Regulatory B Cell (B10 Cell) Expansion During *Listeria* Infection Governs Innate and Cellular Immune Responses In Mice

Mayuka Horikawa[*,1], **Eric T. Weimer**[*,1], **David J. DiLillo**[*], **Guglielmo M. Venturi**[*], **Rosanne Spolski**[‡], **Warren J. Leonard**[‡], **Mark T. Heise**[†], and **Thomas F. Tedder**[*]

[*]Department of Immunology, Duke University Medical Center, Durham, NC 27710

[†]Carolina Vaccine Institute, University of North Carolina, Chapel Hill, NC 27599

[‡]Laboratory of Molecular Immunology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892-1674, USA

Abstract

Pathogens use numerous methods to subvert host immune responses, including the modulation of host IL-10 production by diverse cell types. However, the B cell sources of IL-10 and their overall influence on innate and cellular immune responses have not been well characterized during infections. Using *Listeria* as a model pathogen, infection drove the acute expansion of a small subset of regulatory B cells (B10 cells) that potently suppress inflammation and autoimmunity through the production of IL-10. Unexpectedly, spleen bacteria loads were 92–97% lower in B10 cell-deficient CD19$^{-/-}$ mice, in mice depleted of mature B cells, and in mice treated with CD22 mAb to preferentially deplete B10 cells before infection. By contrast, the adoptive transfer of wild type B10 cells reduced bacterial clearance by 38-fold in CD19$^{-/-}$ mice through IL-10-dependent pathways. B10 cell depletion using CD22 mAb significantly enhanced macrophage phagocytosis of *Listeria* and their production of IFN-γ, TNF-α, and nitric oxide *ex vivo*. Accelerated bacteria clearance following B10 cell depletion significantly reduced Ag-specific CD4$^+$ T cell proliferation and cytokine production, but did not alter CD8$^+$ T cell responses. B10 cell regulatory function during innate immune responses was nonetheless dependent on cognate interactions with CD4$^+$ T cells since B10 cells deficient in IL-10, MHC-II or IL-21 receptor expression did not influence *Listeria* clearance. Thus, *Listeria* manipulates immune responses through a strategy of immune evasion that involves the preferential expansion of endogenous B10 cells that regulate the magnitude and duration of both innate and cellular immune responses.

Keywords

B cells; *Listeria monocytogenes*; innate immunity; regulatory B cells; B10 cells

INTRODUCTION

Infectious organisms use numerous methods to subvert host immune responses, including the modulation of host cytokine production (1, 2). *Listeria monocytogenes* is a frequently

used model for both infection and immune response evasion in mice (3). This facultative Gram-positive bacterium also causes listeriosis in humans. *Listeria* typically replicates within the cytosol of macrophages and epithelial cells, which protects the bacteria from deletion during humoral immune responses. *Listeria* and several other pathogens also induce serum IL-10 (4), a potent cytokine that can facilitate pathogen survival by negatively regulating both innate and acquired host immune responses (5–9). Consequently, IL-10$^{-/-}$ mice are resistant to infection with *Listeria* and other pathogens (7, 10, 11). Multiple cell types are capable of producing IL-10, including activated macrophages, T and B lymphocytes, mast cells, dendritic cells, and keratinocytes (5, 12, 13). How these distinct IL-10 producing cell subsets individually dictate the quality, quantity, and direction of host immune responses is generally unknown.

In addition to the classical immune responses that are induced during pathogen infections, host immunoregulatory pathways may also become activated to limit the magnitude and duration of immune responses and to prevent excessive immunopathology. B cell IL-10 production has been implicated in the negative regulation of immune responses against bacteria, helminths and parasitic protozoa. B cell-derived IL-10 can suppress *Salmonella* immunity by inhibiting neutrophils, natural killer cells, and inflammatory T cells (14). IL-10 produced by adoptively transferred peritoneal cavity B-1 cells also inhibits *Borrelia hermsii* clearance in B cell-deficient mice (15). B cells from *Brudia pahangi* infected mice produce IL-10, which down regulates B cell expression of the B7-1 and B7-2 costimulatory molecules (16). *Schistosoma mansoni* infection also induces spleen IL-10-secreting B cells in mice, which protects against allergic hypersensitivity (17, 18). B cells also produce IL-10 following *Leishmania major* infection, which can inhibit dendritic cell IL-12 production *in vitro* (8). Despite these individual findings, the B cell sources of IL-10 have not been well characterized in most cases and their overall influence on innate and cellular immune responses during infections has not been examined.

A subset of immunoregulatory B cells has been functionally identified in mice and humans by their ability to express IL-10 within 5 h of *ex vivo* stimulation (13, 19, 20). These rare IL-10-competent B cells have been functionally labeled as "B10 cells" to distinguish them from other regulatory B cell subsets that are known to exist (21, 22). Regulatory B10 cells limit inflammation and disease in mouse models of contact hypersensitivity, experimental autoimmune encephalomyelitis, lupus, allergy, and collagen-induced arthritis (20, 23–26). Regulatory B10 cells are found in the spleens of naïve mice at low frequencies (1–5%), where they predominantly represent a subset of the CD1d^{hi}CD5^{+}CD19^{hi} B cell subpopulation (13, 20, 27) that shares overlapping cell surface markers with multiple phenotypically-defined B cell subsets (23, 28, 29). Agonistic CD40 signals or LPS can also "mature" additional CD1d^{hi}CD5^{+} B10 progenitor (B10pro) cells to acquire IL-10 competence (21, 30, 31). Cognate interactions between T cells and B10 cells induce Ag-specific regulatory B10 effector cells that secrete Il-10 and regulate autoimmunity *in vivo* (32). IL-10-competent regulatory B cells that parallel mouse B10pro and B10 cells have also been identified in healthy and autoimmune humans (33). The capacity of human and mouse B10pro and B10 cells to express IL-10 is central to their negative regulation of inflammation, autoimmunity, and adaptive and innate immune responses (13, 20, 26, 30, 32, 34–36). B cell expression of cytoplasmic IL-10 protein parallels both their expression of IL-10 transcripts and secretion of IL-10 as measured by ELISA (13, 20, 26, 30, 32, 34–36). The current studies demonstrate that *Listeria* infection induces acute B10 cell expansion and IL-10 production in mice, which inhibits macrophage activation. As a consequence, bacteria loads are increased, thereby promoting T cell expansion and the development of cellular immunity. Conversely, B10 cell-deficiency or depletion uniquely reshapes the course and magnitude of both innate and cellular immune responses during infections.

MATERIALS AND METHODS

Mice and immunotherapy

C57BL/6, *Il*-10$^{-/-}$ (B6.129P2-*Il10tmlCgn*/J), and Tiger (B6.129S6-*Il10tm1Flv*/J) mice (37) were from The Jackson Laboratory (Bar Harbor, ME). A gene dose-dependent decrease in IL-10 production was not observed in homozygous Tiger mice, which occurs with T cells (37). MHC-II$^{-/-}$ (B6.129-*H2-Ab1tm1Gru*B2m*tmJae*N17) mice (38) were from Taconic Farms, Inc. (Hudson, NY). CD19$^{-/-}$ mice (39) were backcrossed with C57BL/6 mice for 14 generations. CD22$^{-/-}$CD154Tg mice (31) were homozygous at both genetic loci. OT-II transgenic (Thy1.2^{+}) mice (40) were crossed to Thy1.1^{+} mice to generate OT-II/Thy1.1-expressing T cells. The OVA-specific TCR transgenic line (OT-I) was as described (41). CD20$^{-/-}$ and IL-10$^{-/-}$CD20$^{-/-}$ mice were as described (20). IL-21$^{-/-}$ mice were as described (42). All mice were bred in a specific pathogen-free barrier facility and used at 8–12 wk of age.

Total B cells were depleted *in vivo* using sterile, endotoxin-free CD20 mAb (MB20-11, IgG2c) as described (43). B10 cells were depleted *in vivo* using sterile, endotoxin-free CD22 (MB22-10, IgG2c) mAb as described (26, 27). CD20, CD22 or isotype-matched control mAb (250 μg in 200 μl PBS) were injected into the peritoneal cavity. All animal studies and procedures were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Duke University Institutional Animal Care and Use Committee.

Cell preparation and immunofluorescence analysis

Single-cell leukocyte suspensions from spleens and peripheral lymph nodes (pooled bronchial, axillary, and inguinal) were generated by gentle dissection. For macrophage assays, splenocytes were adhered to 6-well plates for 3 h. The plates were then washed twice with sterile PBS. Adherent macrophages (>90% F4/80^{+}CD11b^{+}) were removed by scraping and stimulated with LPS (1 μg/ml) for 4 or 24 h.

FITC-, PE-, PE Cy5-, PE Cy7-, or allophycocyanin (APC)-conjugated CD1d (1B1), CD4 (H129.19), CD8 (53-6.7), CD5 (53-7.3), CD11b (M1/70), CD11c (N418), CD19 (6D5), F4/80 (BM8), CD49b (DX-5), CD80 (16-10A1), CD86 (GL1), Ly6C (AL21), Ly6G (1A8), and Thy1.1 (HIS5.1) mAbs were from BD Biosciences (San Diego, CA). Intracellular cytokine staining was performed using cytofix/cytoperm (BD Biosciences) with mAbs reactive with IL-10 (JES5-16E3), IFN-γ (XMG1.2), and TNF-α (MP6-XT22) (from eBioscience, San Diego, CA). Macrophages were identified by cell surface F4/80 and CD11b expression. For two- to six-color immunofluorescence analysis, single-cell suspensions (10^{6} cells) were stained at 4°C using predetermined optimal concentrations of mAb for 20 min as described (44). Background staining was assessed using nonreactive, isotype-matched control mAbs (Caltag Laboratories, San Francisco, CA). Cells with the forward and side light scatter properties of lymphocytes or macrophages were analyzed using a BD FACSCanto II (BD Biosciences). Dead cells were detected using a LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Life Technologies) before cell surface staining. All flow cytometry experiments were gated on viable, single lymphocytes or macrophages. For macrophage intracellular cytokine staining, splenocytes were harvested 1 d following infection and incubated with brefeldin A (1 μg/ml; eBioscience) for 5 h in the absence of stimulation. For Ag-specific T cell intracellular cytokine staining, lymphocytes were stimulated *in vitro* with OVA$_{329-339}$ or OVA$_{257-264}$ (10 μg/ml; American Peptide, Sunnyvale, CA) in the presence of brefeldin A (1 μg/ml) for 5 h before staining.

B10 cell analysis

Intracellular IL-10 expression was visualized by immunofluorescence staining and analyzed by flow cytometry as described (45). Briefly, isolated leukocytes or purified cells were resuspended (2×10^6 cells/ml) in complete medium (RPMI 1640 media containing 10% FCS, 200 µg/ml penicillin, 200 U/ml streptomycin, 4 mM L-glutamine, and 5×10^{-5} M 2-ME, all from Life Technologies, Grand Island, NY) with LPS (10 µg/ml, *Escherichia coli* serotype 0111:B4; Sigma-Aldrich), phorbol 12-myristate 13-acetate (50 ng/ml; Sigma-Aldrich), ionomycin (500 ng/ml; Sigma-Aldrich, St. Louis, MO), and monensin (2 µM; eBioscience) for 5 h in 48-well flat-bottom plates. In some experiments, the cells were incubated for 48 h with an agonistic anti-mouse CD40 mAb (1 µg/ml; HM40-3; BD Biosciences) as described (45). For IL-10 detection, Fc gamma receptors II and III were blocked with mouse Fc receptor mAb (2.4G2; BD Biosciences), and dead cells were identified using a LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Life Technologies) before cell surface molecule immunofluorescence staining. Cells stained for cell surface molecules were fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences), according to the manufacturer's instructions, and stained with APC-conjugated anti-mouse IL-10 mAb.

Infection, recovery and labeling of bacteria

Mice were infected i.v. with *Listeria* strain 10403S engineered to express chicken ovalbumin (OVA; kindly provided by Dr. Hao Shen, University of Pennsylvania, Philadelphia, PA) (40, 46). For immunizations, bacteria were inoculated into brain heart infusion media supplemented with erythromycin (5 µg/ml) overnight at 37°C. An aliquot (1 ml) of the overnight bacteria culture was washed twice with sterile PBS and diluted in PBS for infections as described (47). Mice received diluted bacteria (8×10^5 cfu, 0.1 LD_{50} in 200 µl) through the lateral tail vein. The inoculum was subsequently serially diluted on brain heart infusion plates and counted to verify the infection dose. At this infection dose, there are no symptoms of weight loss or core body temperature changes.

To recover bacteria from infected mice, spleens were harvested and homogenized using a Tissuemizer (Fischer Scientific, Pittsburgh, PA) in 4 ml of sterile PBS. The spleen homogenates were serially diluted in sterile PBS 10-fold until a dilution of 10^5. A 100 µl aliquot of each dilution was spread on brain heart infusion plates supplemented with erythromycin (5 µg/ml) and incubated at 37°C for 24–48 h before the colonies were counted to determine bacteria numbers.

Bacteria were labeled as previously described (48, 49). Briefly, bacteria were incubated with 100 µM CFSE (Invitrogen) for 10 minutes at 37°C, washed in PBS and immediately used for the *in vitro* infection of macrophages. CFSE-labeling did not affect bacteria viability (data not shown) as described (50). The number of phagocytosed bacteria was determined using a similar protocol as that used for other Gram-positive bacteria. Briefly, macrophages were infected at a multiplicity of infection of 10 for 2 h before the macrophages were washed, harvested, and assessed for CFSE[+] bacteria. Extracellular bacteria were eliminated from the analysis by adding trypan blue (0.2% final) prior to flow cytometry analysis as described (51).

Lymphocyte subset isolation and adoptive transfer experiments

Splenic B cells from naïve wild type, IL-10^{−/−}, CD22^{−/−}CD154Tg, CD20^{−/−} or CD20^{−/−}IL-10^{−/−} mice were isolated using CD19 mAb-coated microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. When necessary, B cells were enriched a second time using a fresh magnetic-activated cell sorting column to obtain >95% cell purities. CD1d^{hi}CD5^{+} and CD1d^{lo}CD5^{−} B cells were identified by immunofluorescence

staining and purified (95–98%) using a FACSAria flow cytometer (BD Biosciences). B cell subset isolation did not induce detectable IL-10 production (data not shown). After purification, 10^6 cells were immediately transferred into appropriate recipient mice through a lateral tail vein. Without appropriate *ex vivo* stimulation, the donor B cells remained IL-10 negative following transfers into recipient mice. Splenic $CD4^+$ or $CD8^+$ T cells, and macrophages were purified using $CD4^+$ T cell isolation kits, $CD8^+$ T cell isolation kits, and CD11b mAb-coated microbeads (Miltenyi Biotec), respectively. When necessary, the cells were enriched a second time using a fresh magnetic-activated cell sorting column to obtain >95% cell purities. Isolated $CD4^+$ or $CD8^+$ T cells from naïve OT-II/Thy1.1$^+$ or OT-I mice were labeled with CFSE (5 µM) for 10 min at 37°C, with $3×10^6$ cells transferred i.v. into recipient mice.

ELISAs

For assessing IFN-γ production, purified macrophages were stimulated *in vitro* with media or LPS (1 µg/ml) for 4 or 24 h. Tissue culture supernatant fluid IFN-γ concentrations were measured by ELISA (BD Biosciences) according to the manufacturer's instructions. For assessing IL-10 secretion, purified $CD19^+$, $CD1d^{hi}CD5^+$, or $CD1d^{lo}CD5^-$ B cells (4 × 10^5/0.2 ml of complete medium) from naïve or *Listeria* infected mice were cultured in triplicate using 96-well flat-bottom tissue culture plates. Culture supernatant fluid IL-10 concentrations were quantified using IL-10 OptEIA ELISA kits (BD Pharmingen) following the manufacturer's protocols.

Statistical analyses

Differences between means were assessed using the Student's t-test (GraphPad Prism software, version 5.00, San Diego, CA). Significant differences between data sets that were not normally distributed or were of unequal variance were determined using the Mann–Whitney rank sum test. P values of <0.05 were considered significant.

RESULTS

B10 cells expand during *L. monocytogenes* infection

The effects of infection on spleen $CD19^+$ B cells was assessed in wild type mice given *L. monocytogenes* engineered to express chicken OVA (40, 46). Following 3 d of infection, there was little change in spleen B cell or $CD4^+$ T cell numbers relative to control mice given PBS (Fig. 1A). B cell numbers increased significantly by day 5 along with a 46% (p=0.03) reduction in $CD4^+$ T cell frequencies, with normal cell numbers by 8 d post-infection, as described (52). *Listeria* infection alone did not induce measurable IL-10 expression in B cells cultured *ex vivo* with monensin alone (Fig. 1B). Therefore, changes in regulatory B10 cell frequencies and numbers during *Listeria* infection were assessed following *ex vivo* B cell stimulation with PMA, ionomycin, and monensin (PIM) for 5 h to induce cytoplasmic IL-10 accumulation that was visualized by immunofluorescence staining with flow cytometry analysis as described (45). LPS was added to the 5 h PIM cultures (LPIM) as it marginally enhances B10 cell enumeration (30). The advantage of using cytoplasmic IL-10 expression to enumerate B10 cells is that this technique demonstrates the frequency and numbers of B cells that are competent to express IL-10 and the level of IL-10 synthesis once induced.

Following *Listeria* infection, spleen B10 cell numbers dropped by day 3, but expanded 2-fold by 5 d post-infection (p 0.03) and remained elevated in the spleen and lymph nodes by d 8 when compared with uninfected control mice. The spleen $CD1d^{hi}CD5^+$ B cell subset was also significantly expanded by 5 d post-infection, while the $CD1d^{lo}CD5^+$ B cell subset did not increase significantly (Fig. 1C). When B10 cell numbers were quantified using Tiger

IL-10/GFP reporter mice (37), similar results were obtained. *Listeria* infection of Tiger mice did not induce detectable GFP expression, but GFP$^+$ cell frequencies and numbers paralleled B10 cell cytoplasmic IL-10 expression in wild type mice following LPIM stimulation (Fig. 1B and D). Thus, B10 cell numbers increased significantly following infection.

Agonistic CD40 signals induce B10pro cell maturation, which renders these cells competent to express IL-10 following 5 h of LPIM stimulation (26, 30). Both B10 and B10pro cells (B10+B10pro) are quantified in this assay, as the B cells that acquire IL-10 competence *in vitro* (e.g., matured B10pro cells) cannot be differentiated from preexisting B10 cells that express cytoplasmic IL-10 after 5 h of LPIM stimulation. Spleen B10+B10pro cell numbers decreased by 87% (p=0.02) on d 3 post-infection, but returned to uninfected levels by 5–8 d post-infection (Fig. 1E). Infection did not significantly affect lymph node B10+B10pro cell numbers.

Listeria infection induced modest but measurable IL-10 secretion by purified spleen B cells cultured *ex vivo* without stimulation (Fig. 1F). LPS stimulation *in vitro* induced significant B cell IL-10 production, with more IL-10 produced by B cells isolated from *Listeria*-infected mice. The CD1d^{hi}CD5$^+$ B cell subset was enriched 10-fold for IL-10-secreting B cells relative to CD1d^{lo}CD5$^-$ B cells (Fig. 1G). Thus, *Listeria* infection was predominantly activating a subset of B cells within the CD1d^{hi}CD5$^+$ B10+B10pro cell pool.

Spleen FoxP3$^+$CD25$^+$CD4$^+$ regulatory T cell (Treg) frequencies or numbers were not significantly changed following *Listeria* infection (d 5, Fig. 1H). However, the frequency and number of activated CD4$^+$ T cells (FoxP3$^-$CD25$^+$) increased by five-fold. Thus, the B10 cell subset preferentially expanded following *Listeria* infection, while the B10pro and Treg cell subsets did not.

B10 cells inhibit *L. monocytogenes* clearance

The impact of B cells and B10 cells on *Listeria* infection was assessed using three different mouse models; CD19$^{-/-}$ mice, CD20 mAb-treated mice, and CD22 mAb-treated mice. Most B cell subsets develop normally in CD19$^{-/-}$ mice (53), but they are deficient in B10 cells (13, 26, 30, 32, 34, 35, 54, 55). Relative to wild type littermates, total spleen B cell numbers were 32% (p=0.006) lower in uninfected and day 5 infected CD19$^{-/-}$ mice (Fig. 2A). However, B10 cell numbers were reduced by 83% (p=0.001) in CD19$^{-/-}$ mice, and this did not change with infection. CD20 mAb treatment depletes all mature B cells and B10 cells (13, 20, 27, 56). Total B cell depletion by CD20 mAb reduced B10 cell and B cell numbers by >97% (p<0.0001) in both control and *Listeria*-infected mice (Fig. 2B). CD22 mAb treatment does not significantly reduce peripheral B cell numbers but depletes the majority of spleen CD1d^{hi}CD5$^+$ B cells, which results in significant B10 cell reductions (26, 31, 57). The term "depletion" as used in conjunction with CD20 and CD22 mAb treatments in this study designates significant reductions in the frequencies and numbers of tissue lymphocytes that either express a mature B cell phenotype (CD20 mAb), a CD1d^{hi}CD5$^+$ phenotype (CD22 mAb), or that are able to express IL-10 (CD20 or CD22 mAb) as published (13, 20, 26, 27, 31, 56, 57). CD22 mAb treatment reduced CD1d^{hi}CD5$^+$ (B10 cell-enriched) cell numbers by 60% (p=0.01) in uninfected mice and by 83% (p=0.003) in infected mice, but did not significantly affect total B cell numbers (Fig. 2C). B10 cell numbers were reduced by 80% (p=0.002) in uninfected mice and by 85% (p=0.03) in infected mice following CD22 mAb treatment. In contrast with CD20 and CD22 mAb-treated mice, B10 cell numbers increased 1.6-fold (p=0.02) in control mAb-treated mice by 5 d post-infection when compared with uninfected littermates (Fig. 2B–C). Thus, CD19-deficiency, total B cell depletion using CD20 mAb, and CD22 mAb treatment each significantly reduced B10 cell numbers following infection.

Spleen bacteria loads were reduced significantly (p 0.01) by CD19-deficiency (97%), total B cell depletion using CD20 mAb (92%), or CD22 mAb treatment (93%, notice different scale on bar graph) as early as 3 d post-infection when compared with wild type or control mAb-treated littermates (Fig. 2D). Spleen bacteria loads also remained 95% lower 5 d post-infection in CD22 mAb-treated mice when compared with control mAb-treated littermates. Similar results were obtained for liver bacteria loads (data not shown). Thus, CD19-deficiency, total B cell depletion, and CD22 mAb treatment significantly accelerated *Listeria* clearance *in vivo*.

Adoptive transfer experiments were used to verify that bacterial clearance was accelerated due to the absence of regulatory B10 cells. Purified spleen $CD1d^{hi}CD5^+$ (B10 cell-enriched) and $CD1d^{lo}CD5^-$ (B10 cell-deficient) B cell subsets from wild type mice were adoptively transferred into B10 cell-deficient $CD19^{-/-}$ mice prior to *Listeria* infection (Fig. 2E). Spleen bacteria loads were 38-fold higher in $CD19^{-/-}$ mice given $CD1d^{hi}CD5^+$ B cells in comparison with littermates given PBS or $CD1d^{lo}CD5^-$ B cells 3 d post-infection (p=0.01). $CD1d^{hi}CD5^+$ and $CD1d^{lo}CD5^-$ B cells from $IL-10^{-/-}$ mice were also transferred into $CD19^{-/-}$ mice before infection. However, spleen bacteria levels were equivalent in mice that received PBS, $IL-10^{-/-}$ $CD1d^{hi}CD5^+$ B cells, or $IL-10^{-/-}$ $CD1d^{lo}CD5^-$ B cells (3 d post-infection; p=0.29). Thus, three independent mouse models where B cells or subsets of B cells were depleted and adoptive transfer experiments demonstrated that B10 cells inhibited *Listeria* clearance through IL-10-dependent mechanisms.

B10 cell depletion enhances macrophage cytokine production

The effect of B10 cell depletion on spleen macrophage and neutrophil numbers and activation was assessed *ex vivo* 1 d after *Listeria* infection. In wild type mice, CD22 mAb treatment did not significantly alter the frequencies or numbers of spleen $CD11b^+Ly6C^+$ macrophages or $CD11b^+Ly6G^+$ neutrophils (Fig. 3A). Similarly, CD22 mAb treatment did not result in spleen $F4/80^+CD11b^+$ macrophage activation as measured by *ex vivo* CD80 and CD86 expression (Fig. 3B). However, CD22 mAb treatment significantly enhanced *ex vivo* macrophage production of IFN-γ (2.6-fold, p=0.02) and TNF-α (40%, p=0.03) when compared with macrophages from control mAb-treated mice (Fig. 3C). By contrast, CD22 mAb treatment did not significantly affect IFN-γ production by $CD11c^+$ (dendritic) or $DX-5^+$ (natural killer) cells (data not shown). Thus, *in vivo* CD22 mAb treatment significantly enhanced macrophage cytokine production.

Adoptive transfer experiments were carried out in parallel with the experiments described above to verify that the absence of regulatory B10 cells following CD22 mAb treatment enhanced infection-induced macrophage cytokine production. First, spleen $CD1d^{hi}CD5^+$ and $CD1d^{lo}CD5^-$ B cells were purified from $CD22^{-/-}CD154Tg$ mice, in which the majority of $CD1d^{hi}$ B cells are B10 cells (31). These B cell subsets were then adoptively transferred into wild type mice that were given CD22 mAb prior to *Listeria* infection. Since the adoptively transferred B cells are CD22-deficient, they are not affected by CD22 mAb treatment (57). IFN-γ and TNF-α production by macrophages from CD22 mAb-treated mice (Fig. 3C) were reduced by 67% and 54% (p=0.01), respectively, by the adoptive transfer of $CD1d^{hi}CD5^+$ $CD22^{-/-}$ B cells but were not affected by the adoptive transfer of $CD1d^{lo}CD5^-$ $CD22^{-/-}$ B cells (Fig. 3D). In fact, IFN-γ and TNF-α production were virtually identical in macrophages from CD22 mAb-treated mice given $CD1d^{hi}CD5^+$ $CD22^{-/-}$ B cells and untreated control mice. Thus, the CD22 mAb-induced effects were directly attributable to $CD1d^{hi}CD5^+$ B cell depletion.

A second set of adoptive transfer experiments were carried out to confirm that $CD1d^{hi}CD5^+$ B cell regulation of macrophage cytokine production was IL-10 dependent. Spleen $CD1d^{hi}CD5^+$ and $CD1d^{lo}CD5^-$ B cells were purified from $CD20^{-/-}$ or $IL-10^{-/-}CD20^{-/-}$

mice and adoptively transferred into wild type mice that were given CD20 mAb prior to *Listeria* infection. Macrophage IFN-γ and TNF-α production were elevated similarly in mice given either CD20 or CD22 mAb relative to macrophages isolated from control mAb-treated *Listeria*-infected mice (Fig. 3C–E). However, the adoptive transfer of CD1d^{hi}CD5$^+$ B cells from CD20$^{-/-}$ mice reduced IFN-γ and TNF-α production in mice given CD20 mAb by 43% and 60%, respectively, in comparison with CD1d^{lo}CD5$^-$ CD20$^{-/-}$ B cells (Fig. 3E). Neither CD1d^{hi}CD5$^+$ nor CD1d^{lo}CD5$^-$ B cells from IL-10$^{-/-}$CD20$^{-/-}$ mice affected IFN-γ and TNF-α production by macrophages from CD20 mAb-treated mice (p=0.8). Thus, endogenous B10 cells found within the CD1d^{hi}CD5$^+$ B cell subpopulation negatively regulated macrophage cytokine production through IL-10-dependent pathways.

To verify that the *ex vivo* changes in macrophage IFN-γ secretion were durable, macrophages purified from CD22 or control mAb-treated mice were cultured with LPS *in vitro*. Following 4 h of LPS stimulation, macrophages from CD22 mAb-treated mice secreted 62% (p=0.03) more IFN-γ than macrophages from control mAb-treated mice (Fig. 3F). After 24 h of culture without LPS stimulation, macrophages from CD22 mAb-treated mice secreted 9.2-fold (p=0.01) more IFN-γ than macrophages from control mAb-treated mice. After LPS stimulation, macrophages from CD22 mAb-treated mice secreted 30% (p=0.03) more IFN-γ than macrophages from control mice. Nitric oxide production by macrophages from CD22 mAb-treated mice was also 40% (p=0.04) higher than for macrophages from control mAb-treated mice (Fig. 3G). Thereby, B10 cell negative regulation of macrophage cytokine and nitric oxide production *in vivo* was persistent and not reversed by *ex vivo* stimulation.

B10 cell depletion enhances macrophage phagocytosis of *Listeria*

Since macrophages play an important role in host defense during *Listeria* infections, the ability of spleen CD11b$^+$ macrophages from mice treated with either CD22 or control mAb for 7 d were analyzed for their ability to phagocytose CFSE-labeled *Listeria ex vivo* in the absence of extrinsic stimulation. Trypan blue was used to quench CFSE fluorescence from cell surface-associated bacteria during flow cytometry analysis as described (51). Within two hours, twice as many macrophages from CD22 mAb-treated mice (p=0.01) had internalized bacteria when compared with macrophages from control mAb-treated mice (Fig. 3H). Furthermore, macrophages from CD22 mAb-treated mice that had internalized bacteria contained twice as many (p=0.01) bacteria as macrophages from control mAb-treated mice. Thus, *in vivo* B10 cell depletion following CD22 mAb treatment significantly enhanced the *ex vivo* phagocytic capacity of spleen macrophages.

B10 cell depletion reduces Ag-specific CD4$^+$ T cell activation

The effect of CD22 mAb-induced B10 cell depletion on cellular immune responses was quantified in wild type mice. CD22 mAb treatment did not significantly affect spleen CD4$^+$ or CD8$^+$ T cells in either uninfected or *Listeria*-infected mice (Fig. 4A). However, as shown in Figure 1A, *Listeria* infection results in a transient reduction in CD4$^+$ T cell numbers, and this effect was blocked in mice given CD22 mAb but not control mAb. Therefore, B10 cell depletion reduced the effect of *Listeria* infection on spleen CD4$^+$ T cell frequencies.

The involvement of B10 cells during Ag-specific CD4$^+$ T cell proliferation and function was quantified *in vivo* using Thy1.1$^+$ congenic mice that express an OVA-specific T cell receptor transgene (40). CD4$^+$ T cells purified from OT-II mice were CFSE-labeled and adoptively transferred into CD22 or control mAb-treated wild type mice 1 d prior to *Listeria* infection. Uninfected control mice were given PBS. Spleen Thy1.1$^+$ OT-II T cell proliferation was assessed 3 d later by measuring CFSE dilution. Relatively few OT-II T cells from CD22 mAb-treated mice diluted CFSE (p<0.001, Fig. 4B), while OT-II T cells

from infected mice given control mAb proliferated vigorously, with 75% of the T cells diluting CFSE. Consistent with these results, half as many OT-II T cells isolated from CD22 mAb-treated mice were induced to produce IFN-γ when stimulated *ex vivo* with OVA$_{323-339}$ peptide in comparison with OT-II T cells from infected mice given control mAb (p=0.005; Fig. 4C). CD4$^+$ OT-II T cell production of IL-2 was equivalent in both CD22 and control mAb-treated mice after infection. Thus, CD4$^+$ T cell proliferation and cytokine production were reduced in mice given CD22 mAb to deplete CD1d^{hi}CD5$^+$ B10 cells.

The effects of B10 cell depletion using CD22 mAb on Ag-specific CD8$^+$ T cell proliferation and function was also quantified *in vivo* using mice that express an OVA-specific T cell receptor transgene (41). CD8$^+$ T cells purified from OT-I mice were CFSE-labeled and adoptively transferred into CD22 or control mAb-treated wild type mice 1 d prior to *Listeria* infection, with spleen OT-I T cell proliferation assessed 3 d later. OT-I T cells proliferated vigorously in infected mice given control or CD22 mAb, with >95% of the T cells diluting CFSE (p=0.4; Fig. 4D). Ag-specific OT-I T cells from B10 cell-depleted or control-treated mice also produced similar amounts of IFN-γ when stimulated *ex vivo* with OVA$_{257-264}$ peptide (p=0.7; Fig. 4E). Thus, B10 cell depletion using CD22 mAb reduced Ag-specific CD4$^+$ but not CD8$^+$ T cell proliferation and cytokine production *in vivo*.

B10 cell regulation is dependent on cognate interactions with CD4$^+$ T cells

B10 cell regulation of T cell-mediated autoimmunity requires cognate interactions with CD4$^+$ T cells through MHC-class II and IL-21 dependent pathways to induce B10 cell effector function and IL-10 production (32). Whether inflammatory signals or bacterial products can induce regulatory B10 cell function independent of cognate T cell interactions was therefore examined using CD19$^{-/-}$ mice infected with *Listeria.* Remarkably, the adoptive transfer of wild type CD1d^{hi}CD5$^+$ B cells resulted in a subsequent 58-fold (p 0.01) increase in spleen bacteria loads following *Listeria* infection when compared to littermates given PBS or CD1d^{lo}CD5$^-$ B cells (Fig. 5A). By contrast, the adoptive transfer of either CD1d^{hi}CD5$^+$ or CD1d^{lo}CD5$^-$ B cells from MHC-II$^{-/-}$ mice, IL-21 receptor deficient (IL-21R$^{-/-}$) mice, or IL-10$^{-/-}$ mice did not significantly affect bacterial clearance. Thus, IL-10, IL-21, and MHC-II expression were required to elicit regulatory B10 cell function during innate immune responses to this bacterial pathogen.

DISCUSSION

The current studies demonstrate that *Listeria* manipulates early immune responses through a strategy of immune evasion that involves preferential B10 cell expansion. Specifically, spleen and lymph node B10 cell frequencies and numbers were acutely expanded by day 5 of infection (Fig. 1), with B10 cell production of IL-10 dramatically reducing bacteria clearance (Fig. 2). Enhanced bacteria clearance in the absence of B10 cells or following their *in vivo* depletion paralleled significantly improved macrophage phagocytosis of bacteria (Fig. 3H) and their significantly increased production of IFN-γ, TNF-α and nitric oxide *ex vivo* (Fig. 3C–G). Remarkably, B10 cell negative regulation of macrophage cytokine production persisted even when the macrophages were restimulated *ex vivo* with LPS (Fig. 3F). Enhanced bacteria clearance following B10 cell depletion using CD22 mAb also significantly reduced Ag-specific CD4$^+$ T cell expansion in response to *Listeria* infection, but did not reduce Ag-specific CD8$^+$ T cell expansion (Fig. 4). Thereby, B10 cell expansion normally inhibits *Listeria* clearance *in vivo* by reducing macrophage activation, which promotes bacteria persistence and the generation of foreign Ags that drive CD4$^+$ T cell expansion. B10 cell expansion thus shapes the qualitative and quantitative nature of both innate and cellular immune responses during acute *Listeria* infections.

In contrast with B10 cell expansion, B10 cell depletion significantly reduced spleen and liver bacteria loads by 82–97% (Fig. 2, data not shown). This was demonstrated in three ways. First, enhanced bacteria clearance was observed in CD19$^{-/-}$ mice that have dramatically reduced numbers of B10 and B10pro cells, but otherwise have near normal B cell numbers (Fig. 2C, ref. 13). Enhanced clearance of *Listeria* in the absence of B10 cells was similar to the rapid clearance of *Listeria* in IL-10$^{-/-}$ mice (7). Second, *Listeria* clearance was also dramatically accelerated in mice depleted of all mature B cells using a potent CD20 mAb (Fig. 2B). This result is consistent with enhanced bacteria clearance in B cell-deficient μMT mice (58). Third, *Listeria* loads were significantly reduced in mice given a ligand-blocking CD22 mAb that deleted most CD1d^{hi}CD5$^+$ B cells and 60–80% of spleen B10 cells (Fig. 2C, refs. 31, 57). However, the vast majority of spleen B cells remain intact after CD22 mAb treatment. The elimination of CD1d^{hi}CD5$^+$ cells and B10 cells by select CD22 mAbs is not a consequence of the initiation of CD22 negative regulation or CD22 removal from the cell surface during B cell Ag receptor signaling (unpublished observations), but likely reflects the diversity of effects elicited by CD22 binding to appropriate *in vivo* ligands (59). Remarkably, CD20 mAb and the ligand-blocking CD22 mAb used in these studies deplete different percentages of B cells and utilize distinct mechanisms of action to deplete B10 cells (43, 57), but their effects on bacteria clearance were identical to those observed in CD19$^{-/-}$ mice without B10 cells. Moreover, B10 cell depletion by CD20 mAb and this ligand-blocking CD22 mAb can also exacerbate cellular immunity and autoimmune disease symptoms similarly (26, 31, 57). Thereby, B cell and particularly B10 cell manipulation has significant effects on bacteria clearance and innate immune responses.

Listeria initially infects macrophages, with the subsequent elicitation of Ag-specific adaptive immunity (60). Macrophages also serve as the primary *in vivo* mediators of *Listeria* clearance (61). Thereby, enhanced *Listeria* clearance in the absence of B10 cells is likely to result from the enhanced ability of macrophages from B10 cell-depleted mice to phagocytose bacteria (Fig. 3H) and express cytokines (Fig. 3C–E) and nitric oxide (Fig. 3G) *in vivo*. That B cell regulation of bacteria clearance by monocytes was B10 cell and IL-10-dependent was confirmed by adoptive transfer experiments. First, the adoptive transfer of wild type CD1d^{hi}CD5$^+$ B cells that are enriched for B10+B10pro cells (13, 32) reduced *Listeria* clearance in CD19$^{-/-}$ mice to levels seen in wild type mice (Fig. 2E). By contrast, the adoptive transfer of IL-10$^{-/-}$ CD1d^{hi}CD5$^+$ B cells or CD1d^{lo}CD5$^-$ B cells was without effect. Second, the adoptive transfer of CD1d^{hi}CD5$^+$ B cells from CD22$^{-/-}$CD154Tg mice, where the majority of CD1d^{hi}CD5$^+$ B cells are B10 cells (31), normalized *in vivo* macrophage TNF-α and IFN-γ production in CD22 mAb-treated mice, while CD22$^{-/-}$CD1d^{lo}CD5$^-$ B cells were without effect (Fig. 3D). Third, the adoptive transfer of CD1d^{hi}CD5$^+$ B cells from CD20$^{-/-}$ mice normalized *in vivo* macrophage TNF-α and IFN-γ production in CD20 mAb-treated mice, while CD20$^{-/-}$CD1d^{hi}CD5$^+$ B cells were without effect (Fig. 3E). Fourth, the adoptive transfer of CD1d^{hi}CD5$^+$ B cells from IL-10$^{-/-}$CD20$^{-/-}$ mice were unable to normalize *in vivo* macrophage TNF-α and IFN-γ production in CD20 mAb-treated mice. Consistent with these findings, small numbers of adoptively transferred B10 cells inhibit macrophage activation through IL-10-dependent mechanisms and thereby dramatically suppress antibody-mediated lymphoma depletion *in vivo*, as well as cytokine production *in vivo* and *in vitro* (56). B10 cells also negatively regulate human macrophage activation and phagocytic activity (33). Exogenous IL-10 also down-regulates macrophage cytokine production *in vitro*, their generation of reactive oxygen intermediates, and cell surface expression of MHC class II and other costimulatory molecules (62–64). B10 cell inhibition of *Listeria* clearance is also consistent with the proposed role for B1 cell-derived IL-10 in the regulation of *B. hermsii* clearance from the blood (15). These collective studies thereby demonstrate that IL-10 production by the B10 cell subset has substantial regulatory effects on macrophage function, including the clearance of *Listeria* and potentially other pathogens.

Acute expansion of the B10 cell subset after *Listeria* infection (Fig. 1B–D) was similar to the rapid increase in B10 cells that occurs during inflammation (13, 20, 26, 56) and the regulatory B cell increases observed with other inflammatory conditions (15–18). By contrast, there was only modest expansion of the FoxP3+CD4+ regulatory T cell subset by day 5 following infection (Fig. 1H). B10 cell expansion at early time-points in the absence of regulatory T cell expansion also occurs during experimental autoimmune encephalomyelitis in mice (26). Thereby, acute *Listeria* infection may preferentially expand B10 cells but not regulatory T cells to manipulate IL-10 levels and increase bacterial loads that may be normally required to drive early Treg and CD4+ T cell expansion. Rapid *Listeria* clearance in the absence of B10 cells following CD22 mAb-treatment may thereby limit the availability of foreign Ags and explain the dramatic reductions in CD4+ T cell proliferation and cytokine production in the current studies (Fig. 4B–C). Alternatively, the absence of B10 cells may facilitate the rapid development of potent CD8+ effector T cells that facilitate bacteria clearance. Reduced bacteria loads with augmented T cell responses have been observed in μMT mice after *Listeria* infection (65). Acute *Listeria*-driven expansion of the B10 cell subset thereby accelerates the accumulation of stimulatory foreign Ags that determine the magnitude of CD4+ but not CD8+ T cell proliferation and cytokine production.

B10 cell function appears Ag-specific in models of inflammation, and Ag receptor specificity and signaling are critical components for B10 cell function *in vivo* (30, 32). For example, B10 cells fail to develop in CD19−/− mice where B cell Ag receptor signaling is muted (Fig. 2A). While B cell Ag receptor signaling appears to direct B10 cell development, cognate interactions with Ag-specific T cells producing IL-21 appear critical for the development of IL-10-producing B10 effector cells (32), which may then negatively regulate cognate CD4+ T cell partners (Fig. 5B). B10 cell expansion may thereby normally limit Ag-specific CD4+ T cell proliferation and cytokine production during acute *Listeria* infections, or may alternatively reshape the repertoire of CD4+ T cells that expand in response to infection. Consistent with this, regulatory B10 cell inhibition of bacteria clearance required that B10 cells express IL-10, MHC class II molecules, and CD21-R (Fig. 5A), arguing that B10 cells require cognate interactions with CD4+ T cells to induce their regulatory effector functions during infections (Fig. 5B) as occurs during T cell-mediated autoimmunity (32). Given this, macrophage function is likely to be regulated downstream of B10 cell interactions with CD4+ T cells. Under these conditions, B10 cell negative regulation of monocyte function would remain Ag-specific and may only affect macrophages within the local microenvironment of ongoing immune responses.

In addition to the current studies with B cells, IL-10 produced by other immune cells is known to modulate host immune responses to diverse pathogens including *Listeria*, *Bordetella pertussis*, lymphocytic choriomeningitis virus, and vaccinia virus (6, 9, 11, 66). Gram-negative bacteria also induce B cells to express IL-10 through a MyD88-dependent pathway, which inhibits immunity (14). It is therefore possible that pathogens also utilize TLR-signaling pathways to induce B10 cell IL-10 production. Consistent with this, inflammatory signals and bacterial products are potent stimulators of B10 cell maturation and IL-10 production (30). However, B10 cells develop normally in both MyD88-deficient and pathogen-deficient mice, and B10 cell function remains Ag-specific in models of inflammation, which suggests that Ag receptor specificity and signaling are also critical components for B10 cell function *in vivo* (30). Given the negative regulatory functions of B10 cells and their capacity to modulate both innate and adaptive immune responses, it is likely that pathogens will utilize multiple diverse mechanisms to manipulate B10 cell function and biology to modulate immune responses to their advantage. Alterations in the numbers and/or proportions of B10 cells during acute infections may thus have major ramifications for quantitatively and qualitatively programming subsequent innate and

cellular immune responses that might be manipulated for the development of vaccines to a variety of pathogens.

Acknowledgments

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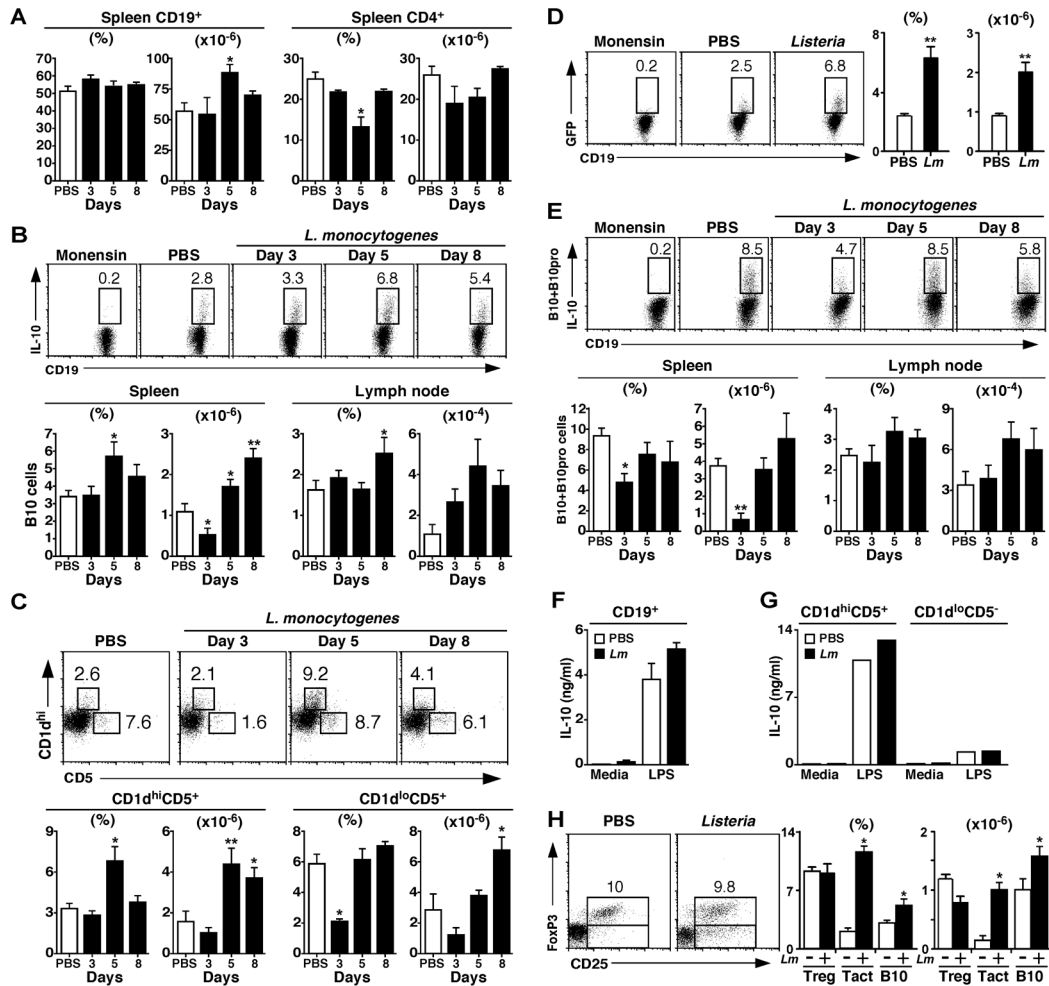
Figure 1. B10 cells expand following *Listeria* infection

Wild type mice were given PBS or infected with 8×10^5 cfu of *Listeria*-OVA on d 0. (**A**) Spleen B and T cell numbers after *Listeria* infection. At the indicated times post-infection, CD19$^+$ B cell and CD4$^+$ T cell frequencies and numbers were determined by immunofluorescence staining with flow cytometry analysis. Data from PBS-treated control mice were unchanged across all time points (d 3, 5, and 8 post-infection) and were therefore pooled. Values represent mean ($\pm$ SEM, n=3–6/group) cell frequencies or numbers. (**B**) B10 cells expand after *Listeria* infection. At the indicated times post-infection, spleen and lymph node lymphocytes were stimulated *ex vivo* with LPS, PMA, ionomycin, and monensin (LPIM) for 5 h and stained for cell surface CD19 and intracellular IL-10. Representative histograms show IL-10 expression by viable and single spleen CD19$^+$ B cells. Cells from infected mice that were cultured with monensin alone served as negative staining controls. Numbers indicate the frequencies of cells within the indicated gates. Data from PBS-treated mice were unchanged across all time points (d 3, 5, and 8 post-infection) and were therefore pooled. Values in the bar graphs represent mean ($\pm$ SEM, n=10) frequencies or numbers of IL-10$^+$ B cells. (**C**) The spleen CD1d^{hi}CD5$^+$CD19$^+$ B cell subset expands after *Listeria* infection. At the indicated times post-infection, CD1d^{lo}CD5$^+$CD19$^+$ and CD1d^{hi}CD5$^+$CD19$^+$ B cell subset frequencies and numbers were determined. The data are presented as in (B, n=7). (**D**) B cell GFP expression in IL-10 reporter Tiger mice. Five d after infection, splenocytes were stimulated *ex vivo* with LPIM for 5 h before staining for

cell surface CD19 and evaluating CD19^{+} B cell intracellular GFP expression by flow cytometry. Numbers indicate cell frequencies within the indicated gates. Values in bar graphs represent mean (± SEM, n=5) frequencies or numbers of CD19^{+}GFP^{+} B cells 5 d post-infection. (**E**) Spleen and lymph node B10+B10pro cell frequencies and numbers following *Listeria* infection. B10+B10pro cells were enumerated after 48 h of stimulation with CD40 mAb, with LPIM added during the final 5 h of culture. Representative histograms show IL-10 expression by viable and single spleen CD19^{+} B cells, with bar graphs as in (B, n=10). (**F–G**) *Listeria* infection enhances B10 cell IL-10 production. Purified spleen (F) CD19^{+} B cells or (G) CD1d^{hi}CD5^{+} and CD1d^{lo}CD5^{−} B cell subsets were isolated from mice 5 d after infection and cultured for 72 h with or without LPS. Culture supernatant fluid IL-10 concentrations were measured by ELISA. Bar graphs indicate mean IL-10 concentrations (± SEM, n=3). (**H**) Treg cells do not expand following *Listeria* infection. Histograms show representative FoxP3 and CD25 expression by splenic CD4^{+} T cells 5 d post-infection. Values in the bar graphs represent mean (± SEM, n=5) frequencies or numbers of CD25^{+}FoxP3^{+} CD4^{+} Treg cells, activated CD25^{+}FoxP3^{−} CD4^{+} T cells (Tact), or B10 cells. (A–H) Significant differences between sample means for mice given PBS or infected are indicated: *; p<0.05; **; p <0.01.
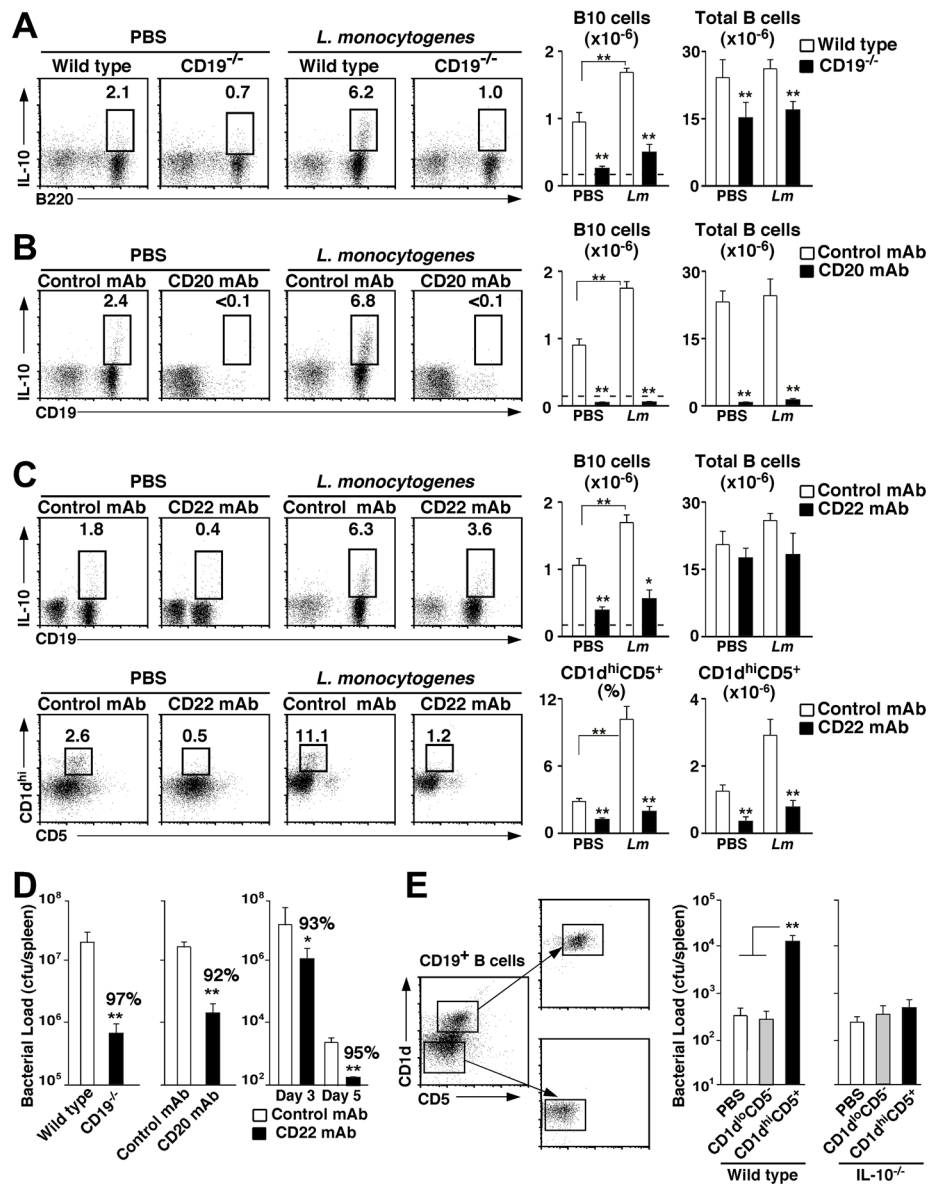
Figure 2. B10 cells inhibit *Listeria* clearance

Spleen B10 cell numbers in (**A**) CD19$^{-/-}$ mice, (**B**) mice given either CD20 or isotype control mAb on d −7 or (**C**) mice given CD22 or isotype control mAb on d −7 and −1 before *Listeria* infection or PBS treatment on d 0. Five d later, splenocytes were stimulated *ex vivo* with LPIM for 5 h before staining for cell surface (A) B220, (B) CD19, or (C) CD19, CD1d, and CD5, and intracellular IL-10 expression. (**D**) CD19-deficiency, total B cell depletion, and CD22 mAb treatment enhance bacterial clearance. Mice treated as in (A–C) were infected with *Listeria* on day 0, and the number of bacteria present within their spleens was quantified 3 d (and 5 d, for CD22 mAb) later. Values represent mean (± SEM, n=8) bacterial loads in each set of mice. (**E**) B10 cells inhibit bacterial clearance through IL-10-dependent mechanisms. Representative fractionation of purified spleen CD19$^+$ B cells from wild type or IL-10$^{-/-}$ mice into CD1d^{hi}CD5$^+$ (B10 cell enriched) or CD1d^{lo}CD5$^-$ B cell populations by cell sorting. The cells were then adoptively transferred into CD19$^{-/-}$ mice. One d later, the recipient mice were treated with PBS or infected with *Listeria*. Bar graphs represent

mean (± SEM, n=7) spleen bacterial loads 3 d later. (A–E) Significant differences between means are indicated: *; p<0.05; **; p <0.01.
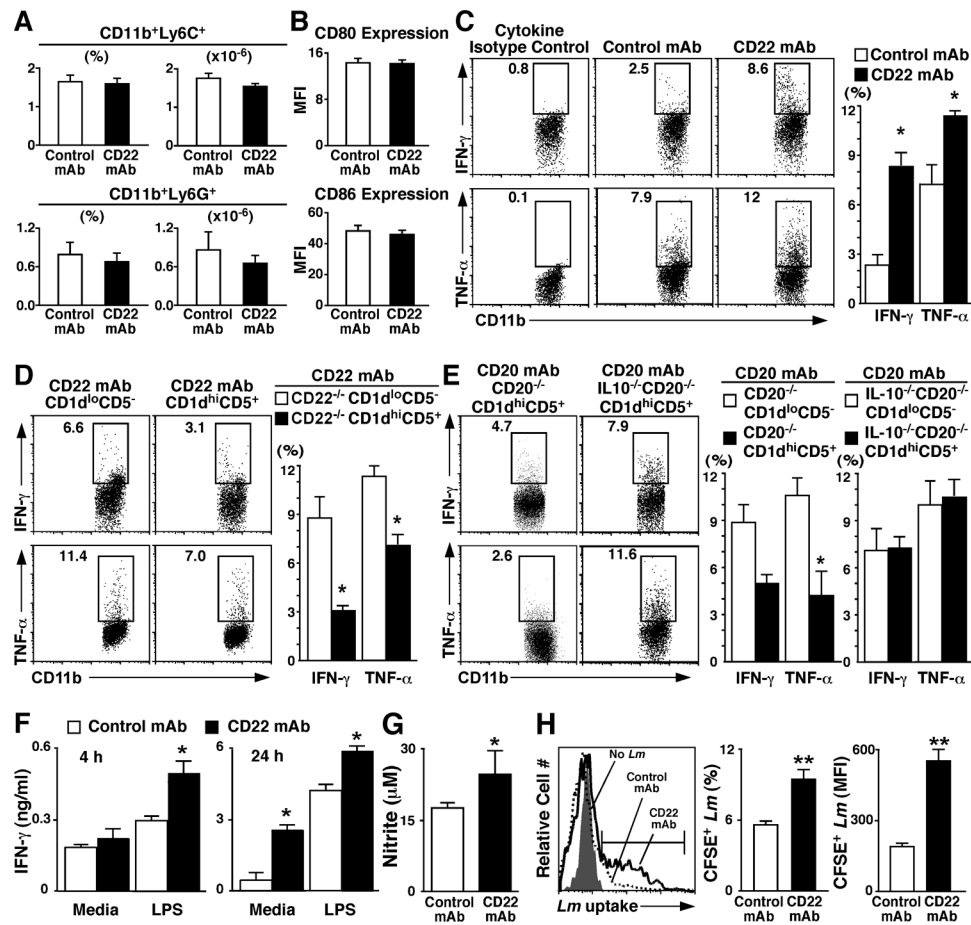
Figure 3. B10 cell depletion enhances macrophage cytokine production and phagocytosis
In all experiments, wild type mice were given CD22 or isotype control mAb on d −7 and −1 and infected with *Listeria* on d 0. (**A**) CD22 mAb-treatment does not alter macrophage or neutrophil numbers *in vivo*. Frequencies and numbers of viable spleen CD11b+Ly6C+ macrophages and CD11b+Ly6G+ neutrophils were quantified *ex vivo* 1 d post-infection with *Listeria* by immunofluorescence staining with flow cytometry analysis. Values represent mean (± SEM, n=3) cell frequencies and numbers among spleen mononuclear cells. (**B**) CD22 mAb-treatment does not induce macrophage activation *in vivo*. Spleen F4/80+CD11b+ macrophage CD80 and CD86 mean fluorescence staining intensities (MFI) were quantified *ex vivo* 1 d post-infection with *Listeria* by immunofluorescence staining with flow cytometry analysis. Values represent mean (± SEM, n=3) MFI values with isotype control mAb background staining subtracted. (**C**) CD22 mAb-treatment enhances macrophage cytokine production. Representative histograms show intracellular IFN-γ and TNF-α expression by viable, single F4/80+CD11b+ spleen macrophages directly *ex vivo* 1 d post-infection with *Listeria* as assessed by immunofluorescence staining. Background staining was determined using isotype-matched control antibodies. (**D**) Adoptively transferred CD1d^hiCD5+ B10 cells inhibit macrophage cytokine production in CD22 mAb-treated mice. In parallel with the experiments in (C), mice were given either control or CD22 mAb. Subsequently, the mice were given purified spleen CD1d^hiCD5+ or CD1d^loCD5−CD19+ B cells from CD22−/−CD154Tg mice. One day later, the recipient mice were infected with *Listeria*, with spleen macrophage IFN-γ and TNF-α expression quantified as in (C). (C,D) Bar graphs show mean (± SEM, n=5) frequencies of cytokine

expressing cells within the indicated gates. (**E**) Spleen CD1d^{hi}CD5$^+$ B cells inhibit macrophage cytokine production through IL-10 production. Wild type mice were given control or CD20 mAb as in figure 2B–C. Subsequently, the mice were given either purified spleen CD1d^{hi}CD5$^+$ or CD1d^{lo}CD5$^-$ CD19$^+$ B cells from CD20$^{-/-}$ or IL-10$^{-/-}$CD20$^{-/-}$ mice. One day later, the recipient mice were infected with *Listeria*, with spleen macrophage IFN-γ and TNF-α expression assessed as in (C). (**F–G**) CD22 mAb-treatment *in vivo* regulates spleen macrophage cytokine and nitric oxide production *ex vivo*. Adherent macrophages isolated from CD22 or control mAb-treated *Listeria*-infected mice generated as in (C) were stimulated with LPS. Mean (± SEM) supernatant fluid (F) IFN-γ (4 or 24 h cultures, n=6) or (G) nitrate (24 h culture, n=6) concentrations were quantified by ELISA. (**H**) CD22 mAb-treatment enhances macrophage phagocytosis of CFSE-labeled *Listeria*. Mice were treated with CD22 or isotype-matched control mAb on d −7 and −1. Spleen CD11b$^+$ macrophages were isolated on d 0 and cultured with CFSE-labeled *Listeria* for 2 h. The histogram shows representative intracellular CFSE fluorescence of macrophages from CD22 or control mAb-treated mice cultured without (No *Lm*) or with CFSE-labeled *Listeria*. The horizontal line indicates the flow cytometry gate used for quantifying CFSE-positive cells. Bar graphs represent mean (± SEM, n=8) frequencies of CFSE$^+$ macrophages or mean fluorescent intensities (MFI) of CD11b$^+$CFSE$^+$ cells. (A–H) Significant differences between the sample means are indicated: *; p<0.05; **; p <0.01.
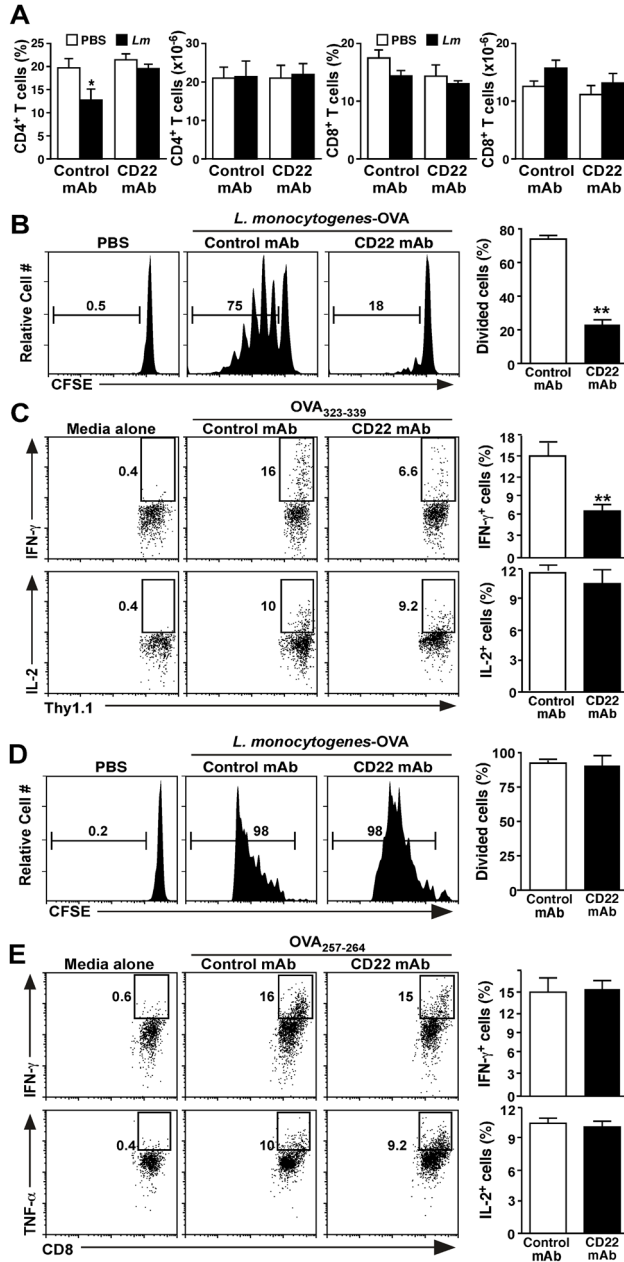
Figure 4. B10 cells regulate Ag-specific CD4^{+} T cell activation
Wild type mice were given CD22 or control mAb on day −7 and −1 and were infected with
Listeria-OVA on day 0. (**A**) CD22 mAb treatment normalizes spleen CD4^{+} T cell numbers
following *Listeria* infection. Values represent mean (± SEM, n=6) frequencies and numbers
of total spleen CD4^{+} and CD8^{+} T cells 5 d post-infection or treatment with PBS. (**B**) CD22
mAb-treatment reduces Ag-specific spleen CD4^{+} T cell proliferation. CD22 or control mAb-
treated and infected mice received CFSE-labeled spleen Thy1.1^{+}OT-II CD4^{+} T cells on d
−1. The *in vivo* proliferation of adoptively transferred CFSE-labeled Thy1.1^{+}OT-II T cells
was assessed by CFSE dilution 3 d following infection. Representative histograms
demonstrate CFSE staining for uninfected mice (PBS) versus infected mice treated with
control or CD22 mAb, with the percentages of dividing cells indicated. Bar graph values
represent mean (± SEM, n=6) frequencies of spleen Thy1.1[+]OT-II T cells with diluted

CFSE in infected mice. (**C**) CD22 mAb-treatment reduces Ag-specific CD4$^+$ T cell cytokine production. Splenocytes from control or CD22 mAb treated mice given Thy1.1$^+$ OT-II T cells as in (B) were harvested 3 d post-*Listeria* infection and stimulated *ex vivo* with media alone or OVA$_{323-339}$ peptide for 4 h in the presence of brefeldin A. IFN-γ, IL-2, and TNF-α production were assessed by intracellular staining with flow cytometry analysis. Representative dot plots show relative frequencies of cytokine expressing spleen Thy1.1$^+$ T cells within the indicated gates. Bar graphs represent mean ($\pm$ SEM, n=6) frequencies of Thy1.1$^+$ OT-II T cells expressing each cytokine. (**D**) B10 cell depletion does not reduce Ag-specific spleen CD8$^+$ T cell proliferation. CD22 or control mAb-treated and infected mice received CFSE-labeled spleen Thy1.1$^+$ OT-I CD8$^+$ T cells on d −1. The *in vivo* proliferation of adoptively transferred CFSE-labeled spleen Thy1.1$^+$ OT-I T cells was assessed as in (B; n=6). (**E**) B10 cell depletion does not reduce spleen Ag-specific CD8$^+$ T cell cytokine production. Cytokine production by adoptively transferred Thy1.1$^+$ OT-I T cells was assessed as in (C) using OVA$_{257-264}$ peptide (n=4). (A–E) Significant differences between the indicated sample means among control mAb and CD22 mAb treated mice are indicated: *; p<0.05; **; p <0.01.
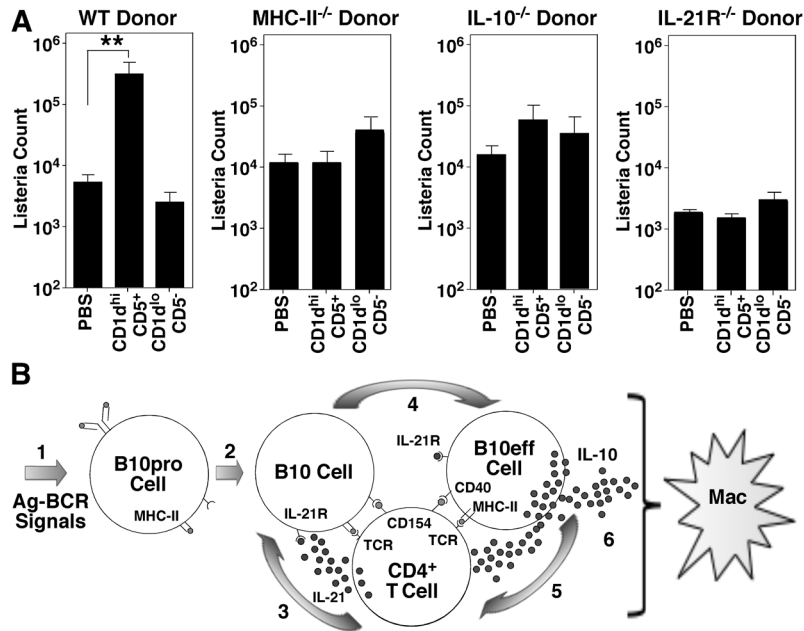
Figure 5.
B10 cell regulation of innate immune responses to *Listeria* requires cognate interactions with CD4^{+} T cells. (**A**) B10 cell MHC class II, IL-10, and IL-21R expression are required for regulation of innate immune responses. CD19^{−/−} mice were either given PBS or purified spleen CD1d^{hi}CD5^{+} or CD1d^{lo}CD5^{−} B cells from naïve wild type (WT), MHC-II^{−/−}, IL-10^{−/−}, or IL-21R^{−/−} mice 1 day before *Listeria* infection. Values represent mean (±SEM) spleen bacterial counts from 4 mice in each group 3 days after infection. Significant differences between the indicated sample means are shown: **, p<0.01. (**B**) Model for pathogen (Ag)-specific B10 cell function. B cells capture Ags that trigger appropriate BCR signals (step 1) and promote IL-10-competent B10pro cell development. During immune responses (step 2), B10pro cells present peptides to Ag-specific CD4^{+} T cells through cognate interactions that induce T cell activation and CD40/CD154 interactions. Activated T cells may produce IL-21 locally, which binds to proximal B10 cell IL-21R (step 3). IL-21R signals induce B10 cell IL-10 production and effector function (B10eff, step 4), which may negatively regulate Ag-specific T cell function (step 5). Indirectly, B10 effector cell production of IL-10 in the local environment negatively regulates macrophage activation and effector function.