Leishmania major-Specific B Cells Are Necessary for Th2 Cell Development and Susceptibility to *L. major* LV39 in BALB/c Mice[1]

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B lymphocytes are considered to play a minimal role in host defense against *Leishmania major*. In this study, the contribution of B cells to susceptibility to infection with different strains of *L. major* was investigated in BALB/c mice lacking mature B cells due to the disruption of the IgM transmembrane domain (μMT). Whereas BALB/c μMT remained susceptible to infection with *L. major* IR173 and IR75, they were partially resistant to infection with *L. major* LV39. Adoptive transfer of naive B cells into BALB/c μMT mice before infection restored susceptibility to infection with *L. major* LV39, demonstrating a role for B cells in susceptibility to infection with this parasite. In contrast, adoptive transfer of B cells that express an IgM/IgD specific for hen egg lysozyme (HEL), an irrelevant Ag, did not restore disease progression in BALB/c μMT mice infected with *L. major* LV39. This finding was likely due to the inability of HEL Tg B cells to internalize and present *Leishmania* Ags to specific T cells. Furthermore, specific Ig did not contribute to disease progression as assessed by transfer of immune serum in BALB/c μMT mice. These data suggest that direct Ag presentation by specific B cells and not Ig effector functions is involved in susceptibility of BALB/c mice to infection with *L. major* LV39. *The Journal of Immunology,* 2008, 180: 4825–4835.

The CD4$^+$ T cells recognize foreign peptides in association with MHC class II molecules at the surface of the APCs. Whereas professional APCs, i.e., macrophages, dendritic cells (DCs),[5] and B cells, are capable of sensitizing T cells, the respective role of an individual subset of APCs in a particular T cell response is yet unclear. Although DCs are clearly the APC involved in primary T cell response, there is some evidence that B cells are important for CD4$^+$ T cell responses, but their role in either initiation or maintenance of such responses is not well established.

In vitro, B cells are able to stimulate CD4$^+$ T cells (1–5); however, a differential responsiveness of CD4$^+$ Th1 and Th2 clones was demonstrated depending on the nature of the APC. Although purified B cells stimulate optimal proliferation of Th2 cells, adherent cells stimulate proliferation of Th1 cells (6). In vivo, mice rendered deficient in B cells by administration of anti-μ chain Abs do not mount a T cell proliferative response in lymph node (LN) cells (7–9), a priming defect reversed by adoptive transfer of B cells before antigenic challenge (7, 9). However, using mice genetically deficient in B cells, the role of B cells as APCs is controversial. In B cell-deficient mice generated by disruption of the IgM transmembrane domain (μMT mice), T cell proliferation and cytokine production to soluble Ags, such as keyhole limpet hemocyanin, purified protein derivative, or to deaggregated human gammaglobulins were identical with proliferation and production found in normal control mice (10, 11). In contrast, primed CD4$^+$ T cells from B cell-deficient mice generated by disruption of the J_h segment of the Ig H chain (J_HD) were unable to produce IL-4 and to provide T cell help for Ab production by B cells (12).

The murine model of infection with *L. major* lends itself for the study of immunity to intracellular pathogens. In this model system, mice from most inbred strains are resistant to infection with *Leishmania major* but mice from the BALB strains develop progressive disease. Genetically determined resistance and susceptibility to infection result from the appearance of parasite-specific CD4$^+$ Th1 or Th2 cells, respectively (13). Thus, if B cells are required for Th2 cell response in BALB/c mice, BALB/c mice deficient in B cell should develop a Th1 response and control the infection. Indeed, there is some evidence that B cells could play a role in the susceptibility to infection with *L. major*. First, anti-IgM-treated BALB/c mice control effectively their infection (14), and BALB/c Xid mice that lack the B1 B cell subset are more resistant to infection than controls (15). Furthermore, administration of IL-7, which increases the number of B cells to BALB/c Xid mice, exacerbated the disease (16). Finally, whereas reconstitution of SCID mice with T cells alone induced resistance to infection, additional transfer of B cells led to susceptibility to *L. major* (17).

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[5] Abbreviations used in this paper: DC, dendritic cell; HEL, hen egg lysozyme; Tg, transgenic; LN, lymph node; LACK, *Leishmania*-activated C kinase; MFI, mean fluorescence intensity.

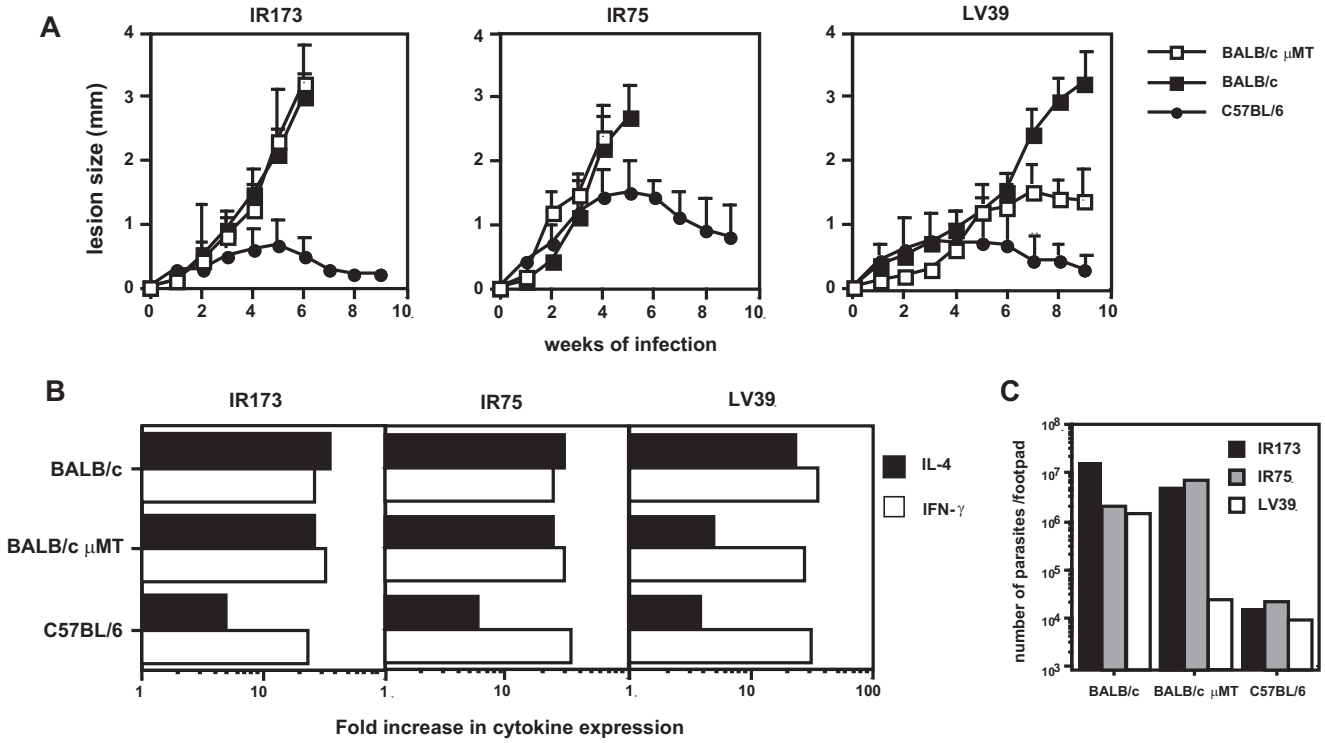

FIGURE 1. BALB/c μMT mice control infection with *L. major* LV39 but not with *L. major* IR173 and IR75. *A*, BALB/c, C57BL/6, and BALB/c μMT mice were infected with 3×10^6 *L. major* IR173, IR75, and LV39 in the footpad, and lesion development was monitored using a Vernier caliper. Mean size and SD of lesions ($n = 5$ mice per group) is shown. Similar results were obtained in two different experiments. *B*, Draining LN from infected mice were isolated from mice described in *A* at the end of infection. RNA was extracted and the levels of IL-4 and IFN-γ mRNA expression were determined by semiquantitative RT-PCR. Results are expressed as the fold increases in cytokine mRNA compared with levels in noninfected mice from the corresponding group. Results were comparable in two independent experiments. *C*, The number of parasites in lesions at the end of infection in mice designated in *A* was quantified as described in *Materials and Methods*. Similar results were obtained in two separate experiments.

Interestingly, B cells from susceptible BALB/c mice were shown to be better Th2 inducer than B cells from resistant C57BL/6 mice (18). However, in contrast with these data, infection of mice genetically deficient in B cells with *L. major* generated conflicting results. Indeed, both wild-type and BALB/c μMT mice were equally susceptible to infection with *L. major* and mounted a similar Th2 cell response (19). Furthermore, in J_HD mice on a BALB/c genetic background, there was no clear evidence for a role of B cells in the development of susceptibility to infection. Indeed, although in some reports these mice were susceptible (20), in others these mice were resistant (21). These discrepancies might be due to either the genetic background of infected mice, i.e., BALB/c μMT or J_HD mice, or to the number or strains of *L. major* used for infection, i.e., strains IR173, Friedlin, and WR309 *L. major*.

Thus, given the different patterns of diseases obtained in B cell-deficient mice infected with *L. major* and the absence of clear evidence for a role of B cells in the susceptibility of BALB/c mice to infection, we analyzed in this study the susceptibility and T helper responses in BALB/c μMT mice infected with *L. major* from different strains. In contrast to infection with *L. major* strains IR173 or IR75, B cells were necessary for susceptibility to infection with *L. major* LV39, and played a critical role as APCs to instruct the development of the Th2 response observed in susceptible BALB/c mice.

Materials and Methods

Mice

The μMT mice and hen egg lysozyme (HEL) transgenic (HEL Tg) MD4 mice on the C57BL/6 background were obtained from Kitamura et al. (22) and Goodnow and colleagues (23), respectively. These mice were backcrossed 10 times to the BALB/c background. Flow cytometry analysis was used to confirm the absence of $B220^+$ and $CD19^+$ cells in the peripheral blood of BALB/c μMT mice, and the expression of the HEL Tg (IgM^a) using biotinylated HEL followed by a streptavidin-FITC. The receptor *Leishmania*-activated C kinase (LACK)-specific (ABLE) TCR-transgenic mice that express a Vβ4-Vα8 TCR recognizing an epitope comprising the aa 156–173 from the LACK Ag in the context of MHC class II $I\text{-}A^d$ molecules were provided by Dr. R. M. Locksley (University of California, San Francisco, San Francisco, CA) (24). DO11.10 mice, which express a transgenic TCR specific for OVA, were obtained from The Jackson Laboratory. Female BALB/c and C57BL/6 were purchased from Harlan. Mice were bred and maintained in the animal facilities of the Swiss Institute for Experimental Cancer Research under pathogen-free conditions. The maintenance and care of mice complied with the guidelines of the University of Lausanne Ethic Committee for the human care of laboratory animals.

Parasites and infection

L. major LV39 (MRHO/SU/59/P), IR173 (MHOM/IR/−173), and IR75 (MRHO/IR/75/ER) were maintained in vivo and grown in vitro as previously described (25). *L. major* LV39 (MRHO/SU/59/P) has been isolated from a gerbil reservoir in southern Russia. *L. major* IR173 (MHOM/IR/−173) and *L. major* IR75 (MRHO/IR/75/ER) have been isolated from patients with localized cutaneous leishmaniasis in Iran. If course of infection (Fig. 1*A*) and parasite number (Fig. 1*C*) within the lesion are considered as marker for virulence, strains IR173 and IR73 *L. major* could be considered as more virulent than the LV39 strain. For infection, mice were injected in one hind footpad with 3×10^6 stationary phase *L. major* promastigotes in a volume of 50 μl of DMEM. Size of footpad lesions were measured with a Vernier caliper and compared with the thickness of the uninfected footpad. Footpad tissues were used to create limiting dilutions for quantification of viable parasite burdens as previously described (25).

Reagents

The *Leishmania* receptor for activated C kinase (LACK) and the recombinant LACK with the major I-A^d epitope (bp 660–713) deleted (ΔLACK) were produced in *Escherichia coli* from the expression plasmid pET3a-δ9-rLACK and purified on Ni-NTA Sepharose as previously described (26). HEL and OVA were purchased from Sigma-Aldrich. HEL, OVA, and purified recombinant LACK were biotinylated with LC-NHS-biotin-EZ linker from Pierce according to the supplier instructions. Following extensive dialysis, the biotinylated molecules were mixed in a 2:2:1 molar ratio with avidin or PE-Cy5-labeled avidin (Molecular Probes) for 60 min at room temperature to obtain HEL-LACK, OVA-LACK, and HEL-OVA complexes. The FITC-conjugated CD19 (1D3, IgG2a) and B220 (RA36B2, IgG2b) mAbs were obtained from BD Biosciences and used in FACS analysis.

MACS and adoptive transfer of B cells to BALB/c μMT mice

Naive B cells were purified from spleen cells from either naive BALB/c or HEL Tg BALB/c mice using MACS (Miltenyi Biotec). Briefly, total spleen cells were incubated with magnetic microbeads conjugated with anti-B220 (RA36B2) and B220$^+$ cells isolated after immobilization with a magnet. This resulted in a cell population consisting of 97% B220$^+$ or CD19$^+$ B cells as determined by flow cytometric analysis using FITC-conjugated anti-B220 or anti-CD19 mAbs. BALB/c μMT mice were reconstituted with 10^7 purified B cells by the i.v. route and infected with *L. major* 3 days later.

Passive serum transfer

Immune serum was collected from BALB/c mice 6 wk after infection with *L. major* LV39. Passive serum transfer was done by injecting BALB/c μMT mice with immune serum i.p. using the following four different regimens: 100 μl on days −6, −3, and +2; 200 μl on days +1, +7, and +14; 200 μl on day +21 of infection as previously described (21, 27); and 200 μl on days −2, 0, +2, +6, and +14.

Leishmania-*specific IgG serum levels*

The levels of *Leishmania*-specific IgG Abs in the sera of mice were analyzed by ELISA at different time points after infection of BALB/c μMT transferred or not with immune serum. Briefly, wells of 96-well plates (MaxiSorb; Nunc) were coated with *L. major* lysate (equivalent to 10^6 promastigotes/well) in PBS at 4°C overnight. After saturating nonspecific binding sites with PBS-10% FCS for 2 h at 37°C, the Ag-coated wells were probed 2 h at 37°C with individual serum samples at different dilutions in PBS-10% FCS. Bound *L. major*-specific IgG Abs were detected with biotinylated goat anti-mouse IgG (dilution 1/2000; Invitrogen Life Technologies) following by streptavidin-peroxidase conjugate (dilution 1/10,000; DakoCytomation). After washing, enzyme activity was detected using the tetramethylbenzidine substrate (Sigma-Aldrich). All samples were set up in duplicates and the absorbance (OD λ = 450 nm) was measured using an ELISA reader (Multiskan Ascent; Thermo LabSystems).

Lymphocyte cultures, proliferation, and detection of cytokines in supernatants

Popliteal LN cells (5×10^6) were stimulated with UV irradiated *L. major* promastigotes (1×10^6) in a final volume of 1 ml. Cells were cultured in DMEM supplemented with 5% heat-inactivated FCS, 2 mM L-glutamine, 5×10^{-5} M 2-ME and 10 mM HEPES in an atmosphere of 7% CO_2 at 37°C. Culture supernatants were collected after 72 h and stored −20°C until use. IFN-γ was measured in supernatants by ELISA as described (28). Mouse recombinant IFN-γ (supernatant of L1210 cells transfected with the murine IFN-γ), a gift of Y. Watanabe (Kyoto University, Kyoto, Japan), was used as standard. IL-10 and IL-4 were measured by ELISA using a commercial kit (BD Biosciences). The limits of detection of these assays were 10 IU/ml for IFN-γ and 20 pg/ml for IL-4 and IL-10.

In designated experiments CD4$^+$ T cells were purified (95% of purity) from spleen cells of ABLE or DO11.10 mice by magnetic cell sorter as described by the manufacturer (Miltenyi Biotec). Depending of experiments, 3×10^4 CD4$^+$ T cells were stimulated with either HEL, OVA, LACK, ΔLACK, HEL-LACK, or OVA-LACK complexes (5 μg/ml) in the presence as APC of either irradiated spleen cells (10^7) or purified B cells (10^6) obtained from either BALB/c mice or HEL Tg BALB/c mice already described. The cells were then pulsed at 48 h with 1 μCi [^{3}H]thymidine, and cell proliferation assessed 18 h later.

Receptor-mediated internalization of the LACK-HEL complex

The internalization of HEL-LACK or OVA-LACK complexes by HEL Tg B cells was analyzed by incubating total splenocytes with specific anti-Fc receptor Ab (2.4G2) for 15 min at 4°C in PBS containing 5% FCS. Following washing, the cells were incubated with Cy5-labeled HEL-LACK or Cy5-labeled OVA-LACK complex for 30 min at 4°C. Alternatively the incubation with fluorescent complexes was performed at 37°C allowing its internalization. Complexes bound to the cell surface were stripped by incubating the cells with a buffer containing 25 mM 2-ME acid (29). B cells were then specifically detected by staining with FITC-B220 Ab (RA36B2) for 30 min at 4°C. The mean fluorescence intensity (MFI) for the stripping efficiency of the complex for B220$^+$ cells was calculated as follow: (100 − (MFI of PE-Cy5 complex in B220$^+$ cells after stripping and incubation at 4°C/MFI of PE-Cy5 complex in B220$^+$ cells before stripping and incubation at 4°C)) × 100. The efficiency of internalization was determined using the following: (100 − (MFI of PE-Cy5 complex in B220$^+$ cells after stripping and incubation at 37°C/MFI of PE-Cy5 complex in B220$^+$ cells before stripping and incubation at 37°C)) × the stripping efficiency. The stripping efficiency was 88% and the internalization by HEL Tg B cells was 87% for HEL-LACK complex and <1% for OVA-LACK complex.

RNA extraction and competitive PCR

Total RNA was extracted from cells of draining LNs as described (26). First strand cDNA synthesis was performed using a first strand cDNA synthesis kit according to the manufacturer's directions (Amersham Biosciences). The polycompetitor plasmid pQRS was used to quantitate amounts of transcripts for IFN-γ, IL-4, and the constitutive expressed HPRT gene, using primers and PCR conditions as previously described (30). The first strand cDNA was used directly as a template in the presence of serial 5-fold dilution of the pQRS competitor. After separation of the PCR products by agarose gel electrophoresis, the ratio of IFN-γ or IL-4 to HPRT transcripts was calculated. The results are shown as the fold increases in cytokine mRNA in mice infected with *L. major*.

Results

B cells are required for Th2 cell maturation in BALB/c mice infected with L. major *LV39 but not with* L. major *IR173 and IR75*

To determine whether B cells are required for susceptibility to infection with *L. major*, groups of BALB/c μMT mice together with resistant C57BL/6 and susceptible BALB/c mice were infected with *L. major* from three different strains, i.e., IR173, IR75, and LV39. Although disease progression with the three different strains of *L. major* was similar in susceptible BALB/c and resistant C57BL/6 mice, different patterns of infection occurred in BALB/c μMT mice. As shown in Fig. 1*A*, BALB/c μMT mice infected with *L. major* IR173 and IR75 expressed a susceptible phenotype identical with BALB/c wild-type mice. In contrast, BALB/c μMT mice infected with LV39 contained partial lesions that remained in a plateau during the time of infection.

Levels of IL-4 and IFN-γ mRNA expression in draining LN cells were measured at the end of the infection. Infection with *L. major* IR173, IR75, and LV39 induced a Th2 response with high levels of IL-4 in BALB/c mice and a Th1 response with low levels of IL-4 in C57BL/6 mice (Fig. 1*B*). In contrast, whereas infection with *L. major* IR173 and IR75 induced a Th2 response in BALB/c μMT mice, these mice developed a Th1 response with low levels of IL-4 after infection with *L. major* LV39 (Fig. 1*B*). Interestingly, levels of IFN-γ were similar in BALB/c μMT and C57BL/6 mice infected with the three different strains. IL-4 and IFN-γ mRNA expression was confirmed by real-time PCR (data not shown).

The number of parasites in the lesions of BALB/c μMT mice was compared with the number in control susceptible BALB/c mice following infection with parasites from the three different strains. After infection with *L. major* IR75 and IR173, parasite numbers were similar in BALB/c μMT and BALB/c mice (Fig. 1*C*). In contrast, lesions from BALB/c μMT mice infected with *L. major* LV39 contained an average of 1000-fold less parasites than

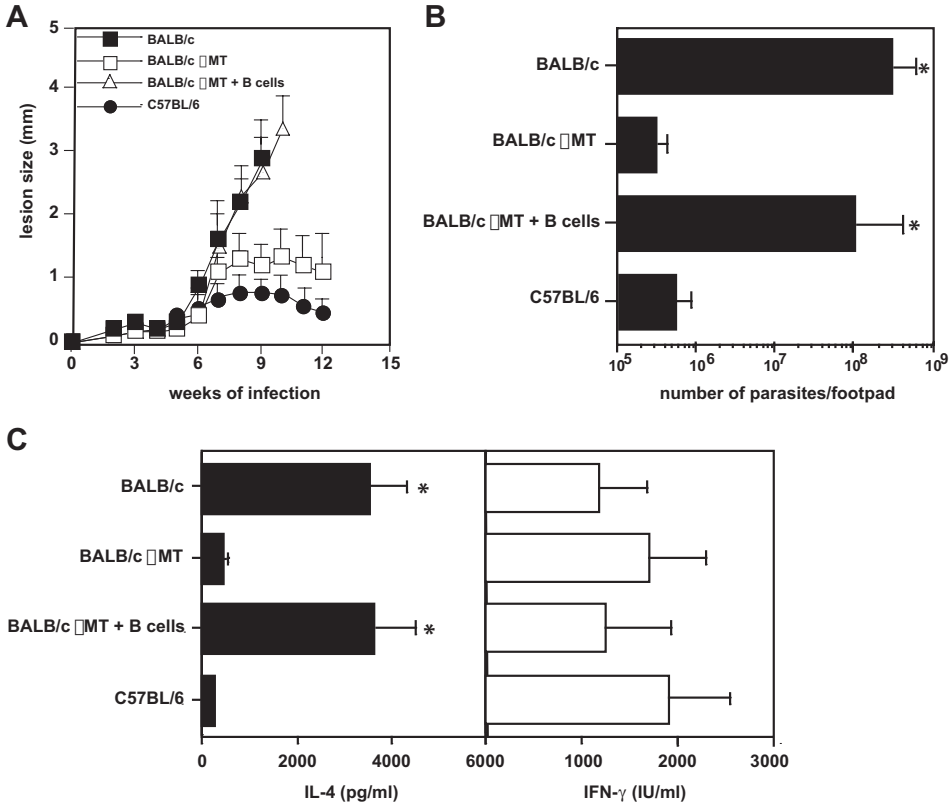

FIGURE 2. B cells are necessary for susceptibility of BALB/c mice to infection with *L. major* LV39. BALB/c μMT mice were reconstituted i.v. with 10^7 B cells from naive BALB/c mice. Three days after the cell transfer, mice were inoculated with 3×10^6 *L. major* LV39. Similarly infected but not reconstituted BALB/c μMT mice, BALB/c, and C57BL/6 mice were used as controls. Results were comparable in five independent infections. *A*, The size of the footpad lesion from designated mice infected with *L. major* LV39 was monitored using a Vernier caliper as in Fig. 1. Mean size and SD of lesions is shown. *B*, The number of parasites in the lesions at the end of infection in designated mice was quantified as described in *Materials and Methods*. *, $p < 0.05$, compared with BALB/c μMT mice. *C*, Draining LN cells obtained at the end of infection in designated mice were stimulated with UV irradiated parasites, and after 72 h of culture, IL-4 and IFN-γ production in supernatants was measured as described in *Materials and Methods*. For each determination, background levels of cytokines in supernatants of cultures without *L. major* were subtracted. *, $p < 0.05$, compared with BALB/c μMT mice.

BALB/c mice, indicating that parasite growth was controlled in BALB/c μMT mice infected with *L. major* LV39 (Fig. 1*C*).

Together the results show that B cells are required for Th2 cell response and consequently susceptibility in BALB/c mice to infection with *L. major* LV39 but not to infection with *L. major* IR75 and IR173.

Adoptive transfer of naive B cells allows the expression of a susceptible phenotype in otherwise resistant BALB/c μMT mice infected with L. major *LV39*

Because BALB/c μMT mice express a partially resistant phenotype after infection with *L. major* LV39, we assessed whether the adoptive transfer of naive B cells into BALB/c μMT before infection could redirect Th2 cell development and susceptibility to infection with *L. major*. Thus 10^7 B cells purified from the spleen of naive BALB/c mice were injected i.v. into BALB/c μMT mice. Three days later, B cell-reconstituted mice and control mice were infected with *L. major* LV39, and the course of infection was monitored. At the time of infection, LN from reconstituted mice contained a mean percentage of B cells of 8.43 ± 4.3% compared with 15.4 ± 3.8% in control mice.

As shown in Fig. 2*A*, BALB/c μMT mice contained the infection, whereas BALB/c μMT reconstituted with naive B cells developed progressive lesions similar to those observed in BALB/c mice. Estimation of the number of parasites in lesions clearly showed that parasites were controlled in BALB/c μMT mice but not in BALB/c μMT mice reconstituted with naive B cells (Fig. 2*B*).

Analysis of the cytokine production by LN cells at the end of infection showed that whereas BALB/c μMT mice reconstituted with B cells developed a strong Th2 response with high levels of IL-4 similar to the response observed in infected BALB/c mice, BALB/c μMT mice not reconstituted with B cells developed a Th1 response with high levels of IFN-γ and very low levels of IL-4 (Fig. 2*C*). Similar results were obtained with B cells purified on the basis of B220 or CD19 marker expression.

These results demonstrate that adoptive transfer of naive B cells into BALB/c mice deficient for B cells restore their capacity to mount a Th2 cell response and render them susceptible to infection with *L. major* LV39.

Specific Abs do not promote the expression of a susceptible phenotype in resistant BALB/c μMT mice

To determine whether the production of Abs could account for the ability of B cells to restore susceptibility to infection with *L. major* in BALB/c μMT mice, we studied the effect of injection of immune serum from infected BALB/c mice on the course of disease in BALB/c μMT mice. Immune serum was administrated to BALB/c μMT mice either on days −6, −3, and +2 or on days + 1, +7, +14 or with *L. major* LV39 infection at day +21. Whatever time of immune serum transfer before and at the first day of the infection in μMT mice, i.e., on days −6 and −3 and on day +1,

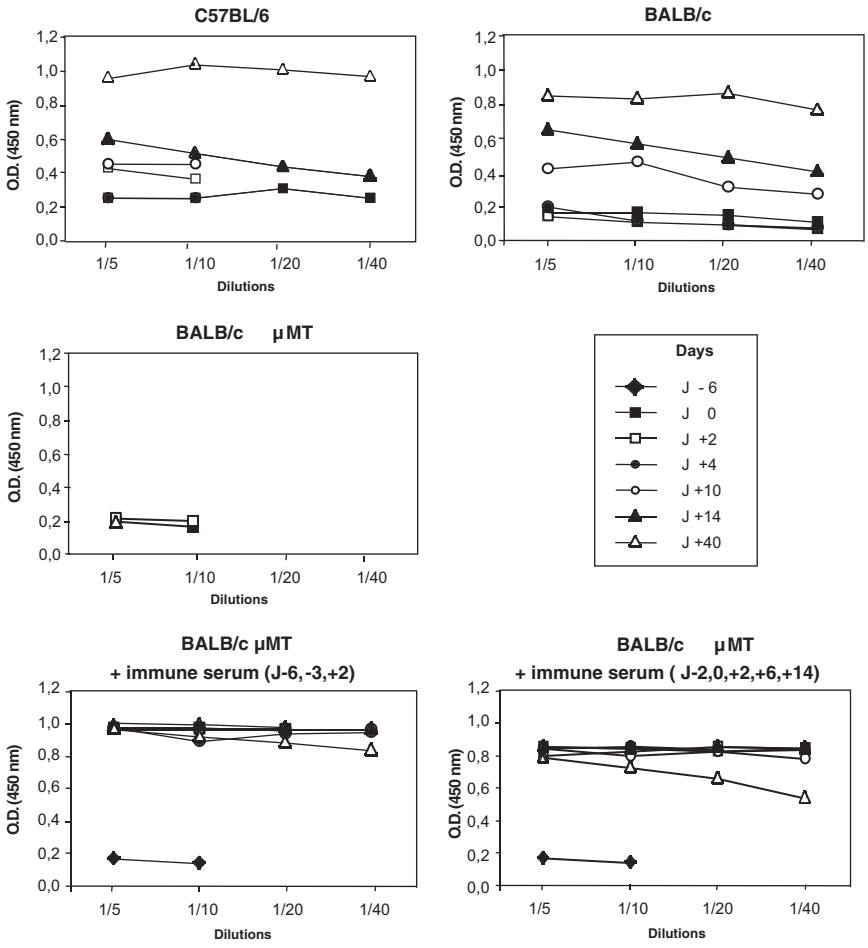

FIGURE 3. Levels of total *L. major*-specific IgG in BALB/c μMT mice after immune serum transfer. *L. major*-specific IgG production in sera of *L. major*-infected BALB/c μMT mice, injected or not with immune serum, and control groups over infection was measured. Results represent OD values (450 nm) of serum dilutions for one representative mouse per group.

only after one injection of immune serum, levels of *L. major*-specific Ab in these mice were similar to levels observed in BALB/c mice at day 40 postinfection and remained stable within time of infection (Fig. 3). Regardless of the protocol used, the administration of serum from infected BALB/c mice to BALB/c μMT mice had no effect on the disease progression. Treated mice developed lesions with the same size and kinetics than nontreated mice (Fig. 4, *A* and *B*) and mounted a typical Th1 response with high levels of IFN-γ and low levels of IL-4 (Fig. 4*C*). Similar results were obtained in mice transferred with immune serum on days -2, 0, $+2$, $+6$, and $+14$ in BALB/c μMT mice (Figs. 3 and 4*D*). Because a role for IL-10 in the progression of lesions after administration of specific immune serum has been described (21), we analyzed the IL-10 production in *L. major*-stimulated LN cells. Although cells from BALB/c μMT mice produced significantly lower levels of IL-10 than BALB/c mice, administration of immune serum did not result in increased IL-10 production (Fig. 4, *C* and *D*).

Adoptive transfer of HEL Tg B cells did not modify the resistance of BALB/c μMT mice infected with L. major

In an attempt to evaluate in vivo the importance of *L. major*-specific B cells in redirecting Th2 cell development and susceptibility to *L. major* of BALB/c μMT mice, B cells from HEL Tg mice were adoptively transferred into BALB/c μMT mice before infection with *L. major* LV39 and the development of lesions monitored. Whereas BALB/c μMT mice reconstituted with wild-type B cells developed progressive disease and Th2 cell responses similar to responses observed in BALB/c mice, BALB/c μMT mice reconstituted with HEL Tg B cells developed less severe lesions and a Th1 response (Fig. 5, *A* and *C*). Estimation of the number of viable parasites in lesions clearly showed that in contrast to BALB/c μMT mice reconstituted with normal B cells, BALB/c μMT mice reconstituted with HEL Tg B cells showed control lesion development (Fig. 5*B*). At the time of parasite burden determination, the percentage of splenic B cells was similar in BALB/c μMT mice reconstituted with wild-type or HEL Tg B cells: $35.1 \pm 4.1\%$ and $42.5 \pm 5.2\%$ of lymphocytes in BALB/c μMT reconstituted with wild-type B cells or HEL Tg B cells, respectively.

These data suggest that *L. major*-specific B cells are required in the development of the Th2 response observed in BALB/c mice infected with *L. major* LV39.

The expression of a susceptible phenotype in resistant BALB/c μMT mice following adoptive transfer of B cells pertains to the APC capacity of the transferred B cells

It is known that the Th2 response developing in BALB/c mice following infection with *L. major* is directed, at least initially, against an immunodominant epitope of the LACK protein (*Leishmania* homolog of mammalian RACK1) (26). Thus, the necessity of parasite-specific B cells for the induction of a Th2 response to *L. major* LV39 could proceed from an advantage of B cells with specific Ig receptors for *L. major* on their surface for presenting LACK epitope to specific cells. Therefore, we compared B cells from wild-type BALB/c mice with B cells from HEL Tg mice for their ability to present LACK to specific T cells.

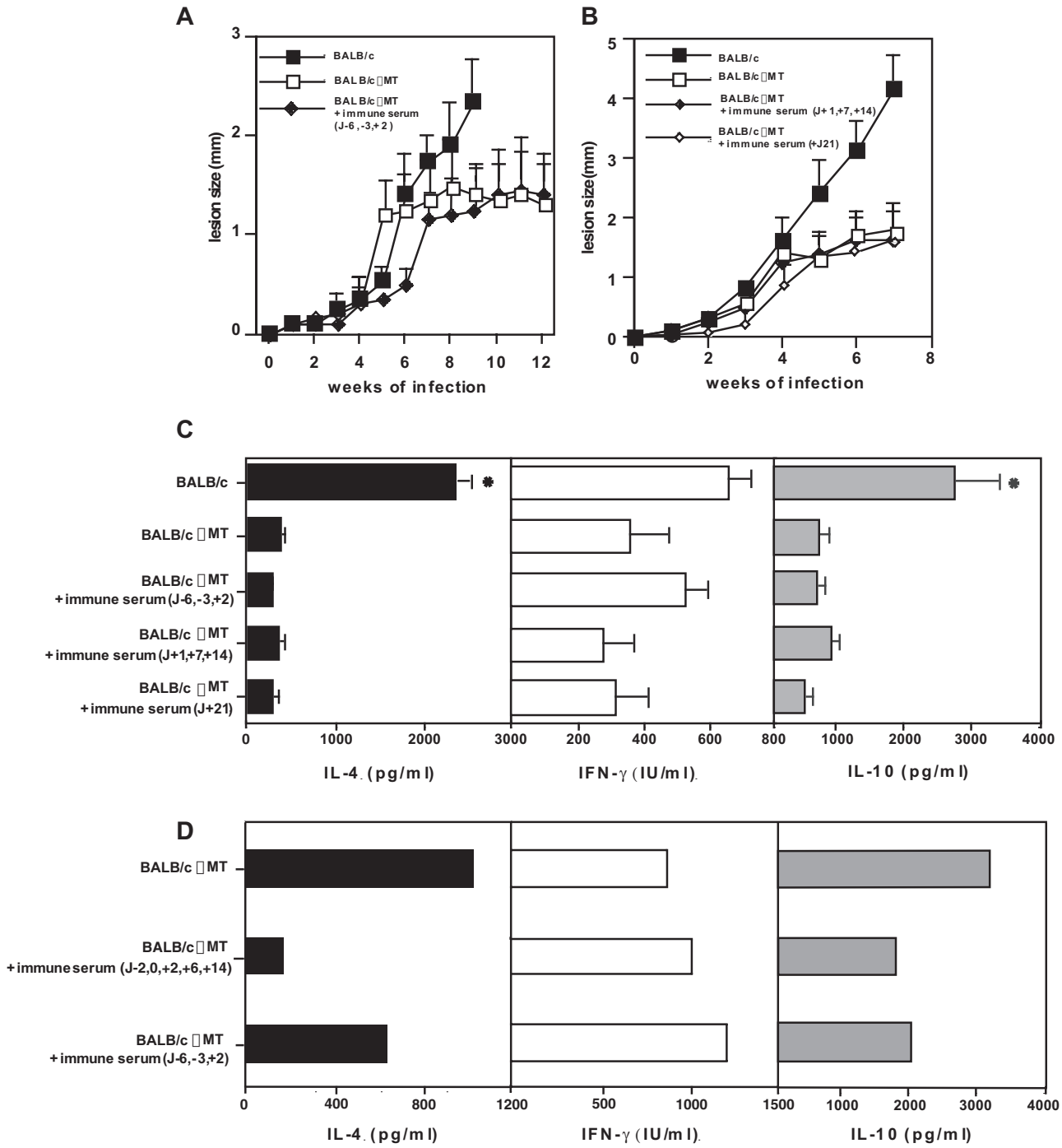

FIGURE 4. Administration of immune serum to BALB/c μMT mice does not promote susceptibility to *L. major* LV39. BALB/c μMT mice were injected i.p. with immune serum obtained from 6-wk-infected BALB/c mice at day −6, −3, and +2 (*A*) or days +1, +7, and +14 or at day + 21 (*B*) of infection with *L. major* LV39. Similarly not treated BALB/c μMT mice and BALB/c mice were used as controls. Results were comparable in three independent experiments. *A* and *B*, The size of the footpad lesion from designated mice infected with *L. major* LV39 was monitored using a Vernier caliper as in Fig. 1. Mean size and SD of lesions is shown. *C*, Draining LN cells obtained at the end of infection in designated mice were stimulated with UV irradiated parasites and after 72 h of culture, IL-4, IL-10, and IFN-γ production were measured in supernatants as described in *Materials and Methods*. For each determination, background levels of cytokines in supernatants of cultures without *L. major* were subtracted. ∗, $p < 0.05$, between with BALB/c and BALB/c μMT mice. *D*, BALB/c μMT mice were injected i.p. with immune serum obtained from 6 wk infected BALB/c mice at day −2, 0, +2, +6, and +14 of infection with *L. major* LV39. Draining LN cells obtained at the end of infection were stimulated with UV irradiated parasites and after 72 h of culture, IL-4, IL-10, and IFN-γ production were measured in supernatants.

The ability of B cells to present LACK was analyzed in vitro in a proliferation assay using $CD4^+$ T cells from mice transgenic for a TCR specific for the I-A^d-dominant epitope of LACK and expressing the Vβ4-Vα8 TCR chains (ABLE mice). $CD4^+$ T cells specific for OVA (DO11.10 mice), an Ag unrelated to *L. major*, were used as controls.

First, we tested the specificity of the proliferation of LACK and OVA specific $CD4^+$ T cells using irradiated BALB/c spleen

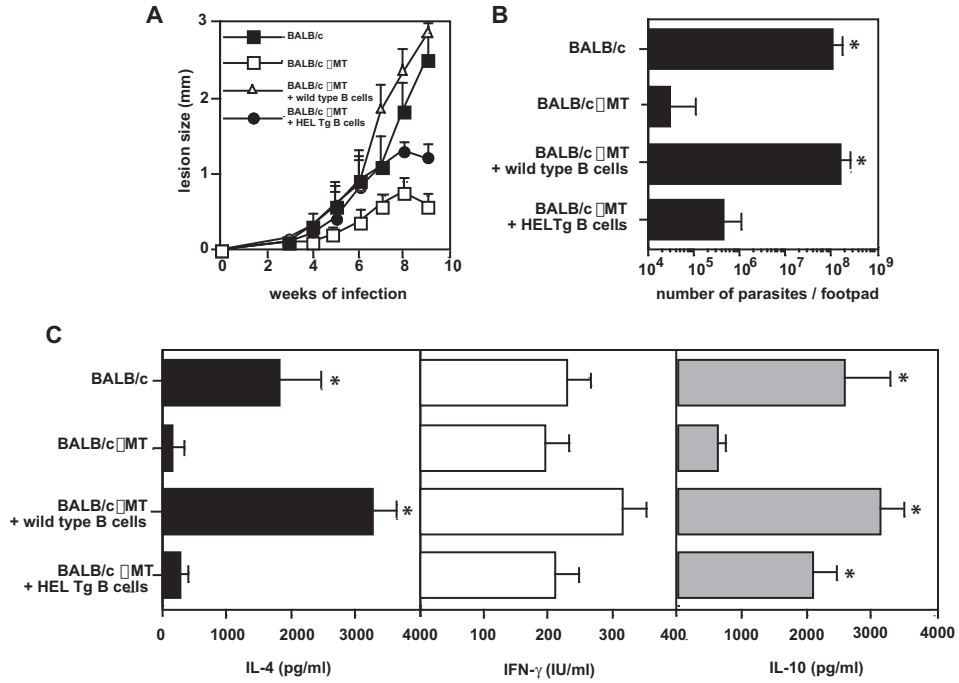

FIGURE 5. HEL Tg B cells are unable to restore susceptibility to *L. major* LV39 in BALB/c μMT mice. BALB/c μMT mice were reconstituted i.v. with 10^7 B cells from either normal or HEL Tg BALB/c mice. Three days after cell transfer, mice were inoculated with 3×10^6 *L. major* LV39. Similarly infected but not reconstituted BALB/c μMT and BALB/c mice were used as controls. Similar results were obtained in three different experiments. *A*, The size of the footpad lesion from designated mice infected with *L. major* LV39 was monitored using a Vernier caliper as in Fig. 1. Mean size and SD of lesions is shown. The results are from one of three experiments giving comparable results. *B*, The number of parasites in the lesions of designated mice at the end of infection was estimated as described in *Materials and Methods*. *, $p < 0.05$, compared with BALB/c μMT mice. *C*, Draining LN cells obtained at the end of infection in designated mice were stimulated with UV irradiated parasites as described in *Materials and Methods*. After 72 h of culture, IL-4 and IFN-γ production in supernatants were measured as described in *Materials and Methods*. For each determination, background levels of cytokines in supernatants of cultures without *L. major* were subtracted. *, $p < 0.05$, compared with BALB/c μMT mice.

cells as source of APCs. As expected, *L. major* and LACK were able to induce proliferation of LACK-specific $CD4^+$ T cells (Fig. 6*A*) but not of OVA-specific $CD4^+$ T cell (Fig. 6*B*). Similarly, OVA was able to induce proliferation of OVA-specific $CD4^+$ T cells (Fig. 6*B*), but not proliferation of LACK-specific $CD4^+$ T cells (Fig. 6*A*).

The capacity of purified B cells to present Ag was similarly determined. B cells from BALB/c mice in the presence of *L. major* and LACK induced proliferation of LACK-specific $CD4^+$ T (Fig. 6*A*), but not proliferation of OVA-specific $CD4^+$ T cells (Fig. 6*B*). As expected, ΔLACK, which is lacking the $I\text{-}A^d$ immunodominant epitope recognized by the LACK-specific $V\beta4\text{-}V\alpha8$ $CD4^+$ T cells, did not induce proliferation in either LACK-specific (Fig. 6*A*) or OVA-specific (Fig. 6*B*) $CD4^+$ T cells. OVA was unable to induce proliferation of LACK-specific $CD4^+$ T cells (Fig. 6*A*), but induced a strong response of OVA-specific $CD4^+$ T cells (Fig. 6*B*). Irradiated spleen cells and B cells from BALB/c mice and T cells alone from either ABLE or DO11.10 mice were unable to proliferate in response to LACK or OVA stimulation (data not shown). Together these results demonstrated that B cells are able to present *L. major* and LACK to specific $CD4^+$ T cells.

To assess the importance of the BCR specificity for presentation of the LACK epitope, the proliferation of LACK- or OVA-specific $CD4^+$ T cells in vitro in the presence of B cells from BALB/c HEL Tg mice that express at their surface only IgM or IgD specific for HEL was analyzed. When HEL Tg B cells were used as APC and in contrast to wild-type B cells, no detectable proliferation of LACK-specific $CD4^+$ T cells in response to stimulation with *L. major* and LACK was observed (Fig. 6C). In contrast, although unable to induce proliferation of LACK-specific $CD4^+$ T cells, OVA induced a significant proliferation of OVA-specific $CD4^+$ T cells in the presence of HEL Tg B cells (Fig. 6*C*).

The inability of HEL Tg B cells to present LACK to specific T cells could result from the inability of these cells to bind LACK through their specific BCR or from another inherent defect in LACK presentation. To distinguish between these two possibilities, we constructed HEL-LACK tetrameric complexes using biotinylated HEL and LACK coupled to a fluorochrome allowing measurement of the binding and internalization of these complexes by HEL Tg B cells. Furthermore, we assessed the capacity of HEL Tg B cells to present LACK following HEL-LACK complex internalization and used OVA-LACK and HEL-OVA complexes as controls.

The specific binding of the HEL-LACK complexes to HEL Tg B cells and to B cells from wild-type BALB/c mice was investigated at 4°C. Fig. 7*A* clearly shows that most of HEL Tg B cells (77% as detected by B220 expression) were able to bind HEL-LACK complex but not to OVA-LACK complex (6% of HEL Tg B cells). In contrast, only 2–3% of B cells from BALB/c mice were able to bind to either HEL-LACK or OVA-LACK complex. As controls, 72% of HEL Tg B cells did bind to the HEL-OVA complexes and <2% of the OVA-LACK complexes (data not shown), thus strongly suggesting that HEL Tg B cells bind specifically to the HEL-LACK complex.

To test whether HEL Tg B cells internalized HEL-LACK complex, B cells were incubated at 37°C with the complexes, a

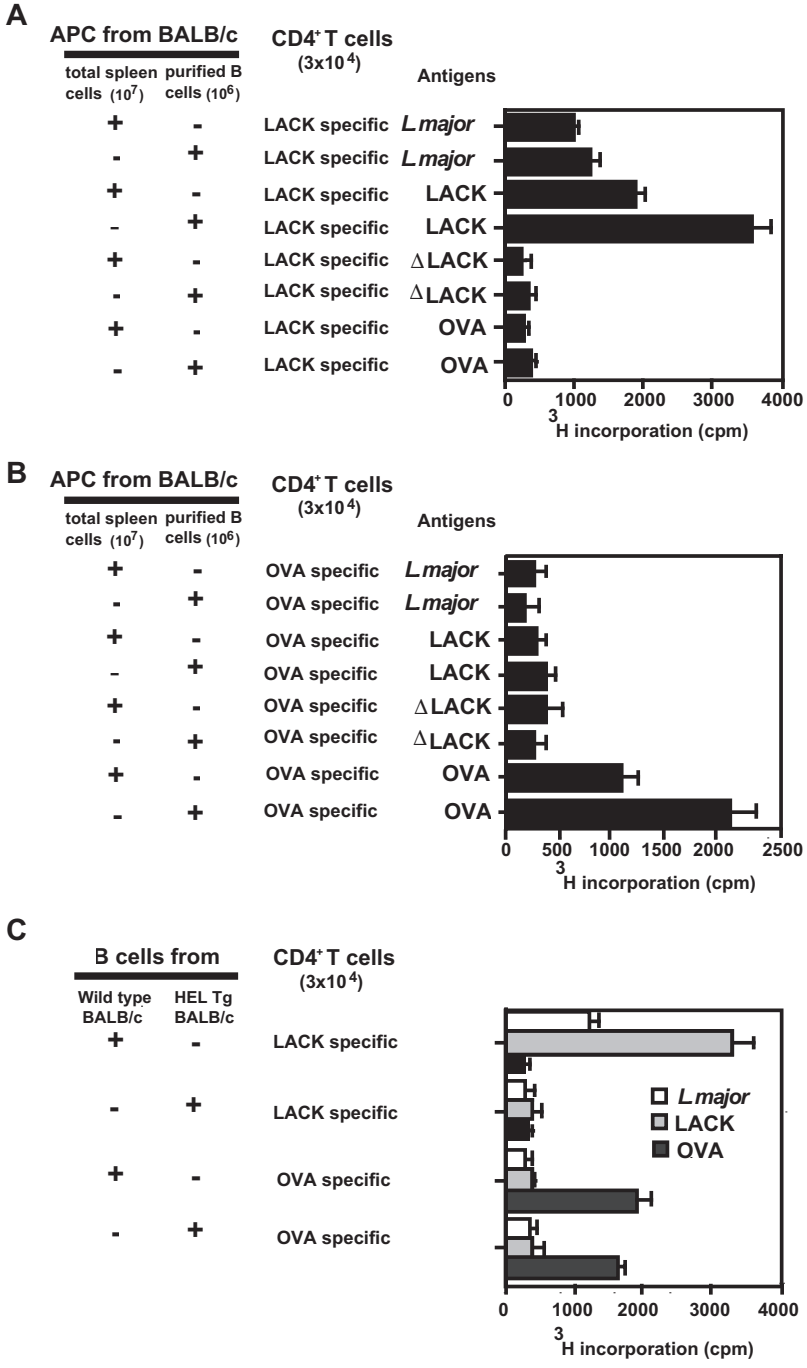

FIGURE 6. B cells from BALB/c mice are able to present LACK to specific T cells. $CD4^+$ T cells from spleen from ABLE mice (LACK-specific T cells) (*A* and *C*) and from DO11.10 mice (OVA-specific T cells) (*B* and *C*) were purified by MACS. A total of 3×10^4 purified $CD4^+$ T cells were stimulated in the presence of UV irradiated *L. major* (10^6/ml), LACK, ΔLACK, and OVA (5 μg/ml) and of either irradiated total spleen cells (10^7) or purified B cells (10^6) from either wild-type BALB/c mice or HEL Tg BALB/c mice. The cells are pulsed at 48 h with 1 μCI [^{3}H]thymidine and cell proliferation assessed 16 h later. For each determination, background levels of proliferation in supernatants of cultures without Ags were subtracted. Results are presented as mean and SD of triplicates and are from one of two experiments given the same results.

condition allowing their internalization, and thereafter treated with a stripping protocol that dissociates the complex from the cell surface. This treatment did not affect the fluorescent staining of B cells indicating that the HEL-LACK complex had been internalized. Following incubation at 4°C, a temperature that prevents internalization and limits the staining to the cell surface, fluorescent HEL-LACK complex was stripped from the surface (up to 88% of stripping efficiency). Thus, at 37°C, HEL Tg B cells efficiently internalized HEL-LACK complex (Fig. 7*B*) as well as HEL-OVA complex (data not shown). Interestingly, although some HEL Tg B cells can bind the OVA-LACK complex (Fig. 7*A*), these cells are unable to internalize it (Fig. 7*B*).

Finally, the capacity of HEL Tg B cells to present the LACK peptide following the internalization of HEL-LACK complex was assessed by measuring proliferation of LACK-specific $CD4^+$ T cells. As shown in Fig. 7*C*, LACK-specific $CD4^+$ T cells proliferated in response to HEL-LACK complex presented by either B cells from wild-type or HEL Tg mice. As controls, HEL-OVA complex did not induce detectable proliferation of LACK-specific cells but they induced, in contrast to HEL-LACK complex, strong proliferation of OVA-specific $CD4^+$ T cells (Fig. 7*C*). It appears therefore that the internalization of the LACK protein by HEL-specific B cells renders these cells able to present LACK epitope to specific T cells.

Together, these results suggest that the inability of HEL Tg B cells to induce the proliferation of LACK-specific $CD4^+$ T cells is resulting from their inability to bind and internalize LACK through their specific BCR.

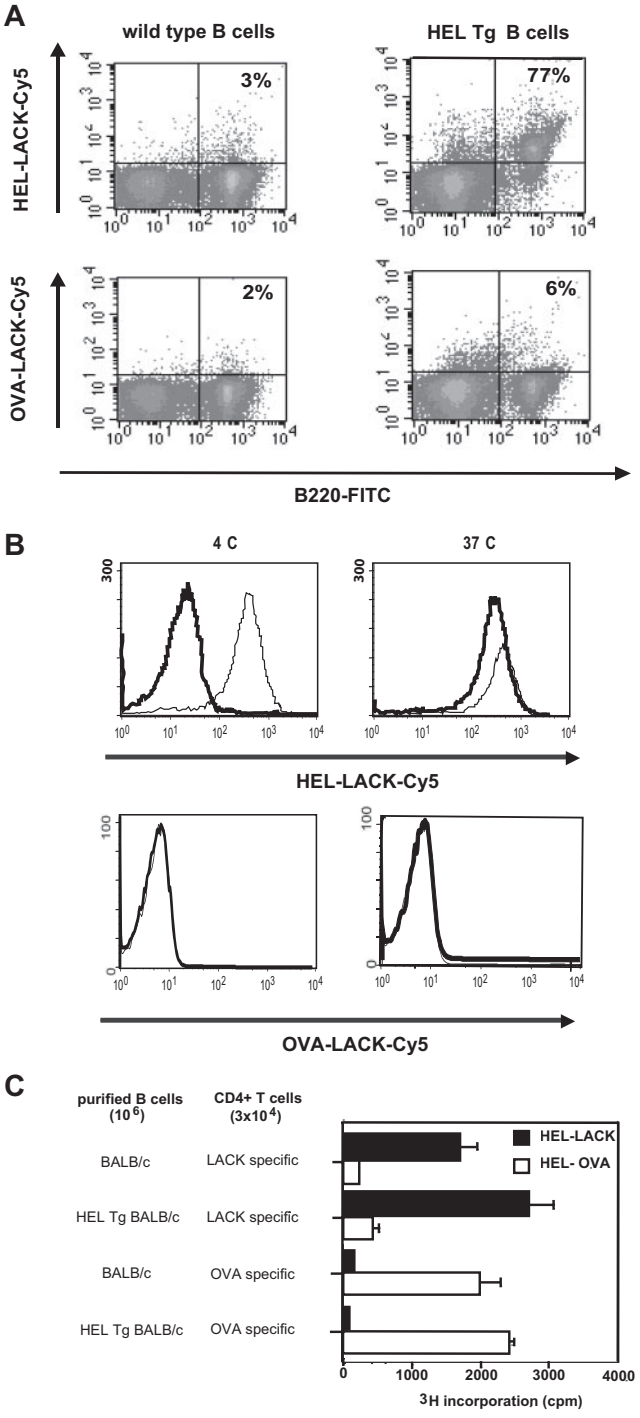

FIGURE 7. The failure of HEL Tg B cells to present LACK to specific T cells results from their inability to internalize LACK. *A*, Binding of the HEL-LACK complex to specific HEL Tg B cells was analyzed by incubating Cy5-labeled HEL-LACK complex with spleen cells from either wild type or HEL Tg BALB/c mice. Cy5-labeled OVA-LACK complex was used as controls. B cells were stained with FITC-conjugated anti-B220 Ab as described in *Materials and Methods*. Results are from one of three experiments with comparable results. *B*, After incubation of Cy5-labeled HEL-LACK complexes with total splenocytes from HEL Tg BALB/c mice at either 4 or 37°C, the complexes bound to the cell surface were stripped by incubation with 2-ME as described in *Materials and Methods*. The presence of HEL-LACK-Cy5 was analyzed on gated B cells stained with FITC-conjugated anti-B220 mAb. Gray line: florescence before stripping. Bold line: fluorescence after stripping. Results are from one of three experiments giving similar results. *C*, $CD4^+$ T cells from spleen from ABLE mice (LACK-specific T cells) and from D11.10 mice (OVA-specific T cells) were purified by MACS. A total of 3×10^4 purified $CD4^+$ T cells were stimulated in the presence of HEL-LACK or HEL-OVA complexes (5 μg/ml) and of purified B cells (10^6) from wild-type or HEL Tg BALB/c mice. The cells are pulsed at 48 h with 1 μCI [^{3}H]thymidine and cell proliferation assessed 18 h later. Results are presented as mean and SD of triplicates and are from one of three experiments with comparable results.

Discussion

The influence of B cells in Th2 cell development and susceptibility to infection with *L. major* was analyzed in BALB/c μMT mice that are deficient in B cells. Results have shown that B cells are required for susceptibility and Th2 cell development in BALB/c mice infected with *L. major* LV39. In contrast to BALB/c mice, BALB/c μMT mice infected with *L. major* LV39 restrict the development of lesions, contain parasites replication and mount a Th1 response. Adoptive transfer of B cells from BALB/c mice in B cell-deficient BALB/c μMT mice before infection redirect susceptibility to infection with *L. major* LV39 and Th2 cell development in these otherwise resistant mice, implying a role of B cells in the susceptibility to infection with this parasite. These results are in agreement with previous data showing that although adoptive transfer of T cells alone in SCID mice induced resistance to *L. major*, transfer of T and B cells induced susceptibility (17). Noteworthy BALB/c mice deficient in B cells are also resistant to other *Leishmania* species such as *L. donovani* (31) or *L. mexicana* (27).

Remarkably, BALB/c μMT mice, although resistant to *L. major* LV39, were fully susceptible to infection with *L. major* from two other strains IR75 and IR173, and developed a Th2 cell response to these parasites. It is noteworthy that contradictory data concerning the outcome of infection in B cell-deficient mice have been obtained using different strains of *L. major* (19, 20). In a similar vein, opposite outcomes of infection have been also observed in IL-4R$\alpha^{-/-}$ BALB/c mice depending upon the strains of *L. major* used for infection: *L. major* LV39 caused progressive lesions, whereas *L. major* IR173 was controlled (32, 33). Moreover, there is evidence that *L. major* from distinct strains have intrinsic differences in their susceptibility to killing by immune-activated macrophages in vitro. Indeed killing of LV39 by macrophages required 25- to 500-fold greater concentrations of IFN-γ than killing of IR173 (33).

Although numerous studies attest that susceptibility of BALB/c mice to *L. major* result from the maturation of Th2 responses regardless of the strain of *L. major* used for infection, the present study results indicate that B cells are required for Th2 cell development only after infection with *L. major* LV39.

The two main functions of B cells are Ig production or Ag presentation to T cells. Because Abs have been shown to play a critical role in the pathology associated with infection with either *L. amazonensis* (27) or *L. major* (21), we studied the possible role of immune serum in restoring susceptibility to *L. major* LV39 in BALB/c μMT mice. Using three different regimens of administration of immune serum, i.e., either around the time of infection (day -6, -2, and $+3$), during the first 2 wk of infection (day $+1$, $+7$, and $+14$), or at day 21, we were unable to alter the course of infection with *L. major* LV39 in BALB/c μMT mice and redirect Th2 cell development. Similar results were observed using a more stringent regimen of administration of immune serum, i.e., days -2, 0, $+2$, $+6$, and $+14$ (data not shown). IL-10 produced by macrophages in response to the ligation of specific Abs to FcγR (21, 34) has been recently reported to exacerbate the disease progression (21). Contrasting with these results, we were unable to detect IL-10 production by *L. major*-stimulated LN cells from BALB/c μMT mice treated with immune serum. Thus, our present

data clearly differ from previous findings showing that specific Ig contribute to susceptibility to infection with *L. major* (21) and *L. amazonensis* (27). Because in the reports showing an effect of specific Abs on the course of disease, amastigotes were used for infection, these discrepancies could be due to the use of promastigotes in our study. The internalization of promastigotes by host cells occurs mainly through the mannose fucose receptor, the fibronectin receptor, and CR1 and CR3 complement receptors (35). In contrast, Ig coated on tissue-derived amastigotes (36) may modulate the uptake of amastigotes by macrophages and induce IL-10 production (21). Nevertheless, supplying immune serum at day 21 after infection in BALB/c μMT mice, a time when the amastigote differentiation has occurred, did neither modify the progression of lesion nor redirected Th2 cell development. Thus, under the conditions used in this study, specific Igs are not responsible for the effect of adoptively transferred B cells on the course of disease in BALB/c μMT mice.

The possible role of B cells in T cell activation is controversial. Indeed, T cells proliferation from B cell-deficient mice has been shown to be either normal (10–12) or lower than in control BALB/c mice (7, 37–39). Results in this report show that, in vitro, $CD4^+$ T cells specific for LACK, an Ag from *Leishmania*, proliferate in the presence of B cells. Noteworthy, LACK-specific $CD4^+$ T cells failed to proliferate in the presence of monoclonal B cells from HEL Tg mice that express at their surface only IgD/IgM specific for HEL, which is an irrelevant Ag. These results suggest that the presence of B cells with specific Ig receptors for either LACK or *L. major* within the population of B cells used as APC was required for the activation of LACK-specific $CD4^+$ T cells. Indeed allowing LACK to bind to the receptors of HEL-specific B cells by using an engineered HEL-LACK construct, enable these cells to specifically stimulate LACK-reactive $CD4^+$ T cells. These results combined with the observations showing that reconstitution of BALB/c μMT mice with HEL Tg B cells, in contrast to wild-type B cells, neither redirect Th2 development nor susceptibility to *L. major*, indicating that promotion of Th2 cell development by B cells requires cognate interactions between these cells. In contrast, the presence of B cells with a specific receptor for OVA is not required for the activation of OVA-specific $CD4^+$ T cells. Indeed and as already described (40), OVA-specific $CD4^+$ T cells were able to proliferate in the presence of B cells from wild-type or HEL Tg mice. Unfortunately, using biotinylated LACK, we were unable to detect LACK-specific B cells in B cells from naive BALB/c mice, suggesting that the frequency of LACK-specific B cells is rather low as determined by FACS analysis (data not shown). The frequency of B cells with a given specificity has been estimated to be 1 of 4×10^4 naive B cells (41). However, only five activated B cells are required for the formation of germinal centres within 6 to 7 h after Ag administration (42), demonstrating that few B cells are sufficient to induce a response. Remarkably, it has recently been reported that Ag-specific B cells residing in the follicles acquire Ag within minutes of injection first in the region closest to the subcapsular sinus where lymph enters the LN. Subsequent T cell activation did not appear to require B cell migration to T cell area (43).

It is well established that IL-4 provides an important signal for Th2 differentiation and susceptibility to infection with *L. major* (44). Because production of IL-4 by B cells has been reported (45), some IL-4 derived from *Leishmania*-activated B cells might play a role in instructing Th2 cell differentiation. However, we were unable to detect IL-4 either in vitro in *L. major* stimulated B cells or ex vivo in purified B cells at different times after infection (data not shown), suggesting that the role of B cells in favoring the development of a Th2 cell response during infection with *L. major* is not due to the IL-4 they could produce but rather to other signals instructing T cells to produce IL-4 (6, 46, 47). In this context, it has been recently shown that during infection with *Nippostrongylus brasiliensis* that the expression of B7-1/B7-2 on B cell is involved in the development of the B cell-dependent Th2 immune response rather than B cell-derived IL-4 (48). Our present data showing that *L. major*-stimulated T cells from BALB/c μMT mice infected with *L. major* LV39 produced low levels of IL-4 and that adoptive transfer of syngeneic B cells from BALB/c mice in BALB/c μMT mice restored high levels of IL-4 strongly support a role for B cells in the T cell-derived IL-4 production detected in susceptible mice. Interestingly, such treatment had no effect on the IFN-γ production.

The molecular basis for the role of B cells in the Th2 cell development following infection with *L. major* is yet unknown. B cells could be necessary for either initiation of T cell responses (7, 9, 49) or clonal expansion of activated T cells (50, 51). In this context, it is admitted that DCs are essential for initiating a T cell response. However, B cells that are the most abundant MHC class II-positive cells within naive LNs might also play this role. It has been reported that in vivo DCs could concentrate, transport, and transfer Ags to naive B cells (52). Furthermore it has been recently shown that B cells could be activated by Ag-bearing DCs through direct membrane interaction as soon as 3 h after immunization and thus possibly influence the T cell responses (53). Visualizing B and T cells interactions in LN, it has been observed that Ag-specific B cells move to the edge of the follicles very rapidly after immunization and present Ag to specific $CD4^+$ T cells that have been activated by DCs 2 days after Ag administration (54). However, a role of B cells in the initiation of the response could not be excluded in this study because whether the $CD4^+$ T cells were primed during their encounter with DCs or with Ag-specific B cells was not addressed. In this context, in a recent study, Ag acquisition by specific B cells did not appear to require exposure to DCs (43). Thus, although we cannot completely exclude a role of B cells in the initiation of the response induced during infection with *L. major*, B cells may have mainly a role in the maintenance of the $CD4^+$ T cell response. Indeed, if the role of DCs is to initiate the immune response, the capacity of DCs to sustain CD4 responses should be limited in time because it is believed that after interaction with lymphocytes, DCs die by apoptosis (55). Furthermore, DCs exit LN 48 h after stimulation (56) leaving activated B cells in large numbers in LN as compared with DCs.

The results presented in this study clearly indicate that B cells are required for polarization of the Th2 cell response and susceptibility to infection with *L. major* LV39. Full understanding of the various parameters of this response might be important for the design of new strategies to prevent pathology during infection with *Leishmania*.

Disclosures

The authors have no financial conflict of interest.

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