.2⁺ γδ T cells, which in cattle have been shown to have a regulatory phenotype. *A. marginale* infection-induced an-
TABLE 10 Average frequencies of different γδ T-cell subsets in PBMC and spleen lymphocytes in uninfected cattle and in cattle before and after infection with Anaplasma

| Infection group and lymphocyte subset | Frequency (mean ± SD) at time wk<sup>a</sup> |
|--------------------------------------|---------------------------------------------|
|                                       | 0    | 1    | 3    | 5    | 13  |
| **A. marginale**                     |      |      |      |      |     |
| PBMC                                 |      |      |      |      |     |
| All γδ T cells                       | 20.1 (11.3) | 13.5 (7.7) | 15.7 (10.5) | 9.6 (3.2) | 9.9 (4.5) |
| CD8<sup>+</sup> γδ T cells           | 1.0 (0.4) | 1.5 (1.0) | 0.9 (0.5) | 0.7 (0.6) | 0.8 (0.6) |
| WC1.2<sup>+</sup> γδ T cells         | 9.9 (6.2) | 6.1 (3.9) | 7.1 (5.1) | 3.6 (1.7) | 4.1 (1.8) |
| WC1.2<sup>-</sup> γδ T cells         | 10.2 (5.3) | 7.4 (2.4) | 8.6 (3.6) | 6.0 (2.0) | 5.8 (2.1) |
| Spleen<sup>b</sup>                   |      |      |      |      |     |
| All γδ T cells                       | 29.9 (13.3) | 20.9 (5.7) | 20.1 (3.8) | 11.2 (2.3) | 9.2 (4.0) |
| CD8<sup>+</sup> γδ T cells           | 3.3 (2.8) | 2.6 (2.3) | 2.7 (3.3) | 0.9 (0.4) | 0.6 (0.6) |
| WC1.2<sup>+</sup> γδ T cells         | 11.9 (4.7) | 8.4 (1.3) | 8.4 (3.1) | 4.4 (2.0) | 3.7 (2.1) |
| WC1.2<sup>-</sup> γδ T cells         | 18.0 (10.4) | 12.5 (5.2) | 11.7 (2.0) | 6.8 (0.4) | 5.9 (2.1) |
| **A. marginale subsp. centrale**     |      |      |      |      |     |
| PBMC                                 |      |      |      |      |     |
| All γδ T cells                       | 23.3 (11.0) | 28.0 (11.0) | 17.4 (7.6) | 15.7 (2.2) | 11.7 (4.2) |
| CD8<sup>+</sup> γδ T cells           | 1.0 (0.2) | 1.0 (0.3) | 1.1 (0.5) | 0.6 (0.3) | 0.4 (0.1)<sup>c</sup> |
| WC1.2<sup>+</sup> γδ T cells         | 12.9 (5.6) | 16.0 (4.1) | 9.2 (3.9) | 7.9 (1.7) | 5.7 (1.6) |
| WC1.2<sup>-</sup> γδ T cells         | 10.4 (5.5) | 12.0 (2.9) | 8.2 (3.8) | 7.8 (5.0) | 6.0 (2.6) |
| Spleen<sup>c</sup>                   |      |      |      |      |     |
| All γδ T cells                       | 20.3 (4.9) | 25.0 (8.5) | 24.4 (6.5) | 16.2 (4.9) | 12.3 (5.5) |
| CD8<sup>+</sup> γδ T cells           | 1.9 (0.8) | 1.6 (0.6) | 1.8 (1.9) | 2.1 (0.7) | 1.7 (0.9) |
| WC1.2<sup>+</sup> γδ T cells         | 10.4 (2.8) | 13.7 (4.7) | 12.4 (3.4) | 5.8 (2.7) | 5.1 (2.6)<sup>d</sup> |
| WC1.2<sup>-</sup> γδ T cells         | 9.9 (2.7) | 11.3 (4.0) | 12.0 (3.5) | 10.4 (2.3) | 7.2 (2.9) |
| **No infection**                     |      |      |      |      |     |
| PBMC                                 |      |      |      |      |     |
| All γδ T cells                       | 14.6 (5.2) | 25.0 (7.6) | 16.7 (8.9) | 11.2 (4.2) | 14.2 (5.0) |
| CD8<sup>+</sup> γδ T cells           | 0.5 (0.1) | 1.4 (0.3)<sup>c</sup> | 1.5 (0.8) | 2.1 (0.7) | 0.7 (0.3) |
| WC1.2<sup>+</sup> γδ T cells         | 7.3 (4.8) | 10.9 (4.1) | 7.8 (4.3) | 4.5 (3.5) | 5.9 (1.0) |
| WC1.2<sup>-</sup> γδ T cells         | 7.3 (1.8) | 14.1 (7.1) | 8.9 (5.7) | 6.7 (1.7) | 8.3 (4.6) |
| Spleen<sup>d</sup>                   |      |      |      |      |     |
| All γδ T cells                       | 14.5 (8.0) | 15.3 (5.1) | 9.8 (0.5) | 11.8 (7.7) | 6.8 (3.9) |
| CD8<sup>+</sup> γδ T cells           | 1.9 (1.8) | 1.0 (0.4) | 0.7 (0.4) | 0.9 (0.0) | 0.9 (1.0) |
| WC1.2<sup>+</sup> γδ T cells         | 6.2 (1.8) | 9.2 (2.8) | 5.2 (0.4) | 4.2 (1.6) | 3.3 (2.3) |
| WC1.2<sup>-</sup> γδ T cells         | 8.3 (6.7) | 6.1 (2.3) | 4.6 (0.9) | 7.6 (6.2) | 3.5 (1.7) |

<sup>a</sup>Frequency indicates the percentage of PBMC or spleen cells that were TcR<sup>+</sup> (all γδ T cells), and the percentage of PBMC or spleen cells that expressed TcR1 and were CD8<sup>+</sup>, WC1.2<sup>+</sup>, or WC1.2<sup>-</sup> after subtracting any background staining obtained with the isotype control antibody.

<sup>b</sup>There were cells available from 3 animals in this group for analysis.

<sup>c</sup>There was a significantly lower percentage of cells obtained at week 0, just before infection (P < 0.05, using a 2-tailed Student t test for paired samples and Bonferroni correction for multiple comparisons).

<sup>d</sup>The percentage of cells was significantly greater than that at week 0.

<sup>e</sup>In the no-challenge group, there were only spleen cell samples for three animals at weeks 0 and 13 and for two animals at weeks 1, 3, and 5. Statistical analysis was not performed for this group.

tigen-specific CD4 T-cell exhaustion/deletion represents a mechanism distinct from the antigenic variation in outer membrane proteins to evade the adaptive immune response. Additional studies need to be performed to determine the molecular mechanisms underlying functional CD4 T-cell exhaustion, which should include examining a potential role of inhibitory receptors and their ligands (11, 12, 13).

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