CD1d<sup>hi</sup>CD5<sup>+</sup> B Cells Expanded by GM-CSF In Vivo Suppress Experimental Autoimmune Myasthenia Gravis

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IL-10–competent subset within CD1d<sup>hi</sup>CD5<sup>+</sup> B cells, also known as B10 cells, has been shown to regulate autoimmune diseases. Whether B10 cells can prevent or suppress the development of experimental autoimmune myasthenia gravis (EAMG) has not been studied. In this study, we investigated whether low-dose GM-CSF, which suppresses EAMG, can expand B10 cells in vivo, and whether adoptive transfer of CD1d<sup>hi</sup>CD5<sup>+</sup> B cells would prevent or suppress EAMG. We found that treatment of EAMG mice with low-dose GM-CSF increased the proportion of CD1d<sup>hi</sup>CD5<sup>+</sup> B cells and B10 cells. In vitro coculture studies revealed that CD1d<sup>hi</sup>CD5<sup>+</sup> B cells altered T cell cytokine profile but did not directly inhibit T cell proliferation. In contrast, CD1d<sup>hi</sup>CD5<sup>+</sup> B cells inhibited B cell proliferation and its autoantibody production in an IL-10–dependent manner. Adoptive transfer of CD1d<sup>hi</sup>CD5<sup>+</sup> B cells to mice could prevent disease, as well as suppress EAMG after disease onset. This was associated with downregulation of mature dendritic cell markers and expansion of regulatory T cells resulting in the suppression of acetylcholine receptor–specific T cell and B cell responses. Thus, our data have provided significant insight into the mechanisms underlying the tolerogenic effects of B10 cells in EAMG. These observations suggest that in vivo or in vitro expansion of CD1d<sup>hi</sup>CD5<sup>+</sup> B cells or B10 cells may represent an effective strategy in the treatment of human myasthenia gravis. *The Journal of Immunology, 2014, 193: 2669–2677.*
(tAChR) was used to induce EAMG and as Ag for in vitro studies of immune responses.

Induction and clinical scoring of EAMG

Eight-wk-old female C57BL6/J mice were immunized with 20 μg tAChR/IFA in 100 μl s.c., and boosted with 20 μg tAChR emulsified in IFA in 100 μl injected in the flanks and tail base every 24–30 d. Mice were observed and scored daily or every other day after the first booster. For clinical examination, mice were evaluated for myasthenic weakness and assigned clinical scores as previously described (8, 9). Clinical weakness was graded as follows: grade 0, mouse with normal posture, muscle strength, and mobility at baseline and after exercise; grade 1, normal at rest but with muscle weakness postexercise, as shown by a hunchback posture, restricted mobility, and difficulty in raising the head after exercise; grade 2, mild weakness at baseline, which worsens after exercise; grade 3, dehydrated and moribund with moderate weakness at baseline; and grade 4, dead.

The evaluator was blinded to treatment status for all clinical evaluations.

GM-CSF treatment and adoptive transfer experiments

For adoptive transfer (AT) experiments, donor mice were immunized with tAChR (20 μg tAChR/IFA in 100 μl s.c.) followed by one booster at 24–30 d later (day 0) and treatment with GM-CSF (2 μg daily i.p. for 10 d) or PBS. These donor mice were sacrificed 14 d after GM-CSF treatments (24 d after the booster immunization). Splenic CD19+ B cells were isolated from mice by positive selection using magnetic beads (Miltenyi Biotec, Auburn, CA) with obtained purity ≥95%. CD1dhiCD5+ cells were purified (95–98%) using a FACSaria flow cytometer (BD Biosciences). After purification, CD1dhiCD5+ B cells (1 × 106) were immediately transferred into appropriate recipient mice by tail-vein injection.

All recipient mice received immunizations every 24–30 d at least twice after the initial priming immunization with tAChR. The protocols, specifically the timing of AT and booster immunizations for EAMG prevention and suppression studies, are summarized in Supplemental Table I. Clinical scores were followed daily starting at day 0. All AT experiments were repeated at least twice to ensure reproducibility.

Flow cytometry

Single-cell suspensions of splenocytes were prepared from mice sacrificed upon the completion of the GM-CSF/PBS treatment regimen for flow cytometric analysis. Cells were washed with PBS supplemented with 0.05% BSA, blocked with anti-CD16/CD32 Fc-block (BD Pharmingen, San Jose, CA) on ice for 30 min. Allophycocyanin-conjugated anti-CD19, Pacific Blue–conjugated anti-CD5 and PE-conjugated anti-CD1d (Biolegend), anti-Id (MHC class II), anti-CD40, anti-CD80, anti-CD86, isotype control Abs (BD Pharmingen) were used in flow cytometry and analyzed using FlowJo software (Tree Star, Ashland, OR). Mouse Treg staining kit (w/PE Foxp3) from BD Biosciences was used for intracellular staining of T cells. PE-conjugated anti-CD154 Ab was used for intracellular cytokine staining of T or B cells. For B cells, isolated leukocytes or purified cells were resuspended (2 × 106 cells/ml) in complete medium (RPMI 1640 containing 10% FCS, 200 μg/ml penicillin, 200 μl streptomycin, 4 mM L-glutamine, and 5 × 10–4 M 2-mercaptoethanol) and placed in 24-well plates for 5 h at 37˚C. Cells were fixed and permeabilized using the Cytofix/Cytoperm kit. Permeabilized cells were stained with PE-conjugated anti-IL-10 Ab. For intracellular T cell cytokine analysis, the same protocol was used except LPS was omitted.

In vitro cell proliferation assay, Ab production, and cytokine detection

Splenic CD1dhiCD5+ B cells from donor mice in the PBS/EAMG (AChR immunized mice receiving PBS) and in the GM-CSF/EAMG (AChR-immunized mice receiving GM-CSF) groups were isolated and co-cultured with responder T or B cells from immunized mice at a 1:1 ratio. For proliferation assay, responder CD4+ or CD19+ cells were isolated from mice in untreated EAMG mice using magnetic cell sorting (Miltenyi Biotec, Auburn, CA) and were stained with CFSE at a concentration of 1 μM for 10 min at 37˚C. Cells were washed three times and plated into 96-well, flat-bottom plates at 5 × 103 cells/well. T cell–depleted enriched DCs (1 × 103 cells/well; also accomplished by magnetic cell sorting) were used as feeder cells in these cells. Cells were stimulated with tAChR (5 μg/ml) for 72 h and then harvested for CFSE dilution studies and intracellular cytokine expression using a FACS analyzer (BD Biosciences). For Ab production, total anti-AChR IgG concentrations in culture supernatants in B cell cocultures were measured using a mouse IgG ELISA set (Bethyl Laboratories, Montgomery, TX), according to the manufacturer’s specifications.

ELISA for mouse serum AChR Ab isotopes

Mice were bled via tail vein at day 0 before the AT and at the end of study period. Affinity-purified mouse AChR (0.5 μg/ml) was used to coat 96-well microtiter plates (Corning Costar 96 wells plate; eBioscience, San Diego, CA) with 0.1 M carbonate bicarbonate buffer (pH 9.6) overnight at 4˚C. Serum samples diluted 1:5000 in blocking buffer were added and incubated at 37˚C for 90 min. After four washes, HRP-conjugated goat anti-mouse IgG (Caltag Laboratories, Burlingame, MA) or goat anti-mouse IgG1 (GeneTex, Irvine, CA), diluted in 1:2000 in blocking buffer, were added and incubated at 37˚C for 90 min. Subsequently, TMB substrate solution (eBioscience) was added, and color was allowed to develop at room temperature for the dark for 15 min. The reaction was stopped by adding 2 M H2SO4, and absorbance values were measured at a wavelength of 450 nm using a Bio-Rad microplate-reader (model 550). Results were expressed as OD values.

Determination of mouse muscle AChR content

Mice were sacrificed at end of the study period and muscle AChR was extracted from limb muscles. In brief, mouse muscle (20 ng) was homogenized in 4 vol Tris buffer (25 mM Tris HCl, 150 mM NaCl, pH 7.2) using a Polytron-equipped homogenizer (Model PT 3000; Kinemetic, Littau, Switzerland) on ice. Mechanical homogenization was achieved using a 7-mm tip (generator). Each sample was processed for 1 min at 10 rpm and then centrifuged at 100,000 × g for 30 min (4˚C). Supernatants (100 μl) were used to coat 96-well microtiter plates with coating buffer overnight at 4˚C. tAChR (0.5 μg/ml) with double dilution was coated in the plates as the standard control. Biotin-conjugated α-bungarotoxin (1 μg/ml; Invitrogen, Carlsbad, CA) in blocking buffer was added and incubated at 37˚C for 90 min. After four washes, HRP-conjugated streptavidin (0.1 μg/ml; Invitrogen, Carlsbad CA) in blocking buffer was added and incubated at 37˚C for 90 min. After addition of TMB substrate solution (eBioscience), color was allowed to develop at room temperature in the dark for 15 min. The reaction was stopped as described in the previous section. The muscle content (ng/ml) was measured from the OD value according to the standard curve from tAChR. The percentage of loss of muscle AChR contents from test mice was calculated by comparison with values from control mice (only adjuvant) mice.

Statistical analysis

All statistical analyses were performed using the SPSS software application. Student t test and nonparametric tests such as Mann–Whitney U test were used as appropriate. Significance levels were set at p < 0.05. Experiments were repeated at least twice to ensure reproducibility, and results were pooled for statistical analysis. Unless otherwise specified, data are presented as mean ± SEM.

Results

GM-CSF treatment reduced clinical severity and expanded CD1dhiCD5+ B cells and B10 cells

Before AT studies, we first confirmed that donor EAMG mice receiving GM-CSF (designated as GM-CSF/EAMG group) exhibited less severe disease compared with donor EAMG mice treated with PBS (designated as PBS/EAMG group). Mice (8-wk-old) were treated i.p. with GM-CSF (2 μg daily for 10 d) starting on the day of a first booster immunization with tAChR (designated as day 0). Animals were studied at day 24. Fig. 1A shows an example of pooled clinical data on donor mice used in prevention studies (Supplemental Table I). The effect of GM-CSF on clinical severity in donor EAMG mice was consistent (Supplemental Fig. 1). Next, we investigated whether GM-CSF treatment could effectively expand B10 cells. The percentages of CD1dhiCD5+ and IL-10+ cells among the CD19+ B cells were increased to a greater extent in the spleen of GM-CSF treated EAMG mice compared with naive mice (Fig. 1B). We also studied the expression of B cell markers (IgM, IgD) and surface molecules such as MHCII, costimulatory molecules (CD80, CD86), CD40, and CD23 in CD19+ B cells from GM-CSF–treated and PBS-treated mice.
EAMG animals by flow cytometric analysis (Fig. 1C). There was no difference in the percentage or the absolute number of total CD19+ cells, IgM+ B cells, IgD+ B cells, or CD23+ B cells between the PBS/EAMG group and the GM-CSF/EAMG group (Fig. 1C and Supplemental Table II). The expression of CD40 was significantly increased in the GM-CSF/EAMG group, but the expression of MHCII, as well as CD80 and CD86 (data not shown), was not different from PBS/EAMG mice. We confirmed that GM-CSF–expanded B10 cells were predominantly found within the sorted CD1dhiCD5+ B cell subset, but not detected within the sorted CD1dloCD5+ B cell subset (Fig. 1D). These results indicate that GM-CSF can expand B10 cells in vivo.

Effect of CD1dhiCD5+ B cells on T cell proliferation, Th cytokine profile, and B cell proliferation in vitro

To investigate the functional properties of sorted CD1dhiCD5+ B cells, we examined their effects on in vitro T cell proliferation and cytokine response in the presence of AChR (5 μg/ml). We found that T cells cocultured with CD1dhiCD5+, CD1dhiCD5−, and CD1dhiCD5− in vitro did not show any difference compared with T cells alone (n = 3, data not shown). As shown in Fig. 2A, GM-CSF/EAMG–expanded CD1dhiCD5+ B cells did not inhibit Ag-specific T cell proliferation in cocultures compared with those sorted from the PBS/EAMG group or T cells alone (control). However, CD4 T cell cytokine profile was altered by sorted CD1dhiCD5+ B cells, resulting in decreased Th1 and Th17 cells and increased IL-10+ CD4+ T cells (Fig. 2B). CD1dhiCD5+ B cells from GM-CSF/EAMG mice exhibited a more potent modulatory effect on Th cytokine profile than those isolated from PBS/EAMG mice.

In contrast with the lack of effect of sorted CD1dhiCD5+ B cells on T cell proliferation, we found that CD1dhiCD5+ B cells attenuated B cell proliferation (also a 1:1 ratio for 72 h). The extent of inhibition of B cell proliferation was greater with CD1dhiCD5+ cells sorted from GM-CSF/EAMG mice than those from PBS/EAMG mice and can be prevented by neutralizing anti–IL-10 Ab (20 μg/ml; Fig. 3A). In addition, the production of anti-AChR IgG was reduced by CD1dhiCD5+ B cells from GM-CSF/EAMG and PBS/EAMG mice compared with B cells alone (Fig. 3B). Thus, CD1dhiCD5+ B cells regulate B cell function in vitro through IL-10 production.

GM-CSF/EAMG–expanded CD1dhiCD5+ B cells prevented the development of EAMG upon AT

In view of the earlier in vitro findings, we proceeded to examine the in vivo preventive and suppressive effects of GM-CSF/EAMG–expanded CD1dhiCD5+ B cells in AT experiments (Supplemental Table I). For prevention studies, AT was performed 1 d before first booster (day 0). Recipient animals were divided into 3 groups of 10 mice each: 1) control group: no AT; animals received i.v. PBS; 2) AT with CD1dhiCD5+ B cells from donor PBS/EAMG mice; and 3) AT with CD1dhiCD5+ B cells from donor GM-CSF/EAMG mice. The clinical severity was expressed as mean clinical score. As

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

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shown in Fig. 4A, mice receiving CD1d<sup>hi</sup>CD5<sup>+</sup> B cells experienced less severe disease than the control group. In addition, CD1d<sup>hi</sup>CD5<sup>+</sup> B cells from GM-CSF–treated or PBS-treated EAMG mice (as indicated in parentheses) for 3 d with AChR (5 μg/ml; n = 6, p > 0.05). (B) Changes in cytokine profile corresponding to experiments in (A). A decrease in %Th1 and %Th17 cells and an increase in % IL-10<sup>+</sup> T cells were induced by coculture with CD1d<sup>hi</sup>CD5<sup>+</sup> B cells for 3 d. For Th1 cells, **p < 0.0005, †p < 0.0001 versus CTRL; ‡p < 0.005 versus PBS/EAMG. For Th17 cells, *p < 0.05, †p < 0.005 versus CTRL; ‡p < 0.05 versus PBS/EAMG. For IL-10<sup>+</sup> T cells, *p < 0.001, †p < 0.0001 versus CTRL; *p < 0.05 versus PBS/EAMG; n = 6 each. The percentage of Th1, Th17, and IL-10<sup>+</sup> T cells for each mouse was displayed in the lower panels.
To investigate possible mechanisms for the enhanced potency of GM-CSF/EAMG–expanded CD1d<sup>hi</sup>CD5<sup>+</sup> B cells in vivo, we examined the immunophenotypic properties of splenic DCs, CD4<sup>+</sup> T cell proliferative and cytokine response, and % CD4<sup>+</sup> Tregs (CD25<sup>+</sup>Foxp3<sup>+</sup>) from all three groups of recipient mice. DCs from animals that had received AT of CD1d<sup>hi</sup>CD5<sup>+</sup> B cells showed significantly lower expression of MHCII, CD80, and CD86, which was more dramatic when donor EAMG animals were treated with GM-CSF than with PBS (Fig. 4D). Therefore, AT of CD1d<sup>hi</sup>CD5<sup>+</sup> B cells led to altered DC phenotype from pathogenic to tolerogenic state. We also found that splenic CD4<sup>+</sup> T cell proliferation was decreased by AT of CD1d<sup>hi</sup>CD5<sup>+</sup> B cells, which was more significant when isolated from the GM-CSF/EAMG group than from the PBS/EAMG group. This was accompanied by corresponding increases in the percentage and absolute numbers of splenic CD4<sup>+</sup> Tregs (Fig. 4E and Supplemental Table III).

**FIGURE 4.** AT of in vivo expanded CD19<sup>+</sup>CD1d<sup>hi</sup>B cells before immunization: clinical and immunological effects. (A) Clinical severity. Recipient mice were injected with 1 × 10<sup>6</sup> CD1d<sup>hi</sup>CD5<sup>+</sup> B cells from GM-CSF–treated or PBS-treated donor EAMG group. Results are pooled from two independent experiments (n = 10, *p < 0.05, †p < 0.01 versus CTRL; ‡p < 0.02 versus PBS/EAMG). Day −1 corresponds to the initiation of AT. Booster immunization was performed at days 0 and 24. Animals were sacrificed on day 30. (B) Attenuated loss of muscle AChR contents by AT of CD1d<sup>hi</sup>CD5<sup>+</sup> B cells. *p < 0.02, †p < 0.006 versus CTRL; ‡p < 0.01 versus PBS/EAMG. For IgG, *p < 0.05, †p < 0.006 versus CTRL; ‡p < 0.01 versus PBS/EAMG. For IgG2b, *p < 0.05, †p < 0.001 versus CTRL; ‡p < 0.04 versus PBS/EAMG; n = 10. (C) Induction of tolerogenic state in DCs by AT of CD1d<sup>hi</sup>CD5<sup>+</sup>B cells. For MHCII, *p < 0.05, †p < 0.003 versus CTRL; ‡p < 0.04 versus PBS/EAMG. For CD80, *p < 0.05, †p < 0.003 versus CTRL; ‡p < 0.04 versus PBS/EAMG. For CD86, *p < 0.05, †p < 0.008 versus CTRL; ‡p < 0.007 versus PBS/EAMG. (D) Induction of tolerogenic state in DCs by AT of CD1d<sup>hi</sup>CD5<sup>+</sup>B cells. A lower percentage of MHCII<sup>+</sup>, CD80<sup>+</sup>, and CD86<sup>+</sup> DCs was observed in recipient mice. For MHCII, *p < 0.05, †p < 0.003 versus CTRL; ‡p < 0.04 versus PBS/EAMG. For CD80, *p < 0.05, †p < 0.003 versus CTRL; ‡p < 0.04 versus PBS/EAMG. For CD86, *p < 0.04 versus PBS/EAMG. For CD80, *p < 0.05, †p < 0.003 versus CTRL; ‡p < 0.04 versus PBS/EAMG. For CD86, *p < 0.05, †p < 0.003 versus CTRL; ‡p < 0.04 versus PBS/EAMG. For CD25/Foxp3. *p < 0.05, †p < 0.0001 versus CTRL; ‡p < 0.0007 versus PBS/EAMG. Data were derived from six animals in (D) and (E).
FIGURE 5. AT of in vivo expanded CD1d^{hi}CD5^{+} B cells after onset of EAMG. (A) Alleviation of established EAMG by AT of CD1d^{hi}CD5^{+} B cells, but not by AT of CD1d^{lo}CD5^{+} B cells from GM-CSF/EAMG mice. Day 0 corresponds to first AT (AT1), which was 21d after booster1; day 7: booster2; day 14: second AT (AT2). *p < 0.05 (n = 16). (B) Reduction in serum anti-AChR Ab levels corresponding to experiments shown in (A). *p < 0.05 (n = 10). (C) Clinical score showing more potent suppressive effect of GM-CSF–expanded CD1d^{hi}CD5^{+} B cells than those from PBS/EAMG mice. *p < 0.05, †p < 0.03 versus CTRL; *p < 0.05 versus PBS/EAMG. Results are pooled from two separate experiments (n = 10). Day 0 corresponds to booster1; day 10: AT1; day 30: booster2; day 40: AT2. (D) Muscle AChR contents (ng/ml). AChR content was more significantly preserved in mice receiving CD1d^{hi}CD5^{+} from GM-CSF/EAMG donor mice compared with mice receiving CD1d^{hi}CD5^{+} from PBS/EAMG and control (CTRL; no AT), *p < 0.007, †p < 0.0002 versus CTRL; *p < 0.007 versus PBS/EAMG; n = 10. (E) Effect of AT of CD1d^{hi}CD5^{+} B cells on T cell proliferation, Tregs, and cytokine profile. For T cell proliferation, *p < 0.003, †p < 0.0001 versus CTRL; ‡p < 0.002 versus PBS/EAMG. For Tregs, *p < 0.003, †p < 0.0007 (Figure legend continues)
GM-CSF/EAMG–expanded CD1d<sup>hi</sup>CD5<sup>+</sup> B cells can suppress established EAMG

To investigate the possible therapeutic effect of CD1d<sup>hi</sup>CD5<sup>+</sup> B cells on established EAMG, we first compared the effect of CD1d<sup>hi</sup>CD5<sup>+</sup> B cells and CD1d<sup>lo</sup>CD5<sup>−</sup> B cells from GM-CSF–treated donor EAMG mice. Recipient mice received first AT at day 0 (21 d after first booster), second booster at day 7, and second AT at day 14 (n = 16 each). The average clinical score was 1.6 ± 0.08 just before the first AT. The clinical severity of recipient EAMG mice was reduced after receiving 1 × 10<sup>6</sup> CD1d<sup>hi</sup>CD5<sup>+</sup> B cells, but not after receiving CD1d<sup>lo</sup>CD5<sup>−</sup> B cells (Fig. 5A). This was accompanied by reduction of serum mouse anti-AChR total IgG and IgG2b levels in mice receiving CD1d<sup>hi</sup>CD5<sup>+</sup> B cells, but not in those receiving CD1d<sup>lo</sup>CD5<sup>−</sup> B cells (Fig. 5B).

Next, we compared the suppressive efficacy of GM-CSF/EAMG–expanded CD1d<sup>hi</sup>CD5<sup>+</sup> B cells versus those isolated from PBS/EAMG mice on established EAMG. Three groups of recipient EAMG mice (n = 10 each) received booster injections at days 0 and 30, with AT of CD1d<sup>hi</sup>CD5<sup>+</sup> B cells (1 × 10<sup>6</sup>) from donor EAMG mice performed at days 10 and 40 (Supplemental Table I). The average clinical score was 1.4 ± 0.07 just before first AT. GM-CSF–expanded CD1d<sup>hi</sup>CD5<sup>+</sup> B cells exhibited more potent suppressive action compared with those isolated from donor PBS/EAMG mice (Fig. 5C). The suppressive effect of CD1d<sup>hi</sup>CD5<sup>+</sup> B cells was first detected on day 30 and maintained throughout the course of EAMG. There was a corresponding attenuation of loss of muscle AChR contents (Fig. 5D).

Flow cytometric studies on CD4<sup>+</sup> T cells were performed at the end of the study period (day 60). CD4<sup>+</sup> T cell proliferation and the percentage of Th1 cells were reduced by AT of CD1d<sup>hi</sup>CD5<sup>+</sup> B cells from both donor groups, but the effect was greater with GM-CSF–expanded CD1d<sup>hi</sup>CD5<sup>+</sup> B cells than those from PBS/EAMG mice (Fig. 5E). Conversely, the percentages of IL-10<sup>+</sup> CD4<sup>+</sup> T cells and the proportion of Tregs were increased by AT of CD1d<sup>hi</sup>CD5<sup>+</sup> B cells, which was more dramatic when expanded by GM-CSF in vivo. Effect on absolute numbers of proliferating CD4<sup>+</sup> T cells and Tregs is summarized (Supplemental Table III). Studies on DC markers showed a decrease in the percentage of MHCII<sup>+</sup>, CD80<sup>+</sup>, and CD86<sup>+</sup> cells (Fig. 5F). Overall, the consequence of AT of CD1d<sup>hi</sup>CD5<sup>+</sup> B cells on immune function was similar in the prevention and suppression studies.

Discussion

One of the best-characterized autoantibody-mediated diseases is MG, where effector mechanisms mediated by anti-AChR Abs have been well elucidated, but the triggering factors and regulatory mechanisms remain incompletely understood. Numerous T cell and B cell subpopulations have now been shown to exhibit regulatory activity. It is recognized that Bregs are phenotypically diverse, although most recent studies have been focused on a rare IL-10–competent B cell subset found within the CD19<sup>+</sup> CD1d<sup>hi</sup>CD5<sup>+</sup> population (20, 21, 26, 32, 37, 42, 43). Ag-specific BCR signaling, CD40 ligation, TLR, and BAFF are crucial to the development and maintenance of this population (20, 21, 26, 32, 37, 42, 43). Ag-specific BCR signaling in addition to CD40 engagement for Ag-specific BCR signaling in addition to CD40 engagement is an important mechanism. GM-CSF treatment led to enhanced regulatory function of CD1d<sup>hi</sup>CD5<sup>+</sup> B cells, most likely because of increased B10 cells within this cell population. Although many B cell subsets have the capacity to produce IL-10 upon binding of TLR ligands, B10 cells are the major B cell source of IL-10 (20). Our in vitro findings are consistent with known anti-inflammatory actions of IL-10 such as downregulation of Th1 and Th17 responses, and suppression of activation and function of monocytes/macrophages (49–52).

However, IL-10 has also been reported to exert immunostimulatory properties on human B cells resulting in enhanced proliferation and differentiation into Ab-secreting cells (53, 54). Under our experimental conditions, the immunoregulatory action of IL-10 predominates. Aside from B10 cells, IL-10 is produced by other immune cells, including DCs, T cells (Th1, Th2, Th3, Tregs, Tr1), NK cells, and macrophages (52). IL-10 deficiency often leads to development of enterocolitis and aggravates autoimmune pathology in many animal models such as EAE and experimental autoimmune neuritis (49, 55, 56).

Experimental autoimmune disease is often worse in the absence of B10 cells and other Bregs, as occurs when B cells are depleted in contact hypersensitivity and in EAE (31, 35). B10 cells regulate autoimmunity in an Ag-restricted manner, implying a requirement for Ag-specific BCR signaling in addition to CD40 engagement (29, 31, 35, 45, 47). In EAE, B10 cells predominantly control disease initiation, whereas Tregs inhibit the late phase of disease (57). Although AT of B10 cells at the time of induction of autoimmune disease has been shown to alleviate disease severity in experimental models, this strategy has been less successful in suppressing established, chronic autoimmune diseases (23, 58–61).

We found that AT of GM-CSF/EAMG–expanded B10 cells is an effective therapeutic approach in EAMG, in that it not only prevents but can also suppress established disease. This is associated with preservation of muscle AChR contents and reduction in circulating levels of anti-AChR Abs. A less potent effect was observed using B10 cells isolated from PBS/EAMG mice, which correlates with our in vitro findings on Ag-specific T cell and B cell responses. Note that EAMG has been shown to be aggravated by IL-10 administration and alleviated in IL-10 knockout mice (62, 63). Possible interpretations include: 1) IL-10–independent mechanisms (e.g., cell–cell contact) contribute to the beneficial effect of B10 cells in EAMG; and 2) cell-based targeted delivery of IL-10 to a specific anatomic site or during a specific time window is necessary to suppress disease severity or promote recovery in EAMG and other disease models.

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versus CTRL: ‡ < 0.004 versus PBS/EAMG. For Th1 and IL-10<sup>+</sup> T cells, p < 0.05 comparing PBS/EAMG versus CTRL; p < 0.01 comparing GM-CSF/EAMG versus CTRL, p < 0.03 comparing GM-CSF/EAMG versus PBS/EAMG. (F) Effect of AT of CD1d<sup>hi</sup>CD5<sup>+</sup> B cells on DCs. For MHCII, *p < 0.002, †p < 0.001 versus CTRL; *p < 0.02 versus PBS/EAMG. For CD86, ‡p < 0.04, §p < 0.04 versus CTRL; *p < 0.002 versus PBS/EAMG. For CD80, ‡p < 0.04, †p < 0.001 versus CTRL; *p < 0.04 versus PBS/EAMG. n = 6 for data in (E) and (F).
In parallel to the clinical severity, the expression of MHCII, CD80, and CD86 on DCs cells was decreased and the frequency of CD4+ Tregs was increased by the AT of CD1dCD5+ B cells in EAMG. These results indicate that B10 cells induce a tolerogenic state, and that the regulatory function of DCs and Tregs plays a critical role during the disease initiation and progression of EAMG. Consistent with our findings, B10 cells have been shown to regulate the Ag-presenting capability of DCs in vitro (57). We found that CD1dCD5+ B cells attenuate AChR-specific T cell proliferation in vivo but not in vitro. The apparent discrepancy of these findings may be because: 1) B10 cells act indirectly via other cell types that are not present under our in vitro conditions; or 2) the cytokine microenvironment differs in vivo from in vitro conditions. Our data also suggest that B10 cells may be important in the generation or maintenance of Tregs, similar to findings by other investigators (64–67). However, reports arguing against this concept also exist (23, 68).

In summary, we found that GM-CSF-expanded CD1dCD5+ B cells play a crucial role in the maintenance of immune homeostasis against self-Ags in EAMG. The protective effect of GM-CSF in EAMG has been previously postulated to involve mobilization of semimature or tolerogenic CD8α−DCs from bone marrow, which promotes the expansion of Tregs (8, 11, 69). We have now added another mechanism underlying the suppressive action of GM-CSF in EAMG, that is, by expansion of the CD1dCD5+ B cell subset, which suppresses the immune response against AChRs. Interestingly, a fusokine (GM-CSF fused with IL-15) has been reported to expand B10 cells in vitro (70). That our findings are relevant to human MG is supported by data from two studies. One recent study found that MG patients had fewer B10 cells than controls, which correlated with disease activity and responsiveness to rituximab therapy (71). A case study had shown that treatment with GM-CSF was associated with clinical improvement and expansion of circulating Tregs in a MG patient (72). Therefore, it appears feasible to translate our experimental findings to the clinical setting in vivo in human MG. Furthermore, autologous B10 cells can be expanded in vitro under new use as cellular immunotherapy of MG.

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Disclosures
The authors have no financial conflicts of interest.

References
Figure S1. Amelioration of the clinical severity of donor mice upon GM-CSF treatment. Donor EAMG mice were treated with GM-CSF or PBS for 10 days. Day 0 corresponds to the initiation of treatment at the time of first booster immunization. A. Donor mice for suppression studies shown in Fig. 5A. *p < 0.0005 starting at day 10 (n = 32). B. Donor mice for suppression studies shown in Fig. 5C. *p < 0.04 starting at day 16 (n = 20) (also see Table S1).
Table 1. Summary of treatment protocols and number of animals

<table>
<thead>
<tr>
<th>Figure</th>
<th>Protocols</th>
<th>No. of animals/group in each experiment</th>
<th>No. of experiments</th>
<th>Pooled No. of animals in each group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>Donor mice: booster at Day 0, GM-CSF treatment started at Day 0 for 10 d.</td>
<td>10</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>4A</td>
<td>Booster at Day 0 and Day 24, AT at Day -1.</td>
<td>5</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>5A</td>
<td>Day 0: first AT (at 21d post booster1), second AT at Day 14, booster2 at Day 7.</td>
<td>8</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>5C</td>
<td>Booster at Day 0 and Day 30, AT at Day 10 and Day 40.</td>
<td>5</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>S1A</td>
<td>Donor mice: same as 1A</td>
<td>16</td>
<td>2</td>
<td>32</td>
</tr>
<tr>
<td>S1B</td>
<td>Donor mice: same as 1A</td>
<td>10</td>
<td>2</td>
<td>20</td>
</tr>
</tbody>
</table>

Mice were immunized with tAChR and given booster immunization every 24-30d. At least two booster immunizations were given except in donor mice (Fig. 1A, S1A and S1B) which received only one booster.
### Table 2. Absolute number of B cells and subsets (1x10⁵)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Groups</th>
<th>Naïve</th>
<th>PBS/EAMG</th>
<th>GM-CSF/EAMG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1B</td>
<td>Total B cells</td>
<td>121±9.2</td>
<td>125±10</td>
<td>133±7.1</td>
</tr>
<tr>
<td></td>
<td>CD1d&lt;sup&gt;hi&lt;/sup&gt;CD5&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.92±0.5</td>
<td>3.57±0.2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>6.1±0.3**</td>
</tr>
<tr>
<td></td>
<td>CD19&lt;sup&gt;-&lt;/sup&gt; IL-10&lt;sup&gt;-&lt;/sup&gt;</td>
<td>2.0±0.2</td>
<td>3.6±0.3&lt;sup&gt;*&lt;/sup&gt;</td>
<td>6.4±0.6**</td>
</tr>
<tr>
<td>1C</td>
<td>CD19&lt;sup&gt;+&lt;/sup&gt; CD40&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.6±0.2</td>
<td>3.9±0.6&lt;sup&gt;*&lt;/sup&gt;</td>
<td>p &lt; 0.006 vs PBS/EAMG</td>
</tr>
<tr>
<td></td>
<td>CD19&lt;sup&gt;-&lt;/sup&gt; MHCI&lt;sup&gt;+&lt;/sup&gt;</td>
<td>3.1±0.3</td>
<td>4.0±0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD19&lt;sup&gt;-&lt;/sup&gt; CD23&lt;sup&gt;+&lt;/sup&gt;</td>
<td>8.2±0.9</td>
<td>8.9±1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD19&lt;sup&gt;-&lt;/sup&gt; IgM&lt;sup&gt;+&lt;/sup&gt;</td>
<td>7.3±0.6</td>
<td>7.1±0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD19&lt;sup&gt;-&lt;/sup&gt; IgD&lt;sup&gt;+&lt;/sup&gt;</td>
<td>9.0±0.7</td>
<td>8.8±0.6</td>
<td></td>
</tr>
</tbody>
</table>

Absolute cell number was determined by using total cellularity, determined by trypan blue dye exclusion and percentage of CD19<sup>-</sup> or B cell subsets. Total splenocytes ranged from 6.4x10⁷ to 8.6x10⁷. P values listed were based on comparison with Naïve group unless otherwise specified (n = 6 each).
<table>
<thead>
<tr>
<th>Figure</th>
<th>Cells</th>
<th>Groups</th>
<th>CTRL (no AT)</th>
<th>CD1d⁺CD5⁺ (PBS/EAMG)</th>
<th>CD1d⁺CD5⁺ (GM-CSF/EAMG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4E</td>
<td>Proliferating CD4⁺</td>
<td>1.72±0.05</td>
<td>1.38±0.06*</td>
<td>0.79±0.09**</td>
<td>p &lt; 0.001 vs. CTRL; p &lt; 0.001 vs. PBS/EAMG</td>
</tr>
<tr>
<td></td>
<td>Total CD4⁺</td>
<td>116±3.1</td>
<td>113±2.5</td>
<td>117±1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD25⁻Foxp3⁺</td>
<td>8.5±0.6</td>
<td>11.2±0.8*</td>
<td>14.7±0.3**</td>
<td>p &lt; 0.0001 vs CTRL; p &lt; 0.002 vs. PBS/EAMG</td>
</tr>
<tr>
<td>5E</td>
<td>Proliferating CD4⁺</td>
<td>1.25±0.15</td>
<td>0.71±0.06*</td>
<td>0.53±0.05**</td>
<td>p &lt; 0.00005 vs CTRL; p &lt; 0.0006 vs. PBS/EAMG</td>
</tr>
<tr>
<td></td>
<td>Total CD4⁺</td>
<td>116±2.2</td>
<td>114±3.1</td>
<td>115±2.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD25⁻Foxp3⁺</td>
<td>10.7±0.2</td>
<td>12.9±0.3*</td>
<td>17.5±1.1**</td>
<td>p &lt; 0.0002 vs CTRL; p &lt; 0.005 vs. PBS/EAMG</td>
</tr>
</tbody>
</table>

Absolute cell number was determined by using total cellularity, determined by trypan blue dye exclusion and percentage of CD4⁺ or CD4⁺CD25⁺Foxp3⁺ cells. For all T cell proliferation, 5x10⁵/well of CFSE labeled CD4⁺ cells were used. Total splenocytes ranged from 8.4x10⁷ to 10x10⁷. P values listed were based on comparison with CTRL unless otherwise specified (n = 6 each).