Title

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Journal

Infection and Immunity, 83(1)

ISSN

0019-9567

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Publication Date

2015

DOI

10.1128/iai.02471-14

Peer reviewed

IAI Journals.ASM.org

CD4$^+$ T Cells Promote Antibody Production but Not Sustained Affinity Maturation during *Borrelia burgdorferi* Infection

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CD4 T cells are crucial for enhancing B cell-mediated immunity, supporting the induction of high-affinity, class-switched antibody responses, long-lived plasma cells, and memory B cells. Previous studies showed that the immune response to *Borrelia burgdorferi* appears to lack robust T-dependent B cell responses, as neither long-lived plasma cells nor memory B cells form for months after infection, and nonswitched IgM antibodies are produced continuously during this chronic disease. These data prompted us to evaluate the induction and functionality of *B. burgdorferi* infection-induced CD4 T_{FH} cells. We report that CD4 T cells were effectively primed and T_{FH} cells induced after *B. burgdorferi* infection. These CD4 T cells contributed to the control of *B. burgdorferi* burden and supported the induction of *B. burgdorferi*-specific IgG responses. However, while affinity maturation of antibodies against a prototypic T-dependent *B. burgdorferi* protein, Arthritis-related protein (Arp), were initiated, these increases were reversed later, coinciding with the previously observed involution of germinal centers. The cessation of affinity maturation was not due to the appearance of inhibitory or exhausted CD4 T cells or a strong induction of regulatory T cells. *In vitro* T-B cocultures demonstrated that T cells isolated from *B. burgdorferi*-infected but not *B. burgdorferi*-immunized mice supported the rapid differentiation of B cells into antibody-secreting plasma cells rather than continued proliferation, mirroring the induction of rapid short-lived instead of long-lived T-dependent antibody responses *in vivo*. The data further suggest that *B. burgdorferi* infection drives the humoral response away from protective, high-affinity, and long-lived antibody responses and toward the rapid induction of strongly induced, short-lived antibodies of limited efficacy.

Tick-borne infections with the Lyme disease agent *Borrelia burgdorferi* induce chronic nonresolving infections that result in tissue inflammation, most frequently so-called Lyme arthritis and myocarditis and, in some humans and nonhuman primates, but not mice, the inflammation of the central nervous system (1–3). The presence of gamma interferon (IFN-γ)-producing CD4 T cells has been associated mostly with increased tissue pathology in humans and mice (4–7), and the treatment of mice with anti-interleukin-12 (IL-12) monoclonal antibody (MAb) reduced arthritis development in C3H mice (6). Thus, much focus on CD4 T cell responses to *B. burgdorferi* has been on their pathological and proinflammatory role.

Early studies provided evidence both for and against a positive role of T cells in the course of *B. burgdorferi*-induced disease (4, 8, 9), leading some to conclude that CD4 T cells are largely dispensable for the control of *B. burgdorferi* infection (4, 8). However, while the anti-IL-12 treatment reduced tissue pathology, it also increased the *B. burgdorferi* tissue burden (6), and the lack of IFN-γ was shown to increase joint swelling (10). Others reported that the adoptive transfer of IFN-γ-secreting CD4$^+$ T cells into *B. burgdorferi*-infected T-cell-deficient mice promoted carditis resolution (11). Thus, together these data suggest that CD4 T cells can play an immune-enhancing role against *B. burgdorferi* by activating cellular immune response components, such as macrophages, thereby reducing tissue-spirochete burden, albeit at the cost of causing tissue damage.

Another important function of CD4 T cells is their ability to enhance antibody-mediated immunity by driving affinity maturation and the development of long-lived plasma cells and memory B cells (12, 13). Strong evidence links infection-induced, antibody-mediated immunity to the control of *B. burgdorferi* tissue burden and to disease resolution (4, 14, 15) but not to the clearance of *B. burgdorferi* infection (16, 17). Paradoxically, existing literature suggests that the presence of CD4 T cells does not measurably enhance the disease-ameliorating humoral response to *B. burgdorferi* (8), which may be explained by an induction of strong disease-resolving T cell-independent B cell responses (8, 18). However, it appears unlikely that the protective B cell response to *B. burgdorferi*, a highly complex pathogen expressing many immunogenic surface antigens (19), is confined to T-independent antibody responses alone. Indeed, previous studies identified Arthritis-related protein (Arp; GenBank accession no. AF050212) of *B. burgdorferi* N40 to be dependent on conventional T cell help in C57BL/6 mice (20). Such antibodies were shown previously to resolve arthritis development (21).

Studies with multiple pathogens have demonstrated a specific role for CD4 T follicular helper (T_{FH}) cells in the activation of B cells (22), including the induction of germinal centers, hallmarks of T-dependent B cell responses and birthplaces of long-term humoral immunity (23). Our recent studies suggested that germinal center responses were nonfunctional after primary *B. burgdorferi*

Received 8 August 2014 **Returned for modification** 5 September 2014
Accepted 3 October 2014
Accepted manuscript posted online 13 October 2014
Citation Elsner RA, Hastey CJ, Baumgarth N. 2015. CD4$^+$ T cells promote antibody production but not sustained affinity maturation during *Borrelia burgdorferi* infection. Infect Immun 83:48–56. doi:10.1128/IAI.02471-14.
Editor: S. Ehrt
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doi:10.1128/IAI.02471-14

infection, as long-lived antibody-secreting plasma cells (18) and memory B cells (R. A. Elsner, C. J. Hastey, and N. Baumgarth, unpublished data) were not induced for months after infection (18). Importantly, a coadministered influenza vaccine antigen similarly failed to induce long-term immunity when given during *B. burgdorferi* infection (Elsner et al., unpublished). Thus, these studies pointed to specific deficits in the T-dependent B cell responses against *B. burgdorferi*.

Here, we sought to directly assess the impact of *B. burgdorferi* infection on the induction and functionality of CD4 T cells, particularly the induction of the T_{FH} cells. The study confirms our previous findings on the inability of T-dependent *B. burgdorferi*-specific germinal center-derived antibodies to be maintained in the long term after *B. burgdorferi* infection. While CD4 T cell responses appeared effectively primed and T_{FH} cells were induced following *B. burgdorferi* infection, affecting a reduction of *B. burgdorferi* tissue burden, they differed in functionality from T_{FH} cells induced following immunization with *B. burgdorferi*. Infection-induced T_{FH} cells showed a greater *in vitro* propensity to drive rapid B cell differentiation but not proliferation, mirroring the induction of rapid short-lived, instead of long-lived, T-dependent antibody responses.

MATERIALS AND METHODS

***Borrelia burgdorferi*, recombinant proteins, and qPCR.** Low-passage-number aliquots of *B. burgdorferi sensu stricto* strain cN40 were cultured in modified Barbour-Stoenner-Kelley II medium at 33°C. Spirochetes were enumerated at mid-log phase using a Petroff-Hauser bacterial counting chamber (Baxter Scientific) and were used to infect SCID mice or for *in vitro* assays. Recombinant Arp from *B. burgdorferi* cN40 was generated in-house as previously described (24). Recombinant DbpA from cN40 was produced by GenScript. The detection of the number of FlaB copies per mg of tissue by quantitative PCR (qPCR) was done as previously described (18).

Mice, infections, and immunizations. Female C57BL/6J (B6), BALB/cByJ (BALB/c), male or female B6.CB17-*Prkdc*^{scid}/SzJ (B6-SCID), and CBySmn.CB17-*Prkdc*^{scid}/J (BALB/c-SCID) mice were purchased from The Jackson Laboratory. Breeder pairs of BLIMP-yellow fluorescent protein (YFP) reporter mice (25) were a kind gift of M. C. Nussenzweig (Rockefeller University). TS-1 BALB/c mice (breeder pairs kindly provided by A. Caton, The Wistar Institute) express a transgenic influenza virus hemagglutinin-specific T cell receptor (TCR) (influenza virus strain A/Puerto Rico/34/8, H1N1) (26). B6-SCID and BALB/c-SCID mice were infected by subcutaneous injection between the shoulders with 1×10^4 or 1×10^6 *B. burgdorferi* spirochetes, respectively. After 2 to 5 weeks, ear tissue pieces from infected SCID mice were transplanted beneath the skin of the right hind leg of recipient C57BL/6 or BALB/cByJ mice for study (24). For *B. burgdorferi* immunization, mice were given a single subcutaneous injection in the right hind leg of 5×10^3 heat-killed and sonicated bacteria emulsified in complete Freund's adjuvant (Difco Laboratories). For influenza virus immunization, 1,000 hemagglutinating units (HAU) of influenza virus A/PR8 (H1N1) in 100 μl phosphate-buffered saline (PBS) was mixed with 100 μl of incomplete Freund's adjuvant (Difco Laboratories) or Imject alum (Thermo Scientific), and two subcutaneous injections of 100 μl each were given on both sides of the tail base. Mice were maintained in microisolator cages under specific-pathogen-free conditions (available upon request). The University of California, Davis, Institutional Animal Care and Use Committee approved all experimental protocols.

***In vivo* CD4 depletion.** Three days prior to infection, B6 mice were injected intraperitoneally with 0.3 mg of either rat anti-mouse CD4 (clone GK1.5) MAb or polyclonal rat IgG control Ab (Sigma). Anti-CD4 was purified in-house from tissue culture supernatants by protein G affinity chromatography. Following infection, mice were given twice-weekly 0.1-mg doses of antibodies. On days 0 and 28 postinfection, peripheral blood mononuclear cells (PBMCs) were isolated from tail vein blood to confirm CD4 T cell depletion by fluorescence-activated cell sorter (FACS) analysis with anti-CD4-Alexa 750-allophycocyanin (APC) (clone RM4-5; eBioscience). CD4 T cells also were quantified in spleen and right inguinal lymph node samples at day 60 postinfection.

Flow cytometry. Cells were prepared and stained as previously described (27) with the following antibodies, generated in-house unless otherwise specified: CD19-Cy5-phycoerythrin (PE), CD3-fluorescein isothiocyanate (FITC), CD80-Cy5.5-PE, CD4-Alexa 750-APC (clone RM4-5; eBioscience), biotinylated anti-TS-1-transgene (clone 6.5-1), streptavidin-QDot605 (Molecular Probes), CD3-Alexa 750-APC (eBioscience), CD44-APC (eBioscience), CD11a-Cy7-PE (BD Biosciences), CD4-Cy5.5-PE (in-house and eBioscience), ICOS-FITC (clone 7E.17G9; BD Pharmingen; conjugated in-house), CD3-eFluor780-APC (eBioscience), CXCR5-Biotin (BD Pharmingen), CXCR4-APC (BD Biosciences), PD-1-PE (eBioscience), CTLA-4-PE (eBioscience), CD45R-Cy5.5-PE, CD19-APC, CD4-FITC, and CD4-APC. When indicated, cells were stained for active caspase-3-PE (BD Biosciences) by following the manufacturer's instructions. Surface staining was followed by staining with the dead cell discriminator Live/Dead fixable violet (Invitrogen). Cells were washed and resuspended for FACS analysis. Samples were run on either a FACSAria or Fortessa instrument (BD Biosciences). Data were analyzed using FlowJo software (Tree Star Inc.).

ELISA. *B. burgdorferi* Arp- and DbpA-specific enzyme-linked immunosorbent assays (ELISAs) were performed as described above using 2.5 μg/ml Arp and 0.5 to 1 μg/ml DbpA as a coating, revealing bound serum IgG with goat anti-mouse IgG (Southern Biotech). *B. burgdorferi*-specific IgG was measured using a cocktail of 2.5 μg/ml each of Arp, DbpA, OspC, and BmpA and revealing serum antibodies with goat anti-mouse IgG (Southern Biotech) (18). For index ELISAs, immediately following a 2-h incubation with serum, duplicate wells were incubated with PBS or 6 M urea (in PBS) for 5 min. Detection steps were as described previously (18), except that for all subsequent wash steps, plates were washed 5 times with PBS (omitting the previously described PBS-Tween and distilled water wash steps). The avidity index was calculated as the ratio of antibody detected with urea wash to total antibody detected by PBS wash; in other words, the ratio of high avidity to total antibody in the serum. An avidity index of 1 indicates that antigen-specific antibodies were fully retained during the high-stringency urea wash.

T cell priming. Lymph nodes collected from infected or immunized mice at the indicated days posttreatment were pooled and homogenized between the ends of two frosted glass slides. Live cells were counted by Trypan dye exclusion and plated at the indicated cell numbers. $CD4^+$ cells from spleens of naive TS-1 mice were enriched by depletion of unwanted cells using antibodies against CD8α, CD19, CD11b, and CD49b (BioLegend) and anti-biotin MicroBeads (Miltenyi Biotec). $CD4^+$ cell purities ranged from 85 to 91% as assessed by FACS analysis. $CD4^+$ cells were washed in PBS and labeled with 0.5 μM carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes) for 10 min, immediately followed by adding 10 to 12 ml staining media. Cells were washed once more with staining media and resuspended in cell culture media (RPMI [Gibco] containing 100 U/ml penicillin/100 μg/ml streptomycin/0.292 mg/ml L-glutamate [Gibco] and 10% heat-inactivated fetal bovine serum [Gibco]), and live cells were counted. Graded numbers of $CD4^+$ cells plus 200 HAU heat-killed influenza virus were added to the culture. Cells were cultured for 4 days at 37°C with 5% CO_2. Cells were stained with anti-CD4 and antibody 6.5-2, specific for the TS-1 transgene, and the dead cell exclusion dye Live/Dead fixable violet (Invitrogen).

T cell-B cell coculture. B cells were enriched from day 10-immunized B6 mice by magnetic depletion (autoMACS; Miltenyi Biotech) of unwanted cells (CD4, CD8a, CD11b, and T-cell receptor γ/δ [eBioscience]). T cells were enriched from mice at day 10 of infection or immunization by depletion of unwanted cells (CD19, CD8a, CD11b, and CD49b [eBioscience]). B and T cell purities were determined to be >91%. B cells were labeled with CFSE, washed, and resuspended in cell culture media, and

2.5×10^5 B cells were added per well to 96-well plates. Graded numbers of $CD4^+$ T cells were added to culture wells in triplicate, along with 1×10^6 heat-killed (60°C 1 h) culture-grown *B. burgdorferi*, and cultured for 4 days. Supernatants were collected for antibody detection by ELISA, and cells were washed and stained for flow cytometry. CFSE dilution on $CD45R^+$ B cells was analyzed using the FlowJo Proliferation Platform to determine the frequency of cells that underwent at least one division and the average number of divisions of proliferating cells. For BLIMP-YFP expression, cultures were established as described above but as single rather than triplicate wells, and $ICOS^+$ $CD4^+$ T cells were FACS sorted from *B. burgdorferi*-infected or -immunized mice; influenza virus-immunized mice served as a negative (noncognate) control. For this, 2.5×10^7 *B. burgdorferi*-immunized B cells/ml were pulsed for 1.5 h with either 1×10^8/ml *B. burgdorferi* or 1,000 HAU/ml heat-inactivated influenza virus (noncognate control) and cultured as described above. After 4 days, supernatants were collected to determine antibody concentration by ELISA, and cells were stained for flow cytometry.

Statistical analysis. All graphs and statistical analyses were performed using GraphPad Prism software, version 5 (GraphPad Software). *P* values were calculated as indicated in the figure legends, and $P < 0.05$ was considered significant.

RESULTS

T cells contribute to the control of bacterial burden and affinity maturation but fail to maintain the proportion of high-avidity antibodies. We began our analysis of $CD4^+$ T cell function during *B. burgdorferi* infection by studying the effects of depleting $CD4^+$ cells over the course of 60 days of infection of B6 mice with host-adapted N40 spirochetes (24). The efficiency of depletion was confirmed by FACS analysis (Fig. 1A). Compared to controls, mice depleted of $CD4^+$ cells had significantly higher copy numbers of *B. burgdorferi* flagellin DNA in the urinary bladder and heart base (Fig. 1B). Results in the tibiotarsus did not reach significance. These studies demonstrate that $CD4^+$ T cells are important for the control of bacterial burden.

$CD4^+$ T cells can provide help for the generation of *B. burgdorferi*-specific antibodies after infection, and antibodies are crucial in regulating *B. burgdorferi* tissue burden and disease manifestations (21). The IgG response to a prototypic *B. burgdorferi* antigen, arthritis-related protein (Arp) of *B. burgdorferi*, which was shown previously to protect from arthritis development, was completely dependent on CD4 T cells, as CD4 T cell removal reduced IgG responses to Arp to undetectable levels (Fig. 1C). IgG responses to DbpA appeared T independent, while BmpA-IgG responses were somewhat increased in CD4 T-deficient mice. Consistent with these data, a previous study with T cell-deficient $TCR\beta/\delta^{-/-}$ mice also found a lack of T dependence of the DbpA response (20). Furthermore, repeated immunization with recombinant DbpA from cN40 failed to induce measurable CD4 T cell responses (data not shown). While the responses to OspC were reduced in the CD4-depleted mice, this reduction did not reach statistical significance (Fig. 1C). However, additional experiments with $CD40L^{-/-}$ mice showed this response to be dependent in part on conventional T cell help (data not shown). Thus, CD4 T cells importantly affect the magnitude and composition of the humoral response to *B. burgdorferi*, suggesting that the increased *B. burgdorferi* load observed in CD4-depleted mice (Fig. 1A) likely is due at least in part to altered antigen-specific serum IgG responses (Fig. 1C).

To further address the effects of CD4 T cells on the quality of the *B. burgdorferi*-specific serum IgG responses, we examined to what extent affinity maturation, a T cell-dependent process that occurs largely in germinal centers, is evident after *B. burgdorferi* infection. Due to its complete dependence on CD4 T cells, we focused on IgG responses to Arp. DbpA-specific T cell-independent antibodies served as a control (Fig. 1C) (8, 20). We utilized a method for detecting changes in the overall avidity of *B. burgdorferi* antigen-specific serum antibodies over time by comparing antibody binding after high- and low-stringency washes. Arp-specific antibody avidity increased for the first 6 to 8 weeks of infection of B6 mice but declined for the next 10 weeks despite continued increases in serum levels of these antibodies (Fig. 2A).

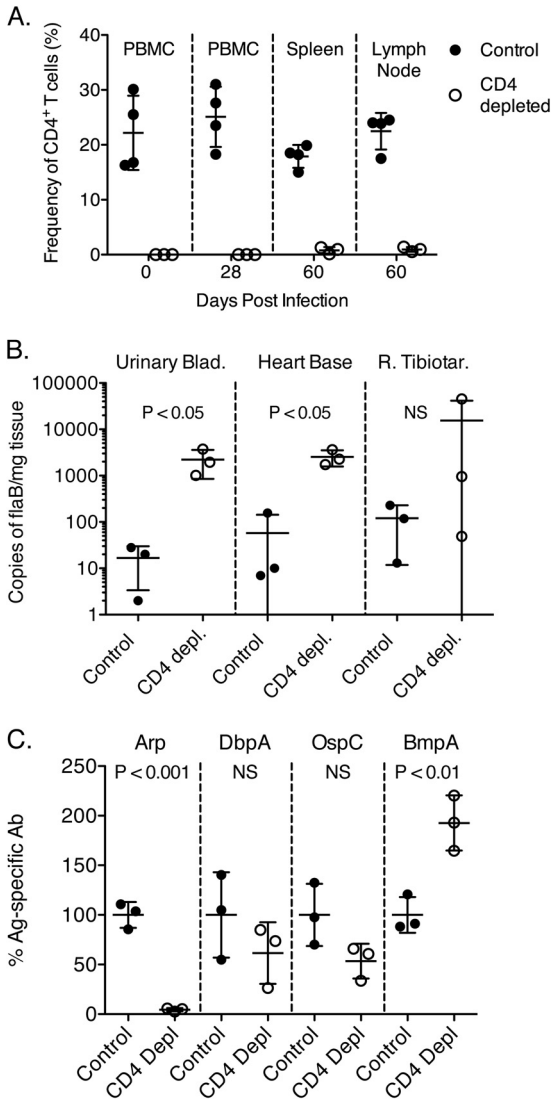

FIG 1 $CD4^+$ T cells affect bacterial burden. C57BL/6 (B6) mice were infected with *B. burgdorferi* and depleted of $CD4^+$ T cells (CD4 depl.; open circles) or treated with rat IgG (control, filled circles) for 60 days. (A) PBMCs, lymph nodes, or spleens were taken at the indicated time points and stained for $CD4^+$ cells to confirm depletion. (B) Bacterial burden was quantified by qPCR for *flaB* copy number in the indicated tissues. Blad., bladder; R. Tibiotar., right tibiotarsus. (C) Serum antigen (Ag)-specific antibodies were quantified by ELISA. The percentage of Ag-specific antibodies for each mouse compared to the means of the control mice is reported. Symbols represent individual mice ($n = 3$ each group), with bars indicating the means ± standard deviations (SD)/group. Significance (NS, not significant) for each tissue was calculated using Student's *t* test.

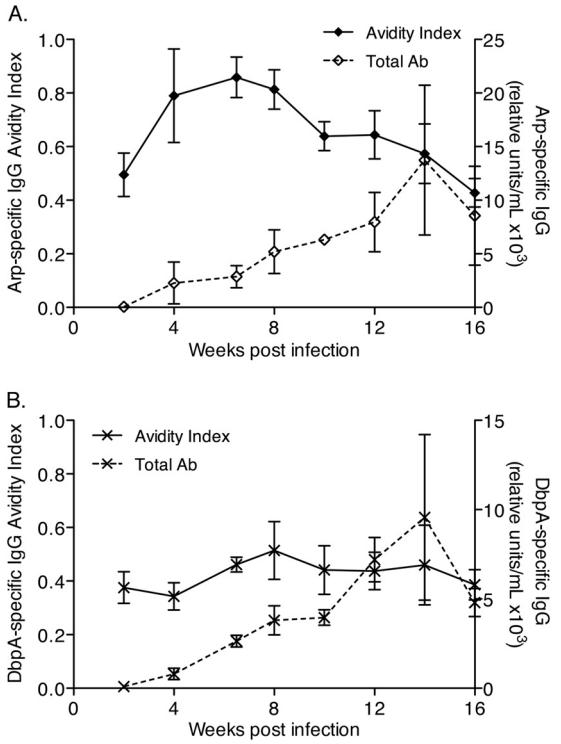

FIG 2 T-dependent Arp-specific antibodies undergo affinity maturation, but high-avidity antibodies are not maintained. (A and B) Arp-specific (A) and DbpA-specific (B) IgG avidity indexes were calculated by establishing the ratio of IgG bound to Ag in the presence/absence of a high-salt wash for serum samples from B6 mice collected at the indicated times after *B. burgdorferi* infection (solid line, left *y* axis). These are compared to the total Arp-specific (A) and DbpA-specific (B) IgG detected in the PBS wash (dashed line, right *y* axis). Points represent means ± SD ($n = 4$) from one experiment.

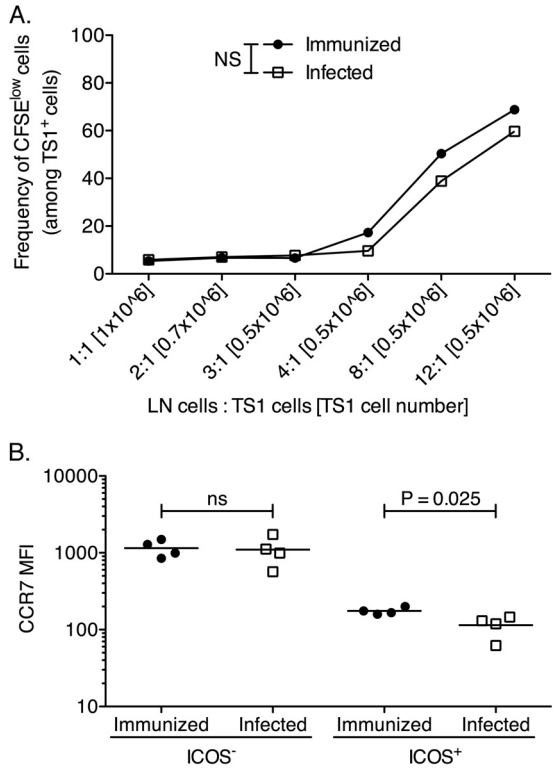

FIG 3 Lymph node cells from *B. burgdorferi*-infected mice support CD4 T cell priming. (A) At day 10 of *B. burgdorferi* infection or immunization of BALB/c mice, lymph node cell suspensions were cultured at the indicated ratios with naive, CFSE-labeled, transgenic T cells specific for influenza virus APR/8 hemagglutinin and heat-killed whole virus to assess the ability of the lymph node antigen-presenting cell environment to prime T cells. The frequency of TS-1 cells that proliferated (in diluted CFSE) is shown. Each symbol represents one culture well, and the *P* value was calculated using Student's *t* test. One representative experiment of three is shown. (B) CCR7 mean fluorescent intensity (MFI) of $ICOS^-$ and $ICOS^+$ lymph node T cells from B6 mice 10 days after *B. burgdorferi* infection (open symbols) or immunization (filled symbols). Symbols represent individual mice ($n = 4$), and bars indicate the means for each group.

As expected, the T-independent DbpA-specific antibody response did not increase in avidity over the course of infection (Fig. 2B).

Thus, affinity maturation of IgG to the T-dependent antigen Arp was induced but not efficiently maintained either because of stronger increases in low-avidity responses or a cessation of high-avidity antibody production. The latter is consistent with our previous findings that germinal center responses decline, starting 3 weeks after *B. burgdorferi* infection, and fail to develop T-dependent B cell memory and long-lived plasma cells (18). These data demonstrate that CD4 T cells provide help for early antibody production and participate in antibody affinity maturation. However, these responses are not sustained, causing only short-lived induction of high-avidity antibody responses. Since high-avidity B cell responses are dependent on effective T cell help, we sought to systematically assess $CD4^+$ T cell priming and expansion and B cell helper function during *B. burgdorferi* infection to determine whether *B. burgdorferi* reduces CD4 priming or affects their functionality during infection.

Lymph node cells from *B. burgdorferi*-infected mice support $CD4^+$ T cell priming. To measure the ability of antigen-presenting cells from lymph nodes of *B. burgdorferi*-infected mice to prime naive T cells, we cultured them with an unrelated antigen (influenza virus) and naive CFSE-labeled influenza virus-specific TS-1 transgenic CD4 T cells *in vitro* and assessed T cell proliferation by FACS. Lymph node antigen-presenting cells from *B. burgdorferi*-immunized mice served as controls. Because the TS-1 transgenic mice are inbred BALB/c mice, these studies used BALB/c mice. TS-1 proliferation was induced in an antigen dose-dependent manner, and priming with antigen-presenting cells from *B. burgdorferi*-infected lymph nodes was indistinguishable from priming induced by lymph node cells from immunized animals (Fig. 3A).

Primed T cells downregulate CCR7 expression to migrate toward B cell follicles (28). Therefore, we quantified CCR7 expression by flow cytometry as a measure of *in vivo* priming efficiency. The results showed that activated $ICOS^+$ T cells downregulated CCR7 expression by day 10 of *B. burgdorferi* infection as efficiently as control T cells from immunized B6 mice (Fig. 3B). Thus, antigen-presenting cells from *B. burgdorferi*-infected mice appeared capable of strong CD4 T cell priming.

***B. burgdorferi* infection induces strong CD4 T cell activation.** We next began to assess the quality of T cell help induced during *B. burgdorferi* infection by examining markers of T cell exhaustion and suppression. Follicular T helper (T_{FH}) cells are the major $CD4^+$ T cell population interacting with B cells. T_{FH} cells ($CD3^+$ $CD4^+$ $ICOS^+$) coexpressing CXCR4 and CXCR5 localize to germinal centers (T_{GC}; $X5^+$ $X4^+$) (27). Thus, the expression of these

two chemokine receptors can distinguish germinal center-resident T_{FH} from non-germinal-center T_{FH} cells, and the latter only express CXCR5 ($X5^+$ $X4^-$) (27). Both T_{FH} and T_{GC} cells were induced by *B. burgdorferi* infection (Fig. 4A).

Because PD-1 overexpression is a sign of T cell exhaustion in chronic infection (29), we monitored its expression on lymph node T cell subsets over the course of *B. burgdorferi* infection in B6 mice. As expected, PD-1 expression was highest on T_{GC} cells, lower on non-germinal-center T_{FH} and total activated $ICOS^+$ cells, and not expressed on the largely naive $ICOS^-$ T cells (Fig. 4A). Germinal center responses drastically decline between days 15 and 60 of *B. burgdorferi* infection (18). The expression of PD-1 on CD4 T cells did not increase over this time (Fig. 4A). We also found that the frequency of $CD4^+$ T cells coexpressing the inhibitory molecule CTLA-4^+ actually was lower at days 21 and 30 than at day 10 of infection (Fig. 4B). Finally, we examined the frequency of active caspase-3^+ apoptotic T cells during germinal center decline and again found that the frequency was, if anything, lower than that on day 10 for each T cell population (Fig. 4C). Therefore, it appears that the failure to maintain germinal center responses during *B. burgdorferi* infection is not due to the presence of exhausted or suppressive T cells or the death of large numbers of activated T cells.

$FoxP3^+$ Treg numbers are comparable to those of immunized controls. Regulatory T_{FH} cells coexpressing FoxP3 have been shown to regulate germinal center B cell responses (30; reviewed in reference 22). The strong induction of regulatory T cells during *B. burgdorferi* infection could lead to a suppression of T-dependent B cell responses. However, no significant difference in the frequency of $FoxP3^+$ $CD4^+$ Treg was notable at day 10 of *B. burgdorferi* infection compared to the level for immunized B6 controls (Fig. 5A). Although the frequency of $FoxP3^+$ cells among T_{GC} and $ICOS^+$ populations did rise slightly over days 21 and 30 (Fig. 5B), the overall number of these cells was very low compared to that of the $ICOS^-$ $FoxP3^+$ cells (Fig. 5C). Collectively, these data do not support a strong role for Treg-mediated suppression after *B. burgdorferi* infection as a mechanism by which germinal center responses might be terminated.

$CD4^+$ T cells promote antibody secretion over B cell proliferation. Since the presence of exhausted or suppressive T cell populations did not seem to explain the failure to sustain germinal center responses *in vivo* after *B. burgdorferi* infection, we next assessed the functional capacity of $CD4^+$ T cells to provide help to B cells *in vitro*. For this, we cultured B cells from lymph nodes of immunized control mice with T cells from either *B. burgdorferi*-infected or *B. burgdorferi*-immunized B6 mice and compared their abilities to provide cognate help for B cell proliferation and differentiation. Similar numbers of B cells initiated division in culture with CD4 T cells from day 10 *B. burgdorferi*-immunized or -infected lymph nodes (Fig. 6C). However, $CD4^+$ T cells from infected mice supported significantly fewer rounds of B cell division than $CD4^+$ T cells from immunized control mice (Fig. 6B and D), resulting in significantly lower frequencies of $CFSE^{low}$ B cells (Fig. 6A and E). This indicated that $CD4^+$ T cells from *B. burgdorferi*-infected mice are less effective in supporting B cell proliferation than those taken from immunized mice.

A mismatch in antigen specificity might have led to decreases in B cell proliferation, as *B. burgdorferi* surface antigen expression in the host-adapted spirochetes used for infections likely is somewhat different from the culture-grown spirochetes used for immunization. However, no significant differences in the initiation of B cell divisions were noted (Fig. 6C), and similarly reduced levels of B cell proliferation were observed in cocultures with both T and B cells from infected B6 mice compared to cocultures with

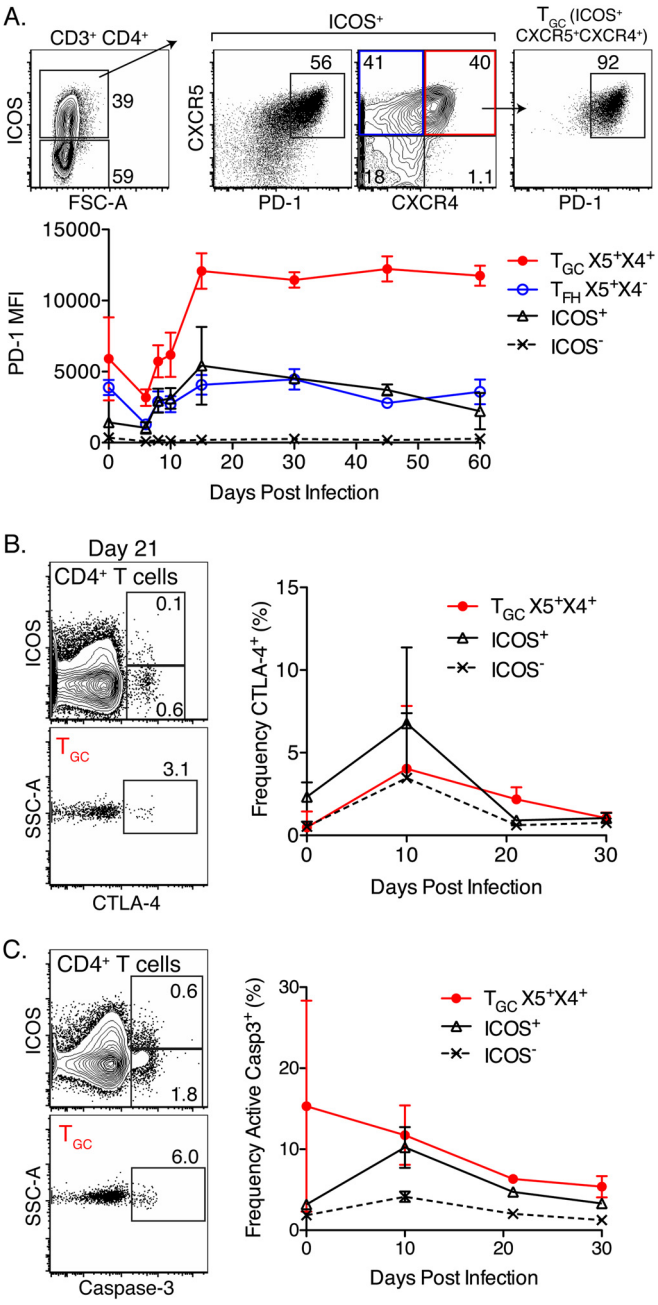

FIG 4 *B. burgdorferi* infection induces strong CD4 T cell activation. (A) Representative FACS plots illustrating $CXCR5^+$ $PD\text{-}1^{hi}$ T_{FH} and $CXCR5^+$ $CXCR4^+$ T_{GC} populations in lymph nodes at day 15 of *B. burgdorferi* infection of B6 mice. Further analysis of $CXCR5^+$ $CXCR4^+$ T_{GC} cells showed a homogeneously $PD\text{-}1^{hi}$ population, with the PD-1 MFI remaining constant during germinal collapse. Time points represent means ± SD from four mice for one experiment. (B) Representative FACS plots from lymph nodes at day 21 of *B. burgdorferi* infection showing CTLA-4 expression on total $CD4^+$ T cells (top) and $CXCR5^+$ $CXCR4^+$ T_{GC} cells (bottom). The frequency of CTLA-4^+ cells among T_{GC} cells compared to naive $ICOS^-$ T cells was determined by FACS analysis. Each symbol and lines represent the means ± SD from four mice for each time point for one experiment. (C) The analysis was done as shown in panel B for active caspase-3.

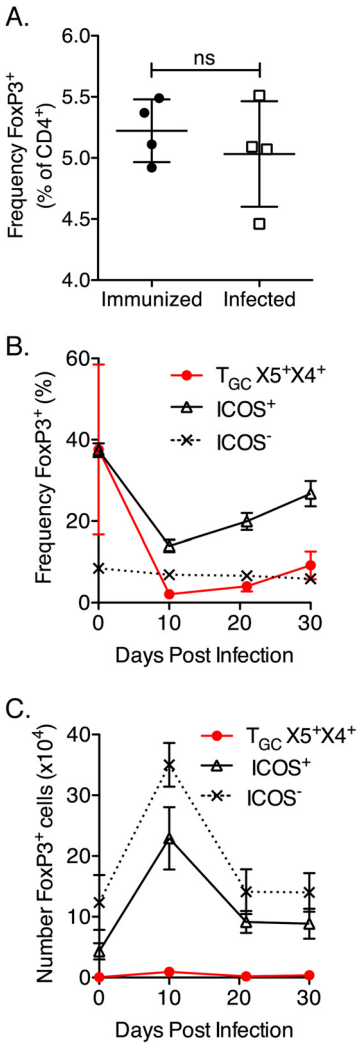

FIG 5 FoxP3$^+$ Treg numbers are comparable to those of immunized controls. (A) FoxP3$^+$ Treg populations were compared in lymph nodes from B6 mice at day 10 after *B. burgdorferi* immunization or infection. Shown are the frequencies of FoxP3$^+$ cells among CD4$^+$ T cells, with each symbol representing one mouse and the means ± SD. Student's *t* test did not show a significant difference between the populations. This was repeated at day 15 after infection/immunization with the same results (data not shown). (B and C) The frequency (B) and number (C) of FoxP3$^+$ cells of each indicated CD4$^+$ T cell subset were quantified by flow cytometry at the indicated days postinfection to assess the kinetics of Treg populations. Symbols and lines represent the means ± SD from four mice.

T cells from immunized mice and B cells from infected mice (data not shown). Furthermore, and remarkably, while CD4$^+$ T cells from infected mice consistently provided poor help for B cell proliferation, they induced significantly more antibody secretion by B cells than their counterparts isolated from *B. burgdorferi*-immunized mice (Fig. 6F). Thus, differences in the quality of T cell help exist between CD4 T cells isolated from *B. burgdorferi*-immunized mice and those from *B. burgdorferi*-infected mice.

Because strong antibody production is found *in vivo* in the lymph nodes of mice early after *B. burgdorferi* infection (18, 24), we aimed to further assess the functional impact of CD4 T cells from infected mice on B cell differentiation to antibody-producing cells. High levels of antibody secretion are associated with differentiation to plasma cells driven by the expression of the transcription factor BLIMP-1 (reviewed in reference 31). Using a Blimp-1 reporter mouse, we showed that T cells from *B. burgdorferi*-infected B6 mice induced more B cell expression of BLIMP-1 than immunized mice. While this difference did not quite reach statistical significance ($P = 0.07$) (Fig. 6H, left), when we focused on CD4 T cell/CD19$^+$ B cell conjugates, which formed strongly in cultures with *B. burgdorferi*-specific T and B cells but not in control cultures with influenza virus-specific CD4 T cells (Fig. 6G), the frequency of BLIMP-1$^+$ B cells among doublets was significantly higher for T cells from *B. burgdorferi*-infected mice ($P < 0.05$) (Fig. 6H, right). Stable B-T conjugate formation previously has been reported to occur *in vivo* between B cells and cytokine-secreting T_{FH} cells (32). These data suggest that helper CD4 T cells in *B. burgdorferi* infection support the rapid differentiation of B cells to antibody-secreting cells over the continued proliferation of B cells. Given the lack of effects noted on B cells from influenza virus-immunized mice, the provided help likely is cognate in nature and not due to nonspecific B cell activation.

DISCUSSION

B cell responses are crucial for host defense against many extracellular pathogens (33), including infections with *B. burgdorferi* (20, 34). *B. burgdorferi*-specific antibodies reduce *B. burgdorferi* tissue burden and disease manifestations, such as arthritis, myocarditis, and neuroinflammation (1–3, 20, 34). We showed previously that T-dependent and T-independent *B. burgdorferi*-specific antibodies are rapidly and strongly induced in extrafollicular areas of the lymph node within the first week of infection, providing early *B. burgdorferi*-specific antibodies (18). T-dependent germinal centers also were established by about 2 weeks after the infection (18). This is consistent with data from our study here that showed strong CD4 T cell activation and their differentiation to T_{FH} cells, including to CXCR4$^+$ CXCR5$^+$ T_{GC} cells (Fig. 4). This initial germinal center response likely is responsible for the generation of affinity-matured serum IgG (Fig. 2). Thus, early T-dependent B cell activation events appear to be initiated in the lymph nodes of *B. burgdorferi*-infected mice.

However, similar to the germinal center responses, which rapidly involute within 3 to 4 weeks after *B. burgdorferi* infection (18), affinity-matured antibody levels begin to decline after about 6 weeks (Fig. 1); thus, it appears that affinity-matured IgG ceases to be produced at about the same time as germinal centers begin to disappear from the lymph nodes of infected mice (18). This is consistent with previous findings that long-lived plasma cells are not induced after *B. burgdorferi* infection. Thus, any antibody secretion from cells that have successfully undergone affinity maturation would be provided by short-lived plasmablasts/cells.

Interestingly, a previous study demonstrated that the passive protective capacity of immune serum initially rises after infection but then begins to decline despite increasing levels of total *B. burgdorferi*-specific IgG (35). While there likely are multiple mechanisms at play, our data suggest that the early outputs of the germinal center responses increased the protective capacity due to their increased avidity, but that the production of such higher avidity antibodies is not maintained. The decline in germinal center responses occurred despite the ongoing presence of antigen (18), and as we show here, it was not associated with a lack or disappearance of T_{GC} cells, a strong induction of regulatory FoxP3-positive T_{GC} cells, or the presence of suppressive or exhausted

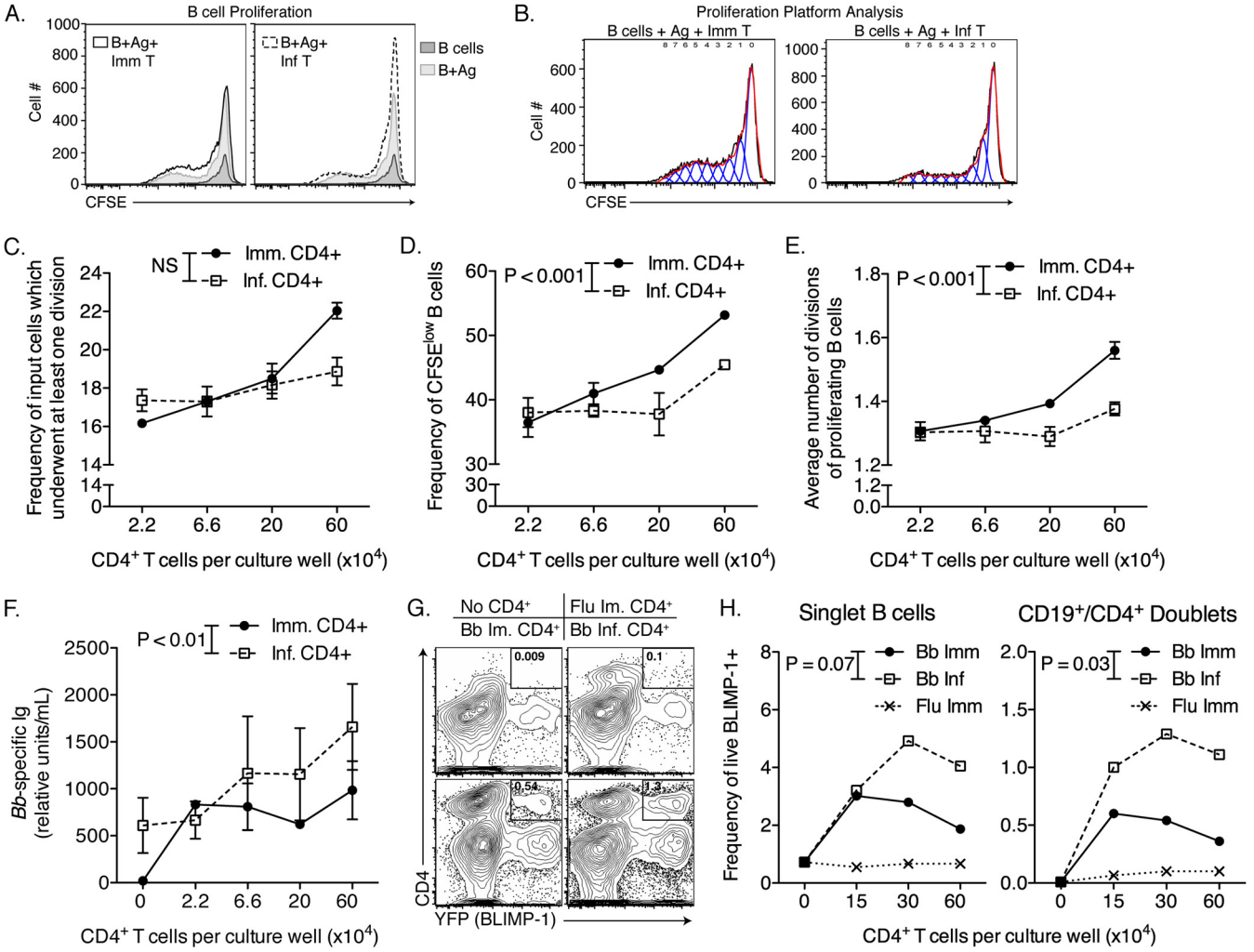

FIG 6 *B. burgdorferi* infection-induced CD4$^+$ T cells promote B cell antibody secretion and differentiation but not B cell proliferation. (A to F) Antigen-pulsed B cells from *B. burgdorferi*-immunized (A to E) or *B. burgdorferi*-infected (F) B6 mice were cultured with the indicated number of T cells from *B. burgdorferi*-immunized (Imm T) or *B. burgdorferi*-infected (Inf T) mice for 4 days. Each symbol represents the means ± SD from triplicate culture wells; *P* values were calculated by two-way analysis of variance (ANOVA). Shown is one representative experiment of three. (A and D) B cell proliferation was assessed by CFSE dilution. FlowJo Proliferation Platform analysis (B) calculated the frequency of B cells in the initial culture that underwent at least one division (C), as well as the average number of divisions of proliferating B cells (E). (F) Antibody concentrations in the supernatants were determined by ELISA. (G) B cells from *B. burgdorferi* (*Bb*)-immunized BLIMP-1-YFP reporter mice were cultured as described for panels A to F, and BLIMP-1 expression was assessed after 4 days. Shown are representative FACS plots pregated on live, CD19$^+$ cells identifying BLIMP-1$^+$ single B cells and CD4$^+$ CD19$^+$ doublets. (H) Frequency of live BLIMP-1$^+$ cells among CD19$^+$ cells (left) and CD19$^+$ CD4$^+$ doublets (right). Each symbol represents one culture well at decreasing numbers of CD4$^+$ T cells. The *P* value was calculated by Student's *t* test using paired observations.

CD4 T cells (Fig. 4). Thus, a lack of T cell activation or T_{FH} cell differentiation is unlikely to be responsible for the previously observed failure to induce functional long-lived high-affinity T-dependent B cell responses during *B. burgdorferi* infection. Studies in humans (36), mice (37), and dogs (38) provide evidence that late in the infection the immune response eventually shifts toward the generation of long-lived antibodies and memory B cells. However, by then the bacteria are disseminated and may no longer be fully accessible to these later responses.

The failure to induce fully functional germinal center responses after *B. burgdorferi* infection explains the clinical observations that humans and animals, particularly those living in areas where the infection is endemic, can be reinfected frequently (39, 40). They also explain the challenge in using serological evaluation of patient sera to test for prior exposure to *B. burgdorferi* (2, 41–43). IgG responses to *B. burgdorferi* are known to severely decline following antibiotic treatment (41), and this in fact is sometimes used as a sign of effective treatment (44). Thus, *B. burgdorferi* serodiagnostics may greatly underestimate the number of prior exposures, not necessarily because the available tests are of low quality but because of the pathophysiology of the *B. burgdorferi* infection-induced B cell responses. Whether CD4 T cell responses, which we show here to be induced by the infection, develop memory and provide a more suitable diagnostic target remains to be determined. The lack of defined major histocompatibility complex class II-restricted CD4 T cell epitopes, for use with studies in mice and humans, currently is hindering progress in this regard.

Consistent with the *in vivo* induction of mainly short-lived

extrafollicular B cell responses resulting in rapid antibody production after *B. burgdorferi* infection, the *in vitro* helper function of the *B. burgdorferi*-specific CD4 T cells appeared to be skewed toward providing effective help for B cell differentiation to plasma cells but less support of ongoing strong B cell proliferation (Fig. 6). When we compared the expression of numerous cytokines, including IL-2, IL-4, IL-5, IL-6, and IL-21, known to be critical for germinal center function from purified $CD4^+$ T cells of lymph nodes at days 10 and 21 after infection and after immunization, we did not observe significant differences (data not shown). Thus, the mechanisms underlying these differential effects of CD4 T cells on B cell help remain to be elucidated. The data provide the intriguing possibility that the interaction of B cells with infection-induced CD4 T_{FH} cells promotes their rapid differentiation and premature exit from germinal centers *in vivo* rather than their ongoing proliferation and resulting affinity maturation.

While this study focused on the B helper functions of CD4 T cells, CD4 T cell's interaction with innate leukocytes, in particular macrophages, likely is crucially affecting *Borrelia* burden in addition to their effects on the humoral response. Macrophages are important for the control of the infection. For example, macrophage activation via IFN-γ or its suppression by IL-10 regulate their ability to phagocytose and destroy spirochetes (45–47). Importantly, the type of inflammatory cytokines elaborated by macrophages depends on the type of receptors engaged in the initial phagocytosis of *B. burgdorferi* (48), and this in turn likely is influenced by the activation stage of the macrophage, which itself is dependent on cytokines, such as IFN-γ, elaborated by CD4 T cells and iNKT cells (47). The outcome of these diverse effects of CD4 T cells on the control of *Borrelia*-induced disease appears strongly tissue dependent. In joint tissue, the ability of CD4 T cells to increase inflammation likely contributes to Lyme arthritis development (4, 49), while in the heart, the elaboration of IFN-γ by CD4 T cells led to carditis resolution (11). In this context, these cells seemed to function in part by a forward-feedback loop in which IFN-γ production by CD4 T cells or innate cells alters chemokine production that favors the accumulation of CD4 T cells and macrophages at the expense of a strongly neutrophilic infiltrate (50). In keeping with the diverse roles of CD4 T cells, their actions in the regulation of both cellular and humoral immune events clearly affect the course of *B. burgdorferi* infection in mammalian hosts.

CD4 T cells and T-dependent antibody responses are targets for attack or evasion by many highly successful viral (51, 52), bacterial (53, 54), and parasitic pathogens (55, 56). Thus, reducing the effectiveness of CD4 T cells during *B. burgdorferi* infection to support long-term humoral immunity and perhaps other mechanisms of immune protection would not be without precedent. This study suggests that the functionality of the helper T cells induced during *B. burgdorferi* infection is responsible in part for the preponderance of strong, low-affinity antibody responses over long-lived, high-affinity antibody responses to *B. burgdorferi* infection. Such a shift in the quality of the humoral response would benefit the pathogen. Ultimately, the question is whether the CD4 T cell function during *B. burgdorferi* infection can be manipulated therapeutically to improve resistance against *B. burgdorferi* infection.

ACKNOWLEDGMENTS

This work was supported by NIH/NIAID grants R01 AI073911 and R01 AI051354 and NIH training grants T32-AI060555 and T32-AI074550.

We thank Stephen Barthold (Center for Comparative Medicine, UC Davis) for ongoing support and expert advice regarding experimental design, as well as a member of his laboratory, Sunlian Feng, for supplying recombinant Arp. We acknowledge Abigail Spinner (California National Primate Research Center) for expert technical assistance with flow cytometry and Peter Barry (Center for Comparative Medicine, UC Davis) and laboratory members William Chang, Kim Schmidt, and Meghan Eberhardt for assistance with the avidity index assay.

We have no conflicts of interest to declare.

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