Regulatory B Cells (B10 cells) and Regulatory T Cells Have Independent Roles in Controlling EAE Initiation and Late-Phase Immunopathogenesis

Regulatory B Cells (B10 cells) and Regulatory T Cells Have Independent Roles in Controlling EAE Initiation and Late-Phase Immunopathogenesis[1]

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Abstract

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 $CD1d^{hi}CD5^{+}$ 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 regulatory $FoxP3^{+}$ T cells (Treg) overlap or influence EAE immunopathogenesis independently has remained unanswered. The current studies demonstrate 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 (MOG_{35-55}) immunization, which paralleled B10 cell regulation of disease initiation. The adoptive transfer of MOG_{33-35}-sensitized B10 cells into wild type mice reduced EAE initiation dramatically. However, B10 cells did not suppress ongoing EAE disease. Rather, Treg cell 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, while Treg cell depletion enhanced late-phase disease. B10 cells did not regulate T cell proliferation during *in vitro* assays, but significantly altered $CD4^{+}$ T cell IFN-γ and TNF-α production. Furthermore, B10 cells down-regulated the ability of DCs to act as antigen-presenting cells and thereby indirectly modulated T cell proliferation. Thus, B10 cells predominantly control disease initiation, while Treg cells reciprocally inhibit late-phase disease, with overlapping B10 cell and Treg cell functions shaping the normal course of EAE immunopathogenesis.

Introduction

Multiple sclerosis (MS) has been classically viewed as a predominantly T cell-dependent autoimmune disease, a feature shared by rheumatoid arthritis, systemic sclerosis, and type 1 diabetes. This conclusion derives from the finding that the adoptive transfer of T cells from diseased animals can initiate disease symptoms in healthy recipients. By contrast, regulatory $CD4^+$ T cells (Treg) are critically important for limiting T cell activation during MS and other autoimmune diseases (1, 2), in part through their production of IL-10 (3). However, B cells also regulate immune responses and can contribute to disease pathogenesis (4, 5) by functioning as cellular adjuvants for $CD4^+$ T cell activation (6) and through the production of cytokines that regulate T cell function and inflammation (7). Furthermore, recent phase I and II clinical trials in MS patients using depleting CD20 mAb (rituximab) suggest that pan-mature B cell depletion has clinical efficacy for the treatment of MS (8, 9), in addition to demonstrated efficacy for other autoimmune disorders (5). Despite these advances, understanding of the complex mechanisms through which B cells influence disease activity in humans and mice remains largely incomplete.

B cell negative regulation of immune responses has been demonstrated in the mouse experimental autoimmune encephalomyelitis (EAE) model of human MS (10-12), and other mouse models of autoimmunity and inflammation (7, 12-20). A relatively rare spleen B cell subset with IL-10-dependent negative regulatory functions has recently been identified that is predominantly contained within the phenotypically-unique $CD1d^{hi}CD5^+CD19^{hi}$ spleen B cell subpopulation in mice (12, 17-19). A specific subset of the $CD1d^{hi}CD5^+$ B cells can be induced to express cytoplasmic IL-10 following 5 h of *in vitro* stimulation with LPS, PMA, and ionomycin, with monensin included in the cultures to block IL-10 secretion (L+PIM stimulation). Given that multiple regulatory B cell subsets are likely to exist, as is now well recognized for T cells, we have specifically labeled the IL-10-competent $CD1d^{hi}CD5^+$ B cells as B10 cells because they appear to only produce IL-10 and they are responsible for most B cell IL-10 production (21). B10 progenitor (B10pro) cells have also been functionally identified in mice (5, 21). Spleen B10pro cells are also found within the $CD1d^{hi}CD5^+$ B cell subpopulation, but these

cells require 48 h of *in vitro* stimulation with LPS or through CD40 before they acquire the ability to express cytoplasmic IL-10 after 5 h stimulation with L+PIM (21). Although B10 cells normally represent only 1-2% of spleen B cells, they dramatically inhibit the induction of antigen-specific inflammatory reactions and autoimmunity (12, 17).

Significant roles for B10 cells and B cells have been reciprocally identified during the initiation and progression of EAE (12). Mature B cell depletion in mice before EAE induction significantly exacerbates disease symptoms, while 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 $CD1d^{hi}CD5^{+}$ 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 Treg cells overlap or influence EAE immunopathogenesis independently has remained unanswered. To address this question, the regulatory effects of adoptively transferring increasing numbers of naïve or EAE-sensitized B10 cells, or IL-10-deficient $CD1d^{hi}CD5^{+}$ B cells into wild type mice at various stages of disease was evaluated, in addition to depleting Treg cells 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. These studies thereby demonstrate that B10 cells have different regulatory functions when compared to Treg cells, as they function at different time points during EAE initiation and disease progression. Moreover, B10 cells directly influenced the production of pro-inflammatory cytokines by $CD4^{+}$ T cells and suppressed the Ag presenting function of DCs. Thereby, independent but overlapping B10 cell and Treg cell functions shape the normal course of EAE immunopathogenesis.

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 (Cederlane, 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 DNaseI (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 APC-conjugated CD1d (1B1), CD3 (17A2), CD4 (H129.19), CD5 (53-7.3), 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 used mAbs reactive with IL-10 (JES5-16E3), IFN-γ (XMG1.2), TNF-α (MP6-XT22), and FoxP3 (FJK-16s) (all from eBioscience) and Cytofix/Cytoperm kits (BD Biosciences). For T cell intracellular cytokine staining, lymphocytes were stimulated *in vitro* with PMA (50 ng/ml; Sigma, St. Louis, MO) and ionomycin (1 μg/ml; Sigma), in the presence of Brefeldin A (BFA, 1 μl/ml; eBioscience) for 5 h before staining. Background staining was assessed using non-reactive, isotype-matched control mAbs (Caltag Laboratories, San Francisco, CA). 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 (25). Blood erythrocytes were lysed after staining using FACS™ Lysing Solution (Becton Dickinson, San Jose, CA). Cells with the forward and side light scatter properties of lymphocytes were analyzed using a FACScan flow cytometer (Becton Dickinson) or BD FACSCanto™ II (BD Biosciences).

Mice and immunotherapy

C57BL/6 and IL-10$^{-/-}$ (B6.129P2-*Il10*^{tmlCgn}/J) mice (26) were from The Jackson Laboratory (Bar Harbor, ME). CD19$^{-/-}$ and hCD19Tg (h19-1 line) mice were backcrossed with C57BL/6 mice for 14 and 7 generations, respectively, as described (27, 28). TCR^{MOG} transgenic mice (Thy1.2^{+}, provided by Dr. V. K. Kuchroo, Harvard Medical School, Boston, MA) were crossed to C57BL/6.Thy1.1 mice to generate Thy1.1-expressing T cells. All mice were bred in a specific pathogen-free barrier facility and used at 6-12 wks of age.

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

EAE induction

Active EAE was induced in 6- to 8-week-old female mice by subcutaneous immunization with 100 μg of MOG$_{35-55}$ peptide (MEVGWYRSPFSRVVHLYRNGK; NeoMPS, San Diego, CA) emulsified in CFA containing 200 μg of heat-killed *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI) on day 0. Additionally, mice received 200 ng of pertussis toxin (List Biological Laboratories, Campbell, CA) i.p. in 0.5 ml of PBS on days 0 and 2. Clinical signs of EAE were assessed daily with a 0 to 6 point scoring system; 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 day 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, animals 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 lumber 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 (NIH, 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 (2×10^6 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-mercaptoethanol (all from Gibco, Carlsbad, CA)] with LPS (10 μg/ml, *Escherichia coli* serotype 0111: B4, Sigma), PMA (50 ng/ml; Sigma), ionomycin (500 ng/ml; Sigma), 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 Pharmingen) as described (21). For IL-10 detection, Fc receptors were blocked with mouse Fc receptor mAb (2.4G2; BD PharMingen) with dead cells detected using a LIVE/DEAD® Fixable Violet Dead Cell Stain Kit (Invitrogen-Molecular Probes) before cell surface staining. Stained cells were fixed and permeabilized using a Cytofix/Cytoperm kit (BD PharMingen) 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 reverse transcription PCR Analysis

Total RNA was extracted from cell sorter purified B cells using Qiagen RNeasy spin columns (Qiagen Ltd., Crawley, UK). 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. Cycler conditions were one amplification cycle of denaturation at 95º C for 3 min followed by 40 cycles of 95º C for 10 s, 59º C for 1 min, and 95º 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'-GGTTGCCAAGCCTTATCGGA-3' and the antisense primer was 5'-ACCTGCTCCACTGCCTTGCT-3'. The sense GAPDH primer was 5'-TTCACCACCATGGAGAAGGC-3' and the antisense primer was 5'-GGCATGGACTGTGGTCATGA-3'.

Lymphocyte subset isolation

MACS (Miltenyi Biotech, 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 Biotech) 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 purities, respectively. Spleen DCs were obtained as previously described with minor modifications (30). Briefly, spleens were minced and incubated with collagenase D (1 mg/ml; Roche Applied Sciences, Indianapolis, IN) and DNaseI (0.2 mg/ml; Roche Applied Sciences) 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 Biotech).

Cell sorting and adoptive transfer experiments

Naïve 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 Biotech). In addition, $CD1d^{hi}CD5^{+}$ and $CD1d^{lo}CD5^{-}$ B cells were isolated using a FACSVantage SE flow cytometer (Becton Dickinson) with purities of 95-98%. After purification, $1x10^{6}$ cells were immediately transferred intravenously into recipient mice.

***In vitro* T cell, B cell and DC co-culture assays**

Splenic $CD1d^{hi}CD5^{+}$ or $CD1d^{lo}CD5^{-}$ 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 ($1x10^{6}$/ml) were cultured alone or with CD40/LPS-stimulated $CD1d^{hi}CD5^{+}$ or $CD1d^{lo}CD5^{-}$ B cells ($1x10^{6}$/ml) in the presence of MOG_{35-55} (25 μg/ml) for 72 h.

In additional experiments, splenic $CD1d^{hi}CD5^{+}$ or $CD1d^{lo}CD5^{-}$ 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 ($1x10^{6}$/ml) with CD40 mAb/LPS-stimulated $CD1d^{hi}CD5^{+}$ or $CD1d^{lo}CD5^{-}$ B cells ($1x10^{6}$/ml) in the presence of MOG_{35-55} (25 μg/ml) for 72 h. DCs were purified by cell sorting after 72 h co-cultures with B cells, and then cultured ($5x10^{4}$/ml) alone or with MACS-purified TCR^{MOG} $CD4^{+}$ T cells ($2x10^{5}$/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's t test.

Results

B10 and Treg cell expansion during EAE

B10 cells and the spleen $CD1d^{hi}CD5^{+}$ 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 MOG_{35-55} immunization, spleen $CD1d^{hi}CD5^{+}$ B cell frequencies and numbers were significantly increased on days 7, 21, and 28 in contrast to naïve mice (Fig. 1A). B10 cell frequencies and numbers were also significantly increased on days 7, 21, and 28 after MOG_{35-55} immunization (Fig. 1B). Increased B cell IL-10 production paralleled B10 cell frequencies (Fig. 1C), while 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 MOG_{35-55} immunization, which resolved, with a subsequent increase during EAE disease onset and resolution.

Since Treg cells negatively regulate EAE symptoms (2), spleen $CD25^{hi}FoxP3^{+}CD4^{+}$ Treg cell numbers were also quantified. Treg cell frequencies and numbers were only significantly higher by day 21 and 28 after MOG_{35-55} immunization in contrast to naïve mice (Fig. 1E). Thereby, B10 cell numbers increased during both the initiation and late phases of EAE progression, while Treg cell 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 human CD19 transgenic (hCD19Tg) mice that overexpress CD19. Spleen $CD1d^{hi}CD5^{+}$ B cells were present at similar frequencies and numbers in wild type and $IL\text{-}10^{-/-}$ mice (Fig. 2A), as described (17). In hCD19Tg mice, $CD1d^{hi}CD5^{+}$ 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. 2B). By contrast,

$CD1d^{hi}CD5^{+}$ 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, while 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 around day 11 after immunization of wild type mice and peaked around day 18, then declined gradually (Fig. 2C). By contrast, EAE severity was significantly diminished in hCD19Tg mice (cumulative EAE score, 17.5±4.7) when compared with wild type mice (38.1±5.0, $p<0.05$). By contrast, EAE severity was significantly enhanced in $CD19^{-/-}$ mice (62.5±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 $CD1d^{hi}CD5^{+}$ or $CD1d^{lo}CD5^{-}$ 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}. $CD1d^{hi}CD5^{+}$ B cells were used for the adoptive transfer experiments since this small subpopulation contains both the B10 and B10pro cell subsets and can be identified without *in vitro* stimulation (17). Transferring $CD1d^{hi}CD5^{+}$ B cells into $CD19^{-/-}$ mice significantly reduced EAE severity (cumulative EAE score, 40.6±5.0) to levels observed in wild type mice (37.2±5.6, Fig. 2D *left panel*) when compared with $CD19^{-/-}$ mice (62.2±3.9, $p<0.01$). By contrast, the adoptive transfer of $CD1d^{lo}CD5^{-}$ B cells into $CD19^{-/-}$ mice before EAE induction did not affect EAE severity (61.8±5.1, Fig. 2D *right panel*). Furthermore, the adoptive transfer of $CD1d^{hi}CD5^{+}$ 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). Since 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 $CD1d^{hi}CD5^{+}$ 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, while control mAb treatment was without effect. Thereby, $CD1d^{hi}CD5^{+}$ B cells and B10 cells could be preferentially removed without eliminating the majority of spleen B cells.

B10 cells regulate EAE initiation while Treg cells 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 days before and 0, 7, 14, and 21 days after EAE induction in order 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 cell depletion before MOG_{35-55} immunization can increase the severity of EAE, while Treg cells accumulate in the CNS during the recovery phase of disease (32-35). Therefore, the

functional contributions of Treg cells to EAE initiation and pathogenesis were measured after their *in vivo* depletion. CD25 mAb was not used for depleting Treg cells since 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 Treg cells expressing high affinity CD25 (36-38). Denileukin diftitox has limited effects on subsequently activated effector T cells due to its short half-life *in vivo*. Spleen $CD25^{hi}FoxP3^{+}CD4^{+}$ Treg cells were maximally reduced 3 days after denileukin diftitox injection, as previously reported (38), but spleen $CD25^{hi}FoxP3^{+}CD4^{+}$ Treg cells had returned by day 6 after the initial injection. Therefore, multiple denileukin diftitox injections were given. Treg cell numbers were significantly reduced (60%) in mice given denileukin diftitox 1, 4 and 7 days before Treg cell numbers were quantified (Fig. 3C). For Treg cell depletion before EAE onset, mice were given denileukin diftitox 1, 4, 7, 10, and 13 days after MOG_{35-55} immunization. This Treg cell depletion strategy delayed EAE onset by 2 days, 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 cell depletion after EAE onset, mice were given denileukin diftitox 14, 17, 20, 23, and 26 days after EAE induction. Late-phase Treg cell 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, while Treg cell function was important for regulating late-phase disease.

Treg cells dominate the CNS after EAE development

Since B10 cells regulated EAE induction, while Treg cell function regulated late-phase disease, the relative frequencies of B10 and Treg cells 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 MOG_{35-55}

immunization (Fig. 4A, *left panels*). By contrast, CNS-infiltrating Treg cell frequencies relative to $CD4^+$ T cells and numbers were dramatically increased on days 7, 21, and 28 after MOG_{35-55} immunization relative to naïve mice (Fig. 4A, *right panels*). Lymph node B10 cell frequencies were slightly increased by 28 days after MOG_{35-55} immunization in contrast to naïve mice, while B10 cell numbers were significantly increased on days 21 and 28 after MOG_{35-55} immunization (Fig. 4B, *left panels*). Treg cell frequencies within lymph nodes were significantly increased on days 21 and 28 after MOG_{35-55} immunization in contrast to naïve mice, while Treg cell 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 days after MOG_{35-55} immunization (Fig. 4C, *left panels*). However, circulating Treg cell frequencies and numbers were not changed during the course of EAE (Fig. 4C, *right panel*). As a consequence, Treg cells far outnumbered B10 cells within CNS tissues after EAE development, although the relative numbers of B10 and Treg cells 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 Treg cells during EAE progression. Thus, B10 cells were far more prevalent than Treg cells within the CNS before disease initiation, with Treg cells 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 $CD1d^{hi}CD5^+$ B cells and $CD1d^{lo}CD5^-$ B cells were purified from naive mice (Fig. 5A, *left panels*), and transferred into mice that were immunized with MOG_{35-55} 24 h after the transfer. In these adoptive transfer experiments, a total of 1.23×10^5 of the transferred $CD1d^{hi}CD5^+$ B cells or 0.05×10^5 of the transferred $CD1d^{lo}CD5^-$ 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 $CD1d^{hi}CD5^+$ B cells from naive mice reduced disease severity (cumulative EAE score, 30.8±2.5) in comparison with mice given $CD1d^{lo}CD5^-$ 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 $CD1d^{hi}CD5^{+}$ B cells and $CD1d^{lo}CD5^{-}$ B cells were purified from mice with EAE on day 28 after MOG_{35-55} immunization. The frequency of B10 cells within the $CD1d^{hi}CD5^{+}$ B cell subsets were 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 x 10^5 of the transferred $CD1d^{hi}CD5^{+}$ B cells or 0.05 x 10^5 of the transferred $CD1d^{lo}CD5^{-}$ 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 $CD1d^{hi}CD5^{+}$ B cells purified from mice with EAE significantly reduced EAE severity (31.6±4.1; day 13-18, $p<0.05$) in recipients when compared with littermates given $CD1d^{lo}CD5^{-}$ B cells (51.4±4.5) or PBS-treated littermates (43.4±4.7; Fig. 5C, *upper-second panel*). Thus, providing naïve mice with B10 cells from antigen-primed mice significantly reduced EAE symptoms, while more modest effects were obtained when the B10 cells were isolated from naïve mice.

The spleen $CD1d^{hi}CD5^{+}$ 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 $CD1d^{hi}CD5^{+}$ B cell subset from naïve mice or mice with EAE (day 28) normally contains ~12% $IL\text{-}10^{+}$ B10 cells after 5 h of L+PIM stimulation (Fig. 5A-B). However, after 48 h of agonistic CD40 mAb stimulation, ~40% of the purified $CD1d^{hi}CD5^{+}$ B cells expressed cytoplasmic IL-10, whereas <2% of purified $CD1d^{lo}CD5^{-}$ B cells produced IL-10 (Fig. 5B, *right panels*). Thereby, the frequencies of IL-10-competent B10 cells among $CD1d^{hi}CD5^{+}$ B cells can be enhanced significantly by B10pro cell maturation *in vitro*.

For subsequent adoptive transfer experiments, $CD1d^{hi}CD5^{+}$ and $CD1d^{lo}CD5^{-}$ B cells were purified from mice with EAE and stimulated 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 x 10^5 of the transferred $CD1d^{hi}CD5^{+}$ B cells or 0.19 x 10^5 of the transferred $CD1d^{lo}CD5^{-}$ 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 $CD1d^{hi}CD5^{+}$ B

cells dramatically inhibited EAE progression (cumulative EAE score, 15.6±6.9; day 12-22, $p<0.05$) in comparison with PBS-treated littermates (42±4, $p<0.05$), while $CD1d^{lo}CD5^-$ 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 third panels*). Furthermore, CD40/LPS-stimulated $CD1d^{hi}CD5^+$ 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 $CD1d^{hi}CD5^+$ 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 exacerbates EAE and results in higher numbers of $CD4^+$ T cells within the CNS, while 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 $CD1d^{hi}CD5^+$ or $CD1d^{lo}CD5^-$ B cells from EAE mice (day 28), and were examined by immunofluorescence staining of lymphocytes or quantitative microscopy. Remarkably, CD40/LPS-stimulated $CD1d^{hi}CD5^+$ B cell transfers significantly reduced both Treg and $CD4^+$ T cell numbers within the CNS, while $CD1d^{lo}CD5^-$ 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 $CD1d^{hi}CD5^+$ 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 $CD1d^{hi}CD5^+$ B cells had profound effects on Treg cell 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 co-culture systems were developed to determine how B10 cells could regulate T cell mediated autoimmune disease *in vivo*. First, purified spleen $CD1d^{hi}CD5^{+}$ B cells or $CD1d^{lo}CD5^{-}$ 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 $CD1d^{hi}CD5^{+}$ or $CD1d^{lo}CD5^{-}$ 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 co-cultured with B cells in the presence of MOG_{35-55} there were significant differences in T cell cytokine induction observed following PMA, ionomycin, and brefeldin A stimulation during the final 5 h of culture. TCR^{MOG} $CD4^{+}$ T cells cultured with $CD1d^{hi}CD5^{+}$ B cells had dramatically reduced IFN-γ and TNF-α production when compared with TCR^{MOG} $CD4^{+}$ T cells that were cultured with $CD1d^{lo}CD5^{-}$ B cells (Fig. 7B). These changes depended on B cell IL-10 production, since $CD1d^{hi}CD5^{+}$ B cells from $IL\text{-}10^{-/-}$ mice did not affect IFN-γ or TNF-α 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 antigen-specific T cell cytokine production.

B10 cells regulate antigen-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).

Since 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 $CD1d^{hi}CD5^+$ or $CD1d^{lo}CD5^-$ 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 co-cultured with the CD40/LPS-stimulated $CD1d^{hi}CD5^+$ or $CD1d^{lo}CD5^-$ 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 where the TCR^{MOG} $CD4^+$ T cells were cultured with DCs co-cultured with $CD1d^{hi}CD5^+$ 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 to DCs cultured with $CD1d^{lo}CD5^-$ B cells ($p<0.05$; Fig. 7D). By contrast, culturing DCs with $CD1d^{hi}CD5^+$ B cells from IL-$10^{-/-}$ mice did not affect their antigen-presenting ability in this T cell activation assay. Thus, IL-10-competent $CD1d^{hi}CD5^+$ B10 cells were able to regulate the antigen-presenting capability of DCs.

Discussion

These studies reveal that B10 cells predominantly reduce disease severity during EAE initiation through the production of IL-10, while Treg cells reciprocally inhibit late-stage EAE immunopathogenesis (Fig. 3). Remarkably, the early expansion in B10 cell numbers and IL-10 production following MOG_{35-55} immunization parallels B10 cell regulation of disease initiation (Fig. 1A-C), while Treg cell expansion during disease progression parallels their negative regulation of late-stage disease (Fig. 1E). The current studies also demonstrate that numbers of endogenous or adoptively transferred B10 cells directly influences the outcome of EAE pathogenesis. Specifically, mice with decreased B10 cell numbers exhibited enhanced disease severity relative to wild type mice, while 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 pathogenesis (Fig. 3A-B). By contrast, the adoptive transfer of antigen-sensitized B10 cells into naïve recipients before MOG_{35-55} immunizations inhibited EAE pathogenesis through the production of IL-10, while increasing B10 cell numbers in mice exhibiting disease symptoms was without significant effect (Fig. 5). Thus, regulatory B10 cells and Treg cells have independent roles in controlling EAE initiation and late-phase immunopathogenesis.

The timing of differential B10 cell and Treg cell expansion within tissues parallels their importance during disease initiation and late-phase EAE pathogenesis. B10 cell and $CD1d^{hi}CD5^{+}$ B cell numbers expanded significantly (~70% increase) by 7 days after MOG_{35-55} immunization, decreased, and expanded to even higher levels during the course of disease progression, with maximum numbers (~130% increase) accumulating as disease resolved (Fig. 1A-B). IL-10 production by spleen B cells and blood B10 cell numbers followed a similar course of expansion, contraction, and expansion (Fig. 4C, Fig. 1C), while lymph node B10 cell numbers expanded most significantly during disease resolution (Fig. 4B). By contrast, Treg cell numbers did not increase following MOG_{35-55} immunization, but increased gradually with EAE progression (Fig. 2C). On a relative frequency basis, B10 cells were 4-times more prevalent in the CNS than Treg

cells before MOG_{35-55} immunization and their numbers remained constant except during disease resolution (Fig. 4A). By contrast, Treg cells were 19-fold more prevalent in the CNS on day 28 than B10 cells, while spleen B10 cell and Treg cell frequencies were equal throughout the course of EAE (Fig. 4D). The absence of Treg cell expansion during EAE initiation provides the likely mechanistic explanation for why B cell depletion by CD20 mAb or B10 cell depletion by CD22 mAb before MOG_{35-55} immunization exacerbated EAE (Fig. 3B, ref. 12). Reciprocally, the dramatic expansion of Treg cells within the CNS during EAE progression explains why the removal of B cells or B10 cells at this time does not enhance disease pathogenesis. This was confirmed when denileukin diftitox-induced Treg cell depletion had no effect on disease initiation but only exacerbated late-phase disease (Fig. 3D). Denileukin diftitox treatment also suppresses active EAE in rats when given early, but results in lethal disease if given later (41). In contrast to the current studies in wild type mice, it has been previously reported that IL-10-producing B cells contribute to EAE recovery in mice that are genetically B cell-deficient (11). However, this is explained by the finding that B cell deficiency delays the emergence of Treg cells and IL-10 in the CNS during EAE (42). Thereby, the relative balance between B10 cell and Treg cell numbers in wild type mice during the course of EAE has dramatic effects on disease outcome.

Antigen-specific B10 cell expansion is required to elicit B10 cell regulatory functions and a diverse repertoire of B cell antigen receptors is required for B10 cell development (12, 17, 21). Thereby, the ability of B10 cells to rapidity 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 where MOG_{35-55}-primed $CD1d^{hi}CD5^{+}$ B cells, but not naïve $CD1d^{hi}CD5^{+}$ B cells, inhibited EAE development (Fig. 5). The spleen of adult C57BL/6 mice normally contains 0.5-1.0 x 10^6 B10 cells and 3.5-4.5 x 10^6 B10+B10pro cells (43). Remarkably, the adoptive transfer of 1.0 x 10^6 $CD1d^{hi}CD5^{+}$ spleen B cells containing ~1.2 x 10^5 B10 cells from naïve mice reduced EAE severity, while 1.0 x 10^6 $CD1d^{hi}CD5^{+}$ spleen B cells containing only ~1.3 x 10^5 B10 cells from

mice with EAE significantly reduced EAE severity (Fig. 5C). The transfer of 1.0 x 10^6 $CD1d^{hi}CD5^+$ spleen B cells from mice with EAE containing ~3.9 x 10^5 *in vitro*-matured B10+B10pro cells was able to dramatically reduce EAE severity. Thereby, the identification of antigen-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-$10^{-/-}$ $CD1d^{hi}CD5^+$ B cells did not affect EAE responses under any conditions tested. Thus, in addition to their pre-programmed 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 studies demonstrated that B10 cells did not directly regulate T cell proliferation (Fig. 7A), but significantly reduced $CD4^+$ T cell IFN-γ and TNF-α production during *in vitro* assays (Fig. 7B). By contrast, non-$CD1d^{hi}CD5^+$ B cells were not able to influence $CD4^+$ T cell IFN-γ and TNF-α production. Lampropoulou et al. have shown that tissue culture supernatant fluid from LPS-stimulated spleen B cells does not suppress $CD4^+$ T cell proliferation in response to CD3 mAb stimulation *in vitro,* but is able to suppress IFN-g secretion by T cells stimulated with CpG-stimulated DC (44). Treg cells isolated from the CNS are also able to suppress T cell IFN-γ production in response to MOG_{35-55} (45). Furthermore, B10 cells may also down-regulate the ability of DCs to act as antigen-presenting cells 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 $CD4^+$ T cells by inhibiting the Ag-presenting capacity of monocytes and DCs (46) as well as inhibiting pro-inflammatory cytokine production by monocytes and macrophages (47). Lampropoulou et al. 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 (44). 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 cell 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 Treg cells 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 cell maintenance during inflammation *in vivo*. However, future studies will be needed to determine whether B10 cells regulate cytokine production by the wide variety of additional T cell subsets that are known to critically influence EAE pathogenesis.

CD22 mAb treatment preferentially depleted spleen B10 cells and exacerbated EAE disease severity (Figs. 3A-B). Since the CD22 mAb used in the current 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 where B10 cell depletion would be advantageous such as in the treatment of cancers, immunosuppression, or vaccination. For example, Treg cell 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 vaccine-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-dependant (56, 57). In addition, one case

report suggests that B cell depletion might have induced relapses in a patient with an 18-year history of MS who developed anti-myelin-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.

The current studies demonstrate 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 up-regulated 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. While the current studies further reveal the regulatory complexities of the immune system, they also open 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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Footnotes

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[3]Abbreviations used: B10 cells, $CD1d^{hi}CD5^{+}$ B cells with the capacity to produce IL-10; B10pro cells, B10 progenitor cells; EAE, experimental autoimmune encephalomyelitis; hCD19Tg, human CD19 transgenic mice; MOG_{35-55}, myelin oligodendrocyte glycoprotein peptide; MS, multiple sclerosis Treg, regulatory $FoxP3^{+}$ $CD4^{+}$ T cells.

Figure Legends

Figure 1. B10 cell and Treg cell numbers increase during EAE. **(A)** $CD1d^{hi}CD5^{+}$ B cell frequencies and numbers increase during the course of EAE. Splenocytes were isolated from mice before, and 7, 14, 21 or 28 days after MOG_{35-55} immunization and analyzed for CD1d, CD5, and CD19 expression by immunofluorescence staining with flow cytometry analysis. Representative results demonstrate the frequency of $CD1d^{hi}CD5^{+}$ B cells within the indicated gates among total $CD19^{+}$ B cells. Bar graphs indicate mean (±SEM) percentages and numbers of $CD1d^{hi}CD5^{+}$ 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 mice before, and 7, 14, 21 or 28 days after MOG_{35-55} 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 reverse transcription 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)** $CD4^{+}CD25^{+}FoxP3^{+}$ 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 $CD4^{+}CD25^{+}FoxP3^{+}$ T cells within the indicated gates among total $CD4^{+}$ T cells. Bar graphs indicate mean (±SEM) percentages and numbers of $CD4^{+}CD25^{+}FoxP3^{+}$ 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 naïve mice. Significant differences between means of naïve and immunized mice are indicated; *$p<0.05$, **, $p<0.01$.

Figure 2. B10 cell frequencies significantly influence EAE severity. **(A)** $CD1d^{hi}CD5^{+}$ B cell frequencies and numbers in wild type, hCD19Tg, $CD19^{-/-}$, and IL-$10^{-/-}$ mice. Representative results demonstrate the frequency of spleen $CD1d^{hi}CD5^{+}$ B cells within the indicated gates among total $CD19^{+}$ or $CD20^{+}$ B cells. Bar graphs indicate mean (±SEM) percentages and numbers of $CD1d^{hi}CD5^{+}$ B cells in one of two independent experiments with 3 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. (A-B) Values in each group represent results from 6 mice from two pooled independent experiments. Significant differences between means from wild type and other mouse groups are indicated; *$p<0.05$, **, $p<0.01$. **(C)** EAE progression and severity in wild type, $CD19^{-/-}$, and hCD19Tg mice immunized with MOG_{35-55} on day 0 and scored daily thereafter for EAE disease severity. Values represent mean (±SEM) EAE clinical scores from ≥5 mice in each group, with similar results obtained in 2 independent experiments. Significant differences between wild type and mutant mice groups are indicated; *$p<0.05$ (wild type versus hCD19Tg mice), †$p<0.05$ (wild type versus $CD19^{-/-}$ mice). **(D)** Adoptively transferred $CD1d^{hi}CD5^{+}$ B cells reduce EAE severity in $CD19^{-/-}$ mice. Splenic $CD1d^{hi}CD5^{+}$ or $CD1d^{lo}CD5^{-}$ B cells were purified from naïve wild type or IL-$10^{-/-}$ mice by cell sorting. Wild type and $CD19^{-/-}$ recipient mice were either given PBS, purified $CD1d^{hi}CD5^{+}$ or $CD1d^{lo}CD5^{-}$ B cells 1 day (arrowheads) before MOG_{35-55} immunizations. Values represent mean (±SEM) results from ≥5 mice in each group, with similar results obtained in 2 independent experiments. Significant differences between the means of EAE clinical scores are indicated: *$p<0.05$ (black diamond versus white diamond).

Figure 3. B10 cells and Treg cells cooperatively regulate EAE severity. **(A)** B10 cell depletion *in vivo*. Spleen $CD1d^{hi}CD5^{+}$ and IL-10^{+} B cell frequencies were determined in wild type mice 7

days after MB22-10 or control mAb treatments (250 μg/mouse). Upper panels, representative results demonstrate the frequency of $CD1d^{hi}CD5^{+}$ B cells within the indicated gates among total $CD19^{+}$ B cells. Lower panels, representative results demonstrate the frequencies of IL-10^{+} cells within the indicated gates among total $CD19^{+}$ B cells. Bar graphs indicate mean (±SEM) percentages and numbers of $CD1d^{hi}CD5^{+}$ or IL-10^{+} B cells from 6 mice in each group from two pooled independent experiments. Significant differences between MB22-10 or control mAb treatment groups are indicated; **, $p<0.01$. **(B)** Early B10 cell deletion exacerbates EAE disease severity. Mice were treated with MB22-10 (closed circles) or control (open circles) mAb (250 μg, arrowheads) from day -7 (days -7, 0, 7, 14, 21), from day 7 (days 7, 14, 21), from day 14 (days 14, 21), or from day 21, as indicated. Values represent mean (±SEM) EAE clinical scores from ≥5 mice in each group, with similar results obtained in 2 independent experiments. Significant differences between MB22-10 and control mAb-treated groups are indicated; *$p<0.05$. **(C)** Treg cell deletion. Representative results demonstrate spleen $CD4^{+}CD25^{+}FoxP3^{+}$ Treg cell frequencies within the indicated gates among total $CD4^{+}$ T cells and Treg cell numbers in wild type mice 7 days after initial denileukin diftitox or PBS treatments. Denileukin diftitox was administrated 1, 3 or 6 days before analysis or 1, 4 and 7 days before analysis as indicated. Bar graphs indicate mean (±SEM) percentages and numbers of $CD4^{+}CD25^{+}FoxP3^{+}$ Treg cells from 6 mice in each group from two pooled independent experiments. Significant differences between denileukin diftitox or PBS treatment groups are indicated; *$p<0.05$, **, $p<0.01$. **(D)** Late Treg cell deletion exacerbates EAE disease severity. Mice were treated with denileukin diftitox (closed circles) or PBS (open circles) before (days 1, 4, 7, 10, 13, arrowheads) or after EAE onset (days 14, 17, 20, 23, 26, arrowheads). Values represent mean (±SEM) EAE clinical scores from ≥5 mice in each group, with similar results obtained in 2 independent experiments. Significant differences between denileukin diftitox and PBS treatment groups are indicated; *$p<0.05$.

Figure 4. Changes in B10 and Treg cell frequencies and numbers within tissues during the course of EAE. Mononuclear cells were isolated from **(A)** CNS tissue, **(B)** inguinal and axillary lymph nodes draining the site of MOG immunization, or **(C)** blood before, and 7, 14, 21 or 28 days 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 cell frequencies among total $CD4^+$ T cells. Bar graphs indicate mean (±SEM) frequencies and numbers of B cells that produced IL-10 or $CD25^+FoxP3^+CD4^+$ Treg cells from 6 mice in each group from two pooled independent experiments. Horizontal dashed lines are provided for reference to naïve mice. **(D)** Treg cells 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 Treg cells within various tissues. (A-D) Significant differences between means of naïve mice and mice with EAE are indicated; *$p<0.05$, **, $p<0.01$. Similar results were obtained in at least 2 independent experiments.

Figure 5. The adoptive transfer of MOG_{35-55}-sensitized B10 cells can reduce EAE disease severity in wild type mice. **(A-B)** Representative B10 cell purification results for adoptive transfer experiments. Purified spleen B cells from naïve mice or mice with EAE (day 28) were separated into $CD1d^{hi}CD5^+CD19^+$ and $CD1d^{lo}CD5^-CD19^+$ B cell populations. The isolated cells were cultured with L+PIM for 5 h, or were cultured with agonistic CD40 mAb for 48 h with L+PIM added during the final 5 h of culture. B10 cell frequencies in the stimulated cell cultures were determined by immunofluorescence staining with flow cytometry analysis. **(C)** The adoptive transfer of purified spleen $CD1d^{hi}CD5^+$ B cells reduces EAE disease severity. Wild type recipient mice were given either PBS, $CD1d^{hi}CD5^+$ B cells, or $CD1d^{lo}CD5^-$ B cells from naïve wild type mice, mice with EAE (day 28), or IL-$10^{-/-}$ mice with EAE (day 28) 1 day before MOG_{35-55} immunizations or 7 or 14 days after MOG_{35-55} immunization as indicated (arrowheads). In some cases, the purified B cell populations were stimulated as indicated with agonistic CD40

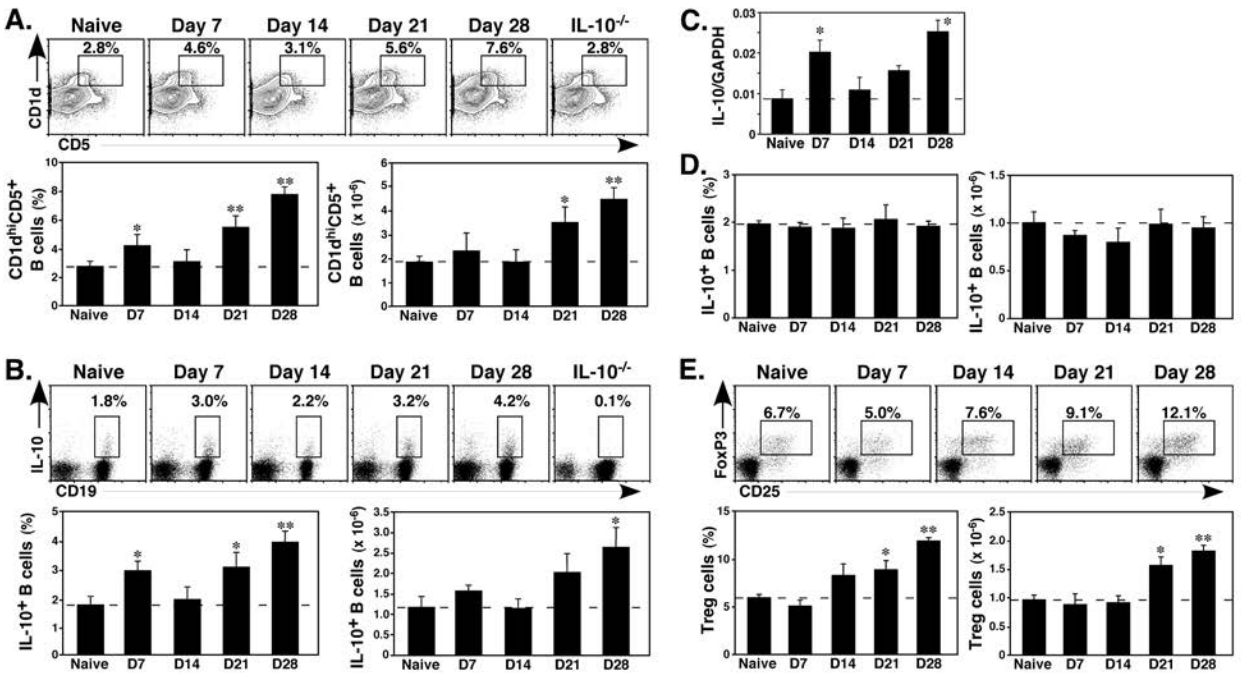
mAb for 48 h, with LPS added during the final 5 h of culture to induce B10pro cell maturation and thereby expand B10 cell frequencies. Values represent means (±SEM) from ≥5 mice in each group, with similar results obtained in 2 independent experiments. Significant differences between the means of EAE clinical scores are indicated: *$p<0.05$ (black diamond versus white circle).

Figure 6. Treg cell and leukocyte infiltration into the CNS following the adoptive transfer of B10 cells. Wild type recipient mice were given PBS, or either CD40/LPS-stimulated $CD1d^{hi}CD5^{+}$ or $CD1d^{lo}CD5^{-}$ B cells from mice with EAE (day 28) 1 day before MOG_{35-55} immunizations as in figure 5C. (**A**) Mononuclear cells within CNS tissues or spleen 18 days after MOG_{35-55} immunizations. Bar graphs indicate mean (±SEM) numbers of $CD4^{+}$ T cells or frequencies/numbers of $CD4^{+}CD25^{+}FoxP3^{+}$ Treg cells among total $CD4^{+}$ T cells (n≥5 mice per group). Significant differences between groups of mice receiving $CD1d^{hi}CD5^{+}$ B cells or PBS are indicated; **$p<0.01$. Similar results were obtained in at least 2 independent experiments. (**B-D**) EAE histopathology following the adoptive transfer of B10 cells. Representative lumbar spinal cord sections were harvested 18 days after MOG_{35-55} immunizations (n≥5 mice per group) with (B) inflammation (H&E staining) and (C) demyelination (Luxol Fast Blue staining) demonstrated. Scale bar is 0.5 mm. B) Arrowheads indicate inflammatory foci. C) Yellow traced areas indicate demyelination. (**D**) Bar graphs indicate mean (±SEM) numbers of inflammatory foci and percentages of spinal cord sections that were demyelinated, with significant differences between groups of mice receiving $CD1d^{hi}CD5^{+}$ B cells or PBS indicated; **$p<0.01$. Similar results were obtained in at least 2 independent experiments.

Figure 7. B10 cells alter T cell cytokine profiles, but not T cell proliferation. Purified splenic $CD1d^{hi}CD5^{+}$ or $CD1d^{lo}CD5^{-}$ 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^{MOG} $CD4^{+}$ T cells were cultured alone or with

CD40/LPS-stimulated $CD1d^{hi}CD5^{+}$ or $CD1d^{lo}CD5^{-}$ B cells in the presence of MOG_{35-55} (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 $CD4^{+}Thy1.1^{+}CFSE^{+}$ 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^{MOG} $CD4^{+}$ T cells ($CD1d^{hi}CD5^{+}$ B cell group, closed bars; $CD1d^{lo}CD5^{-}$ B cell group, open bars) from 6 mice in each group from two pooled independent experiments. (**B**) B10 cells alter $CD4^{+}$ T cell cytokine production. TCR^{MOG} $CD4^{+}$ T cells were cultured with CD40/LPS-stimulated $CD1d^{hi}CD5^{+}$ or $CD1d^{lo}CD5^{-}$ B cells from wild type or $IL\text{-}10^{-/-}$ mice with EAE (day 28) in the presence of MOG_{35-55} (25 μg/ml) for 72 h, with PMA, ionomycin, and BFA added during the final 5 h of culture. For negative controls, TCR^{MOG} $CD4^{+}$ T cells were cultured with CD40/LPS-stimulated $CD1d^{lo}CD5^{-}$ B cells in the presence of MOG_{35-55} (25 μg/ml) for 72 h, with BFA alone (without PMA/ionomycin) added during the final 5 h of culture. Cytokine production by TCR^{MOG} $CD4^{+}$ T cells was determined by intracellular cytokine staining with flow cytometry analysis. Numbers indicate percentages of T cells within the indicated gates among total $CD4^{+}$ T cells. Bar graphs indicate mean (±SEM) percentages of cytokine producing $CD4^{+}$ T cells from 6 mice in each group from two pooled independent experiments. (**C**) Representative IL-10R expression (thick line) by splenic $CD11c^{+}$ DCs, $CD11b^{hi}$ macrophages, $CD19^{+}$ B cells, $CD4^{+}$ T cells, and $CD8^{+}$ T cells from wild type mice before or 7 days 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 6 mice in each group from two pooled independent experiments. (**D**) B10 cells inhibit the ability of DCs to activate $CD4^{+}$ T cells. Splenic $CD1d^{hi}CD5^{+}$ or $CD1d^{lo}CD5^{-}$ 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 $CD1d^{hi}CD5^{+}$ or $CD1d^{lo}CD5^{-}$ B cells in the presence

of MOG_{35-55} (25 μg/ml) for 72 h. DCs purified from the B cell co-cultures were then cultured with TCR^{MOG} $CD4^+$ T cells for 72 h, or the T cells were cultured alone, with CD4 and Thy1.1 expression and CFSE dilution analyzed by flow cytometry. Mean frequencies of dividing CFSE-labeled $CD4^+Thy1.1^+CFSE^+$ cells are indicated (±SEM) in each histogram from 6 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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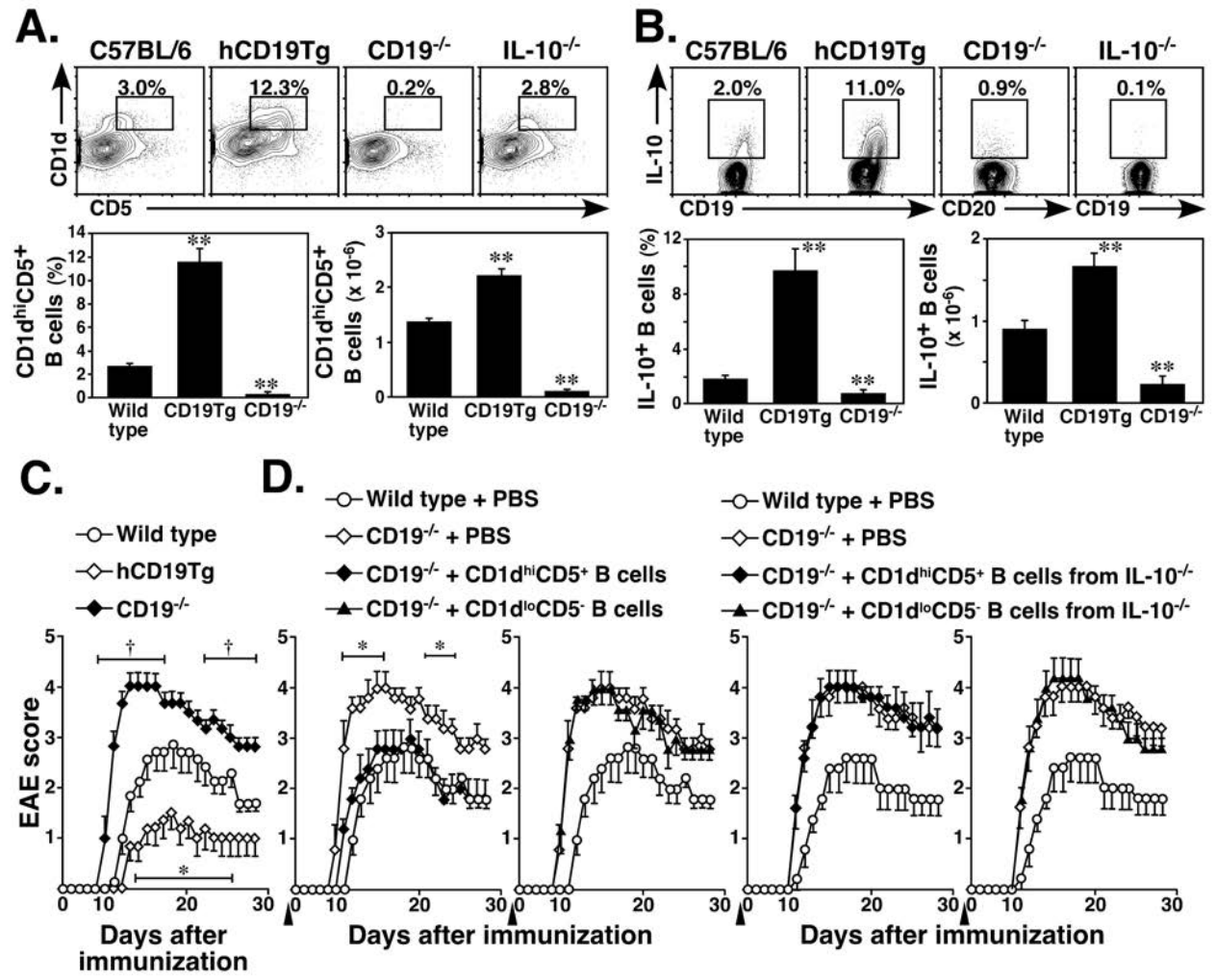
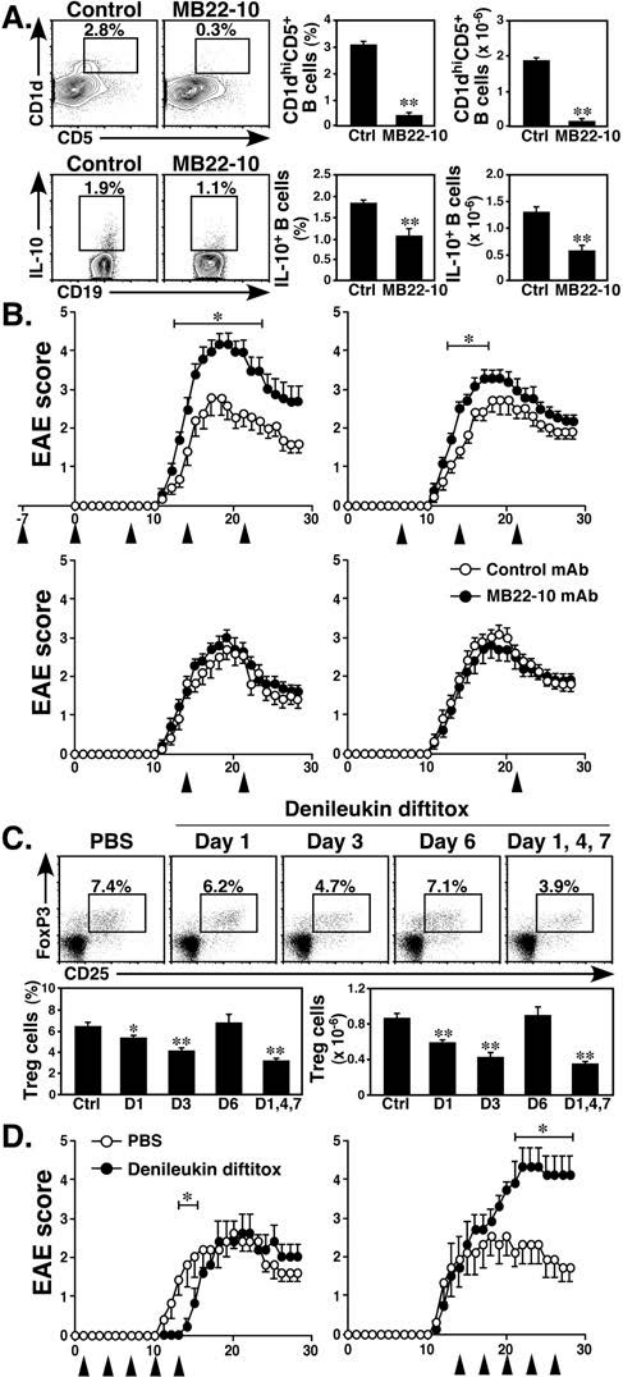
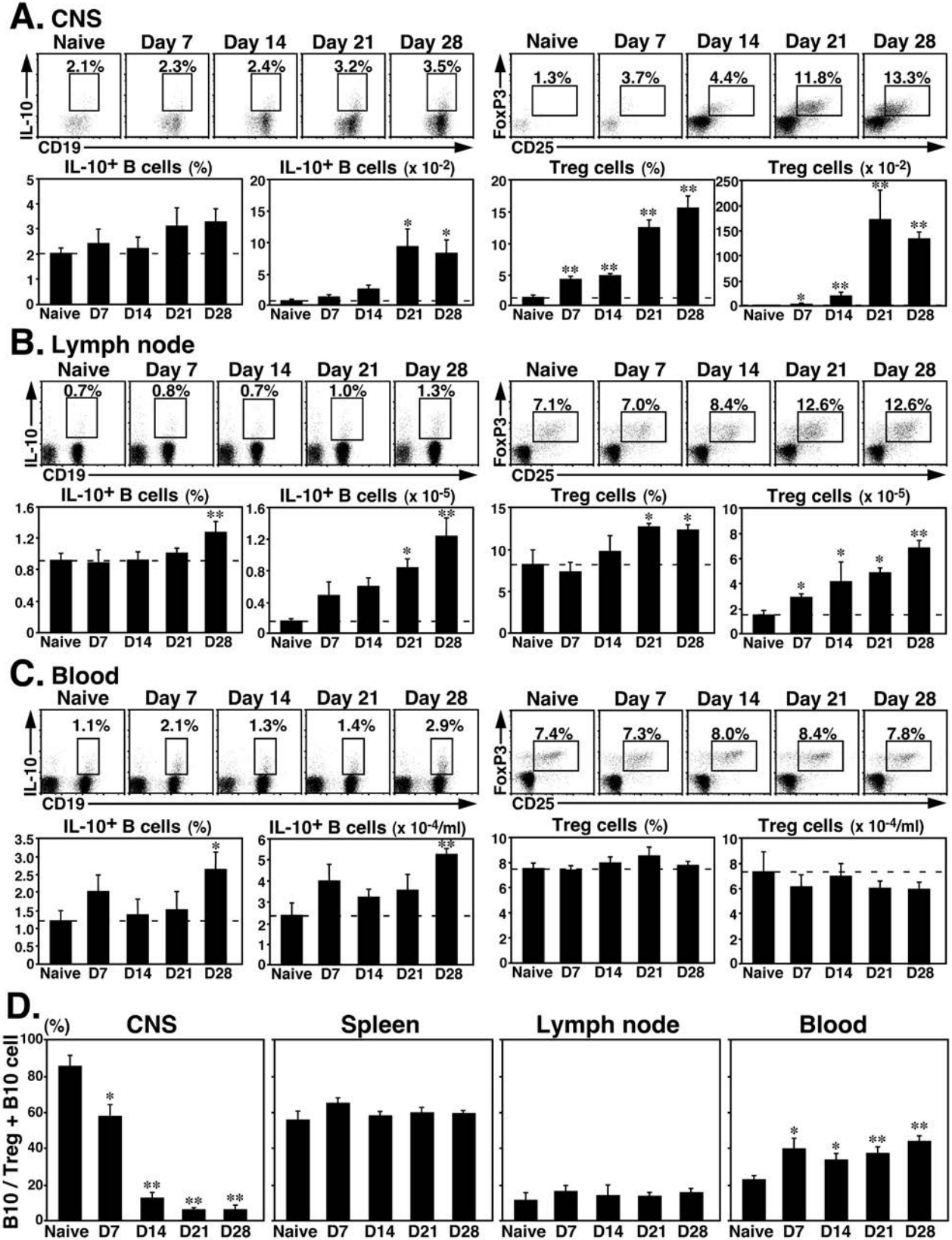
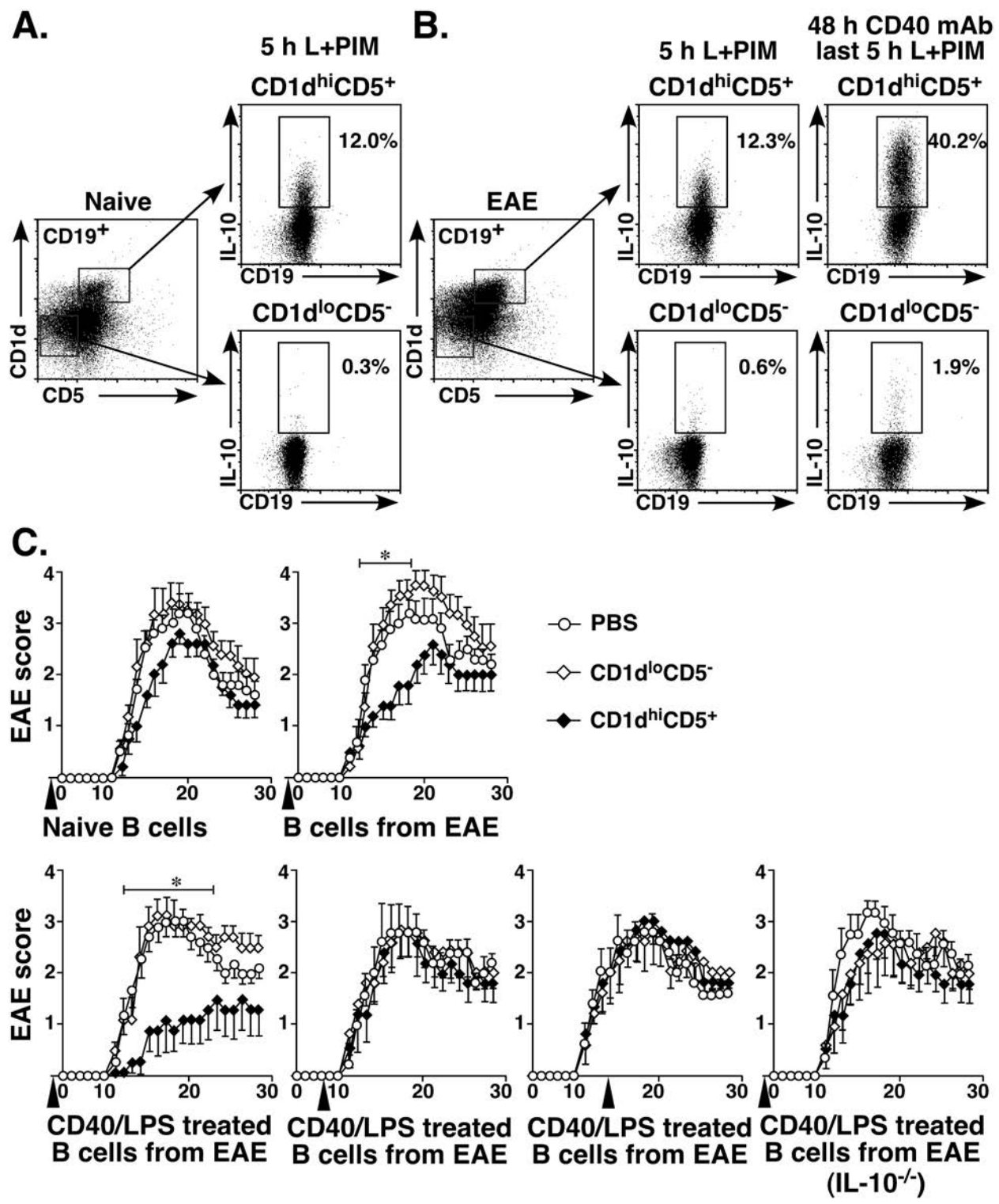
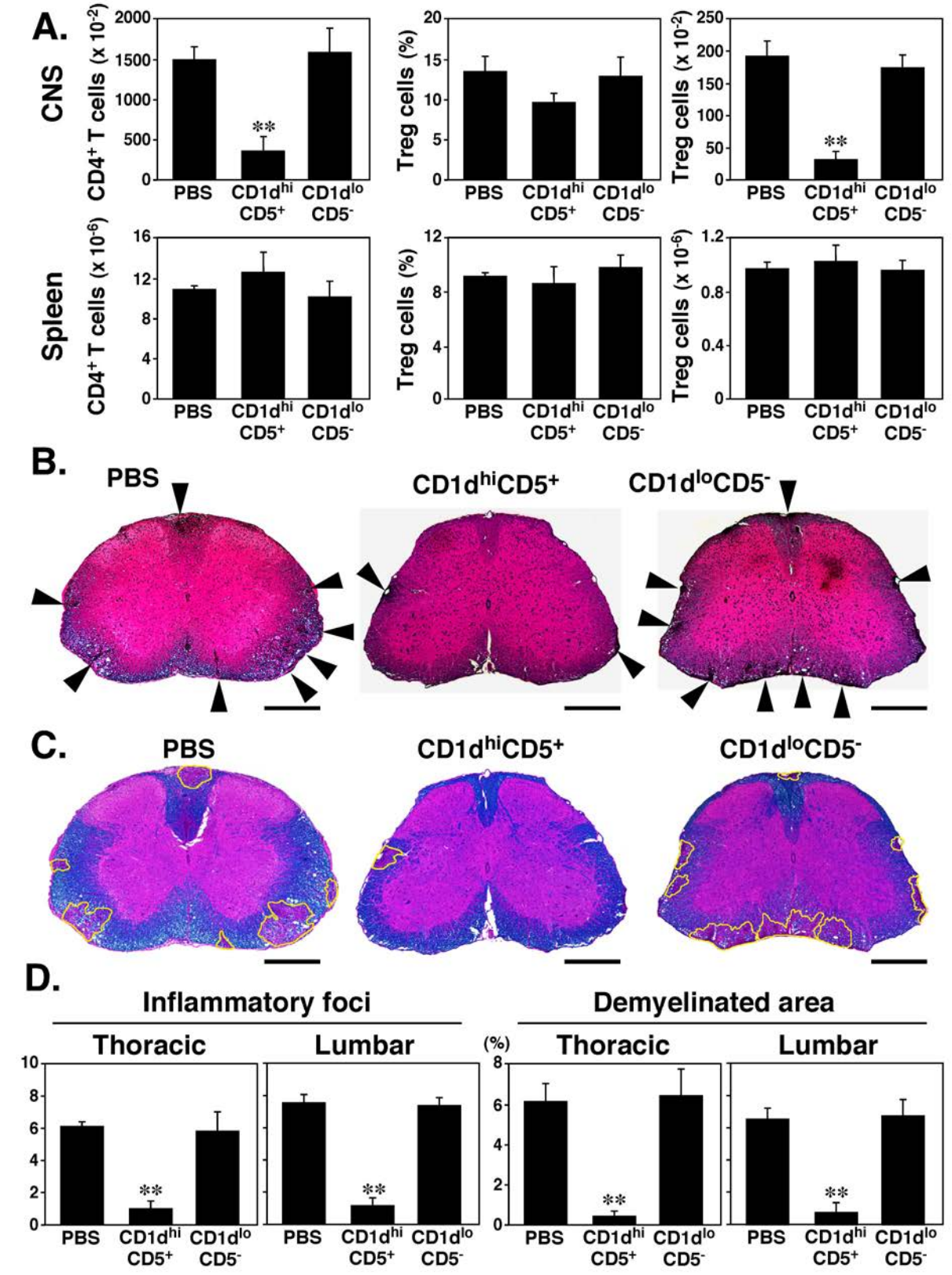
Naive
Day 7
Day 14
Day 21
Day 28
IL-10-/-
2.8%
4.6%
3.1%
5.6%
7.6%
2.8%
CD1d
CD5
CD1dhiCD5+ B cells (%)
CD1dhiCD5+ B cells (x 10-6)
Naive D7 D14 D21 D28
B.
1.8%
3.0%
2.2%
3.2%
4.2%
0.1%
IL-10
CD19
IL-10+ B cells (%)
IL-10+ B cells (x 10-6)
C.
IL-10/GAPDH
D.
E.
6.7%
5.0%
7.6%
9.1%
12.1%
FoxP3
CD25
Treg cells (%)
Treg cells (x 10-6)

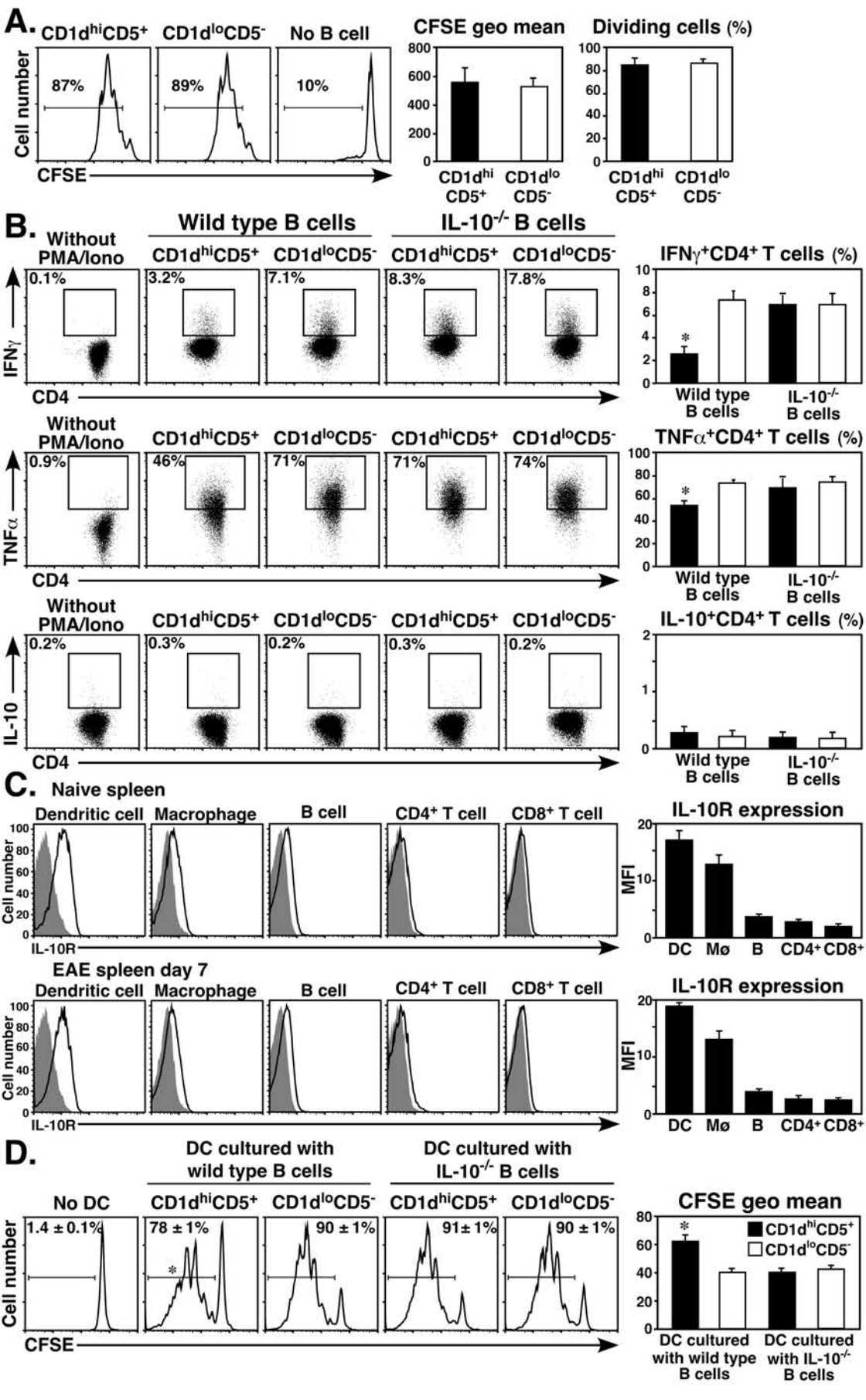

A.
C57BL/6
hCD19Tg
CD19-/-
IL-10-/-
3.0%
12.3%
0.2%
2.8%
CD1d
CD5
CD1dhiCD5+ B cells (%)
CD1dhiCD5+ B cells (x 10-6)
Wild type
CD19Tg
CD19-/-
B.
2.0%
11.0%
0.9%
0.1%
IL-10
CD19
CD20
IL-10+ B cells (%)
IL-10+ B cells (x 10-6)
C.
Wild type
hCD19Tg
CD19-/-
EAE score
Days after immunization
D.
Wild type + PBS
CD19-/- + PBS
CD19-/- + CD1dhiCD5+ B cells
CD19-/- + CD1dloCD5- B cells
CD19-/- + CD1dhiCD5+ B cells from IL-10-/-
CD19-/- + CD1dloCD5- B cells from IL-10-/-


A.
Control
MB22-10
2.8%
0.3%
CD1d
CD5
CD1d^hiCD5^+ B cells (%)
CD1d^hiCD5^+ B cells (x 10^-5)
Ctrl
MB22-10
1.9%
1.1%
IL-10
CD19
IL-10^+ B cells (%)
IL-10^+ B cells (x 10^-6)
B.
EAE score
Control mAb
MB22-10 mAb
Denileukin diftitox
C.
PBS
Day 1
Day 3
Day 6
Day 1, 4, 7
7.4%
6.2%
4.7%
7.1%
3.9%
FoxP3
CD25
Treg cells (%)
Treg cells (x 10^-6)
Ctrl
D1
D3
D6
D1,4,7
D.
PBS
Denileukin diftitox
EAE score


A. CNS
Naive
Day 7
Day 14
Day 21
Day 28
2.1%
2.3%
2.4%
3.2%
3.5%
IL-10
CD19
1.3%
3.7%
4.4%
11.8%
13.3%
FoxP3
CD25
IL-10+ B cells (%)
IL-10+ B cells (x 10-2)
Treg cells (%)
Treg cells (x 10-2)
B. Lymph node
0.7%
0.8%
0.7%
1.0%
1.3%
7.1%
7.0%
8.4%
12.6%
12.6%
IL-10+ B cells (x 10-5)
Treg cells (x 10-5)
C. Blood
1.1%
2.1%
1.3%
1.4%
2.9%
7.4%
7.3%
8.0%
8.4%
7.8%
IL-10+ B cells (x 10-4/ml)
Treg cells (x 10-4/ml)
D.
(%)
B10 / Treg + B10 cell
CNS
Spleen
Lymph node
Blood


A.
5 h L+PIM
CD1d^hiCD5^+
12.0%
Naive
CD19^+
CD1d
CD5
IL-10
CD19
CD1d^loCD5^-
0.3%
B.
5 h L+PIM
48 h CD40 mAb
last 5 h L+PIM
CD1d^hiCD5^+
12.3%
CD1d^hiCD5^+
40.2%
EAE
CD19^+
CD1d^loCD5^-
0.6%
CD1d^loCD5^-
1.9%
C.
EAE score
PBS
CD1d^loCD5^-
CD1d^hiCD5^+
Naive B cells
B cells from EAE
CD40/LPS treated B cells from EAE
CD40/LPS treated B cells from EAE
CD40/LPS treated B cells from EAE
CD40/LPS treated B cells from EAE (IL-10^-/-)


A.
CNS
Spleen
CD4+ T cells (x 10-2)
Treg cells (%)
Treg cells (x 10-2)
CD4+ T cells (x 10-6)
Treg cells (%)
Treg cells (x 10-6)
PBS
CD1dhi CD5+
CD1dlo CD5-
**
B.
PBS
CD1dhiCD5+
CD1dloCD5-
C.
PBS
CD1dhiCD5+
CD1dloCD5-
D.
Inflammatory foci
Demyelinated area
Thoracic
Lumbar
(%)


A.
CD1d^hiCD5+
CD1d^loCD5-
No B cell
87%
89%
10%
Cell number
CFSE
CFSE geo mean
Dividing cells (%)
CD1d^hi CD5+
CD1d^lo CD5-
B.
Without PMA/Iono
Wild type B cells
IL-10^-/- B cells
0.1%
3.2%
7.1%
8.3%
7.8%
IFNγ
CD4
IFNγ+CD4+ T cells (%)
Wild type B cells
IL-10^-/- B cells
0.9%
46%
71%
71%
74%
TNFα
TNFα+CD4+ T cells (%)
0.2%
0.3%
0.2%
0.3%
0.2%
IL-10
IL-10+CD4+ T cells (%)
C.
Naive spleen
Dendritic cell
Macrophage
B cell
CD4+ T cell
CD8+ T cell
IL-10R
IL-10R expression
MFI
DC
Mø
B
CD4+
CD8+
EAE spleen day 7
D.
No DC
DC cultured with wild type B cells
DC cultured with IL-10^-/- B cells
1.4 ±0.1%
78 ±1%
90 ±1%
91± 1%
90 ±1%
CFSE geo mean
DC cultured with wild type B cells
DC cultured with IL-10^-/- B cells