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Regulatory B Cells (B10 Cells) and Regulatory T Cells Have Independent Roles in Controlling Experimental Autoimmune Encephalomyelitis Initiation and Late-Phase Immunopathogenesis

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Experimental autoimmune encephalomyelitis (EAE) is a T lymphocyte-mediated autoimmune disease of the CNS. Significant roles for B cells and a rare IL-10–producing CD1d\(^{hi}\)CD5\(^{hi}\) regulatory B cell subset (B10 cells) have been identified during the initiation and progression of EAE. Whether and how the regulatory functions of B10 cells and FoxP3\(^{+}\) T regulatory cells (Tregs) overlap or influence EAE immunopathogenesis independently has remained unanswered. This study demonstrates that the number of endogenous or adoptively transferred B10 cells directly influenced EAE pathogenesis through their production of IL-10. B10 cell numbers expanded quickly within the spleen, but not CNS following myelin oligodendrocyte glycoprotein\(33-55\) immunization, which paralleled B10 cell regulation of disease initiation. The adoptive transfer of myelin oligodendrocyte glycoprotein\(33-35\) sensitized B10 cells into wild-type mice reduced EAE initiation dramatically. However, B10 cells did not suppress ongoing EAE disease. Rather, Treg numbers expanded significantly within the CNS during disease progression, which paralleled their negative regulation of late-phase disease. Likewise, the preferential depletion of B10 cells in vivo during disease initiation enhanced EAE pathogenesis, whereas Treg depletion enhanced late-phase disease. B10 cells did not regulate T cell proliferation during in vitro assays, but significantly altered CD4\(^{+}\) T cell IFN-\(\gamma\) and TNF-\(\alpha\) production. Furthermore, B10 cells downregulated the ability of dendritic cells to act as APCs and thereby indirectly modulated T cell proliferation. Thus, B10 cells predominantly control disease initiation, whereas Tregs reciprocally inhibit late-phase disease, with overlapping B10 cell and Treg functions shaping the normal course of EAE immunopathogenesis.

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titure B cell depletion in mice before EAE induction significantly exacerbates disease symptoms, whereas B cell depletion during EAE progression dramatically inhibits disease symptoms. B10 cell depletion from mice before disease initiation accounts for exacerbated disease, which can be ameliorated by the adoptive transfer of spleen CD1dhighCD5+ B cells. Similarly, IL-10 deficiency enhances the severity of EAE (22). Thereby, the balance between opposing positive and negative regulatory B cell functions shapes the normal course of EAE immunopathogenesis.

Whether and how the regulatory functions of B10 cells and Tregs overlap or influence EAE immunopathogenesis independently has remained unanswered. To address this question, the regulatory effects of adoptively transferring increasing numbers of naive or EAE-sensitized B10 cells, or IL-10-deficient CD1dhighCD5+ B cells into wild-type mice at various stages of disease were evaluated, in addition to depleting Tregs during both disease initiation and progression. Furthermore, we are the first to show in this study that in vivo CD22 mAb treatment preferentially depletes spleen B10 cells, which dramatically exacerbates EAE severity during the initiation phase of disease. This study thereby demonstrates that B10 cells have different regulatory functions when compared with Tregs, as they function at different time points during EAE initiation and disease progression. Moreover, B10 cells directly influenced the production of proinflammatory cytokines by CD4+ T cells and suppressed the Ag-presenting function of dendritic cells (DCs). Thereby, independent, but overlapping B10 cell and Treg functions shape the normal course of EAE immunopathogenesis.

Materials and Methods

Cell preparation and immunofluorescence analysis

Single-cell leukocyte suspensions from spleens and peripheral lymph nodes (paired axillary and inguinal) were generated by gentle dissection. Blood mononuclear cells were isolated from heparinized blood after centrifugation over a discontinuous lympholyte-Mammal (Cedarlane Laboratories, Hornby, Ontario, Canada) gradient. CNS mononuclear cells were isolated after cardiac perfusion with PBS, as described (23). Briefly, CNS tissues were digested with collagenase D (2.5 mg/ml; Roche Diagnostics, Mannheim, Germany) and DNase (1 mg/ml; Roche Diagnostics) at 37 °C for 45 min. Mononuclear cells were isolated by passing the tissue through 70-mm cell strainers (BD Biosciences, San Diego, CA), followed by Percoll gradient (70/37%) centrifugation. Lymphocytes were collected from the 37/70% interface and washed.

Mouse CD20-specific mAb MB20-11 was used as described (24). FITC-, PE-, PE Cy5-, PE Cy7-, or allophycocyanin-conjugated CD1d (B1), CD3 (17A2), CD4 (H129.19), CD68 (55-7.3), CD8 (53-6.7), CD11b (M1/70), CD11c (N418), CD19 (1D3), CD25 (PC61), B220 (RA3-6B2), and Thy1.1 (OX-7) mAbs were from BD Biosciences. PE-conjugated IL-10R mAb (1B1.3a) was from BioLegend (San Diego, CA). Intracellular staining using mAbs reactive with IL-10 (JES5-16E3), IFN-γ (XMG1.2), TNF-α (MP6-XT22), and FoxP3 (FJK-16s) (all from eBioscience, San Diego, CA) and PE-conjugated anti-CD40 mAb (2.4G2; BD Biosciences) were used to generate cDNA. IL-10 transcriptase (Invitrogen, Carlsbad, CA) were used to generate cDNA for IL-10. Total RNA was extracted from cell sorter-purified B cells using Qiagen RNeasy spin columns (Qiagen, Crawley, United Kingdom). Random hexamer primers (Promega, Madison, WI) and Superscript II RNase H reverse transcriptase (Invitrogen, Carlsbad, CA) were used to generate cDNA. IL-10 transcripts were quantified by real-time PCR analysis using SYBR Green as the detection agent. The PCR was performed with the iCycler iQ system (Bio-Rad, Hercules, CA). All components of the PCR mix were purchased from Bio-Rad and used according to the manufacturer’s instructions. Cycle conditions were one amplification cycle of denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 59°C for 1 min, and 95°C for 1 min.

Mice and immunotherapy

C57BL/6 and IL-10−/− (B6.129P2-H2−/−;129S2/ScSnJ) mice (26) were bred in the specific-pathogen-free barrier facility and used at 6–12 wk of age.

To deplete B10 cells in vivo, sterile CD22 (MB22-10, IgG2c) or isotype-matched mAbs (250 μg) were injected in 200 μl PBS through lateral tail veins (29). To deplete CD4+CD25 FoxP3+ Tregs in vivo, denileukin diftitox (5 μg in 500 μl PBS, Ligand Pharmaceuticals, San Diego, CA) or PBS was injected i.p. The Duke University Animal Care and Use Committee approved all studies.

EAE induction

Active EAE was induced in 6- to 8-wk-old female mice by s.c. immunization with 100 μg myelin oligodendrocyte glycoprotein (MOG)35-55 peptide (MEVGWYRSPFSRVVHLNYGK; NeoMPS, San Diego, CA) emulsified in CFA containing 200 μg heat-killed Mycobacterium tuberculosis H37RA (Difco, Detroit, MI) on day 0. Additionally, mice received 200 ng pertussis toxin (List Biological Laboratories, Campbell, CA) i.p. in 0.5 ml PBS on days 0 and 2. Clinical signs of EAE were assessed daily with a 0- to 6-point scoring system, as follows: 0, normal; 1, flaccid tail; 2, impaired righting reflex and/or gait; 3, partial hind limb paralysis; 4, total hind limb paralysis; 5, hind limb paralysis with partial fore limb paralysis; 6, moribund state (11). Moribund mice were given disease severity scores of 6 and euthanized. Disease scores over the course of the 28-d experiments were totaled for each animal, and the mean for the experimental group was expressed as a cumulative EAE score.

Histology

Following an initial perfusion with PBS, tissues were perfused transcardially with 4% paraformaldehyde and spinal cords were removed. Tissues were processed and blocked in paraffin wax. Two transverse sections of the thoracic and lumbar spinal cord were stained with H&E and Luxol Fast Blue. The number of inflammatory foci that contained at least 20 cells were counted in each H&E-stained section in a blinded fashion. When foci coalesced, estimates were made of the number of foci. Areas of demyelination were assessed for Luxol Fast Blue-stained sections. ImageJ software (National Institutes of Health, Bethesda, MD) was used to manually trace the total cross-sectional area and the demyelinated area of each section. Total demyelination was expressed as a percentage of the total spinal cord area.

B10 cell analysis

Intracellular IL-10 expression was visualized by immunofluorescence staining and analyzed by flow cytometry, as described (17). Briefly, isolated leukocytes or purified cells were resuspended (2 × 106 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, Carlsbad, CA] with LPS (10 μg/ml, Escherichia coli serotype 0111:B4; Sigma-Aldrich), PMA (50 ng/ml; Sigma-Aldrich), ionomycin (500 ng/ml; Sigma-Aldrich), 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 mAb; BD Biosciences), according to the manufacturer’s instructions, and stained with PE-conjugated mouse anti–IL-10 mAb. Leukocytes from IL-10−/− mice served as negative controls to demonstrate specificity and to establish background IL-10–staining levels.

Real-time RT-PCR analysis

Total RNA was extracted from cell sorter-purified B cells using Qiagen RNeasy spin columns (Qiagen, Crawley, United Kingdom). Random hexamer primers (Promega, Madison, WI) and Superscript II RNase H reverse transcriptase (Invitrogen, Carlsbad, CA) were used to generate cDNA. IL-10 transcripts were quantified by real-time PCR analysis using SYBR Green as the detection agent. The PCR was performed with the iCycler iQ system (Bio-Rad, Hercules, CA). All components of the PCR mix were purchased from Bio-Rad and used according to the manufacturer’s instructions. Cycle conditions were one amplification cycle of denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 59°C for 1 min, and 95°C for 1 min.
Specificity of the RT-PCR was controlled by the generation of melting curves. IL-10 expression threshold values were normalized to GAPDH expression using standard curves generated for each sample by a series of four consecutive 10-fold dilutions of the cDNA template. For all reactions, each condition was performed in triplicate. Data analysis was performed using iQ Cycler analysis software. The sense IL-10 primer was 5'-GGTTGCGCAAGCTATCCGGA-3', and the antisense primer was 5'-ACCTGCTCCTACGCTGTGCT-3'. The sense GAPDH primer was 5'-TTCACCATGGAGAAAGGC-3', and the antisense primer was 5'-GGCATGCGACCTTGATCA-3'.

**Lymphocyte subset isolation**

MACS (Miltenyi Biotec, Auburn, CA) was used to purify lymphocyte populations according to the manufacturer’s instructions. CD19 mAb-coated microbeads and CD4+ T cell isolation kits (Miltenyi Biotec) were used to purify B cells and CD4+ T cells, respectively. When necessary, the cells were enriched a second time using a fresh MACS column to obtain >95% cell purity, respectively. Spleen DCs were obtained as previously described, with minor modifications (30). Briefly, spleens were minced and incubated with collagenase D (1 mg/ml) and DNase I (0.2 mg/ml) for 30 min at 37 °C. Cold EDTA was added to a final concentration of 20 mM, and cell suspensions were incubated for 5 min at room temperature before filtering through nylon mesh to remove tissue and cell aggregates. To enrich for cells with a low buoyant density, cellular suspensions were separated over a 30% BSA gradient and cells were collected from the interface. After enrichment, DCs were isolated using CD11c mAb-coated microbeads (Miltenyi Biotec).

**Cell sorting and adoptive transfer experiments**

Naive wild-type mice, wild-type mice with EAE, or IL-10−/− mice with EAE were used as B cell donors. Splenic B cells were first enriched using CD19 mAb-coated microbeads (Miltenyi Biotec). In addition, CD1dhigh CD5+ and CD1dlowCD5- B cells were isolated using a FACS Vantage SE flow cytometer (BD Biosciences) with purities of 95–98%. After purification, 1 × 10^6 cells were immediately transferred i.v. into recipient mice.

In vitro T cell, B cell, and DC coculture assays

Splenocyte B cells from day 28 EAE mice were purified by cell sorting. The sorted cell populations were stimulated with agonistic CD40 mAb for 48 h, with LPS added during the final 5 h of culture. TCR MOG CD4+ T cells were purified by MACS, and CFSE-labeled. CFSE-labeled TCR MOG CD4+ T cells (1 × 10^6/ml) were cultured alone or with CD40/LPS-stimulated CD1dhighCD5+ or CD1dlowCD5- B cells (1 × 10^6/ml) in the presence of MOG35–55 (25 μg/ml) for 72 h.

In additional experiments, splenic CD1dhighCD5+ or CD1dlowCD5- B cells from day 28 EAE mice were purified by cell sorting. The sorted cell populations were stimulated with agonistic CD40 mAb for 48 h, with LPS added during the final 5 h of culture. Splenic DCs from day 10 EAE mice were purified by MACS and cultured (1 × 10^6/ml) with CD40 mAb/LPS-stimulated CD1dhighCD5+ or CD1dlowCD5- B cells (1 × 10^6/ml) in the presence of MOG35–55 (25 μg/ml) for 72 h.

**FIGURE 1.** B10 cell and Treg numbers increase during EAE. A, CD1dhighCD5+ B cell frequencies and numbers increase during the course of EAE. Splenocytes were isolated from naive mice before and day 7, 14, 21, or 28 after MOG35–55 immunization and analyzed for CD1d, CD5, and CD19 expression by immunofluorescence staining with flow cytometry analysis. Representative results demonstrate the frequency of CD1dhighCD5+ B cells within the indicated gates among total CD19+ B cells. Bar graphs indicate mean (±SEM) percentages and numbers of CD1dhighCD5+ B cells. B, IL-10+ B10 cell frequencies and numbers increase during the course of EAE. Splenocytes were cultured with L + PIM for 5 h, stained with CD19 mAb, permeabilized, and stained using IL-10 mAb with flow cytometry analysis. Representative results demonstrate the frequency of IL-10−producing cells within the indicated gates among total CD19+ B cells. Bar graphs indicate mean (±SEM) percentages and numbers of B cells that produced IL-10. C, B cell IL-10 transcript expression during EAE. RNA was isolated from splenic CD19+ B cells purified from naive mice before and 7, 14, 21, or 28 d after MOG35–55 immunization by MACS beads (purities >99%). Values represent relative mean IL-10 transcript levels normalized to GAPDH transcript levels (±SEM) as quantified by real-time RT-PCR analysis. D, CFA immunization does not affect IL-10+ B10 cell frequencies or numbers. Spleen B10 cell frequencies were examined before and after immunization with CFA emulsified in an equal volume of PBS, as outlined in B, E, CD4+CD25FoxP3+ T cell frequencies and numbers increase during the course of EAE. Splenocytes were stained with CD4 and CD25 mAbs, permeabilized, and stained using FoxP3 mAb with flow cytometry analysis. Representative results demonstrate the frequency of CD4+CD25FoxP3+ T cells within the indicated gates among total CD4+ T cells. Bar graphs indicate mean (±SEM) percentages and numbers of CD4+CD25FoxP3+ T cells. A–E, Bar graphs indicate results from one of two independent experiments with ≥5 mice in each group. Horizontal dashed lines are provided for reference to naive mice. Significant differences between means of naive and immunized mice are indicated: ∗p < 0.05; ∗∗p < 0.01.
presence of MOG35–55 (25 μg/ml) for 72 h. DCs were purified by cell sorting after 72-h cocultures with B cells, and then cultured (5 × 10⁵/ml) with MACS-purified TCRMOG CD4⁺ T cells (2 × 10⁵/ml) for 72 h.

Statistical analysis
All data are shown as means (±SEM). The significance of differences between sample means was determined using the Student t test.

Results

B10 and Treg expansion during EAE
B10 cells and the spleen CD1d⁺CD5⁺ B cell subpopulation are significantly expanded in autoimmune prone mice (21). To determine whether B10 cells expand during EAE, B10 cell numbers were quantified after L + PIM stimulation and staining for cytoplasmic IL-10 expression. After MOG35–55 immunization, spleen CD1d⁺CD5⁺ B cell frequencies and numbers were significantly increased on days 7, 21, and 28 in contrast to naive mice (Fig. 1A). B10 cell frequencies and numbers were also significantly increased on days 7, 21, and 28 after MOG35–55 immunization (Fig. 1B).

Increased B cell IL-10 production paralleled B10 cell frequencies (Fig. 1C), whereas immunizations with CFA alone had no effect on B10 cell numbers (Fig. 1D). Thus, there was an initial increase in B10 cell numbers and IL-10 transcripts following MOG35–55 immunization, which resolved, with a subsequent increase during EAE disease onset and resolution.

Because Tregs negatively regulate EAE symptoms (2), spleen CD25⁺FoxP3⁺CD4⁺ Treg numbers were also quantified. Treg frequencies and numbers were only significantly higher by days 21 and 28 after MOG35–55 immunization in contrast to naive mice (Fig. 1E). Thereby, B10 cell numbers increased during both the initiation and late phases of EAE progression, whereas Treg numbers were only increased during late-phase EAE.

B10 cell regulation of EAE
To determine whether quantitative differences in spleen B10 cell numbers influenced EAE, disease initiation and progression were compared in wild-type, CD19-deficient (CD19−/−), and hCD19Tg mice that overexpress CD19. Spleen CD1d⁺CD5⁺ B cells were present at similar frequencies and numbers in wild-type and

![Figure 2](http://www.jimmunol.org/)

**Figure 2.** B10 cell frequencies significantly influence EAE severity. A, CD1d⁺CD5⁺ B cell frequencies and numbers in wild-type, hCD19Tg, CD19−/−, and IL-10−/− mice. Representative results demonstrate the frequency of spleen CD1d⁺CD5⁺ B cells within the indicated gates among total CD19+ or CD20+ B cells. Bar graphs indicate mean (±SEM) percentages and numbers of CD1d⁺CD5⁺ B cells in one of two independent experiments with three mice in each group. B, IL-10⁺ B10 cell frequencies and numbers in wild-type, hCD19Tg, CD19−/−, and IL-10−/− mice. Representative results demonstrate the frequency of spleen IL-10⁺-producing cells within the indicated gates among total CD19⁺ or CD20⁺ B cells. Bar graphs indicate mean (±SEM) percentages and numbers of B cells that produced IL-10. C and D, EAE progression and severity in wild-type, CD19−/−, and hCD19Tg mice immunized with MOG35–55 on day 0 and scored daily thereafter for EAE disease severity. Values represent mean (±SEM) results from 5 mice in each group, with similar results obtained in two independent experiments. Significant differences between the means of EAE clinical scores are indicated; *p < 0.05 (♦ versus ♣).
IL-10$^{-/-}$ mice (Fig. 2A), as described (17). In hCD19Tg mice, CD1d$^{high}$CD5$^+$ B cell frequencies and numbers were 4.4- and 1.6-fold higher than in wild-type littermates, respectively. B10 cell frequencies and numbers were also 5.6- and 1.8-fold higher in hCD19Tg mice, respectively (Fig. 2F). By contrast, CD1d$^{high}$ CD5$^+$ B cell frequencies (93% decrease, $p < 0.01$) and numbers (92% decrease, $p < 0.01$) were reduced in CD19$^{-/-}$ mice when compared with wild-type mice, whereas B10 cell frequencies and numbers were 62 and 76% lower, respectively ($p < 0.01$). Thus, hCD19Tg and CD19$^{-/-}$ mice provided an optimal model system for assessing the importance of B10 cell numbers during EAE.

EAE responses were assessed in MOG$_{35-55}$-immunized CD19$^{-/-}$, hCD19Tg, and wild-type mice. EAE symptoms first appeared on about day 11 after immunization of wild-type mice and peaked on about day 18, then declined gradually (Fig. 2C). By contrast, EAE severity was significantly diminished in hCD19Tg mice (cumulative EAE score, 17.5 $\pm$ 4.7) when compared with wild-type mice (38.1 $\pm$ 5.0, $p < 0.05$). By contrast, EAE severity was significantly enhanced in CD19$^{-/-}$ mice (62.5 $\pm$ 3.3, $p < 0.005$), as reported (31). Thus, enhanced or reduced B10 cell numbers in mice inversely paralleled their disease symptoms.

To confirm that B10 cells regulated EAE responses in CD19$^{-/-}$ mice, spleen CD1d$^{high}$CD5$^+$ or CD1d$^{low}$CD5$^-$ B cells were purified from naive wild-type mice and adoptively transferred (10$^6$ per recipient) into CD19$^{-/-}$ mice 24 h before immunization with MOG$_{35-55}$. CD1d$^{high}$CD5$^+$ B cells were used for the adoptive transfer experiments because this small subpopulation contains both the B10 and B110 cell subsets and can be identified without in vitro stimulation (17). Transferring CD1d$^{high}$CD5$^+$ B cells into CD19$^{-/-}$ mice significantly reduced EAE severity (cumulative EAE score, 40.6 $\pm$ 5.0) to levels observed in wild-type mice (37.2 $\pm$ 5.6; Fig. 2D, left panel) when compared with CD19$^{-/-}$ mice (62.2 $\pm$ 3.9, $p < 0.01$). By contrast, the adoptive transfer of CD1d$^{low}$CD5$^-$ B cells into CD19$^{-/-}$ mice before EAE induction did not affect EAE severity (61.8 $\pm$ 5.1; Fig. 2D, right panel). Furthermore, the adoptive transfer of CD1d$^{high}$CD5$^+$ B cells purified from IL-10$^{-/-}$ mice did not affect EAE severity (Fig. 2D). Thus, B10 cells negatively regulated EAE development through the production of IL-10, with increased B10 cell numbers significantly reducing disease severity.

**Blocking CD22 ligand binding depletes B10 cells in vivo**

To determine whether endogenous B10 cells regulate EAE, methods were developed to preferentially deplete B10 cells. The
in vivo treatment of mice with mAbs that bind CD22 ligand binding domains preferentially depletes splenic B cells with a marginal zone phenotype, while leaving follicular B cells largely intact (29). Because B10 and marginal zone B cells share some overlapping cell surface markers (17), the ability of CD22 mAb (MB22-10) to deplete B10 cells in vivo was quantified. Remarkably, CD22 mAb treatment reduced both the frequency (86%) and number (90%) of CD1d\(^{high}\)CD5+ spleen B cells by day 7 (Fig. 3A). CD22 mAb treatment also reduced both the frequency (48%) and number (54%) of spleen B10 cells by day 7, whereas control mAb treatment was without effect. Thereby, CD1d\(^{high}\)CD5+ B cells and B10 cells could be preferentially removed without eliminating the majority of spleen B cells.

**B10 cells regulate EAE initiation, whereas Tregs regulate late-phase EAE**

The functional contributions of B10 cells to EAE initiation and pathogenesis were measured after depleting B10 cells using CD22 mAb. First, mice were given CD22 mAb 7 d before and 0, 7, 14, and 21 d after EAE induction to deplete B10 cells before and during EAE onset. B10 cell depletion did not accelerate disease onset, but made disease severity significantly worse (cumulative EAE score, 52.3 ± 4.4) in comparison with control mAb-treated littermates (32.0 ± 4.3; p, 0.01; Fig. 3B). In fact, 20% of the CD22 mAb-treated mice became moribund and were euthanized. Mice were also given CD22 mAb on days 7, 14, and 21 after MOG\(_{35-55}\) immunization, which increased disease severity (44.8 ± 2.08) in comparison with control mAb-treated littermates (35.1 ± 2.6). B10 cell depletion in mice given CD22 mAb on days 14 and 21 after MOG\(_{35-55}\) immunization did not alter disease severity (35.1 ± 2.3) in comparison with control mAb-treated littermates (30.9 ± 3.3). B10 cell depletion in mice given CD22 mAb on only day 21 did not alter disease severity. These findings argue that B10 cell regulatory function is critical during disease initiation, but not after disease onset.

Treg depletion before MOG\(_{35-55}\) immunization can increase the severity of EAE, whereas Tregs accumulate in the CNS during the recovery phase of disease (32–35). Therefore, the functional contributions of Tregs to EAE initiation and pathogenesis were measured after their in vivo depletion. CD25 mAb was not used for depleting Tregs because persistent mAb can deplete or inhibit the expansion of activated T cells expressing CD25 and thereby influence EAE pathogenesis independent of Treg depletion. Rather, denileukin diftitox was used, a fusion protein of IL-2, and diptheria toxin that binds to Tregs expressing high-affinity CD25 (36–38). Denileukin diftitox has limited effects on subsequently activated effector T cells due to its short \(t_{1/2}\) in vivo. Spleen CD25\(^{high}\)FoxP3\(^{+}\)CD4\(^{+}\) Tregs were maximally reduced 3 d after

**FIGURE 4.** Changes in B10 and Treg frequencies and numbers within tissues during the course of EAE. Mononuclear cells were isolated from CNS tissue (A), inguinal and axillary lymph nodes (B) draining the site of MOG immunization, or blood (C) before and 7, 14, 21, or 28 d after MOG\(_{35-55}\) immunizations (left panels). Representative results demonstrate the frequencies of IL-10–producing cells after L + PIM stimulation for 5 h within the indicated gates among total CD19\(^{+}\) B cells, as well as CD4\(^{+}\)CD25\(^{+}\)FoxP3\(^{+}\) Treg frequencies among total CD4\(^{+}\) T cells. Bar graphs indicate mean (±SEM) frequencies and numbers of B cells that produced IL-10 or CD25\(^{+}\)FoxP3\(^{+}\)CD4\(^{+}\) Tregs from six mice in each group from two pooled independent experiments. Horizontal dashed lines are provided for reference to naive mice. D, Tregs dominate CNS tissues after EAE development. Bar graphs indicate mean (±SEM) percentages of IL-10\(^{+}\) B10 cells relative to total IL-10\(^{+}\) B10 cells plus Tregs within various tissues. A–D, Significant differences between means of naive mice and mice with EAE are indicated; *p < 0.05; **p < 0.01. Similar results were obtained in at least two independent experiments.
denileukin diftitox injection, as previously reported (38), but spleen CD25hiFoxP3hiCD4+ Tregs had returned by day 6 after the initial injection. Therefore, multiple denileukin diftitox injections were given. Treg numbers were significantly reduced (60%) in mice given denileukin diftitox 1, 4, and 7 d before Treg numbers were quantified (Fig. 3C). For Treg depletion before EAE onset, mice were given denileukin diftitox 1, 4, 7, 10, and 13 d after MOG35–55 immunization. This Treg depletion strategy delayed EAE onset by 2 d, but the severity of disease symptoms was not altered (cumulative EAE score, 29.6 ± 5.5) in comparison with PBS-treated littermates (33.6 ± 5.7; Fig. 3D). For Treg depletion after EAE onset, mice were given denileukin diftitox 14, 17, 20, 23, and 26 d after EAE induction. Late-phase Treg depletion worsened disease (54.0 ± 4.4%) significantly with 20% of the treated mice becoming moribund in comparison with PBS-treated littermates (35.6 ± 5.0, p < 0.05). Thus, B10 cell function was important for regulating EAE induction, whereas Treg function was important for regulating late-phase disease.

**Tregs dominate the CNS after EAE development**

Because B10 cells regulated EAE induction, whereas Treg function regulated late-phase disease, the relative frequencies of B10 and Tregs within the CNS, inguinal and axillary lymph nodes draining the site of MOG immunization, and blood were compared. Within the CNS, B10 cell frequencies relative to other B cells did not change significantly during EAE development, although B10 cell numbers were significantly increased on days 21 and 28 after MOG35–55 immunization (Fig. 4A, left panels). By contrast, CNS-infiltrating Treg frequencies relative to CD4+ T cells and numbers were dramatically increased on days 7, 21, and 28 after MOG35–55 immunization relative to naive mice (Fig. 4A, right panels). Lymph node B10 cell frequencies were slightly increased by 28 d after MOG35–55 immunization in contrast to naive mice, whereas B10 cell numbers were significantly increased on days 21 and 28 after MOG35–55 immunization (Fig. 4B, left panels). Treg frequencies within lymph nodes were significantly increased on days 21 and 28 after MOG35–55 immunization in contrast to naive mice, whereas Treg numbers were significantly increased during all stages of EAE (Fig. 4B, right panels). In blood, B10 cell frequencies and numbers were significantly increased on 28 d after MOG35–55 immunization (Fig. 4C, left panels). However, circulating Treg frequencies and numbers were not changed during the course of EAE (Fig. 4C, right panel). As a consequence, Tregs far outnumbered B10 cells within CNS tissues after EAE development, although the relative numbers of B10 and Tregs within the spleen and lymph nodes were essentially unchanged during the course of EAE (Fig. 4D). By contrast, blood B10 cell numbers increased gradually relative to Tregs during EAE progression. Thus, B10 cells were far more prevalent than Tregs within the CNS before disease initiation, with Tregs dominating during late-phase EAE.

**Adoptively transferred B10 cells inhibit EAE progression**

The effect of increasing B10 cell numbers in wild-type mice on EAE responses was assessed using adoptive transfer experiments. Spleen CD1dhiCD5+ B cells and CD1dloCD5− B cells were...
purified from naive mice (Fig. 5A, left panels), and transferred into mice that were immunized with MOG\textsubscript{35–55} 24 h after the transfer. In these adoptive transfer experiments, a total of $1.23 \times 10^5$ of the transferred CD\textsuperscript{1d\textsubscript{high}}CD\textsubscript{5\textsuperscript{+}} B cells or $0.05 \times 10^5$ of the transferred CD\textsuperscript{1d\textsubscript{low}}CD\textsubscript{5\textsuperscript{−}} B cells expressed cytoplasmic IL-10 after 5 h in vitro stimulation with L + PIM in two independent experiments. In one of two experiments with identical results, the adoptive transfer of CD\textsuperscript{1d\textsubscript{high}}CD\textsubscript{5\textsuperscript{+}} B cells from naive mice reduced disease severity (cumulative EAE score, 30.8 ± 2.5) in comparison with mice given CD\textsuperscript{1d\textsubscript{low}}CD\textsubscript{5\textsuperscript{−}} B cells (42.6 ± 4.5), but this effect was not statistically different from EAE induction or progression in PBS-treated littermates (36.6 ± 3.8; Fig. 5C, upper-left panel). Therefore, spleen CD\textsuperscript{1d\textsubscript{high}}CD\textsubscript{5\textsuperscript{+}} B cells and CD\textsuperscript{1d\textsubscript{low}}CD\textsubscript{5\textsuperscript{−}} B cells were purified from mice with EAE on day 28 after MOG\textsubscript{35–55} immunization. The frequency of B10 cells within the CD\textsuperscript{1d\textsubscript{high}}CD\textsubscript{5\textsuperscript{+}} B cell subsets was comparable when the donors were either naive mice (Fig. 5A) or mice with EAE (day 28; Fig. 5B, middle panels). Thereby, a total of $1.27 \times 10^5$ of the transferred CD\textsuperscript{1d\textsubscript{high}}CD\textsubscript{5\textsuperscript{+}} B cells or $0.05 \times 10^5$ of the transferred CD\textsuperscript{1d\textsubscript{low}}CD\textsubscript{5\textsuperscript{−}} B cells expressed cytoplasmic IL-10 after 5-h stimulation with L + PIM in two independent experiments. In one of two experiments with identical results, transferring CD\textsuperscript{1d\textsubscript{high}}CD\textsubscript{5\textsuperscript{+}} B cells purified from mice with EAE significantly reduced EAE severity (31.6 ± 4.1; days 13–18, $p < 0.05$) in recipients when compared with littermates given CD\textsuperscript{1d\textsubscript{low}}CD\textsubscript{5\textsuperscript{−}} B cells (51.4 ± 4.5) or PBS-treated littermates (43.4 ± 4.7; Fig. 5C, upper-second panel). Thus, providing naive mice with B10 cells from Ag-primed mice significantly reduced EAE symptoms, whereas more modest effects were observed when the B10 cells were isolated from naive mice.

The spleen CD\textsuperscript{1d\textsubscript{high}}CD\textsubscript{5\textsuperscript{+}} B cell subset contains B10pro cells that become competent to express IL-10 after in vitro CD40 engagement for 48 h to induce their maturation into IL-10–competent B10 cells (21). The CD\textsuperscript{1d\textsubscript{high}}CD\textsubscript{5\textsuperscript{+}} B cell subset from naive mice or mice with EAE (day 28) normally contains $\leq12\%$ IL-10$^+$ B10 cells after 5 h of L + PIM stimulation (Fig. 5A,5B). However, after 48 h of agonistic CD40 mAb stimulation, $\geq40\%$ of the purified CD\textsuperscript{1d\textsubscript{high}}CD\textsubscript{5\textsuperscript{+}} B cells expressed cytoplasmic IL-10, whereas $\leq2\%$ of purified CD\textsuperscript{1d\textsubscript{low}}CD\textsubscript{5\textsuperscript{−}} B cells produced IL-10 (Fig. 5B, right panels). Thereby, the frequencies of IL-10–competent B10 cells among CD\textsuperscript{1d\textsubscript{high}}CD\textsubscript{5\textsuperscript{+}} B cells can be enhanced significantly by B10pro cell maturation in vitro.

For subsequent adoptive transfer experiments, CD\textsuperscript{1d\textsubscript{high}}CD\textsubscript{5\textsuperscript{+}} and CD\textsuperscript{1d\textsubscript{low}}CD\textsubscript{5\textsuperscript{−}} B cells were purified from mice with EAE and...
FIGURE 7. B10 cells alter T cell cytokine profiles, but not T cell proliferation. Purified splenic CD1d<sup>high</sup>CD5+ or CD1d<sup>low</sup>CD5− B cells from mice with EAE (day 28) were stimulated with agonistic CD40 mAb for 48 h, with LPS added during the final 5 h of culture. A, B10 cell effects on T cell proliferation. CFSE-labeled TCR<sub>MOG</sub> CD4+ T cells were cultured alone or with CD40/LPS-stimulated CD1d<sup>high</sup>CD5+ or CD1d<sup>low</sup>CD5− B cells in the presence of MOG<sub>35-55</sub> (25 μg/ml) for 72 h. After 72 h, the cultured cells were stained for CD4 and Thy1.1 expression and analyzed for CFSE dilution by flow cytometry. Representative frequencies of dividing CFSE-labeled cells are shown (gated on CD4<sup>+</sup>Thy1.1<sup>+</sup>CFSE<sup>+</sup> cells). Bar graphs (left) indicate CFSE geometric mean fluorescence of the entire histogram, which is inversely proportional to cell divisions. Bar graphs (right) indicate mean (±SEM) frequencies of dividing TCR<sub>MOG</sub> CD4<sup>+</sup> T cells (CD1d<sup>high</sup>CD5+ B cell group, n; CD1d<sup>low</sup>CD5− B cell group, N) from six mice in each group from two pooled independent experiments.

B, B10 cells alter CD4<sup>+</sup> T cell cytokine production. TCR<sub>MOG</sub> CD4<sup>+</sup> T cells were cultured with CD40/LPS-stimulated CD1d<sup>high</sup>CD5+ or CD1d<sup>low</sup>CD5− B cells from wild-type or IL-10<sup>−/−</sup> mice with EAE (day 28) in the presence of MOG<sub>35-55</sub> (25 μg/ml) for 72 h, with PMA, ionomycin, and BFA added during the final 5 h of culture. Cytokine production by TCR<sub>MOG</sub> CD4<sup>+</sup> T cells was determined by intracellular cytokine staining with flow cytometry analysis. Numbers indicate percentages of T cells within the indicated gates among total CD4<sup>+</sup> T cells. Bar graphs indicate mean (±SEM) percentages of cytokine-producing CD4<sup>+</sup> T cells from six mice in each group from two pooled independent experiments.

C, Representative IL-10R expression (thick line) by splenic CD11c<sup>+</sup> DCs, CD11b<sup>high</sup> macrophages, B cells, and CD4<sup>+</sup> T cells from naive spleens and EAE spleens day 7. MFI (mean fluorescence intensity) was assessed for IL-10R expression in each cell population.

D, DCs increased the expression of IL-10R on B cells. DCs were cultured with wild-type or IL-10−/− B cells, and the expression of IL-10R on B cells was assessed by flow cytometry. The MFI was calculated for each group.

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stirulated with agonistic CD40 mAb for 48 h, with LPS added during the final 5 h of culture. In these adoptive transfer experiments, a total of $3.95 \times 10^5$ of the transferred CD1d$^{high}$CD5$^+$ B cells or $0.19 \times 10^5$ of the transferred CD1d$^{low}$CD5$^-$ B cells expressed cytoplasmic IL-10 in vitro after 5-h stimulation with L + PIM in two independent experiments. In one of two experiments with identical results, transferring CD40/LPS-stimulated CD1d$^{high}$CD5$^+$ B cells dramatically inhibited EAE progression (cumulative EAE score, 15.6 ± 6.9; days 12–22, $p < 0.05$ in comparison with PBS-treated littermates (42 ± 4, $p < 0.05$), whereas CD1d$^{low}$CD5$^-$ B cells did not (42 ± 6; Fig. 5C, lower-left panel). However, inhibition of disease was only observed when the transferred cells were given before MOG$_{35-55}$ immunization of recipients, but not on days 7 or 14 after immunization (Fig. 5C, lower-second and lower-third panels). Furthermore, CD40/LPS-stimulated CD1d$^{high}$CD5$^+$ B cells purified from IL-10$^{-/-}$ mice with EAE (day 28) did not affect EAE severity in wild-type recipients (Fig. 5C, lower-right panel). Thus, CD40/LPS-stimulated CD1d$^{high}$CD5$^+$ B cells optimally inhibited EAE initiation in an IL-10-dependent manner.

Adoptively transferred B10 cells inhibit leukocyte infiltration into the CNS

B cell depletion before MOG$_{35-55}$ immunization exacerabtes EAE and results in higher numbers of CD4$^+$ T cells within the CNS, whereas B cell depletion following disease initiation reduces CD4$^+$ T cell infiltration into the CNS (12). Whether the adoptive transfer of B10 cells from EAE mice inhibited T cell infiltration into the CNS of MOG$_{35-55}$-immunized mice was therefore assessed. CNS tissues were collected on day 18 from groups of mice that had been given PBS, or CD40/LPS-stimulated CD1d$^{high}$CD5$^+$ or CD1d$^{low}$CD5$^-$ B cells from EAE mice (day 28), and were examined by immunofluorescence staining of lymphocytes or quantitative microscopy. Remarkably, CD40/LPS-stimulated CD1d$^{high}$CD5$^+$ B cell transfers significantly reduced both Treg and CD4$^+$ T cell numbers within the CNS, whereas CD1d$^{low}$CD5$^-$ B cells were without effect (Fig. 6A). Spleen Treg and CD4$^+$ T cell numbers were not changed among these groups. As quantified by microscopy, CD40/LPS-stimulated CD1d$^{high}$CD5$^+$ B cell transfers reduced leukocyte infiltration (84% decrease thoracic, 84% lumbar; Fig. 6B) and significantly reduced demyelination (93% decrease thoracic, 88% lumbar; Fig. 6C) within CNS tissues when compared with mice given PBS ($p < 0.01$; Fig. 6D). Thus, adoptively transferred CD1d$^{high}$CD5$^+$ B cells had profound effects on Treg and leukocyte infiltration into the CNS.

B10 cells do not inhibit T cell proliferation, but regulate their cytokine production

In vitro T cell–B cell coculture systems were developed to determine how B10 cells could regulate T cell–mediated autoimmune disease in vivo. First, purified spleen CD1d$^{high}$CD5$^+$ B cells or CD1d$^{low}$CD5$^-$ B cells were stimulated with agonistic CD40 mAb (48 h) and LPS (last 5 h of culture), washed extensively, and added to cultures containing MOG$_{35-55}$ and CFSE-labeled CD4$^+$ T cells from TCR$^{MOG}$ transgenic mice whose CD4$^+$ T cells respond to MOG$_{35-55}$ peptide (39). CFSE dilution was assessed 72 h later as a marker for T cell proliferation. TCR$^{MOG}$ CD4$^+$ T cells cultured without B cells or without MOG$_{35-55}$ added to the cultures did not proliferate (Fig. 7A, data not shown). However, TCR$^{MOG}$ CD4$^+$ T cells cultured with either CD1d$^{high}$CD5$^+$ or CD1d$^{low}$CD5$^-$ B cells proliferated equally well in response to MOG$_{35-55}$. Thus, B10 cells did not regulate T cell proliferation in these in vitro assays. However, when the TCR$^{MOG}$ CD4$^+$ T cells were cocultured with B cells in the presence of MOG$_{35-55}$, there were significant differences in T cell cytokine induction observed following PMA, ionomycin, and BFA stimulation during the final 5 h of culture. TCR$^{MOG}$ CD4$^+$ T cells cultured with CD1d$^{high}$CD5$^+$ B cells had dramatically reduced IFN-$


gamma$ and TNF-$

alpha$ production when compared with TCR$^{MOG}$ CD4$^+$ T cells that were cultured with CD1d$^{low}$CD5$^-$ B cells (Fig. 7B). These changes depended on B cell IL-10 production, because CD1d$^{high}$CD5$^+$ B cells from IL-10$^{-/-}$ mice did not affect IFN-$

gamma$ or TNF-$

alpha$ production by TCR$^{MOG}$ CD4$^+$ T cells. IL-10 production by TCR$^{MOG}$ CD4$^+$ T cells was not significantly changed in these culture systems. Thus, B10 cell IL-10 can regulate Ag-specific T cell cytokine production.

B10 cells regulate Ag presentation by DCs in vitro

IL-10R expression is heterogeneous among cells of the immune system (40). Therefore, IL-10R expression by splenic DCs, macrophages, B cells, CD4$^+$ T cells, and CD8$^+$ T cells from wild-type mice was assessed. IL-10R expression was highest on DCs and macrophages, with modest expression by CD4$^+$ T cells, CD8$^+$ T cells, and B cells (Fig. 7C). IL-10R expression was not increased on any of these leukocyte subpopulations during the course of EAE (day 7).

Because high IL-10R expression by DCs may render them more sensitive to the regulatory effects of IL-10 than CD4$^+$ T cells, a role for B10 cells in regulating DC activation of CD4$^+$ T cells was assessed. Briefly, purified splenic CD1d$^{high}$CD5$^+$ or CD1d$^{low}$CD5$^-$ B cells from mice with EAE (day 28) were stimulated with agonistic CD40 mAb for 48 h, with LPS added during the final 5 h of culture. Purified splenic DCs from mice with EAE (day 10) were then cocultured with the CD40/LPS-stimulated CD1d$^{high}$CD5$^+$ or CD1d$^{low}$CD5$^-$ B cells in the presence of MOG$_{35-55}$. After 72 h of culture, the DCs were purified by cell sorting and cultured with TCR$^{MOG}$ CD4$^+$ T cells for 72 h. The TCR$^{MOG}$ CD4$^+$ T cells were then stained for CD4 and Thy1.1 expression and analyzed for CFSE dilution. In cultures in which the TCR$^{MOG}$ CD4$^+$ T cells were cultured with DCs cocultured with CD1d$^{high}$CD5$^+$ B cells, the intensity of CFSE staining was significantly higher and the percentage of dividing TCR$^{MOG}$ CD4$^+$ T cells was significantly reduced when compared with DCs cultured with CD1d$^{low}$CD5$^-$ B cells ($p < 0.05$; Fig. 7D). By contrast, culturing DCs with CD1d$^{high}$CD5$^+$ B cells from IL-10$^{-/-}$ mice did not affect their Ag-presenting ability in this T cell activation

macrophages, CD19$^+$ B cells, CD4$^+$ T cells, and CD8$^+$ T cells from wild-type mice before or 7 d after MOG$_{35-55}$ immunization. Gray histograms represent isotype-matched control mAb staining. Bar graphs indicate average mean linear fluorescence intensities (±SEM) of IL-10R expression by each cell type from six mice in each group from two pooled independent experiments. D. B10 cells inhibit the ability of DCs to activate CD4$^+$ T cells. Splenic CD1d$^{high}$CD5$^+$ or CD1d$^{low}$CD5$^-$ B cells from mice with EAE (day 28) were purified by cell sorting, and cultured with agonistic CD40 mAb for 48 h, with LPS added during the final 5 h of culture. Purified splenic DCs from mice with EAE (day 10) were cultured alone or with CD40/LPS-stimulated CD1d$^{high}$CD5$^+$ or CD1d$^{low}$CD5$^-$ B cells in the presence of MOG$_{35-55}$ (25 µg/ml) for 72 h. DCs purified from the B cell cocultures were then cultured with TCR$^{MOG}$ CD4$^+$ T cells for 72 h. The TCR$^{MOG}$ CD4$^+$ T cells were then stained for CD4 and Thy1.1 expression and analyzed for CFSE dilution. Mean frequencies of dividing CFSE-labeled CD4$^+$Thy1.1$^+$CFSE$^-$ cells are indicated (±SEM) in each histogram from six mice in each group from two pooled independent experiments. Bar graphs indicate CFSE geometric mean fluorescence of the entire histogram, which is inversely proportional to cell divisions. Significant differences between groups indicated; $p < 0.05$.  

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assay. Thus, IL-10–competent CD1d<sup>hi</sup>CD5<sup+</sup> B10 cells were able to regulate the Ag-presenting capability of DCs.

**Discussion**

This study reveals that B10 cells predominantly reduce disease severity during EAE initiation through the production of IL-10, whereas Tregs reciprocally inhibit late-stage EAE immunopathogenesis (Fig. 3). Remarkably, the early expansion in B10 cell numbers and IL-10 production following MOG<sub>35–55</sub> immunization parallels B10 cell regulation of disease initiation (Fig. 1A–C), whereas Treg expansion during disease progression parallels their negative regulation of late-stage disease (Fig. 1E). The current study also demonstrates that numbers of endogenous or adoptively transferred B10 cells directly influence the outcome of EAE pathogenesis. Specifically, mice with decreased B10 cell numbers exhibited enhanced disease severity relative to wild-type mice, whereas mice with enhanced B10 cell numbers had reduced EAE severity (Fig. 2). Likewise, the preferential depletion of B10 cells in vivo by CD22 mAb treatment enhanced EAE (Fig. 3).

**Ag-specific B10 cell expansion is required to elicit B10 cell regulatory functions, and a diverse repertoire of B cell Ag receptors is required for B10 cell development (12, 17, 21).** Thereby, the ability of B10 cells to rapidly expand during EAE initiation and to quickly inhibit disease severity suggests that a sufficient pool of Ag-specific B10 cells exists naturally that are rapidly mobilized to inhibit inflammation. This is supported by adoptive transfer experiments in which MOG<sub>35–55</sub>-primed CD1d<sup>hi</sup>CD5<sup+</sup> B cells, but not naive CD1d<sup>hi</sup>CD5<sup+</sup> B cells, inhibited EAE development (Fig. 5). The spleen of adult C57BL/6 mice normally contains 0.5–1.0 × 10<sup>6</sup> B10 cells and 3.5–4.5 × 10<sup>6</sup> B10 + B10pro cells (43). Remarkably, the adoptive transfer of 1.0 × 10<sup>6</sup> CD1d<sup>hi</sup>CD5<sup+</sup> spleen B cells containing ≈1.2 × 10<sup>5</sup> B10 cells from naive mice reduced EAE severity, whereas 1.0 × 10<sup>6</sup> CD1d<sup>hi</sup>CD5<sup+</sup> spleen B cells containing only ≈1.3 × 10<sup>5</sup> B10 cells from mice with EAE significantly reduced EAE severity (Fig. 5C). The transfer of 1.0 × 10<sup>6</sup> CD1d<sup>hi</sup>CD5<sup+</sup> spleen B cells from mice with EAE containing ≈3.9 × 10<sup>5</sup> in vitro matured B10 + B10pro cells was able to dramatically reduce EAE severity. Thereby, the identification of Ag-specific B10 cells may significantly reduce the number of adoptively transferred B10 cells needed for inhibiting EAE in vivo. Importantly, the adoptive transfer of IL-10<sup−</sup> CD1d<sup>hi</sup>CD5<sup+</sup> B cells did not affect EAE responses under any conditions tested. Thus, in addition to their preprogrammed ability to rapidly proliferate in response to external stimuli (21) and produce IL-10 (17), the size of the endogenous B10 and B10pro cell pool is a critical factor for regulating the magnitude of acute inflammation and the induction of autoimmunity.

B10 cells regulate T cell-mediated inflammatory responses and EAE through IL-10–dependent mechanisms (12, 17). The current study demonstrated that B10 cells did not directly regulate T cell proliferation (Fig. 7A), but significantly reduced CD4<sup+</sup> T cell IFN-γ and TNF-α production during in vitro assays (Fig. 7B). By contrast, non-CD1d<sup>hi</sup>CD5<sup+</sup> B cells were not able to influence CD4<sup+</sup> T cell IFN-γ and TNF-α production. Lampropoulou et al. (44) have shown that tissue culture supernatant fluid from LPS-stimulated spleen B cells does not suppress CD4<sup+</sup> T cell proliferation in response to CD3 mAb stimulation in vitro, but is able to suppress IFN-γ secretion by T cells stimulated with CpG-stimulated DCs. Tregs isolated from the CNS are also able to suppress T cell IFN-γ production in response to MOG<sub>35–55</sub> (45). Furthermore, B10 cells may also downregulate the ability of DCs to act as APCs and thereby indirectly modulate T cell proliferation (Fig. 7). Consistent with these findings, others have found that IL-10 suppresses the proliferation of Ag-specific CD4<sup+</sup> T cells by inhibiting the Ag-presenting capacity of monocytes and DCs (46) as well as inhibiting proinflammatory cytokine production by monocytes and macrophages (47). Lampropoulou et al. (44) have also shown that tissue culture supernatant fluid from LPS-stimulated spleen B cells is able to suppress T cell activation by CpG-stimulated DCs through IL-10–dependent pathways. IL-10 was initially associated with Th2 cells and was described to inhibit Th1 cytokine production (48–50). However, IL-10 is not only involved in the inhibition of Th1 polarization, but also prevents Th2 responses and exerts anti-inflammatory and suppressive effects on most hematopoietic cells. IL-10 produced by monocytes and cells other than T cells is required to maintain Treg-suppressive function and to maintain expression of the FoxP3 transcription factor in mice with colitis (51). However, the adoptive transfer of in vitro matured spleen B10 + B10pro cells from mice with EAE significantly reduced the number of Tregs within the CNS, in addition to reducing the number of inflammatory foci (Fig. 6). It is thereby unlikely that IL-10 produced by B10 cells contributes significantly to Treg maintenance during inflammation in vivo. However, future
CD22 mAb treatment preferentially depleted spleen B10 cells and exacerbated EAE disease severity (Fig. 3A, 3B). Because the CD22 mAb used in this study blocks CD22 ligand binding, it is possible that CD22 engagement is particularly essential for the survival of B10 cells within lymphoid tissues (29, 52). How these mouse findings relate to the therapeutic benefits of a CD22 mAb, epratuzumab, currently in clinical trials is unknown. However, CD22 mAb treatment only exacerbated EAE severity when given to mice before disease induction and did not exacerbate symptoms in mice with ongoing disease. Thereby, CD22 mAb treatment may not worsen disease in patients diagnosed with autoimmunity. However, there may be instances in which B10 cell depletion would be advantageous, such as in the treatment of cancers, immunosuppression, or vaccination. For example, Treg depletion using denileukin diftitox has demonstrated efficacy in the treatment of hematologic malignancies and solid tumors that do not express CD25, in autoimmune disease, and in enhancing vaccination-mediated T cell immunity (37, 38, 53, 54). Although therapeutic B cell depletion has shown clinical efficacy in treating MS patients (8, 9), B cell depletion may also remove B10 cells and exacerbate MS severity, induce disease in some undiagnosed cases, or promote relapses in some rare cases (55). As examples, B cell depletion was recently suggested to exacerbate ulcerative colitis and trigger psoriasis, diseases that are thought to be predominately T cell dependent (56, 57). In addition, one case report suggests that B cell depletion might have induced relapses in a patient with an 18-y history of MS who developed antmyelin-associated glycoprotein polyneuropathy that was treated with rituximab (58). Thereby, the therapeutic benefits of B10 cell and B cell depletion in humans may also depend on the relative contributions and timing of these opposing B cell functions during immune responses.

This study demonstrates that B10 cells expand during autoantigen-specific adaptive immune responses and that adoptively transferred B10 cells are sufficient to blunt EAE induction. Thus, the development of B10 cell-based therapies may be ideal for treating some autoimmune diseases. This could include the isolation, expansion, and return of expanded B10 cells isolated from Ag-sensitized individuals. Other studies have also shown that the adoptive transfer of B cells can have a therapeutic benefit in the treatment of mice with EAE, type 1 diabetes, and collagen-induced arthritis (11, 12, 59, 60). It may also be possible to identify pathways that regulate B10 cell activation, expansion, and function, which will allow this potent B cell subset to be manipulated for therapeutic benefit. In support of this, B cell IL-10 production in MS patients is significantly lower than in healthy controls and is upregulated following therapy (61). In addition, helminth infections induce regulatory B cells in MS patients and suppress disease activity (62), which may explain environment-related suppression of MS in areas with low disease prevalence. Whereas this study further reveals the regulatory complexities of the immune system, it also opens the door for the identification of B10 cell-directed therapies that may be able to reshape the course of autoimmune disease.

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References


