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## A deficiency in the B cell response of C57BL/6 mice correlates with loss of macrophage-mediated killing of *Leishmania amazonensis*

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### Abstract

Infection of C3HeB/FeJ and C57BL/6 mice with *Leishmania major* stimulates a healing cell-mediated immune response, while *Leishmania amazonensis* infection leads to chronic disease. Here we show C3HeB/FeJ mice co-infected with both species of *Leishmania* heal, while co-infected C57BL/6 mice do not. Using an in vitro killing assay we determined B cells from infected C57BL/6 mice are ineffective in promoting parasite killing compared with B cells from infected C3HeB/FeJ mice. Furthermore, infected C57BL/6 mice produce less antigen-specific antibodies compared with infected C3HeB/FeJ mice. These findings suggest B cells play a required role in the cell-mediated immune response against *L. amazonensis*.

### Keywords

Cutaneous leishmaniasis; Murine model; Co-infection; B cells; Macrophages

Leishmaniasis is a zoonotic, vector-borne disease caused by an obligate intracellular protozoan parasite of the genus *Leishmania*. Infection of C3HeB/FeJ (C3H) mice with *Leishmania amazonensis* (*La*) leads to chronic disease with large non-resolving cutaneous lesions and high parasite loads (Jones et al., 2000). The *La*-induced immune response is neither a T helper 1 (Th1) or a T helper 2 (Th2) response, as evidenced by unpolarized CD4<sup>+</sup> T cells that fail to efficiently produce either IFN- $\gamma$  or IL-4 and by dendritic cells that produce little IL-12 (Afonso and Scott, 1993; Jones et al., 2000; Ji et al., 2002; Vanloubbeek et al., 2004; Ramer et al., 2006). Experimental evidence derived using *Leishmania major* (*Lm*) indicates that protection from these parasites requires establishment of a polarized Th1 immune response characterized by production of IL-12 and subsequent activation of IFN- $\gamma$ -producing CD4<sup>+</sup> T cells (Sacks and Noben-Trauth, 2002). More recent studies demonstrated that *La*-specific Th1 CD4<sup>+</sup> T cells were ineffective in killing *La* in vivo and did not promote lesion resolution (Qi et al., 2004; Vanloubbeek et al., 2004). However, our laboratory and others have shown that T<sub>H</sub>1 immunity associated with *Lm* infection provided significant protection against subsequent *La* infection (Veras et al., 1999; Vanloubbeek and Jones, 2004; Gonzalez-Lombana et al., 2008).

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Note: Supplementary data associated with this article

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Similar to the cross-protection observed in C3H mice, C57BL/6 (B6) mice first infected with  $1 \times 10^4$  *Lm* and subsequently challenged with *La* also heal the infection, but interestingly, these mice do not heal a simultaneous infection with both *Lm* and *La* (Gonzalez-Lombana et al., 2008). To better understand the cellular mechanism underlying the productive healing response provided during co-infection of C3H mice and not B6 mice, we performed a simultaneous co-infection with *Lm* and *La* in both the C3H and B6 mouse models. *La* (MHOM/BR/0016/LTB) and *Lm* (MHOM/IL/80/Friedlin) promastigotes were grown in complete Grace's Insect medium (Atlanta Biologicals, Lawrenceville, GA, USA) to stationary phase, harvested, washed in endotoxin-free PBS (Cellgro, Herdon, VA, USA) and diluted to a concentration of  $1 \times 10^8$  parasites per ml. Female C3HeB/FeJ mice (6-8 weeks of age) were bred in-house in a specific pathogen-free environment. The Institutional Animal Care and Use Committee at Iowa State University, USA, approved all protocols involving animals. Female C57BL/6 mice of the same age were obtained from Jackson Laboratories (Bar Harbor, Maine, USA). Mice with a single infection were inoculated with  $5 \times 10^6$  *La* or *Lm* stationary phase promastigotes, while co-infected mice were inoculated with  $2.5 \times 10^6$  stationary phase *Lm* promastigotes plus  $2.5 \times 10^6$  *La* promastigotes, totaling  $5 \times 10^6$  parasites in the left hind footpad. Mice were infected for 12 weeks with weekly monitoring of lesion size. At 12 weeks p.i. the mice were euthanized and parasite quantification in the infected footpad was performed using limiting dilution.

After co-infection with *La* and *Lm*, C3H mice had negligible footpad lesions by 12 weeks p.i., similar to infection with *Lm* alone (Fig. 1A). Parasite load from infected footpads was between  $10^2$  and  $10^3$  parasites (Fig. 1C). In contrast, co-infected B6 mice developed large footpad lesions that persisted for the 12 week period (Fig. 1B) and parasite burdens were  $10^6$  to  $10^7$  parasites per footpad, similar to infection with *La* alone (Fig. 1C). Therefore, co-infection with *Lm* leads to decreased footpad lesion size during concurrent *La* infection in C3H mice, but not in B6 mice.

To better understand the underlying cellular mechanism of the difference observed after co-infection of C3H versus B6 mice, we employed an in vitro assay developed in our laboratory (Mukbel et al., 2006) to assess which immune cell-types, derived from the draining lymph node (DLN) of *Lm*-infected C3H mice, are required to deplete *La* infection. This assay utilizes bone marrow-derived macrophages (BMDM) from C3H mice infected with *La* amastigotes for 24 h in vitro. These infected macrophages are then co-cultured with DLN cells harvested from C3H mice that had been infected with *Lm* for 4 weeks, the point at which footpad lesions are resolving. Our previous studies determined that although antigen-specific  $CD4^+$  T cells are required, they are not sufficient to promote macrophage killing of intracellular *La*. Antigen-specific  $CD19^+$  B cells and their antibodies were found to be additional necessary components for macrophage killing of intracellular *La* amastigotes in this assay (Mukbel et al., 2006).

Using transwell chambers we showed that direct contact between infected macrophages and the lymphocytes was not required for the observed parasite depletion, allowing experiments with syngeneic cells. However, although B cells could be isolated to the upper chamber,  $CD4^+$  T cell function required cell contact with either the macrophage in the lower chamber or the B cell in the upper chamber. This fact precludes the ability to test syngeneic macrophages and B cells with allogeneic  $CD4^+$  T cells without the confounding influence of a mixed lymphocyte response. Based on these findings and the fact that co-infected B6 mice do not heal (Fig. 1B), we hypothesized that  $CD4^+$  T cells and B cells from the DLN of *Lm*-infected B6 mice would not be able to induce macrophages to kill *La* in our in vitro co-culture assay. To test this, BMDM were derived from naïve C3H and B6 mice as described previously (Mukbel et al., 2006). Briefly, after 6 days in culture BMDM were harvested, counted and  $5 \times 10^5$  macrophages were added into the bottom compartment of 24 well transwell plates (Corning Costar, NY, USA), each containing a coverslip (Fisher Scientific, Hanover Park, IL, USA) in complete tissue culture media (CTCM). Macrophages were infected with *La*

amastigotes at a 3:1 parasite to macrophage ratio. Popliteal lymph nodes were harvested from C3H or B6 mice infected for 4 weeks with *Lm*. CD4<sup>+</sup> T cells (Miltenyi Biotech, Auburn, CA, USA) or CD19<sup>+</sup> B cells (MagCollect, R&D system, Minneapolis, MN, USA) were purified via depletion using an autoMACS™ separator (Miltenyi Biotech, Auburn, CA, USA). The CD4<sup>+</sup> T cells and B cells were added to the top compartment of a 0.4 μm diameter transwell together with freeze-thawed *Lm* promastigote antigen, as indicated in Fig. 2. When allogeneic B cells were assessed they were placed separately into the upper chamber and the syngeneic CD4<sup>+</sup> T cells and macrophages were placed together in the bottom chamber, necessitating the use of BMDM from both C3H (Fig. 2A) or B6 (Fig. 2B) mice. All experimental conditions were performed in duplicate. Coverslips were harvested after 5 days of culture, fixed with 100% methanol and stained with the HEMA 3 stain set (Fisher Scientific, Hanover Park, IL, USA). Data analysis was performed by counting the coverslips via light microscopy and examining three areas at 100× magnification. In each area, 100 macrophages were examined assessing the number of infected macrophages and the number of parasites per 100 infected macrophages.

Fig. 2 shows CD4<sup>+</sup> T cells and B cells isolated from *Lm*-infected B6 mice do not reduce the percentage of infected macrophages in vitro compared with the same cells isolated from *Lm*-infected C3H mice. This phenomenon is not affected by the mouse strain from which the macrophages are derived (Fig. 2A, first and second bars; Fig. 2B, first and third bars). Furthermore, B cells from C3H mice promoted killing of *La* even in combination with CD4<sup>+</sup> T cells from B6 mice (Fig. 2B, second bar). In contrast, B cells from B6 mice did not promote killing even with CD4<sup>+</sup> T cells isolated from C3H mice (Fig. 2A, third bar). These results demonstrate that the inability of B6 mice to control the co-infection tracks with an inability of B6 B cells to aid in the killing of *La* in our in vitro killing assay system. Thus B cells from *Lm*-infected B6 mice are not functionally equivalent to those from *Lm*-infected C3H mice.

Based on the conclusion that B cells from C3H and B6 mice did not demonstrate equivalent responses in vitro, we wanted to determine whether there were differences in their B cell responses in vivo. Therefore we compared parasite-specific total IgG and IgG2a or IgG2c (Martin et al., 1998) antibody production during infection of C3H or B6 mice, respectively. ELISA was performed on serum for antibody isotype titers. All samples from both C3H and B6 mice were positive for total IgG, IgG2a or IgG2c, at 1:10,000 dilutions at 5 and 12 weeks post-infection, indicating readily detectable serum antibodies. Western blot analysis was performed with serum (1:25 dilution) from mice infected for 5 or 12 weeks hybridized against freeze-thawed *La* and *Lm* stationary phase promastigote parasite antigen. C3H mice infected with *Lm* alone or co-infected produced readily demonstrable parasite-specific total IgG and the Th1-associated IgG2a isotype to both *La* and *Lm* antigens (Fig. 3) at both 5 and 12 weeks p.i. No parasite specific IgG2a was detected after *La* infection at 5 weeks with a small amount seen at 12 weeks p.i. (Fig. 3B). In contrast, B6 mice produced only small amounts of parasite-specific total IgG (Fig. 3A) and almost undetectable amounts of the Th1-associated IgG2c isotype, which was *Lm*-specific even after co-infection (Fig. 3B). These results were recapitulated using amastigote-derived antigen preparations for Western blot analyses (data not shown).

Our results support the premise that B cells play a necessary role in an effective Th1-mediated immune response towards *La* and that the inability of B6 mice to heal a co-infection correlates with a defect in the B cell response, rather than an exclusive defect in the CD4<sup>+</sup> T cell response. Although B cells and the production of protective antibodies are classically considered part of a productive Th2 immune response, several reports indicate a protective role for B cells and/or antibodies during *Leishmania* infection. Recently, B cell production of antibodies has been shown to be important for phagocytosis of *Lm* by dendritic cells. Without antibodies, *Lm*-infected mice had larger lesion sizes, higher parasite loads, low IFN-γ production and a

decreased T cell response, indicating antibodies supported resolution of the infection (Woelbing et al., 2006). In contrast, other studies have indicated the importance of B cells in enhancing immunopathology during *Leishmania* infection. Miles et al. (2005) showed that in the absence of IgG, *Lm*-infected BALB/c mice had smaller lesions with fewer parasites as compared with infected wild-type mice (Miles et al., 2005). Recently, another study using mice that lack functional B cells and antibodies showed *La*-infected mice had a delayed onset of disease and developed small lesions (Wanasen et al., 2008). Kima et al. (2000) also described limited infections with both *L. amazonensis* and *Leishmania pifanoi* in the absence of circulating antibodies, and infection of Fc gamma receptor (Fc  $\gamma$ R) knockout mice resulted in similarly limited lesions (Kima et al., 2000). In addition, work by Thomas and Buxbaum showed Fc  $\gamma$ RIII knockout mice were resistant to *Leishmania mexicana* infection. These mice healed their lesions with a high level of IFN- $\gamma$  production, indicating antibody stimulation of Fc  $\gamma$ RIII was detrimental during *L mexicana* infection (Thomas and Buxbaum, 2008).

Our results are not in disagreement with these studies. Our previous and current work suggests that any effective immune response against *La* in addition to other factors, must include a productive antigen-specific B cell response that can promote macrophage-mediated parasite killing. Following inoculation, *Leishmania* promastigotes are phagocytosed by mammalian macrophages and dendritic cells located at the site of infection. Macrophages express Fc  $\gamma$ R on their surface that bind the Fc portion of antibodies; specifically IgG antibodies. After Fc  $\gamma$ R-antibody binding, macrophages can become activated to produce effector molecules including NADPH-oxidase dependent superoxide production via immunoreceptor tyrosine-based activation motifs (ITAMs) (Hulett and Hogarth, 1994). A recent study demonstrated that superoxide generation was a required factor in killing *La* in human cases of chronic disease (Khouri et al., 2009). In the C3H mouse model, our laboratory has shown that B cells and IgG antibodies together with CD4<sup>+</sup> T cells from an established *Lm* infection are necessary for providing effective stimulation to macrophages for superoxide dependent killing of *La* (Mukbel et al., 2006). Neutralization of IgG2a in vitro negates killing of *La* within infected macrophages (Supplementary Fig. S1). However, replacement of C3H B cells with serum from *Lm*-infected C3H mice is not sufficient for killing of *La* in vitro (unpublished observations).

Our results indicate B6 mice produce detectable but low levels of *Lm* parasite-specific total IgG and IgG2c antibodies during infection, compared with total IgG and parasite-specific IgG2a produced by C3H mice. This overall low level of parasite-specific IgG2c antibody production may result in inadequate stimulation of Fc $\gamma$  receptors, limiting superoxide production. Overall, our findings indicate that antibodies are necessary, but not sufficient, for killing *La* and that antibodies are just one of several critical immune components required for killing. The known complexities of antigen-antibody and Fc $\gamma$ R-mediated immunostimulatory versus immunoregulatory mechanisms (reviewed in Hjelm et al., 2006) suggests that simple serum or B cell transfer experiments may not adequately recapitulate all of the immune components required for killing *La* either in vivo or in vitro, consistent with our own unpublished observations.

In conclusion, the inability of B6 mice to heal a co-infection of *Lm* and *La* correlates with a defect in the B cell response, rather than the CD4<sup>+</sup> T cell response, as defined by our in vitro killing assay. These results suggest that both effective CD4<sup>+</sup> T cells and B cells are required for a protective, healing, cell-mediated immune response to *La*.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

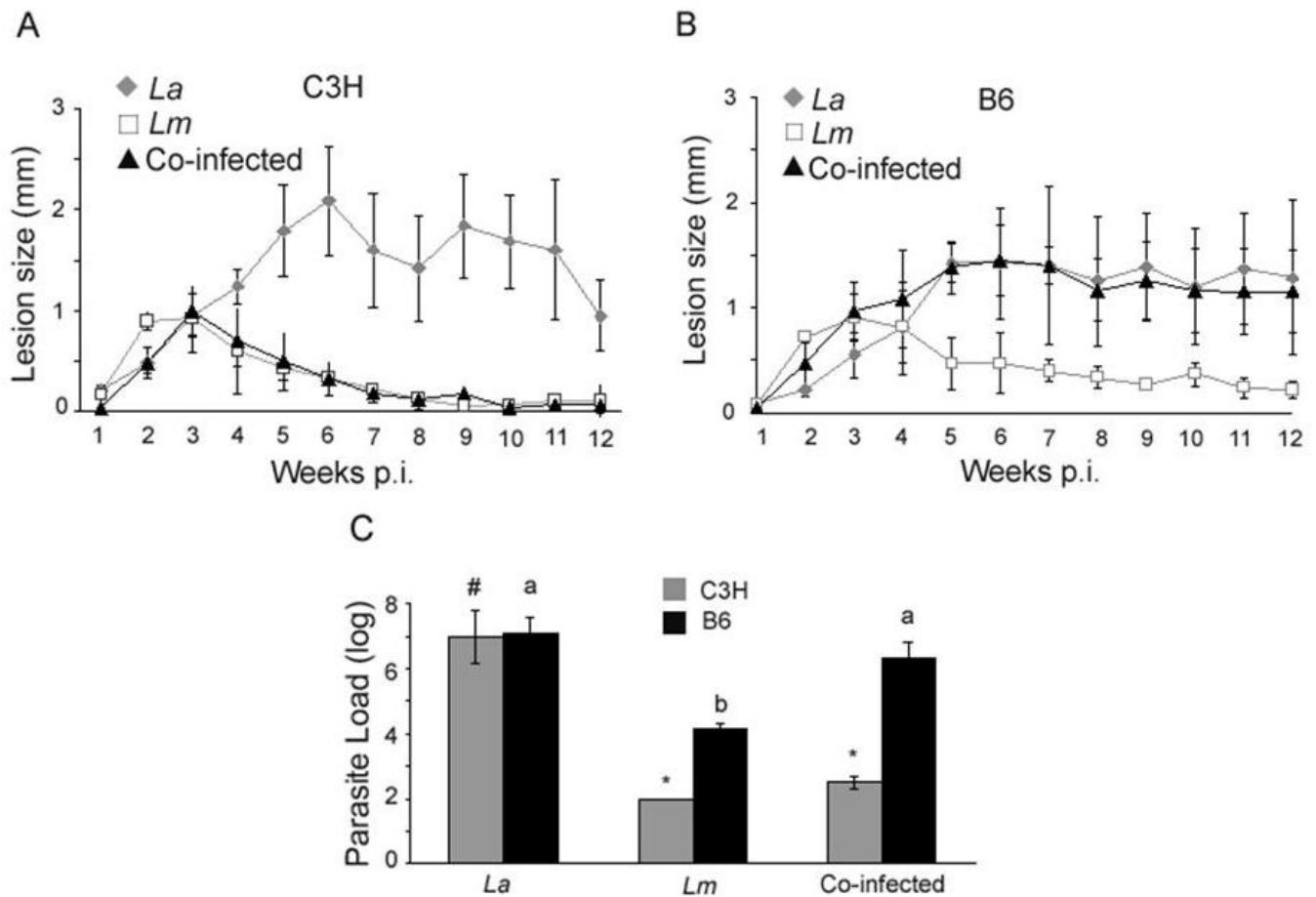
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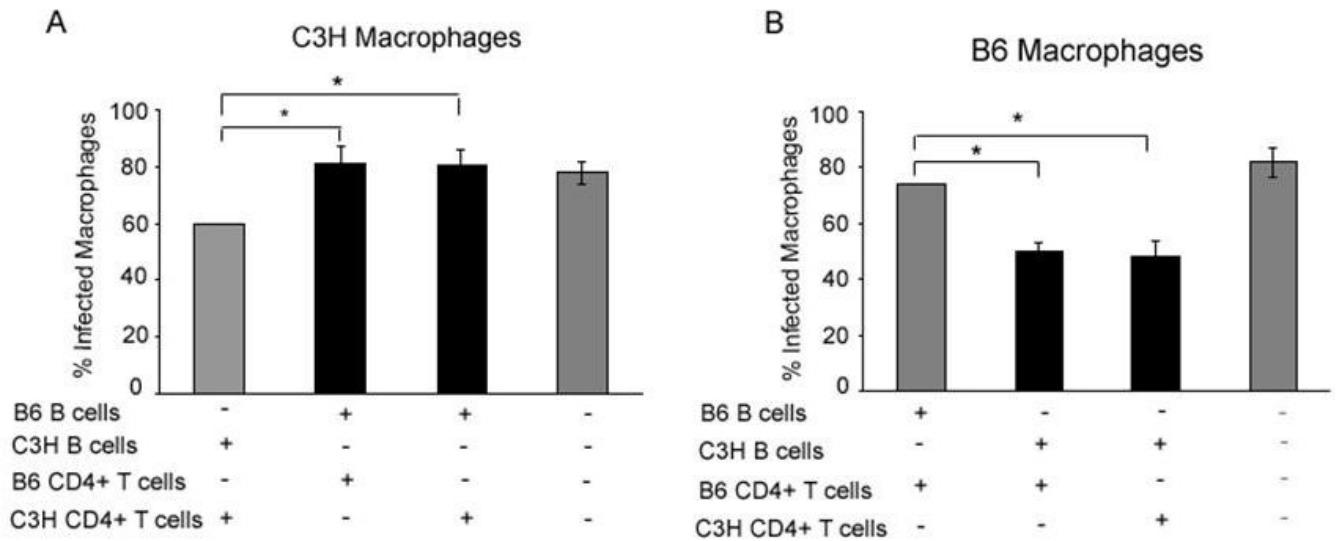
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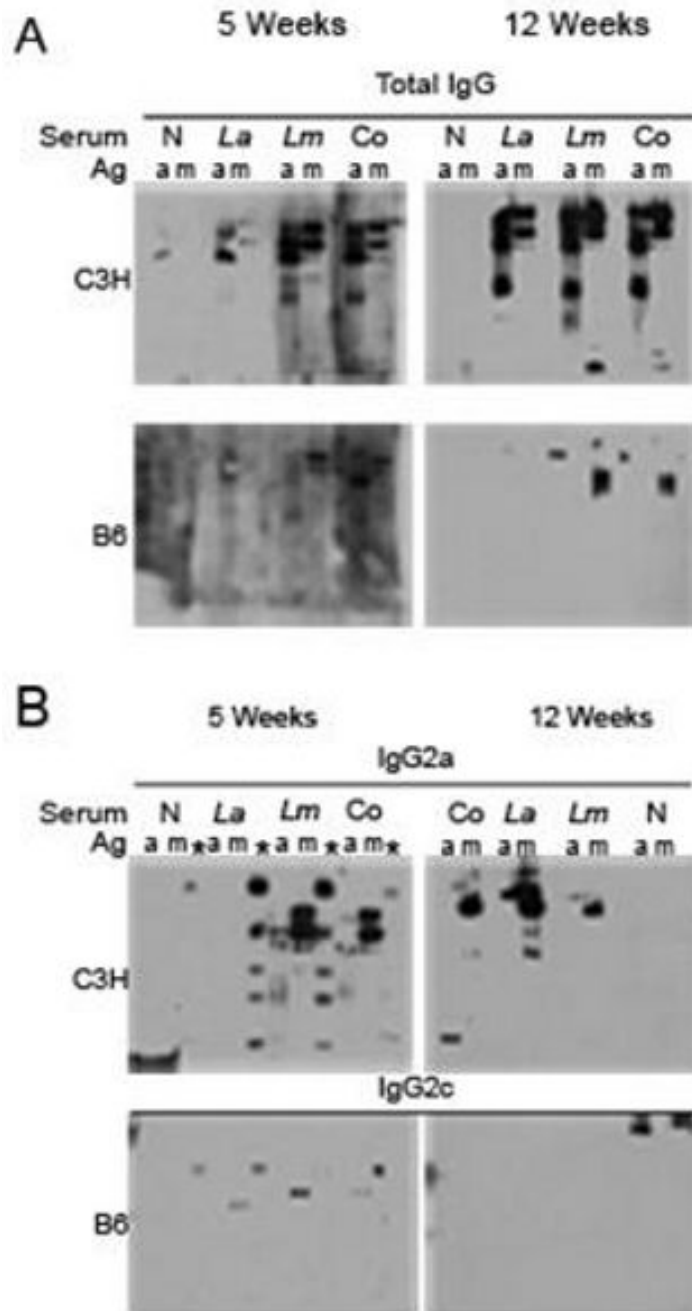


**Fig. 1.** Simultaneous co-infection with both *Leishmania major* (*Lm*) and *Leishmania amazonensis* (*La*) allows for lesion resolution in C3H but not B6 mice. A) Lesion size of co-infected C3H mice was significantly different from C3H mice infected with *La* alone (grey diamonds) ( $P < 0.001$ ), while B) co-infected B6 mice were significantly different from the B6 mice infected with *Lm* alone (open squares) ( $P < 0.001$ ). Lesion size was determined by measuring the infected footpad and comparing that with the non-infected footpad. Repeated measure ANOVA was used for statistical analysis. Results are representative of three separate experiments. C) The number of parasites in the lesions of co-infected C3H and B6 mice. Infected footpads were harvested and parasite suspensions were serially diluted in Complete Grace's medium and incubated at 27°C for 5 to 7 days. Different symbols (\*, #) represent a statistically significant difference ( $P < 0.001$ ) within the C3H infection groups and different letters (a, b) represent significant differences ( $P < 0.001$ ) within the B6 infection groups. ANOVA and Scheffe pairwise comparisons using Stat View software were used for statistical analyses. Results are the mean and standard error from three separate experiments.

**Fig. 2.**

B cells isolated from *Leishmania major* (*Lm*)-infected C57BL/6 (B6) mice do not promote killing of *Leishmania amazonensis* (*La*) in vitro. A) CD19<sup>+</sup> B cells and CD4<sup>+</sup> T cells, purified from the draining lymph node (DLN) of C3H or B6 mice infected with *Lm* for 4 weeks, were placed into the upper chamber of a transwell plate with *La*-infected C3H-bone marrow-derived macrophages (BMDM) in the lower chamber as indicated above (first and second bars, respectively). B cells from B6 mice were isolated to the upper chamber and CD4<sup>+</sup> T cells from C3H mice were placed in the bottom chamber (third bar). All wells contained *Lm* freeze-thawed antigen and were incubated for 5 days at 34°C. Black shading indicates the presence of B cells from B6 mice. B) Same as A except with BMDM derived from B6 mice and B cells from C3H mice were isolated to the upper chamber (second bar) as indicated. Black shading indicates the presence of B cells from C3H mice. \* represent statistically significant differences ( $P < 0.001$ ) as determined by ANOVA and Scheffe pair-wise comparison. Results are the mean and standard error from three separate experiments.





**Fig. 3.** Western blot analyses of parasite-specific production of total IgG and IgG isotypes IgG2a and IgG2c. Freeze-thawed *Leishmania amazonensis* (La) (a) or *Leishmania major* (Lm) (m) antigen was separated on a polyacrylamide gel and protein was transferred to a polyvinylidene fluoride (PVDF) membrane. The blots were subsequently hybridized with mouse serum (1:25 dilution) pooled from four C3H mice or B6 mice (as indicated) that were non-infected (N) or infected for 5 or 12 weeks with *Lm*, *La* or both parasites (Co) as indicated. Following serum hybridization, the membranes were probed with goat anti-mouse antibodies to (A) total IgG or with goat anti-mouse antibodies to (B) IgG2a (C3H), or IgG2c. \* identifies lanes with

commercial molecular weight markers that cross-reacted with serum antibodies. Results are from one experiment at 5 weeks p.i. and representative of two experiments at 12 weeks p.i.