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Suppression of Experimental Autoimmune Myasthenia Gravis by Granulocyte-Macrophage Colony-Stimulating Factor Is Associated with an Expansion of FoxP3+ Regulatory T Cells

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Dendritic cells (DCs) have the potential to activate or tolerize T cells in an Ag-specific manner. Although the precise mechanism that determines whether DCs exhibit tolerogenic or immunogenic functions has not been precisely elucidated, growing evidence suggests that DC function is largely dependent on differentiation status, which can be manipulated using various growth factors. In this study, we investigated the effects of mobilization of specific DC subsets—using GM-CSF and fms-like tyrosine kinase receptor 3-ligand (Flt3-L)—on the susceptibility to induction of experimental autoimmune myasthenia gravis (EAMG). We administered GM-CSF or Flt3-L to C57BL/6 mice before immunization with acetylcholine receptor (AChR) and observed the effect on the frequency and severity of EAMG development. Compared with AChR-immunized controls, mice treated with Flt3-L before immunization developed EAMG at an accelerated pace initially, but disease frequency and severity was comparable at the end of the observation period. In contrast, GM-CSF administered before immunization exerted a sustained suppressive effect against the induction of EAMG. This suppression was associated with lowered serum autoantibody levels, reduced T cell proliferative responses to AChR, and an expansion in the population of FoxP3+ regulatory T cells. These results highlight the potential of manipulating DCs to expand regulatory T cells for the control of autoimmune diseases such as MG. The Journal of Immunology, 2006, 177: 5296–5306.

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utoimmune myasthenia gravis (MG) is a T cell-dependent, Ab-mediated, organ-specific autoimmune disease. Autoantibodies targeted to the skeletal muscle acetylcholine receptor (AChR) impair neuromuscular transmission resulting in muscle weakness (1). Current therapies for MG produce nonspecific immune suppression, must usually be continued lifelong to maintain disease control, and are associated with significant chronic side effects and enhanced risk for infection and malignancy.

The ideal therapy for MG would eliminate or suppress the autoimmune response to the AChR specifically without otherwise affecting the immune system. At least in theory, the design of an Ag-specific treatment for MG should be attainable since the autoantigen and immunopathogenesis are relatively well-characterized. Unfortunately, the best available evidence indicates that the autoimmune T cell and Ab responses in MG are highly heterogeneous (2–4). Thus, targeting a discreet population of critical T or B cells for therapeutic immunomodulation is, at best, a highly challenging proposition, particularly in light of the vast adaptability of the immune system. To ultimately achieve the goal of an Ag-specific therapy for MG, it is likely that the immune system itself will have to be harnessed and used as a tool to effectively recognize and suppress the heterogeneous repertoire of autoreactive immune cells.

Dendritic cells (DCs) are the most potent APCs of the immune system, and are critically involved in the initiation of immune responses. DCs are involved in the earliest phase of an immune response and their interactions with T cells can profoundly affect subsequent immunity or tolerance. Thus, in addition to their role in activating lymphocytes, DCs also tolerate T cells to Ags, thereby minimizing autoreactive immune responses (5). The tolerogenic properties of DCs appear to be linked to their differentiation status, such that mature DCs promote immunity, while immature or “semimature” DCs promote tolerance (6). One of the relevant roles for tolerogenic DCs is the induction and maintenance of Ag-specific regulatory T cells (Tregs) (7–9). The administration of hematopoietic growth factors has been shown to modulate DC phenotype and functional status. Specifically, the administration of GM-CSF was recently shown to suppress a T cell-mediated autoimmune disease, experimental autoimmune thyroiditis, while administration of fms-like tyrosine kinase receptor 3-ligand (Flt3-L) had opposite effects (10). The observed suppression associated with GM-CSF treatment was dependent upon the presence of IL-10-producing CD4+CD25+ Tregs induced by tolerogenic DCs (11).

In the present study, we investigated the effects of mobilization of different subsets of DCs using Flt3-L and GM-CSF on the induction of an Ab-mediated autoimmune disease, experimental autoimmune MG (EAMG). We show that Flt3-L accelerates EAMG development while GM-CSF effectively suppresses clinical disease induction. Significantly, the protective effect of GM-CSF was associated with a selective expansion of CD11c+CD8α− DCs,
Materials and Methods

Mice

Eight-week-old female C57BL/6J mice were purchased from The Jackson Laboratory. Mice were housed in the Biologic Resources Laboratory facilities at the University of Illinois and provided food and water ad libitum. All mice were cared for in accordance with the guidelines set forth by the University of Illinois Animal Care and Use committee.

Purification of Torpedo AChR (tAChR) and induction of EAMG

tAChR was purified from the electric organs of Torpedo californica by affinity chromatography using a conjugate of neurotoxin coupled to agarose as described previously (12). Purity of the isolated product was tested by SDS-PAGE. The purified tAChR was used to induce EAMG and as Ag for in vitro testing of immune responses.

To induce EAMG, mice were immunized with 40 μg of tAChR emulsified in CFA in a total volume of 200 μl s.c. along the back and at the base of the tail. The mice were boosted with 20 μg of tAChR emulsified in IFA in 200 μl of volume injected in the flanks and tail base 20 and 50 days after the first immunization. Control mice received an equal volume of PBS in CFA or IFA.

Clinical scoring of EAMG

For clinical examination, mice were observed on a flat platform for a total of 2 min. They were then exercised by gently dragging them suspended by the base of the tail across a cage top grid repeatedly (20–30 times) as they attempted to grip the grid. They were then placed on a flat platform for 2 min and again observed for signs of EAMG. Clinical muscle 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 characteristically shown by a hunchback posture, restricted mobility, and difficulty in raising the head after exercise; grade 2, grade 1 symptoms without exercise during observation period; grade 3, dehydrated and moribund with grade 2 weakness; and grade 4, dead.

Flt3-L and GM-CSF treatment

Recombinant mouse GM-CSF and Flt3-L were purchased from BioSource International and Cell Science, respectively. Mice were randomly divided into four groups of eight mice per group: 1) control; 2) tAChR; 3) tAChR plus GM-CSF; and 4) tAChR plus Flt3-L. Mice were treated with GM-CSF (5 μg), Flt3-L (10 μg), and PBS as follows. For mice in groups 1 and 2, PBS was administered i.p. on days 1–5, and PBS was injected IP on days 6–9. Mice in group 4 received Flt3-L, administered i.p. on days 1–9. All mice, except for the control group, were immunized with tAChR (40 μg/mouse) emulsified in CFA on day 10 as described above; the control group received CFA alone. Treatment with GM-CSF, Flt3-L, or PBS was given i.p. on days 1–5, and PBS was injected IP on days 6–9. Mice in the control group, were immunized with tAChR (40 μg/mouse) emulsified in IFA on day 10 as described above; the control group received CFA alone. Treatment with GM-CSF, Flt3-L, or PBS was given i.p. on days 1–5, and PBS was injected IP on days 6–9.

Flow cytometric analysis of DC and Treg phenotypic markers

Single-cell suspensions of spleen and lymph nodes were prepared from mice sacrificed 48 h after completion of the Flt3-L/GM-CSF/PBS treatment regimen for DC analysis. Cells were washed with PBS supplemented with 2% FBS and blocked with anti-CD11c/CD32 Fc-Block (BD Pharmingen) on ice for 30 min. FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 Abs were used for intracellular staining for Foxp3 expression. Data were collected on a FACSCalibur (BD Biosciences), and analyzed using CellQuest software (BD Biosciences).

RT-PCR

DCs were isolated from spleen samples using a CD11c isolation kit (Miltenyi Biotec). The resulting DC preparation was >90% pure. RNA was extracted using TRIzol and cDNA were synthesized using a ThermoScript RT-PCR System (Invitrogen Life Technologies). A multiplex RT-PCR kit (Maxim-Bio) was used to detect cytokine transcripts. All samples were subjected to electrophoresis using a 2% agarose gel to confirm the specificity of the PCR, and relative quantification of the resulting bands assessed as the ratio of the cytokine transcript to the housekeeping gene, GAPDH. The above experiment was repeated three times to ensure reproducibility.

Confocal microscopy to detect IgG and C3 deposition at the neuromuscular junction (NMJ)

Forelimb muscle samples were obtained from mice in each of the four experimental groups (three mice per group). Muscle samples were frozen in liquid nitrogen and stored at −80°C. Sections (10 μm) were taken and stained using a multiplex beads immunoassays (BioSource International) and analyzed with a Luminex 100 instrument (Bio-Rad). The suggested minimal detection levels using this kit is 15 pg/ml for IL-10, and 1 pg/ml for IFN-γ.
allowed to air-dry, then fixed in cold acetone for 10 min. After washing with PBS, the sections were blocked with PBS/5% goat serum (Cappel; MP Biomedicals) for 1.5 h and then washed with PBS, three times for 15 min. The slides were incubated with tetramethylrhodamine-conjugated anti-BTx (Molecular Probes) (1/500 dilution) for 1 h at room temperature to label the NMJ, then incubated with goat anti-mouse IgG (Chemicon International), goat anti-mouse complement C3 (Cappel; MP Biomedicals) to colocalize IgG or complement deposits on the NMJ. Anti-IgG and anti-C3 Abs were FITC-conjugated (1/500 dilution). The sections were washed with PBS three times for 15 min and viewed using a fluorescence microscope (Zeiss LSM510 laser scanning microscopy). Endplate areas were identified as regions of tetramethylrhodamine-conjugated anti-BTx staining. A minimum of 15 sites per muscle to two with three endplates per site was evaluated. The presence of IgG and C3 staining was ascertained visually, and the percentage of endplate regions with visible C3 or IgG staining was determined for each of the experimental groups. All sections were stained and processed in parallel to avoid interassay variations.

Electron microscopy
At the end of the experiment, mice from each of the four experimental groups (three mice per group) were anesthetized with 50 mg/kg sodium pentobarbital and sacrificed. The tibialis anterior muscle and diaphragm were removed and fixed using 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) in 4°C. The samples were sectioned and post fixed with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4), dehydrated through a graded ethanol series, and embedded in epoxy resin. Endplates were located in toluidine blue-stained 1-μm semithin sections from the central region of each muscle. Ultra-thin sections (Leica ultracut UCT; Leica Microsystems) from selected areas were contrasted with uranyl acetate and lead citrate and viewed with an electron microscope (JEM-1220 Electron Microscope; JEOL USA). At least five endplate regions with clearly defined nerve terminals and postsynaptic membranes were photographed and evaluated per muscle. Digital micrographs were taken with Gatan slow-scan charge-coupled device camera. Endplates were graded visually as “normal” or “abnormal” by an evaluator who was not aware of the identity of the specimen. A “normal” endplate had the following features: readily visualized nerve terminal closely opposed to a postsynaptic membrane with a normal postsynaptic folded pattern. Postsynaptic membrane regions without an associated nerve terminal in the microscopic field were not included in the analysis. The criteria for an “abnormal” endplate required a readily visible simplification of the postsynaptic membrane structure with reduction or loss of the normal postsynaptic folding pattern in a photographed endplate region with a clearly defined nerve terminal and postsynaptic membrane.

Statistical analysis
Mean, SD, SE, and statistical significance were calculated using SPSS software applications. Nonparametric Wilcoxon signed test was used for statistical analysis of clinical severity; Fisher’s exact test was used for percentage of endplates with IgG/complement deposition and for clinical incidence. Student’s t test was used for ELISA, proliferation assays, and flow cytometry. A value of p ≤ 0.05 was considered significant.

Results
GM-CSF and Flt3-L modulate initial susceptibility to EAMG, and GM-CSF has a sustained suppressive effect on disease incidence and severity
We immunized three groups of mice (eight mice per group) with tAChR on days 0, 20, and 50. Mice were pretreated with GM-CSF, Flt3-L, or PBS before each immunization. We measured mouse strength on an every other day basis beginning immediately after the first immunization. Mice treated with Flt3-L developed more frequent and more severe myasthenic weakness compared with tAChR-immunized, untreated controls. Interestingly, this effect persisted until ~7 wk after the initial immunization, but then disease incidence and severity equalized in this group compared with the control animals. In contrast, mice treated with GM-CSF developed significantly less frequent and severe disease, compared with untreated, tAChR-immunized animals. This relative protection from disease induction persisted until the end of the observation period. By the end of the observation period, 10% of the GM-CSF treated animals had clinical disease compared with 90% in both the Flt3-L and the tAChR-immunized control groups. No animals in the GM-CSF group developed a clinical score more than one at any point during the nine-week observation period. Fig. 1A shows the incidence of EAMG over the 9-wk observation period; Fig. 1B shows the average clinical scores over the same period (tAChR = 8, Flt3-L = 8, GM-CSF = 8, CFA = 6). Disease incidence at the end of the observation period in GM-CSF-treated mice compared with tAChR-immunized controls showed a significant difference (p < 0.01), as did average disease severity (p < 0.01).

Activation of DCs
To determine the effects of GM-CSF and Flt3-L treatment on DC phenotype, we analyzed the expression of CD11c, MHC class II, and costimulatory molecules, as well as the production of proinflammatory cytokines from GM-CSF-treated, Flt3-L-treated, and untreated mice after tAChR immunization. Spleens from mice treated with GM-CSF and Flt3-L had relatively equal percentages of CD11c+ cells and comparable levels of expression of MHC class II, and CD80 compared with untreated tAChR-immunized mice and control (CFA) mice (data not shown). GM-CSF-treated mice, however, showed an increase in CD80+CD11c+ cells: 3.55% compared with 1.82% in the untreated group, and 2.05% in...
the Flt3-L-treated group (Fig. 2A). GM-CSF-treated mice also had relatively higher levels of expression of CD86 (data not shown). However, levels of proinflammatory cytokines, such as TNF-α, IL-12, and IL-1β, evaluated by RT-PCR, were low in GM-CSF-treated mice compared with untreated tAChR-immunized controls and Flt3-L-treated mice (Fig. 2B).

Serum anti-tAChR and anti-mouse AChR Abs

The effects of GM-CSF and Flt3-L treatment on the serum anti-AChR Ab responses were monitored at different time points. We measured the serum concentration of anti-tAChR (Fig. 3A) and anti-mouse AChR Abs (Fig. 3B) by ELISA preimmunization and day 28 and day 56 postimmunization. The titer of anti-tAChR and anti-mouse Abs did not correlate with disease severity (data not shown). Interestingly, treatment with Flt3-L did not significantly affect serum anti-tAChR or anti-mouse AChR Ab levels compared with untreated tAChR-immunized controls. In contrast, treatment with GM-CSF resulted in a decrease in total anti-tAChR IgG Ab response compared with untreated tAChR-immunized controls and had a particularly significant effect on lowering levels of anti-mouse AChR IgG. This decrease in total anti-mouse AChR IgG levels reflected a prominent decrease in complement-fixing IgG2b isotypes, while IgG1 isotypes were relatively unaffected. These differences were evident on day 28 postimmunization and persisted relatively unchanged at day 56. The anti-IgG2a mAbs yielded a relatively small signal that also decreased in GM-CSF-treated animals, likely resulting from cross-reactivity with homologous IgG2c Abs (13).

GM-CSF and Flt3-L exert differential effects on tAChR-specific T cell proliferative responses

Spleen cells from mice treated with Flt3-L showed a significantly higher (p = 0.007) proliferative response to stimulation with tAChR, while spleen cells from mice treated with GM-CSF had a significantly lower (p = 0.0025) proliferative response to tAChR compared with immunized controls (Fig. 4). Comparable results were obtained using draining lymph node cells (data not shown). No differences were observed in the GM-CSF-treated, Flt3-L-treated, and untreated mice with nonspecific stimulation using the lectin Con A (data not shown).

GM-CSF and Flt3-L treatment affect cytokine production by CD4+ lymphocytes

We analyzed cytokine production by assessing intracellular cytokine expression profiles in splenic lymphocytes obtained from mice treated with the described growth factors and untreated mice 14 days after the initial immunization with tAChR (Fig. 5). The percentages shown represent cytokine expression by isolated CD4+ cells. There were no significant differences in the amounts of IFN-γ, TNF-α, IL-4, or IL-10 expressed by spleen cells from Flt3-L-treated and untreated tAChR-immunized mice. In contrast, spleen cells from GM-CSF-treated mice expressed lower amounts of IFN-γ (p < 0.05) and TNF-α (p < 0.05) compared with control mice and higher amounts of IL-4 (p < 0.05). Although the percentage of IL-10-producing cells in GM-CSF-treated mice was not significantly different compared with controls, analysis of cell-free culture supernatants showed significantly higher IL-10 production (p < 0.01) by cells from GM-CSF-treated mice, as well as decreased production of IFN-γ (p < 0.05).

GM-CSF treatment induces an expansion of the population of CD4+CD25+ and FoxP3-expressing T cells

To determine whether treatment with GM-CSF and Flt3-L affects the relative numbers of Tregs, we tested spleen cells for expression of known Treg surface markers by FACS (Fig. 6). The numbers of CD4+CD25+ and FoxP3+ cells were not affected by treatment with Flt3-L and were comparable to those observed in untreated tAChR-immunized control mice. Interestingly, we found significantly increased numbers of CD4+CD25+ cells in mice treated
with GM-CSF (Fig. 6A) compared with untreated tAChR-immunized control animals ($p < 0.05$). We also found an expansion in the percentage of both CD25$^+$ and CD25$^-$ cells expressing the transcription factor FoxP3 in GM-CSF-treated mice compared with untreated tAChR-immunized controls, which was also statistically significant ($p < 0.05$). We observed similar results when the analysis was performed on splenocytes isolated from the animals at the end of the experiment as well as when the analysis was...
performed in lymph node cells (data not shown), suggesting that these cells could have an important regulatory role in the GM-CSF-mediated suppression of the disease.

Deposition of IgG and C3 is reduced in the NMJs of GM-CSF-treated mice

To examine the effects of GM-CSF and Flt3-L treatment on the deposition of IgG and complement at the NMJ, we performed immunofluorescence studies on forelimb muscles isolated from mice in each experimental group as described. Endplates from Flt3-L-treated and untreated tAChR-immunized controls showed strong staining for C3 and IgG which colocalized to endplate regions as defined by staining with tetramethylrhodamine-conjugated anti-BTx, while GM-CSF-treated mice had little or no deposition of C3 or IgG in the majority of endplates regions assessed. Specifically, the percentages of visualized endplates showing immunoreactivity for C3 in each experimental group is as follows: tAChR, 94.1%; Flt3-L, 83.3%; GM-CSF, 35%. For IgG: tAChR, 93.3%, Flt3-L, 81.3%; GM-CSF, 28.5% (Fig. 7A). Deposition of IgG and C3 was significantly reduced in endplates from GM-CSF-treated mice (p < 0.001). Representative images from the four experimental groups are shown in Fig. 7B.

GM-CSF protects the muscle endplate from anti-AChR Ab-induced morphologic damage

To determine whether the clinical and immunologic changes observed in GM-CSF-treated mice were associated with prevention of the hallmark pathologic findings of destruction of the postsynaptic membrane in EAMG, we performed electron microscopic observations and morphometric analysis on diaphragm muscle obtained from three mice from each experimental group. In mice treated with FLt3-L and in untreated mice endplates showed morphological abnormalities characterized by simplification of the membrane structure and reduction or loss of the postsynaptic folding pattern. In contrast, the endplate regions in GM-CSF-treated mice were largely normal in appearance. Specifically, the percentage of endplates showing clear morphological abnormalities as described for each of the experimental groups is as follows: tAChR control: 66%; Flt3-L-treated: 71.4%; GM-CSF-treated: 27%. Representative endplate regions for each of the four experimental groups are shown in Fig. 8.

Discussion

The immunopathogenesis of EAMG involves the production of high-affinity anti-AChR Abs whose synthesis is modulated by, and dependent upon AChR-specific CD4+ T cells (14–16). The activation of AChR-specific T cells is, in turn, determined by their interactions with APCs. As the most potent APC of the immune system, DCs play an important role in MG by presenting self-Ags and promoting the priming and/or boosting of AChR-specific T cells (17–19). Furthermore, DCs have been shown to have potent capabilities to tolerate T cells in an Ag-specific manner (20, 21). Recently, a number of studies have shown that autologous DCs modified in a number of different ways in vitro and administered to rodents with EAMG can have a protective effect on the development and progression of disease (18, 22, 23). Li et al. (24) have reported that immature DCs (generated in low doses of GM-CSF and pulsed in vitro with AChR) can induce tolerance to EAMG in the rat model of the disease, while mature DCs pulsed with AChR and unpulsed mature or immature DCs had no effect. In contrast to these studies, we induced tolerance to EAMG by direct administration of GM-CSF to experimental mice, an intervention that could readily be applied to human MG. The observed effect of GM-CSF on DC phenotype in our study was not consistent with shift to an “immature” phenotype according to the classical model of DC maturation since there was no effect on DC expression of MHC and costimulatory molecules. The recent realization that DCs capable of promoting tolerance can express substantial levels of costimulatory and other maturation markers has led some investigators to classify DCs as “immunogenic” or “tolerogenic” based on DC effector function (25). DCs with a tolerogenic phenotype express relatively high levels of MHC class II and costimulatory molecules, and produce low levels of proinflammatory cytokines (26), as was observed in DCs isolated from GM-CSF-treated mice in our study.

In secondary lymphoid organs, two additional DC subsets are characterized by their expression of the marker CD8α (27, 28), and the administration of GM-CSF and Flt3-L can induce differential activation of these subsets (29). Flt3-L has been shown to mobilize both CD8α+ and CD8α− subsets of DCs and to promote a Th1 type of immune response to protein Ags (30–33). Th1 cells produce proinflammatory cytokines, which are important for cell-mediated responses and production of IgG subclasses that bind complement (34, 35). GM-CSF is a potent growth factor for CD8α− DCs and has been shown to promote a Th2 response (32, 33, 36, 37). Th2 cells induce production of IgA, IgE, and IgG subclasses that do not fix complement and produce anti-inflammatory cytokines, which may down-modulate immune responses, possibly by acting as growth and differentiation factors for Tregs (34, 35). Similar to previous findings (10, 11, 38) in the T cell-mediated disease experimental autoimmune thyroiditis (EAT), we have shown that treatment with GM-CSF results in mobilization of CD11c+/CD8α− DCs and a skewing of the immune response to a Th2 polarization in the EAMG mouse model. Accordingly, GM-CSF-treated mice showed only slight increases in the intracellular expression of IFN-γ and TNF-α by splenocytes compared with nonimmunized control (CFA) mice, which had significantly reduced levels of these cytokines, and increased production of IL-4 and IL-10 compared with control tAChR-immunized mice. The resultant suppressive effect of GM-CSF treatment on clinical and immunologic disease in EAMG suggests that the differentiation of anti-AChR Th2 cells down-regulated the pathogenic anti-AChR Th1 immune response.
This hypothesis is complicated by the unclear roles of Th1 and Th2 cell subsets in the immunopathogenesis of EAMG. CD4+ Th1 cells are known to be involved in the development of EAMG, promoting the synthesis of anti-AChR Abs that bind and activate complement and cause destruction of the NMJ (39–41). IFN-γ and TNF-α are Th1, proinflammatory cytokines known to be crucial to the induction and development of EAMG (13, 42–44). Furthermore, the Th2 cytokine, IL-4, may have a protective role in EAMG as mice deficient in IL-4 are more susceptible to EAMG induction (45). In addition, STAT6 deficient BALB/c mice, which have impaired IL-4-mediated functions are more susceptible to EAMG compared with STAT4-deficient mice having reduced Th1 responses (46).

Conversely, the actions of IL-4 and IL-10 collectively induce activated B lymphocytes to proliferate, switch isotype, and ultimately differentiate into Ab-producing plasma cells (47). IL-10 has even been reported to aggravate EAMG through induction of Th2 and B cell responses to the AChR (48), and the transgenic expression of IL-10 in T cells facilitates the development of EAMG (49). Thus, the enhanced production of IL-10 in GM-CSF-treated animals might be expected to promote the synthesis of anti-AChR Abs and possibly make EAMG worse. In fact, the opposite effect was observed in our study.

One possible explanation for this apparent paradox, may be that Ab isotype switching resulted in a shift to less pathogenic (non-complement-fixing) isotypes, a hypothesis that is supported by our finding of reduced circulating levels of IgG2b Abs, as well as reduced IgG and complement deposition at the NMJs in GM-CSF-treated animals. However, total IgG levels were significantly reduced in GM-CSF-treated mice, suggesting that the observed switch to a Th2 immune response in these animals did not result in enhanced B cell responses, at least with regard to autoreactive B cell responses.

In fact, the suppressive effect of GM-CSF on the induction of EAMG may be best explained by the over-riding effects of the expanded population of Tregs induced by tolerogenic DCs (7–9) on the proliferation of Tregs induced by tolerogenic DCs (7–9) on the proliferation of AChR specific T cells and the synthesis of anti-AChR Abs. It has been shown that in vitro, Ag-stimulated, T cell-proliferative responses suppressed by GM-CSF treatment can be restored by depletion of CD4+CD25+ T cells in experimental

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**FIGURE 5.** Cytokine response to tAChR stimulation. Mice were treated with GM-CSF or Flt3-L before immunization and sacrificed 14 days later. Cytokine response was measured using single-cell suspensions of splenocytes by intracellular cytokine staining (A) and/or by a multiplex suspension assay system (B). A, Intracellular staining for cytokine expression was conducted using FITC-labeled anti-CD4 and PE-labeled anti-IFN-γ, PE-labeled anti-IL-4, PE-labeled anti-IL-10, or PE-labeled anti-TNF-α Abs followed by analysis by FACS. The percentages shown represent cytokine expression by isolated CD4+ cells. With GM-CSF treatment, significantly lower expression of IFN-γ and TNF-α was observed, whereas higher levels of expression of IL-4 and IL-10 (not significant) were found. B, The above splenocytes were also cultured for 48 h, and cell-free culture supernatants were assayed using a multiplex bead immunoassay for IFN-γ and IL-10. Significantly lower levels of IFN-γ and significantly higher levels of IL-10 were detected in GM-CSF-treated animals. A and B, Results are expressed as mean ± SE (*, p < 0.05).
autoimmune thyroiditis, a T cell-mediated autoimmune disease (10). Furthermore, adoptive transfer of CD4^+CD25^- T cells from GM-CSF-treated mice into thyroglobulin-primed mice resulted in significant suppression of thyroglobulin-specific T cell proliferation (11). These findings suggest that the expanded CD4^+CD25^- Treg populations specifically inhibit autoimmune effector T cell functions, which in EAT are thyroglobulin-specific effector T cells.

Similar to this, our studies indicate that GM-CSF-treated mice, having relatively increased numbers of CD4^+CD25^- and FoxP3 expressing T cells, are protected against the induction of EAMG, an Ab-mediated, T cell-dependent, autoimmune disease. Despite persistent exposure to endogenous AChR Ag in all the experimental groups, this expansion of cells with a regulatory phenotype occurred only with GM-CSF pretreatment. Previous reports support the existence of an AChR-specific, immunoregulatory T cell population in EAMG, which plays a role in determining the quality and magnitude of the autoimmune response to the AChR (50, 51). Furthermore, we not only observed an expansion of CD25^-FoxP3^- cells, but also CD25^-FoxP3^+ cells having a phenotype consistent with type 1 Tregs (Tr1). This may be consistent with the hypothesis that naturally occurring CD4^+CD25^-FoxP3^- cells play a role in the induction and differentiation of Tr1 cells, which are induced upon Ag exposure under certain tolerogenic conditions (52–54). We plan to investigate the precise roles of these two Treg subsets in GM-CSF's suppression of EAMG in future studies.

It is possible that in EAMG, other cytokines may also be important in the immunoregulatory properties of GM-CSF. For example, it is known that IL-6, a key cytokine required for the final maturation of plasma cells (55) is critically involved in the immunopathogenesis of EAMG (56), and acts in concert with IL-10 to

**FIGURE 6.** Effects of GM-CSF and Flt3-L on CD25^- and FoxP3^- T cells. Mice were sacrificed at 14 days following treatment with GM-CSF, Flt3-L, and PBS. Splenocytes were isolated from animals and stained with FITC-labeled anti-mouse CD4, APC-labeled anti-mouse CD25, and PE-labeled anti-Foxp3. Representative plots from three separate experiments (n = 3/group) showing the percentage of CD4^-CD25^+ (A) and FoxP3^- (B) Tregs are shown. A, Gated CD4^+ cells were analyzed. Significantly increased numbers of CD4^-CD25^+ cells were detected in mice treated with GM-CSF compared with untreated tAChR-immunized control animals (p = 0.004). B, Gated CD4^+ cells are again shown and the expression of CD25 or FoxP3 was analyzed. A statistically significant expansion in the percentage of FoxP3-expressing (both CD25^- and CD25^+) cells was observed in GM-CSF-treated mice compared with untreated tAChR-immunized controls (p < 0.05). The numbers of CD4^-CD25^- and FoxP3^- cells were not affected by treatment with Flt3-L and were comparable to those observed in untreated tAChR-immunized control mice. The results for CD25^- cells in each of the four experimental groups are also shown in graphic form in A. Each value represents the mean ± SE of triplicate values. B, The values in the table represent the mean values of three separate experiments with the range of values given in parentheses.
promote B cell proliferation and survival. Recently, a newly recognized subset of IL-17-producing cells (Th17), mobilized by the combined presence of IL-6 and TGF-β, have been noted to have a role in the induction of autoimmune tissue injury (57). These cells appear to oppose the effects of Tregs, perhaps by inhibiting the expression of FoxP3 (58). The present study did not specifically examine the production of IL-6 or IL-17 in GM-CSF-treated vs untreated animals, but future studies examining this and using Abs to block the effects of selected cytokines (IL-4, IL-10, IL-6, and IL-17), as well as specifically assessing B cell responses to the administration of GM-CSF may clarify the immunological mechanisms underlying our observations and determine whether the observed effects are dependent on specific cytokines.

The important role of complement-mediated alteration of end-plate region morphology and/or destruction has been confirmed by the finding of complement components in the neuromuscular junctions of patients with MG and in mice with EAMG (59–64). Furthermore, as previously noted, IL-12-deficient mice, which are resistant to EAMG induction, have IgG deposits at their endplates but no deposition of complement, suggesting that binding of the anti-AChR Ab at the neuromuscular junction may not be sufficient for the production of myasthenic symptoms (41). In C57BL/6 mice, predominantly IgG2b isotype activates and binds complement (65), and it is this isotype of anti-AChR that was most significantly suppressed by GM-CSF treatment in our study.

In conclusion, we have shown that the differential activation of specific subsets of DCs using GM-CSF effectively protects against the induction of EAMG. This effect was likely due to a shift in the cytokine milieu to a Th2 polarization and the generation of increased numbers of CD4+CD25+ and FoxP3-expressing Tregs,

**FIGURE 7.** Effects of GM-CSF and Flt3-L on deposition of C3 and IgG at the NMJs of EAMG mice. Forelimb muscle specimens obtained from three mice from each of the four experimental groups were analyzed by standard immunohistochemical analysis. Cryosections of muscles were double-stained with tetramethylrhodamine-conjugated anti-BTx (second column) and goat anti-mouse IgG or goat anti-mouse C3 (first column); merge on the right. A. The percentages of visualized endplates showing immunoreactivity for C3 in each experimental group is shown in graphic form. B. Endplates from Flt3-L-treated and untreated Tachr-immunized controls showed strong staining for C3 and IgG, which colocalized to endplate regions, while GM-CSF-treated mice had little or no deposition of C3 or IgG in the majority of endplates regions assessed. The immunofluorescence data shown represents one of at least 15 sites analyzed for each experimental group with approximately two to three endplates visualized per site. Original magnification for all images is 63 x 10 x 2.5; bars are 20 μm. All images were taken under similar conditions.

**FIGURE 8.** Electron microscopic examination of the postsynaptic folds of motor endplates in GM-CSF-treated, Flt3-L-treated, and untreated EAMG mice. NMJs from the isolated diaphragm muscle from mice in each of the four experimental groups (CFA, tAChR, Flt3-L, and GM-CSF) are shown. Damaged postsynaptic membranes with a reduction or absence of the normal folded pattern was commonly seen in the tAChR and Flt3-L groups, whereas intact or normal postsynaptic folds were observed in the CFA and GM-CSF groups. Arrows indicate the postsynaptic membranes; nerve terminals are labeled NT; scale bars are 0.8 μm.
which suppressed anti-AChR T cell and Ab responses. These findings extend the observations previously reported for a T cell-mediated autoimmune disease (EAT), to a well-characterized Ab-mediated, organ-specific, autoimmune disease. Determining the effectiveness of this approach in reversing established disease and defining the precise mode of function, cytokine-dependency, and Ag-specificity of the observed expanded population of Tregs will further our understanding of the immunopathogenesis of EAMG and may lead to new therapeutic avenues for human MG.

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