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Marginal Zone B Cells Regulate Antigen-Specific T Cell Responses during Infection

Rashmi Bankoti,* Kshitzit Gupta,†‡ Andre Levchenko,†‡ and Simona Stäger*§

Marginal zone B cells (MZB) participate in the early immune response to several pathogens. In this study, we show that in μMT mice infected with Leishmania donovani, CD8 T cells displayed a greater cytotoxic potential and generated more effector memory cells compared with infected wild type mice. The frequency of parasite-specific, IFN-γ* CD4 T cells was also increased in μMT mice. B cells were able to capture parasites, which was associated with upregulation of surface IgM and MyD88-dependent IL-10 production. Moreover, MZB presented parasite Ags to CD4 T cells in vitro. Depletion of MZB also enhanced T cell responses and led to a decrease in the parasite burden but did not alter the generation of effector memory T cells. Thus, MZB appear to suppress protective T cell responses during the early stages of L. donovani infection. 


B cells are mainly known for their role in the production of Abs aimed at facilitating pathogen and/or other Ag clearance. However, an increasing body of literature has now shown that they may also regulate adaptive T cell responses by various Ab-independent mechanisms, such as cytokine production, costimulation, and Ag presentation (1–4). Recent studies in various infectious disease models have demonstrated that B cells can enhance Th2 responses (5–7). Conversely, B cells were also shown to support Th1 responses (6). In contrast, IL-10–producing B cells can suppress CD4 T cell responses (8) and prevent the induction of autoimmune diseases in several mouse models (9, 10). These reports suggest that depending on the disease model, B cells can both enhance and/or suppress CD4 T cell responses.

The role of B cells in regulating CD8 T cell responses is less clear, but also seems to depend on the disease model. In some models, B cells are not critical for the priming of CD8 T cells (11–13) or for memory generation (14). In contrast, in LCMV- (13, 15) and L. monocytogenes- (12) infected mice, the absence of B cells seems to affect memory generation. In addition, TGF-β-secreting B cells can induce anergy in CD8 T cells (16). Moreover, B cells were shown to inhibit CD4 and CD8 T cells in tumor-induced immunity by a yet unknown mechanism (17).

Various studies have now demonstrated that B cells play a negative role in several experimental models of leishmaniasis (5,

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Abbreviations used in this article: BMDC, bone marrow-derived dendritic cell; Breg, regulatory B cell; DC, dendritic cell; FoB, follicular B cell; MZB, marginal zone B cell; RT, room temperature; Tc45, central memory T cell; TcEm, effector memory T cell; VL, visceral leishmaniasis; WT, wild type.

Copyright © 2012 by The American Association of Immunologists, Inc. 0022-1767/12/S16.00/18–20. B cell depletion was shown to enhance resistance to Leishmania tropica and Leishmania mexicana in BALB/c mice (18), and cotransfer of B cells converts T cell–reconstituted, Leishmania major-resistant, C.B-17 scid mice into a susceptible phenotype (21). Furthermore, C57BL/6 B cell-deficient mice are highly resistant to Leishmania donovani infection (20). The mechanism by which B cells exacerbate Leishmania infections is yet unknown. A recent study has suggested that IL-10 derived from L. major-induced regulatory B cells (Bregs) skews the balance toward unprotective Th2 responses (5). In contrast, Deak et al. (19) recently proposed that IgM production and polyclonal B cell activation, which requires activation of complement, are the main cause of disease exacerbation in Leishmania infantum–infected mice rather than IL-10 production by B cells.

In this study, we investigated the role of B cells in the regulation of Ag-specific CD8 T cell responses in a murine model of visceral leishmaniasis (VL). CD8 T cells are very important effector cells in the immune response to the protozoan parasite L. donovani, a causative agent of VL (22, 23). However, we have previously reported that mice infected with L. donovani developed deficient parasite-specific CD8 T cell responses, characterized by limited clonal expansion and functional exhaustion during chronic disease (23). In this study, we demonstrate that marginal zone B cells (MZB) suppress Ag-specific CD8 and CD4 T cell responses during the early stages of VL. Suppression of NK1.1* cell functions appears to be, in part, mediated by MyD88–dependent IL-10 production. Moreover, B cells inhibit the generation of effector memory CD8 T cells after L. donovani infection.

Materials and Methods

Mice and parasite

All experiments were approved by and conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee of The Johns Hopkins University School of Medicine (Protocol No. M008M501).

Six- to 8-wk-old C57BL6N/Cr and CD45.1-C57BL6 female mice were obtained from National Cancer Institute (Frederick, MD). C57BL/6-IItö*/–, B6.129S7-Rag1tm1J/J, and C57BL6-Tg(OT-I)-RAG1tm1J/J mice were obtained from The Jackson Laboratory. μMT mice were provided by Dr. S. Desiderio (The Johns Hopkins University, Baltimore, MD). C57BL/6-MdD88–/– mice were a gift from Dr. F. Zavala (The Johns Hopkins University). Ly5.1-OT-I mice were generated by crossing CD45.1-C57BL6 with C57BL6-Tg(OT-I)-RAG1tm1J/J mice. Mice were housed at the animal facility of The Johns Hopkins University under specific pathogen-free conditions.
Adoptive transfer of OT-I cells

CD45.1-OT-I transgenic mice, expressing an MHC class I-restricted TCR specific for chicken ovalbumin, were used for adoptive transfers. CD8 T cells were enriched from the spleen of naive OT-IRAG1 mice using MACS following manufacturer’s instructions (Miltenyi Biotech). Naive CD8 T cells were then sorted to >98% purity using FACSVantage (Becton Dickinson) based on the CD62L and CD44 expression. A total of 2 × 10^6 sorted cells were then injected via the tail vein of mice.

Ab blockade and depletion

For the IL-10 blockade, mice were treated biweekly with 200 μg anti-IL-10R Ab (clone IB.3A) or with isotype control. MZB were depleted using the mixture of Abs described previously (24, 25). In brief, mice were injected with a single dose of 100 μg of either isotype or anti-LFA-1α (clone M17/4; eBioscience) and anti-CD49d Ab (clone R1-2; eBioscience). Mice were infected 1 wk after the administration of anti-LFA-1α and anti-CD49d. To deplete B cells, we injected mice with 250 μg MB20-11 Ab (26), which was a kind gift from Dr. Thomas Tedder (Duke University). Mice were infected 6 d after B cell depletion.

Sera reconstitution

WT mice were infected with PINK. Sera from infected mice were collected between days 12 and 14 p.i. and pooled. μMT mice were administered with 200 μl of the pooled sera the day before infection. Control μMT mice received naive sera.

PKH67 labeling of parasitites

L. donovani parasites were stained with PKH67 (Sigma) following manufacturer’s instructions. Mice received 5 × 10^6 PKH67-labeled parasites. Spleens from naive and infected mice were harvested 20 h later and surface stained for flow cytometric analysis.

B cell in vitro studies

Naive B cells were purified using the naive B cell isolation kit (Miltenyi Biotech) according to manufacturer’s protocol. Purified B cells (purity 90–92%) were then incubated either alone, with different ratios of parasitites, or with 1 μg/ml CpG (InvivoGen). For cross-linking of IgM and C3dg receptor (CD21), latex beads were incubated either alone, with anti-IgM (5 μg/ml), or with anti-CD21 (5 μg/ml) for 2 h at room temperature (RT). The beads were washed twice with PBS and then incubated with B cells at 37°C to remove unbound Abs. IL-10 production by B cells was assessed at 24 h using the IL-10 secretion kit from Miltenyi Biotech following the manufacturer’s protocol.

Flow cytometry

Adoptively transferred OT-I T cells were identified by staining the splenocytes with biotinylated anti-CD45.2 and/or anti-CD45.1 Ab followed by streptavidin-PerCP, or with anti-CD45.1-FITC. Other Abs used to further characterize the B cells were CD8-allophycocyanin, CD8-Pacific blue, CD69-PE, CD44-allophycocyanin, CD44-biotin, CD62L-PE, CD62L-allophycocyanin, CD122-PE, CD127-PE, and KLRG1-allophycocyanin. All Abs unless specified were from BD Pharmingen. For intracellular staining, splenocytes were stimulated with the SIINFEKL peptide (Geno-sphere Biotechnologies) and IL-2 (Amgen) in the presence of brefeldin A (BD Pharmingen) for 4 h at 37°C followed by surface staining for anti-CD45.2 and -CD8 (23). The cells were then fixed, permeabilized, and stained with either isotype controls or anti-granzyme B–PE (Catag, anti-IFN-γ-allophycocyanin, anti–IL-2–PE (BD Pharmingen), and anti–TNF-α–PE-Cy7 (eBioscience). More than 1 million cells per sample were acquired with an LSRII (Beckton Dickinson), and analysis was done using FACS-Diva software.

Endogenous CD4 T cell responses were analyzed as follows. Bone marrow–derived dendritic cells (BMDCs) were pulsed with fixed parasites for 24 h at 37°C. Splenocytes were added to BMDCs and incubated for 2 h at 37°C. Brefeldin A (BD Pharmingen) was then added for a further 4 h. Cells were then stained with biotinylated anti-CD3 followed by PerCP-conjugated streptavidin, FITC-conjugated anti-CD4, and allophycocyanin-conjugated anti–IFN-γ (all BD Bioscience). For B cell analysis, samples were surface stained with anti-CD19-allophycocyanin-Cy7 (1D3), anti-CD5-FITC (53-7.3), anti-CD21 PE (7G6; BD Pharmingen), anti-CD1d-PE (1B1), and anti-CD23-PE-Cy7 (B3B4; eBioscience). Flow cytometric analysis was performed with an LSRII flow cytometer (Becton Dickinson). A total of 350,000 cells per sample were acquired and analyzed with the FACSDiv software.

The expression of costimulatory molecules by B cells 24 h after incubation with parasitites was assessed with the following Abs (BD Pharmingen): anti-CD19-allophycocyanin-Cy7, anti-CD86-PE (GL1), anti-CD80-PerCP-Cy5.5 (16-10A1), anti–MHC class II–FITC (2G9), anti–CD40-biotin (3–23), and anti–SA-PE-Cy7.

Immunofluorescence staining

L. donovani parasites were stained with PKH67 (Sigma) following manufacturer’s instructions. Naive B cells were purified using the naive B cell isolation kit (Miltenyi Biotech) according to manufacturer’s protocol. The purity of the B cells was 90–92%. One million purified B cells were then incubated either alone or with 1:5 or 1:10 ratios of parasitites in a 48-well plate for 24 h. B cells in culture were cytokin on glass slides, allowed to air-dry, and then fixed with cold acetone for 10 min. The slides were then washed with PBS and stained as follows: the fixed B cells were incubated for 35 min at RT with 100 μl PBS with naive mouse serum (diluted 1:500) followed by the washed with PBS. The slides were then incubated with streptavidin-PerCP-Cy7 (BD Pharmingen) at 37°C for 1 h, washed, and incubated with rabbit anti-rat Alexa Fluor 647 (BD Pharmingen) for 30 min at RT. The slides were subsequently washed and mounted with mounting media. Images were taken at 600× with an Olympus BX51 microscope.

Staining of parasitites

Cytopsin of parasitites were prepared and fixed with cold acetone. The parasitites were stained with anti-C3 (Cedarlane) or with anti-mouse IgG2a and conjugated with rabbit anti-rat AF594 (Invitrogen). The slides were washed and mounted with mounting media. Images were taken at 1000× with an Olympus BX51 microscope.

L. donovani amastigotes were incubated with supernatants collected from the B cell in vitro studies (naive B cells, infected B cells, CpG stimulated B cells) for 20 min on ice. They were then washed, incubated with biotinylated anti-IgM for 20 min on ice, and subsequently with streptavidin allophycocyanin (BD Pharmingen). Samples were fixed with 2% paraformaldehyde and analyzed by flow cytometry.

Live B cell imaging

Glass-bottomed petri dishes (Mattek, Ashland, MA) were coated with 10 μg/ml fibronectin for 3 h and washed once with PBS. Isolated naive B cells were seeded on the dish at a density of 25,000 cells/ml and allowed to settle for 30 min before adding parasitites at a final density of 100,000 cells/ml. Imaging was started within 1 min of addition of the parasites to the medium with Zeiss Axiovert 2000 microscope using a 40× apochromatic lens (numerical aperture = 1.5). Images were acquired at 37°C, 5% CO2 every 30 s for 4 h using Slidebook 4.2 (Intelligent Image Innovations, Denver, CO). Movies were created using Slidebook and ImageJ (National Institutes of Health, Bethesda, MD).

T cell activation assay

KZO and B3Z hybridomas were kind gifts from Dr. N. Shastri (University of California, Berkeley, CA). KZO is a CD4 T cell hybridoma that recognizes OVA263-266 in the context of I-Aκ. B3Z is a CD8 T cell hybridoma specific for the OVA257-264 epitope in the context of H-2Kd. Both hybridomas have been transfected with an NFAT-Jac2 reporter construct (27). MZB were sorted from a starting population of naive splenic B cells (enriched as described earlier) to >98% purity using FACSVantage (Becton Dickinson) based on the expression of CD21 and CD23. A total of 10^5 sorted MZB were incubated at 37°C with PINK and/or L9V (multiplicity of infection 1.5) and 10^5 KZO and/or B3Z. OVA (for KZO) or SIINFEKL (for B3Z) were added to the culture for the positive control groups. Eighteen hours later, the cells were loaded with fluorescein di-dioleatoacetoxymethyl (Invitrogen) and analyzed for luc2 expression on a flow cytometer as described by Karttunen and Shastri (28). MZB were gated out of the analysis based on the relative small size compared with T cell hybridomas.

RNA extraction and real-time PCR analysis

WT and μMT mice were infected with 2 × 10^7 PINK amastigotes. Spleens were harvested on days 1, 3, and 6 p.i. Spleens were collagenase digested...
followed by isolation of CD11c+ dendritic cells (DCs) using anti-CD11c beads (Miltenyi Biotec) as previously described (29). RNA was extracted using RNeasy mini kit (Qiagen) as per manufacturer’s instruction. Reverse transcription was performed using iScript cDNA synthesis kit (Bio-Rad) using manufacturer’s protocol. Real-time PCR analysis was performed on cDNA using iQ SYBR Green supermix kit (Bio-Rad). IL-12p35, IL-12/IL-23 p40, and actin were amplified using primers described previously (29). All PCRs were carried out with an iCycler (Bio-Rad).

**Generation of mix bone marrow chimera**

C57BL/6 mice were irradiated with two doses of 5.5 Gy within a 2-h interval. Irradiated mice were reconstituted with bone marrow mixed from μMT, IL-10−/−, and C57BL/6. The bone marrow was mixed at a ratio of 3:1 for the following combinations: 75% μMT with 25% IL10−/− and 75% C57BL/6 with 25% IL10−/−. Irradiated mice were reconstituted with 10^7 bone marrow cells from the earlier combinations. Mice were provided with antibiotics in drinking water for 4 wk. Engraftment was allowed to proceed for 6 wk. A group of mice was checked for reconstitution 6 wk after bone marrow transfer.

**Statistical analysis**

Results were analyzed using unpaired Student t test. A p value <0.05 was considered significant. Real-time PCR results were analyzed using an unpaired Student t test. All experiments were repeated at least twice.

**Results**

**B cell deficiency results in enhanced CD8 and CD4 effector functions**

It is known that B cells exacerbate Leishmania infections (5, 18–20). B cell-deficient (μMT) mice are indeed more resistant than WT mice to infection with L. donovani (see Supplemental Fig. 1A) (20). However, the mechanisms by which B cells help to exacerbate disease have not yet been clarified. Hence we first wanted to determine whether B cells interfere with the priming and development of protective, Ag-specific CD8 T cell responses in vivo. Thus, OT-I T cells, which recognize an H-2Kb–restricted CD8 T cell epitope of OVA257–264, were adoptively transferred into μMT mice. These mice were subsequently infected with OVA-transgenic L. donovani amastigotes. Between days 3 and 7 p.i., OT-I T cells undergo clonal expansion during L. donovani infection. This is typically followed by clonal contraction (days 9–14) (23). We first compared the number of OT-I T cells that engrafted after the adoptive transfer in both types of host and found similar numbers of OT-I cell in the spleen of both mouse strains 7 d after adoptive transfer (1240 ± 549 cells in the spleen of naive μMT mice, and 1213 ± 528 in WT mice). Next, we compared the expansion of OT-I T cells in μMT and WT mice. B cells did not seem to affect the proliferative capacity of OT-I T cells. Indeed, the number of OT-I T cells present in the spleen of WT mice was similar to that in μMT mice (Fig. 1A). Interestingly, a greater percentage of OT-I T cells expressed granzyme B in μMT mice compared with WT mice (Fig. 1B, Supplemental Fig. 1B). A similar increase in granzyme B expression was observed in the endogenous CD8 T cell population (Fig. 1C, Supplemental Fig. 1C). Furthermore, no difference was observed in the absence of B cells (Fig. 1H, Supplemental Fig. 1C), suggesting that B cells are not only interfering with CD8 T cell responses during VL, but also with CD4.

Because IgM is thought to contribute to disease exacerbation in BALB/c mice infected with L. infantum (19), we reconstituted μMT mice with sera from days 12–14 infected WT mice before adoptive transfer of OT-I T cells, and infected them with L. donovani. μMT mice reconstituted with sera had a similar parasite burden when compared with unreconstituted μMT mice (see Supplemental Fig. 2A). Furthermore, no difference was observed in the number or effector function of OT-I T cells present in the spleen of both groups of mice (data not shown). Taken together,

![FIGURE 1](http://www.jimmunol.org/)

**Enhanced CD8 and CD4 T cell responses in μMT mice infected with L. donovani.** (A) A total of 2 × 10^6 OT-I T cells were adoptively transferred before infection with PINK amastigotes. OT-I T cells were identified by gating on CD8+ Ly5.1+ cells. Graphs represent the average number ± SE of OT-I T cells in the spleen of individual mice on days 7 and 13 p.i. (B–G) Splenocytes from infected WT and μMT mice were restimulated and stained for granzyme B, IFN-γ, TNF-α, and IL-2. (B) Graph represents the percentage of OT-I T cells producing granzyme B. (C) granzyme B-producing endogenous CD8 T cells, (D) NK1.1+ granzyme B-producing cells, (E) IFN-γ–producing OT-I T cells, (F) TNF-α– and IFN-γ–producing OT-I T cells, and (G) IFN-γ– and IL-2–producing OT-I cells. (H) For endogenous CD4 T cell responses, infected WT and μMT splenocytes were incubated with BMDCs pulsed with fixed parasites for 2 h and then an additional 4 h with brefeldin. Graph represents the percentage of IFN-γ–producing CD4 T cells. Data represent mean percentages ± SE, representative of four independent experiments, n = 3–5. *p < 0.05, **p < 0.01.
these results show that B cells appear to be involved in the regulation of CD8 and CD4 T cell responses during the early stages of VL.

**B cells suppress the generation of effector memory T cells in L. donovani-infected mice**

In a previous study, we reported that CD8 T cells fail to generate effector memory cells (TEM) during VL. Further, we reported that at the end of contraction, ~70–80% of the OT-I T cells displayed a central memory cell (T<sub>CM</sub>)-like phenotype and 20–30% were effector cells (23). Hence we next characterized the phenotype of adoptively transferred OT-I T cells in μMT mice at two different time points: during clonal expansion (day 7) and at the end of contraction (day 13). Phenotypes that were considered were: effector cells (CD62L<sup>lo</sup>CD127<sup>+</sup>), TEM (CD62L<sup>lo</sup>CD127<sup>+</sup>), and T<sub>CM</sub> (CD62L<sup>hi</sup>CD127<sup>+</sup>). No difference in the percentage of OT-I T cells expressing an effector phenotype (CD62L<sup>lo</sup>CD127<sup>+</sup>) was observed at day 7 p.i. (Fig. 2A, 2B, Supplemental Fig. 2B). In contrast, when we looked at the OT-I T cell responses at day 13 p.i., we noticed a significant increase in the frequency of CD62L<sup>lo</sup> OT-I T cells present in the spleen of μMT mice compared with WT mice (Fig. 2A). Nevertheless, the percentages of CD127<sup>neg</sup> OT-I T cells at day 13 p.i. were similar in both groups of mice (Fig. 2B). Moreover, there was no difference in the percentages of CD44<sup>+</sup> OT-I T cells between both groups (data not shown). Hence 40–50% of the adoptively transferred OT-I T cells that survived contraction in μMT mice was CD44<sup>+</sup>CD62L<sup>lo</sup>CD127<sup>+</sup>, a phenotype typically associated with TEM. OT-I T cells did not generate TEM in infected WT mice. We also looked at the KLRG1 expression at days 7 and 13 p.i., which is a marker for short-lived effector cells (30). In WT mice, only 10–14% of the OT-I T cells expressed KLRG1 at both time points; in contrast, ~25% at day 7 and 35% of the OT-I T cells at day 13 were positive for KLRG1 in μMT mice (Fig. 2C, Supplemental Fig. 2B). A recent study demonstrated that the generation of KLRG1<sup>+</sup> effector CD8 T cells is governed by IL-12 (31). Thus, we assessed the IL-12 p35 and IL-12/IL-23 p40 mRNA expression by splenic CD11c<sup>hi</sup> DCs at days 1, 3, and 6 after L. donovani infection.

**FIGURE 2.** TEM are generated in μMT mice but not in WT mice after infection with L. donovani. OT-I T cells were identified by gating on CD8<sup>+</sup> Ly5.1<sup>+</sup> cells. Graphs represent the percentage ± SE of gated cells that were (A) CD62L<sup>lo</sup>, (B) CD127<sup>+</sup>, and (C) KLRG1<sup>+</sup> on days 7 and 13 p.i. (D and E) Splenic DCs were MACS enriched after infection with parasite on days 1, 3, and 6. Fold gene induction was calculated after normalization to the housekeeping gene actin. Expression of (D) IL-12p35 and (E) IL-12p40 in naive WT and naive μMT was taken as 1 and compared with the respective infected WT and μMT mice. (F–K) B cells were depleted in naive C57BL/6 mice with the anti-CD20 Ab. OT-I T cells were then adoptively transferred into depleted and control undepleted mice. Animals were infected a day after transfer with L. donovani. Graphs represent (F) the splenic parasite burden, (G) granzyme B-producing endogenous CD8<sup>+</sup> T cells, (H) NK1.1<sup>+</sup> granzyme B-producing cells, and (I) IFN-γ production by CD4 T cells. Graphs represent the percentage of OT-I T cells that were (J) KLRG1<sup>+</sup>, (K) CD62L<sup>lo</sup> CD127<sup>+</sup>, CD62L<sup>hi</sup>CD127<sup>+</sup>, and CD62L<sup>lo</sup> CD127<sup>+</sup>. Data represent mean percentages ± SE, representative of two to four independent experiments, n = 3–5. *p < 0.05, **p < 0.01.
a moderate and transient increase in IL-12p35 mRNA levels in DCs, which peaks at 5 h p.i (32). As shown in Fig. 2D, IL-12p35 mRNA levels gradually decreased from day 1 to 6 p.i. in DCs isolated from infected WT mice. In contrast, in infected μMT mice, IL-12p35 expression was induced at greater levels compared with WT mice and sustained over the first 6 days of infection (Fig. 2D). The expression levels for the IL-12/IL-23 p40 mRNA were comparable in DCs isolated from infected WT and μMT mice, with the exception of day 3, when p40 was expressed at greater levels in infected WT mice (Fig. 2E).

To test whether Abs were involved in suppressing the generation of TEM, we reconstituted a group of μMT mice with serum collected at days 12–14 p.i. from infected WT mice. TEM OT-I T cells were generated in infected, serum-reconstituted μMT mice at a similar frequency to that observed in μMT (Supplemental Fig. 2C, 2D). Adoptively transferred OT-I T cells in these mice also had similar KLRG1 expression at days 7 and 12 p.i. (Supplemental Fig. 2E). This implies that Abs alone may not play a role in the regulation of TEM responses; nevertheless, we cannot exclude the participation of Abs in the suppressive effects mediated by B cells.

To exclude the fact that our observations were mainly due to differences in the splenic architecture, cellular composition, and physiology between μMT and C57BL/6 mice rather than to the lack of B cells, we depleted B cells in C57BL/6 mice using a depleting Ab directed against CD20 (Supplemental Fig. 2F). We then infected these mice and control undepleted C57BL/6 mice with L. donovani. Like μMT mice, B cell-depleted C57BL/6 mice had a similar parasite burden to the undepleted group at day 6 p.i. By day 12 p.i., though, B cell-depleted mice showed a superior capacity in controlling parasite growth and had nearly cleared infection (Fig. 2F). B cell depletion also resulted in an increase in granzyme B expression in CD8 T cells (Fig. 2G) and NK1.1+ cells (Fig. 2H) at day 6 p.i. No difference in granzyme B production by both cell populations was observed at day 12 p.i. Similarly, B cell-depleted mice had a significantly higher frequency of IFN-γ+ CD4 T cells at day 6 p.i. compared with the control group (Fig. 2I); no differences were detected at day 12 p.i.

As already observed in L. donovani-infected μMT mice, the frequency of KLRG1+ OT-I T cells was significantly greater in B cell-depleted mice compared with the undepleted group (Fig. 2J). Moreover, OT-I T cells generated TEM in anti-CD20–treated mice at day 12 p.i., but not in the control group (Fig. 2K, Supplemental Fig. 2G).

**B cells are activated on exposure to L. donovani**

To understand how B cells may interfere with the development of CD8 T cell responses, we monitored the interaction between naive splenic B cells and L. donovani amastigotes over 24 h. One to 2 h after exposure to the parasites, some B cells already had amastigotes attached to their surface and were projecting protrusions (see Supplemental Video). This was a transient phenomenon that was only observed at the early time points after exposure, suggesting that B cells were activated (33). Moreover, some B cells had formed clusters within 3–5 h after exposure to the parasite. At 48 h, most of the B cells that had clustered were dead (data not shown).

To better understand the B cell–parasite interaction, we exposed B cells to fluorescently labeled L. donovani. After 24 h, B cells were labeled with an anti-IgM Ab and analyzed by immunofluo-
rescence. As shown in Fig. 3A (1–3), most of the B cells carrying parasites had formed clusters after 24 h in culture. Interestingly, the IgM staining seemed to form pockets that partially surrounded the parasite aggregates. This suggests that IgM was not only increasingly expressed on the surface of B cells but probably also secreted. We next investigated whether the interaction between B cells and parasites resulted in B cell activation. Hence we incubated parasites with naive B cells and monitored the surface modulation of the costimulatory molecules CD86, CD80, and CD40 24 h after exposure with L. donovani. The B cell–Leishmania interaction resulted in the upregulation of CD86 (Fig. 3B). The majority of these cells also expressed high levels of MHC class II and CD80 (Fig. 3B). CD40 was not upregulated after coculture with L. donovani (data not shown).

To determine whether B cells were also capturing parasites in vivo, we infected C57BL/6 mice i.v. with PKH67-labeled L. donovani and sacrificed them 20 h later. As expected from the results obtained in vitro, a small percentage (0.2–0.3%) of splenic L. donovani and sacrificed them 20 h later. As expected from the results obtained in vitro, a small percentage (0.2–0.3%) of splenic parasites had formed clusters after 24 h in culture. Interestingly, though, despite the increased expression of CD83 and CD86 (Fig. 3B), we next investigated whether MZB were able to present parasite-derived Ags to T cells. Hence we coincubated sorted MZB and PINK parasites with the KZO (ova-specific CD4 T cell hybridoma) and/or B3Z (ova-specific CD8 T cell hybridoma) (27, 28, 36) overnight. As shown in Fig. 5A, presentation of the SIIFKEL peptide by MZB led to the activation of the B3Z cells, as measured by the lacZ expression. However, when MZB were incubated with PINK, we did not detect any activation in the B3Z cell population, suggesting that MZB were not able to present parasite-derived ova to B3Z (Fig. 5A). We also assessed whether MZB were capable of presenting parasite-derived OVA to ova-specific CD4 T cell hybridoma (KZO). As a positive control, we coincubated MZB and OVA with KZO, which showed increased lacZ expression (Fig. 5B). Interestingly, when we coincubated MZB with PINK, we also noticed a significant increase in T cell activation compared with the negative controls (Fig. 5B). We next investigated whether follicular B cells (FoB) were able to present parasite-derived OVA to KZO and B3Z cells. Neither B3Z (Fig. 5C) nor KZO (Fig. 5D) were activated on coincubation with FoB and PINK, suggesting that FoB are not capable of presenting parasite-derived OVA to B3Z or KZO.

IL-10 suppresses expansion of OT-I T cells

Next, we evaluated whether IL-10 could mediate the suppressive effects observed on the T cell responses in infected mice. Hence we treated C57BL/6 mice with an anti–IL-10R Ab and monitored the development of adoptively transferred OT-I T cells. In vivo IL-10R blockade resulted in a 3- to 4-fold increased expansion of OT-I cells at day 6 p.i. (Fig. 6A). Interestingly, though, despite the increased expansion at day 6, OT-I T cells in mice treated with the anti–IL-10R Ab contracted to about the same number at day 9 p.i. IL-10R blockade did not improve IFN-γ production by CD8 OT-I T cells (data not shown); however, we noticed an increase in granzyme B production by OT-I T cells (Fig. 6B, Supplemental Fig. 3B) and in the frequency of endogenous CD4 T cells producing IFN-γ (Fig. 6C, Supplemental Fig. 3B) at day 13 p.i. in infected mice treated with the anti–IL-10R Ab. However, IL-10R blockade failed to restore the generation of T EM in L. donovani-infected mice (see Supplemental Fig. 3C, 3D), but we observed a significant increase in the frequency of KLRG1+ cells in anti–IL-10R–treated, infected mice (Fig. 6D). Taken together, these data show that IL-10R blockade enhanced the effector functions of CD8 and CD4 T cells, and KLRG1 expression by CD8 T cells; however, it did not induce T EM, suggesting that the pathway leading to the generation of T EM is not governed by IL-10.

CD5+CD1d−/−CD23+ and CD5+CD1d−CD23hi B cells produce IL-10 after exposure to L. donovani

Recent literature has demonstrated that some Leishmania species activate B cells and induce IL-10 production (5, 19). Thus, we investigated whether B cells also produce IL-10 after L. donovani infection. Purified naive splenic B cells were incubated with L. donovani amastigotes for 24 h and IL-10 production was assessed by FACS. As expected, B cells stimulated with CpG produced IL-10 (34) (Fig. 4A); the majority of the CpG-stimulated cells producing IL-10 were CD1d−CD5+CD23hi, a phenotype that has been associated to Bregs. Of the B cells exposed to L. donovani amastigotes, 2.3% also produced IL-10. However, the IL-10 producers were divided into two populations, both of which were CD21+ (data not shown): CD19+CD21+CD1d+ and CD19−CD21−CD1d−CD23hi, cells, a phenotype similar to MZB, and CD19−CD21+CD1d+CD23hi cells, which have been described as Bregs (4). In the following, we investigated the role of MZB in the production of IL-10. Thus, we depleted MZB (25) in naive mice, purified naive splenic B cells, and exposed them to L. donovani in vitro. Strikingly, the IL-10 production was reduced by ~60% after incubation with the parasite (Fig. 4B) and most of the cells that were still producing IL-10 in the MZB-depleted group were CD1d+CD5+CD23hi B cells (Fig. 4B). The IL-10 production by B cells after exposure with CpG was also severely reduced after depletion of MZB (Fig. 4B).

IL-10 production by B cells is MyD88 dependent

Given the quick kinetics of IL-10 production by B cells exposed to L. donovani, we investigated the mechanism that leads to IL-10 secretion. We have previously reported that HASPB1, a surface protein of L. donovani, is recognized by natural Abs and complement (35); and in Fig. 3A, we have shown that B cells dramatically upregulate IgM expression after coinfection with parasites. Thus, we first coated beads with anti-IgM Abs to cross-link IgM on B cells. Of the B cells incubated with anti-IgM–coated latex beads, 3.7% produced IL-10, and the majority of these cells showed a phenotype similar to MZB (Fig. 4C). Because amastigotes purified from Rag1−/− mice were coated with complement C3 (see Supplemental Fig. 3A), we next assessed whether cross-linking of CD21 (complement receptor 2), would also induce IL-10 production. Hence we incubated B cells with anti-CD21–coated latex beads for 24 h and monitored IL-10 production. Only 1.8% of cells produced IL-10, the majority of which displayed a MZB-like phenotype. These results suggest that Leishmania may be inducing IL-10 production by cells with an MZB phenotype via cross-linking of surface IgM and/or CD21. However, neither mechanism induced IL-10–producing B cells expressing CD1d+CD5+CD23hi, implying that there may be an additional pathway of IL-10 induction. Thus, we investigated whether the adaptor protein MyD88 was involved in the induction of IL-10 secretion by B cells after exposure with L. donovani. Naive B cells were purified from Myd88−/− mice and coincubated with L. donovani or CpG. As expected, no IL-10 production was detected in culture treated with CpG in Myd88−/− B cells (Fig. 4D). Similarly, IL-10 was also nearly completely abrogated after incubation of Myd88−/− B cells with L. donovani (compare Fig. 4A and 4D).

MZB present parasite-derived OVA to CD4 T cells

Because L. donovani was captured by cells with an MZB-like phenotype (Fig. 3C) and this interaction resulted in the upregulation of CD86 (Fig. 3B), we next investigated whether MZB were able to present parasite-derived Ags to T cells. Hence we coincubated sorted MZB and PINK parasites with the KZO (ova-specific CD4 T cell hybridoma) and/or B3Z (ova-specific CD8 T cell hybridoma) (27, 28, 36) overnight. As shown in Fig. 5A, presentation of the SIIFKEL peptide by MZB led to the activation of the B3Z cells, as measured by the lacZ expression. However, when MZB were incubated with PINK, we did not detect any activation in the B3Z cell population, suggesting that MZB were not able to present parasite-derived ova to B3Z (Fig. 5A). We also assessed whether MZB were capable of presenting parasite-derived OVA to ova-specific CD4 T cell hybridoma (KZO). As a positive control, we coincubated MZB and OVA with KZO, which showed increased lacZ expression (Fig. 5B). Interestingly, when we coincubated MZB with PINK, we also noticed a significant increase in T cell activation compared with the negative controls (Fig. 5B). We next investigated whether follicular B cells (FoB) were able to present parasite-derived OVA to KZO and B3Z cells. Neither B3Z (Fig. 5C) nor KZO (Fig. 5D) were activated on coinfection with FoB and PINK, suggesting that FoB are not capable of presenting parasite-derived OVA to B3Z or KZO.
MZB suppress T cell functions but not the generation $T_{EM}$ during the first week of infection

We then wanted to determine which B cell population was involved in the regulation of T cell responses during the early stages of $L. donovani$ infection. Thus, we proceeded to deplete MZB before infection with $L. donovani$ (see Supplemental Fig. 4A). MZB depletion did not affect OT-I T cell expansion in infected mice (Fig. 7A) and did not promote the generation of $T_{EM}$ at day 14 p.i. (Fig. 7B, 7C, Supplemental Fig. 4B). However, the frequency of KLRG1+ OT-I T cells was significantly increased in mice depleted of MZB compared with undepleted mice (Fig. 7D, Supplemental Fig. 4B). Moreover, the percentage of granzyme B producing OT-I T cells was also increased at day 7 p.i. in mice depleted of MZB compared with the control group (Fig. 7E, Supplemental Fig. 4C). No differences were observed at day 14 p.i. Endogenous CD8 T cells also expressed more granzyme B in infected MZB-
depleted mice at day 7 p.i. (Fig. 7F, Supplemental Fig. 4C) and the frequency of IFN-γ–producing CD4 T cells was also slightly greater in these mice compared with undepleted mice (Fig. 7G, Supplemental Fig. 4C). More importantly, depletion of MZB not only improved T cell responses, but also resulted in a significantly greater parasite burden at day 12 p.i. compared with the WT/Il10−/− group (Fig. 7F). Taken together, these results imply that B cell-derived IL-10 does not affect CD8 T cell responses during VL. In contrast, when we analyzed granulocyte B expression in NK1.1+ cells, we noticed a significant increase in granulocyte B+ NK1.1+ cells in the μMT/H10−/− group at day 6 p.i. (Fig. 8E). As observed in infected mice depleted of MZB (Fig. 7G), the frequency of IFN-γ+ CD4 T cells was also slightly greater in the μMT/H10−/− group at day 6 p.i. (Figs. 8B and 8C). By day 13 p.i., though, both groups of mice had comparable frequencies of IFN-γ+ CD4 T cells. Interestingly, μMT/H10−/− mice had a significantly lower parasite burden at day 12 p.i. compared with the WT/H10−/− group (Fig. 8G). Taken together, our data suggest that IL-10 production by B cells does not interfere with the development of CD8 T cell responses; however, it significantly reduces the cytotoxic capacity of NK and/or NKT cells, and slightly contributes to delay the onset of Th1 responses and to exacerbate disease during the early stages of infection.

**Discussion**

In this study, we show that MZB are involved in the suppression of CD8 and CD4 effector functions during the early stages of *L. donovani* infection, and that this suppression contributes to disease...
exacerbation. Moreover, we demonstrate that B cells also prevent the generation of T EM by a yet unidentified mechanism. We have previously shown that *L. donovani* induces defective CD8 T cell responses with limited expansion capacity (23). Interestingly, although most of the CD8 T cells during peak expansion are CD62L lo, they do not express other markers typically associated with effector cells, such as PD-1 (23), Fas (data not shown), or KLRG1, and the majority of the CD62L lo effectors are uncharacteristically highly positive for Bcl2 (data not shown). This phenotype is not typically associated with effector CD8 T cells. Moreover, 70–80% of the CD8 surviving contraction are T CM-like cells and T EM are not generated (23). Thus, it seems that there is a bias toward the development of T CM-like cells rather than effectors during the early stages of *L. donovani* infection. The transition of effector to memory CD8 T cells is affected by ex-
tracellular stimuli such as costimulation, the strength and timing of TCR–Ag interactions, and inflammatory cytokines (37–39). In the experimental model for VL, Ag may only be available in low quantities during the first few days of infection, because parasites are quickly segregated into macrophages. Moreover, the parasite actively suppresses IL-12 production in macrophages (40, 41), and DCs only transiently express this cytokine (42). Hence parasite-specific CD8 T cells are primed in a low-Ag, IL-12–poor environment. This could explain why only a small percentage of effector CD8 T cells expresses KLRGB1 and 70–80% of the CD8 surviving contraction are TCM-like cells (31).

However, in B cell-deficient mice, IL-12 production by DCs was sustained during the first week of infection. Consequently, in agreement with the literature (31), a larger percentage of effector CD8 T cells expressed KLRGB1, and CD8 TEM were generated in those mice. L. donovani infection in μMT mice also resulted in a stronger Th1 response compared with WT mice. CD4 T cells are known to provide help during CD8 T cell priming, among others by secreting IL-2. A recent study has shown that priming of CD8 T cells in an IL-2–deficient environment results in decreased KLRGB1 and granzyme B expression by effector CD8 T cells and in premature upregulation of CD127 and CD62L (43). Hence Leishmania-specific CD8 T cells in WT mice may be primed in an environment in which both IL-12 and IL-2 are present only at very low levels.

The mechanism by which B cells interfere with the development of T cell responses is still not clear and is only partly mediated by IL-10, which is known to suppress IL-12 production by DCs and also Th1 effector functions (44). MZB depletion and IL-10R blockade resulted in increased KLRGB1 and granzyme B expression by effector CD8 T cells and in premature upregulation of CD127 and CD62L (43). Hence Leishmania-specific CD8 T cells in WT mice may be primed in an environment in which both IL-12 and IL-2 are present only at very low levels.

The IL-10-rich environment present during the first week of infection may explain the partial suppression on T cell functions by MZB, which contributes to suppress NK1.1+ cells, CD8, and CD4 T cell responses during the early stages of infection, which then contribute to suppress protective Th1 responses. A recent study has shown that signaling via MyD88 in B cells suppresses NK cells and T cell responses in Salmonella typhimurium-infected mice (56). In this study, IL-10 was an essential mediator of the B cell inhibitory effects on NK and T cells, and MyD88 was crucial for mediating these effects. Inhibition of T cell functions by B cells has also been reported in tumor immunity (17). A recent vaccination study that used CpG as an adjuvant has shown that B cells suppressed CD8 T cell responses (57).

In conclusion, we have identified a novel inhibitory function for MZB, which contribute to suppress NK1.1+ cells, CD8, and CD4 T cell responses during the early stages of L. donovani infection. Suppression of NK1.1+ cells and CD4 T cells is only partly mediated by MyD88-dependent production of IL-10 by B cells. Furthermore, depletion of MZB results in increased resistance to infection.

Hence the early recognition of some pathogens by MZB contributes to shape the development of adaptive T cell responses and may help the establishment of chronic infections.

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Disclosures

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References
