Immunization-Induced *Anaplasma marginale*-Specific T-Lymphocyte Responses Impaired by *A. marginale* Infection Are Restored after Eliminating Infection with Tetracycline

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Infection of cattle with *Anaplasma marginale* fails to prime sustained effector/memory T-cell responses, and high bacterial load may induce antigen-specific CD4 T exhaustion and deletion. We tested the hypothesis that clearance of persistent infection restores the exhausted T-cell response. We show that infection-induced T-cell exhaustion, characterized as loss of antigen-specific proliferation, and gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) production are partially restored in cattle following clearance of persistent infection with tetracycline.

For a little over a decade, it has been appreciated that certain chronic infectious diseases and cancers result in a loss of T-cell function that has been termed T-cell exhaustion (reviewed in references 1–5). T-cell exhaustion is a progressive loss of effector T-cell functions, beginning with production of interleukin 2 (IL-2), followed by tumor necrosis factor alpha (TNF-α), then gamma interferon (IFN-γ), and eventually leading to T-cell death (2). This has been shown to occur for both CD8 and CD4 T cells (5, 6), 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 (2, 3, 4, 7, 8, 9). Furthermore, in a noninfection mouse model where CD4 T cells were exposed to persistent antigen (moth cytochrome c), a dose- and time-dependent persistence of antigen beyond the T-cell expansion phase resulted in a dysfunctional memory response. Following removal of the antigen, the T cells regained proliferative capacity (10).

Evasion of innate and adaptive immune responses occurs with many pathogenic bacteria that establish chronic infection (11–13). *Anaplasma marginale* is a tick-borne intraerythrocytic rickettsial pathogen that causes acute bacteremia and anemia, which resolves into a lifelong persistent infection in the ruminant host (14). One well-described strategy of immune escape shared by pathogenic bacteria is antigen variation (15). During *A. marginale* infection, variation in the major surface protein 2 (MSP2) enables the bacteria to escape a variant-specific antibody response (16). However, antigenic variation in MSP2 is not sufficient to explain the inability to clear infection. When cattle were immunized with native MSP2 comprised of a complement of variant MSP2s and infected with bacteria expressing the same major MSP2 variants, there was no protection against infection with bacteria expressing these MSP2 variants (17). This occurred even though immunization with native MSP2 induced CD4 proliferative and IFN-γ–secreting T-cell and IgG responses specific for the major MSP2 variant (18).

To further explore the failure of MSP2 to protect against infection in the face of variant-specific immune responses, T-cell responses were monitored throughout the course of infection in the MSP2 vaccinees, and it was discovered that near the peak of bacteremia during acute infection, T-cell responses specific for *A. marginale*, but not other vaccine antigens, were lost (17). Responses to *A. marginale* were not detected for a year postinfection. This loss of an immunization-induced CD4 T-cell response following infection was also reported for recombinant MSP1a, where the loss of CD4 T-cell responses was paralleled by a rapid loss of MSP1a epitope-specific T cells measured by major histocompatibility complex (MHC) class II tetramer staining (19). In both studies, the CD4 T cells failed to proliferate and make IFN-γ, and in the latter study, they apparently underwent deletion, as MSP1a

**TABLE 1** Proliferative responses to *A. marginale* OM following immunization with OM

| Animal no. | Stimulation indices of PBMC in response to antigena |
|------------|---------------------------------------------------|
|            | OM (pre) | TCGF (pre) | OM (post) | TCGF (post) |
| 35113      | 0.9 ± 0.7 | 166.0 ± 42.0 | 13.7 ± 2.3 | 5.3 ± 0.3 |
| 35141      | 0.7 ± 0.4 | 11.5 ± 3.5  | 5.4 ± 0.7  | 2.4 ± 0.4 |
| 35160      | 4.3 ± 2.6 | 30.2 ± 5.7  | 42.8 ± 0.8 | 37.4 ± 1.7 |
| 35280      | 2.7 ± 0.4 | 23.9 ± 10.1 | 35.1 ± 5.9 | 18.6 ± 3.1 |
| 583        | 0.7 ± 0.2 | 5.2 ± 1.2   | 8.7 ± 0.2  | 1.5 ± 0.1 |
| 35967      | 6.8 ± 1.9 | 37.3 ± 6.0  | 31.6 ± 9.2 | 16.5 ± 4.1 |
| 36092      | 0.5 ± 0.3 | 31.4 ± 0.5  | 12.7 ± 2.6 | 5.0 ± 0.4 |
| 36345      | 1.5 ± 0.5 | 34.5 ± 4.7  | 36.0 ± 7.1 | 9.3 ± 1.3 |
| 36381      | 1.9 ± 0.3 | 9.9 ± 0.4   | 7.7 ± 1.9  | 6.4 ± 0.8 |
| 36413      | 1.16 ± 0.2 | 22.7 ± 2.4 | 52.6 ± 4.0 | 21.6 ± 0.9 |

a PBMC were obtained before immunization (pre) and 3 weeks following the last immunization (post) and were simultaneously tested for proliferation to *A. marginale* OM and TCGF as a positive control for cell viability. Results are presented as SI ± 1 SD, calculated as the mean cpm of triplicate cultures with 10 μg/ml OM or 10% TCGF/mean cpm of triplicate cultures with 10 μg/ml URBBC. Significant responses compared to URBBC are highlighted in bold type, where *P* ≤ 0.05 using a one-tailed Student *t* test with Bonferroni correction.
epitope-specific CD4 T cells were no longer detected above background levels in blood. However, a small percentage of tetramer-staining T cells remaining in the spleens and liver were unresponsive to antigen, suggesting these had not been completely deleted but were exhausted (19). Together, these results suggest *A. marginale* infection leads to exhaustion and deletion of antigen-specific CD4 T cells. This is another mechanism by which *A. marginale* maintains a persistent infection.

Like some chronic viral infections, anaplasmosis is characterized by a high antigen load, with $10^9$ or more bacteria per ml of blood during acute infection and recurring peaks of up to $10^7$ bacteria per ml of blood throughout persistence (17, 19, 20). We
TABLE 2 Chlortetracycline levels in serum of treated cattle

| Animal no. | −3  | 14   | 35   |
|-----------|-----|------|------|
| 35113     | <20 | 326  | 43   |
| 35141     | <20 | 319  | 34   |
| 35160     | <20 | 315  | 34   |
| 35280     | <20 | 211  | 21   |
| 583       | <20 | 319  | 38   |

Mean ± SD 298 ± 49 34 ± 8

a Serum samples were obtained 3 days before drug was administered and on days 14 and 35 after the initial oral dose. Drug was given in the feed for 30 days.

hypothesize that the exhausted A. marginale-specific CD4 T-cell response to infection is dependent on the presence of bacteria and will be restored in calves following clearance of persistent infection with antibiotic therapy. Bacterial outer membrane (OM)-immunized cattle infected with homologous St. Maries strain of A. marginale and then treated with tetracycline to clear the infection were used to test this hypothesis, as it was shown earlier that the response to OM is mediated by CD4 T cells (19–21). Ten Holstein cattle were repeatedly immunized with 60 μg purified bacterial OM (22), and proliferation assays were performed with peripheral blood mononuclear cells (PBMC) to confirm the response, as described previously (17, 19). Preimmunization PBMC did not respond to OM, with the exception of animal 35280, which had a weak response (stimulation index [SI] 2.7), and animal 35967 (SI, 6.8 to 10 μg/ml OM) (Table 1). In contrast, PBMC from all cattle proliferated to bovine T-cell growth factor (TCGF). In all animals, the postimmunization response to OM was significantly higher than that observed before immunization.

For infection, A. marginale-infected Dermacentor andersoni ticks were applied for a transmission feed (23). Tick infection was confirmed by performing msp5-specific nested PCR on salivary glands dissected from ticks after transmission feeding (24, 25). All animals developed clinical anaplasmosis, with maximum bacteremias of 0.1% to 9.0%, and anemia, as measured by a decrease in packed erythrocyte cell volumes of 11.4% to 66.7%, between days 35 to 45 postinfection. Lymphoproliferative responses were measured through the acute infection and into persistence, for 19 weeks postinfection. Compared to preimmunization values, responses were generally significant at 13 or 14 weeks preinfection and up to 6 weeks postinfection, but thereafter were not significant, with the exception of animal 36345, which had a positive response during persistent infection at week 19 (Fig. 1). T-cell responses to TCGF and Vision 7 killed Clostridium sp. vaccine (Merck Animal Health, Summit, NJ, USA) were maintained (data not shown) as shown previously (17, 19).

To determine if A. marginale-specific T-cell responses recovered once the pathogen load was eliminated, the infection was cleared in five cattle following tetracycline therapy (26). This consisted of a single 20-mg/kg of body weight intramuscular injection of oxytetracycline (Tetradure 300; Merial Limited, Duluth, GA, USA) followed 3 days later by 30 days of 8.8 mg/kg of body weight of granular chlortetracycline calcium complex (Aureomycin 50 Granular; Alpharma, Inc.) given once per day in the feed. Sera were collected from animals 3 days before oral treatment and at 14 and 35 days after the beginning of treatment, and were assayed for the presence of chlortetracycline by the Veterinary Diagnostic Laboratory at Iowa State University (27). Oral administration of chlortetracycline resulted in a mean serum concentration of 298 ng/ml on day 14, halfway through the treatment period. By 5 days following the last dose, the drug levels expectedly fell to one-tenth this concentration (Table 2).

To confirm clearance of Anaplasma, genomic DNA extracted from whole blood was used as a template to amplify the msp5 gene (25). Blood from an uninfected calf provided negative-control genomic DNA, while A. marginale St. Maries strain genomic DNA was used for a positive control. PCR products were visualized on Southern blots by hybridizing with a digoxigenin-labeled msp5 probe. Animals were monitored from the beginning of treatment through 11 weeks posttreatment (Fig. 2). All animals were positive by Southern blotting for the msp5 gene at 9.5 weeks and at 3 days (week 0) before oral treatment began. There was no evidence of infection in animals 35113, 35141, and 35160 at 2 weeks after treatment was begun and throughout the remainder of the study. Animals 35280 and 583 remained positive through 7 weeks posttreatment but were negative by 8 weeks and remained negative thereafter, an indication that Anaplasma had been cleared from each animal. To verify clearance, 20 ml of blood from each animal was transfused into a splenectomized calf. Over a 45-day period, clinical disease was not observed, blood smears remained negative, and Anaplasma infection in this calf was not detected using msp5-specific nested PCR, showing that the infection was cleared in the five drug-treated animals.

During the treatment and posttreatment phases of the experiment, proliferative responses of PBMC specific for OM were mea-
sured and compared to the response at the beginning of treatment, week 19 postinfection (Fig. 3). Significant OM-specific proliferation was first observed in animal 583, 26 weeks after infection (7 weeks after beginning oral tetracycline treatment). Three animals had significant responses by week 30 (11 weeks posttreatment), and the fifth animal responded by week 34 (15 weeks posttreatment). By the end of the experiment, at 38 weeks postinfection and 19 weeks posttreatment, all animals in the treatment group had significant responses to OM.

It was possible that we would also observe a restored response in untreated cattle, so the experiment was controlled by similarly monitoring PBMC proliferative responses in OM-immunized cattle that were infected but not treated with tetracycline. Two animals had significant responses at 26 and 30 weeks postinfection, and two animals had significant responses at 30 weeks postinfection (Fig. 4). These responses, however, were not sustained throughout the total 38-week time period following infection. This sporadic and transient type of response was previously observed in unimmunized cattle infected with one of two different strains of A. marginale by either needle inoculation of infected blood or tick transmission (20). The transient appearance of antigen-specific T cells during persistent infection may result from an intermittent escape of CD4 T cells from immune suppression such as that imposed by regulatory T cells (28). Alternatively, the transiently responding T cells may represent newly primed T cells specific for subdominant OM antigens (22), as has been observed in other chronic infections (29, 30).

Because the hallmark of T-cell exhaustion during chronic infection is the progressive loss of cytokine secretion in addition to proliferation, we also measured IL-2, IFN-γ, and TNF-α at several time points over the course of immunization, infection, and treatment. ELISAs were performed to measure bovine IL-2 and TNF-α with DuoSet ELISA kits from R&D Systems, and bovine IFN-γ was measured with an ELISA VetSet kit from Kingfisher Biotech, Inc., according to manufacturer’s specifications. A SpectraMax190 Microplate Reader was used and the data analyzed with SoftMax Pro (v. 5.2) software (both from Molecular Devices). Cryopreserved PBMC were obtained at four time points: preimmunization, postimmunization and 13 to 15 weeks preinfection, 16 or 19 weeks postinfection and pretreatment, and 34 or 38 weeks postinfection and 15 or 19 weeks posttreatment. PBMC were cultured at 2.5 x 10⁶ cells/ml in complete RPMI medium with 3 μg/ml A. marginale OM for 72 h, and supernatants harvested by centrifugation were stored at −20°C until

![FIG 3 Sustained A. marginale-specific proliferative responses of PBMC in tetracycline-treated cattle. Three days following an oxytetracycline injection, on week 19 postinfection, five cattle began treatment with oral tetracycline for 30 days as indicated by an arrow. PBMC obtained at the indicated weeks postinfection were tested with 10 μg/ml OM or URBC and the results are presented as stimulation indices ± 1 SD of triplicate cultures (where stimulation index is equal to the mean cpm of PBMC cultured with OM/mean cpm of PBMC cultured with URBC). Significant proliferative responses from week 21 to week 38 postinfection were compared to pretreatment values (week 19 postinfection) determined by two-way ANOVA and a Dunnett’s multiple-comparison test across the time points. Asterisks indicate significant responses with a P value of <0.05.](http://cvi.asm.org/)
use. Thawed, undiluted supernatants were tested by enzyme-linked immunosorbent assay (ELISA), and the results are shown in Table 3 for both treated and untreated groups. All of the supernatants had IL-2 levels below the detection limits of the assay, where the standard curve ranged from 0.156 to 10.0 ng/ml. However, all cattle responded to immunization at weeks 13 to 15 pre-infection by producing IFN-γ and all but two produced TNF-α, but only cattle that were treated with tetracycline maintained one or both cytokine responses after treatment and clearance of infection. According to a Student one-tailed paired t test, there were significant IFN-γ and TNF-α responses postimmunization compared to preimmunization in each group (P < 0.05), and a value of 0 was assigned to all negative ELISA readings. By week 16 or 19, the responses were no longer significant in either group. By week 34 or 38, significant responses were detected only in the treated group (P < 0.032). When the two groups were compared using a two-tailed t test, there were no significant differences in postimmunization levels of IFN-γ (P = 0.41) or TNF-α (P = 0.46). In contrast, there were significantly higher levels of IFN-γ (P = 0.021) and TNF-α (P = 0.005) when cytokines were compared at week 34 or 38 postinfection in the two groups. These data support the hypothesis that infection with *A. marginale* leads to functional T-cell exhaustion, which is reversed upon clearance of the bacterial infection.

A recent study by Hammac et al. (31) used a mutant *A. marginale* St. Maries strain, which expressed the green fluorescence protein (gfp) gene and had slower growth kinetics in vitro than the parental strain, to determine protection against the wild-type bacteria. In this study, the mutant bacterial strain failed to cause clinical disease, but as expected, the animals were protected against virulent challenge. This was expected because it is well known that persistently infected cattle are protected against a homologous strain challenge, termed premunition (32, 33). It is not clear why the mutant strain had an attenuated phenotype in vivo, but it was maintained in *Ixodes scapularis* ISE6 cell culture and had reduced expression of genes coding for an iron transporter, an RNA cleaving enzyme, and genes involved in translation, translation elongation, and purine biosynthesis (34). Lack of virulence by the mutant strain and induction of protection against virulent St. Maries strain bacteria that had not been passaged in tick cells are possibly explained by the inability of the mutant bacteria to rapidly replicate to high levels, so that protective immune responses were induced before the infection could become acute. As T-cell and antibody responses in the cattle were not determined, it is not known whether CD4 T-cell responses were induced and maintained or experienced exhaustion similar to what we have described. However, these results are not incompatible with our results showing that immunization-induced CD4 T-cell responses become ex-

![FIG 4 Lack of sustained *A. marginale*-specific proliferative responses of PBMC in untreated cattle. PBMC obtained at the indicated weeks postinfection were tested with 10 μg/ml OM or URBC and the results are presented as stimulation indices ± 1 SD of triplicate cultures (where stimulation index is equal to the mean CPM of PBMC cultured with OM/mean CPM of PBMC cultured with URBC). Significant proliferative responses from week 21 to week 38 postinfection were compared to pretreatment values (week 19 postinfection), determined by two-way ANOVA and a Dunnett’s multiple-comparison test across the time points. Asterisks indicate significant responses with a P value of <0.05.](http://cvi.asm.org/)
TABLE 3 Cytokine responses over the course of immunization and infection in treated and untreated cattle

| Animal no. and time pointa | Cytokine production (ng/ml) (SD)b | IFN-γ | TNF-α |
|---------------------------|----------------------------------|-------|-------|
| **Treated cattle**         |                                   |       |       |
| 35113 Pre                  | Negc                            | Neg   | Neg   |
| −14 0.795 (0.082) 0.135 (0.001) |       |       |
| 19 0.217 (0.101) 0.078 (0.016) |       |       |
| 38 0.402 (0.063) 0.289 (0.037) |       |       |
| 35141 Pre                  | Negc                            | Neg   | Neg   |
| −15 0.397 (0.109) 0.137 (0.061) |       |       |
| 19 Neg Neg Neg             |       |       |
| 38 0.153 (0.101) 0.453 (0.084) |       |       |
| 35160 Pre                  | Negc                            | Neg   | Neg   |
| −15 0.452 (0.105) 0.121 (0.061) |       |       |
| 19 Neg Neg Neg             |       |       |
| 34 0.265 (0.040) 0.699 (0.062) |       |       |
| 35280 Pre                  | Negc                            | Neg   | Neg   |
| −14 0.211 (0.024) Neg      |       |       |
| 19 Neg Neg Neg             |       |       |
| 38 Neg 0.206 (0.035)       |       |       |
| 583 Pre                    | 0.141 (0.057) Neg               | Neg   |       |
| −15 1.794 (0.339) 0.643 (0.038) |       |       |
| 19 Neg Neg Neg             |       |       |
| 38 0.216 (0.049) 0.187 (0.001) |       |       |
| **Untreated cattle**       |                                   |       |       |
| 35967 Pre                  | Negc                            | Neg   | Neg   |
| −13 0.536 (0.174) Neg      |       |       |
| 16 Neg Neg Neg             |       |       |
| 38 Neg Neg Neg             |       |       |
| 36092 Pre                  | Negc                            | Neg   | Neg   |
| −13 0.321 (0.067) 1.063 (0.008) |       |       |
| 16 Neg Neg Neg             |       |       |
| 34 Neg Neg Neg             |       |       |
| 36345 Pre                  | Negc                            | Neg   | Neg   |
| −13 0.287 (0.122) 0.508 (0.004) |       |       |
| 19 Neg Neg Neg             |       |       |
| 34 Neg Neg Neg             |       |       |
| 36381 Pre                  | Negc                            | Neg   | Neg   |
| −13 0.406 (0.147) 0.204 (0.047) |       |       |
| 16 Neg Neg Neg             |       |       |
| 34 Neg Neg Neg             |       |       |
| 36413 Pre                  | Negc                            | Neg   | Neg   |
| −13 0.410 (0.201) 0.543 (0.007) |       |       |
| 16 Neg Neg Neg             |       |       |
| 38 Neg Neg Neg             |       |       |

a PBMC were obtained preimmunization, postimmunization, 13 to 15 weeks before infection, 16 or 19 weeks postinfection (before tetracycline treatment), and 34 or 38 weeks postinfection (15 to 19 weeks after tetracycline treatment was initiated).
b Cytokine concentrations in 72-h supernatants from PBMC were determined by ELISA performed in duplicate and using a standard curve ranging from 0.125 to 8.0 ng/ml IFN-γ or TNF-α.
c Neg indicates that optical density readings were below the range of the standard curve.

during acute infection. They are also not contradictory to our results showing that during virulent *A. marginale* infection in immunologically naive animals, CD4 T-cell proliferative responses were sporadic and transient in both blood and spleen, and IFN-γ-secreting cell numbers were not significantly increased in response to antigen (20). However, in a study by Han et al. (20), there was paradoxically a strong IgG2 response by day nine postinfection, and acute infection was controlled in spite of undetectable antigen-specific CD4 T-cell responses in blood and spleen. These cells had an exhausted phenotype defined in our studies, including the one reported here, as lack of proliferation (IL-2 production) and IFN-γ secretion in response to *A. marginale* (17, 19, 20).

In summary, we provide evidence that immunization of cattle with *A. marginale* OM failed to provide complete protection against *D. andersoni*-transmitted infection with the homologous strain from which OM were prepared. Immunization-induced T-cell proliferative responses were uniformly lost after the peak of acute infection, as we previously reported for cattle immunized with a native or recombinant MSP. In addition, IFN-γ and TNF-α responses were generally lower or lost by 16 to 19 weeks postinfection, before tetracycline therapy was initiated. The loss of response is regulated by the infection itself, as clearance of infection with tetracycline restored the antigen-specific response determined as proliferation and production of IFN-γ and/or TNF-α in all treated cattle. Thus, our results suggest the bacterial infection is required to maintain a suppressed, dysfunctional T-cell response during persistent anaplasmosis.

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