Immunopathology and Infectious Diseases

Promotion of a Functional B Cell Germinal Center Response after Leishmania Species Co-Infection Is Associated with Lesion Resolution

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Co-infection of C3HeB/FeJ (C3H) mice with both *Leishmania major* and *Leishmania amazonensis* leads to a healed footpad lesion, whereas co-infection of C57BL/6 (B6) mice leads to non-healing lesions. This inability to heal corresponds to a deficiency in B cell stimulation of the macrophage-mediated killing of *L. amazonensis in vitro* and a less robust antibody response. The mechanism that leads to healing of these lesions is not completely known, although our studies implicate the B cell response as having an important effector function in killing *L. amazonensis*. To understand more completely this disparate clinical outcome to the same infection, we analyzed the draining lymph node germinal center B cell response between co-infected C3H and B6 mice. There were more germinal center B cells, more antibody isotype-switched germinal center B cells, more memory B cells, and more antigen-specific antibody-producing cells in co-infected C3H mice compared to B6 mice as early as 2 weeks postinfection. Interleukin (IL)-21 production and IL-21 receptor expression in both mouse strains, however, were similar at 2 weeks, suggesting that the difference in the anti-*Leishmania* response in these mouse strains may be due to differences in T follicular cell commitment or intrinsic B cell differences. These data support the idea that functional B cells are important for healing *L. amazonensis* in this infectious disease model. (*Am J Pathol 2012, 180: 2009–2017; DOI: 10.1016/j.ajpath.2012.01.012*)

Leishmaniasis is a vector-borne disease caused by an obligate intracellular protozoan parasite of the genus Leishmania.

Both Leishmania (L.) major and *L. amazonensis* cause cutaneous leishmaniasis, characterized by focal to multi-focal cutaneous ulcerations, which can occur after infection of a sand fly bite. Infection of C3HeB/FeJ (C3H) mice with *L. major* resolves within 8 to 12 weeks and is dependent on development of a polarized CD4+T helper 1 (Th1) immune response, which is critical for activation of infected macrophages to kill internalized parasites.[1] Infection of the same mouse strain with *L. amazonensis* leads to large, non-healing lesions, and the immune response is not polarized to either a Th1 or Th2 response.[1,2]

Prior infection of C3H mice with *L. major* leads to protection against subsequent *L. amazonensis* infection.[3,4] Using an *in vitro* model of Leishmania infection developed in our laboratory, we identified that CD4+ T cells and CD19+ B cells from *L. major*-infected C3H mice were necessary to kill *L. amazonensis* within infected macrophages.[5] More recently it was described that co-infection with both *L. major* and *L. amazonensis* in the same footpad led to significantly higher lesion size and parasite load in co-infected C57BL/6 (B6) mice when compared to C3H mice.[6] Using an *in vitro* assay with cell depletion and reconstitution it was determined that B cells from *L. major*-infected B6 mice fail to activate infected macrophages to kill *L. amazonensis*.[6] It was also shown that co-infected B6 mice did not make significant levels of Leishmania-antigen specific antibodies as compared to co-infected C3H mice, suggesting an important primary defect in the B cell response in B6 mice.[6] In addition, although studies have demonstrated that B cells contribute to the immunopathology of a non-healing response toward Leishmania infection, our findings suggest that there can be an important role for B cells during characteristically effective T-cell-mediated immunity.

To understand the different biological response to an identical infectious challenge, we now show differences in the B cell germinal center response between C3H and

B6 mice co-infected with *L. major* and *L. amazonensis*. There were significant differences in C3H versus B6 B cell responses during Leishmania co-infection. Co-infected C3H mice had increased germinal center B cells that were isotype switched, increased memory B cells, and increased antigen-specific antibody-producing cells as early as 2 weeks postinfection as compared to B6 mice. We also demonstrated that IL-21 production, location of IL-21 producing cells in the lymph node, and expression of the IL-21 receptor are similar, indicating that IL-21 expression does not necessarily translate to effective germinal center formation. Our findings establish a fundamental difference in the immune response to Leishmania infection between these two mouse strains and provide additional evidence that the B cell response, in part, determines immune effectiveness toward *L. amazonensis* infection.

Materials and Methods

Mice

Female C57BL/6 (B6) and female C3HeB/FeJ (C3H) mice (6 to 8 weeks of age) were obtained either from Jackson Laboratories (Bar Harbor, Maine) or from an in-house breeding colony. Mice were maintained in a specific pathogen-free facility. Mice were infected with either 5×10^6 stationary-phase *L. major*, *L. amazonensis* or 2.5×10^6 *L. major* and 2.5×10^6 *L. amazonensis* promastigotes in 50 μL of PBS in the left hind footpad. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Iowa State University.

Parasites and Antigens

L. amazonensis (MHOM/BR/00/LTB0016) and *L. major* (MHOM/IL/80/Friedlin) promastigotes were grown in complete Grace's Insect medium (Atlanta Biologicals, Lawrenceville, GA) to stationary phase, harvested, washed in endotoxin-free PBS (Cellgro, Herdon, VA) and prepared to a concentration of 1×10^8 parasites/mL. Freeze-thawed Leishmania antigen was obtained from stationary phase promastigotes as previously described.[7]

Lymph Node Cell Culture and Sorting

Total lymph node (TLN) cells were obtained from the left popliteal lymph node draining the site of left footpad infection from C3H and B6 mice infected for 2 or 5 weeks with *L. major*, *L. amazonensis*, or co-infected with both species. Lymph nodes from each mouse were kept separate and harvested into 2 mL of complete tissue culture medium (RPMI 1640, 2 mmol/L L-glutamine, 100 U penicillin, 100 μg streptomycin/mL, 25 mmol/L HEPES, 0.05 μm 2-mercaptoethanol and 10% fetal bovine serum). A single cell suspension was created using a 2 mL tissue homogenizer. Cells were passed through a 40-μm nylon cell strainer (BD Falcon, Bedford, MA) and washed with 10 mL of complete tissue culture medium at $250 \times g$, 4°C for 10 minutes. After washing, the cells were resuspended in 0.5 mL complete tissue culture medium and counted.

Flow Cytometry

For analysis of surface molecule expression, 0.5×10^6 TLN cells were washed in 2 mL of fluorescence-activated cell sorting buffer (FACS) (0.1% sodium azide and 0.1% bovine serum albumin in phosphate buffer saline). Fcγ receptors were blocked with 10% purified rat anti-mouse CD16/CD32 antibody (BD Pharmingen, San Diego, CA) in 1 mg/mL rat IgG (Sigma, St. Louis, MO) for 20 minutes at 4°C to prevent nonspecific antibody binding. TLN cells were then incubated with appropriate primary antibody or isotype control for 30 minutes on ice. The antibodies used include phycoerythrin-labeled CD19(1D3), Cy5-labeled CD19 (1D3), biotin-labeled CD69, biotin-labeled IgM (b7-6), biotin-labeled IgD, (11–26) biotin-labeled major histocompatibility complex (MHC) class II (M5114 for B6 or I-A^k for C3H), fluorescein isothiocyanate-labeled CD86 (GL1), biotin-labeled CD40, fluorescein isothiocyanate-labeled peanut agglutinin (PNA), phycoerythrin-labeled CD23 (B2B4), Cy5-labeled B220 (6B2) phycoerythrin-labeled IL-21 receptor, and biotin-labeled CD95. CD69, CD95, IL-21 receptor and MHC class II (I-A^k) were purchased from eBiosciences (San Diego, CA), PE-CD19 was purchased from BD Pharmingen (San Diego, CA) and the remainder of antibodies were prepared as previously described.[8] Following incubation, cells were washed twice in 2 mL of FACS buffer and incubated with appropriate secondary reagent, if necessary, for 30 minutes at 4°C. Secondary reagents included phycoerythrin-labeled streptavidin (as previously described[8]) and fluorescein isothiocyanate-labeled streptavidin (BD Pharmingen, San Diego, CA). After secondary incubation, cells were washed twice in 2 mL Fluorescence-activated cell sorting (FACS) buffer, fixed in 200 μL of 1% paraformaldehyde, and stored at 4°C in the dark until analysis. Then the analysis was performed on a BD FACScanto flow cytometer (Becton Dickinson, San Jose, CA) and data analysis was performed using FlowJo software V8.5.2 (Tree Star, Inc., Ashland, OR).

Antigen-Specific Enzyme-Linked Immunosorbent Spot

IgG1, IgG2a, and IgG2c enzyme-linked immunosorbent spot (ELISPOT) were performed on TLN cells. Immulon 2 plates (Fischer, Fair Lawn, NJ) were coated with 5 μg/mL of freeze-thawed Leishmania parasite antigen overnight at 4°C. After washing with PBS, commercially available biotinylated anti-IgG1, IgG2a, and IgG2c antibodies (Jackson ImmunoResearch West Grove, PA) were added at a 1:10,000 dilution in 5% fetal bovine serum overnight at 4°C. ELISPOT were developed using 2-amino-2-methyl-1-propanol (ICN Biomedicals Inc., Aurora, OH) and 5-bromo-4-chloro-3-indoly-phosphate (Fisher, Fair Lawn, NJ).

For IL-21 ELISPOT, a similar procedure was used as previously described on TLN cells using the mouse IL-21

DuoSet kit, according to manufacturer's instructions (R&D Systems, Minneapolis, MN).

Lymph Node Histopathology and Immunohistochemistry

Popliteal lymph nodes from the left hind leg draining the site of infection were harvested and placed in cassettes in 10% neutral buffered formalin for histological and immunohistochemical analyses. Histological examination was performed on paraffin-embedded tissues cut at 5-μm thickness onto positively charged slides and stained with H&E. For immunohistochemistry, slides were de-paraffinized and blocked with 20% normal rat serum. The sections were then incubated with either an anti-mouse B220/CD45R antibody (BD Harlingen, San Diego, CA) at a concentration of 1:400, a biotin-labeled PNA (Vector Laboratories, Burlingame, CA) at a concentration of 1:500, or with IL-21 antibody (Millipore, Billerica, MA) at a concentration of 1:500 with 10% normal rat serum. The slides were rinsed with PBS and then incubated with biotin-labeled goat anti-rat IgG (KPL, Gaithersburg, MD) at a concentration of 1:300 in 10% normal donkey serum when appropriate. Slides were washed and incubated with peroxidase-conjugated streptavidin (BioGenex, San Ramon, CA) for 45 minutes. After 2 PBS washes, the color was developed with Nova Red (KPL, Gaithersburg, MD). The slides were then counterstained with Harris' hematoxylin, then dehydrated and mounted with coverslips. A semiquantitative scoring scale for PNA staining was used as defined by: 0, no PNA staining; 1, 1-2 PNA-positive germinal centers, 2, 3-4 PNA-positive germinal centers; 3, 5 or more PNA-positive germinal centers per lymph node section. All evaluations were made based on the average of one lymph node section from three animals and two separate experiments and assessed blindly by a board-certified veterinary pathologist.

Statistics

Statistical analysis was performed with Prism4 (Graph-Pad Software Inc., La Jolla, CA). Differences between groups were determined using two-way analysis of variance with Tukey post hoc test or a Mann-Whitney U-test when appropriate. P values <0.05 were considered statistically significant.

Results

Increased Germinal Center B Cells and Isotype Switched Germinal Center B Cells during Co-Infection of C3H Mice Compared to B6 Mice

We previously demonstrated that C3H mice co-infected with *L. major* and *L. amazonensis* heal their footpad lesions by 10 to 12 weeks postinfection.[6] Co-infected C57BL/6 (B6) mice, in comparison, have persistent non-healing lesions (Figure 1) and a significantly higher footpad parasite burden (data not shown).[6] Using an *in vitro* co-culture assay, we have shown that B cells harvested
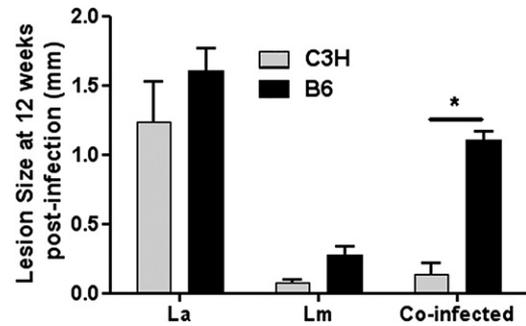

Figure 1. Simultaneous co-infection with both *Leishmania major* and *L. amazonensis* allows for lesion resolution in co-infection of C3HeB/FeJ (C3H), but not C57BL/6 (B6) mice at 12 weeks postinfection. Mice with a single infection were inoculated with *L. amazonensis* (La) or *L. major* (Lm) stationary phase promastigotes, whereas co-infected mice (Co) were inoculated with Lm and La in the left hind footpad. Mice were infected for 12 weeks with weekly monitoring of lesion size. The lesion size was determined by measuring the infected footpad and comparing that to the non-infected footpad. Data are represented as the mean ± SEM of three separate experiments. *$P \leq 0.05$.

from *L. major*-infected B6 mice cannot promote killing of intracellular *L. amazonensis* in contrast to B cells from *L. major*-infected C3H mice.[6] Based on these previous findings, we hypothesized that B cells from co-infected B6 are phenotypically and functionally different from B cells from co-infected C3H mice. We assessed the number of germinal center B cells and isotype-switched germinal center B cells via flow cytometric analysis using draining TLN cells from mice infected for 2 and 5 weeks with *L. amazonensis*, *L. major* or both species of parasites.

On entering the germinal center, B cells typically display PNA lectin and up-regulate CD95 surface expression.[9] There were significantly more germinal center positive (B220$^+$, PNA$^+$) B cells in the draining lymph nodes of co-infected C3H mice as compared to co-infected B6 mice at both 2 and 5 weeks postinfection (Figure 2A). As expected, naïve mice of both strains had negligible numbers of germinal center B cells (Figure 2A).

The germinal center functions as the primary location for isotype switching of activated B cells.[10] To assess the population of B cells that have undergone isotype switching, we assessed the B220$^+$, PNA$^+$ cell populations for expression of IgM via FACS analysis of cells from the draining lymph nodes of *L. amazonensis*, *L. major*, and co-infected C3H and B6 mice. We determined that co-infected C3H mice have more germinal center B cells that are IgM$^-$ and therefore have undergone isotype switching at 2 and 5 weeks postinfection (Figure 2B).

On activation, B cells will also up-regulate surface expression of MHC class II, CD80, CD86, and CD40.[11] To assess B cell activation status, flow cytometry was used to determine surface expression of these markers. Draining lymph node cells were first gated on the B220$^+$ (also known as CD45R) population and were then analyzed for B cell activation makers. Infection led to measurable activation in all infected groups, as determined by increases in mean fluorescent intensity over naïve controls for MHC class II (Figure 2C), CD86 (Figure 2D), or CD40 (data not shown) at either 2 or 5 weeks. All groups, however, expressed similar fold increases in mean fluo-
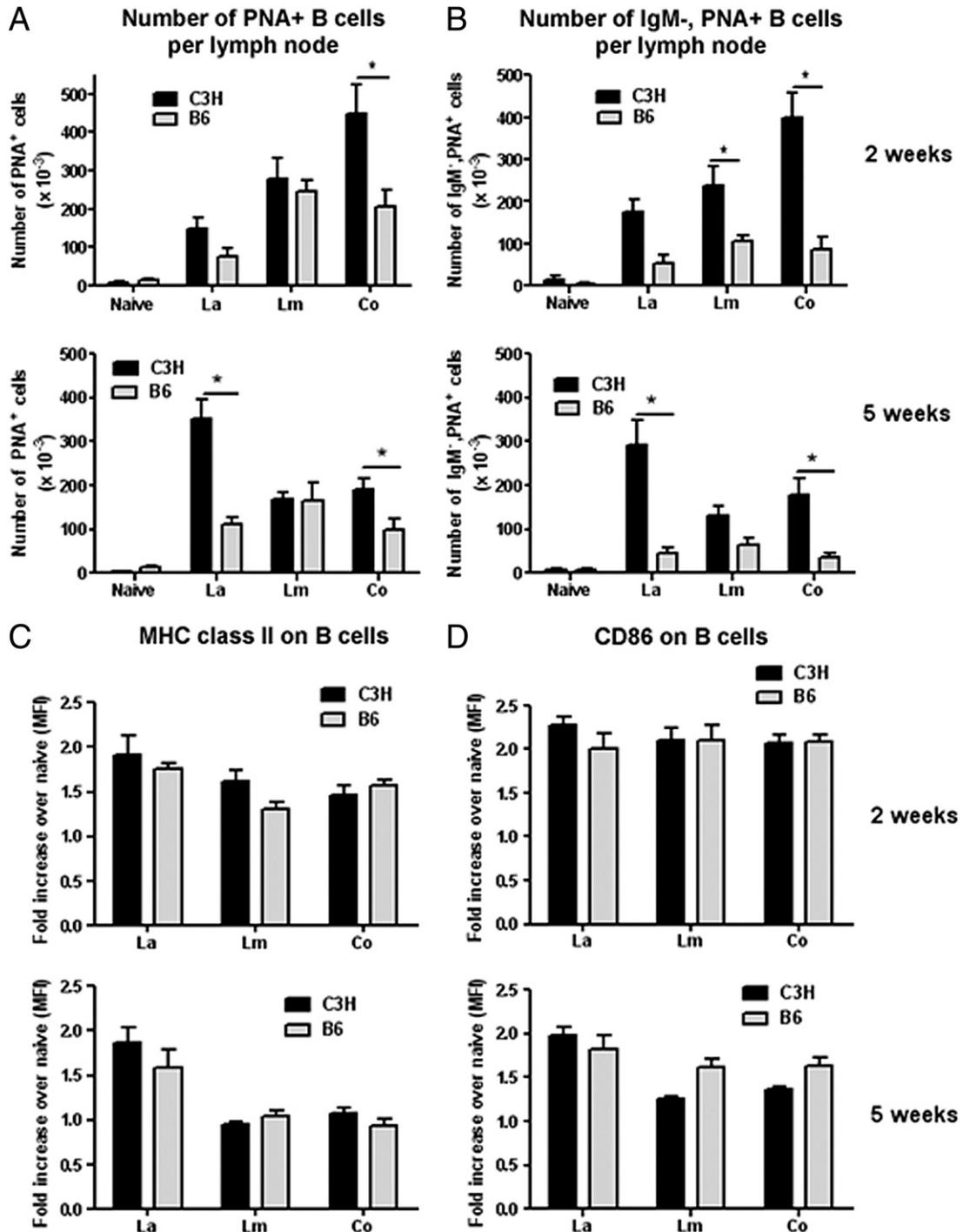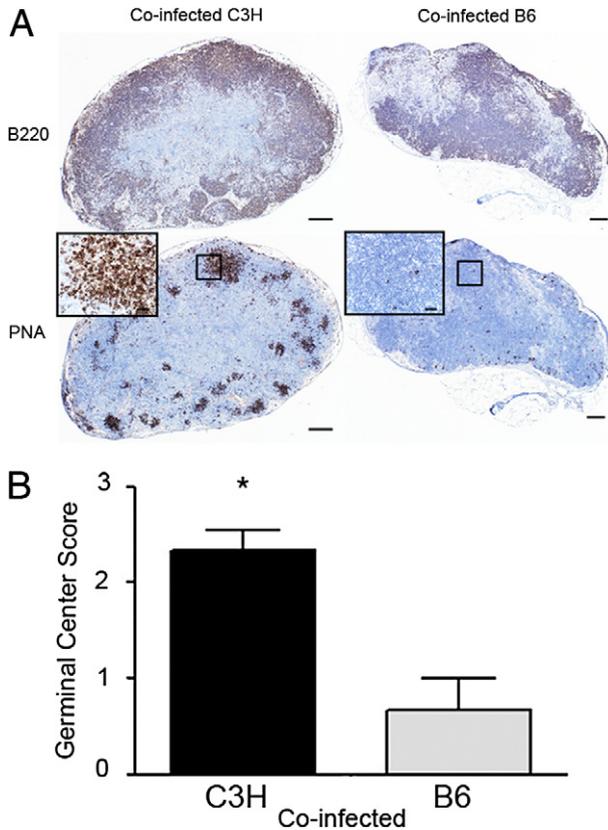
Figure 2. Increased number of total germinal center B cells and germinal center B cells undergoing isotype switching in co-infection of C3HeB/FeJ (C3H) mice. C3H and C57BL/6 (B6) mice were infected with *Leishmania amazonensis* (La), *L. major* (Lm), or co-infected (Co) with both species of parasites. Total draining lymph node cells were harvested at 2 and 5 weeks postinfection. Cells were analyzed via fluorescence-activated cell sorting (FACS) and first gated on the B220[+] population and analyzed for binding to peanut agglutinin (PNA) (**A**), PNA binding (**B**), surface expression of major histocompatibility complex (MHC) class II (**C**), and surface expression of CD86, as indicated by mean fluorescence intensity (MFI) (**D**). Cell number was determined based on the percentage of cells within the gated population and the total number of lymph node cells recovered. Data are represented as the mean ± SEM of three separate experiments. *$P \leq 0.05$.

rescent intensity over naïve controls, suggesting no measurable difference in overall B cell activation status between co-infected B6 and C3H mice.

To confirm our flow cytometric findings regarding differences in B cell germinal center formation, we performed immunohistochemistry using anti-B220 and bio-

tin-labeled PNA on draining lymph nodes of co-infected mice 2 weeks postinfection. The pattern of immunoreactivity for B220 demonstrated that lymph nodes from co-infected C3H mice have active cortices with multiple, large follicles, and distinct germinal center formation, as compared to co-infected B6 mice, which had less distinct

Figure 3. Increased number of germinal centers in the draining lymph node following co-infection of co-infection of C3HeB/FeJ (C3H) mice with *Leishmania major* and *L. amazonensis*. **A:** Photomicrographs of lymph node sections labeled with anti-mouse B220 and biotin-peanut agglutinin (PNA) from C3H and C57BL/6 (B6) mice co-infected for 2 weeks. Bar = 200 μm. Designated areas magnified in the **insets** which highlight PNA immunoreactive cells. Scale bars: 40 μm. **B:** Histological germinal center scores for PNA immunoreactivity at 2 weeks. Score is based on the number of PNA$^+$ germinal centers within a single draining lymph node. Data are representative of two separate experiments $\pm$ SEM. *P = 0.0087.

follicles and rare germinal centers (Figure 3A, top panels). PNA staining confirmed there were more germinal centers in co-infected C3H mice, whereas draining lymph nodes of co-infected B6 mice had germinal centers were fewer in number (Figure 3, A [bottom panels] and B). Together the data indicate that the germinal center B cell response at both 2 and 5 weeks postinfection in co-infected B6 mice was less robust. It is known that the germinal center is the site in which B cells become either memory B cells or antibody-secreting plasma cells.[12] These findings suggest that C3H mice co-infected with *L. major* and *L. amazonensis* have more memory B cells and/or more antibody-secreting cells.
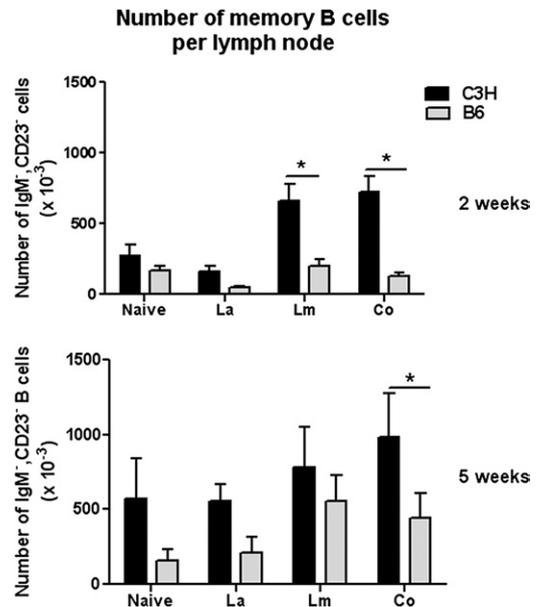
Increased Memory B Cells during Leishmania Co-Infection in C3H Mice Compared to B6 Mice

We wanted to determine whether the difference in the number of germinal center B cells would lead to a downstream alteration in the memory B cell population between these two mouse strains after co-infection. Cells from the draining lymph nodes were analyzed via flow

cytometry with anti-CD19 to identify B cells, anti-IgM, and anti-CD23. Anti-CD19, instead of anti-B220, was used to differentiate B cells from plasmacytoid dendritic cells, which also express B220. Nonswitched, mature B cells within the lymph node have been previously shown to express surface IgM and CD23, whereas memory B cells are CD19$^+$, IgM$^-$, and CD23$^-$.[8] At both 2 and 5 weeks after co-infection, we found that C3H mice had increased numbers of memory B cells as compared to B6 mice (Figure 4). These memory B cells include both germinal center memory B cells and postgerminal center switched memory B cells. The relatively low numbers of memory B cells and isotype switched effector B cells in co-infected B6 mice is consistent with poor germinal center formation in these mice.

Increased Antigen-Specific Antibody Production in Co-Infected C3H Mice Compared to Co-Infected B6 Mice

To determine whether the observed differences in germinal center formation and B cell effector phenotype lead to differences in B cell antibody production, we analyzed the number of antigen-specific antibody-producing B cells during co-infection of C3H and B6 mice. ELISPOT analysis was performed on draining TLN cells for antigen-specific antibody production of IgG2a (C3H), IgG2c (B6), and IgG1 from draining lymph nodes of co-infected C3H and B6 mice. B6 mice express the Igh1-b allele, which encodes for and leads to IgG2c antibody isotype produc-

Figure 4. Increased number of memory B cells (B220$^+$, IgM$^-$, and CD23$^-$) in the draining lymph node of co-infected C3HeB/FeJ (C3H) mice. C3H and C57BL/6 (B6) mice were infected with *Leishmania amazonensis* (La), *L. major* (Lm), or co-infected (Co) with both species of parasites. Total draining lymph node cells were harvested at 2 and 5 weeks postinfection. Via flow cytometry, surface marker expression was determined based on a CD19$^+$ population. Surface expression of both IgM and CD23 was determined based on the percentage of cells within the gated population as compared to the total lymph node cells recovered. Data are representative of two separate experiments $\pm$ SEM. *$P \le 0.05$.
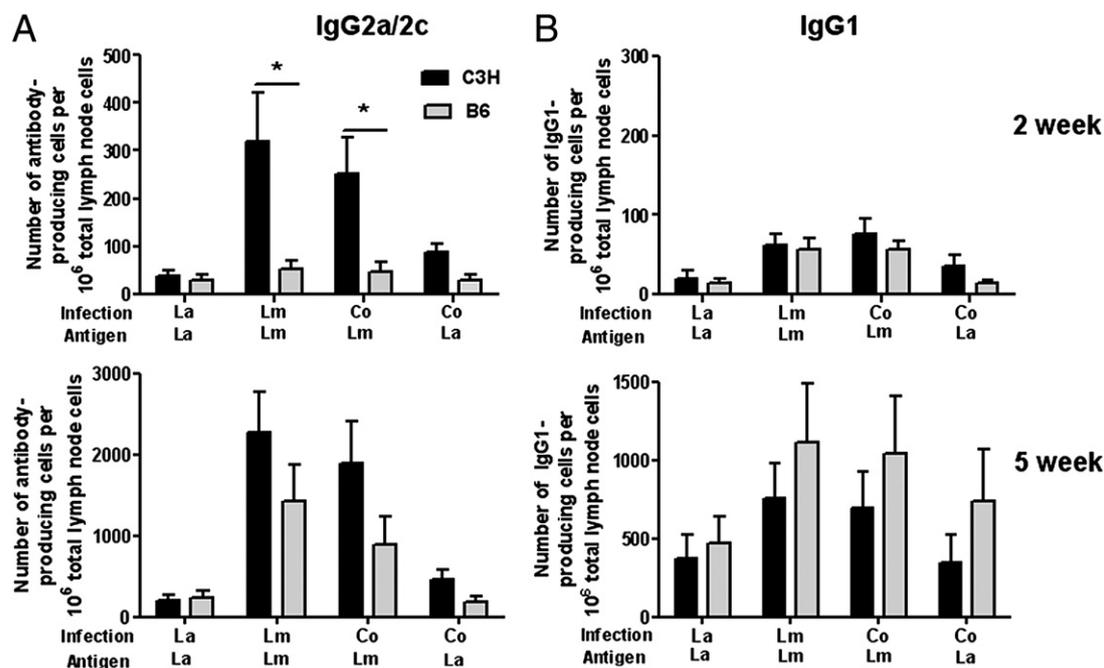
Figure 5. Increased number of antigen-specific IgG2a-producing cells following co-infection in C3HeB/FeJ (C3H) mice. C3H and C57BL/6 (B6) mice were infected with *Leishmania amazonensis* (La), *L. major* (Lm), or co-infected (Co) with both species of parasites. Total draining lymph node cells were harvested at 2 and 5 weeks postinfection (please note scales). Number of IgG2a (C3H) and IgG2c (B6)-producing cells (**A**), and IgG1-producing cells (**B**), as determined by enzyme-linked immunosorbent spot (ELISPOT) analysis of total draining lymph node cells stimulated with freeze-thawed Leishmania promastigote antigen, as previously indicated. Data are represented as the mean ± SEM of three separate experiments. *$P \leq 0.05$.

tion, whereas C3H mice carry the Igh1-a allele, and therefore produce IgG2a.[13] Co-infected C3H mice produce significantly more *L. major*-specific IgG2a than B6 mice produce *L. major*-specific IgG2c at 2 weeks postinfection (Figure 5A). No significant differences were noted in the production of antigen-specific IgG1 (Figure 5B). These results indicate a differential production of antigen-specific antibodies when C3H and B6 mice are compared at the 2-week time point. Not statistically significant, however, at 5 weeks postinfection there is a similar trend in which smaller numbers of antigen-specific antibodies in B6 mice are compared to C3H mice.

IL-21 Production, Location of Production, and IL-21 Receptor Expression Are Similar between C3H and B6 Mice Co-Infected for 2 Weeks

After B6 mice were co-infected in the footpad with both *L. major* and *L. amazonensis*, we observed an overall decrease in the number of lymph node germinal centers, isotype switched germinal center B cells, memory B cells, and antigen-specific IgG2a/c antibody-producing cells than co-infected C3H mice. We sought to determine whether these differences were intrinsic to B cell function or if they were due to differential production of IL-21 from the draining lymph node of co-infected C3H versus B6 mice. IL-21 is primarily produced by T follicular helper (Tfh) cells within germinal centers, but they can also be produced by T helper 17 cells and other lineages of CD4$^+$ T cells.[14] IL-21 has been shown to function in B cell proliferation and production of plasma cells.[15] The important role of IL-21 in B cell function has been demonstrated

in B cells deficient in IL-21 receptor expression, which have an impaired ability to undergo isotype switching and maintain germinal center organization.[16,17] We performed IL-21 ELISPOT on TLN cells from co-infected C3H versus B6 mice. There was a significant increase in the number of IL-21-producing cells after co-infection of either B6 or C3H mice as compared to *L. amazonensis* infection alone. There was no difference in the number of IL-21 producing cells at 2 weeks postinfection between C3H and B6 mice with or without antigen stimulation (Figure 6A [data not shown]). IL-21 immunohistochemistry was used to confirm these results, and again we did not observe any differences in the distribution of IL-21 immunoreactive cells in the draining lymph nodes of co-infected C3H versus B6 mice (Figure 6C). Therefore, a paucity of IL-21 producing cells is not responsible for the differences we have described in germinal center B cell responses after co-infection of B6 mice versus C3H mice. We then determined if the expression of B cell IL-21 receptor was different between co-infected C3H and B6 mouse strains at 2 weeks postinfection. Using flow cystometry, we gated on B220+ cells and determined that expression of the IL-21 receptor was not significantly different between co-infection or single parasite infection (Figure 6B).

Discussion

The immune factors required to heal cutaneous leishmaniasis caused by *L. amazonensis* are unknown. In this article, we demonstrated that co-infection with both *L. major* and *L. amazonensis* led to a healing phenotype in C3H
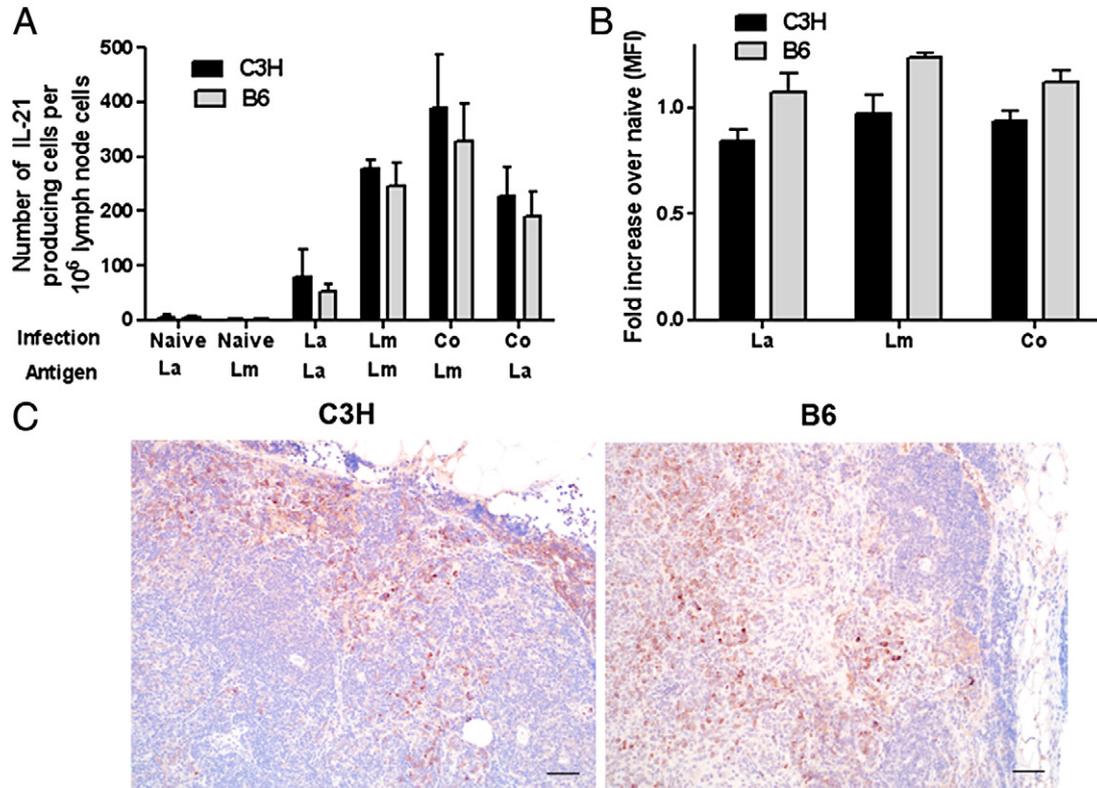
Figure 6. No difference in the number of IL-21 producing cells, IL-21 receptor expression, or site of IL-21 production in draining lymph node cells from co-infected C3HeB/FeJ (C3H) and C57BL/6 (B6) mice. **A:** Number of IL-21-producing cells was determined by enzyme-linked immunosorbent spot (ELISPOT) analysis of total draining lymph node cells stimulated with freeze-thawed Leishmania promastigote antigen. C3H and B6 mice were infected with *Leishmania amazonensis* (La), *L. major* (Lm), or co-infected (Co) with both species of parasites. Total draining lymph node cells were harvested at 2 weeks postinfection. Data are represented as the mean ± SEM of two (for B6) or three (for C3H) separate experiments. **B:** Cells were first gated on the B220$^+$ population and surface expression of IL-21 receptor was measured as indicated by mean fluorescence intensity (MFI). The MFI for each marker presented as fold increase over naïve control. Total draining lymph node cells were harvested at 2 weeks postinfection. Data are represented as the mean ± SEM of two separate experiments. **C:** Photomicrographs of lymph node sections labeled with anti-mouse IL-21 from C3H and B6 mice co-infected for 2 weeks. Bar = 50 μm.

mice, whereas B6 mice develop non-healing, persistent lesions (Figure 1).[6] Using an *in vitro* model that mimics co-infection, we have previously demonstrated that B cells from infected B6 mice do not function as effectively as B cells from C3H mice to kill intracellular *L. amazonensis*.[6] Here, for the first time we describe a significant difference in the germinal center B cell response between healing C3H and non-healing B6 mice co-infected with *L. major* and *L. amazonensis*, as early as 2 weeks postinfection. Germinal centers, formed within secondary lymphoid organs, are the site for early B cell expansion.[12] After proliferation, signal-dependent isotype switching occurs within the germinal center changing the B cell receptor surface expression from IgD or IgM to IgG, IgA, or IgE.[12] At 2 and 5 weeks following infection there were more germinal center B cells and more isotype switched germinal center B cells in healing co-infected C3H mice compared to B6 mice (Figure 2, A and B). We also observed increased germinal centers within the draining lymph nodes of co-infected C3H mice compared to co-infected B6 mice at 2 weeks postinfection (Figure 2, A and B). B cell memory is also formed in germinal centers,[18] and it was not surprising that there are more memory B cells at both 2 and 5 weeks post co-infection in C3H mice compared to B6 mice (Figure 3).

Little has been documented regarding the germinal center response during Leishmania infection. Histological studies in mice have reported enlarged or hyperplastic germinal centers with apoptotic cells up to 40 days postinfection with *L. amazonensis*.[19] More recently, using *L. major* infection of BALB/c mice, it was determined that Tfh cells in germinal centers produce cytokines that influence the affinity and isotype of the antibody response.[20] Our data indicate that there are significantly more antigen-specific IgG2a-producing cells in the draining lymph node of co-infected C3H mice as compared to antigen-specific IgG2c-producing cells from co-infected B6 mice at 2 weeks (Figure 5). These antibody isotypes are important, as they are the predominant antibodies produced during a polarized Th1 immune response.[21] The presence of robust germinal center formation in C3H mice versus B6 mice after co-infection and the downstream effector function of B cells, as measured by isotype switching, memory cell formation, and production of antigen-specific antibodies all suggest a robust B cell response is part of productive immunity and healing of co-infection with *L. major* and *L. amazonensis*.

The role of B cells during infection with Leishmania is controversial. Some reports describe a negative effect of antibody production during Leishmania infection. When

IgG-negative BALB/c mice were infected with *L. major*, there were smaller lesions with lower parasite loads compared to infected mice with IgG.[21] A more recent study showed *L. amazonensis*-infected mice, which lacked functional B cells, and therefore antibodies had a delayed onset of disease and developed small lesions.[22] It has also been shown that there were limited infections with both *L. amazonensis* and L. pifanoi in the absence of circulating antibodies, and infection of Fc gamma receptor (Fc γR) knockout mice or Fc γRIII knockouts resulted in similarly limited lesions.[23,24] In addition, L. mexicana infection of Fc γRIII$^{-/-}$ mice produced higher levels of IFN- γ.[25] In contrast, other studies have described a protective role for both B cells and antibodies during Leishmania infection. Scott et al[26] showed that eliminating B cells in neonatal mice using an anti-IgM antibody impaired the T cell-mediated immune response following *L. major* infection. Antibody production has also been shown to be a key factor for phagocytosis of *L. major* by dendritic cells and infected, B cell-deficient mice had larger lesions, higher parasite loads, lower IFN-γ production and a decreased T cell response.[27] In our co-infection system, we have previously demonstrated an important role for B cells *in vitro*[6] and here we demonstrate that the *in vivo* B cell response is consistent with the hypothesis that these cells have an important role for cutaneous lesion resolution in C3H mice with *L. amazonensis* as part of a co-infection and that B cells from B6 mice do not play an effective role in co-infected animals.

T cells have a critical role in germinal center formation and maintenance. Tfh cells are a T helper cell subset that is specialized in regulating the effector and memory responses of B cells.[17] Within the germinal center Tfh cells produce IL-21, which has been shown to promote B cell proliferation and production of plasma cells.[14] Tfh cells not only produce IL-21, they can also produce interferon-γ, IL-4, or IL-17.[17] B cells express the IL-21 receptor and B cells deficient in this receptor have an impaired ability to undergo isotype switching and lose germinal center organization.[17] We hypothesized that differential IL-21 production could have been responsible for germinal center phenotypic differences between co-infected C3H and B6 mice. There was no difference, however, in the number of IL-21-producing cells at 2 weeks postinfection between these two mouse strains following antigenic stimulation (Figure 5A). IL-21 receptor expression was measured and again no differences in receptor expression were observed at 2 weeks postinfection in co-infected C3H versus B6 mice (Figure 5B). We also used immunohistochemistry for IL-21 and determined that IL-21 immunoreactive cells were present within the cortex, as well as within the subcapsular sinus and medullary sinuses (Figure 5C). These findings confirmed our ELISPOT data showing that there were no differences in IL-21 production in the two mouse strains. These results indicate that IL-21-producing cells may be necessary for robust germinal center formation and function, although IL-21 production per se is not sufficient to generate a germinal center response.

Here we describe for the first time a difference in the draining lymph node germinal center B cell response between co-infected C3H and B6 mice. These findings are consistent with our *in vitro* data indicating that a productive B cell response is required for healing a co-infection with *L. amazonensis* and *L. major* in the mouse. Based on this, we expect that broadly effective anti-Leishmania vaccinations or treatments require not only a productive T cell response, but also a productive B cell response.

Acknowledgments

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