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Regulatory B Cells Shape the Development of Th2 Immune Responses in BALB/c Mice Infected with *Leishmania major* Through IL-10 Production

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Recent evidence indicates that B cells are required for susceptibility to infection with *Leishmania major* in BALB/c mice. In this study, we analyzed the role of the IL-10 produced by B cells in this process. We showed that B cells purified from the spleen of BALB/c mice produced IL-10 in response to stimulation with *L. major* in vitro. In vivo, early IL-10 mRNA expression is detected after *L. major* infection in B cells from draining lymph nodes of susceptible BALB/c, but not of resistant C57BL/6 mice. Although adoptive transfer of naive wild-type B cells prior to infection in B cell-deficient BALB/c mice restored Th2 cell development and susceptibility to infection with *L. major* of these otherwise resistant mice, adoptive transfer of IL-10^{-/-} B cells mice did not. B cells stimulated by *L. major*, following in vitro or in vivo encounter, express the CD1d and CD5 molecules and the IL-10 produced by these cells downregulate IL-12 production by *L. major*-stimulated dendritic cells. These observations indicate that IL-10 secreting B cells are phenotypically and functionally regulatory B cells. Altogether these results demonstrate that the IL-10 produced by regulatory CD1d⁺ CD5⁺ B cells in response to *L. major* is critical for Th2 cell development in BALB/c mice. *The Journal of Immunology*, 2010, 184: 886–894.

In addition to their Ig secreting function, B cells have been demonstrated to play a role as APCs. Recently, using live imaging techniques, the APC function of B cells was confirmed by showing that Ag-specific B cells present in lymph nodes (LNs) acquire, process, and present soluble Ag to specific CD4⁺ T cells with a greater efficiency than dendritic cells (DCs) (1).

B cells are also capable of cytokine production. Two populations of effector B cells named Be1 and Be2 that produced distinct patterns of cytokines (i.e., IL-12/IFN- γ and IL-4, respectively) have been detected in vitro and in vivo. The cytokines produced by Be1 and Be2 have been described to modulate the differentiation of CD4⁺ T cells toward Th1 or Th2 cells, respectively (2). Indeed, IFN- γ -producing and IL-12-producing B cells have been identified in mice infected with pathogens that induce Th1 immunity (i.e., *Toxoplasma gondii*) (2, 3), whereas IL-4-producing B cells were detected in mice infected with parasites that induce Th2 immunity (i.e., *Heligmosomoides polygyrus*) (2, 4).

B cells are also able to produce regulatory cytokines such as IL-10 in vitro (5–7). The in vivo role of IL-10-producing B cells was first demonstrated in a murine model of experimental autoimmune encephalomyelitis (EAE). In this model, the exacerbated encephalomyelitis that develops after immunization with myelin oligodendrocyte glycoprotein peptide in B cell-deficient mice was demonstrated to be the consequence of a deficiency of IL-10-producing B cells (8). Indeed, in the absence of IL-10 production by B cells, the proinflammatory type 1 immune response persisted, and mice did not recover (8). The involvement of the IL-10-producing B cells named regulatory B (Breg) cells was then extended to immunopathology developing in other autoimmune diseases such as collagen induced arthritis, ulcerative colitis, and lupus (9–11).

Recently, IL-10 produced by B cells was also described during parasitic diseases. B cells from mice infected with *Schistosoma mansoni* proliferate, secrete IL-10, and promote the development of Th2 immune response (12). Purified B cells from BALB/c mice infected with *Leishmania major*, *Brugia malayi*, or *Brugia pahangi* produced IL-10 in response to restimulation with soluble *Leishmania* or microfilarial extract, respectively (5, 13). In addition, it was recently described that stimulation of naive splenic BALB/c B cells with *Leishmania infantum* trypanolysin lead to IL-10 production (14). Altogether, these observations demonstrate that B cells could produce IL-10 in response to parasite Ags. However, the exact role of the IL-10 producing B cells in vivo in parasitic disease is not yet established.

Studies in vivo, using the murine model of infection with *L. major*, have established that resistance and susceptibility to infection with this protozoan parasite are correlated with the activation of parasite-reactive CD4⁺ Th1 or Th2 cells, respectively (15). IL-4 has been shown for a long time to play a predominant role in directing the functional differentiation of *Leishmania*-reactive CD4⁺ Th2 T cells observed in susceptible BALB/c mice postinfection with *L. major* (16). However, the use of IL-4R α ^{-/-} BALB/c mice has generated controversial results. Indeed, IL-4R α ^{-/-} BALB/c mice infected with *L. major* either contained

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Abbreviations used in this paper: Breg, regulatory B; BMDC, bone marrow dendritic cell; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; LN, lymph node; SLA, soluble *Leishmania* Ag; SN, supernatant; Treg, regulatory T; WT, wild-type.

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infection (17) or still developed progressive disease (18). These observations suggest that another cytokine than IL-4 might be involved in susceptibility to infection with *L. major*. Results showing that IL-10^{-/-} and IL-4R α ^{-/-} IL-10^{-/-} BALB/c mice control parasite replication demonstrate that IL-10 signaling contribute to susceptibility to *L. major* infection (19).

We have recently demonstrated that B cells are required for susceptibility and Th2 cell development in BALB/c mice infected with *L. major* LV39 (20). We (20) and others (21) have also established that the ability of B cells to direct the immune response in BALB/c mice toward a Th2 phenotype was dependant upon their capacity to present Ag to T cells rather than upon their production of specific IgG Abs.

The aim of the current study was to analyze the role of cytokine secreted by B cells, particularly IL-10, in the polarization of the Th2 cell response and the susceptibility of BALB/c mice to infection with *L. major* LV39. We clearly demonstrated that IL-10 produced by B cell is necessary for susceptibility to infection with *L. major* LV39 in BALB/c mice and for the Th2 cell development. The effect of IL-10 producing B cells was shown, at least in vitro, to stem IL-12 production by DCs.

Materials and Methods

Mice

μ MT mice on the C57BL/6 background were obtained from Kitamura et al. (22). These mice were backcrossed 10 times to the BALB/c background. Flow cytometry analysis was used to confirm the absence of CD19⁺ cells in the peripheral blood of these mice. IL-10^{-/-} BALB/c mice were kindly provided by Dr. J. P. Farrell (University of Pennsylvania, Philadelphia, PA). Female BALB/c and C57BL/6 were purchased from Harlan (Horst, The Netherlands). Mice were bred and maintained in the animal facilities of the Swiss Institute for Experimental Cancer Research under pathogen-free conditions (Epalinges, Switzerland). The maintenance and care of mice complied with the guidelines of the University of Lausanne Ethic Committee.

Parasites and infection

L. major LV39 (MRHO/SU/59/P), isolated from a gerbil reservoir in southern Russia, were maintained in vivo and grew in vitro as previously described (23). For infection, mice were injected in hind footpads with 3 \times 10⁶ stationary phase *L. major* promastigotes in a volume of 50 μ l of DMEM. Size of footpad lesions was 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 (23).

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

B cells were purified from spleen cells from either naive BALB/c or IL-10^{-/-} BALB/c mice using Magnetic Activated Cell Sorting (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, total spleen cells were incubated with a mixture of biotin-conjugated Abs and anti-Biotin microbeads against CD43 (Ly-48), CD4 (L3T4), and Ter-119 to remove T cells, NK cells, DCs, macrophages, granulocytes, and erythroid cells. After immobilization of all these cells with a magnet, untouched CD19⁺ cells were recovered. Purity of B cells was >98% as determined by flow cytometry analysis using anti-CD19 mAbs (1D3) from eBioscience (San Diego, CA). BALB/c μ MT mice were reconstituted with 10⁷ purified B cells by the i.v. route and infected with *L. major* 3 d later.

Lymphocyte stimulation and detection of cytokines in supernatants

Total LN cells. Popliteal LN cells (5 \times 10⁶) were stimulated with UV-irradiated *L. major* promastigotes (1 \times 10⁶) in a final volume of 1 ml. Cells were cultured in DMEM supplemented with 5% heat inactivated FCS, 2 mM L-glutamine, 5 \times 10⁻⁵ M 2- β ME, and 10 mM Hepes in an atmosphere of 7% CO₂ at 37°C. All media and FCS used in this study were certified endotoxin free. Culture supernatants were collected after 72 h and stored at -20°C until use.

B cells. B cells purified by MACS (2 \times 10⁶ cells) were stimulated in vitro with complete DMEM medium alone, *L. major* live promastigotes (1–5 parasites/cell), LPS (1 μ g/ml, *Escherichia coli*, serotype 055:B5; Sigma-Aldrich, Epalinges, Switzerland), CpG (1 μ g/ml, ODN1826; InvivoGen,

Toulouse, France). Culture supernatants were collected after 24 h and stored at -20°C until use.

ELISA. IFN- γ was measured in supernatants by ELISA as described (20). 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 commercial kits (BD Biosciences, Allschwill, Switzerland). The limits of detection of these assays were 10 IU/ml for IFN- γ and 20 pg/ml for IL-4 and IL-10. An ELISA kit with a sensitivity of 15 pg/ml was used for the detection of IL-12p70 (eBioscience).

FACS analysis

Single-cell suspensions were first incubated 10 min with an anti-Fc γ R/III mAb (Fcblock, 2.4G2), followed by a 20 min exposure to cell surface staining performed with the following Abs: CD19 (1D3), CD21 (7G6), CD23 (B3B4), IgM^a (DS-1), and IgD^b (AMS9.1). mAbs were obtained from BD Biosciences, and CD5 (53-7.3) and CD1d (1B1) were provided by eBioscience. PE-cychrome5-streptavidin was purchased from BD Biosciences. Cells were analyzed in PBS containing 3% FCS using a FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (TreeStar, Ashland, OR).

Flow cytometry analysis of IL-10-producing B cells

Splenic or LN B cells were purified by MACS and were stimulated in medium alone, or with either *L. major* or with CpG, during 24 h. IL-10-producing cells were isolated using the IL-10 secretion assay in accordance to the manufacturer's instructions (Miltenyi Biotec). Briefly, a rat anti-IL-10 Ab conjugated to cell surface marker specific Ab (IL-10 catch reagent) was attached at the surface of B cells. Cells were then incubated 45 min at 37°C and IL-10 secreted bound to IL-10 catch reagent on the secreting cells. Then the cytokine is labeled with an IL-10-detecting mAb conjugated with PE. Finally, IL-10-producing cells were purified using anti-PE microbeads. Thereafter, enriched IL-10-secreting B cells were stained for specific markers (CD1d, CD5, IgM, IgD, CD21, CD23) to phenotype further IL-10⁺ B cells.

RNA extraction, cDNA preparation, and real-time RT-PCR

Total RNA was isolated from total LN cells or purified cells using RNeasy Mini Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions doing the optional DNase treatment. cDNA preparation was carried out following standard procedures using dNTPs (Roche, Basel, Switzerland) and Superscript II reverse transcriptase (Invitrogen, Basel, Switzerland) according to the manufacturer's instruction. PCR amplification was prepared using LightCycler Faststart DNA Master SYBR Green I kit (Roche Diagnostics AG, Rotkreuz, Switzerland) and quantitative PCR amplifications were performed with the LightCycler 1.5 system (Roche) according to the manufacturer's instructions. The primers for the real-time PCR were the following: HPRT Fw 5'-GTTGGATATGCCCTTGAC-3'; Rev 5'-AGGACTAGAACACCTGCT-3'; IL-10 Fw 5'-ACCTGCTCCA CTGCCTTGCT-3'; and IL-10 Rev 5'-GGTTGCCAAGCCTTATCGGA-3'. The results were analyzed using the comparative threshold cycle method ($\Delta\Delta C_T$) for relative quantification of gene expression normalized to the housekeeping gene HPRT. In some experiments, results are expressed as the fold change of the IL-10 mRNA in cells from infected mice compared with naive cells, which was defined as the reference.

Dendritic cell generation and coculture

Bone marrow cells were flushed from long bones with RPMI. Erythrocytes were lysed with RBC lysis buffer (150 mM NH₄Cl, 1.0 mM KHCO₃, and 0.1 mM Na₂EDTA). DCs were generated by plating of bone marrow progenitors (2 \times 10⁵ cells/ml) in complete RPMI medium supplemented with 30% conditioned medium from GM-CSF-transduced AO-2 cells as previously described (24). After 3 d, fresh DC medium was added. On day 6, DCs were recovered and stimulated with *L. major* promastigotes (ratio 5:1) or LPS (1 μ g/ml).

In some experiments, DCs were stimulated in a final volume of 400 μ l during 24 h with 200 μ l medium or *L. major* promastigotes in presence of 200 μ l of B cell supernatants that have been stimulated with medium alone or with *L. major*.

Results

B cells produce IL-10 after stimulation by *L. major* in vitro

Cytokine production by B cells purified from spleens of naive BALB/c mice stimulated by *L. major* promastigotes (from one to five parasites/cell) was analyzed. Although IL-10 was already produced by B cells from naive BALB/c mice in response to

stimulation with *L. major* at one parasite/cell (data not shown), optimal IL-10 production was detected with five parasites/cells (Fig. 1). In addition, soluble *Leishmania* Ags (SLA) induce similar IL-10 secretion by purified B cells than live *L. major* promastigote (Fig. 1). In contrast, whatever the number of parasites used for the stimulation, we were unable to detect IFN- γ , IL-4, and IL-12p70 secretion in the supernatant of stimulated B cells (data not shown). B cells from naive BALB/c mice produced IL-10 but no IL-4, IFN- γ , and IL-12 p70 upon stimulation with LPS or CpG (data not shown).

CD19⁺ cells from BALB/c mice produced IL-10 in vivo during the first days of infection with L. major

It has been clearly established that, in the murine model of infection with *L. major*, the cytokine environment during the early phases of infection is critical for the differentiation of CD4⁺ T cells toward the Th1 or Th2 phenotype (25, 26). Thus, to determine whether IL-10 was produced during this critical window of time, susceptible BALB/c and resistant C57BL/6 mice were infected in the footpads with *L. major* promastigotes and the kinetics of IL-10 mRNA expression in draining LN cells determined. Results in Fig. 2A show that IL-10 mRNA expression was detected in both susceptible BALB/c and resistant C57BL/6 mice during the first 3 d of infection with a peak on day 1 that rapidly returned to the baseline levels at day 3.

To determine whether B cells are responsible for the IL-10 mRNA burst detected postinfection with *L. major*, B cells were purified from draining LN cells of both BALB/c and C57BL/6 mice and analyzed for IL-10 mRNA expression. Results clearly showed an increase of IL-10 mRNA transcripts 1 d postinfection with *L. major* exclusively in CD19⁻ cells in C57BL/6 mice but in both CD19⁺ and CD19⁻ cells in BALB/c mice (Fig. 2B). These data suggest that B cells are a source of IL-10 in BALB/c mice infected with *L. major* LV39. However, it is necessary to note that only living parasites induce this phenotype, because when BALB/c mice were inoculated with SLA, a peak of IL-10 mRNA transcripts was observed in draining LN cells but mainly in CD19⁻ cells (data not shown).

Transfer of IL-10^{-/-} B cells into μ MT BALB/c mice did not modify the resistance of these mice to infection with L. major

We have previously reported that adoptive transfer of naive B cells into otherwise resistant BALB/c μ MT mice restored susceptibility to infection with *L. major* (20). In an attempt to determine whether the IL-10 produced by B cells was necessary or not to redirect susceptibility to *L. major* in BALB/c mice, we analyzed the effect of the adoptive transfer of naive B cells from IL-10^{-/-} BALB/c mice into BALB/c μ MT on the course of infection and the pathway of Th cell differentiation.

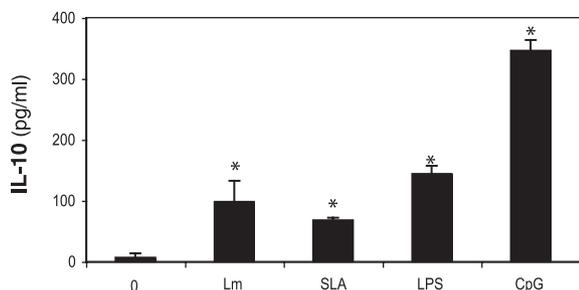


FIGURE 1. B cells from BALB/c mice secrete IL-10 after stimulation in vitro by *L. major* LV39. Purified splenic B cells from naive BALB/c mice were cultured in presence of medium alone, *L. major* LV39 (five parasites/cell), SLA, or 1 μ g/ml of LPS or CpG. After 24 h of stimulation, IL-10 production in culture supernatants was measured by ELISA. Data are the mean \pm SD of 10 experiments. * p < 0.05 compared with unstimulated B cells.

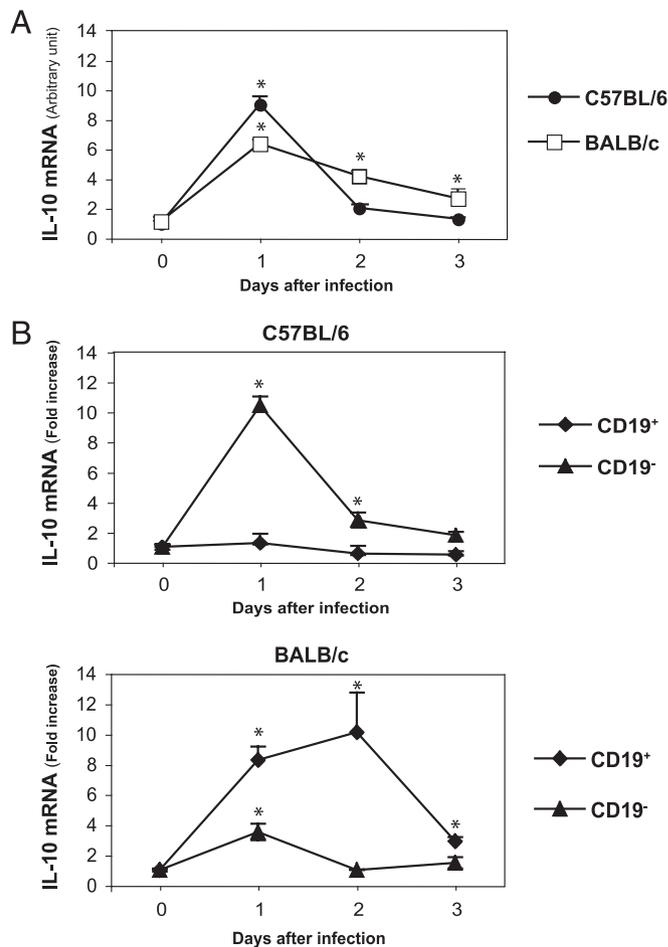


FIGURE 2. IL-10 mRNA expression increase in LN cells postinfection with *L. major*. BALB/c or C57BL/6 mice were infected with 3×10^6 *L. major* LV39 in the footpad, and draining LN cells were collected during the first 3 d of infection for IL-10 mRNA expression analysis by real-time PCR. Three mice per group were sacrificed at each time point. A, IL-10 mRNA expression in draining LNs was determined during the first 3 d of infection. mRNA levels were normalized to the level in noninfected control mice for each background. Data are the mean \pm SD of IL-10 mRNA values from one experiment representative of three. B, CD19⁺ and CD19⁻ cells were purified from draining LNs of C57BL/6 and BALB/c mice by MACS as described in *Materials and Methods*. IL-10 mRNA expression was analyzed daily during the first 3 d of infection. In each background, results are presented as fold increase of IL-10 transcripts in CD19⁺ and CD19⁻ cells postinfection as compared with CD19⁺ and CD19⁻ cells from day 0, respectively, defined as the reference (fold change 1). * p < 0.05 compared with uninfected cells (day 0).

As already demonstrated, BALB/c μ MT mice infected with *L. major* LV39 were resistant to infection. Whereas BALB/c μ MT mice reconstituted with wild-type (WT) B cells developed progressive lesions similar to those observed in BALB/c mice, BALB/c μ MT mice reconstituted with IL-10^{-/-} B cells controlled the development of lesions (Fig. 3A). At the time of parasite burden determination, the percentages of draining LN B cells were: $23.4\% \pm 3.8\%$ and $26.6\% \pm 2.3\%$ of lymphocytes in BALB/c μ MT reconstituted with WT B cells or IL-10^{-/-} B cells, respectively.

Estimation of the number of parasites in lesions clearly showed that parasites growth was controlled in BALB/c μ MT mice reconstituted with IL-10^{-/-} B cells but not in BALB/c μ MT mice reconstituted with WT B cells (Fig. 3B).

Analysis of cytokines production in supernatants of specifically stimulated draining LN cells at the end of infection (day 48) revealed that whereas BALB/c μ MT mice reconstituted with WT B cells

mounted a typical Th2 cell response with high levels of IL-4 similar to those observed in infected BALB/c mice, BALB/c μ MT mice reconstituted with IL-10^{-/-} B cells developed a Th1 response with high levels of IFN- γ and low levels of IL-4 (Fig. 3C). IFN- γ and IL-4-producing cells were analyzed by intracellular staining. The absolute number of IFN- γ ⁺-producing cells among the CD4⁺ T cells in the draining LNs was similar among all the group of mice. In contrast, five times more CD4⁺ IL-4⁺ cells were measured in BALB/c mice and μ MT mice reconstituted with WT B cells than in μ MT mice and μ MT mice reconstituted with IL-10^{-/-} B cells (data not shown).

IL-10 production was also analyzed in supernatants of draining LN stimulated with *L. major*. First and interestingly, cells from BALB/c μ MT produced lower IL-10 levels than cells from BALB/c mice. In addition, whereas draining LN cells from BALB/c μ MT mice reconstituted with WT B cells produced IL-10 in response to stimulation with *L. major* in a similar amount compared to cells from BALB/c mice, cells from BALB/c μ MT mice reconstituted with IL-10^{-/-} B cells did not (Fig. 3C).

These results indicate that the IL-10 produced by B cells is involved in the induction of a Th2 cell response and in susceptibility to *L. major* LV39 in BALB/c mice.

IL-10 produced by B cells downregulates IL-12 production by DCs in vitro after L. major stimulation

IL-10 is a potent antiinflammatory cytokine, which was identified as a Th1 inhibitor due to its ability to prevent IL-12 secretion by DCs (27). Thus, we analyzed the effect of the IL-10 produced by B cells stimulated with *L. major* stimulation on the capacity of DCs to produce IL-12 in response to this parasite.

First, we analyzed IL-12p70 production by bone marrow DCs (BMDCs) derived from WT BALB/c mice in response to *L. major* stimulation. As shown in Fig. 4A, BMDCs from BALB/c mice did not produce IL-12p70 in response to *L. major* stimulation. As a control, substantial amount of IL-12p70 (500 pg/ml) was produced by BMDCs cells in response to stimulation by LPS (Fig. 4A). Interestingly, stimulation of BMDCs from BALB/c mice with *L. major* induced some IL-10 production (Fig. 4A). As IL-10 is described as a cytokine able to prevent IL-12 production by DCs, similar DC stimulations were done with BMDCs from IL-10^{-/-} BALB/c. As shown in Fig. 4B, DCs from IL-10^{-/-} BALB/c mice produced significant amount of IL-12p70 in response to *L. major*. These results suggest that autocrine IL-10 produced by BMDCs in response to *L. major* stimulation downregulate IL-12p70 production.

As BMDCs from IL-10^{-/-} BALB/c produced significant amount of IL-12p70 after *L. major* stimulation, these cells were further used to analyze the suppressive effect of IL-10 secreted by B cells on DC activity.

First, we analyzed the effect of IL-10 produced by WT B cells in vitro in response to *L. major* on the capacity of BMDCs from IL-10^{-/-} BALB/c mice to produce IL-12p70. Thus, BMDCs from IL-10^{-/-} BALB/c mice were stimulated during 24 h with *L. major* in the presence of supernatants of either unstimulated or *L. major*-stimulated B cells. Fig. 4C clearly shows that in contrast to supernatants of unstimulated B cells, supernatants of BALB/c B cells stimulated with *L. major* inhibited IL-12p70 production of IL-10^{-/-} BMDCs stimulated with *L. major*. The same results were obtained with supernatants of LPS-stimulated B cells (data not shown).

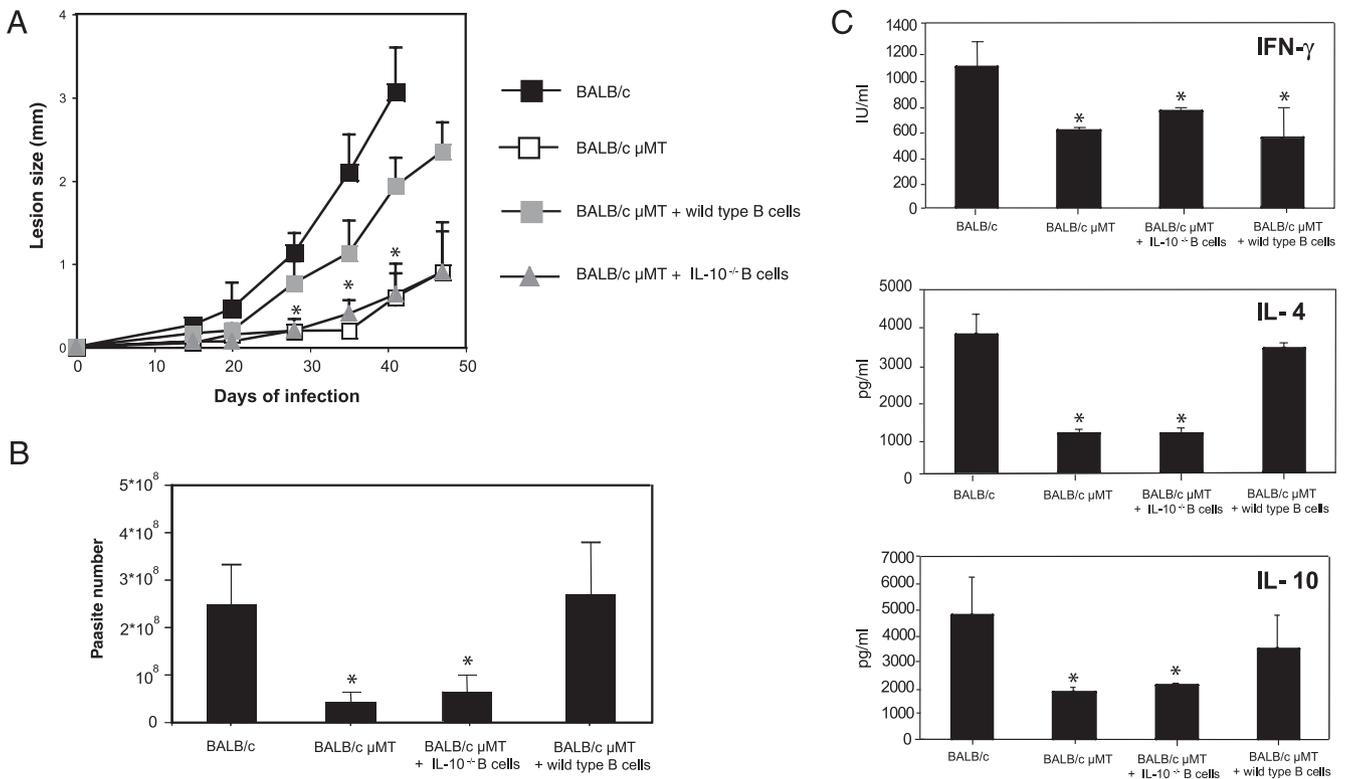


FIGURE 3. IL-10-producing B cells are necessary for susceptibility of BALB/c mice to infection with *L. major* LV39. BALB/c μ MT mice were reconstituted i.v. with 10⁷ B cells from either WT or IL-10^{-/-} BALB/c mice. Three days after the cell transfer, mice were inoculated with 3 × 10⁶ *L. major* LV39 in the footpad. Similarly infected but not reconstituted BALB/c μ MT mice, BALB/c, and C57BL/6 mice were used as controls. Results were comparable in three independent infections. Results represent the mean ± SD of five individual mice per group. **A**, Size of the footpad lesion from the designated mice infected with *L. major* LV39, monitored using a Vernier caliper. **B**, Numbers of parasites in lesions at the end of infection in the designated mice were quantified as described in *Materials and Methods*. **C**, Draining LN cells obtained at the end of infection in the designated mice were stimulated with UV-irradiated *L. major*. After 72 h of culture, IFN- γ , IL-4, and IL-10 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 compared with BALB/c mice.

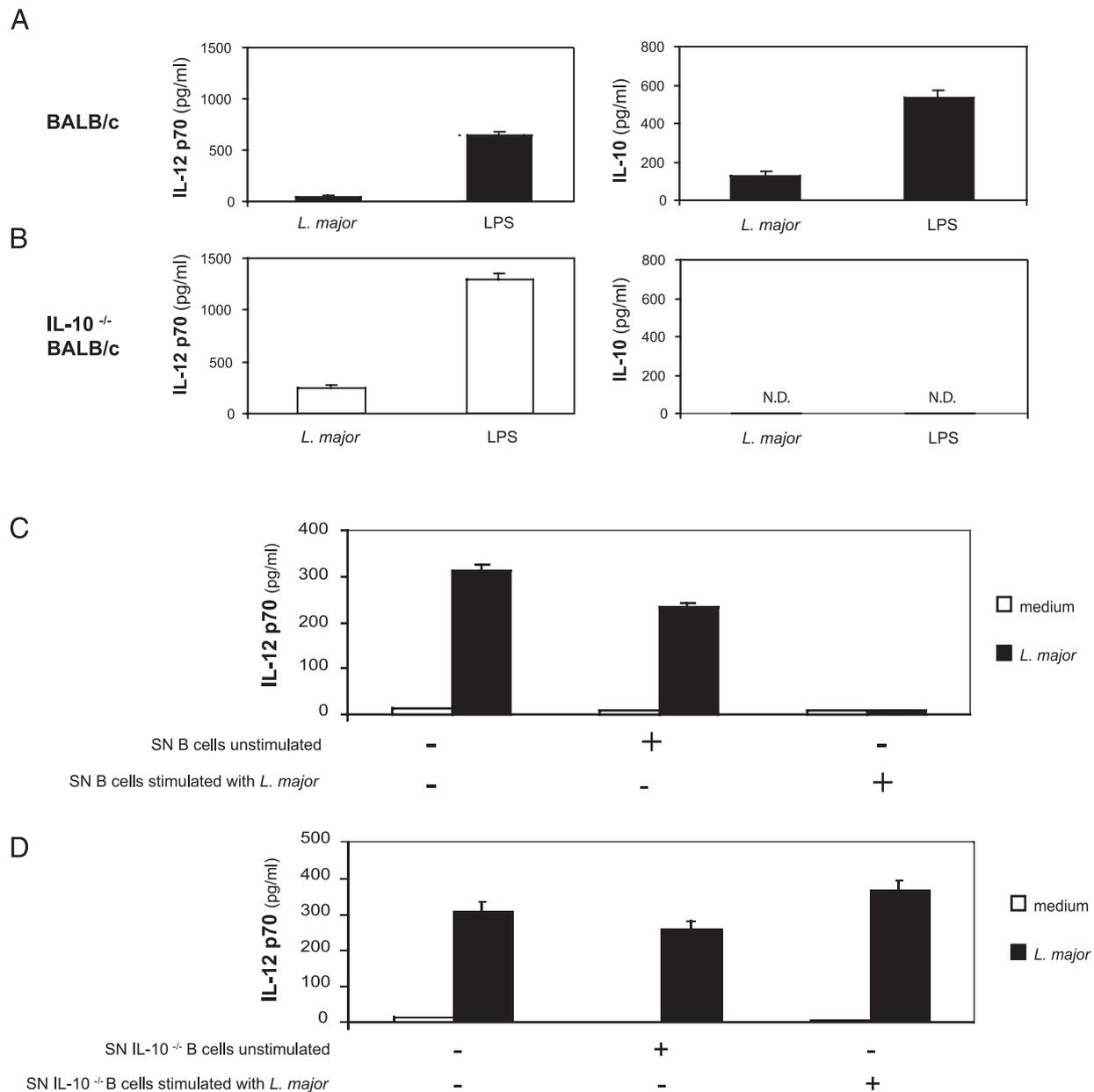


FIGURE 4. IL-10-producing B cells prevent IL-12 secretion by DCs stimulated with *L. major*. *A* and *B*, BMDCs were generated from WT (*A*) or IL-10^{-/-} (*B*) BALB/c mice and stimulated in vitro either with *L. major* or LPS (1 μ g/ml). After 24 h of stimulation, levels of IL-12p70 and IL-10 produced in the supernatants of cultures were determined by ELISA. For each determination, background levels of cytokines detected in supernatants of unstimulated cultures were subtracted. Bars represent the mean \pm SD of triplicate determinations. Similar results were obtained in three individual experiments. *C* and *D*, BMDCs were generated from IL-10^{-/-} BALB/c mice and stimulated during 24 h in vitro with *L. major* in presence of supernatant of either unstimulated or *L. major*-stimulated WT B cells (*C*) or IL-10^{-/-} B cells (*D*). Secretion of IL-12p70 by B cells was determined by ELISA. Bars represent the mean \pm SD of triplicate determinations. Results presented are from one of three experiments with comparable results. SN, supernatants.

To confirm that IL-10 present in supernatants of B cells stimulated by *L. major* was indeed responsible for IL-12p70 downregulation observed above in IL-10^{-/-} BMDCs (Fig. 4C), IL-10^{-/-} BMDCs were stimulated by *L. major* in the presence of supernatants from IL-10^{-/-} B cells stimulated with *L. major*. Fig. 4D clearly shows that supernatants of IL-10^{-/-} B cells stimulated with *L. major* did not inhibit the IL-12p70 produced by IL-10^{-/-} BMDCs stimulated by *L. major*. Altogether, these results demonstrated that IL-10 produced by B cells in response to *L. major* downregulate the IL-12p70 produced by BMDCs.

Unfortunately, we were unable to study the regulation by B cells derived IL-10 on IL-12p70 production by DCs in vivo during the early steps of infection, because induction of IL-12p70 in *L. major*

infected mice is restricted to few numbers of DCs during the first days of infection (28).

Characterization of B cell-producing IL-10 in response to *L. major*

In order to determine the phenotype of B cells secreting IL-10 after *L. major* stimulation in vitro, we enriched the IL-10-producing B cells with an IL-10 secretion assay kit (Miltenyi Biotec) and analyzed the expression of specific markers of B cells on the IL-10 negative and positive enriched B cell population.

As shown in Fig. 5A, IL-10-producing B cells were mostly CD5⁺ after stimulation with *L. major* (76.5%) or with CpG (70.2%). Interestingly, the few IL-10-producing B cells detected

in unstimulated culture were also CD5⁺ (71.2%). However, whereas 35.9% of IL-10–producing cells are CD5⁺ and CD1d⁺ after *L. major* stimulation, only 16.6% of IL-10–producing B cells in responses to CpG were CD5⁺ and CD1d⁺ (Fig. 5A). As a control, without any stimulation, 8.8% of IL-10–producing B cells are CD1d⁺ and CD5⁺ (Fig. 5A). In addition, non-IL-10–producing B cells are CD5[−] and CD1d[−] whatever the stimulation used.

Expression of characteristic markers of B cell subsets such as CD21, CD23, IgD, and IgM were also analyzed in IL-10–producing B cells. A downregulation of CD21 and CD23 expression was observed in B cells stimulated with *L. major* as compared with unstimulated or CpG stimulated B cells (58.2% versus 92.7% and 91%, respectively, for CD21; 53.2% versus 90.9% and 76.5%, respectively, for CD23). Whereas no difference was observed in IgM expression in B cells whatever the stimulus used (*L. major* or CpG), a downregulation of IgD was detected in IL-10–producing B cells after *L. major* and CpG stimulation as compared with unstimulated B cells.

Altogether these results demonstrated that in vitro IL-10–producing B cells after *L. major* are CD5⁺, CD1d⁺, CD21^{low}, and CD23^{low} and that this phenotype is specific to *L. major* because IL-10–producing B cells after stimulation with CpG are CD5⁺, CD1d[−], CD21^{high}, and CD23^{high}.

To demonstrate that B cells producing IL-10 also expressed CD5 and CD1d in vivo, purified B cells were isolated from the draining LN 1 d postinfection with *L. major* and characterized for their expression of CD5 and CD1d markers. In accordance with the phenotype of IL-10–producing B cells after *L. major* stimulation in vitro, 14.1% of IL-10–producing B cells 1 d postinfection with *L. major* are CD5⁺ and CD1d⁺ (Fig. 5B). As controls, only 0.91% non-IL-10–producing B cells are CD5⁺ and CD1d⁺ 1 d postinfection with *L. major*.

In conclusion, IL-10 production by B cells after *L. major* stimulation is confined to a small, discrete population of CD5⁺ and CD1d⁺ B cells.

Discussion

Results obtained in this study showed that the IL-10 produced by B cells in response to *L. major* LV39 plays an important role in the development of susceptibility to the infection. First, we described that purified B cells stimulated in vitro with *L. major* secrete IL-10. Moreover, IL-10 expression is detected in purified CD19⁺ cells from draining LNs in susceptible but not in resistant mice in the first days postinfection, a window of time particularly important for Th differentiation (15). Furthermore, in contrast to the effect of adoptive transfer of B cells from BALB/c mice into BALB/c μ MT mice prior to infection with *L. major* LV39 (20), transfer of B cells from IL-10^{−/−} BALB/c mice into BALB/c μ MT mice restore neither disease progression nor Th2 cell development in these otherwise resistant mice. But, it is important to note that the effect of B cell IL-10 may be limited to the LV39-specific strain of *L. major*.

It has been described that IL-4 plays a dominant role in the susceptible phenotype observed in the BALB/c mice postinfection with *L. major* (16). However, experiments using IL-4R α ^{−/−}-deficient mice have generated controversial results. Indeed, IL-4R α ^{−/−} mice infection with *L. major* either contained infection during the first 3 mo (17) or developed progressive disease with similar kinetics to WT BALB/c mice (19). These results suggest that cytokines, in addition to IL-4, are responsible for susceptibility to infection with *L. major*. Results showing that IL-4R α ^{−/−} IL-10^{−/−} mice control parasite replication and displayed a resistance phenotype comparable to that of C57BL/6 mice demonstrated that IL-10 might play a role in susceptibility to infection with *L. major*. Moreover, IL-10-deficient or WT mice treated with anti-IL-10R mAb were resistant to *L. major*

LV39 infection but still less so than the IL-4R α ^{−/−} IL-10^{−/−} mice (19). Taken together, these observations indicate that both IL-10 and IL-4 participate in the susceptibility of BALB/c mice.

Several different populations of cells have been described as IL-10 producers after *L. major* infection. Following infection with *L. major*, IL-10 mRNA were detected in CD4⁺ T cells from BALB/c mice (29). Among T cells, CD4⁺CD25⁺ T cells named regulatory T (Treg) cells have been shown to have suppressive activity related to their production of IL-10. Indeed, in *L. major*-resistant C57BL/6 mice, Treg cells have been shown to inhibit effector's responses by IL-10-dependent and -independent mechanisms (30) to maintain low levels of persistent parasites and consequently CD4⁺ T cell memory (31). However, recently it has been demonstrated that the IL-10 produced by CD4⁺ T cells other than Treg cells is responsible for susceptibility to infection (32, 33).

In addition to T cells, macrophages have also been described as a source of IL-10 in response to *Leishmania*. After ligation and internalization of IgG-opsonized parasites through their FcRs, macrophages secrete IL-10 (34–38). However, we have already demonstrated that IL-10 production by LN-stimulated cells from BALB/c μ MT mice reconstituted with immune serum do not differ from those observed in unreconstituted mice, suggesting that specific IgG do not contribute to susceptibility to infection with *L. major* (20).

Recently, in an experimental model of visceral leishmaniasis induced by *Leishmania donovani*, NK cells were described as a novel source of IL-10 (39). Although the role of IFN- γ produced by NK cells is still debated in cutaneous leishmaniasis (40, 41), the role of IL-10 potentially produced by NK cells has not yet been evaluated.

Although B cells have also been described as producing IL-10 in response to *L. major* stimulation in vitro (5, 14), the exact role of this cytokine produced by B cells in vivo has not been established. In this report, we clearly demonstrate that after *L. major* infection, B cells are also a source of IL-10 and that this IL-10 production influences the susceptibility of infection with *L. major*. Indeed, whereas otherwise BALB/c μ MT mice reconstituted with WT B cells develop progressive lesions similar to those observed in BALB/c mice, BALB/c μ MT mice reconstituted with IL-10^{−/−} B cells contain the development of lesions and develop a Th1 response. The results presented in this paper showing that IL-10 produced by B cells, a potential APC, is necessary for susceptibility to infection with *L. major* are in agreement with the observations demonstrating that IL-10 produced by APCs is needed for susceptibility to infection with *L. major* (42).

The effect of IL-10 on susceptibility of BALB/c mice to *L. major* infection appears critically dependent on the nature of its cellular source. In this context, it has been demonstrated that Tg C57BL/6 mice expressing IL-10 under the control of the IL-2 promoter, directing the expression of IL-10 in T cells, remained resistant to *L. major* infection and developed a Th1 response comparable to that of WT C57BL/6 mice (43). In contrast, C57BL/6-resistant mice that express an IL-10 transgene under the control of the MHC II E α promoter, directing the expression of IL-10 mainly in APCs, were susceptible to *L. major* (42).

The absence of IL-10 production by B cells in the first days of infection in resistant mice also suggests that in susceptible mice, B cells facilitate lesion development by suppressing the differentiation of protective Th1 effector cells. A recent report showed that CpG-activated neonatal B cells inhibited IL-12 secretion by DC through an IL-10–dependent mechanism (44). Furthermore, in the EAE model, recovery was associated with the suppression of IL-12 production by DCs by IL-10 secreted by B cells (8, 45). In accordance with these results, we demonstrated that *L. major*-activated

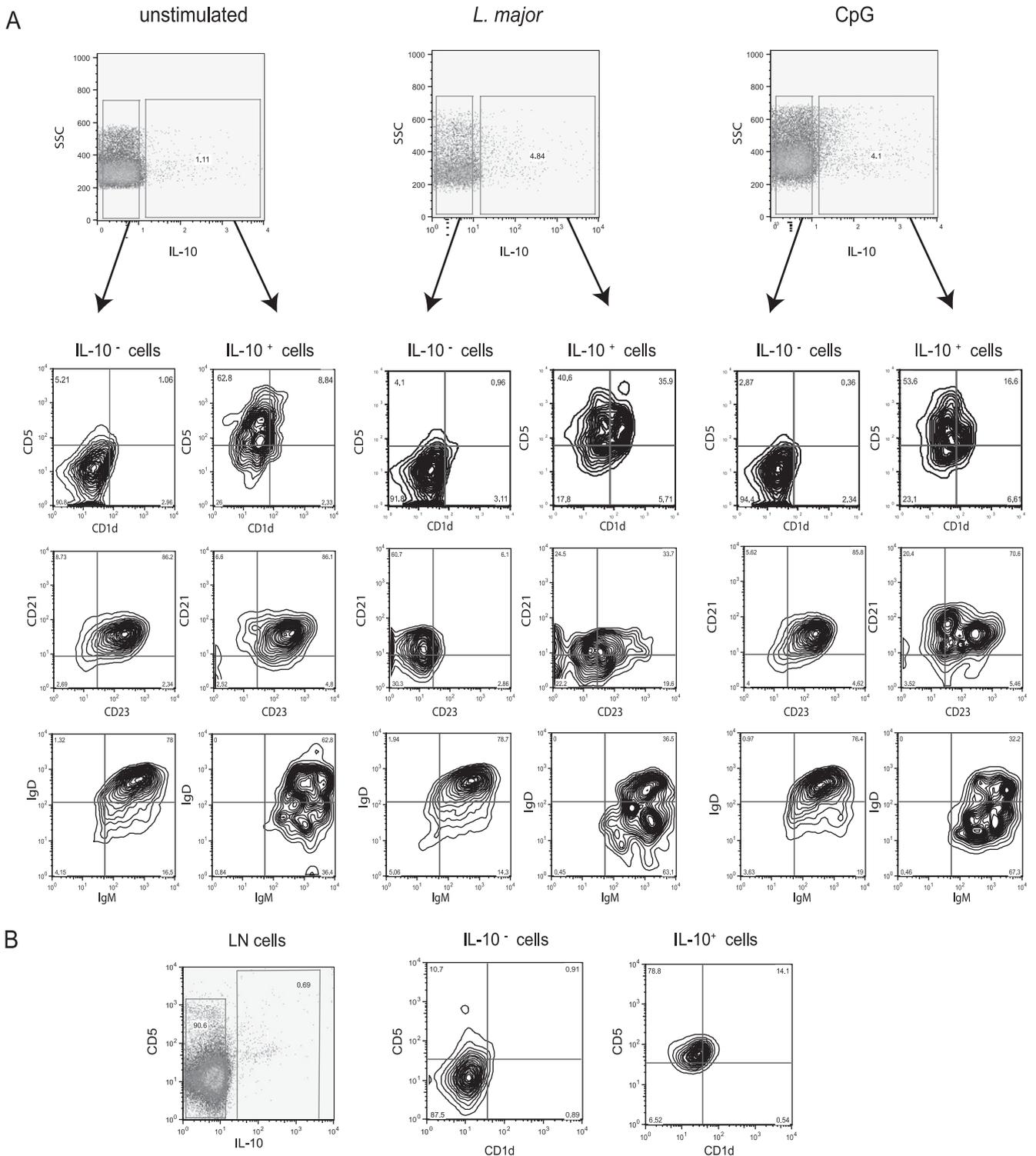


FIGURE 5. CD5⁺CD1d⁺ B cell subsets secrete IL-10 after stimulation by *L. major*. **A**, Phenotype of IL-10⁺ or IL-10⁻-producing splenic B cells in vitro. Purified CD19⁺ splenocytes were stimulated by medium alone, *L. major*, and CpG for 16 h. Thereafter, IL-10-secreting cells were enriched using the IL-10 secretion assay strategy, and cell surface molecule expression on IL-10⁺ and IL-10⁻ B cells was determined by FACS analysis. Similar results were obtained in three individual experiments. **B**, Phenotype of IL-10-producing B cells in the draining LNs 1 d postinfection with *L. major*. CD19⁺ cells were purified and IL-10-producing cells were enriched using the IL-10 secretion assay strategy. CD1d and CD5 expression was analyzed by FACS analysis on IL-10⁺ and IL-10⁻ B cells. Similar results were obtained in two individual experiments.

B cells limit the capacity of DCs to produce IL-12. First, supernatants of *L. major* stimulated B cells from BALB/c mice that contained IL-10 inhibit IL-12p70 production of DCs stimulated with *L. major*. Secondly, in contrast to adoptive transfer of WT B cells, adoptive transfer of IL-10^{-/-} B cells did not promote the development of the Th2 immune response in infected mice.

Recently, Breg cells have been identified in experimental models of autoimmunity, cancer, and infections. The regulatory function of these B cells was shown to be mediated by the production of IL-10. Different subsets of Breg cells distinguished by their surface phenotype and their immunoregulatory properties (i.e., down-regulating IL-12 production or costimulatory molecules on APCs

or even inducing Treg cells) have been described (46). The phenotype of IL-10-producing B cells after *L. major* stimulation is clearly CD19⁺, CD5⁺, CD1d⁺, CD21^{low}, and CD23^{low}. These B cells share some phenotypic markers with the B cell subsets already described: CD5⁺ B1 cells, CD1d⁺ CD23⁺ IgM⁺ IgD⁺ transitional T2, and CD1d⁺ CD23[±] IgM⁺ IgD[±] marginal zone B cells. Interestingly, all these B cell subpopulations have been described as potential Breg cells (47). We can rule out that IL-10-producing B cells after *L. major* stimulation are B1 cells because purification of B cells was done by negative selection using a mAb against CD43, a marker expressed on B1-B cells (48). In addition, using B1-depleted BALB/c mice, Babai et al. (49) have clearly demonstrated that B1-B cells do not contribute to the susceptibility of BALB/c mice to infection with *L. major*.

With respect to the phenotype of the IL-10-producing B cells after *L. major* stimulation, we cannot conclude whether these cells are T2 or MZ B cells. Interestingly, recent data clearly demonstrated that a distinct subset of CD19⁺ CD1d^{high} CD5⁺ cells have potent regulatory function (50). This was confirmed recently in the murine model of EAE. Indeed, adoptive transfer of splenic IL-10 producing CD1d^{high} CD5⁺ CD19⁺ B cells (named B10 cells) normalizes EAE in B cell-depleted mice (51), showing that IL-10 produced by B cells that express CD5⁺ CD1d⁺ are able to modulate the differentiation of CD4⁺ Th1 cells. The fact that IL-10-producing B cells express CD1d and CD5 either in vitro after *L. major* stimulation or in vivo 1 d postinfection with *L. major* strongly suggests that these IL-10-producing B cells correspond to the described B10 cells.

The results reported in this study clearly indicate that IL-10 secreted by CD5⁺ CD1d⁺ B cells is required for polarization of Th cell response toward Th2 phenotype and susceptibility to infection with *L. major* LV39. This could be the result of the regulation of the IL-12 production. Understanding molecular mechanisms involved in controlling B cell regulatory functions remains an important issue that deserves further studies to design new strategies for manipulating the development of effectors responses to the host's benefit.

Disclosures

The authors have no financial conflicts of interest.

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