ANTIGEN-SPECIFIC $CD4^+$ T CELLS IN

ANAPLASMA MARGINALE INFECTION OF CALVES

By

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Abstract

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Acquired T-cell immunity is central for protection against intracellular infection. However, the role of antigen-specific T cells in persistent systemic disease is poorly understood. We investigated this with the ruminant bacterium *Anaplasma marginale*, which replicates to 10^9 bacteria/ml of blood during acute infection and 10^6 bacteria/ml throughout persistent anaplasmosis. We established that immunization-specific $CD4^+$ T cell responses were rapidly and permanently lost following challenge. To determine the fate of antigen-specific $CD4^+$ T cells we enumerated specific T cells with *DRB3**1101 MHC class II tetramers and FACS. Immunization produced antigen-specific T cell frequencies of 0.025% of $CD4^+$ T cells that rapidly declined near peak bacteremia to 0.0003%. Low frequencies of $tetramer^+$ $CD4^+$ T cells in spleen, liver, and lymph nodes 9-12 weeks post-challenge indicated lack of sustainable antigen-specific responses in these lymphoid compartments. Infection of cattle with *A. marginale*, therefore, caused rapid, permanent loss of antigen-specific T cells, which may be a strategy of immune modulation for *A. marginale*. We next examined the role of specific-$CD4^+$ T cells primed by infection by observing serum, PBMC, and spleen for antigen-specific humoral and $CD4^+$ T cell responses during acute and persistent infection of naïve calves. Calves developed

high titers of antigen-specific IgG day 9 post-infection, but specific T cells appeared in PBMC day 39 and as late as day 112 post-infection. Responding T cells were predominantly $CD4^+$ T cells that did not produce IFN-γ, and responses were transient, disappearing rapidly and reappearing sporadically 8 or less times in one year. Splenocytes developed initial antigen-specific T cell responses days 48 and 89 post-infection, and responses were similarly transiently with no evidence of sequestration during infection. In conclusion, *A. marginale*-specific $CD4^+$ T cells primed by infection occur in PBMC and spleen during acute and persistent infection well after emergence of high titers of specific IgG, and cells are continuously down regulated similar to immunization-specific T cells, in the face of persistently high level bacteremia.

TABLE OF CONTENTS

Page

CHAPTER TWO

LIST OF TABLES

Page

LIST OF FIGURES

Page

GENERAL INTRODUCTION

Pathogens that cause chronic infection have evolved many different strategies to persist in the immunocompetent host, which involve subversion or evasion of host innate or acquired immune responses (1-4). Continual exposure to high antigen load during persistent viral infection can lead to induction of regulatory T cells that suppress protective effector T cells, and to a poorly understood progressive dysfunction whereby T cells lose effector function in response to antigen, and are eventually deleted (4, 5). Furthermore, with certain persistent viral infections, such as hepatitis C virus, antigen-specific T cells can be compartmentalized to the liver (5). There are very few examples in the literature of how bacteria block acquired immune responses (reviewed in ref. 2 and 3), and specifically the consequences of high antigen load during persistent infection with blood-borne bacterial or protozoal pathogens such as *Anaplasma, Borrelia,* and *Trypanosoma* on antigen-specific memory and effector T cell responses have not been well characterized.

Anaplasma marginale is a tick-borne, intra-erythrocytic rickettsial pathogen that causes significant morbidity and mortality, and life-long persistent infection in ruminants. Notably, naïve cattle infected with *A. marginale* develop high levels of bacteremia, reaching 10^9 organisms/ml of blood, within two to five weeks post-infection. The infection results in anemia, abortion, and mortality as high as 30%, but cattle that survive acute disease remain persistently infected for life with cyclically peaking, but subclinical levels of bacteremia averaging 10^6 organisms/ml of blood (6, 7). Persistence is due, at least in part, to extensive antigenic variation in immunodominant and abundant major surface protein (MSP2) 2 and MSP3 (8). Antigenic variation in MSP2, which is rich in T and B lymphocyte epitopes, allows the organism to continually escape specific adaptive immune responses (9-13).

In a previous study, we unexpectedly observed the rapid loss of immunization-induced MSP2-specific $CD4^+$ T lymphocyte responses from peripheral blood following *A. marginale* challenge (13). Proliferation and IFN-γ responses disappeared coincident with peak bacteremia, and remained undetectable for up to one year, when the study was terminated. In contrast, responses to unrelated *Clostridium* sp. vaccine antigen administered prior to MSP2 immunization, remained intact throughout the course of the study (13). This rapid loss of antigen-specific T-cell responses following infection contradicts the paradigm that once established, memory $CD4^+$ T cells rapidly expand and execute effector functions upon infection. Furthermore, the modulation of specific host immunity by high antigen load during infection may represent an important mechanism to facilitate persistent infection by *A. marginale.* Whether high antigen load drives antigen-specific cells to physical deletion via activation induced cell death, to anergy from exposure of specific-T cells to antigenically variant epitopes (14, 15), or to sequestration in peripheral lymphoid tissues, such as the spleen, is unknown. To distinguish among these possibilities, and to determine if ablation of the pre-existing immune response following infection occurs with another non-variant immunogen, the study in chapter one tracked antigen-specific $CD4^+$ T lymphocytes in calves immunized with a conserved T cell epitope from *A. marginale* MSP1a, peptide F2-5 (16, 17), before and after homologous strain challenge. F2-5-specific $CD4^+$ T cell responses were measured and specific T cells were enumerated by analyzing $CD4^+$ T lymphocytes stained with a bovine major histocompatibility (MHC) class II tetramer linked to the F2-5B peptide epitope (18). The results are consistent with an *A. marginale*-induced rapid death of antigen-specific $CD4^+$ T cells that results in a loss of functional memory and, more broadly, may represent an important means for persistence of this pathogen in the immunocompetent host.

Our initial study showed that immunization-induced antigen-specific $CD4^+$ T cell responses are vulnerable to challenge with live organism; however, the paradigm of immune control of natural *A. marginale* infection in naïve cattle is thought to be dependent on establishment of antigen-specific $CD4^+$ T lymphocytes early in infection upon initial antigen exposure. These primed $CD4^+$ T cells are assumed to be instrumental in activating B cells, macrophages, and inducing isotype switching to antigen-specific IgG1 and IgG2 antibody, a prominent feature of cattle that successfully control bacteremia (6, 13, 19, 20, 21, 22, 23). Interestingly, in the two previous studies, all immunized and control calves infected with *A. marginale* Florida strain, successfully controlled clinical anaplasmosis and developed early and long-term antigen-specific IgG1 and IgG2 antibody targeted to immunodominant outer membrane proteins, but never developed detectable $CD4^+$ T cell responses specific for *A. marginale* lysate in the PBMC (13, 24). This suggests that infection with *A. marginale* not only down regulated pre-existing $CD4^+$ T cell responses induced by immunization, but also impaired the response primed by infection to other antigens not present in the immunogen.

These results challenge the current paradigm of the role of $CD4^+$ T cells in immune control of *A. marginale.* However, the lack of detectable antigen-specific $CD4^+$ T cell responses in blood may be due to the sampling interval or the strain of bacteria used (non-tick transmissible Florida strain). Furthermore, during acute infection, specific $CD4^+$ T cells may be sequestered in secondary lymphoid organs such as the spleen. We therefore, hypothesized that *A. marginale* infection of the naïve calf is characterized by early generation of antigen-specific $CD4^+$ T lymphocytes in the PBMC and spleen. In chapter two we investigated these possibilities by measuring *A. marginale*-specific T cell and antibody responses in calves during the acute and persistent phases of infection with either tick transmissible South Idaho strain or non-tick transmissible Florida strain organisms. In the first case, calves were monitored weekly for

antigen-specific humoral (IgG) and T-lymphocyte responses in the PBMC for nearly one year. In the second case, calves were monitored for antigen-specific humoral and lymphocyte responses every 3 to 7 days in blood and in aspirates collected from exteriorized spleens for 4 months following infection.

In all calves, regardless of route of infection and infected strain, *A. marginale* antigen-specific $CD4^+$ T lymphocyte responses were detected in the PBMC following the peak of infection concurrent with decreasing or basal levels of bacteremia. T cell responses, measured as proliferation, were found to be transient, immediately disappearing from the PBMC in the subsequent 1 to 2 weeks and recurring sporadically. Antigen-specific T cell responses in spleens were similar though less frequent, showing there was no sequestration of antigen-specific $CD4^+$ T cells in the spleen during acute or chronic infection. Antigen-specific IgG responses, however, occurred very early in infection and high titers persisted long-term throughout the course of the study. Our results are consistent with down regulation of *A. marginale*-specific $CD4^+$ T lymphocytes primed during acute infection. This regulation or deletion of specific-T cell responses occurs in the face of high titers of *A. marginale*-specific IgG which is present throughout infection.

REFERENCES

1. Sacks, D., and A. Sher. 2002. Evasion of innate immunity by parasitic protozoa *Nat. Immunol.* 3: 1041-1047.
2. Hornef, M. W., M. J. Wick, M. Rhen, and S. Normark. 2002. Bacterial strategies for overcoming host innate and adaptive immune responses. *Nat. Immunol.* 3: 1033-1040.
3. Finlay, B. B., and G. McFadden. 2006. Anti-immunology: evasion of the host immune system by bacterial and viral pathogens. *Cell* 124: 767-782.
4. Belkaid, Y. 2007. Regulatory T cells and infection: a dangerous necessity. *Nat. Rev. Immunol.* 7: 875-888.
5. Klenerman, P., and A. Hill. 2005. T cells and viral persistence: lessons from diverse infections. *Nat. Immunol.* 6: 873-879.
6. Palmer, G. H., F. R. Rurangirwa, K. M. Kocan, and W. C. Brown. 1999. Molecular basis for vaccine development against the ehrlichial pathogen *Anaplasma marginale. Parasitol. Today* 15: 281-286.
7. Futse, J. E., K. A. Brayton, M. J. Dark, D. P. Knowles, and G. H. Palmer. 2008. Superinfection as a driver of genomic diversification in antigenically variant pathogens. *Proc. Natl. Acad. Sci. USA* 105: 2123-2127.
8. Palmer, G. H., and K. A. Brayton. 2007. Gene conversion is a convergent strategy for pathogen antigenic variation. *Trends Parasitol.* 23:.408-413.
9. French, D. M., T. F. McElwain, T. C. McGuire, and G. H. Palmer. 1998. Expression of *Anaplasma marginale* major surface protein 2 variants during persistent cyclic rickettsemia. *Infect. Immun.* 66: 1200-1207.
10. French, D. M., W. C. Brown, and G. H. Palmer. 1999. Emergence of *Anaplasma marginale* antigenic variants during persistent rickettsemia. *Infect. Immun.* 67: 5834-5840.

11. Brown, W. C., K. A. Brayton, C. M. Styer, and G. H. Palmer. 2003. The hypervariable region of *Anaplasma marginale* major surface protein 2 (MSP2) contains multiple immunodominant $CD4^+$ T lymphocyte epitopes that elicit variant-specific proliferative and IFN- responses in MSP2 vaccinates. *J. Immunol.* 170: 3790-3798.

12. Abbott, J. R., G. H. Palmer, C. J. Howard, J. C. Hope, and W. C. Brown. 2004. *Anaplasma marginale* major surface protein 2 $CD4^+$- T-cell epitopes are evenly distributed in conserved and hypervariable regions 1 (HVR), whereas linear B-cell epitopes are predominantly located in the HVR. *Infect. Immun.* 72: 7360-7366.

13. Abbott, J. R., G. H. Palmer, K. A. Kegerreis, P. F. Hetrick, C. J. Howard, J. C. Hope, and W. C. Brown. 2005. Rapid and long-term disappearance of $CD4^+$ T lymphocyte responses specific for *Anaplasma marginale* major surface protein-2 (MSP2) in MSP2 vaccinates following challenge with live *A. marginale. J. Immunol.* 174: 6702-6715.

14. Bielekova, B., and R. Martin. 2001. Antigen-specific immunomodulation via altered peptide ligands. J. Mol. Med. 79: 552-565.

15. Plebanski, M., E. A. Lee, and A. V. Hill. 1997. Immune evasion in malaria: altered peptide ligands of the circumsporozoite protein. Parasitology 115(Suppl.): S55-S66.

16. Brown, W. C., T. C. McGuire, W. Mwangi, K/ A. Kegerreis, H. Macmillan, H. A. Lewin, and G. H. Palmer. 2002. Major histocompatibility complex class II DR-restricted memory $CD4^+$ T lymphocytes recognize conserved immunodominant epitopes of *Anaplasma marginale* major surface protein 1a. *Infect. Immun.* 70:5521-5532.

17. Mwangi, W., W. C. Brown, G. A. Splitter, C. J. Davies, C. J. Howard, J. C. Hope, Y. Aida, Y. Zhuang, B. J. Hunter, and G. H. Palmer. 2007. DNA vaccine construct incorporating intercellular trafficking and intracellular targeting motifs effectively primes and induces memory B- and T-cell responses in outbred animals. *Clin. Vaccine Immunol.* 19 14: 304-311.

18. Norimine, J., S. Han, and W. C. Brown. 2006. Quantitation of *Anaplasma marginale* major surface protein (MSP)1a and MSP2 epitope-specific $CD4^+$ T lymphocytes using bovine DRB3*1101 and DRB3*1201 tetramers. *Immunogenetics* 58: 726-739.

19. Tebele, N., T. C. McGuire, and G. H. Palmer**.** 1991. Induction of protective immunity using *Anaplasma marginale* initial body membranes. *Infect. Immun*. **59:**3199–3204.

20. Stich, R. W., L. K. M. Shoda, M. Dreewes, B. Adler, T. W. Jungi, and W. C. Brown. 1998. Stimulation of nitric oxide production in macrophages by *Babesia bovis*. *Infect. Immun.* 66:4130–4136.

21. Palmer, G. H., and T. C. McGuire. 1984. Immune serum against *Anaplasma marginale* initial bodies neutralizes infectivity for cattle. *J. Immunol*. 133: 1010–1015.

22. Palmer G.H., and T.F. McElwain. 1995. Molecular basis of vaccine development against anaplasmosis and babesiosis. *Vet Parasitology*. 57: 233-253.

23.Gale K.R., G. Leatch, M. Gartside, and C.M. Dimmock. 1992. *Anaplasma marginale*: Failure of sera from immune cattle to confer protection in passive-transfer experiments. *Parasitol Res.* 78: 410-415.

24. Han S., J. Norimine, G.H. Palmer, W. Mwangi, K.K. Lahmers, and W.C. Brown. 2008. Rapid deletion of antigen-specific $CD4^+$ T cells following infection represents a strategy of immune evasion and persistence for *Anaplasma marginale*. *J. of Immunol*. 181: 7759-7769.

25. Semmo N., C.L. Day, S.M. Ward, M. Lucas, G. Harcourt, A. Loughry, and P. Klenerman. 2004. Preferential loss of IL-2-secreting CD4+ T helper cells in chronic HCV infection. *Hepatology*. 41: 1019-1028.

26. Fuller M.J., and A.J. Zajac. 2003. Ablation of CD8 and CD4 cell responses by high viral

loads. *J. Immunol*. 170: 477-486.

27. Millington K.A, J.A. Innes, S. Hackforth, T.S.C. Hinks, J.J. Deeks, D.P.S. Dosanjh, V. Guyot-Revol, R. Gunatheesan, P. Klenerman, and A. Lalvani. 2007. Dynamic relationship between IFN-γ and IL- 2 profile of *Mycobacterium tuberculosis*-specific T cells and antigen-load. *J Immunol*. 178: 5217-5226.
28. Sathler-Avelar R., D.M. Vitelli-Avelar, A. Teixeira-Carvalho, and O.A. Martins-Filho. 2009. Innate immunity and regulatory T-cells in human Chagas disease: what must be understood? *Mem Inst Oswaldo Cruz*. 104: 246-251.
29. Mebius R.E., and G. Kraal. 2005. Structure and function of the spleen. *Nature Rev. Immunol*. 5: 606-616.

CHAPTER ONE

INTRODUCTION

Rapid deletion of antigen-specific CD4$^+$ T cells following infection represents a strategy of immune evasion and persistence for *Anaplasma marginale*

Pathogens that cause chronic infection have evolved many different strategies to persist in an immunocompetent host, which involve subversion or evasion of host innate or acquired immune responses (1-4). Continual exposure to high antigen load during persistent viral infection can lead to induction of regulatory T cells that suppress protective effector T cells, and to a poorly understood progressive dysfunction whereby T cells lose effector function in response to antigen, and are eventually deleted (4, 5). Furthermore, with certain persistent viral infections, such as hepatitis C virus, antigen-specific T cells can be compartmentalized to the liver (5). There are very few examples in the literature of how bacteria block acquired immune responses (reviewed in ref. 2 and 3), and specifically the consequences of high antigen load during persistent infection with blood-borne bacterial or protozoal pathogens such as *Anaplasma, Borrelia,* and *Trypanosoma* on antigen-specific memory and effector T cell responses have not been well characterized. *Anaplasma marginale* is a tick-borne, intra-erythrocytic rickettsial pathogen that causes significant morbidity and mortality, and life-long persistent infection in ruminants. Notably, naïve cattle infected with *A. marginale* develop high levels of bacteremia, reaching 10^9 bacteria per ml of blood, within two to five weeks post-infection. The infection results in anemia, abortion, and mortality as high as 30%, but cattle that survive acute disease remain persistently infected for life with cyclically peaking, but subclinical levels of bacteremia averaging 10^6 bacteria per ml of blood (6, 7). Persistence is due, at least in part, to extensive antigenic variation in immunodominant and abundant major surface protein (MSP2) 2 and MSP3

(8). Antigenic variation in MSP2, which is rich in T and B lymphocyte epitopes, allows the organism to continually escape specific adaptive immune responses (9-13).

In a previous study, we unexpectedly observed the rapid loss of immunization-induced MSP2-specific $CD4^+$ T lymphocyte responses from peripheral blood following *A. marginale* challenge (13). Proliferation and IFN- responses disappeared coincident with peak bacteremia, and remained undetectable for up to one year, when the study was terminated. In contrast, responses to unrelated *Clostridium* sp. vaccine antigen administered prior to MSP2 immunization, remained intact throughout the course of the study (13). This rapid loss of antigen-specific T-cell responses following infection contradicts the paradigm that once established, memory $CD4^+$ T cells rapidly expand and execute effector functions upon infection. Furthermore, the modulation of specific host immunity by high antigen load during infection may represent an important mechanism to facilitate persistent infection by *A. marginale.* Whether high antigen load drives antigen-specific cells to physical deletion via activation induced cell death, to anergy from exposure of specific-T cells to antigenically variant epitopes (14, 15), or to sequestration in peripheral lymphoid tissues, such as the spleen, is unknown. To distinguish among these possibilities, and to determine if ablation of the pre-existing immune response following infection occurs with a non-variant immunogen, the present study tracked antigen-specific $CD4^+$ T lymphocytes in calves immunized with a conserved T cell epitope from *A. marginale* MSP1a, peptide F2-5 (16, 17), before and after homologous strain challenge. F2-5-specific $CD4^+$ T cell responses were measured and specific T cells were enumerated by analyzing $CD4^+$ T lymphocytes stained with a bovine major histocompatibility (MHC) class II tetramer linked to the F2-5B peptide epitope (18). The results are consistent with an *A. marginale*-induced rapid death of antigen-specific $CD4^+$ T cells that results in a loss of

functional memory and, more broadly, may represent an important means for persistence of this pathogen in the immunocompetent host.

MATERIALS AND METHODS

Cattle

Holstein calves serologically negative for *A. marginale* received Vision 7® killed *Clostridium* spp. vaccine (Intervet) prior to the start of the study. All calves were heterozygous for the MHC class II *DRB3*1101* gene haplotype, which optimally presents the MSP1a F2-5 T cell epitope GVSYNDGNASAARSVLETLAGHVDALGIS (16, 19). Calves were immunized intradermally as described with the mammalian VR-1055 vector encoding the DRB3*1101-restricted 30-mer F2-5 epitope (17). Control calves were inoculated intradermally with empty vector. All calves were boosted on day 105 post-immunization with 20 μg MSP1a F2-5 peptide in alum (Pierce) diluted in PBS pH 7.2, and administered subcutaneously. Blood was collected very 1-2 wks and PBMC were cryopreserved in liquid nitrogen. All animal studies were conducted using an approved Institutional Animal Care and Use Center protocol.

Challenge with A. marginale

Eleven months after the primary immunization, four immunized and four control calves were challenged intravenously with 1 x 10^6 erythrocytes infected with viable Florida strain *A. marginale*. The packed cell volume, percent infected erythrocytes, determined by light microscopy of Giemsa-stained blood films, and general health were monitored daily. Blood was collected weekly, and PBMC purified by Histopaque® (Sigma) density gradient centrifugation were either used immediately for proliferation assays or cryopreserved for additional assays.

Spleen, liver and lymph node lymphocytes

All immunized and two control calves were euthanized between 63 and 89 days post-challenge and samples of spleen, liver, and inguinal lymph nodes (LN) were collected. Lymphocytes were purified from gently disrupted spleen and LN samples by Histopaque® gradient centrifugation, repeatedly washed with HBSS, and resuspended in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum, 100 mM L-glutamine, 5 x 10^{-5} β-M mercaptoethanol, 24 mM HEPES buffer, and 0.05 mg/ml gentamicin sulfate (complete medium). A sample of liver was perfused with cold PBS, ground in a meat grinder with RPMI-1640 medium, digested with 0.002% DNase I (Sigma) and 0.05% bovine collagenase IV (Sigma) in RPMI medium for 50 min at 37^{o} C, and lymphocytes were extracted by repeated Histopaque® centrifugation and washing in HBSS. Lymphocytes from the tissues were used directly in proliferation assays and extra cells were cryopreserved.

Lymphocyte proliferation and IFN-γ ELISPOT assays

Lymphocyte proliferation assays were performed in triplicate using 2.5 x 10^5 viable cells/well in complete medium (12, 13), and unless indicated otherwise, freshly obtained PBMC were used. Cryopreserved PBMC were used for the day 144 post-immunization time point for the data shown in Fig. 4. Antigens consisted of 1 and 10 μg/ml *A. marginale* Florida strain homogenate, uninfected erythrocyte membranes (URBC), MSP1a peptide F2-5, and negative control peptides *B. bovis* rhoptry associated protein-1 (RAP-1) peptide P2 [(LRFRNGANHGDYHYFVTGLLNNNVVHEEGT; (20)], and *A. marginale* MSP2 peptide P1 [MSAVSNRKLPLGGVLMALVAAVAPIHSLLA; (21)] designated MSP2 P1. T cell growth factor diluted 1:10 and *Clostridium* spp. vaccine antigen diluted 1:100 or 1:1000 in complete medium were positive controls. After 6 days, cells were labeled with 0.25 μCi [^{3}H] thymidine for

8 hr, harvested, and counted in a counter. Statistical significance was determined using a one-way ANOVA with a Tukey correction for multiple comparisons, and results were considered significant when $P < 0.05$. For IFN-γ ELISPOT assays (12, 13, 17), cryopreserved PBMC from multiple time points were tested in the same assay to eliminate assay-to-assay variation. Cells were thawed, purified over Histopaque, and cultured in triplicate at 0.5 x 10^6 cells/well with 0.5 x 10^6 irradiated PBMC/well from a donor homozygous for *DRB3*1101,* and 1 or 10 μg/ml peptide F2-5 or negative control peptide RAP-1 P2. A mixture of PHA (Sigma) (1 μg/ml) plus 0.01ng/ml recombinant human IL-12 (Genetics Institute) was a positive control. Results are presented as the mean number (no.) of spot forming cells (SFC)/10^6 PBMC after subtracting the background mean no. SFC from cells cultured with RAP-1 P2 peptide. Significant responses to peptide F2-5 were determined using a Student's one-tailed t-test where $P < 0.05$

*Construction of DRB3*1101-F2-5B and DQA*1001/DQB*1002-F3-3 tetramers*

Construction of the phycoerythrin (PE)-labeled DRB3*1101-F2-5B tetramer was previously described (18). The *Anaplasma marginale* MSP1a F3-3 peptide (30-mer) was previously identified as a $CD4^+$ T cell epitope restricted by MHC class II DQA*1001/DQB*1002 (16, 19) and the epitope was further defined to a 15-mer peptide, PAGQEKTAEPEHEAA. To construct the DQA*1001/DQB*1002-F3-3 tetramer, eukaryotic expression vectors (pcDNA3.1) expressing the DQA*1001-basic leucine zipper-biotinylation sequence (DQA*1001-blz-bs) and the DQB*1002-acidic leucine zipper-FLAG (DQB*1002-abz-FLAG) were generated using the same PCR method as previously described (18). The templates for the PCR were *pCR3.1BoLADQA*1001* and *pCR3.1BoLADQB*1002*, respectively (19). The DQB*1002-abz-FLAG construct was further modified by linking the T-cell epitope,

PAGQEKTAEPEHEAA, between the signal sequence and the N-terminal end of matured DQB*1002 sequence with 16 amino acid linker using the same protocol as previously described (18). The signal sequence was later replaced with the DRB3*1201 leader sequence (18) for better expression. The primers used for the construction of the DQA*1001/DQB*1002F3-3 tetramer are listed in Table I or were previously described (18). Production and purification of soluble DQA*1001/DQB*1002F3-3 dimers and construction of the PE-labeled tetramer were performed as previously described (18), and this tetramer was used as a negative control.

Enumeration of F2-5-specific $CD4^+$ T cells by tetramer staining

The previously described PE-labeled DRB3*1101-F2-5B tetramer (18) was used to enumerate F2-5-specific T cells in PBMC and tissue lymphocytes, after selecting $tetramer^+$ cells with anti-PE microbeads (Miltenyi Biotec) (22). The 16-mer F2-5B peptide (ARSVLETLAGHVDALG) T-cell epitope in peptide F2-5, is presented by DRA/DRB3*1101(16, 18, 19). For each animal, cryopreserved PBMC from multiple time points were tested in the same assay to eliminate assay-to-assay variation. A number of samples were also independently stained by two individuals to verify reproducibility of the staining. PBMC (up to 20 x 10_6) were purified by Histopaque® gradient centrifugation, washed 3 times and resuspended in complete medium, mixed with 20 μg/ml of PE-labeled DRB3*1101-F2-5B tetramer, and incubated in the dark at room temp for 1 h. Cells were then stained with a mixture of FITC-conjugated mouse anti-bovine $CD4^+$ monoclonal antibody (MAb) MCA1365 (Serotec), Alexa Fluor (AF)-647 conjugated mouse anti-human $CD14^+$ MAb MCA1568A647 (Serotec), and MAbs obtained from the Washington State University MAb Center specific for bovine $CD8^+$ (7C2B), $CD21^+$ (GB25A), and the TCR chain (GB21A), each conjugated with AF-647 (Invitrogen) and used at a final concentration of 10 μg/ml. 7-amino-actinomycin D (10 μg/ml)

(Sigma) was added to label and later exclude any dead cells. Cells were incubated in the dark at room temp for 20 min, washed three times with MACS buffer (1X PBS, Ph 7.2, 0.5% bovine serum albumin, and 2 mM EDTA). Cells (10%) were set aside for FACS analysis, and 90% were resuspended to 160 µl of bead buffer, mixed with 40 µl mouse anti-PE microbeads (Miltenyi Biotec), incubated in the dark for 20 min at 4^{o} C, and passed through a MS column placed in a magnetic field (Miltenyi Biotec) to positively select PE-tetramer stained cells. Cells were analyzed using four-color flow cytometry (18), and frequencies of F2-5-specific $CD4^{+}$ T cells were derived by dividing the number of $Tetramer^{+}$ $CD4^{+}$ cells by the total number of $CD4^{+}$ T cells in the starting population, reported as a percentage of this population of cells. Significant differences in frequencies of $tetramer^{+}$ $CD4^{+}$ T cells in the vaccinates at different times and in different tissues were determined using one-tailed, paired t-tests where $P<0.05$.

RESULTS

Loss of A. marginale peptide F2-5-specific IFN-γ-secreting cells following infection

We wanted to determine if the previously observed sudden loss of effector responses following infection is specific to the highly abundant and antigenically variable MSP2 (13), or is reproducible for other *A. marginale* antigens. We used animals immunized with a DNA construct that had developed strong $CD4^{+}$ T cell responses directed against a very immunogenic epitope in the conserved MSP1a protein, peptide F2-5 (17). Cattle immunized with the construct encoding the MSP1a F2-5 T cell epitope were infected 11 months later, monitored for anaplasmosis, and F2-5-specific IFN-γ-secreting cells in PBMC obtained weekly after challenge were enumerated by ELISPOT. Because calves were immunized with a construct encoding just one T-cell epitope and one B-cell epitope from MSP1a, we did not expect any protection against challenge. As predicted, all four vaccinates and four controls developed acute anaplasmosis, with bacteremias reaching maximal levels of 1.0-3.3% infected erythrocytes between 25-37 days post-infection

(data not shown). Infection was associated with anemia determined as a 33-51% decrease in packed cell volumes. All cattle resolved acute bacteremia by seven weeks post-infection. Weekly total leukocyte and differential white blood cell counts indicated no evidence of leucopenia or lymphopenia (data not shown). Overall, there was no significant change in $CD4^+$ T cell percentages throughout the course of infection, although all calves experienced a mild decline in the percentage of $CD4^+$ T cells during acute infection at the peak of bacteremia (Fig. 1). Additionally, histologically prepared tissue sections of liver and spleen from immunized calf 3959 and control calf 3989 at 11 weeks post-challenge lacked significant lesions (data not shown). Previous results from these animals showed that after a single DNA immunization, significant numbers of $CD4^+$ IFN-γ- secreting cells were detected in all vaccinates, and specific responses were significantly elevated following a peptide boost (17). At the time of challenge, significant numbers of IFN-γ- secreting cells were still detected in PBMC (Table II). In three animals, infection appeared to expand the number of IFN-γ- secreting cells two weeks post-infection. However, this response was no longer significant in any animal by four weeks post-infection, concomitant with peak bacteremias. These results are similar to those reported earlier for animals immunized repeatedly with MSP2 (13), except that in all MSP2 vaccinates there was a decreased frequency of IFN-γ- secreting cells between two to three weeks post-challenge (13). F2-5-specific IFN-γ-secreting cell numbers remained at background levels, similar to those of two control animals that were also followed, until the animals were euthanized five to nine weeks later. Thus, our results show that following *A. marginale* infection, the loss of antigen-specific T lymphocyte recall responses elicited by repeated immunization is not unique for the immunodominant and variable MSP2 protein, but occurs similarly in animals primed to a defined and conserved $CD4^+$ T cell epitope of MSP1a. Additionally, the route and type of immunization used to establish a specific T lymphocyte response was not a factor in the loss of the recall

response, as the results were similar when $CD4^+$ T lymphocyte responses were generated using protein inoculation with multiple protein boosts (13) or a single DNA inoculum with one protein boost (17). However, neither of these experiments can distinguish between a loss of effector function (i.e. IFN-γ secretion) or T cell anergy and a physical loss of antigen-specific T cells. Because we immunized with an MSP1a construct that contained a single T cell epitope, we were able to use this model to ask whether the loss of antigen-specific responses was caused by physical deletion of cells from the peripheral blood by staining F2-5-specific T cells with a bovine MHC class II tetramer.

*DRB3*1101-F2-5B tetramer staining detects $CD4^+$ T cells in PBMC*

We have previously shown that bovine MHC class II tetramers were able to detect antigen-specific T cells in cell lines or clones expanded from PBMC in vitro, and the specificity of the DRB3*1101-F2-5B tetramer was also demonstrated (18). This tetramer specifically stained T cells from an MSP1-immunized steer that were expanded ex vivo after several rounds of stimulation with native MSP1a and peptide F2-5B, whereas the same tetramer loaded with an irrelevant peptide did not (18). However, the frequency of antigen-specific $CD4^+$ T cells is too low in peripheral blood for direct detection by tetramer staining. We therefore enriched PE-labeled $Tetramer^+$ cells in PBMC with anti-PE beads prior to analysis using a method established for staining human PBMC with tetramers (22). To verify that this technique would enable direct detection of F2-5-specific T cells in PBMC, cryopreserved PBMC obtained before and after DNA immunization, and after the protein boost on day 105 were stained with the tetramer, enriched, and analyzed by flow cytometry. Cells obtained from multiple time points from an individual animal were stained in one experiment to eliminate any assay-to-assay variation. Fig. 2A presents results from animal 3959 from before immunization to day 298 post-immunization.

The numbers in the upper right quadrants are the calculated percentages of tetramer$^+$ CD4$^+$ T cells of the total number of CD4$^+$ T cells. In this animal, tetramer$^+$ CD4$^+$ T cells increased from a baseline of 0.0005% to 0.022 % by day 25 post-immunization, fell to about 50% of this level by day 95, and increased rapidly in response to the peptide boost on day 105 so that by 18 days post-boost, there was a frequency of 0.08% tetramer$^+$ CD4$^+$ T cells. This then declined to 0.025% on day 193 post-boost. To confirm the reproducibility of tetramer staining using cryopreserved T cells, and to document the specificity of staining, PBMC obtained at day 133 post-immunization were thawed and stained again with the DRB3*1101-F2-5B tetramer or with an irrelevant tetramer composed of DQA/DQB molecules and peptide MSP1a-F3-3 (16, 19, 23) not found in the immunogen (Fig. 2B). In this experiment, 0.0294% of the CD4$^+$ T cells were stained with the DRB3*1101-F2-5B tetramer, whereas a background level of staining was observed with the non-specific DQ-F3-3 tetramer. In contrast, the DQ tetramer stained 87.9% of cells in a T cell line from MSP1a-immunized animal 4919 (23) cultured with MSP1a for six weeks (data not shown). Thus, the specificity of the tetramer is documented by the lack of specific staining of PBMC obtained prior to immunization, and by the lack of non-specific staining by a tetramer containing a non-stimulatory peptide. The other three vaccinated animals had a similar pattern of expansion of peptide F2-5-specific T cells following immunization and boost, with baseline frequencies of tetramer$^+$ CD4$^+$ T cells of 0.0003% (animal 3937), 0.00196% (animal 3943) and 0.0009% (animal 3892) and reaching maximum frequencies of 0.051% (3937), 0.075% (3943) and 0.100% (3892) of the total CD4$^+$ T cell population following the peptide boost (Fig. 3). The increase in tetramer$^+$ CD4$^+$ cells in PBMC obtained following the peptide boost is consistent with significantly elevated CD4$^+$ T cell proliferation and numbers of IFN-γ secreting cells from these animals after the boost (17). Therefore, we proceeded to enumerate antigen-specific T cells after infection with *A. marginale.*

Loss of antigen-specific responses corresponds with loss of tetramer$^+$ CD4$^+$ T cells in PBMC

In addition to measuring IFN-γ secreting cells, proliferation was also used to measure antigen-specific T lymphocyte responses before and after challenge. Because the majority of proliferation assays were performed with fresh cells, whereas IFN-γ ELISPOT and tetramer staining experiments were performed with cryopreserved cells, there was a possibility that cryopreserved cells do not adequately represent the response of fresh cells. However, a direct comparison of lymphocyte proliferation to peptide F2-5 using fresh PBMC and cryopreserved PBMC obtained at the same time and tested 17 months later demonstrates that there was no loss of antigen-specific proliferation when the same number of PBMC were tested in each assay (Table III). Antigen-specific proliferation was compared with the frequency of antigen-specific cells, detected by tetramer staining, in peripheral blood of all four vaccinates from day 144 post-immunization, until challenge on day 321 and thereafter until acute bacteremias had resolved and the animals were euthanized (Fig. 4A-D). As expected, immunized calves had significant F2-5-specific T lymphocyte proliferative recall responses prior to challenge on day 321 and at 5 days post-challenge (day 326). On days 12 and 19 post-challenge, only PBMC from animals 3943 and 3959 still had significant proliferation to peptide F2-5, but by day 26 post-challenge (at or just preceding peak levels of bacteremia, day 340 post immunization) and thereafter F2-5-specific T lymphocyte proliferative responses had sharply decreased in all immunized animals to background levels. Control animals had no response to peptide F2-5 at any time point during infection (Fig. 4E-H). These results show that both IFN-γ secreting cells and proliferating cells specific for peptide F2-5 dramatically decline to background levels following challenge, as observed previously for MSP2-vaccinated and challenged calves (13). Proliferative responses to *A. marginale* homogenate were also significant in all immunized animals at both time points before challenge and at 5 days post-challenge (Fig. 5A). On days 12 and 19 post-challenge, only

PBMC from animals 3937 and 3943 had significant proliferation to *A. marginale,* and thereafter all animals had insignificant responses when compared to either URBC or medium. Neither control animal tested had significant proliferation to *A. marginale* at any time point (Fig. 5B). In contrast, proliferative responses to a *Clostridium* sp. vaccine antigen remained significantly positive throughout acute and resolving bacteremia when compared to either medium or URBC (Fig. 6A). The response to *Clostridium* vaccine is $CD4^+$ T cell mediated (Fig. 6B), indicating that *A. marginale* infection does not simply induce a generalized suppression of $CD4^+$ T-cell responses. Importantly, the sharp loss of antigen-specific responses by all vaccinates following infection was mirrored by a rapid decline in $tetramer^+$ $CD4^+$ T cells in PBMC (Fig. 2 lower panel, Fig. 4, and Table II). For example, on day 369 (48 days post-challenge), the frequencies of antigen-specific cells ranged from 0.0002-0.0008%, which were not significantly different from pre-immunization levels (P=0.177), but were ~100-fold less than those near the time of challenge, which was significant (P=0.014). Reproducibility of the tetramer staining with two or more aliquots of cryopreserved PBMC is documented in Table IV. The replicate samples were stained, in most cases, by different individuals and show similar results, and the mean values were used in Fig. 4 where more than one tetramer staining assay was performed. PBMC from the control animals showed background levels of staining with the tetramer, which further supports the specificity of this tetramer for staining antigen-specific T cells (Fig. 4E-H). The rapid drop in antigen-specific T cells from peripheral blood of vaccinates cannot be attributed to natural homeostasis of memory $CD4^+$ T cells, which in mice have a reported half-life of ~400 days in the late contractual phase beyond 180 days post-infection (24). These results are most consistent with rapid physical deletion of antigen-specific T cells following high levels of bacteremia during acute infection, but there was a possibility that antigen-specific cells sequestered out of the peripheral blood, presumably to the spleen where blood-borne pathogens are typically

removed (25). To address this question, antigen-specific lymphocytes from the spleen, a peripheral LN, and the liver were examined.

Antigen-specific T-cells in spleen, inguinal LN, and liver

To determine if effector $CD4^+$ T cells sequestered to the spleen or other tissues, all 10 vaccinates and two control animals were euthanized from 9-13 weeks post-challenge, and antigen-specific responses of spleen, liver, and inguinal LN lymphocytes were determined. Weak, but statistically significant F2-5-specific lymphocyte proliferative responses were detected in lymphocytes derived from fresh spleen samples of two vaccinates (3943 and 3959) and from fresh liver samples of three vaccinates (3892, 3943, and 3959) (Table V). However, we did not detect antigen-specific proliferation with cryopreserved spleen or liver cells from animals 3943 and 3892 (data not shown), whereas cryopreserved cells from animal 3959 continued to have significant responses (Table V). Responses were not detected in the LN of any vaccinate or from any tissue derived from the two controls. To determine whether a low frequency of responding T lymphocytes in liver and spleen could be expanded, we repeatedly attempted to culture cryopreserved spleen and liver lymphocytes from animals 3892, 3943, and 3959 with antigen, or with antigen plus human recombinant IL-2, but were unsuccessful. In contrast, short-term cultures of peptide F2-5-specific lymphocytes were easily derived from responsive PBMC from the same donors (data not shown) with techniques normally used to establish $CD4^+$ T cell lines (11, 16). These data suggest that the weak and inconsistent responses detected in spleen and liver, which could not be expanded in culture, may either represent cells undergoing the early stages of apoptosis, or that specific cells in these tissues are present but anergic. Activated antigen-specific human and mouse $CD8^+$T lymphocytes commonly traffic to the liver where they undergo apoptosis, resulting in the removal of these cells from the peripheral blood, and it has

been proposed that activated antigen-specific $CD4^+$ T lymphocytes undergo similar trafficking and apoptosis (26). To try to distinguish these possibilities cryopreserved lymphocytes from spleen, LN, and liver were examined by flow cytometry after staining with the DRB3*1101-F2-5B tetramer and enriching the stained cells with anti-PE beads. Representative dot plots showing the frequency of F2-5-specific $CD4^+$ T lymphocytes in mononuclear cells from tissues of vaccinate 3959 are shown in Fig. 7, and the results from all animals are summarized in Table VI. For comparison, we have included the baseline frequencies of $tetramer^+$ $CD4^+$ cells in PBMC obtained prior to immunization, and either on the day of challenge or 5-12 days later (time points where enough cells were available for analysis). Compared with PBMC at the time of euthanasia, all immunized animals had approximately 10-fold higher frequencies of F2-5B-specific T cells in liver, which was significant (P=0.008), but significant differences were not found in spleen or lymph node.

DISCUSSION

The current understanding of how pathogens evade and/or modulate the immune system of an immunocompetent host to achieve persistent and even lifelong infection is still in its infancy. However, unraveling these mechanisms is vital to the eventual control of many persistent infections in people and animals. In this study we demonstrate that following infection with *A. marginale,* a rickettsial pathogen that replicates to high numbers in erythrocytes during acute and persistent infection, antigen-specific $CD4^+$ T cells elicited by prior immunization rapidly disappear from peripheral blood near the peak of rickettsemia. This discovery likely explains the previously reported sudden loss of MSP2-specific $CD4^+$ T cell-mediated IFN-γ and proliferative responses after infection (13). Additionally, the loss of antigen-specific $CD4^+$ T cell responses upon infection occurs with T cells specific for a conserved (MSP1a) as well as

antigenically variable (MSP2) membrane protein, in animals immunized by subcutaneous inoculation of protein antigen in adjuvant or by intradermal DNA immunization followed by a peptide boost.

MSP2 is an immunodominant and highly abundant protein that is one of the primary targets of T cell and antibody recognition in animals immunized with outer membranes (27, 28) and is the major antigen recognized by antibody during infection (29). MSP2-immunized animals mounted strong antigen specific responses to multiple epitopes on the protein, in both conserved and hypervariable regions (11-13, 21). Both proliferation and IFN-γ- secreting cells, measured by ELISPOT assay, were rapidly lost after infection (13). Thus in this experiment, the loss of T cells is unlikely to be explained by lack of sufficient MSP2 presentation to enable survival of the primed T cells. Similarly, the loss of antigen-specific $CD4^+$ T cells documented in the present study does not likely result from a failure of MSP1a F2-5-specific T cells to receive survival signals due to insufficient antigen presentation during infection. First, PBMC from calves immunized with the protective outer membrane fraction proliferate to native MSP1, a heterodimer of MSP1a and MSP1b (27), and MSP1b is poorly immunogenic for T cells (30). Second, $CD4^+$ T cell clones derived from two calves immunized with native MSP1 and specific for MSP1a F2-5 peptide presented by DRA/DRB3*1101 (19) proliferated strongly to both peptide and *A. marginale* lysate (16). Finally, we have shown that in the present study, MSP1a F2-5-specific T cells obtained before challenge from immunized animals proliferated strongly to both the immunizing peptide and to *A. marginale* homogenate. These observations demonstrate that *A. marginale* contains sufficient MSP1a to elicit a recall response in vitro, which should also be sufficient for presentation to T cells by APC in vivo following infection.

Exosomes are small membrane vesicles released from APC that contain MHC molecules and pathogen epitopes, and have been shown to directly activate antigen-specific T cells in vitro

in a variety of models (31-35). It is therefore highly unlikely that exosomes containing *A.marginale* MSP1a F2-5 released in vivo during peak bacteremia from APC are binding to specific T cells and blocking tetramer binding. If such exosomes were present, the T cells should be activated to proliferate and secrete IFN-γ, but instead we observed a rapid loss of these effector functions.

Because *A. marginale* is a blood borne intraerythrocytic pathogen and is presumably removed from the circulation by phagocytosis in the spleen, the possibility that circulating antigen-specific T cells would traffic to the spleen was evaluated. However, only weak and unsustainable antigen-specific T lymphocyte proliferation was detected in the spleens of two of the four immunized calves at the time of necropsy, which was consistent with an insignificant frequency of tetramer staining cells. Additionally, neither antigen-specific T cell responses nor tetramer$^+$ CD4$^+$ T cells were detected in the inguinal lymph node. Thus, the present study shows that infection-induced loss of *A. marginale*-specific memory T cell responses from peripheral blood is not due to induction of anergy in circulating T cells or sequestration to the spleen or LN, because antigen-specific T cells disappeared from blood and could not be accounted for in the spleen or LN where effector memory (spleen) or central memory (LN) T cells would be expected to reside.

It is interesting that ~10-fold higher frequencies of tetramer$^+$CD4$^+$ T cells were detected in liver samples when compared with PBMC, spleen, or LN obtained at the same time. Lymphocytes obtained from fresh liver samples from three vaccinates proliferated to antigen in vitro, and cryopreserved liver lymphocytes from animal 3959 proliferated as well, showing the cells were not dead (Table V). Nevertheless, the antigen specific cells could not be expanded from viable cryopreserved PBMC of animals 3892, 3943, or 3959 by antigen stimulation in vitro, whereas antigen-specific cells could readily be expanded from cryopreserved PBMC obtained at

time points when cells did respond. Together, our findings are most consistent with activation induced cell death of antigen-specific $CD4^+$ T cells upon exposure to continuous high antigenic load during infection and pre-apoptotic cells that have migrated to this organ to be removed (26). Attempts to stain $tetramer^+$ $CD4^+$ T cells from various tissues and at different time points for markers of apoptosis (annexin V) were unsuccessful due to the high background staining on the cells, even with cells which had strong proliferative responses pre-challenge (data not shown). Staining with annexin V does not necessarily indicate cell death; rather annexin V stains cells at the early stage of apoptosis when membrane perturbations occur. The high background staining with annexin V likely reflects the procedures used to freeze and thaw the cells and stain them with the tetramer, as it is well known that any procedure that affects the integrity of the plasma membrane will result in cells positive for annexin V. Enrichment of MHC class II tetramer positive cells using the microbead selection method has proved to be a very useful tool to enable detection of low frequency $CD4^+$ T cells in peripheral blood (22). However, the enrichment process may make detection of early stage apoptosis technically difficult.

Studies by others have also followed the fate of antigen-specific T cells in response to either antigen or pathogen challenge. Using a T-cell receptor transgenic mouse model, Hayashi et al. (36) observed that adoptively transferred, or in vivo-primed, antigen-specific T cells rapidly became activated, expressed IFN-γ, and divided following an intravenous antigen challenge. However, within 48 hours, the frequency of specific cells in blood, spleen, LN, and liver rapidly decreased to barely detectable levels, and residual cells remained anergic. The mechanism of T cell depletion in this study was not determined. In a different model, malaria-specific, protective $CD4^+$ T cells were expanded ex vivo from immunized mice and adoptively transferred to naïve mice (37). Following challenge with *Plasmodium,* the antigen-specific cells were rapidly deleted from blood and tissues, which was dependent on IFN- γ, but not on TNF or Fas pathways.

However, in both of these rodent models, abnormally high numbers of antigen-specific T cells were present, which could result in competition for antigen and affect normal cell signaling. In a third example, $CD4^+$ T cells from mice infected with *Brugia pahangi* microfilariae displayed defective antigen-specific T cell proliferative responses and underwent apoptosis when stimulated with antigen ex vivo (38). T cell apoptosis was shown to be IFN-γ dependent using IFN-γ knockout mice, and in vitro studies further demonstrated apoptosis was dependent on nitric oxide (NO) produced by macrophages. However, antigen-specific T cells in the *B. pahangi* infected mice did produce IFN-γ , indicating persistence of functional antigen-specific T cells, unlike the T cells isolated during acute and persistent *A. marginale* infection in the present study. In another example of systemic Bacille Calmette-Guérin (BCG) infection in mice, antigen-specific $CD4^+$ T cells rapidly expanded after infection, and then underwent homeostatic contraction to normal levels (39). The contraction phase was shown to be due to IFN-γ - dependent T cell apoptosis, and was also dependent on NO production by macrophages when examined in vitro. However, this study also differs from ours in that not all effector cells were deleted, and considerable numbers remained in the spleens of wild-type mice following contraction of the effector cells (39). More recently, the same group reported that in this model, IFN-γ acts directly on $CD4^+$ T cells to upregulate mRNA and protein expression from genes encoding intrinsic apoptosis machinery involved in promoting damage to the cell mitochondria (40). It was further shown that IFN-γ also induced expression of cell-extrinsic pro-apoptotic signals TRAIL and TNF-α from $CD4^+$ T cells as well as NO and TNF-α from macrophages (40). These studies demonstrate a key role of IFN-γ produced by antigen-specific $CD4^+$ T cells in response to antigen or infection in directly or indirectly promoting T cell apoptosis, to restore a normal homeostatic response.

Our studies have not yet defined a mechanism of antigen-specific T cell deletion following *A. marginale* infection, so we cannot rule out the potential importance of IFN-γ secreted by the T cells themselves in promoting apoptosis. However, our studies differ from the mouse studies, in that tetramers were used to track the specific T cells in vivo, and our results show that the specific T cells in peripheral blood are at background pre-immunization levels by 9-12 weeks post-challenge, which is coincident with insignificant proliferation and numbers of IFN-γ secreting cells. Thus, we were unable to detect IFN-γ- secreting cells using ELISPOT assays at the same time points when proliferation was undetectable. Our studies also differ from the mouse pathogen models described above because even one year after infection, immunization-induced T cell responses could not be elicited ex vivo (13), indicating that functional memory cells were absent. Furthermore, unlike BCG (39-41), malarial parasites (42), and *Babesia bovis*, an intraerythrocytic cattle pathogen that does induce a strong febrile and inflammatory response in vivo and inflammatory cytokines in vitro (43-45), there is no evidence that intraerythrocytic *A. marginale* similarly activates a potent inflammatory response. For example, during acute infection, clinical signs are predominantly related to anemia. Splenomegaly does occur, but cattle do not reproducibly demonstrate sustained elevated body temperatures (13). Furthermore, *A. marginale* lacks LPS and peptidoglycan (46), two well known pathogen-associated molecular patterns present in many other gram-negative bacteria that activate macrophages through toll like receptors. Finally, in the two animals examined, no evidence of inflammation was observed in spleen or liver biopsies obtained at necropsy. Our results, therefore, extend the mouse studies that describe apoptosis of $CD4^+$ T cells during infection with several different pathogens known to activate an inflammatory response. We document in a large out bred animal, the rapid loss of a physiologically relevant number of antigen-specific T cells following infection with a naturally occurring pathogen that induces

minimal inflammation, and persists indefinitely. The rapid deletion of immunization-induced *A. marginale*-specific $CD4^+$ T cells in response to infection may, importantly, indicate another mechanism by which *A. marginale* modulates the immune response. It is well known that antigenic variation of MSP2 results in immune evasion (10, 11). As new antigenic variants of MSP2 and MSP3 continually arise during persistent infection when bacteria can reach 10^7 per ml of blood (7-9), ascending bacteremia may cause rapid antigen-induced deletion of $CD4^+$ T cells specific for new variants. Our data showing a lack of $CD4^+$ T cell responses to *A. marginale* and immunodominant MSP2 following infection of control animals that were not previously immunized with any *A. marginale* protein (13) and a similar lack of response to *A. marginale* in our vaccinated and control animals following infection in the present study support this hypothesis.

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REFERENCES

1. Sacks, D., and A. Sher. 2002. Evasion of innate immunity by parasitic protozoa *Nat. Immunol.* 3: 1041-1047.
2. Hornef, M. W., M. J. Wick, M. Rhen, and S. Normark. 2002. Bacterial strategies for overcoming host innate and adaptive immune responses. *Nat. Immunol.* 3: 1033-1040.
3. Finlay, B. B., and G. McFadden. 2006. Anti-immunology: evasion of the host immune system by bacterial and viral pathogens. *Cell* 124: 767-782.
4. Belkaid, Y. 2007. Regulatory T cells and infection: a dangerous necessity. *Nat. Rev. Immunol.* 7: 875-888.
5. Klenerman, P., and A. Hill. 2005. T cells and viral persistence: lessons from diverse infections. *Nat. Immunol.* 6: 873-879.
6. Palmer, G. H., F. R. Rurangirwa, K. M. Kocan, and W. C. Brown. 1999. Molecular basis for vaccine development against the ehrlichial pathogen *Anaplasma marginale. Parasitol. Today* 15: 281-286.
7. Futse, J. E., K. A. Brayton, M. J. Dark, D. P. Knowles, and G. H. Palmer. 2008. Superinfection as a driver of genomic diversification in antigenically variant pathogens. *Proc. Natl. Acad. Sci. USA* 105: 2123-2127.
8. Palmer, G. H., and K. A. Brayton. 2007. Gene conversion is a convergent strategy for pathogen antigenic variation. *Trends Parasitol.* 23:.408-413.
9. French, D. M., T. F. McElwain, T. C. McGuire, and G. H. Palmer. 1998. Expression of *Anaplasma marginale* major surface protein 2 variants during persistent cyclic rickettsemia. *Infect. Immun.* 66: 1200-1207.

10. French, D. M., W. C. Brown, and G. H. Palmer. 1999. Emergence of *Anaplasma marginale* antigenic variants during persistent rickettsemia. *Infect. Immun.* 67: 5834-5840.

11. Brown, W. C., K. A. Brayton, C. M. Styer, and G. H. Palmer. 2003. The hypervariable region of *Anaplasma marginale* major surface protein 2 (MSP2) contains multiple immunodominant $CD4^+$ T lymphocyte epitopes that elicit variant-specific proliferative and IFN- responses in MSP2 vaccinates. *J. Immunol.* 170: 3790-3798.

12. Abbott, J. R., G. H. Palmer, C. J. Howard, J. C. Hope, and W. C. Brown. 2004. *Anaplasma marginale* major surface protein $CD4^+$ T-cell epitopes are evenly distributed in conserved and hypervariable regions (HVR), whereas linear B-cell epitopes are predominantly located in the HVR. *Infect. Immun.* 72: 7360-7366.

13. Abbott, J. R., G. H. Palmer, K. A. Kegerreis, P. F. Hetrick, C. J. Howard, J. C. Hope, and W. C. Brown. 2005. Rapid and long-term disappearance of $CD4^+$ T lymphocyte responses specific for *Anaplasma marginale* major surface protein-2 (MSP2) in MSP2 vaccinates following challenge with live *A. marginale. J. Immunol.* 174: 6702-6715.

14. Bielekova, B., and R. Martin. 2001. Antigen-specific immunomodulation via altered peptide ligands. J. Mol. Med. 79: 552-565.

15. Plebanski, M., E. A. Lee, and A. V. Hill. 1997. Immune evasion in malaria: altered peptide ligands of the circumsporozoite protein. Parasitology 115(Suppl.): S55-S66.

16. Brown, W. C., T. C. McGuire, W. Mwangi, K.A. Kegerreis, H. Macmillan, H. A. Lewin, and G. H. Palmer. 2002. Major histocompatibility complex class II DR-restricted memory $CD4^+$ T lymphocytes recognize conserved immunodominant epitopes of *Anaplasma marginale* major surface protein 1a. *Infect. Immun.* 70:5521-5532.

17. Mwangi, W., W. C. Brown, G. A. Splitter, C. J. Davies, C. J. Howard, J. C. Hope, Y. Aida, Y. Zhuang, B. J. Hunter, and G. H. Palmer. 2007. DNA vaccine construct incorporating intercellular trafficking and intracellular targeting motifs effectively primes and induces memory B- and T-cell responses in outbred animals. *Clin. Vaccine Immunol.* 14: 304-311.

18. Norimine, J., S. Han, and W. C. Brown. 2006. Quantitation of *Anaplasma marginale* major surface protein (MSP)1a and MSP2 epitope-specific $CD4^+$ T lymphocytes using bovine DRB3*1101 and DRB3*1201 tetramers. *Immunogenetics* 58: 726-739.

19. Norimine, J., and W. C. Brown. 2005. Intrahaplotype and interhaplotype pairing of bovine leukocyte antigen DQA and DQB molecules generate functional DQ molecules important for priming $CD4^+$ T-lymphocyte responses. *Immunogenetics* 57: 750-762.

20. Norimine, J., J. Mosqueda, C. Suarez, G. H. Palmer, T. F. McElwain, G. Mbassa, and W. C. Brown. 2003. Stimulation of T-helper cell gamma interferon and immunoglobulin G responses specific for *Babesia bovis* rhoptry-associated protein 1 (RAP-1) or a RAP-1 protein lacking the carboxy-terminal repeat region is insufficient to provide protective immunity against virulent *B. bovis* challenge. *Infect. Immun.* 71: 5021-5032.

21. Brown, W. C., T. C. McGuire, D. Zhu, H. A. Lewin, J. Sosnow, and G. H. Palmer. 2001. Highly conserved regions of the immunodominant major surface protein 2 of the genogroup II ehrlichial pathogen *Anaplasma marginale* are rich in naturally derived $CD4^+$ T lymphocyte epitopes that elicit strong recall responses. *J. Immunol.* 166: 1114-1124.

22. Scriba, T. J., M. Purbhoo, C. L. Day, N. Robinson, S. Fidler, J. Fox, J. N. Weber, P. Klenerman, A. K. Sewall, and R. E. Phillips. 2005. Ultrasensitive detection and phenotyping of $CD4^+$ T cells with optimized HLA class II tetramer staining. *J. Immunol*. 175: 6334-6343.

23. Macmillan, H., J. Norimine, K. A. Brayton, G. H. Palmer, and W. C. Brown. 2008. Physical linkage of naturally complexed bacterial outer membrane proteins enhances immunogenicity. *Infect. Immun.* 76: 1223-1229.

24. Homann, D., L. Teyton, and M. B. Oldstone. 2001. Differential regulation of antiviral Tcell immunity results in stable $CD8^+$ but declining $CD4^+$ T-cell memory. *Nat. Med.* 7: 913-919.

25. Mebius, R. E., and G. Kraal. 2005. Structure and function of the spleen. *Nat. Rev. Immunol.* 5: 606-616.

26. Crispe, I. N., T. Dao, K. Klugewitz, W. Z. Mehal, and D. P. Metz. 2000. The liver as a site of T-cell apoptosis: graveyard, or killing field? *Immunol. Rev.* 174: 47-62.

27. Brown, W. C., V. Shkap, D. Zhu, T. C. McGuire, W. Tuo, T. F. McElwain, and G. H. Palmer. 1998. $CD4^+$ T-lymphocyte and immunoglobulin G2 responses in calves immunized with *Anaplasma marginale* outer membranes and protected against homologous challenge. *Infect. Immun.* 66: 5406-5413.

28. Lopez J. E., W. F. Siems, G. H. Palmer, K. A. Brayton, T. C. McGuire, J. Norimine, and W. C. Brown. 2005. Identification of novel antigenic proteins in a complex *Anaplasma marginale* outer membrane immunogen by mass spectrometry and genomic mapping. *Infect. Immun.* 73: 8109-8118.

29. Palmer, G. H., A. F. Barbet, A. J. Musoke, J. M. Katende, F. Rurangirwa, V. Shkap, E. Pipano, W. C. Davis, and T. C. McGuire. 1988. Recognition of conserved surface protein epitopes on *Anaplasma centrale* and *Anaplasma marginale* isolates from Israel, Kenya, and the United States. *Int. J. Parasitol.* 18: 33-38.

30. Brown, W. C., G. H. Palmer, H. A. Lewin, and T. C. McGuire. 2001. $CD4^+$ T lymphocytes from calves immunized with *Anaplasma marginale* major surface protein 1 (MSP1), a

heteromeric complex of MSP1a and MSP1b, preferentially recognize the MSP1a carboxyl terminus that is conserved among strains. *Infect. Immun.* 69: 6853-6862.

31. Kovar, M., O. Boyman, X. Shen, I. Hwang, R. Kohler, and J. Sprent. 2006. Direct stimulation of T cells by membrane vesicles from antigen-presenting cells. *Proc. Natl.Acad. Sci. USA* 103: 11671-11676.
32. Admyre, C., S. M. Johansson, S. Paulie, and S. Gabrielsson. 2006. Direct exosome stimulation of peripheral human T cells detected by ELISPOT. *Eur. J. Immunol.* 36: 1772-1781.
33. Colino, J., and C. M. Snapper. 2006. Exosomes from bone marrow dendritic cells pulsed with diptheria toxoid preferentially induce type I antigen-specific IgG responses in naïve recipients in the absence of free antigen. *J. Immunol.* 177: 3757-3762.
34. Giri, P. K., and J. S. Schorey. 2008. Exosomes derived from *M. bovis* BCG infected macrophages activate antigen-specific $CD4^+$ and $CD8^+$ T cells in vitro and in vivo. *PLoS ONE* 3: e2461.
35. Schorey, J. S., and S. Bhatnagar. 2008. Exosome function: from tumor immunology to pathogen biology. Traffic 9: 871-881.
36. Hayashi, N., D. Liu, B. Min, S. Z. Ben-Sasson, and W. E. Paul. 2002. Antigen challenge leads to in vivo activation and elimination of highly polarized TH1 memory T cells *Proc. Natl. Acad. Sci. USA* 99: 6187-6191.
37. Xu, H., J. Wipsa, H. Yan, M. Zeng, M. O. Makobongo, F. D. Finkelman, A. Kelso, and M. F. Good. 2002. The mechanism and significance of deletion of parasite-specific $CD4^+$T cells in malaria infection. *J. Exp. Med.* 195: 881-892.

38. Jenson J. S., R. O'Connor, J. Osborne, and G. B. Glasgow. 2002. Infection with *Brugia microfilariae* induces apoptosis of $CD4^+$ T lymphocytes: a mechanism of immune unresponsiveness in filariasis. *Eur. J. Immunol.* 32: 858-867.

39. Dalton D. K., L. Haynes, C.Q. Chu, S. L. Swain, and S. Wittmer. 2000. Interferon eliminates responding $CD4^+$ T cells during mycobacterial infection by inducing apoptosis of activated $CD4^+$ T cells. *J. Exp. Med.* 192: 117-122.

40. Li X., K. K. McKinstry, S. L. Swain, and D. K. Dalton. 2007. IFN-γ acts directly on activated $CD4^+$T cells during mycobacterial infection to promote apoptosis by inducing components of the intracellular apoptosis machinery and by inducing extracellular proapoptosis signals. *J. Immunol.* 179: 939-949.

41. Demangel, C., and W. J. Britton. 2000. Interaction of dendritic cells with mycobacteria: where the action starts. *Immunol. Cell. Biol.* 78: 318-324.

42. Grau, G. E., P.–F. Piguet, P. Vassalli, and P. H. Lambert. 1989. Tumor necrosis factor and other cytokines in cerebral malaria: experimental and clinical data. *Immunol. Rev.*112: 49-70.

43. Wright, I. G., and B. V. Goodger. 1988. Pathogenesis of babesiosis. In *Babeosis of Domestic Animals and Man.* M. Ristic, ed. CRC Press, Boca Raton, FL. 99-118.

44. Gale, K. R., D. J. Waltisbuhl, J. M. Bowden, W. K. Jorgensen, J. Matheson, I. J. East, H. Zakrzewski, and G. Leatch. 1988. Amelioration of virulent *Babesia bovis* infection in calves by administration of the nitric oxide synthase inhibitor aminoguanidine. *Parasite Immunol.* 20: 441-445.

45. Shoda, L. K. M., G. H. Palmer, J. Florin-Christensen, M. Florin-Christensen, D. L. Godson, and W. C. Brown. 2000. *Babesia bovis*-stimulated macrophages express interleukin-1, interleukin-12, tumor necrosis factor-β, and nitric oxide and inhibit parasite replication in vitro. *Infect. Immun.* 68: 5139-5145.

46. Brayton, K. A., L. S. Kappmeyer, D. R. Herndon, M. J. Dark, D. L. Tibbals, G. H. Palmer, T. C. McGuire, and D. P. Knowles, Jr. 2005. Complete genome sequencing of *Anaplasma marginale* reveals that the surface is skewed to two superfamilies of outer membrane proteins. *Proc. Natl. Acad. Sci. USA* 102: 844-849.

TABLES

Table I. ***Primers used for constructing the DQA*1001/DQB*1002-F3-3 tetramer***

Construct	Name	Primer sequence (5' to 3')
DQA*1001-blz-bs		
	DQA-Blzf	CTATGTCAGAGCTGACAGAGTCGTCAGCAGACCTGGTTCC
	DQA-bLZR	GGAACCAGGTCTGCTGACGACTCTGTCAGCTCTGACATAG
DQB*1002F3-3-alz-FLAG		
	DQB-aLZF	CTGAATCTGCCCAGAGCAAGTCGTCAGCAGACCTGGTTCC
	DQB-aLZR	GGAACCAGGTCTGCTGACGACTTGCTCTGGGCAGATTCAG
	DQBRBR	TACACGAAATCCTTTGGGATCTCCCTGGCCCAG
	DRBQBF	CTGGGCCAGGGAGATCCCAAAGGATTTCGTGTA
	DQBLinkerF	TGGGTCCCCAAAGGATTTCGTGTA
	DQBLinkerR	TACACGAAATCCTTTGGGGACCCACCTCCTCCAGAGCCCCGTGG
	DQBF3-3R1	TTTCTCTTGCCCCGCAGGGATCTCCCTGGCCCAGGC
	DQBF3-3R2	TGCTCAGGTTCGGCAGTTTTCTCTTGCCCCGCAGG
	DQBF3-3R3	TGAGCCACCACCTCCCGCAGCCTCATGCTCAGGTTCGGCAGT

Table II. ***Comparison of IFN-γ secreting cells in PBMC before and after challenge***

	No. SFC per 10^6 PBMC from animals in the following group[a]:					
	Immunized				Control	
Aliquot	3892	3937	3943	3959	3962	3989
Pre-challenge[b]	**128**	**377**	**833**	**521**	20	-97
Post-challenge[c]						
2 wk	**179**	**163**	**827**	**683**	91	5
4 wk	290	71	17	-18	-15	2
6 wk	84	-23	-19	-55	94	-25
9-12 wk	180	17	104	-72	-79	-260

[a]Cryopreserved PBMC from immunized or control animals were stimulated in triplicate wells for 40 hr with 1.0 µg/ml of F2-5 peptide (PBMC) or negative control RAP-1 P2 peptide. Results are presented as the mean number of SFC per 10^6 PBMC after subtracting the response to negative control peptide. Significantly greater responses to F2-5 peptide are indicated in bold, determined by a Student's one-tailed paired t-test where $P<0.05$.

[b]Pre-challenge PBMC were obtained the day of challenge, except for animal 3943, where PBMC were obtained 3 wk before challenge. The response to F2-5 had too many spots to count so the no. SFC was estimated at 600 per 5 x 10^5 cells/well.

[c]PBMC were obtained from animals euthanized between 9-12 weeks post challenge.

Table III. ***Comparison of antigen-specific proliferation using fresh and cryopreserved pre-challenge PBMC***

Animal[a]	Proliferation (mean CPM +/- 1 SD) against the following antigens:[b]			
	MSP1a F2-5	MSP2 P1	MSP1a F2-5	MSP2 P1
Immunized	*Fresh PBMC*		*Cryopreserved PBMC*	
3982	**39,752 +/- 3,352**	Neg[c]	**48,564 +/-5,784**	715 +/- 790
3937	**13,802 +/- 2,021**	2,522 +/- 1,472	**27,744 +/- 6,736**	442 +/- 395
3943	**43,608 +/- 8,025**	Neg	**57,972 +/- 6,898**	Neg
3959	**39,360 +/- 1,828**	761 +/- 223	**44,298 +/- 6,426**	733+/- 755
Controls	*Fresh PBMC*		*Cryopreserved PBMC*	
3962	Neg	Neg	114 +/- 61	10 +/- 13
3989	Neg	Neg	194 +/- 156	367 +/- 766
3960	Neg	Neg	83 +/- 49	778 +/- 1,084
3991	744 +/- 1,555	1,223 +/- 2,231	198 +/- 259	16 +/- 82

[a] PBMC were obtained on day 144 post immunization from vaccinates or controls and were tested immediately in a proliferation assay or cryopreserved in liquid nitrogen until they were tested approximately 17 months later.

[b] Peptides were used at a final concentration of 10 μg/ml. Results are presented as the mean CPM +/- 1 SD of triplicate samples after subtracting the background response to medium. Responses significantly greater than the negative control MSP2 P1 peptide are in bold.

[c] Neg indicates the responses was < 0 after subtracting the medium control.

Table IV. ***Reproducibility of tetramer staining***

Cell source[a]	Frequency (%) of tetramer$^+$ CD4$^+$ cells from animal[b]:	
	3892	3959
PBMC		
Day 326	0.0078 +/- 0.0014	ND[c]
Day 333	0.0063 +/- 0.0018	0.023 +/- 0.0034
Day 340	0.0029 +/- 0.0025	ND
Day 348	0.0005 +/- 0.0005	0.0008 +/- 0.0006
Day 355	ND	0.0028 +/- 0.0002
Day 369	0.0008 +/- 0.0007	0.0004 +/- 0.0003
Day 384	0.0004 +/- 0.0003	ND
Liver	0.0019 +/- 0.008	ND
Spleen	0.0004 +/- 0.00005	ND

[a] Cells were cryopreserved from PBMC or tissues at the indicated days post-immunization.
[b] Results are presented as the mean frequency +/- 1 SD of two to four replicate aliquots.
[c] Not determined for more than one sample at these time points.

Table V. ***F2-5 specific T lymphocyte proliferative responses from PBMC, spleen, LN and liver at euthanasia***

Proliferation (Stimulation Index) from the following group[a]

		Immunized					Control	
Cells[b]	Antigen	3892	3937	3943	3959	3959[c]	3962	3989
PBMC	F2-5 (1)	1.3	1.8	1.0	3.1	2.0	0.7	0.7
	F2-5 (10)	1.0	0.3	0.9	1.9	2.7	1.2	1.0
Spleen	F2-5 (1)	1.5	1.9	**12.2**	1.0	**4.3**	0.9	1.0
	F2-5 (10)	2.0	2.0	**13.2**	**2.1**	**4.7**	1.3	1.8
LN	F2-5 (1)	0.8	1.6	0.5	0.5	0.9	2.4	0.9
	F2-5 (10)	0.7	1.8	1.8	0.2	2.4	1.2	1.4
Liver	F2-5 (1)	**11.3**	0.8	**2.8**	0.7	**10.2**	0.8	1.0
	F2-5 (10)	**8.3**	1.3	**3.4**	**5.0**	**7.0**	1.0	0.9

[a] Peptides F2-5 and MSP2 P1 were tested at 1 and 10 μg per ml. Results are shown as stimulation index calculated as mean CPM of triplicate cultures of peptide F2-5 peptide/mean CPM of triplicate cultures with medium. Responses significantly greater than those to medium and negative control peptide MSP2 P1($P<0.05$) are bolded.

[b] Lymphocytes were obtained at euthanasia and used immediately.

[c] Lymphocytes from animal 3959 were also used after cryopreservation.

Table VI. ***Frequencies of tetramer$^+$ CD4$^+$ cells in PBMC, spleen, LN, and liver at euthanasia***

Frequency (%) of tetramer+ CD4+ cells from the following group[a]						
	Immunized				Control	
Cell source[b]	3892	3937	3943	3959	3962	3989
PBMC (pre-immunization)	0.0009	0.0003	0.0020	0.0005		
PBMC (challenge)	**0.0361**	**0.0090**	**0.0324**	**0.0206**	0.0015	0.0001
PBMC (death)	0.0003	0.0002	0.0003	0.0003	0.0006	0.0002
Spleen (death)	0.0004	0.0018	0.0048	0.0003	0.0006	ND[c]
LN (death)	0.0013	0.0001	0.0026	0.0003	0.0018	0.0007
Liver (death)	**0.0019**	**0.0027**	**0.0032**	**0.0021**	ND	0.0009

[a] Cryopreserved lymphocytes were thawed, and viable cells stained with the *1101-F2-5B tetramer. The percentage of tetramer$^+$ CD4$^+$ cells of total CD4$^+$ T cells was calculated. Numbers in bold represent significantly higher mean frequencies of tetramer$^+$ CD4$^+$ T cells in compared with those in PBMC pre-immunization using a one-tailed paired t-test (P= 0.008 for liver and P= 0.014 for PBMC at the time of challenge).

[b] Cryopreserved PBMC were obtained before immunization, on the day of challenge (day 321 post immunization; animals 3892, 3937, 3989, 3962), 5 days post-challenge (animal 3943), 12 days post-challenge (animal 3959), and at the time of death (day 63, animals 3892 and 3962; day 69, animal 3937; day 75, animal 3943; day 84, animal 3959, and day 89 post-challenge, animal 3989).

[c] Not determined because of insufficient cell numbers.

FIGURES

Figure 1.

Enumeration of $CD4^+$ cells in PBMC during *A. marginale* infection of vaccinates. Cryopreserved PBMC from the four immunized animals (indicated by symbols) were stained with FITC-conjugated anti-CD4 MAb MCA1365 (Serotec) and analyzed by FACS. PBMC were obtained just prior to challenge on day 321 (arrow), and at select time points thereafter until infection was controlled. A one-way ANOVA for multiple comparisons showed no significant difference in the percentages of $CD4^+$ cells in the animals before and after infection (P=0.35).

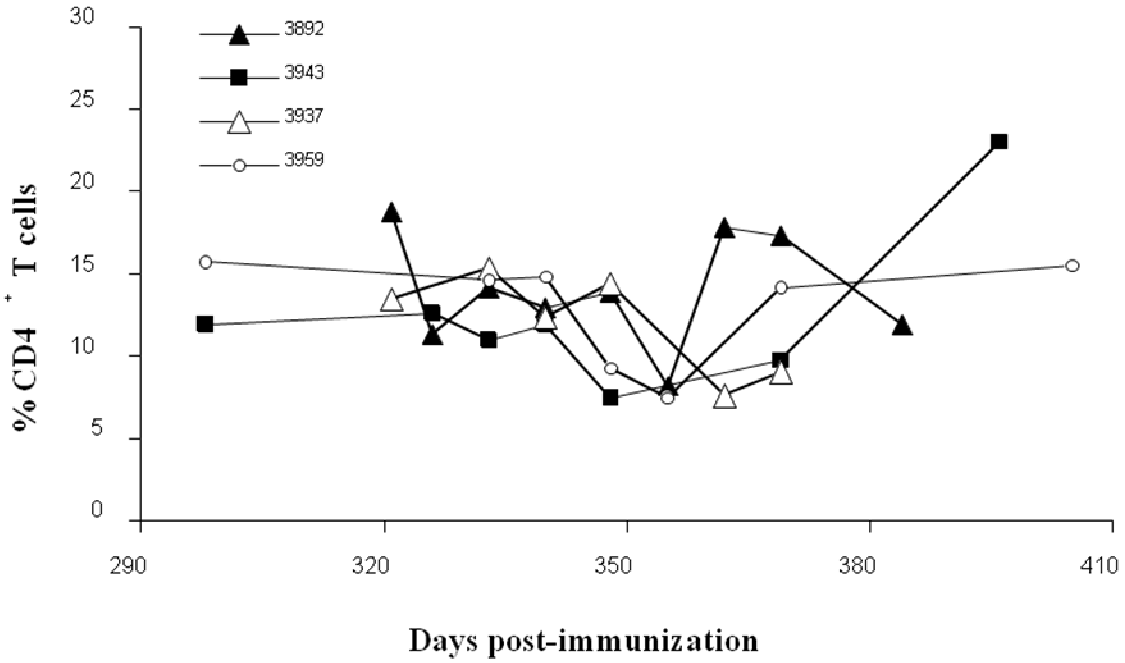
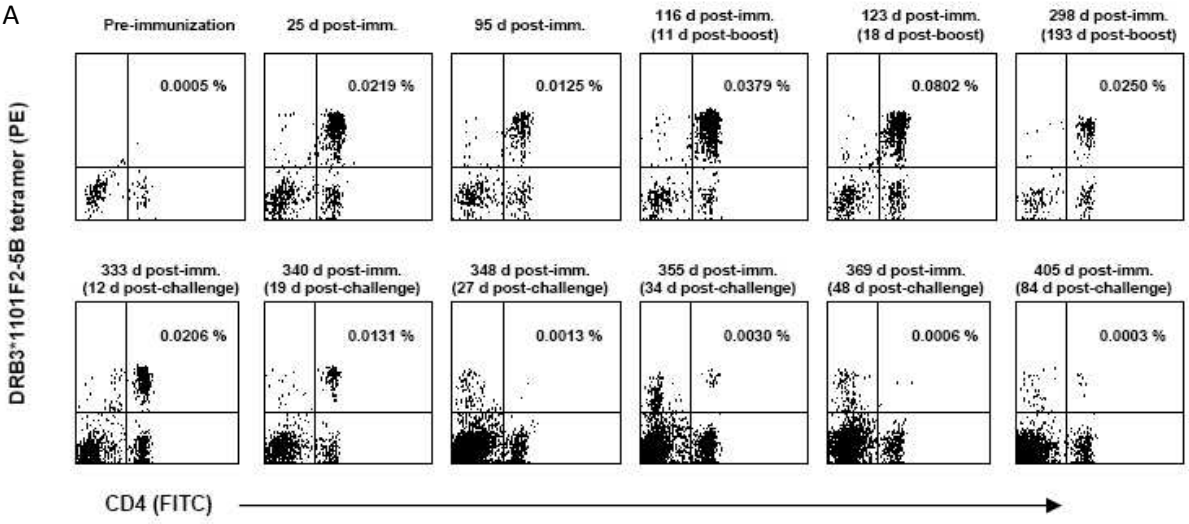


Figure 2.

Enumeration of antigen-specific $CD4^+$ T cells in peripheral blood of animal 3959 before and after immunization and *A. marginale* infection. *A,* Cryopreserved PBMC were stained with the DRB3*1101-F2-5B tetramer at the indicated time points and analyzed by FACS. Dot plots indicate the percentage of $tetramer^+$ $CD4^+$ T cells after anti-PE magnetic bead selection. The upper panel shows cells obtained before and after immunization and after the peptide boost on day 105. The bottom panel shows cells obtained 12-84 days following *A. marginale* challenge. *B,* Cryporeserved PBMC obtained 12 days post-challenge were thawed and stained with either the DRB3*1101 F2-5B tetramer or an unrelated DQA*1101/DQB*1102 F3-3 tetramer after anti-PE magnetic bead selection. The numbers in the upper right quadrants are the calculated percentage of $tetramer^+$ $CD4^+$ T cells of the total $CD4^+$ T cells in the samples.


A
Pre-immunization
25 d post-imm.
95 d post-imm.
116 d post-imm. (11 d post-boost)
123 d post-imm. (18 d post-boost)
298 d post-imm. (193 d post-boost)
0.0005 %
0.0219 %
0.0125 %
0.0379 %
0.0802 %
0.0250 %
333 d post-imm. (12 d post-challenge)
340 d post-imm. (19 d post-challenge)
348 d post-imm. (27 d post-challenge)
355 d post-imm. (34 d post-challenge)
369 d post-imm. (48 d post-challenge)
405 d post-imm. (84 d post-challenge)
0.0206 %
0.0131 %
0.0013 %
0.0030 %
0.0006 %
0.0003 %
DRB3*1101F2-5B tetramer (PE)
CD4 (FITC)

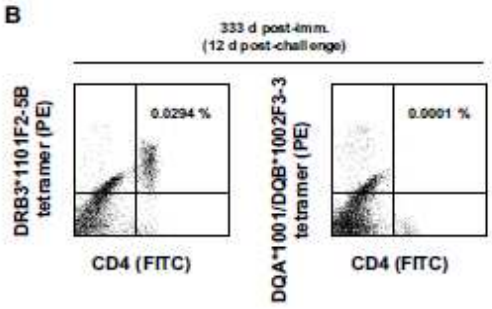

B
333 d post-imm. (12 d post-challenge)
0.0294 %
0.0001 %
DRB3*1101F2-5B tetramer (PE)
DQA*1001/DQB*1002F3-3 tetramer (PE)
CD4 (FITC)
CD4 (FITC)

Figure 3.

Enumeration of tetramer$^+$ CD4$^+$ cells in PBMC from all vaccinates before and after immunization and peptide boost. Cryopreserved PBMC were stained with the tetramer and analyzed by FACS. PBMC were obtained 3 days before immunization (arrow) and at several time points thereafter and following the peptide boost on day 105 (arrow). Individual animals are indicated. The data represent the percentage of tetramer$^+$ CD4$^+$ cells of the total CD4$^+$ T cells.

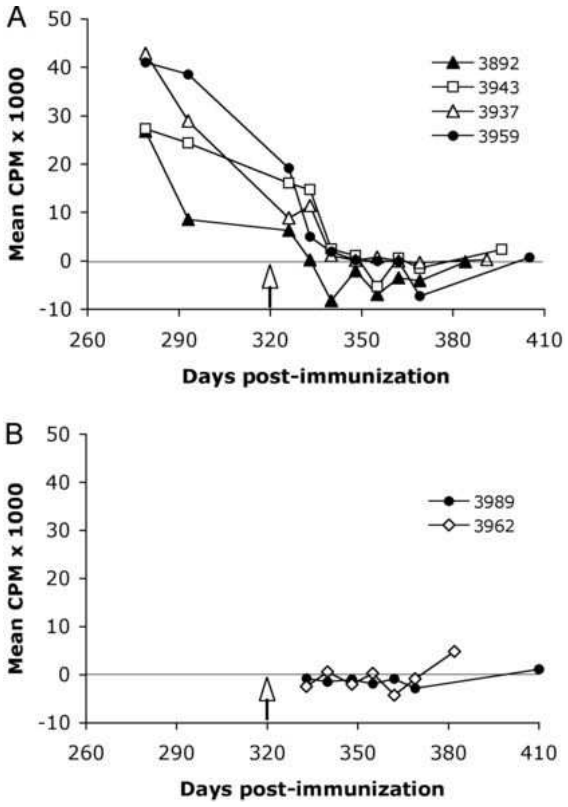


Figure 4.

T lymphocyte proliferation and enumeration of tetramer$^+$ CD4$^+$ cells in PBMC from vaccinates and controls before and after *A. marginale* infection. PBMC were obtained on day 141 post-immunization and at select time points thereafter and following challenge on day 321 (arrow). Cryopreserved PBMC were stained with the tetramer and analyzed by FACS. *A-D,* Results are presented for all vaccinates. *E-H,* Results are presented for all control animals. For proliferation assays, lymphocytes from animals 3960 and 3991 were cryopreserved, whereas lymphocytes from remaining animals were fresh. The data represent the percentages of tetramer$^+$ CD4$^+$ cells of the total CD4$^+$ T cells (black circles) or the mean CPM of triplicate cultures with 1 μg/ml peptide F2-5 after subtracting the background mean CPM in response to medium (white triangles).

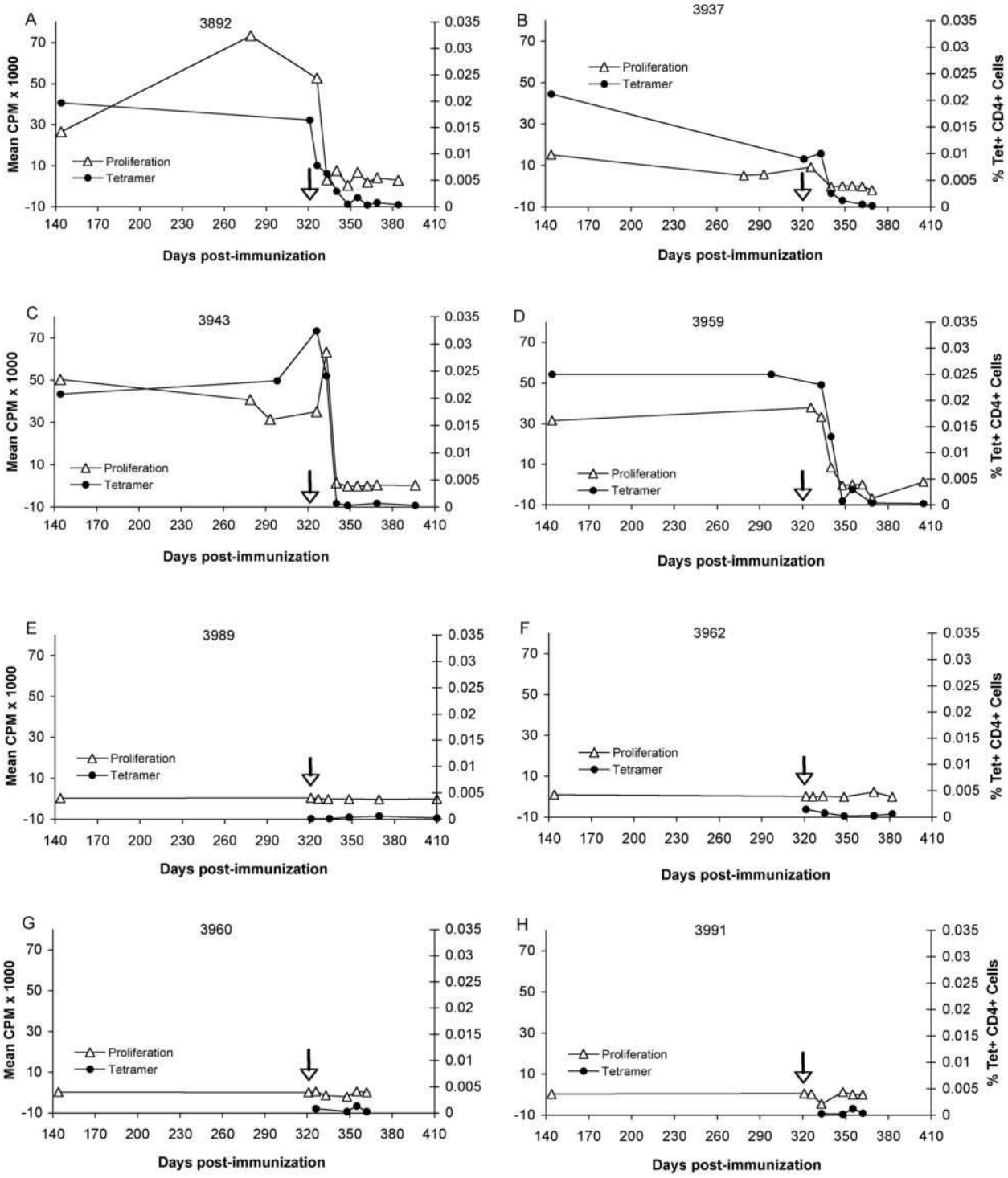
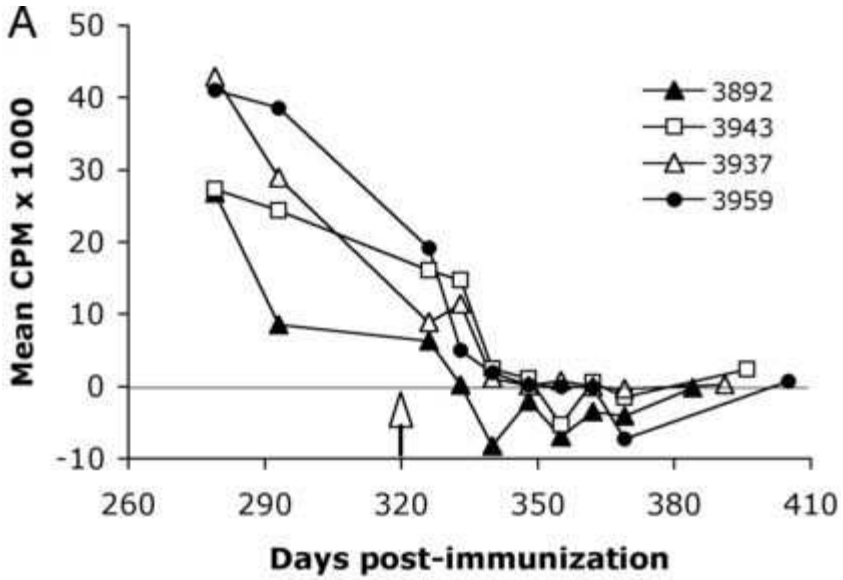

A
3892
B
3937
C
3943
D
3959
E
3989
F
3962
G
3960
H
3991
Mean CPM x 1000
% Tet+ CD4+ Cells
Proliferation
Tetramer
Days post-immunization

Figure 5.

Proliferation of PBMC against *A. marginale* homogenate before and after *A. marginale* infection. *A,* PBMC from the four vaccinates or *B,* two controls (indicated by symbols) were obtained before and at various time points after challenge on day 321 (arrow) and fresh cells were cultured in triplicate for 6 days with *A. marginale* homogenate at 10 μg/ml. Data are presented as the mean CPM after subtracting background responses to 10 μg/ml URBC antigen. Responses were significantly greater than those to either medium or URBC in all vaccinates on both days before and on day 5 post-challenge. There was no significant response to *A. marginale* in either control animal at any time point.


A
50
40
30
20
10
0
-10
Mean CPM x 1000
3892
3943
3937
3959
260
290
320
350
380
410
Days post-immunization

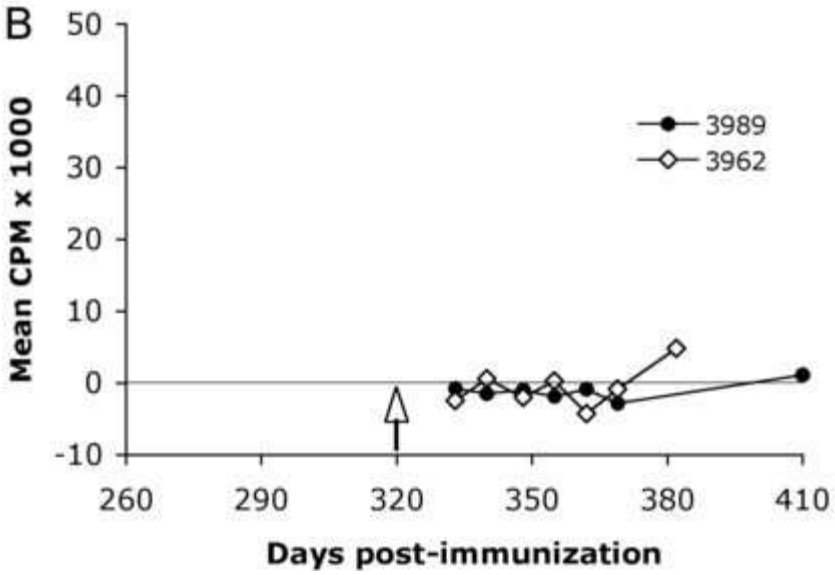
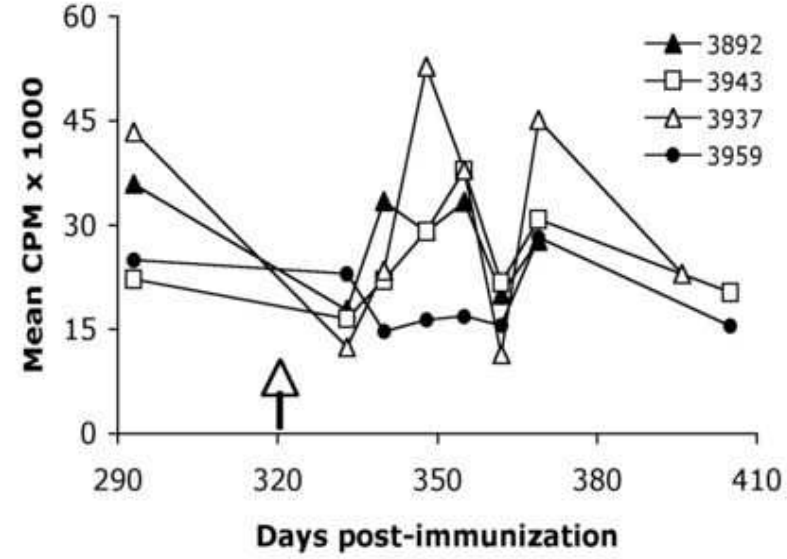

B
50
40
30
20
10
0
-10
Mean CPM x 1000
3989
3962
260
290
320
350
380
410
Days post-immunization

Figure 6.

Proliferation of PBMC from immunized animals to *Clostridium* sp. vaccine antigen before and after *A. marginale* infection. *A*, PBMC from the four immunized animals (indicated by symbols) were obtained before and at various time points after challenge on day 321 (arrow) and fresh cells were cultured in triplicate for 6 days with *Clostridium* sp. antigen diluted 1:1,000. Data are presented as the mean CPM after subtracting background responses to medium. Responses were significantly greater than those to medium or URBC for all animals at all time points. *B,* $CD4^+$ T cells were positively selected from cryopreserved bovine PBMC with anti-8 CD4 MAb ILA-11 bound to goat anti-mouse IgG beads (Miltenyi Biotec) passed over a magnetic field. Cells were stained for $CD4^+$ cells by FACS using MAb CACT138A. Undepleted PBMC (28.2% $CD4^+$ T cells), enriched $CD4^+$ T cells (79.1% $CD4^+$ T cells), and PBMC depleted of $CD4^+$ T cells (3.4% $CD4^+$ T cells) were tested in a proliferation assay using *Clostridium* sp. antigen diluted 1:100 and 1:1,000, with 2.5 x 10^5 PBMC or CD4-depleted PBMC/well, 2.5 x 10^5 $CD4^+$- enriched cells, plus 2 x 10^5 irradiated PBMC as APC/well, or APC alone. Results are presented as the stimulation index, calculated as the mean CPM of cells cultured with 15 μg/ml antigen/mean CPM of cells cultured with complete medium. Statistically significant responses to antigen are indicated by asterisks, where $p<0.05$.

A


60
45
30
15
0
Mean CPM x 1000
290
320
350
380
410
Days post-immunization
3892
3943
3937
3959

B

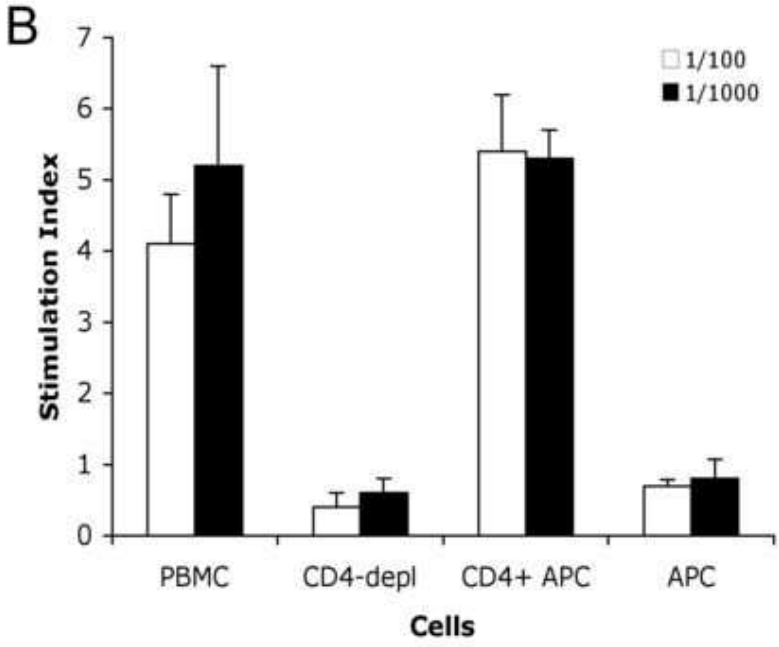
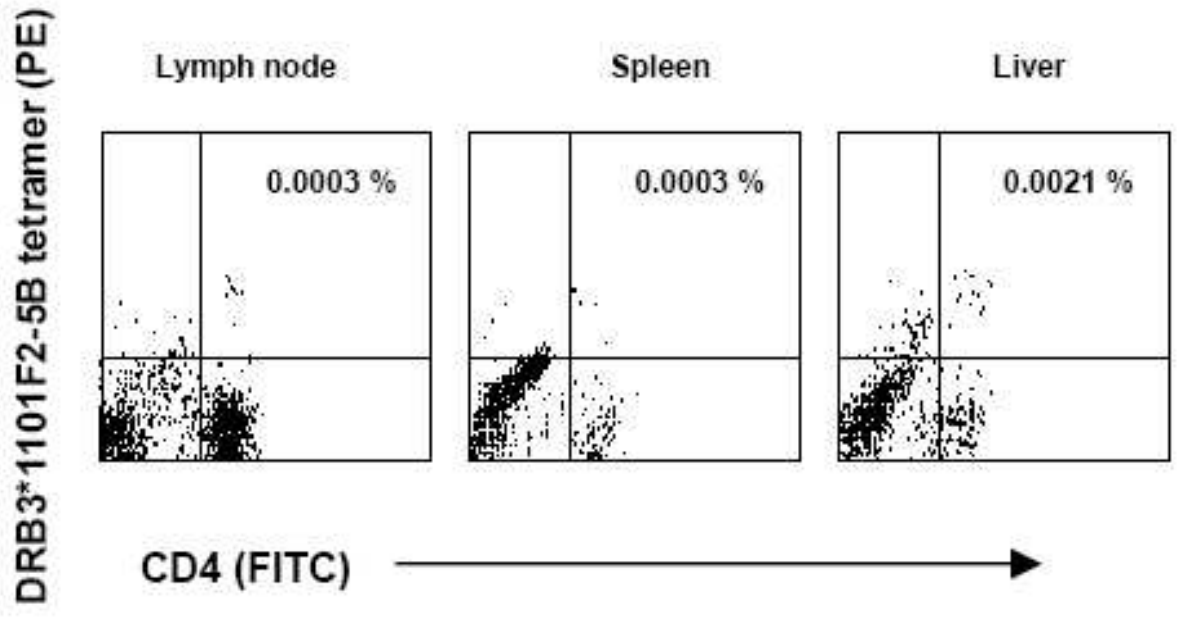

7
6
5
4
3
2
1
0
Stimulation Index
1/100
1/1000
PBMC
CD4-depl
CD4+ APC
APC
Cells

Figure 7.

Enumeration of antigen-specific $CD4^+$ T cells in the inguinal LN, spleen, and liver from animal 3959. Cryopreserved lymphocytes were stained with the DRB3*1101-F2-5B tetramer and analyzed by FACS. The dot plots indicate the $tetramer^+$ $CD4^+$ cells after selection on anti-PE magnetic beads. The numbers in the upper right quadrants are the calculated percentages of $tetramer^+$ $CD4^+$ T cells of total $CD4^+$ T cells.



ATTRIBUTIONS

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Rapid deletion of antigen-specific CD4$^+$ T cells following infection represents a strategy of immune evasion and persistence for Anaplasma marginale.

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Sushan Han co-authored this paper with Junzo Norimine. I originally helped design this project and submitted the proposal as an NIH K08 grant. I was responsible for all cattle work including challenge of the calves and sample collection from the time of challenge through euthanasia, which included daily monitoring of calves and veterinary support during acute anaplasmosis. At euthanasia I performed necropsies, tissue collection, and tissue lymphocyte purification from all calves. I performed all proliferation assays, bovine ELISPOT assays, tetramer staining, and flow cytometry analysis from prior to challenge to the completion of the study. I analyzed, graphed, and performed statistical analyses as well as repeated assays, on all data. I drafted the original manuscript, which was later combined with the contribution of Junzo Norimine. I made revisions and performed additional negative control assays for satisfaction of the reviewers from *Journal of Immunology* and for eventual publishing of this manuscript.

Junzo Norimine co-authored this paper with Sushan Han. He developed the first bovine MHC class II tetramer and developed the negative control and MSP1a F2-5b tetramers used in this study. He performed the tetramer staining and flow cytometry analysis on all calves from pre-immunization until pre-challenge which was presented in the manuscript and standardized our method for generating the frequency of antigen-specific T cells. He oversaw each step of this study and helped interpret our results. He helped write the manuscript and made many of the revisions necessary for final publishing in the *Journal of Immunology*.

Guy Palmer helped design this project and helped in the writing of the original grant proposal which funded the work, and the final manuscript.

Waithaka Mwangi designed the F2-5 DNA immunization that was successfully used on these calves.

Kevin K. Lahmers helped to euthanize and necropsy calves at the termination of the study.

Wendy C. Brown is the principal investigator in our laboratory and specifically on this project. She helped design this study, helped to draft and submit the grants that funded this work, and oversaw each assay and helped interpret all data published in the manuscript. She was fundamental in reviewing and writing the manuscript and revisions as published. Additionally, she supplied extensive knowledge and guidance throughout this study.

CHAPTER TWO

INTRODUCTION

Calves infected with *Anaplasma marginale* have persistent high-load bacteremia that induces sustained high IgG titers associated with delayed and transient T lymphocyte responses

Pathogens capable of persistent infection in humans and animals present a unique opportunity to investigate the subtle interplay between the host immune system and the unique survival mechanisms of the organism. Intracellular organisms, in particular, utilize numerous mechanisms of cellular and humoral immune evasion/modulation tactics in order to maintain a suitable environment for replication and long-term survival within a stable host environment (1). *Anaplasma marginale* is a persistent pathogen that evades the immune response by undergoing continual antigenic variation in two outer membrane proteins (OMP), major surface protein (MSP)2 and MSP3 (2). Transmitted by *Ixodes* ticks, *A. marginale* is an intraerythrocytic rickettsial pathogen of ruminants, which causes an acute infection where bacteremia can reach approximately 10^9 organisms/ml of blood. Clinical disease is associated with anemia, fever, and malaise. Animals that survive acute infection develop a lifelong persistent infection in which the immunocompetent and clinically healthy host controls, but fails to clear bacteremia, with levels averaging 10^6 organisms/ml of blood (3,4). Persistently infected cattle, therefore, provide an important reservoir of bacteria within endemic areas.

Mechanisms of immune control of *A. marginale* have not been completely elucidated; however, experimentally infected animals consistently produce high levels of OMP-specific IgG1 and IgG2 (3, 5-9), which are thought to be $CD4^+$ T cell dependent (3,10,11). Accordingly,

the current paradigm of immune control of *A. marginale*, as with most intracellular bacteria, is that infection induces antigen-specific $CD4^+$ T lymphocyte priming and activation, driving a predominantly Th1-type response characterized principally by lymphocyte proliferation and IFN-γ secretion. IFN-γ is thought to activate macrophages to enhance phagocytosis and NO production, both of which are assumed to be important for the removal of infected erythrocytes and killing of intracellular bacteria (3, 12).

Interestingly, two previous studies have shown that calves immunized with whole OMP of *A. marginale*, MSP2 (8) and the F2-5 peptide of MSP1a (13), generated robust Th1-type antigen-specific memory $CD4^+$ T lymphocyte proliferation and IFN-γ secretion, but antigen-specific $CD4^+$ T responses rapidly disappeared when calves were challenged with homologous Florida strain bacteria (8, 13). In the MSP2 vaccinates, MSP2- and *A. marginale*-specific effector/memory $CD4^+$ T cell responses remained undetectable for one year despite continual production of high titers of specific IgG (8). Using bovine major histocompatibility (MHC) class II tetramers specific for the MSP1a peptide F2-5, a similar loss of antigen-specific $CD4^+$ T cell responses was shown to be due to rapid physical deletion of antigen-specific $CD4^+$ T cells from the blood during acute infection (13). Furthermore, following infection of both immunized and control cattle in each of these studies, T cell responses specific for *A. marginale* lysate were not observed in PBMC (8, 13). This suggested that infection with *A. marginale* not only down regulated pre-existing $CD4^+$ T cell responses induced by immunization, but also impaired the response to additional antigens primed by infection.

These results challenge the current paradigm of the role of $CD4^+$ T cells in immune control of *A. marginale*. However, the lack of detectable antigen-specific $CD4^+$ T cell responses in blood may have been due to the sampling interval or the strain of bacteria used (non-tick

transmissible Florida strain). Furthermore, during acute infection, specific $CD4^+$ T cells may be sequestered in the spleen where blood-borne pathogens are removed from the circulation (14). In this study we investigated these possibilities by measuring T cell and antibody responses in calves during the acute and persistent phases of infection with either tick transmissible South Idaho strain or non-tick transmissible Florida strain organisms. In the first case, calves were monitored weekly for antigen-specific humoral (IgG) and T-lymphocyte responses in PBMC for nearly one year. In the second case, calves were monitored for humoral and lymphocyte responses every 3-7 days in blood and exteriorized spleens for 4 months following infection. Our results are consistent with down regulation of *A. marginale*-specific $CD4^+$ T lymphocyte responses primed during acute infection, and with continual regulation of T cell responses that may arise during persistent infection, in the face of high titers of *A. marginale*-specific IgG maintained throughout infection.

Materials and Methods

Calves

Four Holstein calves, C1028b1**,** C1030b1, 33875, and 33901, were serologically negative for *A. marginale* and received Vision 7 killed *Clostridium* sp. vaccine (Intervet) prior to onset of the study. The MHC class II haplotype of all calves was determined by restriction fragment length polymorphism and selected calves were determined to be heterozygous for the MHC class II phenotype BoLA *DRB3*1101*. This haplotype is genetically linked the *DQA*2206 /DQB*1402* intrahaplotype phenotype, which was verified to be expressed by sequencing DNA from 2 of the 4 calves. Blood was collected by jugular venipuncture in all animals weekly to

three times per week starting at the time of infection to the termination of the study, which varied from 110 to 320 days. PBMC were purified from blood and used fresh or cryopreserved in liquid nitrogen. Prior to the study, calves 33875 and 33901 underwent a surgery to marsupialize the body and tail of the spleen. The spleen was exteriorized through an incision in the abdominal wall and a window in the 11^{th} rib, into a lateral abdominal subcutaneous pouch (15) and splenic lymphocytes were purified from aspirates collected by direct biopsy of the exteriorized spleen. Splenic aspiration was performed as described (16). Aspirates were collected weekly to 3 times per week at the time of infection to 110 days post-infection. Splenic lymphocytes were used fresh or cryopreserved in liquid nitrogen. All animal studies were conducted using an approved Institutional Animal Care and Use Center (Washington State University, Pullman, WA) protocol.

Infection with A. marginale

Calves C1028b1 and C1030b1 were infected with *A. marginale* via transmission feeding of acquisition-fed *Dermacentor andersoni* adult male ticks. Ticks used for infection were acquisition fed for 48h on an *A. marginale* South Idaho strain infected carrier calf near peak bacteremia. Calf C1028bl was infected with 133 ticks which were applied beneath a dermal patch placed on the dorsal back of the calf. Ticks were allowed to transmission feed for 7 days and were removed. Calf C1030bl was similarly infected with 134 male ticks derived from the same acquisition feeding. Calves 33875 and 33901 were infected intravenously into the jugular vein with *A. marginale* Florida strain, a non-tick transmissible strain of *A. marginale*, using $1.0X10^6$ infected fresh red blood cells from an acutely infected donor calf C1285bl near peak bacteremia (24% percent parasitized erythrocytes) diluted in Hank's balanced salt solution (HBSS). Following infection the rectal temperature, pack cell volume, percent infected

erythrocytes as determined by light microscopy of Giemsa-stained blood films, and general health were monitored daily in all infected calves from the time of infection until 42 days post-infection and all animals recovered from acute infection without additional therapy. Before and throughout infection blood and spleen biopsies were collected weekly and up to 3 times per week. PBMC were purified by Histopaque® (Sigma-Aldrich) density gradient centrifugation and repeated washing in HBSS. Splenocytes were purified from splenic aspirates by gently plunging aspirates through glass tissue homogenizers and purifying cells with Histopaque® density gradient centrifugation and washing with Dulbecco's medium (16). All cells were maintained in complete RPMI 1640 (8, 17) and used immediately in proliferation assays and for generating cell lines, or cryopreserved for later assays.

A.marginale-specific antibody quantitation

Serum was collected weekly during the course of infection and antibody specific to MSP5 *A. marginale* was measured to determine the time course and magnitude of the antibody response during acute and persistent infection. Sera were stored at -20^{o}C until the assays were performed simultaneously on all samples. MSP5 antibody was detected using a competitive enzyme-linked immunosorbent assay (C-ELISA) commercial kit (VMRD) as previously described (18) and following the manufacturer's instructions. Briefly, 70 µl of undiluted room temp serum was adsorbed on commercial adsorption / transfer plates and transferred to a 96-well plate coated with recombinant *A. marginale* MSP5, incubated 1 h at room temperature, washed twice, incubated with 50 µl of MSP5-specific antibody-peroxidase conjugate and incubated at room temp for 20 min, washed, and developed with commercial Substrate Solution in the dark. After stopping the reaction, the optical density (O.D.) was read in a plate reader at 650 nm

wavelength with standards. The percent inhibition (%I) of commercial MSP5-peroxidase conjugated antibody was calculated as: %I = 100-[(Sample O.D. x 100) ÷ (Mean Negative Control O.D.)], and results were interpreted as sera with < 30% inhibition as negative for *A. marginale*-specific antibody, and sera with >30% inhibition were considered positive for *A. marginale*-specific antibody. For selected sera, antibody titers specific for *A. marginale* were determined by immunoblotting with sera diluted 1:100, 1:1,000, 1:3,000, 1:30,000, 1:100,000, and 1:1,000,000 in blocking buffer (I-block®, Applied Biosystems) casein based solution containing 5% bovine serum albumin and 0.5% Tween 20 (New England Biolabs). For blots 200 µg of *A. marginale* lysate was boiled 5 min in 1X sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.025 mM Tris-HCl pH 6.8, 2% SDS, 15% glycerol, 2.5% B-mercaptoethanol, 0.02% bromophenol blue), electrophoresed on 10% Tris-HCl polyacrylamide Criterion gels (Bio-Rad) under denaturing conditions for 1 h at 100 V, and protein was transferred to 0.45 µm-pore-size nitrocellulose membranes electro-blotting at 100 V for 30 min. Membranes were cut into individual strips, dried over night, and blocked with blocking buffer. Each strip was incubated with individual dilutions of serum for 1.5 h rocking at room temp. Membranes were washed and blocked with blocking buffer, and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-bovine IgG diluted 1:10,000 in blocking buffer for 1.5 hours rocking at room temp. Membranes were washed several times with blocking buffer and developed with ECL Western Blotting Substrate (Thermo Scientific Pierce) as directed. Standard radiographic film (Kodak) was exposed to membranes and developed in an automatic film processor (Kodak). Pre-immune sera and uninfected red blood cells were used as negative controls, and serum from an *A. marginale* South Idaho strain infected calf (C894b1) with a known *A. marginale*-specific IgG titer, was used as a positive control antibody for each immunoblot to standardize between immunoblots. IgG1 and IgG2 titers were determined as

described (19) using serially diluted serum samples. Secondary antibodies were mouse-anti-bovine IgG1 (Serotec), or mouse-anti-bovine IgG2 (Serotec) used at a 1:100 dilution in blocking buffer, rocking for 1.5 h at room temp. Membranes were washed repeatedly and incubated with HRP-conjugated goat anti-mouse IgG (Kirkegaard and Perry Laboratories), washed several times with blocking buffer, and developed with ECL Western Blot Substrate as previously described (19).

Real time (RT)-PCR for detection and quantitation of bacteria

To quantitate *A. marginale* bacterial numbers in whole blood during infection, a previously described real-time TaqMan® (Applied Biosystems) assay based on the copy number of the *msp5* gene from *A. marginale* St. Maries strain was performed (20, 21). Peripheral blood was collected weekly during infection and samples were washed three times in phosphate buffered saline (PBS, pH 7.2) with centrifugation to remove leukocytes. Washed erythrocytes were diluted 1:1 in PBS and frozen at -20^{o} C until sample were processed. DNA was extracted from 300 µl of washed erythrocytes using a genPURE® kit (Qiagen) following manufacturer instructions. RT-PCR reactions were performed in triplicate using 100 ng of template DNA per reaction in a total volume of 50 µl including 25 µl Taqman Universal buffer, 1.25 µl *msp5* forward primer, 1.24 µl *msp5* reverse primer, 0.125 µl of MSP5 probe, and 17.4 µl of nuclease free water. Once bacterial copy number was determined for the 100 ng template DNA, the number of organisms per ml of whole blood was calculated.

Lymphocyte proliferation assays

Lymphocytes from PBMC and spleen were used fresh or thawed from cryopreserved aliquots and assays were performed in triplicate using 2.5 x 10^5 viable cells/well in complete RPMI 1640 medium (Gibco) (8, 17). Cells were stimulated with 15.0 μg/ml of each antigen using *A. marginale* South Idaho strain and Florida strain lysate (matching infection strain) and negative control uninfected erythrocyte membranes derived from a non-infected calf (URBC). T cell growth factor (TCGF) diluted 1/10 and *Clostridium* sp. vaccine antigen diluted 1:100 or 1:1,000 in complete RPMI 1640 medium were positive controls. *Clostridium* sp. antigen acts as a positive control as all calves were immunized with this vaccine prior to the study and vaccination induced antigen-specific $CD4^+$ cell responses detectable by proliferation assays (13). After 6 days of culture at 37°C in 5% CO_2 in air, cells were labeled with 0.25 μCi [^{3}H] thymidine for 8 h, harvested, and counted in a beta counter. In South Idaho infected calves one week cell lines were developed for use in proliferation assays. Cell lines were established in seven-day cultures using 4.0 x 10^6 cryopreserved PBMC per well in a 24-well plate, in 1.5 ml total volume complete RPMI 1640 medium, and stimulating with 5 μg/ml of South Idaho strain lysate. Proliferation assays using cell lines were performed in triplicate in 96-well plates using 2.0 x 10^4 lymphocytes with 2.5 x 10^5 fresh irradiated PBMC from the same calf, and cultured with 15.0 μg/ml of *A. marginale* South Idaho strain lysate, or positive or negative control antigens, as previously described, in a total volume of 100 μl of complete RPMI 1640 medium. Cells were cultured at 37°C for 4 days, pulsed with 0.25 μCi [^{3}H] thymidine for 8 h, harvested, and counted in a beta counter. In some experiments frozen PBMC or cell lines were depleted of $CD4^+$, $CD8^+$, or γδ T cells and cultured in 4 or 6 day proliferation assays. Depletion was accomplished by incubating cells with monoclonal antibody (MAb) specific for $CD4^+$ (ILA-11) (Washington Monoclonal Antibody Center), $CD8^+$ (7C2B) (Washington Monoclonal Antibody Center), and

γδ T (GB21A) (Washington Monoclonal Antibody Center) at 15 μg MAb per 10 x 10^6 cells rotating at 4°C for 30 min. Cells were then repeatedly washed with compete 1640 RPMI medium and MAC bead buffer (Miltenyi) with 5% bovine serum albumin (BSA). MAb labeled cells were incubated with 80 μl of goat anti-mouse IgG magnetic micro-beads (Miltenyi) per 10 x 10^6 cells, on ice for 20 min. MAb and bead labeled cells were depleted from the aliquot by filtration through a LS magnetic column (Miltenyi) under a strong magnetic field, washed with 3 ml of MACs buffer with BSA. Successfully depleted and enriched aliquots of cells were verified by flow cytometry prior to culture, and aliquots were cultured with antigen in 4 and 6 day cultures as described above for whole PBMC or cell lines, respectively. Statistical significance was determined within each proliferation assay by comparing the mean counts per minute (CPM) of antigen to the mean CPM of negative control antigen at the same concentration using unpaired Student t-tests with a Welch correction for variation in the standard deviation. Aliquots of lymphocytes and splenocytes with significant response ($p < 0.05$) to *A. marginale* were then compared over the course of infection using a one-way ANOVA with a Bonferroni correction for multiple comparisons, and results were considered significant when $p < 0.05$.

Bovine IFN-γ ELISPOT

Bovine IFN-γ ELISPOT assays were performed with purified and cryopreserved PBMC and splenocytes as described (8, 17). Briefly, viable cells were purified on a Histopaque® gradient, and cultured in triplicate using 1.0 X 10^6 cells/well with 15.0 μg/ml *A. marginale* South Idaho strain lysate or Florida strain lysate, and URBC and medium as a negative control. A mixture of PHA (Sigma-Aldrich) (1 μg/ml) plus 0.01 ng/ml recombinant human IL-12 (Genetics Institute) was used as the positive control. Results were assessed by counting the mean number

of spot-forming cells (SFC) cultured with *A. marginale* lysate minus the mean number of SFC cultured with medium, and the results were statistically analyzed using a Student's one-tailed *t*-test where $p < 0.05$ was considered a significant response.

RESULTS

Infection of naïve calves with A. marginale South Idaho strain

In two previous studies, we observed that antigen-specific $CD4^+$ T cell responses elicited by immunization were eliminated at the peak of bacteremia following intravenous infection with the non-tick transmissible Florida strain (8, 13). Additionally, we observed that non-immunized control calves infected with *A. marginale* also failed to develop significant $CD4^+$ T cell responses specific for *A. marginale* throughout the course of the study (nearly one year in some cases). To extend our observations, we systematically followed antigen-specific lymphocyte responses in two calves infected by transmission feeding of *A. marginale* South Idaho strain infected *Dermacentor andersoni* ticks, the natural vector. Calves (C1028b1 and C1030b1) developed acute anaplasmosis with fever, malaise, and clinical anemia that was concurrent with peak blood levels of bacteria (Fig.1A, B). Bacteremia peaked on day 34 post-infection with 2.37×10^7 and 1.27×10^7 organisms/ml blood in calves C1028b1 and C1030b1, respectively. Anemia resulted in a decrease in day 0 percent PCV of 34.5% (C1028b1) and 43.8% (C1030b1). Acute phase anaplasmosis was followed by a persistent phase characterized by cyclical waves of bacteremia in both calves, reaching levels of $1\text{-}2 \times 10^5$ organisms/ml of blood until day 336 post-infection at the termination of the study.

Antigen-specific antibody and lymphoproliferative responses during acute and persistent phase anaplasmosis in South Idaho infected calves

Serologic responses specific for *A. marginale* were monitored monthly and antigen-specific cellular immune responses were monitored weekly using lymphocyte proliferation assays and IFN-γ ELISPOT assays. Using an MSP5 C-ELISA assay, both calves were shown to develop significant levels of *A. marginale*-specific antibody (>30% competitive inhibition) by day 28 post-infection. Levels peaked on day 55 (calf C1028b1) and day 48 (calf C1030b1) post-infection, after which specific-antibody levels remained high (> 50% inhibition) until the termination of the study (Fig. 1C, D). IgG serum titers, representing time points associated with acute and persistent phase anaplasmosis were determined by Western blotting using *A. marginale* South Idaho strain organisms as an antigen (Table I). *A. marginale*-specific IgG was first detected on day 9 post-infection with titers of 1,000 (calf C1028b1) and 10,000 (calf C1030b1). Serum from day 9 to day 274 post-infection consistently maintained positive titers of antigen-specific IgG between 1,000 and 1,000,0000, consistent with C-ELISA data.

To evaluate the antigen-specific T lymphocyte response during infection, fresh PBMC obtained weekly was tested for response to *A. marginale* South Idaho bacterial lysate (Fig. 2A, B). *A. marginale* South Idaho infected calves developed significant lymphocyte proliferative responses first observed on day 106 (C1028b1) and 112 (C1030b1) post-infection. Responses in both calves were transient and not significant the following one to two weeks. These initial responses occurred once bacteremias had fallen below 10^6 organisms/ml of blood. Between days 106 and 336 post-infection, antigen-specific lymphocyte proliferation was detected seven and eight times in calves 1030b1 and C1028b1, respectively, with no obvious association with level or pattern of fluctuating bacteremia, or changes in specific-humoral responses. In contrast, T

lymphocytes responded to T cell growth factor throughout the study, showing that the lack of T cell proliferation to *A. marginale* was not because cells were generally incapable of responding (data not shown).

To characterize the lymphocytes responding to *A. marginale* at time points where significant responses were observed, proliferation was repeated using one week cell lines derived from cryopreserved PBMC cultured with *A. marginale* South Idaho strain lysate. Cell lines were then depleted of either $CD4^+$ cells, or other lymphocyte subsets capable of proliferation and cytokine production in response to antigen, notably $CD8^+$ cells and γδ T cells. Depletions were performed using magnetic beads coated with specific lymphocyte-specific MAb (Fig. 2C, D). *A. marginale*-specific lymphocyte proliferation was maintained when cell lines were enriched for $CD4^+$ T lymphocytes by depleting either $CD8^+$ cells or γδ T cells, but responses were lost when cell lines were depleted of $CD4^+$ T lymphocytes. These results demonstrate that *A. marginale*-specific lymphocyte proliferation in persistent anaplasmosis was due primarily to antigen-specific $CD4^+$ T lymphocytes.

We next determined if antigen-specific $CD4^+$ lymphocyte proliferation was associated with production of IFN-γ, typical of Th1-type responses, and to enumerate these antigen-specific cells. PBMC obtained at both responsive and unresponsive time points were used in IFN-γ ELISPOT assays with *A. marginale* lysate. PBMC from time points that had antigen-specific $CD4^+$ T lymphocyte proliferation did not show significantly elevated numbers of IFN-γ producing cells, and the only significant numbers of *A. marginale*-specific SFC were noted one time in each calf on a day that lacked significant proliferative responses (Table II). These results demonstrate that $CD4^+$ antigen-specific T lymphocyte responses occurred during persistent *A. marginale* infection, although responses were sporadic and transient and were usually not

associated with significant IFN-γ secretion, inconsistent with a Th1-type response. Interestingly, background numbers of IFN-γ secreting cells (measured as mean SFC cultured with medium) tended to increase in animal C1028b1over the course of infection, and were relatively high by day 21 in animal C1030b1 and remained high throughout infection (Table II). Of interest too is the finding that in the majority of cases, the number of SFC was lower in response to *A. marginale* than to medium (Table II). Previous studies using similar assay conditions also detected significant numbers of SFC in response to medium alone in immunized cattle following challenge with *A. marginale* (8, 13).

The lack of antigen-specific $CD4^+$ T lymphocyte proliferation during acute infection is also difficult to reconcile with early isotype switching to IgG and development of high and persistent titers, which is typically dependent on $CD4^+$ T lymphocyte help. This led us to determine whether antigen-specific $CD4^+$ T lymphocyte responses are found in the spleen, where infected erythrocytes are presumably removed (14). It was also possible that we failed to detect antigen-specific lymphocyte proliferation by examining PBMC weekly during acute anaplasmosis. Lastly, it was possible that exposure to ticks induced an early immune suppression to *A. marginale* (22).

Infection of spleen-marsupialized calves with A. marginale Florida strain

Since transient antigen-specific $CD4^+$ T lymphocyte responses were detected in PBMC of South Idaho strain infected calves only after acute infection was resolved, but antigen-specific IgG, which is traditionally dependent on $CD4^+$ T lymphocyte help, occurred day 9 post-infection, we suspected that early antigen-specific $CD4^+$ T lymphocyte responses occurred in PBMC, but in the face of rapidly rising bacterial levels, and hence, high antigen load, responses may have

been more rapidly transient, and we may have missed significant antigen-specific $CD4^+$ T lymphocyte responses with our previous sampling interval. Therefore, in this second study group, we chose to examine PBMC of calves infected with *A. marginale* of the Florida strain more closely by increasing the sampling interval to every 2 to 3 days during acute infection specifically incorporating days with rising, peak, and post-peak levels of bacteria. Additionally, we suspected that antigen-specific $CD4^+$ T lymphocyte may be undetectable in the PBMC due to sequestration in the spleen. Therefore, we surgically exteriorized the spleens of these calves prior to infection to facilitate frequent needle biopsies and examination of splenocytes throughout infection. Holstein calves 33875 and 33901, also expressing the MHC class II haplotype *DRB3*1101*, were used for this second phase of the study. Calves were infected intravenously with non-tick-transmissible *A. marginale* Florida stain which also served to eliminate possible suppression by tick infestation. Typical acute infection was observed in both calves as demonstrated by fever, malaise, and bacteremias reaching peak levels of 4.78×10^9 organisms/ml of blood (calf 33875) and 6.58×10^9 organisms/ml of blood (calf 33901) on days 31 and 26 post-infection, respectively. Anemia was quite severe, and associated with decreases in PCV of 61.8% (calf 33875) and 62.3% (calf 33901) (Fig. 3A, B). Both calves resolved anemia and remained persistently infected with bacteremia ranging from 10^4 to 10^8 organisms/ml of blood. In addition to weekly sampling of PBMC, serum, and splenocytes we intensively collected PBMC and splenocytes every two to three days for a period of three weeks spanning peak bacteremia to examine whether transient antigen-specific $CD4^+$ T lymphocyte responses could be detected in early acute anaplasmosis. Sera and lymphocytes were monitored for antigen-specific responses to *A. marginale* Florida strain antigen.

Antigen-specific antibody and lymphoproliferative responses in A. marginale Florida infected calves

Similar to tick-transmitted South Idaho strain infected calves, Florida strain infected calves had antigen-specific antibody responses detected by MSP5 C-ELISA as early as day 20 post-infection (Fig. 3C, D). *A. marginale*-specific antibody levels increased sharply and reached a plateau by day 27 post-infection in both calves, remaining between 83.49 and 94.56% inhibition for 110 post-infection until the termination of the study. IgG titers were determined in serum samples from all phases of infection by Western blotting using *A. marginale* Florida strain lysate and sera diluted from 1:100 to 1:1,000,000 (Table I). Calves had specific IgG titers of 3,000 and 30,000 (33875 and 33901, respectively) by day 13 post-infection and both had titers of 1,000,000 on day 110, consistent with C-ELISA data. To sub-classify IgG isotypes, titers of *A. marginale*-specific IgG1 and IgG2 were also determined. On day 13, specific IgG1 and IgG2 titers of 3,000 (33901) and 30,000 (33875) were measured, and on day 20 post-infection titers were similar with 3,000 IgG1 and IgG2 (33875) and 3,000 IgG1 and 30,000 IgG2 (33901). On day 79 post-infection, IgG1 and IgG2 were detected in each calf at a titer of 3,000. Our findings show that antigen-specific IgG was comprised of approximately equivalent amounts of IgG1 and IgG2 antibody during both early and late acute anaplasmosis.

To determine antigen-specific T lymphocyte responses, PBMC and splenocytes were collected weekly or every two to three days during ascending, peak, and descending bacteremia. Significant *A. marginale*-specific lymphocyte proliferation in PBMC was detected earlier than in the South Idaho infected calves, on day 36 (33875) and day 48 (33901) post-infection, concurrent with declining levels of bacteremia (Fig. 4A, B). Similar to South Idaho infected calves, *A. marginale*-specific proliferation was transient, immediately ceasing within one to two

weeks following initial detection and recurring sporadically concurrent with levels of bacteremia below 10^7 organism/ml on days 69 and 82 post-infection (calf 33875) and day 69 (calf 33901) post-infection. Cryopreserved cells from all responsive time points were retested and similar proliferation results were obtained at least twice (data not shown). Results indicate that antigen-specific T cell responses occur in the PBMC during acute infection, although responses are transient, detectable for no more than two consecutive weeks, and then undetectable for several weeks. Additionally, responses prior to the peak of infection, in the face of ascending and very high bacteremia, could not be detected despite increasing the sampling interval to every two to three days.

In spleens, the first significant antigen-specific lymphocyte proliferation was detected on day 48 post-infection in calf 33901, concurrent with significant proliferative responses in the PBMC, and day 89 post-infection in calf 33875. Lymphocyte proliferative responses were found to be similarly transient immediately disappearing the following week and recurring only on day 104 (33875) and days 89 and 104 (33901) (Fig. 4C, D). These responses were repeatable with cryopreserved cells (data not shown). All PBMC and splenocytes were cultured with 1:100 dilution of *Clostridium* sp. vaccine as a positive control antigen. *Clostridium* sp. antigen served as a positive non-*A. marginale* $CD4^+$ T cell control as calves had been immunized with *Clostridium* sp. prior to the investigation and all calves maintained significant *Clostridium*-specific T cell responses detectible in proliferation assays at all time points of infection, as had been demonstrated in our previous studies (8, 13). Study calves maintained significant proliferative responses to *Clostridium* sp. at all time points throughout infection, indicating that memory $CD4^+$ T cell responses to non-*A. marginale* antigens were intact and unaffected (Fig. 4E through H). Our results show that antigen-specific lymphocyte proliferative responses do occur within the PBMC and spleen during the early stage of infection, but are detectable only after the

peak of bacteremia. These results suggest that antigen-specific cells do not sequester long-term in the spleen, but rather occur transiently similar to PBMC, with responses disappearing rapidly and reoccurring sporadically during infection.

To further characterize antigen-specific proliferative lymphocyte responses in PBMC, proliferation assays were repeated using PBMC enriched for either $CD4^+$ T cells, or for $CD8^+$ cells and γδ T cells (Fig. 4I). PBMC from responding time points maintained *A. marginale*-specific proliferation when enriched for $CD4^+$ T lymphocytes, but lost the ability to respond when PBMC were depleted of $CD4^+$ T cells (enriched for $CD8^+$ and γδ T cells), indicating that antigen-specific lymphocyte proliferation detected early and late in infection was primarily due to antigen-specific $CD4^+$ T lymphocytes. Similarly, enrichment of lymphocyte subsets was attempted using cryopreserved splenocytes from responding time points or by developing one-week cell lines derived from responding cryopreserved splenocytes by culturing with *A. marginale* Florida strain lysate, but due to low numbers of cells assay attempts were unsuccessful (data not shown). To determine if responding and non-responding time points of PBMC produced IFN-γ, IFN-γ ELISPOT assays were performed using cryopreserved PBMC cultured with *A. marginale* Florida strain lysate to enumerate cells producing IFN-γ in an antigen-specific manner. Similar to South Idaho infected calves, at no responsive time point was *A. marginale*-specific IFN-γ secretion detected in PBMC (Table II). We concluded that infection with *A. marginale* therefore induced transient antigen-specific $CD4^+$ T cell proliferative responses that were not consistent with a Th-1 type response.

DISCUSSION

As with most intracellular parasites, control of *A. marginale* infection is presumed to be based on effective antigen-specific cellular and humoral immune responses characterized by early priming and proliferation of antigen-specific $CD4^+$ T lymphocytes that release key cytokines, such as IL-2 and IFN-γ, which in turn drive additional activation and proliferation of $CD4^+$ T lymphocytes and activation of macrophages for NO production and phagocytosis of infected host cells (3). Supporting evidence for this paradigm includes a study in which calves immunized with *A. marginale* OMP were completely protected from challenge infection with live *A. marginale*, and protection correlated with OMP-specific $CD4^+$ T cell proliferation, IFN-γ production, and IgG2 titers (26). Additionally, it is well accepted that antigen-specific $CD4^+$ T cells are instrumental in activating B cells via antigen-presentation by B cell MHC class II molecules to T cells, and via CD40-CD40L interaction (23, 24). Antigen-specific T cell-B cell interactions and cytokine production are critical for generating high-affinity IgG isotypes and memory B cell responses, whereas T- independent B cell responses generally consist of low affinity IgM production and short-lived B cell proliferation with limited memory (25). This accepted paradigm; however, has never been fully demonstrated during *A. marginale* infection of cattle.

In contrast to the readily observed $CD4^+$ T cell response primed by immunization with whole OMP or specific OM proteins using a variety of adjuvants and delivery systems (8, 13, 17, 26-33), the nature of the $CD4^+$ T cell response elicited by *A. marginale* infection is poorly characterized. In two separate studies, a rapid decline of antigen-specific $CD4^+$ T cell responses, originally induced by immunization with specific OMP, was observed after the animals were infected intravenously with the Florida strain (8, 13). We have demonstrated that precipitous

loss of OMP-specific T cell responses was explained by physical loss in antigen-specific, MHC class II tetramer-positive $CD4^+$ T cells in PBMC that coincided with rapidly rising and peak levels of bacteremia (13). In this experiment, sequestration of antigen-specific T cells was not observed in the spleen, liver, or lymph nodes, although only a single time point could be examined (13). Furthermore, following infection of both immunized and non-immunized control cattle in each of these studies, T cell responses specific for *A. marginale* lysate were not observed in PBMC (8, 13). This suggested that infection with *A. marginale* not only down regulated pre-existing $CD4^+$ T cell responses induced by immunization, but also regulated responses primed to new antigens by infection. However, responses of PBMC in non-immunized control animals during infection was monitored less frequently and spleen responses were determined at a single time point at necropsy when acute infection had resolved, in one of the two studies. The current study was therefore conducted to systematically follow the *A. marginale*-specific T lymphocyte response in PBMC in cattle weekly for nearly one year following tick-transmitted infection with the South Idaho strain, and to evaluate immune responses in both PBMC and spleens sampled as often as every two to three days during acute infection with the Florida strain.

The results presented here show that significant antigen-specific $CD4^+$ lymphocyte proliferation was detected in both the blood and spleen during acute anaplasmosis, but only following the peak of bacteremia, as levels of antigen decreased. Furthermore, responses were transient, disappearing rapidly in the following one to two weeks, and reoccurring sporadically with no predictable association to level or fluctuation in bacteremia or antibody. The transient response in blood does not appear to be explained by sequestration of antigen-specific T cells in the spleen in either Florida strain-infected calf. The transient nature of antigen-specific T cell responses in the PBMC and spleen might be explained by down regulation of $CD4^+$ T cells,

newly primed by *A. marginale* infection, in response to high antigen load via possibly activation induced cell death (AICD) from chronic high-affinity antigen-stimulation of the TCR resulting in apoptosis and deletion of antigen-specific T cell clones, or possibly due to mechanisms related to antigen-specific T regulatory cells (34). These mechanisms could occur during acute infection as bacteremia rises to 10^9 organisms/ml of blood, and could also occur during persistent infection, where levels of bacteria fluctuate but are maintained persistently high between 10^4 to 10^7 organisms/ml of blood. The characteristic pattern of cycling levels of bacteremia during persistent infection is in part due to the emergence of organisms that express novel antigenic variants of MSP2 capable of escaping pre-existing antibody response (35). It is theoretically possible that naïve T cells are likewise continually primed to new antigenic variants of both MSP2 and MSP3 during the course of infection (36, 37). Additionally, delayed priming to subdominant OMP epitopes may occur during persistent anaplasmosis as has been described in chronic viral (38) and bacterial infections (39).

In persistent infections characterized by chronic high-antigen load such as human immunodeficiency virus (HIV) (40), experimental LCMV in mice (41), *Mycobacterium* sp. in mice (42) a progressive dysfunction of antigen-specific T cells has been described whereby cells retain the ability to express and produce IFN-γ, but lose significant IL-2 expression, resulting in a short-lived effector phenotype incapable of proliferation, and subsequently undetectable in antigen-specific proliferation assays. Such cells have been successfully rescued in vitro by culturing with IL-2 (40). A similar mechanism of IL-2 dysfunction may occur in *A. marginale* under chronic antigen stimulation, although similar in vitro culture assays designed to rescue unresponsive PBMC with IL-2 failed to demonstrate antigen-specific proliferation (data not shown). To further investigate whether antigen-specific effector and memory T cells are present but unresponsive, or are deleted during the course of infection due to antigen load, we will need

to develop a model whereby bacteremia can be controlled to very low levels to determine whether potentially responsive cells are present. Alternatively, intermittent T cell responses could be explained by periodic escape from a regulatory environment, such as that imposed by regulatory T cells; a mechanism that has been well described in many other persistent bacterial and viral infections (43). This too will need to be explored in future experiments.

In studies using mouse models of persistent pathogens where antigen-specific T cell responses are impaired or specific $CD4^+$ T cells undergo apoptosis, one common theme is the production of IFN-γ which was in some cases shown to be mechanistically involved in the dysfunctional response and is a critical factor in driving intrinsic and extrinsic pathways of apoptosis (44-48). In our study, significant frequencies of IFN-γ-producing cells that responded specifically to *A. marginale* were not detected in any of the four calves at time points where antigen-specific proliferation was found, nor was there antigen-specific IFN-γ secretion at non-proliferative time points during infection. Furthermore, the background IFN-γ secreting cell response following the peak of infection was variable between animals and time points, although there was a tendency for baseline numbers of IFN-γ secreting cells in PBMC to increase over the course of infection particularly during the persistent phase. From this we conclude that undetectable antigen-specific proliferative responses early in infection, and transient responses detected thereafter, are not obviously related to high numbers of IFN-γ secreting cells. Chronically increased baseline levels of IFN-γ may reciprocally play a role in regulation of antigen-specific $CD4^+$ T cells.

In spite of the inability to detect T cell responses early in infection, all animals produced high titers of antigen-specific IgG antibody as early as 9 days post-infection and maintained high titers of specific antibody up to day 336. Early and late phase antibody consisted of both IgG1

and IgG2 isotypes occurring between days 9 and 13 post-infection. Several possibilities may explain isotype switching and high titers of IgG in the absence of detectable antigen-specific T cell responses. One is that antigen-specific T cells were present in other lymphoid organs not sampled, such as the lymph nodes, lungs, liver, or gut. Additionally, we cannot exclude the possibility that antigen-specific $CD4^+$ T lymphocyte responses in the PBMC and spleen, which were not generally detected by our methods or at our sampling time points, are sufficient to activate B cells to differentiate and to induce isotype switching. It is also possible that antigen-specific $CD4^+$ T cells in PBMC and spleen play a limited role in the early control of *A. marginale* and that the IgG is produced in a $CD4^+$ T cell independent (TI) manner. TI-B cell activation leading to IgG production has been documented in viral and bacterial infections of several species including calves. For example, in calves, immune control of foot and mouth disease virus and class switching to IgG directed against the viral capsid, occurred in the complete absence of $CD4^+$ and γδ T lymphocytes (49). Evidence of TI-B cell activation was also described in mice, where infection with the rickettsial pathogen *Ehrlichia muris* resulted in IgG2b, IgG2c, and IgG3 production in the absence of $CD4^+$ T cells or MHC class II expression (50). Antigens that induce B cell activation in the absence of T cell help are thought to have rigid and highly repetitive organized structures, such as lipopolysaccharide, that can engage and cross-link B cell receptors (BCR), leading to concomitant B cell signaling and activation, and predominantly antigen-specific IgM production (51). IgG isotype switching can be induced partially by TNF-family secreted and expressed factors such as BAFF, expressed by splenic macrophages and marginal B cells that have been activated by blood-borne antigen (52). However, isotype switching of the magnitude that we observed during acute anaplasmosis, with high titers of IgG, cannot be completely explained by this mechanism. Additionally, with the exception of MSP1a, which often contains two or more repetitive B cell epitopes in the N-

terminus (53), OMP of *A. marginale* does not contain repetitive epitopes. However, it is of interest that the TI IgG response in *E. muris* infected mice was directed against the immunodominant OMP-19 which also lacked polysaccharide-like motifs (50). In *A. marginale* the immunodominant OMP MSP2 is highly abundant (30), and its ability to cross-link the BCR is currently unknown. Interestingly, in a study where calves were thymectomized and $CD4^+$ T cells were severely reduced by treatment with specific monoclonal antibody, infection with *A. marginale* Florida strain resulted in development of specific IgG1 and IgG2 with subsequent control of bacteremia similar to that observed in non-thymectomized and untreated control calves (54). This supports the possibility that early *A. marginale*-specific humoral responses may be primed with minimal antigen-specific $CD4^+$ T cell help.

In summary, our results show that naïve calves infected with *A. marginale* of different strains mount early and robust antigen-specific IgG responses without detectable antigen-specific $CD4^+$ T cells in the PBMC and spleen. Infected calves maintain *A. marginale*-specific IgG at high titers throughout acute and persistent anaplasmosis, concurrent with successful control of peak bacteremia. *A. marginale* infection is unique in that persistent infection is characterized by basal levels of organisms as high as 10^7 organisms/ml of blood, inducing a state of continuous high-load antigenic stimulation on the host immune system. Possibly due to high bacterial load, antigen-specific $CD4^+$ T cells developed in the PBMC and spleen only after peak bacteremia stabilized to subclinical levels, and thereafter, antigen-specific $CD4^+$ T cell responses were transient, rapidly disappearing and reoccurring infrequently and unpredictably during persistent infection. This irregular pattern of antigen-specific $CD4^+$ T cell responses during *A. marginale* infection may be indicative of continual down-regulation of newly primed or memory *Anaplasma*-specific T cells throughout infection, as has been observed with *Anaplasma*-specific T cell responses primed by immunization. Down-regulation of the host T cell response may play

a critical role in preventing prolonged and likely deleterious, systemic inflammation in the infected host in response to continual high levels of bacteria. Thereby safeguarding the host, but simultaneously permitting lifelong infection of the host and efficient transmission of bacteria to other naïve animals within endemic areas.

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REFERENCES

1. Bodgan, C. 2008 Microreview: Mechanisms and consequences of persistence of intracellular pathogens: leishmaniasis as an example. *Cellular Microbiol*. 10: 1221-1234.

2. Palmer, G.H., and K.A. Brayton. 2007. Gene conversion is a convergent strategy for pathogen antigenic variation. *Trends Parasitol*. 23: 408-413.

3. Palmer, G. H., F. R. Rurangirwa, K. M. Kocan, and W. C. Brown. 1999. Molecular basis for vaccine development against the ehrlichial pathogen *Anaplasma marginale*. *Parasitol*. *Today*. 15:281–286.

4. Futse, J.E., K.A. Brayton, S.D. Nydam, and G.H. Palmer. 2009. Generation of antigenic variants via gene conversion: Evidence for recombination fitness selection at the locus level in *Anaplasma marginale*. *Infect Immunol.* 77:3181-3187.

5. Tebele, N., T. C. McGuire, and G. H. Palmer**.** 1991. Induction of protective immunity using *Anaplasma marginale* initial body membranes. *Infect. Immun*. 59:3199–3204.

6. Palmer, G. H., and T. C. McGuire. 1984. Immune serum against *Anaplasma marginale* initial bodies neutralizes infectivity for cattle. *J. Immunol*. 133: 1010–1015.

7. Palmer, G.H., and T.F. McElwain. 1995. Molecular basis of vaccine development against anaplasmosis and babesiosis. *Vet. Parasitol*. 57: 233-253.

8. Abbott, J.R., G.H. Palmer, K.A. Kegerreis, P.F. Hetrick, C.J. Howard, J.C. Hope, and W.C. Brown. 2005. Rapid and long-term disappearance of $CD4^+$ T lymphocyte responses specific for *Anaplasma marginale* major surface protein-2 (MSP2) in MSP2 vaccinates following challenge with live *A. marginale*. *J. Immunol*. 174:6702-6715.

9. Zhuang, Y., J.E. Futse, W.C. Brown, K.A. Brayton, and G.H. Palmer. 2007. Maintenance of antibody to pathogen epitopes generated by segmental gene conversion is highly dynamic during long-term persistent infection. *Infect. Immun*. 75: 5185-5190.

10. Stich, R. W., L. K. M. Shoda, M. Dreewes, B. Adler, T. W. Jungi, and W. C. Brown. 1998. Stimulation of nitric oxide production in macrophages by *Babesia bovis*. *Infect*. *Immun*. 66:4130–4136.

11. Gale, K.R., M.G. Gartside, C.M. Dimmock, H. Zakizewski, and G. Leatch. 1996. Peripheral blood lymphocyte proliferative responses in cattle infected with or vaccinated against *Anaplasma marginale*. *Parasitol. Res*. 82: 551-562.

12. Flynn, J.L., J. Chan, K.J. Triebold, D.K. Dalton, T.A. Stewart, and B.R. Bloom. 1993. An essential role of interferon γ in resistance to *Mycobacterium tuberculosis* infection. *J. Exp. Med.* 178: 2249-2254.

13. Han, S., J. Norimine, G.H. Palmer, W. Mwangi, K.K. Lahmers, and W.C. Brown. 2008. Rapid deletion of antigen-specific $CD4^+$ T cells following infection represents a strategy of immune evasion and persistence for *Anaplasma marginale*. *J. Immunol*. 181: 7759-7769.

14. Mebius, R.E., and G. Kraal. 2005. Structure and function of the spleen. *Nature Rev. Immunol*. 5:606-616.

15. Varma, S., and A.M. Shatry. 1980. A technique for partial marsupialization of the spleen in calves. *Vet. Rec*. 106: 127-128.

16. Goff, W.L., W.C. Johnson, C.R. Wyatt, and C.W. Cluff. 1996. Assessment of bovine mononuclear phagocytes and neutrophils for induced L-arginine-dependent nitric oxide production. *Vet. Immunol. Immunopathol*. 55: 45-62.

17. Abbott,J.R., G.H. Palmer, C.J. Howard, J.C. Hope, and W.C. Brown. 2004. *Anaplasma marginale* major surface protein 2 $CD4^+$ -T cell epitopes are evenly distributed in conserved and hypervariable regions (HVR), whereas linear B-cell epitopes are predominantly located in the HVR. *Infect. Immun*. 72: 7360-7366.

18. Knowles, D.P., S. Torioni de Echaide, G.H. Palmer, T.C. McGuire, D. Stiller, and T.F. McElwain. 1996. Antibody against an *A. marginale* MSP5 epitope common to tick and erythrocyte stages identifies persistently infected cattle. *J. Clin. Microbiol.* 34: 2225-2230.

19. Zhang, Y., G.H. Palmer, J.R. Abbott, C.J. Howard, J.C. Hope, and W.C. Brown. 2003. CpGODN 2006 and IL-12 are comparable for priming Th1 lymphocyte and IgG responses in cattle immunized with a rickettsial outer membrane protein in alum. *Vaccine*. 21: 3307-3318.

20. Futse, J.E., M.W. Ueti, D.P. Knowles, and G.H. Palmer. 2003. Transmission of *Anaplasma marginale* by *Boophilus microplus*: retention of vector competence in the absence of vector-pathogen interaction. *J. Clin. Microbiol*. 41: 3829-3834.

21. Ueti, M.W., J.O. Reagan, D.P. Knowles, and G.A. Scoles. 2007. Identification of midgut and salivary glands as specific and distinct barriers to efficient tick-borne transmission of *Anaplasma marginale*. *Infect Immun*. 75: 2959-2964.

22. Wikel, S.K. 1999. Tick modulation of host immunity: an important factor in pathogen transmission. *Intrl. J. Parasitol.* 29: 851-859.

23. Elgueta, R., M.J. Benson, V.C. de Vries, A. Wasiuk, Y. Guo, and R.J. Noelle. 2009. Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunol. Reviews*. 229: 152-172.

24. Milicevic, N.M., K. Nohroudi, Z. Milicevic, H.J. Hedrich, and J. Westermann. 2005. T cells are required for the peripheral phase of B-cell maturation. *Immunology*. 116: 308-317.

25. Tangye, S.G., and D.M. Tarlinton. 2009. Memory B cells: Effectors of long-lived immune responses. *Eur. J. Immunol.* 39: 2065-2075.

26. Brown, W.C., V. Shkap, D. Zhu, T.C. McGuire, W. Tuo, T.F. McElwain, and G.H. Palmer. 1998. $CD4^+$ T-lymphocyte and immunoglobulin G2 responses in calves immunized with *Anaplasma marginale* outer membranes and protected against homologous challenge. *Infect. Immun*. 66: 5406-5413.

27. Brown, W.C., D. Zhu, V. Shkap, T.C. McGuire, E.F. Blouin, K.M. Kocan, and G.H. Palmer. 1998. The repertoire of *Anaplasma marginale* antigens recognized by $CD4^+$ T-lymphocyte clones from protectively immunized cattle is diverse and includes major surface protein 2 (MSP-2) and MSP-3. *Infect Immunol*. 66: 5414–5422.

28. Brown, W.C., G.H. Palmer, H.A. Lewin, and T.C. McGuire. 2001. $CD4^+$ T lymphocytes from calves immunized with *Anaplasma marginale* major surface protein 1 (MSP1), a heteromeric complex of MSP1a and MSP1b, preferentially recognize the MSP1a carboxyl terminus that is conserved among strains. *Infect. Immun*. 69: 6853-6862.

29. Brown, W.C., K.A. Brayton, C.M. Styer, and G.H. Palmer. 2003. The hypervariable region of *Anaplasma marginale* major surface protein 2 (MSP2) contains multiple immunodominant $CD4^+$ T lymphocyte epitopes that elicit variant-specific proliferative and IFN-γ responses in MSP2 vaccinates. *J. Immunol*. 170: 3790-3798.

30. Lopez, J.E., W.F. Siems, G.H. Palmer, K.A. Brayton, T.C. McGuire, J. Norimine, and W.C. Brown. 2005. Identification of novel antigenic proteins in a complex *Anaplasma marginale* outer membrane immunogen by mass spectrometry and genomic mapping. *Infect. Immun*. 73: 8109-8118.

31. Lopez, J.E., G.H. Palmer, K.A. Brayton, M.J. Dark, S.E. Leach, and W.C. Brown. 2007. Immunogenicity of *Anaplasma marginale* type IV secretion system proteins in a protective outer membrane vaccine. *Infect. Immun*. 75: 2333-2342.

32. Lopez, J.E., P.A. Beare, R.A. Heinzen, J. Norimine, K.K. Lahmers, G.H. Palmer, and W.C. Brown. 2008. High-throughput identification of T-lymphocyte antigens from *Anaplasma marginale* expressed using in vitro transcription and translation. *J. Immunol. Methods*. 332: 129-141.

33. Mwangi, W., W.C. Brown, G.A. Splitter, C.J. Davies, C.J. Howard, J.C. Hope, Y. Aida,Y. Zhuang, B.J. Hunter, and G.H. Palmer. 2007. DNA vaccine construct incorporating intercellular trafficking and intracellular targeting motifs effectively primes and induces memory B- and T-cell responses in outbred animals. *Clin. Vaccine Immunol*. 14: 301-311.

34. Klenerman, P., and A. Hill. 2005. T cells and viral persistence: lessons from diverse infections. *Nature Immunol. Rev.* 6: 873-879.

35. French, D.M., W.C. Brown, and G.H. Palmer. 1999. Emergence of *Anaplasma marginale* antigenic variants during persistent rickettsemia. *Infect Immun*. 67: 5834-5840.

36. Brayton, K.A., D.P. Knowles, T.C. McGuire, and G.H. Palmer. 2001. Efficient use of a small genome to generate antigenic diversity in tick-borne ehrlichial pathogens. *Proc. Natl. Acad. Sci. U.S.A.* 98:4130-4135.

37. Meeus, P.F., M.A. Brayton, G.H. Palmer, and A.F. Barbet. 2003. Conservation of gene conversion mechanism in two distantly related paralogues of *Anaplasma marginale. Mol. Microbiol*. 47:633-643.

38. Snyder, C.M., K.S. Cho, E.L. Bonnett, S. van Dommelen, G.R. Shellam, and A.B. Hill. 2008. Memory inflation during chronic viral infection is maintained by continuous production of short-lived, functional T cells. *Immunity*. 29: 650-659.

39. Winslow, G.M., A.D. Roberts, M.A. Blackman, and D.L. Woodland. 2003. Persistence and turnover of antigen-specific $CD4^+$ T cells during chronic tuberculosis infection in the mouse. *J. Immunol*. 170: 2046-2052.

40. Younes, S.A., B. Yassine-Diab, A.R. Dumont, M.R. Boulassel, Z. Grossman, J.P. Routy, and R.P. Sekaly. 2003. *J. Exp. Med.* 198: 1909-1922.

41. Fuller, M.J., and A.J. Zajac. 2003. Ablation of $CD8^+$ and $CD4^+$ T cell responses by high viral loads. *J. Immunol.* 170: 477-486.

42. Millington, K.A., Innes, J.A., S. Hackforth, T.S.C Hinks, J.J. Deeks, D.P.S. Dosanjh, V. Guyot-Revol, R. Gunatheesan, P. Klenerman, and A. Lalvani. 2007. Dynamic relationship between IFN-γ and IL-2 profile of *Mycobacterium tuberculosis*-specific T cells and antigen load. 178: 5217-5226.

43. Belkaid, Y. 2007. Regulatory T cells and infection: a dangerous necessity. *Nat. Rev. Immunol*. 7:875-888.

44. Hayashi, N., D. Liu, B. Min, S.Z. Ben-Sasson, and W.E. Paul. 2002. Antigen challenge leads to in vivo activation and elimination of highly polarized TH1 memory T cells. *Proc. Natl. Acad. Sci.* 99:6187-6191.

45. Xu, H., J. Wipsa, H. Yan, M. Zeng, M.O. Makobongo, F.D. Finkelman, A. Kelso, and M.F. Good. 2002. The mechanism and significance of deletion of parasite-specific $CD4^+$ T cells in malaria infection. *J. Exp. Med.* 195: 881-892.

46. Jensen, J.S., R. O'Connor, J. Osborne, and G.B. Glasgow. 2002. Infection with *Burgia* microfilariae induces apoptosis of $CD4^+$ T lymphocytes: a mechanism of immune unresponsiveness in filariasis. *Eur. J. Immunol.* 32: 858-867.

47. Dalton, D.K., L. Haynes, C.Q. Chu, S.L. Swain, S. Wittmer. 2000. Interferon-γ eliminates responding CD4 T cells during mycobacterial infection by inducing apoptosis of activated CD4 T cells. *J. Exp. Med.* 192: 117-122.

48. Li, X., K.K. McKinstry, S.L. Swain, and D.K. Dalton. 2007. IFN-γ acts directly on activated $CD4^+$ T cells during mycobacterial infection to promote apoptosis by inducing components of the intracellular apoptosis machinery and by inducing extracellular proapoptosis signals. *J. Immunol.* 179: 939-949.

49. Juleff , N., M. Windsor, E.A. Lefevre, S. Gubbins, P. Hamblin, E. Reid, K. McLaughlin, P.C.L Beverley, I.W. Morrison, and B. Charleston. 2009. Foot-and-mouth disease virus can induce a specific and rapid $CD4^+$ T-cell-independent neutralizing and isotype class-switched antibody response in naïve cattle. *J. Virol*. 83: 3626-3636.

50. Bitsaktsis, C., N. Bisweswar, R. Racine, K.C. MacNamara, and G.Winslow. 2007. T-cell-independent humoral immunity is sufficient for protection against fatal intracellular *Ehrlichia* infection. *Infect Immun*. 75:4933-4941.

51. Bachmann, M.F., and R.M. Zinkernagel. 1997. Neutralizing antiviral B cell responses. *Ann. Rev. Immunol.* 15: 235-270.

52. Martin, F., A.M. Oliver, and J.F. Kearney. 2001. Marginal zone B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity*. 14: 617-629.

53. McGarey, D.J., A.F. Barbet, G.H. Palmer, T.C. McGuire, and D.R. Allred.1994. Putative adhesions of *Anaplasma marginale*: major surface polypeptides 1a and 1b. *Infect. Immun.* 62: 4594-4601.

54. Valdez, R.A., T.C. McGuire, W.C. Brown, W.C. Davis, J.M. Jordan, and D.P. Knowles. 2002. Selective in vivo depletion of $CD4^+$ T lymphocytes with anti-CD4 monoclonal antibody during acute infection of calves with *Anaplasma marginale*. *Clin. Diagn. Lab. Immunol*. 9: 417-424.

TABLES

Table I. ***A. marginale-specific serum IgG titers post-infection determined using Western blots and serum samples[a] from South Idaho and Florida strain-infected calves***

A. marginale-specific IgG titers from the following animals and days PI:					
PI	**C1028b1**	**C1030b1**	**PI**	**33875**	**33901**
0	ND	ND	0	<100[b]	<100
9	1000	10,000	13	30,000	3,000
29	10,000	30,000	20	30,000	3,000
49	30,000	100,000	40	30,000	30,000
71	3,000	10,000	76	100,000	100,000
94	3,000	30,000	89	30,000	30,000
120	10,000	100,000	110	1,000,000	1,000,000
140	3,000	3,000			
163	3,000	3,000			
189	1,000	30,000			
239	30,000	3,000			
250	10,000	10,000			
274	3,000	1,000,000			

[a] Serum was collected on specified days PI and frozen at -20°C until Western blots were performed simultaneously. Blots were prepared with 10 µg of *A. marginale* South Idaho strain or Florida strain.

[b] Serum was diluted 1:100 to 1:1,000,000 with I-block® . <100 denote a non-detectable antigen-specific response.

Table II. ***Frequency of IFN-γ-producing mononuclear cells in PBMC over the course of infection***

Animals

C1028b1			C1030b1			33875			33901		
Day PI[b]	Media[c]	A. marg[d]	Day PI	Media	A.marg	Day PI	Media	A. marg	Day PI	Media	A. marg
21	146	80	21	480	212	0	62	172	0	5	11
28	27	**112**	49	688	354	18	276	289	18	33	24
49	20	20.6	56	292	142	27	215	165	27	104	118
63	286	354	126	676	497	34	286	354	32	95	75
84	57	122	133	476	349	36	86	53	41	244	229
119	854	274	168	637	264	55	269	196	48	106	120
126	272	123	175	431	132	76	175	105	55	174	82
140	84	52	196	925	765	89	500	492	76	180	145
175	217	269	231	663	403	104	270	193	89	154	92
224	103	72	238	445	448				104	150	158
231	138	39	252	374	140						
238	206	74	259	596	415						
273	130	89	273	384	348						
280	174	206	280	200	103						
294	222	28	294	325	**492**						
302	164	109	301	345	264						
309	265	103									
315	48	28									

[a] Cryopreserved PBMC were thawed and used simultaneously to reduce assay to assay variation.

[b] Days post-infection

[c] IFN-γ response to medium was performed using complete 1640 RPMI medium without antigen cultured with 1 x 10^6 cells per well in triplicate for 48 h at 37ºC. Results are presented as the mean of triplicate wells of spot forming cells (SFC) with bolded values showing aliquots with SFC counts significantly different than SFC of medium alone (p <0.05).

[d] *A. marginale* lysate used was the same as the infecting strain, and assays were prepared using 15 µg/ml of lysate in triplicate with 1 x 10^6 cells per well and cultured as described for medium. Results are the mean of triplicate SFC.

FIGURES

FIGURE 1. Bacteremias, anemia, and antibody responses during the course of infection with *A. marginale* South Idaho strain**.** Calves C1028b1 and C1030b1 were infected via transmission feeding of *A. marginale* South Idaho strain infected *Ixodes* ticks. *A-B*, Bacteremias were determined by quantitative RT-PCR analysis of the single copy *msp5* gene from whole blood, presented as $\log_{10}$ organisms/ml of blood (open triangles), and anemia was determined as % PCV (closed circles). *C-D*, *A. marginale* antibody response was measured weekly throughout infection using an MSP5 C-ELISA and results are presented as the %inhibition (closed squares), where values >30% inhibition were considered significant. Results are presented with bacteremias.

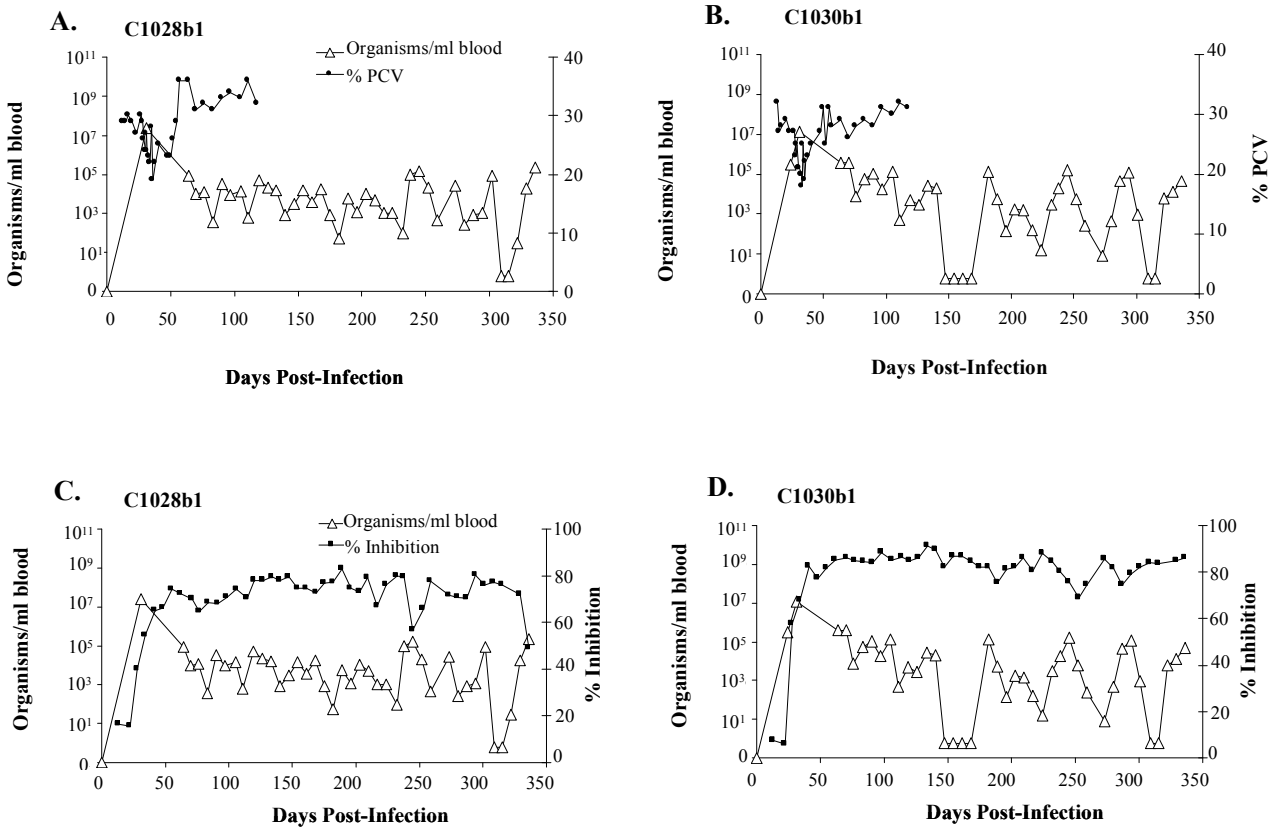
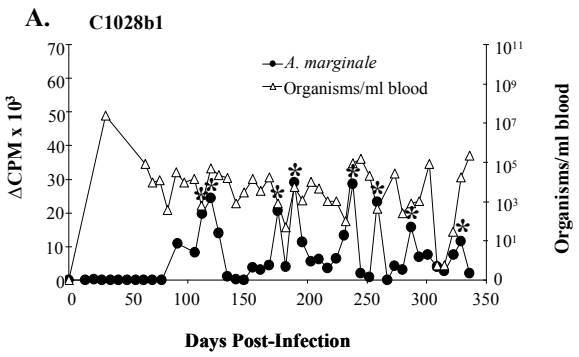
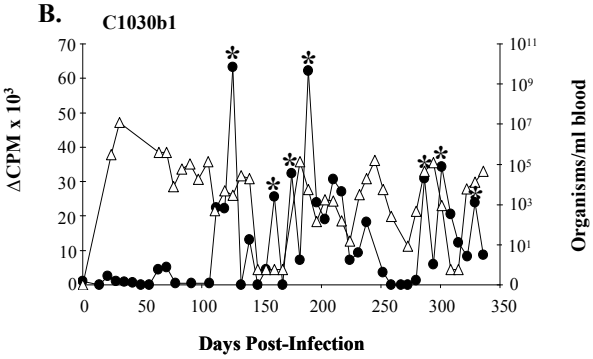
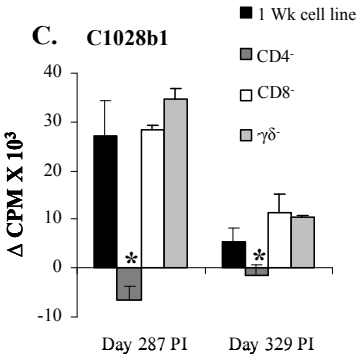


FIGURE 2. *A. marginale*-specific proliferative responses of PBMC collected weekly over the course of acute and persistent infection in South Idaho strain infected calves. *A-B*, Proliferative responses were calculated as the mean counts per minute (CPM) of triplicate wells to 15 μg/ml of *A. marginale* (South Idaho strain) lysate minus the mean CPM of triplicate wells to 15 μg/ml URBC (closed circles) and shown in relation to bacteremias. Responses significantly greater than responses to URBC are indicated by asterisks. *C-D*, Proliferative responses of short-term cell lines established from cryopreserved PBMC obtained at selected time points, and deleted of $CD4^+$ cells, $CD8^+$cells, or γδ T cells. Cell subsets were depleted from one week-cell lines using MAbs and magnetic bead selection, and depleted cells were cultured in duplicate wells with 15 μg/ml *A. marginale* lysate or medium. Results are shown as the mean CPM +/- 1 SD of duplicate wells to antigen after subtracting the mean CPM to medium. Asterisks indicate the response is significantly less than the response by undepleted PBMC, where $p < 0.05$ using the one-tailed Student t- test.


A.
C1028b1
A. marginale
Organisms/ml blood
ΔCPM x 10³
Organisms/ml blood
Days Post-Infection


B.
C1030b1
ΔCPM x 10³
Organisms/ml blood
Days Post-Infection


C.
C1028b1
1 Wk cell line
CD4-
CD8-
γδ-
Δ CPM X 10³
Day 287 PI
Day 329 PI

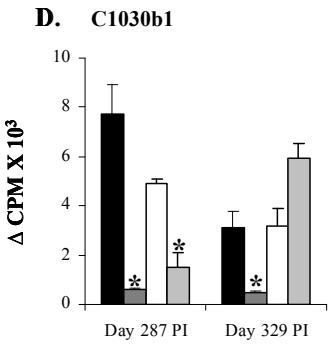

D.
C1030b1
Δ CPM X 10³
Day 287 PI
Day 329 PI

FIGURE 3. Bacteremias, anemia, and antibody responses during the course of infection with *A. marginale* Florida strain **c**alves 33875 and 33901 were infected intravenously with the Florida strain of *A. marginale*. *A-B*, Bacteremias were determined by quantitative RT-PCR analysis of the single copy *msp5* gene from whole blood , presented as $\log_{10}$ organisms/ml of blood, and anemia was determined as % PCV. *C-D*, *A. marginale* antibody response was measured weekly throughout infection using an MSP5 C-ELISA and results are presented as the % inhibition, where values >30% inhibition considered significant. Results are presented with bacteremias.

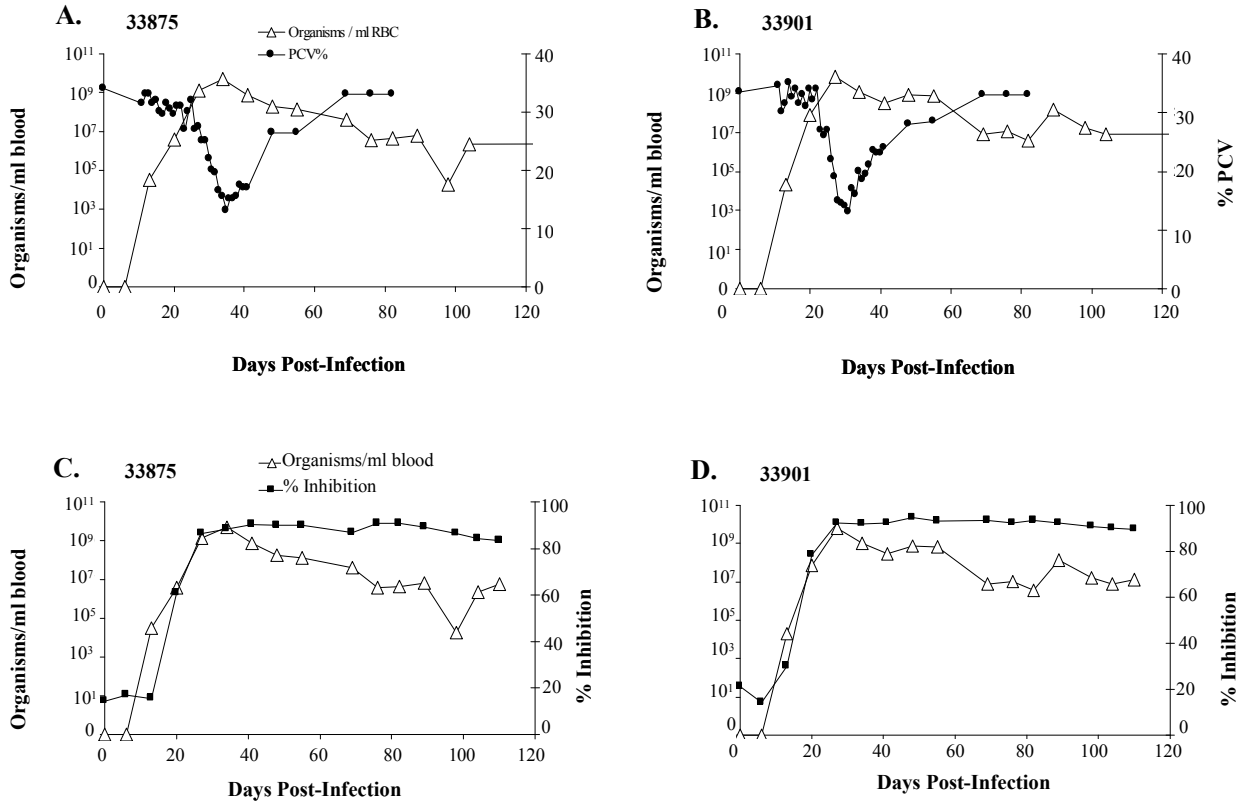
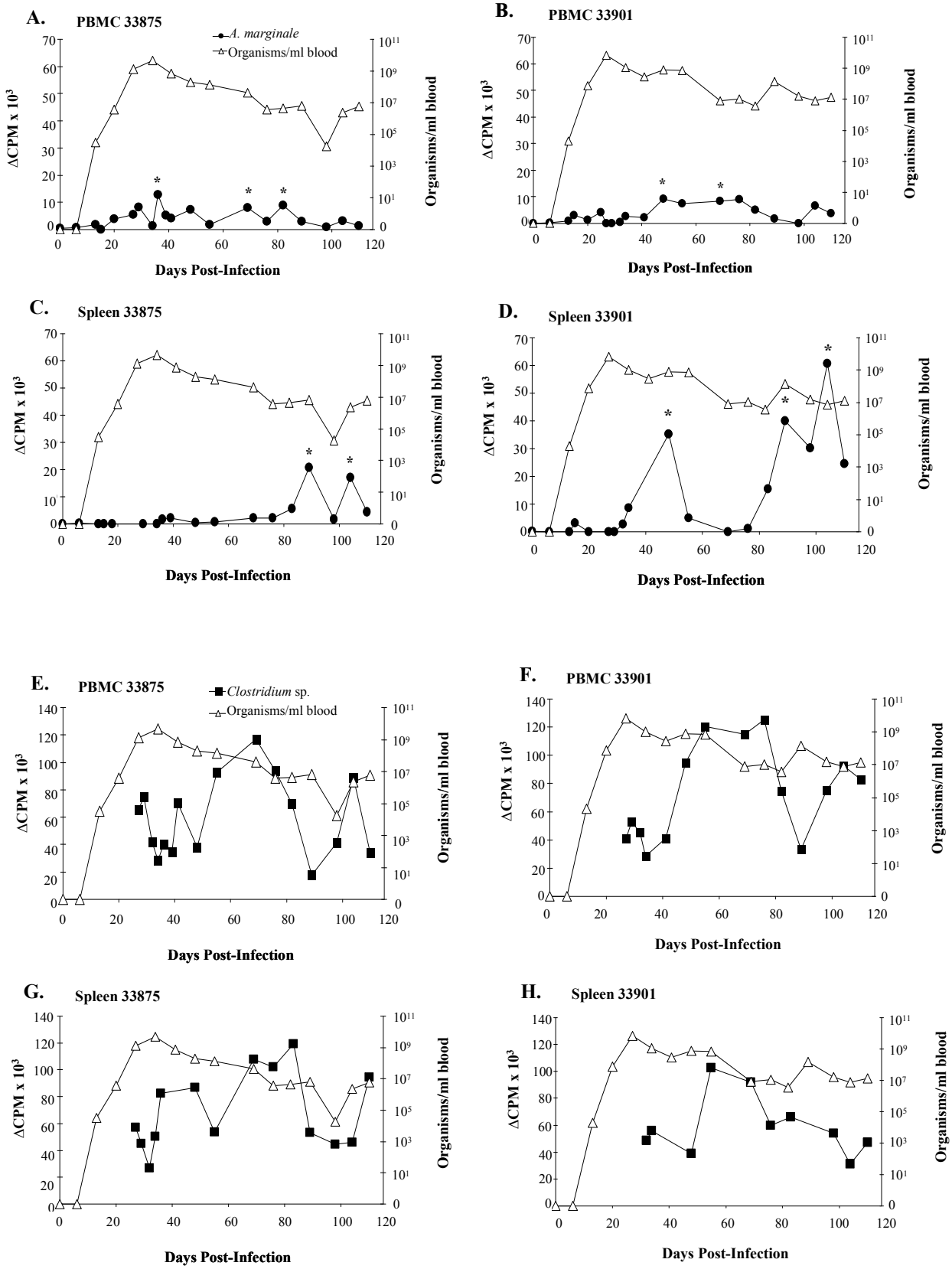
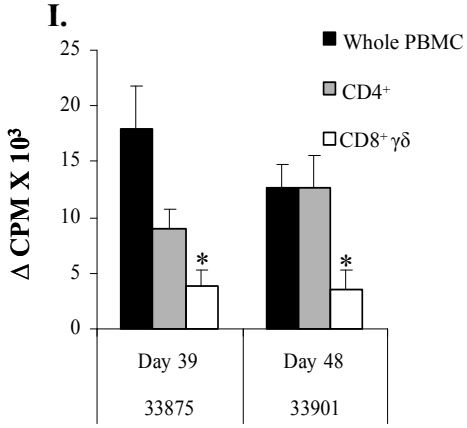


FIGURE 4. *A. marginale*-specific proliferative responses of PBMC and spleen cells collected weekly over the course of acute and persistent infection in Florida strain infected calves. *A-D*, Proliferative responses of PBMC and spleen cells, sampled every 3 days to 1 week intervals, were calculated as the mean CPM of triplicate wells to 15 μg/ml of *A. marginale* (Florida strain) lysate minus the mean CPM of triplicate wells to 15 μg/ml URBC (closed circles) and shown in relation to bacteremias (open triangles). Responses significantly greater than responses to URBC are indicated by asterisks. *E-H*, Proliferative responses to *Clostridium* sp. antigen were calculated as the mean CPM of triplicate wells to 15 μg/ml of a 1:100 dilution of the vaccine antigen minus the mean CPM of triplicate wells to 15 μg/ml URBC (closed squares) and shown in relation to bacteremias. Responses significantly greater than those to URBC are indicated by asterisks. *I*, Responses of $CD4^+$ cell-enriched or $CD8^+$ and γδ T cell-enriched PBMC to *A. marginale*. Cryopreserved PBMC from selected time points were enriched for $CD4^+$ T cells by depleting $CD8^+$ cells and γδ T cells or enriched for $CD8^+$ cells and γδ T cells by depleting $CD4^+$ cells with MAb and magnetic bead selection. Results are presented as the mean CPM +/- 1SD of triplicate cultures to 15 μg/ml of *A. marginale* (Florida strain) lysate minus the mean CPM of triplicate cultures to medium. Responses significantly less than responses by undepleted PBMC are indicated by asterisks, where $p<0.05$ determined by the one-tailed Student's t- test.


A.
PBMC 33875
A. marginale
Organisms/ml blood
ΔCPM x 10³
Organisms/ml blood
Days Post-Infection
B.
PBMC 33901
ΔCPM x 10³
Organisms/ml blood
Days Post-Infection
C.
Spleen 33875
ΔCPM x 10³
Organisms/ml blood
Days Post-Infection
D.
Spleen 33901
ΔCPM x 10³
Organisms/ml blood
Days Post-Infection
E.
PBMC 33875
Clostridium sp.
Organisms/ml blood
ΔCPM x 10³
Organisms/ml blood
Days Post-Infection
F.
PBMC 33901
ΔCPM x 10³
Organisms/ml blood
Days Post-Infection
G.
Spleen 33875
ΔCPM x 10³
Organisms/ml blood
Days Post-Infection
H.
Spleen 33901
ΔCPM x 10³
Organisms/ml blood
Days Post-Infection

I.


Whole PBMC
CD4+
CD8+ γδ
Δ CPM X 10³
25
20
15
10
5
0
*
*
Day 39
Day 48
33875
33901

ATTRIBUTIONS

The above manuscript is in the process of submission to the *Journal of Clinical Vaccine Immunology*.

Calves infected with *Anaplasma marginale* have persistent high-load bacteremia that induces sustained high IgG titers associated with delayed and transient T lymphocyte responses

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Department of Veterinary Microbiology and Pathology[1], School for Global Animal Health[2], Washington State University, Pullman, WA 99164

Sushan Han: I helped design this study, helped to select and prepare the study calves, and performed all procedures on, collected all samples from, and administered all care and treatment for the *A. marginale* Florida infected calves. I performed all lymphocyte proliferation assays, IFN-γ ELISPOT assays, Western blots, serum titers, C-ELISAs, and RT-PCR on Florida infected calves, as well as cell enrichment assays with proliferation assays. I performed approximately one quarter of proliferation assays and cell enrichment assays with proliferation on South Idaho infected calves. I compiled all data from study calves and wrote this manuscript including all revisions incorporating ideas from additional authors.

Junzo Norimine: Offered valuable advice on assays, interpretation of results, and writing of the manuscript.

Kelly Brayton: Provided the technical assistance for RT-PCR on the South Idaho strain infected calves.

Guy Palmer: Helped interpret the results of this study, reviewed and helped revise the manuscript, and provided financial assistance to perform this project.

Glen Scoles: Provided acquisition fed ticks for infection of two study calves with *A. marginale* South Idaho strain.

Megan LaFollett: Performed Western blots on the South Idaho strain infected calves.

Wendy Brown: Is the principal investigator of this laboratory and provided the finances to perform this project, was integral in designing the project, reviewed and assisted in writing this manuscript, and provided valuable interpretation of the data and current literature.

CONCLUSION

Acquired T-cell immunity is central for protection against infection. However, the immunological consequences of exposing memory T cells to high antigen loads during persistent systemic infection are poorly understood. We investigated this using infection with *Anaplasma marginale*, a ruminant pathogen that replicates to levels of 10^9 bacteria per ml of blood during acute infection and maintains mean bacteremia levels of 10^6 per ml during long-term persistent infection. We established that immunization-induced antigen-specific peripheral blood $CD4^+$ T cell responses were rapidly and permanently lost following infection.

To determine whether these T cells were anergic, sequestered in the spleen, or physically deleted from peripheral blood, $CD4^+$ T lymphocytes from the peripheral blood specific for a major surface protein (MSP)1a T-cell epitope were enumerated by DRB3*1101 tetramer staining and FACS analysis throughout the course of immunization and challenge. Immunization induced significant epitope-specific T lymphocyte responses that rapidly declined near peak bacteremia to background levels. Concomitantly, the mean frequency of $tetramer^+$ $CD4^+$ cells decreased rapidly from an average of 0.025% before challenge to a pre-immunization level of approximately 0.0003% of $CD4^+$ T cells. Low frequencies of $tetramer^+$ $CD4^+$ T cells in spleen, liver, and inguinal lymph nodes sampled 9-12 weeks post-challenge were consistent with undetectable or unsustainable antigen-specific responses and the lack of T-cell sequestration. Thus, infection of cattle with *A. marginale* leads to the rapid loss of immunization-induced antigen-specific T cells and immunologic memory, which may be a strategy for this pathogen to modulate the immune response and persist.

Interestingly, loss of immunization-induced antigen-specific $CD4^+$ T cells did not affect the outcome of disease in immunized animals, and like control calves they successfully controlled peak bacteremia and remained persistently infected. Control of acute bacteremia

corresponds with IgG specific for outer membrane proteins (OMP). However, OMP-specific $CD4^+$ T cells induced by immunization were rapidly deleted following infection and failed to develop during either acute or persistent infection in immunized as well as naïve infected calves. Lack of evidence of *A. marginale*-specific $CD4^+$ T cells contradicts this paradigm of immune control of anaplasmosis. To investigate the role of newly primed antigen-specific $CD4^+$ T cells in response to infection, naïve calves were experimentally infected with *A. marginale* by tick transmission (South Idaho strain), or intravenous inoculation of infected blood (Florida strain). *A. marginale*-specific T cell responses were not detected in the blood of South Idaho strain-infected animals until weeks fifteen to sixteen and transient cyclic T cell responses occurred thereafter. More frequent sampling of blood and spleens from calves infected with Florida strain revealed the first antigen-specific $CD4^+$ T cell responses at five and seven weeks, but responses were similarly transient. All calves mounted high titers of *A. marginale*-specific IgG early and maintained titers throughout infection. Our results indicate that the poor T cell response observed in blood during acute infection is not due to sequestration of antigen-specific T cells in the spleen, and are consistent with continual down regulation of newly primed or memory *Anaplasma*-specific T cells throughout infection, as observed for T cells primed by immunization prior to infection.