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IL-10-Producing CD4⁺CD25⁺ Regulatory T Cells Play a Critical Role in Granulocyte-Macrophage Colony-Stimulating Factor-Induced Suppression of Experimental Autoimmune Thyroiditis¹

Eryn Gangi,* Chenthamarakshan Vasu,† Donald Cheatem,* and Bellur S. Prabhakar²*

Our earlier study showed that GM-CSF has the potential not only to prevent, but also to suppress, experimental autoimmune thyroiditis (EAT). GM-CSF-induced EAT suppression in mice was accompanied by an increase in the frequency of CD4⁺CD25⁺ regulatory T cells that could suppress mouse thyroglobulin (mTg)-specific T cell responses in vitro, but the underlying mechanism of this suppression was not elucidated. In this study we show that GM-CSF can induce dendritic cells (DCs) with a semimature phenotype, an important characteristic of DCs, which are known to play a critical role in the induction and maintenance of regulatory T cells. Adoptive transfer of CD4⁺CD25⁺ T cells from GM-CSF-treated and mTg-primed donors into untreated, but mTg-primed, recipients resulted in decreased mTg-specific T cell responses. Furthermore, lymphocytes obtained from these donors and recipients after adoptive transfer produced significantly higher levels of IL-10 compared with mTg-primed, untreated, control mice. Administration of anti-IL-10R Ab into GM-CSF-treated mice abrogated GM-CSF-induced suppression of EAT, as indicated by increased mTg-specific T cell responses, thyroid lymphocyte infiltration, and follicular destruction. Interestingly, in vivo blockade of IL-10R did not affect GM-CSF-induced expansion of CD4⁺CD25⁺ T cells. However, IL-10-induced immunosuppression was due to its direct effects on mTg-specific effector T cells. Taken together, these results indicated that IL-10, produced by CD4⁺CD25⁺ T cells that were probably induced by semimature DCs, is essential for disease suppression in GM-CSF-treated mice. The Journal of Immunology, 2005, 174: 7006–7013.

¹ Abbreviations used in this paper: EAT, experimental autoimmune thyroiditis; DC, dendritic cell; FasL, Fas ligand; HT, Hashimoto’s thyroiditis; mTg, mouse Tg; Tg, thyroglobulin; Tr1, type 1 regulatory T cell; Treg, regulatory T cell.

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CD4⁺CD25⁺ Treg suppress autoimmunity is not fully understood, suppressor cytokines, such as IL-10, have been implicated (28–33). In GM-CSF-treated mice, there was a considerable increase in the levels of IL-10, and neutralization of IL-10 in lymphocyte cultures derived from GM-CSF-treated mice restored mTg-specific T cell responses. Furthermore, lymphocytes from GM-CSF-treated mice that were depleted of CD4⁺CD25⁺ T cells showed enhanced mTg-specific proliferation, with a concomitant decrease in the levels of IL-10 in vitro, suggesting that these cells were the source of IL-10 (24). These data implied a role for IL-10 in GM-CSF-induced suppression of EAT.

In the current study we investigated the direct role of CD4⁺CD25⁺ T cells and IL-10 in GM-CSF-induced suppression of EAT. We show that adoptive transfer of CD4⁺CD25⁺ T cells from GM-CSF-treated mice into mTg-primed mice can suppress mTg-specific proliferation, and cells from recipient mice can produce higher levels of IL-10. Furthermore, in vivo blockade of IL-10R can abrogate GM-CSF-induced suppression and restore mTg-specific T cell responses, resulting in the development of EAT. Moreover, we observed an increase in DCs with a semimature phenotype in GM-CSF-treated mice, which suggested a putative mechanism for the induction of Treg. These data show the critical role that CD4⁺CD25⁺ T cells and IL-10 play in GM-CSF-induced suppression of EAT.

Materials and Methods

Mice
Six- to 8-wk-old female CBA/J were purchased from The Jackson Laboratory. Mice were housed at the Biological Resources laboratory facility at University of Illinois and were provided food and water ad libitum. Animals were cared for in accordance with the guidelines set forth by the University of Illinois animal care and use committee. All mice were used as 8–10 wk of age.

GM-CSF, Abs, and mTg
Recombinant mouse GM-CSF was purchased from Cell Sciences. FITC-conjugated anti-CD11c and PE-conjugated anti-I-Ak (MHC class II), anti-CD8a, anti-CD80, anti-CD86, and anti-CD40 (BD Pharmingen) and PE-conjugated anti-CD11c and PE-conjugated anti-I-Ak (MHC class II), anti-CD40 (BD Pharmingen) were used as a control to ensure equivalent amounts of RNA in the assay. They were killed on day 35, spleen and lymph node cells were collected, and CD4⁺CD25⁺ T cell were isolated from mice in the GM-CSF/mTg group as described above. Effector T cells were isolated from spleen and lymph node cells from mice in the mTg control group using magnetic cell sorting (Miltenyi Biotec) 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 0.5 × 10⁵ cells/well. Isolated CD4⁺CD25⁺ T cells from group 3 were added to cultures at a 5:1 effector:T cell ratio. T cell-depleted spleen cells (0.5 × 10⁵ cells/well) or enriched DCs (0.1 × 10⁵ cells/well) from naïve mice (both accomplished by magnetic cell sorting; Miltenyi Biotec) were used as feeder cells in this study. Cells were harvested after 7 days in culture and were tested for CFSE dilution using a FACs analyzer (BD Biosciences).

Anti-IL-10R Ab treatment
Six groups (group 1, CFA controls; group 2, mTg controls; group 3, GM-CSF/isotype controls; group 4, GM-CSF/anti-IL-10R no. 1; group 5, GM-CSF/anti-IL-10R no. 2; and group 6, GM-CSF/anti-IL-10R no. 3) were included in this set of experiments, with four or five mice per group. Groups 1–3 correspond with those mentioned above, except that animals in group 3 received i.p. injections of rat IgG isotype control Ab (0.5 mg/mouse) on days 6, 11, 20, 25, and 32. Animals in groups 4–6 were treated with GM-CSF and immunized with mTg as described above and received i.p. injections of anti-IL-10R (0.5 mg/mouse) on days 6, 11, 20, 25, and 32; days 6 and 20; and days 11, 25, and 32, respectively. All animals were killed on day 45, and lymph nodes, spleen, thyroids, and sera were collected to evaluate thyroiditis.

Effects of GM-CSF treatment on thyroid microenvironment
CFA control, mTg control, and GM-CSF/mTg mice were used in this study. Three mice from each group were killed on day 21. Thyroids were collected, pooled within groups, and digested with collagenase D (0.5 mg/ml) for 1 h at 37°C to prepare single cell suspensions. Cells were washed with PBS supplemented with 2% FBS and blocked with anti-CD16/CD32 Fc-Block (BD Pharmingen) on ice for 30 min. Cells were stained with FITC-conjugated anti-mouse CD4 along with PE-conjugated anti-mouse CD25 mAbs on ice for 15 min, washed, and analyzed using a FACs analyzer (BD Biosciences) and CellQuest software. At least 10,000 cells/sample were analyzed. To determine cytokine/chemokine production, thy- road cell suspensions were maintained in RPMI 1640 medium containing 2% normal mouse serum for 36 h. Cell-free supernatants were collected from these cultures, and spontaneous cytokine (IL-4, IL-10, and IFN-γ) and chemokine (MCP-1 and RANTES) productions were detected by a multiplex cytokine/chemokine assay kit using Luminex technology at the Luminex core facility of Pitié-Salpetrière Hospital (University of Delaware). The suggested lowest detection levels using this kit are 5 pg/ml for IL-4, 1 pg/ml for IFN-γ, 15 pg/ml for IL-10, 5 pg/ml for MCP-1, and 5 pg/ml for RANTES.

To assess apoptotic molecule expression on thyrocytes, thyrocytes were separated from other resident cells, and mRNA was isolated using an mRNA isolation kit, following the manufacturer’s instructions (Macherey-Nagel). RT-PCR was conducted using mRNA and gene-specific primers for Fas, Fas ligand (Fasl), and caspase-8 (Maxim Biotec). β-Actin was used as a control to ensure equivalent amounts of RNA in the assay.
mTg-specific T cell proliferation

Mouse splenocytes or lymph node cells (5 × 10^6 cells/well) were plated in 96-well, flat-bottom tissue culture plates in triplicate in RPMI 1640 containing 2% normal mouse serum at a final volume of 0.25 ml/well. The mTg was added at a concentration of 20 μg/ml. Con A (1 μg/ml) was used as a positive control. Cells were incubated for 72 h at 37°C in a CO₂ incubator. Cells were pulsed with 1 μCi of [³H]thymidine/well for the last 16 h of culture, transferred into 96-well, U-bottom tissue culture plates, washed twice with PBS, lysed in water, and dried overnight at 37°C. Scintillation fluid was added to these wells (50 μl/well) and counted using a 96-well plate (Microbeta counter; PerkinElmer Wallac). To evaluate and to test the T cell pattern of proliferation, cells were stained with CFSE as described above, plated in 96-well, flat-bottom tissue culture plates in the presence or the absence of mTg (20 μg/ml) in RPMI 1640 containing 2% normal mouse serum at a final volume of 0.25 ml/well, maintained for 7 days, harvested, and tested for CFSE dilution using FACS (BD Biosciences).

Measurement of cytokine production

Spleen or lymph node cells (5 × 10^6 cells/well; 12-well plate) were incubated in the presence or the absence of mTg (20 μg/ml) in 1.5 ml of RPMI 1640 medium supplemented with 2% normal mouse serum for 36 h. Cell-free culture supernatants were collected after 36 h by centrifugation. Cytokine levels in cell-free supernatants were assayed by ELISA, using paired Abs for detection of IL-2, IL-10, IL-4, and IFN-γ, following the manufacturer’s instructions (eBioscience), and the OD₅₅₀ was recorded using a Microplate reader (Bio-Rad). The amount of cytokine was determined using corresponding cytokine standards. The suggested lowest detection levels using this kit are 2 pg/ml for IL-2, 4 pg/ml for IL-4, 15 pg/ml for IFN-γ, and 15 pg/ml for IL-10.

Evaluation of EAT

Thyroids collected from mice at the time of death were fixed in formalin, embedded in paraffin, sectioned, and stained with H&E. Thyroids were scored for the extent of thyroid lymphocytic infiltration, as a marker of disease severity, using a scale of 1 + to 5 +. An infiltrate of at least 125 cells in one or several foci was scored 1 +, 10–20 foci of cellular infiltration involving up to 25% of the gland was scored 2 +, an infiltration involving up to 25–50% of the gland was scored 3 +, destruction of >50% of the gland was scored 4 +, and near-complete destruction of the gland, with few or no remaining follicles, was scored 5 +.

**FIGURE 1.** Effects of GM-CSF treatment on DC maturation. CBA/J mice were left untreated or were treated with GM-CSF for 5 consecutive days starting on days 1 and 15 as described in Materials and Methods. In addition, mice were immunized with mTg emulsified in CFA on days 6 and 20. Mice were killed before (days 6 and 20) and after (days 8 and 22) first and second mTg immunizations to obtain spleens. A, Splenocytes isolated from mice killed on day 8 were stained with FITC-anti-CD11c and with either PE-anti-MHC class II or PE-anti-CD8α and analyzed by FACS. B, The same cells were stained with FITC-anti-CD11c and with PE-anti-B7.1, PE-anti-B7.2, or PE-anti-CD40 and analyzed by FACS. Black lines indicate the isotype control, dark gray lines indicate the mTg control, and light gray lines indicate GM-CSF-treated mice. C, DCs were isolated from spleens before or after mTg immunization, using magnetic column separation. mRNA was isolated and used in a multiplex RT-PCR assay to detect cytokine transcripts. The results shown are representative of two independent experiments using two mice per group.

**Statistical analysis**

Mean, SD, and statistical significance were calculated using an SPSS application. Statistical significance was determined using the nonparametric Wilcoxon signed test. In most cases, values of individual treated and immunized groups were compared with those of untreated but immunized groups. A value of p ≤ 0.05 was considered significant.

**Results**

**GM-CSF-induced DCs maintain semimature phenotype**

To determine the effects of GM-CSF treatment on the maturation of DCs, we analyzed the expression of MHC class II and costimulatory molecules as well as the production of proinflammatory cytokines from DCs isolated from GM-CSF-treated and untreated mice before and after mTg immunization. Spleens from mice treated with GM-CSF showed increased numbers of CD11c+ cells (7.51%) compared with untreated controls (3.61%; Fig. 1A). Despite an increase in the number of DCs, expression levels of MHC class II, B7.1, B7.2, and CD40 were comparable in GM-CSF-treated and untreated mice after immunization with mTg (Fig. 1B). However, levels of proinflammatory cytokines, such as TNF-α, IL-12, and IL-1β, evaluated by RT-PCR, were significantly higher in DCs from untreated, mTg-immunized mice than in DCs from GM-CSF-treated, mTg-immunized mice (Fig. 1C). These data suggest that DCs from GM-CSF-treated, but not untreated, mice maintain a semimature status after mTg immunization.

**CD4+CD25+ T cells from GM-CSF-treated mice suppress anti-mTg response in vivo**

To determine whether CD4+CD25+ T cells from GM-CSF-treated mice can suppress mTg-specific autoimmune responses in vivo, purified CD4+CD25+ T cells from GM-CSF-treated and mTg-primed mice were adoptively transferred to untreated mice that were primed with mTg. As shown in Fig. 2A, mice receiving...
IL-10 produced by CD4<sup>+</sup>CD25<sup>+</sup> T cells is required to suppress mTg-specific proliferation.

**Treatment with anti-IL-10R mAb abolishes GM-CSF-induced suppression of EAT**

Next, we investigated the role of IL-10 in GM-CSF-induced suppression of EAT. The effects of IL-10 were blocked by the administration of saturating concentrations of anti-IL-10R mAb to GM-CSF-treated mice at various times during disease induction. Regardless of the time of administration, almost all animals that received anti-IL-10R mAb, with the exception of some mice treated with anti-IL-10R mAb immediately after GM-CSF treatment (i.e., GM-CSF/anti-IL-10R no. 2), showed increased mTg-specific proliferation compared with mice that received GM-CSF and isotype control mAb. A significant increase in proliferation was seen in mice that received anti-IL-10R mAb 5 days after GM-CSF treatment (i.e., GM-CSF/anti-IL-10R no. 3) or throughout the course of the disease (i.e., GM-CSF/anti-IL-10R no. 1; p = 0.001 and p = 0.005, respectively; Fig. 4A). Interestingly, we observed an increase in the frequency of CD4<sup>+</sup>CD25<sup>+</sup> T cells in all GM-CSF-treated mice regardless of the time of administration of anti-IL-10R mAb (Fig. 4B), suggesting that blocking IL-10 had no effect on the expansion of these cells by GM-CSF-induced DCs.

As shown in Fig. 4C and Table I, thyroids from anti-IL-10R mAb groups of mice, with the exception of some mice treated with anti-IL-10R mAb immediately after GM-CSF treatment (i.e., GM-CSF/anti-IL-10R no. 2), showed more severe lymphocytic infiltration compared with thyroids from GM-CSF/isotype control mice. Taken together, these results indicated that IL-10 is the primary mediator of GM-CSF-induced disease suppression.

**Effect of GM-CSF treatment on thyroid microenvironment**

To test the effects of GM-CSF on the target organ, we investigated the cell type and cytokine production in the thyroids of treated mice. GM-CSF treatment resulted in the expansion of CD8α<sup>+</sup> DCs in the periphery (Fig. 1A); however, this expansion was not reflected within the thyroid (data not shown). In contrast, there was an increase in the percentage of CD4<sup>+</sup>CD25<sup>+</sup> T cells in the thyroids of GM-CSF-treated mice relative to untreated mice (24.57 and 20.06%, respectively; Fig. 5A). Previous studies had shown that MCP-1 preferentially attracts CD4<sup>+</sup>CD25<sup>+</sup> T cells to the thyroid, whereas RANTES preferentially attracts CD4<sup>+</sup> effector T cells (34). Therefore, we tested for the levels of these two chemokines. MCP-1 production was comparable among all experimental groups, whereas RANTES was undetectable (data not shown), suggesting that these chemokines could not account for the observed increase in CD4<sup>+</sup>CD25<sup>+</sup> T cell frequency in GM-CSF-treated thyroids.

Next, we quantified cytokine production by thyrocyte and thyroid-resident lymphocytes. Although a slight increase in IL-10 production with a very small decrease in IFN-γ production were observed in GM-CSF-treated mice compared with mTg control mice (Fig. 5B), these differences were not significant.

Several studies have suggested that thyrocyte destruction in HT is due to Fas-mediated apoptosis through increased caspase expression. Therefore, we tested for the expression levels of Fas, FasL, and caspase 8 on thyrocytes by RT-PCR. Although we observed a slight increase in Fas expression in GM-CSF-treated mice compared with CFA and mTg control mice, there was no detectable FasL expression in any of the groups. Furthermore, there was no substantial difference in the expression levels of caspase 8 among the groups of mice (Fig. 5C).
CD4+ T cells from untreated mice were purified from pooled spleen and lymph node cells using the magnetic separation method. Effector T cells were stained with CD4. Mice were killed on day 35 to obtain lymph node and spleen cells. CD4 and Methods

Materials
GM-CSF for 5 consecutive days starting on days 1 and 15 and were immunized with mTg emulsified in CFA on days 6 and 20 as described in Materials and Methods. Mice were killed on day 35 to obtain lymph node and spleen cells. CD4 and Methods

GM-CSF can expand DCs and maintain them in a semimatured status in vivo, promote expansion of CD4+CD25+ T cells, and induce higher levels of IL-10 production required for EAT suppression. These results further extend our earlier studies (24) in which we showed that GM-CSF treatment can expand CD8a− DCs and CD4+CD25+ Treg and suppress EAT.

Although DC function is traditionally associated with the induction of primary T cell responses, there is increasing evidence that they play a critical role in peripheral tolerance (10–14). DCs pass through several stages of maturation (10), and earlier studies have shown that semimatured DCs play a critical role in the induction and expansion of Treg (10, 18–23). Because GM-CSF treatment led to an increase in the frequency of CD4+CD25+ T cells with regulatory properties (24), we asked whether GM-CSF exerted its effects by affecting DC maturation. We found that DCs from GM-CSF-treated mice displayed a semimature phenotype, as indicated by high levels of expression of MHC class II and B7 molecules, but low levels of expression of proinflammatory cytokines compared with untreated mTg control mice. This suggested that GM-CSF treatment most likely induced and/or promoted tolerance through the expansion of semimature DCs, which are known to aid in the generation of Treg (10, 18–23).

In fact, an earlier study showed that DCs generated by culturing bone marrow precursor cells in low concentrations of GM-CSF are maturation resistant, and inoculation of these DCs pulsed with allopeptides could prolong allograft survival in vivo (35). Generation of tolerogenic DCs capable of preventing autoimmune diseases and allograft rejection have been reported extensively (10–14, 36, 37). One of the major properties of such DCs is their ability to induce generation of IL-10-producing type 1 Treg (Tr1) that do not express significant levels of CD25 unless they are activated (38, 39). However, other studies have clearly shown that immature and other tolerogenic DCs can help expand IL-10-producing CD4+CD25+ Treg (36, 37, 40), which may play an important role in the induction and differentiation of Tr1 cells (28, 41, 42).

Although several types of Treg have been described, each with a specific surface phenotype and a cytokine profile, naturally occurring CD4+CD25+ Treg, which constitute 5–10% of peripheral CD4+ T cells, are the predominant suppressors of autoreactive T cells that escape central tolerance (43–45). Previously we (24) demonstrated that CD4+CD25+ T cells from GM-CSF-treated mice could suppress the mTg-specific proliferative response of effector T cells in vitro. However, CD4+CD25+ T cells from untreated, but mTg-primed, mice failed to show similar suppression of mTg-specific responses. More interestingly, depletion of CD4+CD25+ T cells from in vitro cultures of lymphocytes from GM-CSF-treated mice restored mTg-specific proliferation (24). This showed that effector T cells were generated in GM-CSF-treated mice as they were in untreated, mTg-primed mice, but their function was suppressed by CD4+CD25+ T cells that were induced/expanded in GM-CSF-treated mice. In this study, adoptive transfer of CD4+CD25+ T cells from GM-CSF-treated mice into mTg-primed mice resulted in a significant suppression of mTg-specific proliferation compared with mTg-primed nonrecipients. Although we cannot rule out the possibility that the transferred CD4+CD25+ T cell population contained some activated effector T cells, the suppressive property observed suggested that the population was primarily composed of CD4+CD25+ Treg. Furthermore, lymphocytes from recipient mice, upon in vitro stimulation

**FIGURE 3.** Role of IL-10 in CD4+CD25+ T cell-induced suppression of mTg-specific T cell proliferation. CBA/J mice were treated with or without GM-CSF for 5 consecutive days starting on days 1 and 15 and were immunized with mTg emulsified in CFA on days 6 and 20 as described in Materials and Methods. Mice were killed on day 35 to obtain lymph node and spleen cells. CD4+CD25+ T cells from GM-CSF-treated mice and T cells (effector cells) from untreated mice were purified from pooled spleen and lymph node cells using the magnetic separation method. Effector T cells were stained with CFSE, cocultured with isolated CD4+CD25+ cells (5:1 effector:Treg ratio), and stimulated with mTg in the presence of saturating concentrations of anti-IL-10R or isotype control mAb. Either T cell-depleted spleen cells (A) or enriched DCs (B) from naive mice were used as APCs. The proliferative response to mTg was assessed by CFSE dilution, as determined by FACS on day 7. Histograms shown are gated on the CD4 T cell population. Results shown are representative of two independent experiments conducted in triplicate. * Statistically significant difference compared with the isotype control mice shown in the middle panel.
FIGURE 4. In vivo effects of anti-IL-10R Ab on GM-CSF-induced suppression of EAT. GM-CSF-treated mice were treated with anti-IL-10R mAb as described in Materials and Methods and killed on day 45 along with control mice to obtain lymph nodes and spleen cells. A, Splenocytes were stained with CFSE and stimulated with mTg for 7 days. The proliferative response to mTg was assessed by CFSE dilution, as determined by FACS. Histograms shown are gated on CD4+/H11001 T cell population. B, Splenocytes were stained with FITC-labeled anti-mouse CD4 and PE-labeled anti-mouse CD25 Abs and analyzed using FACS. Ranges in parentheses shown in A and B correspond to values for four or five individual mice. C, Representative photomicrographs of H&E-stained thyroid sections for different groups (described in Table I) are shown. (original, ×40). Numbers shown in parentheses depict corresponding thyroiditis cellular infiltration index.

with mTg, produced higher levels of IL-10 and IL-4 than mTg-primed controls. This indicated that adoptively transferred CD4+CD25+ T cells exerted suppressive effects on recipient effector T cells, as seen in GM-CSF-treated donor mice.

To explore the mechanism of suppression of mTg-specific responses by GM-CSF-induced CD4+CD25+ T cells, we conducted additional studies. Because in an earlier study we had ruled out a critical role for IL-4 in EAT suppression (24), and IL-10 is a critical mediator of Treg-induced suppression of effector T cell function (28–33), we tested the role of IL-10 in both the expansion and the function of CD4+CD25+ T cells in GM-CSF-treated mice. Blockade of IL-10 function in vivo using anti-IL-10R Ab reversed the suppressive effects of CD4+CD25+ T cells from GM-CSF-treated mice on mTg-specific T cell responses in vitro and suggested a critical role for this cytokine in GM-CSF-induced suppression of EAT. Furthermore, we showed that blockade of IL-10 function in vivo completely abolished the disease-suppressive effects of GM-CSF and allowed development of EAT. Initiation of treatment with anti-IL-10R Ab at different time points during disease development allowed us to address two major questions; namely, whether IL-10 is required for the induction and/or expansion of CD4+CD25+ T cells in vivo, and whether it is required for merely suppressing autoreactive effector T cell function, resulting in consequent suppression of EAT. Our results showed that regardless of the time of treatment, blocking IL-10 abolished the EAT-suppressive capacity in a majority of mice. Interestingly, the number of CD4+CD25+ T cells was higher in all GM-CSF-treated mice, compared with untreated mice regardless of anti-IL-10R Ab treatment. Consistent with previous reports (46, 47), our results showed that IL-10 is not essential for the expansion of CD4+CD25+ T cells. However, IL-10 produced by these Treg is critical for the suppression of effector T cells.

IL-10 is a key regulator of inflammation, and it can inhibit both Th1- and Th2-type immune responses through the suppression of proinflammatory cytokines and T cell proliferative responses (48). One of the major mechanisms of IL-10-mediated suppression of T cells is through selective inhibition of the CD28 costimulatory pathway (46). However, in thyroiditis, alternative mechanisms of action of IL-10 have been proposed (7, 9, 49–52). Injection of cDNA expression vectors encoding IL-10 into the thyroid can significantly inhibit lymphocyte infiltration and development of EAT and prevent progression of the disease (50). This suppressive effect of IL-10 is mediated either through enhancement of FasL expression on thyrocytes and induction of activation-induced cell death of thyroid-infiltrating T lymphocytes (51) or through potent up-regulation of antiapoptotic molecules, such as cellular FLIP and

### Table 1. Effect of anti-IL-10R Ab on GM-CSF-induced EAT suppression

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*a Mice were treated with GM-CSF, immunized with mTg, and further treated with anti-IL-10R mAb or isotype control Ab as described in Materials and Methods. These mice were sacrificed along with control mice on day 45, thyroids were collected from mice, at the time of sacrifice, were fixed in formalin, embedded in paraffin, and sectioned for histological H&E staining. Thyroiditis cellular infiltration index was determined as described in Materials and Methods. Values of p were calculated by comparing anti-IL-10R mAb-treated mice with isotype control mice.
IL-10-PRODUCING CD4+CD25+ CELLS IN PREVENTING EAT

primarily due to the direct effects of IL-10 on mTg-specific effector T cells. Studies using SCID and TCR transgenic mice are underway, and they should help elucidate the direct effects of GM-CSF on DCs and/or T cells.

In summary, it is likely that GM-CSF induced the expansion of semimatured DCs, and Tg peptide presentation by these DCs led to the expansion of CD4+CD25+ Treg. IL-10 produced by these Treg inhibited the autoimmune effector functions of mTg-specific T cells with consequent suppression of EAT. These results show the therapeutic potential of GM-CSF in EAT and other autoimmune diseases with pathogenesis similar to that of EAT.

Disclosures
The authors have no financial conflict of interest.

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Bcl-xL, which can prevent CD95-induced apoptosis of thyrocytes (7, 9). Conversely, direct injection of IL-1 and TNF-α into the thyroids of mTg-primed mice induced thyrocyte apoptosis, indicating that proinflammatory cytokines play a critical role in thyroid destruction (6). This raised the possibility that IL-10 might be mediating its effects through suppression of proinflammatory cytokine production.

To determine whether the increased IL-10 response in GM-CSF-treated mice had any effect on the thyroid microenvironment, we tested the levels of expression of various proapoptotic molecules in the thyroids of GM-CSF-treated mice. Although there was an increase in the frequency of CD4+CD25+ T cells and IL-10 production in the thyroids of GM-CSF-treated mice, there was no significant difference in the expression of proapoptotic molecules between the thyroids of GM-CSF-treated and untreated mTg control mice. In this context it is interesting to note that our results to date clearly show that GM-CSF-mediated suppression of EAT is

FIGURE 5. Effects of GM-CSF treatment on thyroid microenvironment. CFA control, mTg control, and GM-CSF/mTg groups of mice (described in Materials and Methods) were used for this study. Three mice were killed on day 21 to obtain thyroids. A, Lymphocytes obtained from thyroids were stained with FITC-labeled anti-mouse CD4 and PE-labeled anti-mouse CD25 Abs and analyzed by FACS. The results shown are representative of three independent experiments with three mice per group. B, Total cells obtained from collagenase-treated thyroids were maintained in anti-mouse CD25 Abs and analyzed by FACS. The results shown are representative of two independent experiments. The mean values of 100 data points are shown. C, Thyrocytes were isolated from thyroid single-cell suspensions, mRNA was obtained and subjected to RT-PCR to detect Fas, FasL, and caspase 8 using a specific set of primers. β-Actin was included as an internal control. The control lane represents RT-PCR using positive control mRNA and specific primers.


