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CD1d^{hi}CD5^{+} B Cells Expanded by GM-CSF In Vivo Suppress Experimental Autoimmune Myasthenia Gravis

Jian Rong Sheng, Songhua Quan, and Betty Soliven

Myasthenia gravis (MG) is a T cell–dependent, B cell–mediated autoimmune disease in which autoantibodies are produced that target the skeletal muscle acetylcholine receptor (AChR) (1, 2). Experimental autoimmune MG (EAMG) can be induced in mice by immunization with AChR purified from the electric organs of the electric ray, *Torpedo californica* (3, 4). In both MG and EAMG, anti-AChR Abs bind to the AChR at the neuromuscular junction, activate complement, and accelerate AChR destruction, thus leading to neuromuscular transmission failure and fatigable muscle weakness (5–7).

EAMG has been used to study immune mechanisms and to develop new therapeutic strategies such as the use of GM-CSF to enhance tolerance (8–11). GM-CSF is capable of both stimulating the immune response and alternatively suppressing the immune response by favoring the development of immature dendritic cells (DCs) that induce/expand regulatory T cells (Tregs) (12–15). In experimental autoimmune encephalomyelitis (EAE), disease is augmented by local administration of GM-CSF and is severely impaired in GM-CSF–deficient mice (16–18). In contrast, GM-CSF attenuates the severity of EAMG, which is accompanied by downregulation of AChR-specific T cell and humoral responses, and expansion of Ag-specific CD4^{+} Tregs (8, 11). Whether GM-CSF also expands other regulatory immune cells such as regulatory B cells (Bregs) or CD8^{+} Tregs has not been studied.

B cells are generally considered to positively regulate immune responses by producing autoantibodies, and they play a central role in the pathogenesis of MG. The regulatory role of B cells in autoimmune diseases was first reported by Janeway and colleagues (19) in EAE. The existence of Bregs was subsequently confirmed by other investigators (20–24). These studies indicate that, like their T cell counterparts, B cells can be divided into functionally distinct regulatory subsets capable of inducing immune tolerance (20, 25–29). One of the Breg subsets is the so-called IL-10–producing B cells (B10 cells), which comprise 1–3% of splenic B cells in wild-type naive mice and are predominantly found within a phenotypically unique CD1d^{hi}CD5^{+}CD19^{+} subset (20, 23, 30, 31).

The goal of this study was to investigate the functional properties of CD1d^{hi}CD5^{+} B cells/B10 cells in EAMG, and whether this Breg subset can be expanded by GM-CSF. B10 cells can be expanded in vitro by stimulation with LPS for 5 h or with CD40 agonists for 48 h (32). B10 cell function requires IL-10 expression and IL-21 signaling, as well as CD40 and MHCII interactions (26, 33–37). There is some evidence that susceptible mouse strains such as NOD mice (38–40) and MRL/lpr mice contain greater numbers of B10 cells than C57BL/6 mice (36, 38–42). However, strategies to expand B10 cells to suppress autoimmunity in vivo are currently limited. In this study, we have provided evidence that the expansion of CD1d^{hi}CD5^{+} B cells/B10 cells by GM-CSF in vivo may represent an effective therapeutic approach to restore tolerance in an Ab-mediated disease like EAMG.

Materials and Methods

*Mice and purification of Torpedo AChR*

Eight-wk-old female C57BL/6j mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed and bred in the Animal Resources Center at the University of Chicago and were provided food and water ad libitum. All animal use procedures were conducted in strict accordance to the National Institutes of Health and University of Chicago institutional guidelines. AChR was purified from the electric organs of *Torpedo californica* by affinity chromatography using a conjugate of neurotoxin coupled to agarose, as previously described (9). Purified *Torpedo AChR*
(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 C57BL/6J mice were immunized with 20 µg tAChR/ CFA in 100 µl s.c. and boosted with 20 µg tAChR emulsified inIFA 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 evaluation, 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/CFA 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<sup>+</sup> B cells were isolated from mice by positive selection using magnetic beads (Miltenyi Biotec, Auburn, CA) with obtained purity ≥95%. CD1<sub>dhi</sub>CD5<sup>+</sup> and CD1<sub>dlo</sub>CD5<sup>−</sup> B cells were purified (95–98%) using a FACSaria flow cytometer (BD Biosciences). After purification, CD1<sub>dhi</sub>CD5<sup>+</sup> B cells (1 x 10<sup>6</sup>) 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-IaB (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-Cy7 anti–CD25, FITC anti–IL-27, PE-Cy5 anti–ICOS, and APC anti–Foxp3, eBioscience, San Diego, CA) was used for intracellular cytokine staining. PE-conjugated Abs were used for intracellular cytokine detection of T cells or B cells. For B cells, isolated leukocytes or purified cells were resuspended (2 x 10<sup>6</sup> cells/ml) in complete medium (RPMI 1640 containing 10% FCS, 200 µg/ml penicillin, 200 U/ml streptomycin, 4 mM L-glutamine, and 5 x 10<sup>−5</sup> M 2-ME; all from Invitrogen, Carlsbad, CA) in the presence of LPS (10 µg/ml; Sigma, St. Louis, MO), PMA (50 ng/ml; BD Pharmingen), ionomycin (500 ng/ml; BD Pharmingen), and monensin (2 µM; BD Pharmingen) in 48-well, flat-bottom 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 CD1<sub>dhi</sub>CD5<sup>+</sup> B cells from donor mice in the PBS/EAMG (tAChR-immunized mice receiving PBS) and in the GM-CSF/EAMG (tAChR-immunized mice receiving GM-CSF) groups were isolated and cocultured with responder T or B cells from immunized mice at a 1:1 ratio. For proliferation assay, responder CD4<sup>+</sup> or CD19<sup>+</sup> 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 x 10<sup>5</sup> cells/well. T cell–depleted enriched DCs (1 x 10<sup>5</sup> cells/well; also accomplished by magnetic cell sorting) were used as feeder cells in these studies. 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 isotypes**

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 IgG<sub>1</sub> (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 for 15 min. The reaction was stopped by adding 2 M H<sub>2</sub>SO<sub>4</sub>, 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 the end of the study period and muscle AChR was extracted from limb muscles. In brief, muscle (20 mg) 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; Kinematica, 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 x g for 30 min (4°C). Supernatants (100 µl) were used to coat 96-well microtiter plates with coating buffer (0.2 µg/ml at 4°C; TMB/CFA 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 CD1<sub>dhi</sub>CD5<sup>+</sup> 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 CD1<sub>dhi</sub>CD5<sup>+</sup> and IL-10<sup>+</sup> cells among the CD19<sup>+</sup> B cells were increased to a greater extent in the spleen of EAMG animals that received GM-CSF compared with the PBS/ EAMG group and 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<sup>+</sup> B cells from GM-CSF–treated and PBS-treated...
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+ and CD1dloCD5+ 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 Th10–Th17 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
shown in Fig. 4A, mice receiving CD1d^{hi}CD5^{+} B cells experienced less severe disease than the control group. In addition, CD1d^{hi}CD5^{+} 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^{+} T cells were induced by coculture with CD1d^{hi}CD5^{+} 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^{+} 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^{+} T cells for each mouse was displayed in the lower panels.

FIGURE 3. Suppression of B cell proliferation and Ab production by CD1d^{hi}CD5^{+} B cells via IL-10 in vitro. (A) Histograms of CFSE fluorescence. Responder CD19^{+} B cells labeled with CFSE were cocultured with CD19^{+}CD1d^{hi}CD5^{+} cells from GM-CSF–treated and PBS-treated EAMG mice at a 1:1 ratio in the presence or absence of neutralizing anti–IL-10 Ab. The percentage of B cells with diluted CFSE is indicated in the histograms. *p < 0.05, †p < 0.005 versus CTRL; ‡p < 0.03 versus PBS/EAMG; n = 6. (B) CD19^{+}CD1d^{hi} B cells attenuated the production of anti-AChR Ab production expressed as OD450 levels, which was reversed by neutralizing anti–IL-10 Ab. *p < 0.01, †p < 0.002 versus CTRL; ‡p < 0.04 versus PBS/EAMG; n = 6.
To investigate possible mechanisms for the enhanced potency of GM-CSF/EAMG–expanded CD1d\(^{hi}\)CD5\(^{+}\) B cells in vivo, we examined the immunophenotypic properties of splenic DCs, CD4\(^{+}\) T cell proliferative and cytokine response, and % CD4\(^{+}\) Tregs (CD25\(^{+}\)Foxp3\(^{+}\)) from all three groups of recipient mice. DCs from animals that had received AT of CD1d\(^{hi}\)CD5\(^{+}\) 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\(^{hi}\)CD5\(^{+}\) B cells led to altered DC phenotype from pathogenic to tolerogenic state. We also found that splenic CD4\(^{+}\) T cell proliferation was decreased by AT of CD1d\(^{hi}\)CD5\(^{+}\) 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\(^{+}\) Tregs (Fig. 4E and Supplemental Table III).

**FIGURE 4.** AT of in vivo expanded CD19\(^{+}\)CD1d\(^{hi}\)B cells before immunization: clinical and immunological effects. (A) Clinical severity. Recipient mice were injected with \(1 \times 10^{6}\) CD1d\(^{hi}\)CD5\(^{+}\) 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\(^{hi}\)CD5\(^{+}\) 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; \(n = 10\). (D) Induction of tolerogenic state in DCs by AT of CD1d\(^{hi}\)CD5\(^{+}\) B cells. A lower percentage of MHCII\(^{+}\), CD80\(^{+}\), and CD86\(^{+}\) DCs cells 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, *\(p < 0.05\), †\(p < 0.003\) versus CTRL; ‡\(p < 0.04\) versus PBS/EAMG. For CD25/Foxp3, *\(p < 0.008\), †\(p < 0.0002\) versus CTRL; ‡\(p < 0.007\) versus PBS/EAMG. Data were derived from six animals in (D) and (E).
FIGURE 5. AT of in vivo expanded CD1d<sup>hi</sup>CD5+ B cells after onset of EAMG. (A) Alleviation of established EAMG by AT of CD1d<sup>hi</sup>CD5+ B cells, but not by AT of CD1d<sup>lo</sup>CD5− B cells from GM-CSF/EAMG mice. Day 0 corresponds to first AT (AT1), which was 21 d 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<sup>hi</sup>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<sup>hi</sup>CD5+ from GM-CSF/EAMG donor mice compared with mice receiving CD1d<sup>hi</sup>CD5+ from PBS/EAMG and control (CTRL; no AT), *p < 0.007, †p < 0.00002 versus CTRL; *p < 0.007 versus PBS/EAMG; n = 10. (E) Effect of AT of CD1d<sup>hi</sup>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 MHCI<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 induction of B10 cells (32, 42, 44–48). Studies on B10 cells are limited to some extent by lack of a specific marker or master regulator, by low abundance in vivo, and by paucity of defined methods to expand the B10 cells for adoptive cell therapy strategies. In this study, we found that treatment of EAMG mice with GM-CSF led to expansion of CD1d<sup>hi</sup>CD5<sup>+</sup> B cell subset and B10 cells in the spleen of these animals. This was associated with an increase in the percentage of CD40<sup>+</sup> B cells. It is possible that treatment with GM-CSF leads to an expansion of B10 progenitor cells, which mature to B10 cells through CD40 ligation.

Data from cocultures revealed that CD1d<sup>hi</sup>CD5<sup>+</sup> B cells from EAMG mice regulate the immune response by suppressing Th1 response without affecting Ag-specific T cell proliferation. Perhaps more importantly, they significantly attenuated B cell proliferation and production of anti-AChR Abs via an IL-10–dependent 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.

versus CTRL; *p < 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 MHCI, *p < 0.002, p < 0.001 versus CTRL; †p < 0.002 versus PBS/EAMG. For CD80, ‡p < 0.002 versus PBS/EAMG. For CD86, ‡p < 0.04, †p < 0.04 versus CTRL; *p < 0.002 versus PBS/EAMG. For CD86, ‡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 CD1dhiCD5+ 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 CD1dhiCD5+ B cells attenuated 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 CD1dhiCD5+ 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 CD80–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 CD1dhiCD5+ 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 human MG. Furthermore, autologous B10 cells can be expanded in vitro under useful conditions for cellular immunotherapy of MG.

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Disclosures

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References


