Published in final edited form as:
J Immunol. 2014 September 15; 193(6): 2669–2677. doi:10.4049/jimmunol.1303397.

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

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Abstract

IL-10-competent subset within CD1d^{hi}CD5^{+} 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 granulocyte macrophage-colony stimulating factor (GM-CSF), which suppresses EAMG, can expand B10 cells *in vivo*, and whether adoptive transfer of CD1d^{hi}CD5^{+} B cells would prevent or suppress EAMG. We found that treatment of EAMG mice with low-dose GM-CSF increased the proportion of CD1d^{hi}CD5^{+} B cells and B10 cells. In vitro co-culture studies revealed that CD1d^{hi}CD5^{+} B cells altered T cell cytokine profile but did not directly inhibit T cell proliferation. On the other hand, CD1d^{hi}CD5^{+} B cells inhibited B cell proliferation and its autoantibody production in an IL-10-dependent manner. Adoptive transfer of CD1d^{hi}CD5^{+} 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 (AChR)-specific T cell and B cell responses. Thus, our data have provided significant insights into the mechanisms underlying the tolerogenic effects of B10 cells in EAMG. These observations suggest that *in vivo* or *in vitro* expansion of CD1d^{hi}CD5^{+} B cells or B10 cells may represent an effective strategy in the treatment of human myasthenia gravis.

Keywords

Autoimmune disease; regulatory B cells; cytokines; acetylcholine receptor; immune tolerance; Tregs

Introduction

Myasthenia Gravis (MG) is a T cell-dependent, B cell-mediated autoimmune disease in which autoantibodies are produced targeting 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 antibodies bind to the AChR at the

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Disclosure
The authors have no financial conflict of interest.

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 granulocyte macrophage-colony stimulating factor (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 antigen-specific $CD4^+$ Tregs (8, 11). Whether GM-CSF also expands other regulatory immune cells such as regulatory B cells or $CD8^+$ Tregs has not been studied.

B cells are generally considered to positively regulate immune responses by producing autoantibodies and 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 in EAE (19). The existence of regulatory B cells 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 regulatory B cell 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 the current study was to investigate the functional properties of $CD1d^{hi}CD5^+$ B cells / B10 cells in EAMG, and whether this regulatory B cell subset can be expanded by GM-CSF. B10 cells can be expanded *in vitro* by stimulation with LPS for 5 hrs or with CD40 agonists for 48 hrs (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 limited at this time. Here, 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 antibody-mediated disease like EAMG.

Materials and Methods

Mice and Purification of Torpedo AChR (tAChR)

Eight-week old female C57BL6/J mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Mice were housed and bred in the Animal Resources Center (ARC) at the University 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 tAChR was used to induce EAMG and as antigen for *in vitro* studies of immune responses.

Induction and clinical scoring of EAMG

Eight-week old female C57BL6/J mice were immunized with 20 μg of tAChR/CFA in 100 μl subcutaneously, and boosted with 20 μg of tAChR emulsified in IFA in 100 μl injected in the flanks and tail base every 24–30 days. 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 post-exercise, 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 (20μg of tAChR/CFA in 100 μl subcutaneously followed by one booster at 24–30 days later (day 0) and treatment with GM-CSF (2 μg daily IP for 10 days) or PBS. These donor mice were sacrificed 14 days after GM-CSF treatments (24 days 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%. $CD1d^{hi}CD5^+$ and $CD1d^{lo}CD5^-$ B cells were purified (95–98%) using a FACSAria flow cytometer (BD Biosciences). After purification, $CD1d^{hi}CD5^+$ B cells (1×10^6) were immediately transferred into appropriate recipient mice by tail vein injection.

All recipient mice received booster immunizations every 24–30 days 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 Table S1. 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 antiCD16/CD32 Fc-Block (BD PharMingen, San Jose, CA) on ice for 30 min. APC-conjugated anti-CD19, PB-conjugated anti-CD5 and PE-conjugated anti-CD1d (BioLegend), anti-IAb (MHC class II), anti-CD40, anti-CD80, anti-CD86, isotype control antibodies (Abs) (BD PharMingen) were used in flow cytometry and analyzed using FlowJo software (Treestar, Ashland, OR). Mouse regulatory T cell staining kit (w/ PE FoxP3 FJK-16s, FITC CD4, APC CD25 Abs) (eBioscience) was used for intracellular staining for FoxP3 expression. PE-conjugated antibodies against IL-10, IL-17A, and IFN-γ were used for intracellular cytokine staining of T or B cells. For B10 cells, isolated leukocytes or purified cells were resuspended (2×10^6

cells/ml) in complete medium (RPMI 1640 containing 10% FCS, 200 μg/ml penicillin, 200 U/ml streptomycin, 4 mM L-glutamine, and 5×10^{-5} M 2-ME; all from Invitrogen, Carlsbad, CA, USA) in the presence of lipopolysaccharide (LPS) (10 μg/ml; Sigma, St. Louis, MO, USA), phorbol 12-myristate 13 acetate (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-IL10 antibody. For intracellular T cell cytokine analysis, the same protocol was used except LPS was omitted.

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

Splenic $CD1d^{hi}CD5^{+}$ 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 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 ºC. Cells were washed three times and plated into 96-well, flat-bottom plates at 5×10^5 cells/well. T cell-depleted enriched DCs (1×10^5 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 hrs, and then harvested for CFSE dilution studies and intracellular cytokine expression using a FACS analyzer (BD Biosciences). For antibody production, total anti-AChR IgG concentrations in culture supernatants in B cell co-cultures were measured using a mouse IgG enzyme-linked immunosorbent assay (ELISA) set (Bethyl Laboratories, Montgomery, TX), according to the manufacturer's specifications.

ELISA for mouse serum AChR antibody isotypes

Mice were bled via tail vein at day 0 prior to the adoptive transfer 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 ºC. Serum samples diluted 1:5000 in blocking buffer were added and incubated at 37 ºC for 90 min. After four washes, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Caltag Laboratories, Burlingame, MA) or goat anti-mouse IgG_{2b} (GeneTex, CA), diluted in 1:2000 in blocking buffer were added and incubated at 37 ºC for 90 min. Subsequently, TMB substrate solution (eBioscience) was added, and color was allowed to develop at room temperature in the dark for 15 min. The reaction was stopped by adding 2 M H_2SO_4 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 study period and muscle AChR was extracted from limb muscles. Briefly, mouse muscle (20 mg) was homogenized in 4 volumes of 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 × *g* for 30 min (4 °C). Supernatants (100 μl) were used to coat 96-well microtiter plates with coating buffer overnight at 4 °C. Torpedo AChR (0.5 μg/ml) with double dilution was coated in the plates as the standard control. Biotin-conjugated alpha-bungarotoxin (Invitrogen, Carlsbad CA) (1 μg/ml) 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 above section. The muscle content (ng/ml) was measured from the OD value according to the standard curve from Torpedo AChR. 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's t test and nonparametric tests such as Mann Whitney test were utilized 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 ± standard error of the mean (SEM).

Results

GM-CSF treatment reduced clinical severity and expanded CD1d^{hi}CD5^{+} B cells and B10 cells

Prior to adoptive transfer studies, we first confirmed that donor EAMG mice receiving GM-CSF (designated as GM-CSF/EAMG group) exhibited less severe disease compared to donor EAMG mice treated with PBS (designated as PBS/EAMG group). Mice (8 weeks old) were treated i.p. with GM-CSF (2 μg daily for 10 days) starting on the day of a first booster immunization with tAChR (designated as Day 0). Animals were studied at Day 24. Figure 1A shows an example of pooled clinical data on donor mice used in prevention studies (Table S1). The effect of GM-CSF on clinical severity in donor EAMG mice was consistent (Fig. S1). Next, we investigated whether GM-CSF treatment could effectively expand B10 cells. The percentages of CD1d^{hi}CD5^{+} and IL-10^{+} cells among the CD19^{+} B cells were increased to a greater extent in the spleen of EAMG animals that received GM-CSF compared to PBS/EAMG group and naïve mice (Fig. 1B). We also studied the expression of B cell markers (IgM, IgD) and surface molecules such as MHCII, co-stimulatory molecules (CD80, CD86), CD40 and CD23 in CD19^{+} 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 GM-CSF/EAMG group (Fig. 1C, Table S2). The expression of CD40 was significantly increased in GM-CSF/EAMG group, but the expression of MHCII, CD80 and CD86 of CD19^{+} was not different from PBS/ EAMG mice. We confirmed that GM-CSF expanded B10 cells were predominantly found within the sorted CD1d^{hi}CD5^{+} B cell subset, but not detected within the sorted CD1d^{lo}CD5^{-} B cell subset (Fig. 1D). These results indicate that GM-CSF can expand B10 cells *in vivo*.

Effect of CD1d^{hi}CD5^{+} B cells on T cell proliferation, Th cytokine profile and B cell proliferation in vitro

To investigate the functional properties of sorted CD1d^{hi}CD5^{+} 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 co-cultured with CD1d^{hi}CD5^{-}, CD1d^{lo}CD5^{+} and CD1d^{lo}CD5^{-} *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 CD1d^{hi}CD5^{+} B cells did not inhibit antigen-specific T cell proliferation in co-cultures compared to those sorted from PBS/EAMG group, or T cells alone (control). However, CD4 T cell cytokine profile was altered by sorted CD1d^{hi}CD5^{+} B cells, resulting in decreased Th1 and Th17 CD4^{+} T cells and increased IL-10^{+} CD4^{+} T cells (Fig. 2B). CD1d^{hi}CD5^{+} 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 to the lack of effect of sorted CD1d^{hi}CD5^{+} B cells on T cell proliferation, we found that CD1d^{hi}CD5^{+} B cells attenuated B cell proliferation (also 1:1 ratio for 72 hrs). The extent of inhibition of B cell proliferation was greater with CD1d^{hi}C5^{+} 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 CD1d^{hi}CD5^{+} B cells from GM-CSF/EAMG and PBS/EAMG mice compared to B cells alone (Fig. 3B). Thus, CD1d^{hi}CD5^{+} B cells regulate B cell function *in vitro* through IL-10 production.

GM-CSF/EAMG-expanded CD1d^{hi}CD5^{+} B cells prevented the development of EAMG upon adoptive transfer (AT)

In view of the above *in vitro* findings, we proceeded to examine the *in vivo* preventive and suppressive effects of GM-CSF/ EAMG-expanded CD1d^{hi}CD5^{+} B cells in AT experiments (Table S1). For prevention studies, AT was performed one day prior to first booster (day 0). Recipient animals were divided into three groups of 10 mice each: 1) ctrl group: no AT; animals received i.v. PBS; 2) AT with CD1d^{hi}CD5^{+} B cells from donor PBS/EAMG mice; 3) AT with CD1d^{hi}CD5^{+} 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 developed less severe disease than the ctrl group. In addition, CD1d^{hi}CD5^{+} B cells from GM-CSF/EAMG mice had a more potent effect than those from PBS/EAMG mice. This was accompanied by preservation of muscle AChR contents, and decreased serum levels of anti-AChR Abs, as measured by ELISA (Fig. 4B, C).

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 significant 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, more significant when isolated from GM-CSF/EAMG group than from PBS/EAMG group. This was accompanied by corresponding increase in the percentage and absolute numbers of splenic $CD4^{+}$ Tregs (Fig. 4E, Table S3).

GM-CSF/EAMG-expanded $CD1d^{hi}CD5^{+}$ B cells can suppress established EAMG

To investigate possible therapeutic effect of $CD1d^{hi}CD5^{+}$ B cells on established EAMG, we first compared the effect of $CD1d^{hi}CD5^{+}$ B cells and $CD1d^{lo}CD5^{-}$ B cells from GM-CSF-treated donor EAMG mice. Recipient mice received first AT at Day 0 (21 days 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 prior to the first AT. The clinical severity of recipient EAMG mice was reduced after receiving 1×10^6 $CD1d^{hi}CD5^{+}$ B cells, but not after receiving $CD1d^{lo}CD5^{-}$ B cells (Fig. 5A). This was accompanied by reduction of serum mouse anti-AChR total IgG and IgG2b levels in mice receiving $CD1d^{hi}CD5^{+}$ B cells, but not in those receiving $CD1d^{lo}CD5^{-}$ B cells (Fig. 5B).

Next, we compared the suppressive efficacy of GM-CSF/EAMG-expanded $CD1d^{hi}CD5^{+}$ B cells vs those isolated from PBS/EAMG mice on established EAMG. Three groups of recipient EAMG mice (n = 10 each) received booster injections at day 0 and day 30, with AT of $CD1d^{hi}CD5^{+}$ B cells (1×10^6) from donor EAMG mice performed at day 10 and day 40 (Table S1). The average clinical score was 1.4 ± 0.07 just prior to first AT. GM-CSF-expanded $CD1d^{hi}CD5^{+}$ B cells exhibited more potent suppressive action compared to those isolated from donor PBS/EAMG mice (Fig. 5C). The suppressive effect of $CD1d^{hi}CD5^{+}$ 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 $CD4^{+}$ T cells were performed at the end of study period (Day 60). $CD4^{+}$ T cell proliferation and the percentage of Th1 cells were reduced by AT of $CD1d^{hi}CD5^{+}$ B cells from both donor groups, but the effect was greater with GM-CSF-expanded $CD1d^{hi}CD5^{+}$ B cells than those from PBS/EAMG mice (Fig 5E). Conversely, the percentages of IL-10^{+} $CD4^{+}$ T cells and the proportion of Tregs were increased by AT of $CD1d^{hi}CD5^{+}$ B cells, which was more dramatic when expanded by GM-CSF *in vivo*. Effect on absolute numbers of proliferating $CD4^{+}$ T cells and Tregs is summarized (Table S3). Studies on DC markers showed a decrease in the percentage of $MHCII^{+}$, $CD80^{+}$, and $CD86^{+}$ cells (Fig. 5F). Overall, the consequence of AT of $CD1d^{hi}CD5^{+}$ B cells on immune function was similar in the prevention and suppression studies.

Discussion

One of the best characterized autoantibody-mediated diseases is myasthenia gravis 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 regulatory B cells (Bregs) are phenotypically diverse, though most recent studies have been focused on a rare IL-10-competent B cell subset found within $CD19^{+}$ $CD1d^{hi}CD5^{+}$ population (20, 21, 26, 32, 37, 42, 43). Ag-specific B cell receptor signaling, CD40 ligation, Toll-like receptor (TLR) and B cell activating factor (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 $CD1d^{hi}CD5^{+}$ B cell subset and B10 cells in the spleen of these animals. This was associated with an increase in the percentage of $CD40^{+}$ 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 co-cultures revealed that $CD1d^{hi}CD5^{+}$ 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 $CD1d^{hi}CD5^{+}$ B cells, most likely due to increased B10 cells within this cell population. While 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, 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 (EAN) (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). While adoptive transfer of B10 cells at the time of induction of autoimmune disease has been shown to ameliorate disease severity in experimental models, this strategy has been less successful in suppressing established, chronic autoimmune diseases (23, 58–61).

We found that adoptive transfer 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 antibodies. 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; 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.

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 adoptive transfer of $CD1d^{hi}CD5^+$ B cells in EAMG. These results indicate that B10 cells induce a tolerogenic state, and that the regulatory function of DCs and Tregs cells 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 $CD1d^{hi}CD5^+$ B cells attenuated AChR-specific T cell proliferation *in vivo* but not *in vitro*. The apparent discrepancy of these findings may be due to: 1) B10 cells act indirectly via other cell types that are not present under our *in vitro* conditions; or 2) cytokine microenvironment differs *in vivo* from i*n 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 $CD1d^{hi}CD5^+$ B cells play a crucial role in the maintenance of immune homeostasis against self-antigens in EAMG. The protective effect of GM-CSF in EAMG has been previously postulated to involve mobilization of semi-mature or tolerogenic $CD8\alpha^-$ 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 $CD1d^{hi}CD5^+$ 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* for subsequent use as cellular immunotherapy of MG.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. M. Meriggioli and Dr. B. Prabhakar for their advice during the early stages of this project.

This work was supported by the Muscular Dystrophy Association (MDA 157286 and MDA 234223) to JRS, and by the NIH NINDS RO1 NS064059 to BS.

Abbreviations used in this paper

MG	myasthenia gravis
EAMG	experimental autoimmune myasthenia gravis
Breg	regulatory B cell
DC	dendritic cell
AChR	acetylcholine receptor
Treg	regulatory T cell
Foxp3	forkhead box p3.

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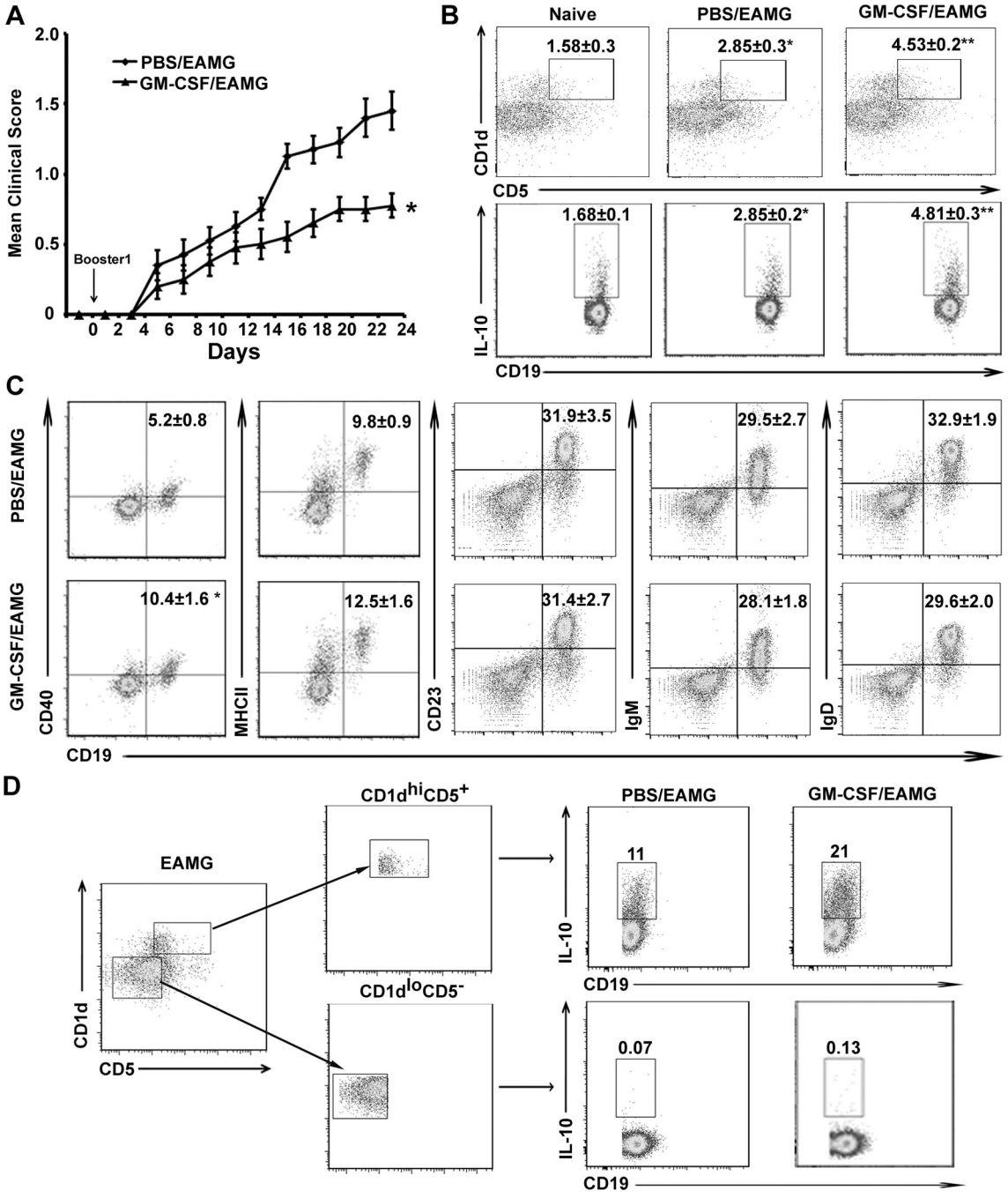

Figure 1. Effects of GM-CSF treatment on the clinical severity and B cell subsets in donor EAMG mice

A) Mean clinical score of donor EAMG mice during days 0–24. Day 0 corresponds to initiation of treatment (GM-CSF vs. PBS) for 10 days at the time of booster immunization. *$p < 0.0002$ starting at day 16 (n = 20). B) Percentage of $CD1d^{hi}CD5^{+}$ B cells within $CD19^{+}$ B cells and $IL10^{+}$ $CD19^{+}$ cells (B10 cells) analyzed at day 24. Splenocytes were stimulated with PMA, ionomycin, monensin and LPS (PIM+L) for 5 hours prior to staining for cell surface CD19 and cytoplasmic IL-10 expression. Values within scatterplots represent mean (±SEM) percentages of $CD1d^{hi}CD5^{+}$ and $IL\text{-}10^{+}$ B cells. *$p < 0.02$; **$p < 0.0004$ (a), $p <$

0.0001 (b) for $CD1d^{hi}CD5^{+}$ B cells and *p < 0.0007; **p < 0.000006 (a), p < 0.0005 (b) for B10 cells (n = 6). Data from PBS/EAMG were compared to naïve group, while data from GM-CSF/EAMG group were analyzed statistically vs. naïve group (a) and vs. PBS/EAMG (b). C) Expression of B cell markers IgM, IgD and surface molecules MHCII, CD40, and CD23 on $CD19^{+}$ B cells isolated at day 24. Values in the scatterplots represent % positive cells (mean ± SEM) (n = 6). *p < 0.002 for CD40. D). Representative scatterplots showing that B10 cells are predominantly found within the $CD1d^{hi}CD5^{+}$ B cell subsets isolated from PBS/EAMG and GM-CSF/ EAMG mice. $CD1d^{hi}CD5^{+}CD19^{+}$ and $CD1d^{lo}CD5^{-}CD19^{+}$ B cells were isolated from spleens of 5 mice each and sorted by FACS. Values in the scatterplots represent % positive cells from one representative experiment.

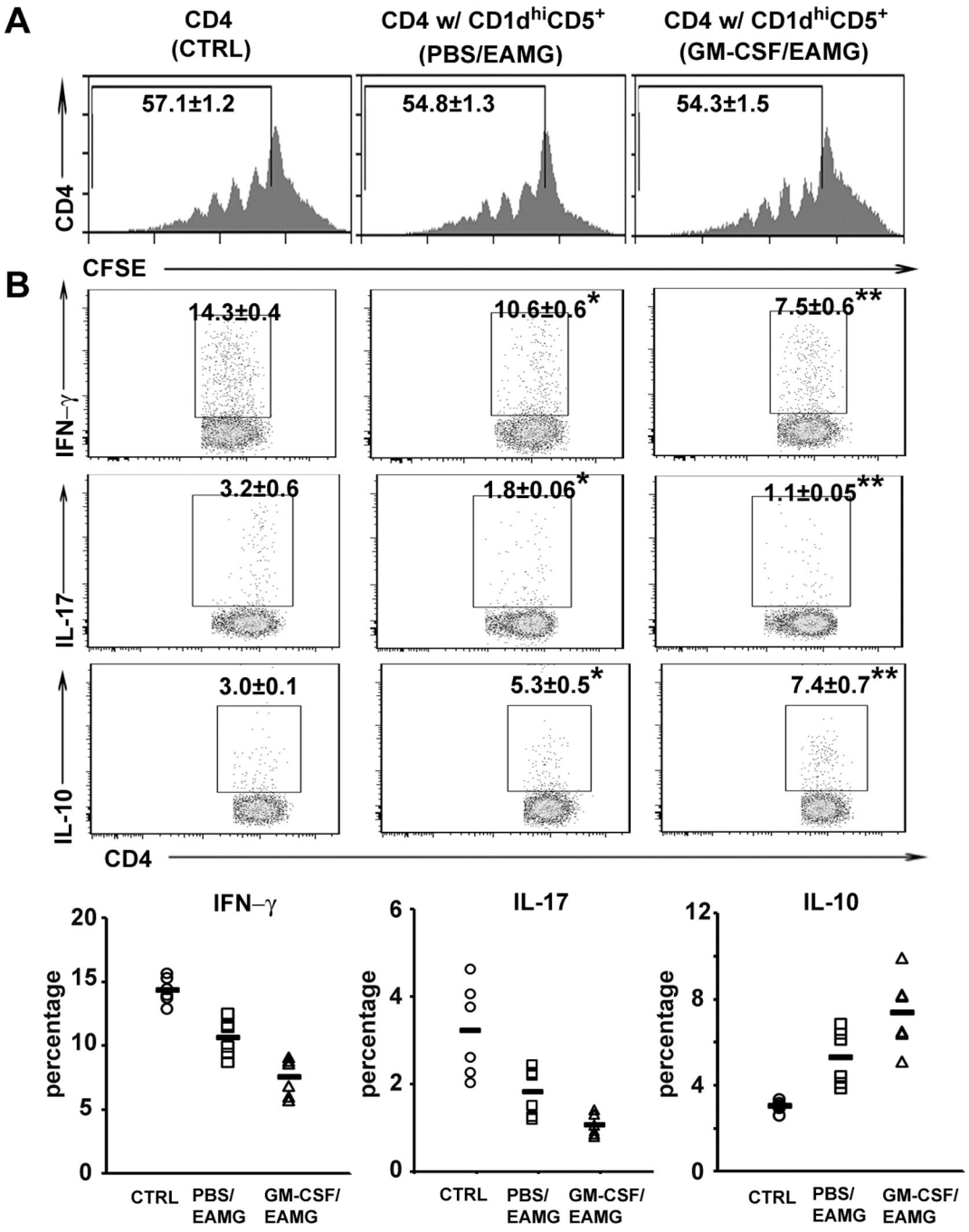

Figure 2. CD1d^{hi}CD5^{+} B cells alter the cytokine profile, but not T cell proliferation *in vitro*

A) Histograms showing T cell proliferative response to AChR stimulation, as determined by % cells with CSFE dilution. Responder CD4^{+} cells (CFSE-labeled) from EAMG mice were cultured alone [control (CTRL)] or with CD1d^{hi}CD5^{+} B cells (1:1 ratio) from GM-CSF-treated or PBS-treated EAMG mice (as indicated in parenthesis) for 3 days with AChR (5 μg/ml) (n = 6, $p > 0.05$). B) Changes in cytokine profile corresponding to panel A experiments. A decrease in %Th1 and %Th17 cells and an increase in % IL-10^{+} T cells were induced by co-culture with CD1d^{hi}CD5^{+} B cells for 3 days. For Th1 cells, *$p < 0.0005$; **p

< 0.000004 (a), $p < 0.005$ (b); for Th17 cells, *$p < 0.05$; **$p < 0.006$ (a), $p < 0.02$ (b); for IL-10^+ T cells, *$p < 0.001$; **$p < 0.0001$ (a), $p < 0.04$ (b); n = 6 each. The percentage of Th1, Th17 and IL-10^+ T cells for each mouse was displayed in the lower panels. Data from PBS/EAMG were compared to CTRL, while data from GM-CSF/EAMG group were analyzed statistically vs. CTRL (a) and vs. PBS/EAMG (b) for this figure and subsequent figures unless otherwise specified.

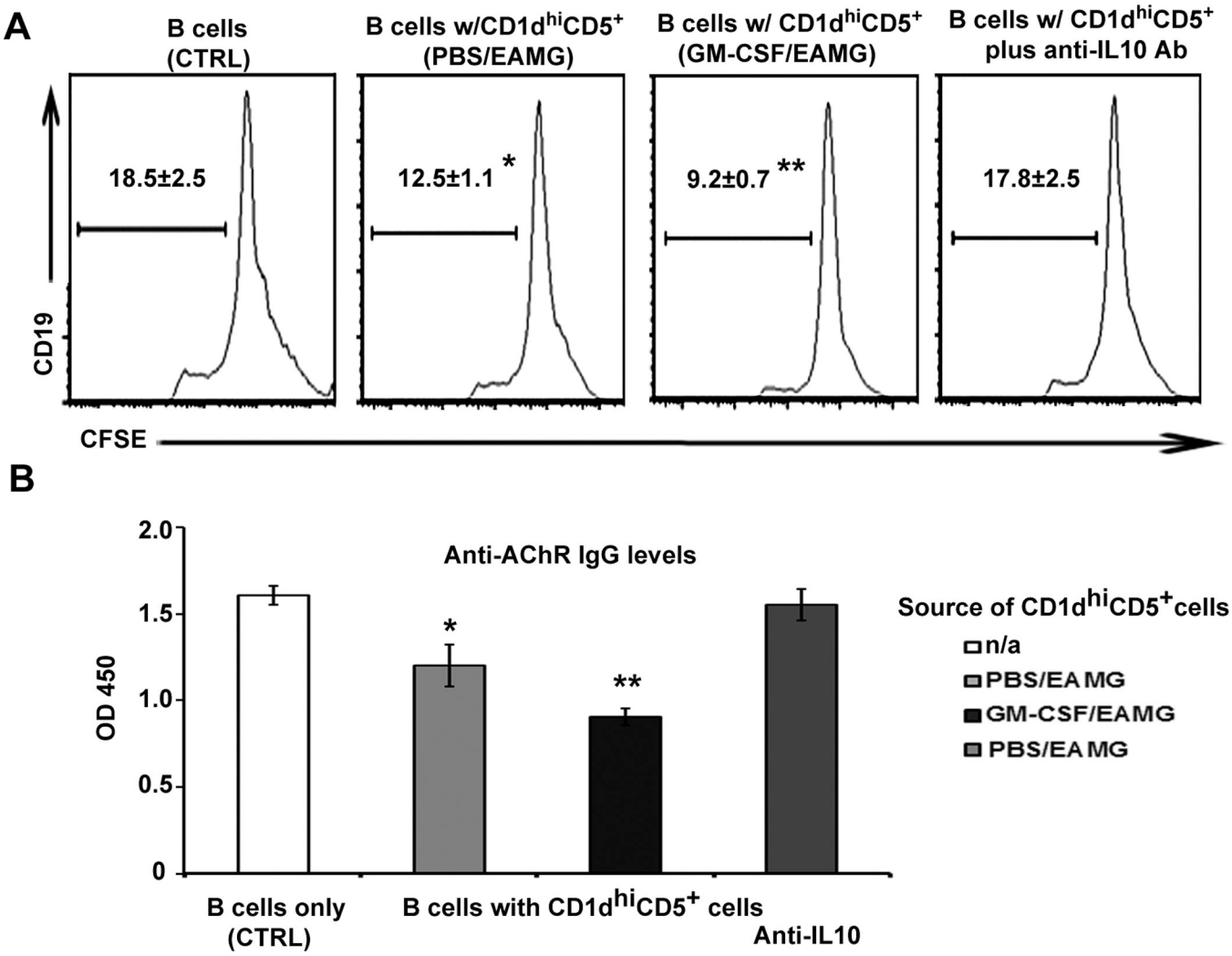

Figure 3. Suppression of B cell proliferation and Ab production by CD1d ^{hi}CD5^{+} B cells via IL-10 *in vitro*

A) Histograms of CFSE fluorescence. Responder CD19^{+} B cells labeled with CFSE were co-cultured with CD19^{+}CD1d^{hi}CD5^{+} cells from GM-CSF-treated and PBS-treated EAMG mice at 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. *$p < 0.05$; **$p < 0.005$ (a), $p < 0.03$ (b) (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. *$p < 0.01$; **$p < 0.002$ (a), $p < 0.04$ (b) (n = 6).

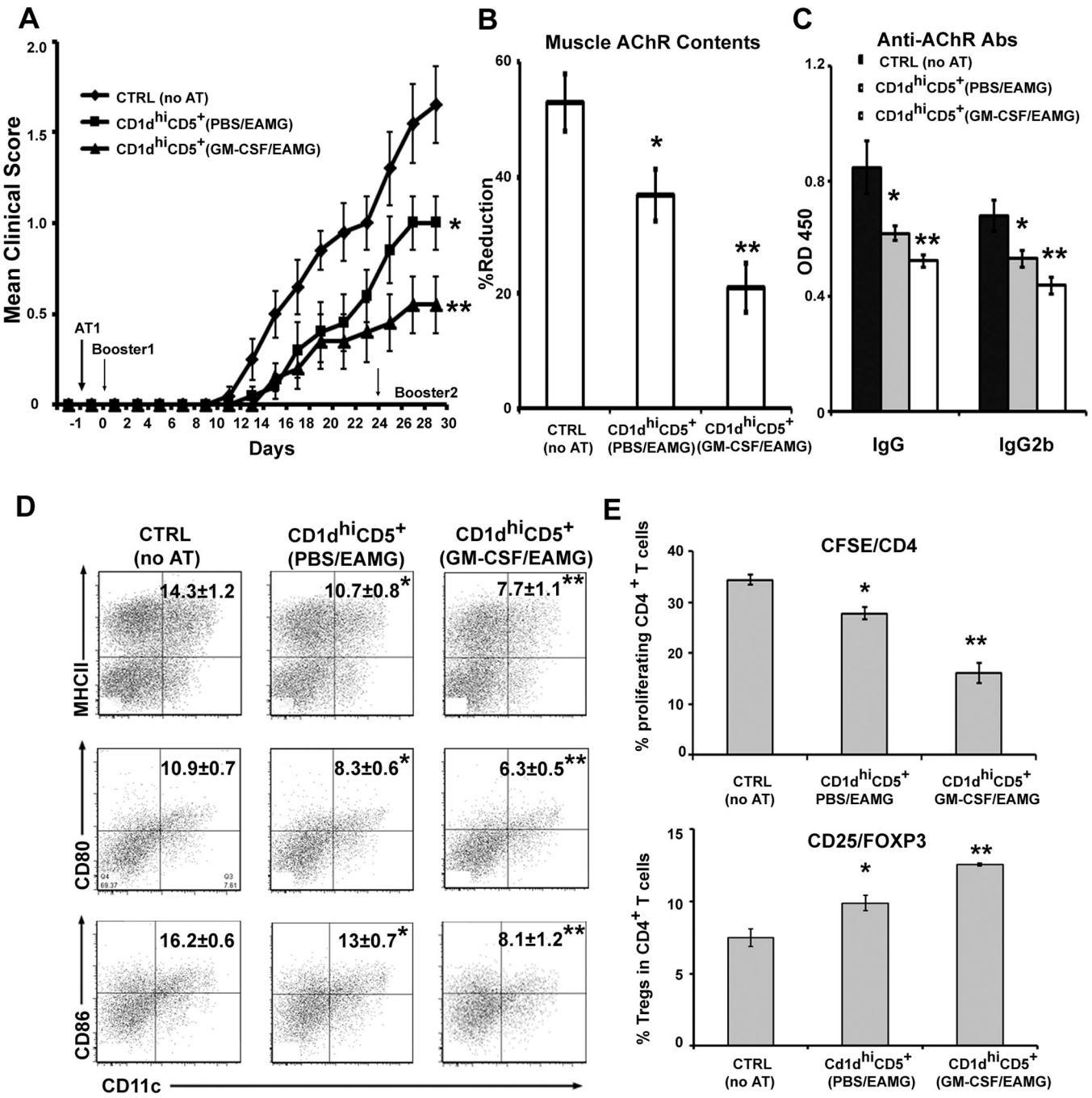

Figure 4. Adoptive transfer (AT) of *in vivo* expanded CD19$^+$CD1d ^{hi} B cells prior to immunization: clinical and immunological effects

A) Clinical severity. Recipient mice were injected with 1 × 10^6 of 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 < 0.02; **p < 0.01 (a), p < 0.02 (b). Day -1 corresponds to the initiation of AT. Booster immunization was performed at day 0 and at day 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 < 0.001 (a), p < 0.01 (b) (n = 10). C) Decreased serum anti-AChR Ab production by AT of CD1d^{hi} CD5$^+$ B cells. For IgG, *p < 0.04; **p <

0.006 (a), $p < 0.01$ (b); for IgG2b, *$p < 0.03$; **$p < 0.001$(a), $p < 0.04$ (b) ($n = 10$). 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, *$p < 0.05$; **$p < 0.003$ (a), $p < 0.05$ (b); for CD80, *$p < 0.02$; **$p < 0.0008$ (a), $p < 0.04$ (b); for CD86, *$p < 0.008$; **$p < 0.0002$ (a), $p < 0.007$ (b). E) Decreased T cell proliferation and increased percentage of Tregs in recipient mice. For T cell proliferation, *$p < 0.002$; **$p < 0.00001$ (a), $p < 0.0006$ (b); for Tregs, *$p < 0.02$; **$p < 0.00001$(a), $p < 0.0007$ (b). Data were derived from 6 animals in figure 4D & E.

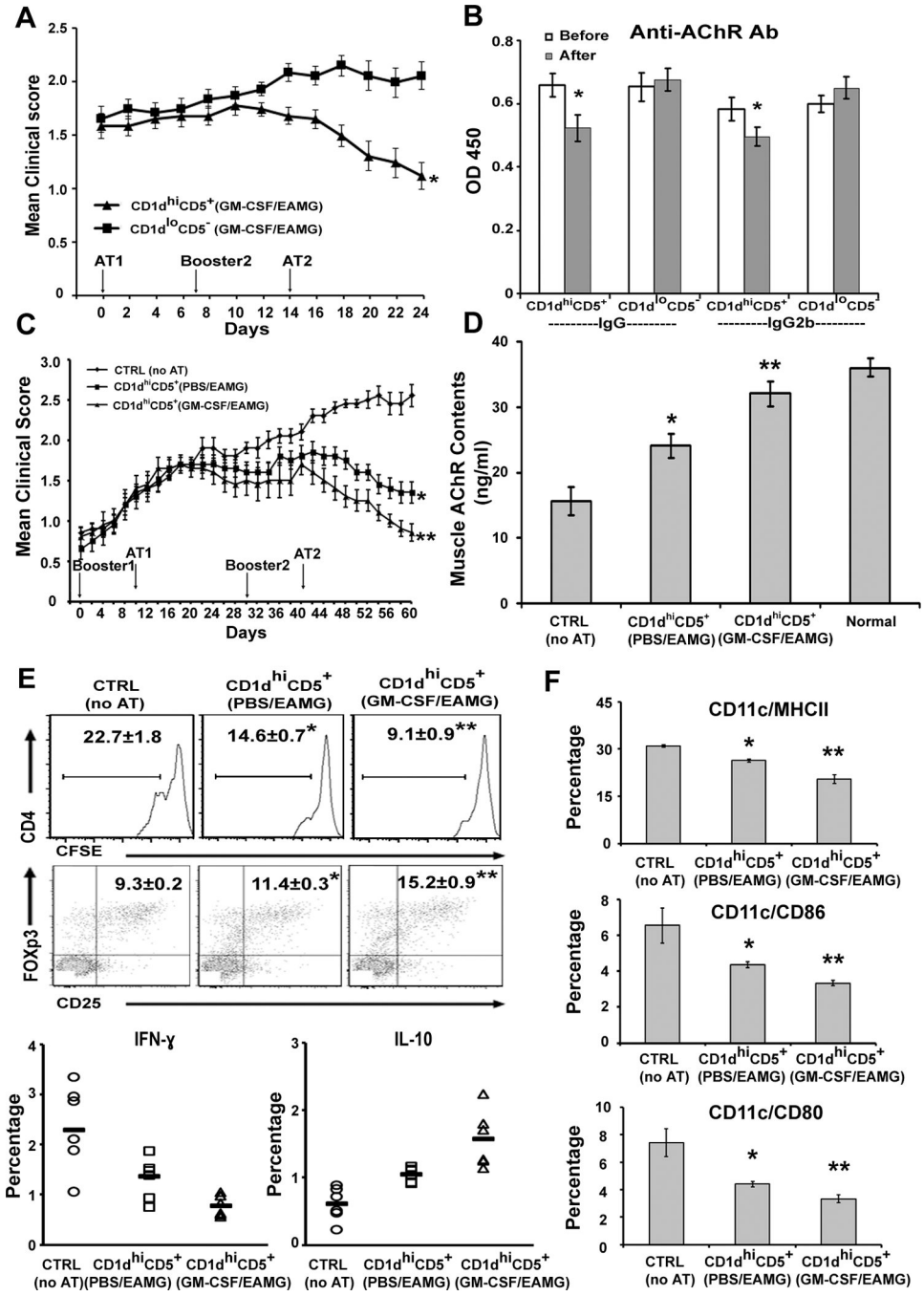

Figure 5. Adoptive transfer of *in vivo* expanded CD1d ^{hi}CD5^{+} B cells after onset of EAMG

A) Amelioration 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 21 d post 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. *$p < 0.05$, **$p < 0.03$ (a); $p < 0.05$ (b). Results are pooled from two separate experiments (n = 10). Day 0 corresponds

to booster1; day 10: AT1; day30: 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 CTRL (no AT), *$p < 0.008$; **$p < 0.00002$ (a), $p < 0.007$ (b) ($n = 10$). E) Effect of AT of $CD1d^{hi}CD5^{+}$ B cells on T cell proliferation, Tregs and cytokine profile. For T cell proliferation, *$p < 0.002$; **$p < 0.00005$ (a), $p < 0.0006$ (b); for Tregs, *$p < 0.003$; **$p < 0.0007$ (a), $p < 0.004$ (b). For Th1 and IL-10^{+} T cells, $p < 0.05$ comparing PBS/EAMG vs CTRL; $p < 0.01$ comparing GM-CSF/EAMG vs CTRL, $p < 0.03$ comparing GM-CSF/EAMG vs PBS/EAMG. F) Effect of AT of $CD1d^{hi}CD5^{+}$ B cells on DCs. For MHCII, *$p < 0.002$; **$p < 0.001$ (a), $p < 0.002$ (b); for CD86, *$p < 0.04$; **$p < 0.02$ (a), $p < 0.001$ (b); for CD80, *$p < 0.02$; **$p < 0.001$ (a), $p < 0.01$ (b). N = 6 for data in figure 5E & F.