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Granulocyte-Macrophage Colony-Stimulating Factor Prevents Diabetes Development in NOD Mice by Inducing Tolerogenic Dendritic Cells that Sustain the Suppressive Function of CD4^+CD25^+ Regulatory T Cells

Simon Gaudreau, Chantal Guindi,^2 Michaël Ménard,^2 Gilles Besin, Gilles Dupuis, and Abdelaziz Amrani^3

Autoimmune diabetes results from a breakdown of self-tolerance that leads to T cell-mediated β-cell destruction. Abnormal maturation and other defects of dendritic cells (DCs) have been associated with the development of diabetes. Evidence is accumulating that self-tolerance can be restored and maintained by semimature DCs induced by GM-CSF. We have investigated whether GM-CSF is a valuable strategy to induce semimature DCs, thereby restoring and sustaining tolerance in NOD mice. We found that treatment of prediabetic NOD mice with GM-CSF provided protection against diabetes. The protection was associated with a marked increase in the number of tolerogenic immature splenic DCs and in the number of Foxp3^+CD4^+CD25^+ regulatory T cells (Tregs). Activated DCs from GM-CSF-protected mice expressed lower levels of MHC class II and CD80/CD86 molecules, produced more IL-10 and were less effective in stimulating diabetogenic CD8^+ T cells than DCs of PBS-treated NOD mice. Adoptive transfer experiments showed that splenocytes of GM-CSF-protected mice did not transfer diabetes into NOD.SCID recipients. Depletion of CD11c^+ DCs before transfer released diabetogenic T cells from the suppressive effect of CD4^+CD25^+ Tregs, thereby promoting the development of diabetes. These results indicated that semimature DCs were required for the sustained suppressive function of CD4^+CD25^+ Tregs that were responsible for maintaining tolerance of diabetogenic T cells in NOD mice. The Journal of Immunology, 2007, 179: 3638–3647.

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^4 Abbreviations used in this paper: DC, dendritic cell; iDC, immature DC; Treg, regulatory T cell; PLN, pancreatic lymph node.

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Autoimmune diabetes results from a breakdown of self-tolerance culminating in T cell-mediated beta-cell destruction (14, 15). Numerous studies have attributed the breakdown of tolerance in NOD mice to defects in the number and/or function of DCs (16–19) and CD4⁺CD25⁺ Tregs (20, 21). The defects include an impaired development of DCs from myeloid progenitors (bone marrow iDCs) and abnormal levels of expression of costimulatory molecules under proinflammatory conditions (16, 17). These combined alterations lead to an increased capacity of DCs to stimulate CD4⁺ and CD8⁺ T cells, thereby promoting Th1 or Th1 differentiation in vitro (18, 19, 22, 23). Consequently, the level of iDCs that induce and maintain peripheral T cell tolerance is not sufficient to trigger a sustained immunoregulatory T cell response in NOD mice. With respect to Tregs, it has been shown that the pool of CD4⁺CD25⁺ Tregs decreases with progression of diabetes (21, 24) and that these cells can afford protection against the development of diabetes (25, 26).

In the present study, we have investigated whether reestablishing a sufficient number of DCs possessing tolerogenic functions would maintain CD4⁺CD25⁺ Treg number and function, thereby providing protection against the development of diabetes in NOD mice. Our data showed that treatment of NOD mice with GM-CSF increased the number of iDCs acquiring a semimature phenotype and that these iDCs had less capability to activate diabetogenic CD8⁺ T cells. We further showed that these tolerogenic DCs did not undergo complete maturation and were required to maintain permanent diabetes protection through CD4⁺CD25⁺ Tregs.

Materials and Methods

Mice and treatment

Female NOD and NOD.SCID mice were purchased from the The Jackson Laboratory. The 8.3-NOD mice, obtained from Dr. P. Santamaria (University of Calgary, Calgary, Alberta, Canada), have been described (27). In vivo treatments of 3-week-old female NOD mice were done by i.p. injection of 100 ng/mouse of recombinant murine GM-CSF (R&D Systems) or PBS. Mice were injected three times per week for the first 3 wk, followed by two injections per week up to 52 wk of age. All the mice were housed and bred in our animal facilities under specific pathogen-free conditions and were used according to guidelines of the Institutional Animal Care Committee of the University of Sherbrooke (Sherbrooke, Quebec, Canada).

Monitoring of diabetes

Diabetes was monitored by a urine glucose test using Uristix (Bayer) and confirmed by measuring hyperglycemia with an Advantage Accu-Check glucometer (Roche Diagnostics). The animals were considered diabetic confirmed by measuring hyperglycemia with an Advantage Accu-Check (Roche) glucometer (Roche Diagnostics). The animals were considered diabetic confirmed by measuring hyperglycemia with an Advantage Accu-Check (Roche) glucometer.

Cell lines and Abs

Anti-CD8α-PE (clone 53-6.7), anti-CD4-FTC/biotin (clone GK1.5), anti-CD25-FTC (clone 7D4), anti-CD11b-FTC (clone M1/70), anti-CD45R/biotin (clone 10-3.6), anti-CD25-FITC (clone 7D4), anti-CD11b-FITC (clone M1/70), anti CD45R/biotin (clone 10-3.6), Abs and streptavidin-PerCP (used for all biotin-conjugated Abs) were from BD Biosciences. The anti-Foxp3-FTC (FJK-16s) and anti-rat IgG2a (eBR2a) Abs were from eBioscience. The biotin (clone 10-3.6) Abs and streptavidin-PerCP (used for all biotin-conjugated Abs) were from BD Biosciences. The anti-Foxp3-FTC (FJK-16s) and anti-rat IgG2a (eBR2a) Abs were from eBioscience. The 145-2C11 hybridoma (anti-CD3) was obtained from Dr. P. Santamaria (FJK-16s) and anti-rat IgG2a (eBR2a) Abs were from eBioscience. The biotin (clone 10-3.6) Abs and streptavidin-PerCP (used for all biotin-conjugated Abs) were from BD Biosciences. The anti-Foxp3-FTC (FJK-16s) and anti-rat IgG2a (eBR2a) Abs were from eBioscience. The 145-2C11 hybridoma (anti-CD3) was obtained from Dr. P. Santamaria (FJK-16s) and anti-rat IgG2a (eBR2a) Abs were from eBioscience.

Histopathology

Pancreata were fixed in formalin, embedded in paraffin, sectioned, and stained with H&E. Islet insulitis was scored as follows: 0, healthy islet; 1, peri-insulitis; 2, leukocytic infiltration of up to 25% of islet mass; 3, leukocytic infiltration of up to 75% of the islet mass; and 4, >20% of islet mass remaining.

Peptides

The NRP-A7 and TUM peptides were from C. Servis (Biochemistry Institute, Lausanne University, Lausanne, Switzerland). They were synthesized using fMOC chemistry, purified by reverse phase HPLC to 90% purity as confirmed by MALDI-TOF mass spectrometry.

T cell and splenic DC isolation

Cell (CD4⁺ and CD8⁺ T cells and DCs) purification was done using Ab-coated magnetic beads from Miltenyi Biotec. Briefly, DCs were purified from collagenase D-digested spleens (two or three organs) using anti-CD11c-coated beads (Miltenyi Biotec). In the case of CD8α⁺ and CD8α⁺ DC purification, spleen cells were enriched for CD11c⁺ cells by depletion of T, B, and NK cells using a mixture of Abs (Miltenyi Biotec), incubated with anti-CD8α microbeads, and sorted by MACS into CD8α⁻ and CD8α⁺CD11c⁺ fractions (Miltenyi Biotec). Purity was >85% for CD11c⁺, CD8α⁺, and CD8α⁻ cells as determined by FACS analysis. In the case of CD4⁺, CD4⁺CD25⁺, CD4⁺CD25⁻, and CD8⁺ T cell enrichment, splenocytes were freed of RBC by hemolysis and the respective T cells were isolated by magnetic beads separation using an anti-CD4⁺CD25⁻ isolation kit for CD4⁺ T cell purification or with anti-CD8α-coated magnetic beads for CD8⁺ T cell purification, according to the manufacturer’s protocol (Miltenyi Biotec).

Bone marrow-derived DCs

Bone marrow-derived DCs from NOD mice were generated by culturing bone marrow cells in the presence of recombinant murine GM-CSF and IL-4 (5 ng/ml) (28).

Flow cytometric analysis

All analyses were performed using a FACSCalibur flow cytometer using the CellQuest Pro software (BD Biosciences). In brief, cells were washed with PBS containing 0.05% sodium azide and 2.5% heat-inactivated FBS (FACS buffer). The cells were then labeled in the same buffer for 30 min at 4°C using the various Abs described in the appropriate experiments, washed, and analyzed by flow cytometry. Foxp3 expression was determined as follows. The cells were stained with a combination of anti-CD4 and anti-CD25 mAb before fixation with 4% paraformaldehyde for 1 h at 4°C. After treatment with FACS buffer containing 0.1% saponin (for intracellular staining), the cells were stained with FITC-labeled anti-Foxp3 mAb or anti-rat IgG2a for 30 min at 4°C, washed, and analyzed by FACS.

Proliferation assays

Assays of 8.3-CD8⁺ T cell activation by DCs were performed as follows. Naïve splenic 8.3-CD8⁺ T cells (2 × 10⁵ lymphocytes/well) were incubated with NRP-A7- or TUM-pulsed (1 μg/ml), irradiated splenic DCs (2 × 10⁶ cells/well), from GM-CSF- or PBS-treated mice, in 96-well plates for 3 days at 37°C. The supernatants were collected after 48 h and assayed for cytokine content by ELISA using commercially available kits (R&D Systems). During the last 18 h, cultures were pulsed with 1 μCi of [³H]thymidine. The regulatory activity of CD4⁺CD25⁺ Tregs purified from GM-CSF- or PBS-treated NOD mice was measured by adding preactivated (2 days with anti-CD3 (5 μg/ml) and IL-2 (10 U/ml) and IL-12 (10 U/ml) and IL-4 (5 μg/ml) and IL-12 (10 U/ml) and IL-4 (5 μg/ml)) CD4⁺CD25⁺ T cells (2 × 10⁶ to 4 × 10⁵ lymphocytes/well) to 8.3-CD8⁺ T cells (2 × 10⁶ lymphocytes/well) and NRP-A7-pulsed (1 μg/ml) irradiated APC (1 × 10⁶ cells/well) cocultures.

Cytokine assays

Measurement of cytokines released in the culture medium by the different T cell populations was done by ELISA (R&D systems) following activation of the cells using anti-CD3-coated 96-well plates (5 μg/ml) with or without IL-2 (10 U/ml, Roche) for 2 days at 37°C in a humidified 5% CO₂ incubator.

RT-PCR

CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells of GM-CSF- and PBS-treated NOD mice were purified using an anti-CD4⁺CD25⁻ magnetic beads isolation kit (Miltenyi Biotec). RNA was isolated from the sorted cells using TRIzol reagent (Invitrogen Life Technologies). cDNA was generated using a first-strand DNA synthesis kit (Invitrogen Life Technologies) and amplified by PCR. The sequences of the primers used were as follows: Foxp3 5'-CAG CTG CCT ACA GTG CCC CTA G-3' and 5'-CAT TTG CCA GCA GTG GAT AG-3'; and HPRT, 5'-GT TGG ATA CAC GGC AGA CTT TGT TG-3' and 5'-GAA GGG TAG GTC GCC CTA TAG GCT-3' (29). All samples were subjected to electrophoresis using a 2% agarose gel.

Adaptive cell transfers

Whole spleen cells or DC-depleted (CD11c⁻/-depleted) spleen cells from GM-CSF- or PBS-treated diabetic mice were injected into the tail vein of
Materials and Methods

PBS (n/H11005 days after cell transfer. mice were monitored for diabetes by measuring urine glucose, starting 20 obtained from a diabetic NOD mice to NOD. SCID only mild inflammation of islets at 32 wk of age (Fig. 2 sive insulitis (Fig. 2, displayed typical inflammation, with both peri-insulitis and inva- mice exhibited little or no insulitis, whereas control (PBS) mice creas showed that, at up to 16 wk of age, GM-CSF-treated NOD development of insulitis. Results of histopathology of pan- diabetes resistance induced by GM-CSF was associated with re- test). All GM-CSF-protected mice remained normoglycemic with 21% of GM-CSF-treated mice be- treatment with GM-CSF significantly decreased the progression of results were consistent with the interpretation that GM-CSF-treat- FIGURE 1. GM-CSF treatment significantly reduced the incidence of diabetes in NOD mice. NOD mice were treated with GM-CSF (n = 27) or PBS (n = 24) and monitored for diabetes development as described in Materials and Methods.

4- to 6-wk-old NOD.Scid hosts (8 x 10⁶ cells). In some experiments, CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells (0.5 x 10⁶ cells) from GM-CSF- or PBS-treated NOD mice were cocultured with splenocytes (8 x 10⁶ cells) obtained from a diabetic NOD mice to NOD.Scid recipients. Recipient mice were monitored for diabetes by measuring urine glucose, starting 20 days after cell transfer.

Statistical analyses

Student’s t test and χ² test were used to determine the statistical significance of differences between groups, which was set at the 95% confidence level. Data reported are representative of a minimum of three independent experiments. Results are shown as the mean ± SEM represented by the error bars.

Results

In vivo treatment of NOD mice with GM-CSF reduces insulitis and protects against diabetes

The effect of GM-CSF on diabetes development was investigated in 3-wk-old prediabetic NOD mice. The mice were treated for 49 wk with GM-CSF (100 ng/mouse) or PBS (control), and the development of diabetes and insulitis was monitored over time. Results showed that the treatment with GM-CSF significantly decreased the progression of the disease (Fig. 1). At 52 wk of age, 21% of GM-CSF-treated mice developed diabetes (n = 27), whereas 86% of PBS-treated mice be- FIGURE 2. Islet inflammation is dramatically reduced in GM-CSF-treated NOD mice. A, Pancreata from GM-CSF- or PBS-treated animals were scored for insulitis as described in Materials and Methods. Data represent the average ± SEM. B, Evolution of islets inflammation in GM-CSF- or PBS-treated NOD mice with time. A minimum of 30 islets was scored for each group of six animals (p < 0.0001). C, Representative microphotographs of H&E-stained pancreatic sections from GM-CSF- or PBS-treated NOD mice.

GM-CSF-treatment expands DCs with low Ag-presenting capacity

To examine whether GM-CSF treatment stimulated the expansion of DCs in NOD mice, we analyzed splenocytes for the presence of cells expressing high levels of the CD11c⁺ marker. Results showed a marked increase in the number of cells expressing CD11c⁺ (2.27 ± 0.26 x 10⁶ cells, n = 12) in the case of DCs from GM-CSF-treated mice in comparison with CD11c⁺ DCs (1.36 ± 0.17 x 10⁶, n = 11) from PBS-treated mice (Fig. 3A). Furthermore, there was a consistent and significant (p < 0.02) higher percentage of purified CD11c⁺ DCs per spleen in the case of GM-CSF-treated mice (2.46 ± 0.27%, n = 14) as compared with PBS control mice (1.63 ± 0.12%, n = 10) (Fig. 3B).

The phenotypes and functions of splenic DCs isolated from GM-CSF-treated NOD and control (PBS-treated) mice were determined in the next sets of experiments. Freshly isolated splenic DCs from groups of mice exhibited an immature phenotype characterized by low levels of expression of CD80 and CD86 and MHC class II molecules (data not shown). Although the mean fluorescence intensity for CD80 and CD86 on DCs from GM-CSF-treated mice appeared to be higher than on DCs from control PBS-treated mice, the differences did not reach statistical significance (data not shown). The DCs from both groups of mice also expressed comparable levels of MHC class II molecules (data not shown). Interestingly, NRP-A7-pulsed DCs from GM-CSF-treated mice were not as efficient as DCs from PBS-treated NOD mice to induce the proliferation of diabetogenic 8.3-CD8⁺ T (p < 0.006) (Fig. 3C). The lower response of 8.3-CD8⁺ T cells to NRP-A7 pulsed DCs from GM-CSF-treated mice was not due to the anergy of CD8⁺ T cells because activated 8.3-CD8⁺ T cells produced equal amounts of IL-2 and IFN-γ as compared with DCs from PBS-treated mice (Fig. 3D). The lower proliferative response of 8.3-CD8⁺ T cells was not accompanied by the production of IL-4 (Fig. 3D), IL-10, or TGF-β (data not shown).
Inhibition of full maturation and Ag-presenting function of DCs from GM-CSF-protected mice

We next examine whether the GM-CSF treatment altered splenic DCs maturation. Splenic DCs of GM-CSF- and PBS-treated mice were exposed to LPS for 48 h and their phenotype and function were analyzed. As expected, LPS-activated DCs from PBS-treated NOD mice expressed high levels of CD80, CD86, and MHC class II, indicating that these DCs had undergone a full process of maturation (Fig. 4A). In contrast, the levels of expression of MHC class II and CD80/CD86 molecules were lower in LPS-activated DCs from GM-CSF-treated mice (Fig. 4A). These observations indicated that DCs from GM-CSF-treated mice did not undergo a full maturation process in response to LPS treatment in contrast to DCs from PBS-treated mice. These results suggested that DCs from GM-CSF-treated mice had acquired a semimature phenotype, a property characteristic of tolerogenic DCs. In addition, the levels of IL-10, a cytokine known to be involved in the tolerogenic functions of DCs, were significantly increased in DCs from GM-CSF-treated mice as compared with PBS-treated mice (Fig. 4B). Splenic DCs of both groups of mice produced similarly low levels of IL-12p70 (Fig. 4C). Under similar conditions of assay, LPS-activated bone marrow-derived DCs of NOD mice produced large amounts of IL-12p70, whereas bone marrow-derived DCs generated from GM-CSF-treated mice produced much lower amounts of IL-12p70 (Fig. 4D). Interestingly, 8.3-CD8+ T cells showed a significantly lower (p < 0.01) proliferative response to stimulation with NRP-pulsed LPS-activated DCs from GM-CSF-treated mice when compared with the response from PBS-treated mice (Fig. 4E). The 8.3-CD8+ T cells stimulated with LPS-activated DCs of GM-CSF-treated mice produced significantly lower amounts of IL-2 and IFN-γ (p < 0.03 and p < 0.004, respectively) (Fig. 4F). Together, these results suggested that DCs from GM-CSF-treated, but not from PBS-treated NOD mice do not undergo full maturation process and are poor Ag-specific CD8+ T cell stimulator.

Splenic CD8α+ DCs from GM-CSF-treated mice display markers and function of tolerogenic cells

We have observed that the number of splenic DCs was significantly increased in GM-CSF-treated NOD mice (Fig. 3, A and B) and asked which DC subset was affected by GM-CSF. We have found that myeloid (CD11c+CD8α−) and lymphoid (CD11c+CD8α+) splenic DC numbers were increased in GM-CSF-treated NOD mice (Fig. 3A). To determine which of these DC subsets display tolerogenic function, splenic CD8α+ and CD8α− DCs from GM-CSF- or PBS-treated mice were purified and tested for their abilities to induce proliferation of 8.3-CD8+ T cells and their cytokines production. We found no significant difference (p > 0.05) in the proliferation of 8.3-CD8+ T cells in response to CD8α+ DCs from GM-CSF- or PBS-treated NOD mice (Fig. 5A). However, a marked decrease (p < 0.001) in the proliferation of 8.3-CD8+ T cells was observed in the case of CD8α− DCs of GM-CSF-treated NOD mice (Fig. 5B). The analysis of cytokines production indicated that there were no significant differences in the amounts of IL-2, IFN-γ, and IL-4 produced by 8.3-CD8+ T cells activated either with CD8α+ or CD8α− DCs from GM-CSF- or PBS-treated mice.
These results suggested that in GM-CSF-treated mice, myeloid CD8α−/H9251/CD8α−/H11001 DCs were endowed with a tolerogenic function by reducing proliferation of diabetogenic CD8α+/H11001 T cells without affecting their cytokine production.

**Phenotype and function of T cells in GM-CSF-treated NOD mice**

T cells from NOD mice were analyzed to determine whether diabetes resistance induced by GM-CSF was associated with alterations in their phenotype and function. The number of spleen cells was not increased in GM-CSF-treated NOD mice as compared with PBS-treated mice (data not shown). In addition, there were no differences in the number, percentage, or ratio of CD4+ and CD8α+ T cell populations in spleen, pancreatic lymph node (PLN), and mesenteric lymph node of GM-CSF- and PBS-treated mice (data not shown).

The possibility that the protective effect of GM-CSF was the result of immune deviation toward Th2 or Tc2 responses was investigated by comparing the proliferative response and cytokine production of splenic CD4+ and CD8α+ T cells in response to anti-CD3 stimulation. Results showed that CD8α+ T cells of GM-CSF- and PBS-treated mice displayed similar proliferative responses and produced the same amounts of IL-2 and IFN-γ (Fig. 6, A and B).

**FIGURE 5.** Low capacity of myeloid DCs from GM-CSF-treated NOD mice to activate diabetogenic 8.3-TCR transgenic CD8α+ T cells in vitro. A, Number of CD11c+CD8α− and CD11c+CD8α− DCs among purified splenic CD11c+ DCs from PBS- (n = 10) and GM-CSF-treated (n = 12) NOD mice as determined by FACS. B, Proliferation of naive 8.3-CD8α+ T cells (2 × 10⁴ lymphocytes/well) in response to peptide-pulsed (NRP-A7 or TUM, 1 μg/ml) irradiated CD11c+CD8α− and CD11c+CD8α− splenic DCs (2 × 10⁴ cells/well). C, Production of IL-2, IFN-γ, and IL-4 by naive 8.3-CD8α+ T cells cultured under the same conditions. Data are representative of four independent experiments. Results are shown as the mean ± SEM.

**FIGURE 6.** CD4+ and CD8α+ T cells function in GM-CSF-treated NOD mice. A and B, Proliferation and cytokine production by CD8α+ T cells from GM-CSF- or PBS-treated mice. C and D, Proliferation and production of IL-2, IFN-γ, IL-4, and IL-10 by activated CD4+ T cells (1 × 10⁵ lymphocytes/well) from GM-CSF- or PBS-treated mice. CD4+ and CD8α+ T cells were exposed to anti-CD3 (2 days, 5 μg/ml) surface-bound mAb and cytokine production was determined in supernatants by ELISA. Data are representative of three independent experiments.
However, there was an absence of production of IL-4 or IL-10 in response to anti-CD3 stimulation (data not shown). These results indicated that the treatment with GM-CSF did not alter CD8\(^+\) T cell function and suggested that diabetes resistance induced by GM-CSF was not a consequence of immune suppression or immune deviation toward a Tc2 response. Splenic CD4\(^+\) T cells of GM-CSF-treated mice responded vigorously and produced higher amount of IL-4 and IL-10 than CD4\(^+\) T cells from PBS-treated NOD mice (Fig. 6, C and D). However, no differences were noted in the production of IL-2 and IFN-\(\gamma\) by CD4\(^+\) T cells of both groups of mice (Fig. 6D). These results indicated that CD4\(^+\) T cells from GM-CSF-treated NOD mice proliferate vigorously in vitro and secrete increased amounts of the anti-inflammatory cytokines IL-10 and IL-4 as compared with PBS-treated mice, and suggest that CD4\(^+\) T cells are acquiring a Th2-like phenotype.

**GM-CSF treatment increases the Foxp3\(^+\) CD4\(^+\) CD25\(^+\) Treg population without altering their in vitro suppressive function**

We next investigated whether GM-CSF treatment affected the number and function of CD4\(^+\) CD25\(^+\) Tregs in NOD mice. In a first series of experiments, the effect of GM-CSF on the pool of CD4\(^+\) CD25\(^+\) Treg was investigated in the spleen and PLNs of GM-CSF-treated and control (PBS-treated) NOD mice. Flow cytometry analysis revealed a significant increase in the size of the CD4\(^+\) CD25\(^+\) Treg population in the spleen and PLNs of GM-CSF-treated mice as compared with PBS-treated mice. Results were 10.5 \(\pm\) 0.8% (\(n=9\)) and 5.9 \(\pm\) 0.6% (\(n=13\)), respectively (\(p<0.001\)), in the case of splenocytes, and 14.0 \(\pm\) 1.7% (\(n=7\)) and 8.3 \(\pm\) 0.6% (\(n=15\)), respectively (\(p<0.005\)), in the case of PLNs (Fig. 7A). In agreement with these results, the CD4\(^+\) CD25\(^+\) Treg marker Foxp3 was expressed in a significantly (\(p<0.02\)) higher percentage of CD4\(^+\) T cells in GM-CSF-treated mice (19.1 \(\pm\) 0.8%) as compared with PBS-treated (13.7 \(\pm\) 0.7%) mice (data not shown). The analysis of the expression of Foxp3 by FACS and by RT-PCR (Fig. 7, B and C) revealed that freshly purified CD4\(^+\) CD25\(^+\) T cells from PBS-treated and GM-CSF-treated mice were Foxp3 positive, supporting the idea that they are Tregs rather than activated CD4\(^+\) T cells. In addition, splenic and PLNs Foxp3\(^+\) CD4\(^+\) CD25\(^+\) Treg populations of GM-CSF- and PBS-treated mice contained normal levels of CD62 ligand and expressed high levels of CTLA-4 and glucocorticoid-induced TNFR (data not shown).

There was a significant (\(p<0.02\)) increase of Foxp3-positive CD4\(^+\) CD25\(^+\) T cells in the spleen of GM-CSF-treated (17.9 \(\pm\) 0.8%) mice as compared with PBS-treated (13.8 \(\pm\) 0.6%) mice (Fig. 7B, top panels). Interestingly, RT-PCR showed the induction of expression of transcription factor Foxp3 gene in CD4\(^+\) CD25\(^+\) T cells from GM-CSF-treated mice (Fig. 7C). When activated with combination of an anti-CD3 mAb and IL-2, CD4\(^+\) CD25\(^+\) T cells of both groups of mice produced the same amount of IL-4 and IL-10, whereas CD4\(^+\) CD25\(^+\) T cells from GM-CSF-treated mice produced significantly increased amounts of IL-4 (\(p<0.001\)) and IL-10 (\(p<0.001\)) (Fig. 7D).

A hallmark of CD4\(^+\) CD25\(^+\) Tregs is their ability to suppress the proliferation of T cells stimulated through their TCR, at least in vitro. We therefore investigated whether Foxp3\(^+\) CD4\(^+\) CD25\(^+\) Tregs from GM-CSF-treated mice displayed a better suppressive activity on the proliferation of diabetogenic CD8\(^+\) T cells than Foxp3\(^+\) CD4\(^+\) CD25\(^+\) Tregs from PBS-treated mice. To this end, in vitro-activated splenic CD4\(^+\) CD25\(^+\) and CD4\(^+\) CD25\(^+\) T cells from GM-CSF- or PBS-treated mice were assessed for their ability to inhibit proliferation and cytokine production of a highly diabetogenic 8.3-CD8\(^+\) T cell clone in a coculture system. Data showed that CD4\(^+\) CD25\(^+\) Tregs from GM-CSF- and PBS-treated mice...
suppressed Ag-specific proliferation of 8.3-CD8+ T cells to a similar extent (Fig. 7E). The suppressive effect was also observed on IL-2 and IFN-γ release by NRP-A7-activated 8.3-CD8+ T cells in vitro (Fig. 7E). However, CD4+CD25+ T cells did not inhibit NRP-A7-specific proliferation of 8.3-CD8+ T cells or their production of cytokines (Fig. 7E). Together, these results suggested that GM-CSF induced diabetes resistance by increasing the frequency of Foxp3+CD4+CD25+ Tregs without affecting their suppressive function, at least in vitro. In addition, the data confirmed that CD4+CD25+ Tregs in NOD mice display normal regulatory functions in vitro when they are properly activated.

Absence of effect of GM-CSF treatment on the suppressive function of Foxp3+CD4+CD25+ Tregs of NOD mice in adoptive transfer experiments

The in vivo suppressor function of CD4+CD25+ Tregs accumulating in GM-CSF-treated mice was tested by evaluating the ability of these cells to control autoimmune diabetes mediated by effector diabetogenic splenocytes from NOD mice after transfer into NOD. SCID recipient mice. CD4+CD25+ Tregs from GM-CSF-treated mice as well as from PBS-treated mice were able to significantly (p < 0.002) delay the onset of diabetes by transfer of diabetogenic T cells into NOD.SCID mice (51 ± 4 days and 52 ± 6 days, respectively) (Fig. 8A). In contrast, recipients of CD4+CD25− T cells from both groups of mice with diabetogenic cells developed diabetes within an average of 3–4 wk posttransfer (Fig. 8A). Adoptive transfer experiments repeated with cotransfer of DCs and diabetogenic T cells showed no direct impact of DCs, purified from GM-CSF-treated or control (PBS-treated) NOD mice, on effector function of diabetogenic splenocytes (Fig. 8B). Similar results were obtained in cotransfer experiments using spleen cells from 6- to 8-wk-old mice (data not shown). These results suggested that CD4+CD25+ Tregs from diabetes-free GM-CSF-treated mice or PBS-treated mice delayed transfer of diabetes but were not efficient to prevent, on a long-term basis, the transfer of the disease.

In vivo requirement of DCs to maintain long-term suppressive functions of CD4+CD25+ Tregs in GM-CSF-protected mice

The findings that Foxp3+CD4+CD25+ Tregs or DCs from GM-CSF-treated mice were not able to provide a long-term protection from diabetogenic spleen cells in adoptive transfer experiments led to investigate whether spleen cells of diabetes-free GM-CSF-treated NOD mice could transfer diabetes into NOD.SCID mice. The experiments were done by transfusing NOD.SCID recipients with 8 × 106 spleen cells from GM-CSF- or PBS-treated mice. Results showed that spleen cells from 32-wk-old GM-CSF-protected mice did not transfer diabetes into NOD.SCID mice (0/8 for 3 mo posttransfer) (Fig. 8C). However, transfer of diabetes was observed when spleen cells of PBS-treated diabetic NOD mice were used (10/10 within 3–4 wk post transfer) (Fig. 8C). The transfer of diabetes was also observed when spleen cells of prediabetic PBS-treated NOD donors (6- to 8-wk-old) (6/6 within 3–4 wk posttransfer) were used (data not shown). These results suggested that cells that reside in the spleen of GM-CSF-treated mice contained a sufficient number of cells (Tregs and/or DCs) with tolerogenic capacity to prevent the development of diabetes. Depletion of DCs from the splenocytes of GM-CSF- or PBS-treated mice were done before transfer into NOD.SCID mice to further dissect the role of splenic DCs and CD4+CD25+ Tregs in the protection from diabetes in GM-CSF-treated mice. NOD.SCID mice that received CD11c+-depleted splenocytes of PBS-treated mice developed diabetes slightly but not significantly later than recipients transferred with diabetogenic splenocytes (Fig. 8C). Interestingly, spleen cells from 32-wk-old GM-CSF-treated mice that had been depleted of DCs were not able to provide long-term protection against diabetes in recipient mice (Fig. 8C). In these mice, diabetes incidence was not significantly decreased and the onset of diabetes occurred significantly later as compared with the mice transferred with DC-depleted splenocytes from PBS-treated mice (74 ± 0.6 days (n = 10) as compared with 34 ± 0.9 days (n = 9), p < 0.0001), further emphasizing the DC-dependent role of Tregs in a long-term protection against diabetes in NOD mice.

Kared et al. (30) have reported that G-CSF prevent diabetes development in NOD mice over a period of 30 wk. The protective effect of GM-CSF was also tested using similar protocol. Female NOD mice received seven consecutive daily injections of GM-CSF (100 or 500 ng/mouse) or PBS at ages 4, 8, 12, and 16 wk, and the development of diabetes was monitored over time. As reported in the case of G-CSF treatment (30), NOD recipients of GM-CSF (500 ng/ml) were significantly (p < 0.02) protected from diabetes at 30 wk of age (Fig. 9). The lower dose of GM-CSF (100 ng/ml) was less efficient and 60% of the animals had become diabetic compared with 80% in PBS-treated mice at this time (Fig. 9).
FIGURE 9. Effect of two different GM-CSF doses on diabetes development in NOD mice. NOD mice (n = 10 per group) were injected i.p. with GM-CSF (100 or 500 ng/mouse) or PBS for seven consecutive days at ages 4, 8, 12, and 16 wk. The development of diabetes was monitored over time.

Discussion

We showed in this study that GM-CSF, a cytokine that has been previously shown to increase the number of DCs and CD4+CD25+ Tregs in vivo (12, 13, 31), provided significant protection against the development of diabetes in NOD mice. Our data provided the first evidence that such a protective effect was dependent on expanded DCs acquiring semimature phenotype under maturation stimuli. These DCs produced high amounts of IL-10 and were required to maintain a critical number of immunoregulatory CD4+CD25+ Tregs that were important for long-term protection against diabetes.

GM-CSF is a key mediator of the development of DCs (32). It expands myeloid CD11c+CD8α− DCs and increases the number of CD11c+CD11b+ DCs (33), two subsets of DCs that play a critical role in induction of tolerance (34). We found that the treatment of NOD mice (before islet insult) with GM-CSF protected NOD mice from diabetes and increased 1.7-fold the number of splenic CD11c+ DCs, most of these having a myeloid phenotype (CD11c+CD11b+CD8α−). This effect of GM-CSF on DC phenotype in NOD mice was consistent with the fact that the protection from other autoimmune diseases such as thyroglobulin-induced autoimmune thyroiditis (12, 13) and experimental autoimmune myasthenia gravis (31), by GM-CSF treatment was associated with expansion of CD11c+CD8α− DCs. Steptoe et al. (19) have shown that autoimmune diabetes could be suppressed by transfer of Gr-1+ myeloid progenitor cells. The Gr-1+ myeloid cells were preferentially retained in the spleen, continued to express CD11b but had lost Gr-1 expression. Furthermore, many of these myeloid cells had acquired a substantial expression of MHC class II, CD80, CD86, and CD11c, a characteristic of resting DCs. In this study, we do not know whether expanded splenic DCs originated from myeloid Gr-1+ precursor.

Although there is controversy with respect to the subsets of DCs responsible for peripheral tolerance, it remains that DCs must be in a steady or semimature state to initiate and maintain tolerance (35, 36). In this study, we found that freshly isolated splenic DCs from both groups of animals expressed low levels of CD80, CD86, and MHC class II molecules, and that total and myeloid but not lymphoid splenic DCs from GM-CSF-treated mice were less effective in inducing proliferation of diabetogenic CD8+ T cells without affecting their cytokine production. These differences in the capacity to activate proliferation of CD8+ T cells could not be explained by the costimulation pathway because the low levels of CD80, CD86, and MHC class II molecules were not significantly different from those of control NOD mice. One possible explanation is that iDCs from GM-CSF-treated mice use an additional inhibitory pathway such as PD-1/PD-L1, which competes with CD80/CD86-dependent costimulation. In support of this interpretation, it has been reported that GM-CSF increased the expression of PD-L1 on bone marrow-derived DCs (37). In addition, it has been observed that PD-1/PD-L1 inhibits CD4+ and CD8+ T cell priming (38) and its blockade accelerates the development of diabetes in prediabetic NOD mice (39). Finally, a recent study has shown that CTLA-4 and PD-1/PD-L1 played a synergic role in iDCs-dependent induction of peripheral CD8+ T cell tolerance (36). The contribution of this pathway in the induction of tolerance in NOD mice treated with GM-CSF is currently under investigation in our laboratories.

Because DCs undergo abnormal maturation in NOD mice, we investigated whether the treatment with GM-CSF interfered with this process and restored DCs tolerogenic properties. We found that LPS-activated splenic DCs from GM-CSF-treated mice did not undergo a full process of maturation as compared with control mice that showed increased levels of expression of MHC class II and costimulatory molecules (CD80 and CD86). Of interest, LPS-activated DCs from GM-CSF-treated mice were less efficient in Ag presentation and activation of diabetogenic CD8+ T cells as compared with DCs from PBS-treated NOD mice. In agreement with our findings, others have reported that LPS-activated bone marrow-derived DCs generated with GM-CSF expressed low levels of CD80/CD86 molecules and suppressed T cell activation (4). In addition, semimature DCs maintained self-tolerance by sustaining a population of CD4+CD25+ Tregs (40). Similarly, exposure of bone marrow-derived DCs to low doses of GM-CSF led to the development of iDCs that were resistant to maturation stimuli in vitro. These iDCs induced T cell unresponsiveness and prolonged cardiac and islet allograft survival (41, 42). Our results suggested that the GM-CSF-associated prevention from diabetes in NOD mice was largely dependent on the state of maturation of DCs. Because DCs from GM-CSF-treated mice produced amounts of IL-10 greater than DCs from control mice, it is likely that IL-10 produced in the vicinity of DCs influenced their function and their process of maturation. This argument was supported by the findings that autocrine IL-10 prevented the spontaneous maturation of DCs, limited LPS- and CD40-mediated maturation and increased IL-10 production by DCs (43).

Previous studies have shown that GM-CSF is a potent growth factor of DCs and a promoter of Th2 response in other model of autoimmunity (12, 13, 31). In agreement with previous findings in T cell-mediated autoimmune thyroiditis, we have shown that treatment with GM-CSF results in mobilizing CD11c+ and a skewing of the immune response to Th2-like cells. Unlike Th2 cells, Th2-like cells had increased production of IL-4 and IL-10, but continued to produce similar amounts of IFN-γ and IL-2 as compared with PBS-treated mice. Interestingly these CD4+CD25− T cells exhibited higher levels of Foxp3 as compared with CD4+CD25− of PBS-treated mice. The role of CD4+CD25− Foxp3+ T cells in protection from diabetes in GM-CSF-protected NOD mice is not fully known. However, there is evidence that Tregs can be thymically derived or peripherally induced from CD4+CD25−Foxp3− T cells (29, 44–46). Furthermore, retroviral transduction of Foxp3 in naïve CD4+ T cells convert these cells toward a Treg phenotype (47). These observations support the interpretation that peripheral CD4+CD25− Foxp3+ T cells of GM-CSF-treated mice may originate from naïve CD4+CD25− T cells that are going to be converted to CD4+CD25+Foxp3+ suppressor T cells. Therefore, it is likely that CD4+CD25+Foxp3+ cells constitute a reservoir of committed regulatory T cells that gain CD25 expression in GM-CSF-protected mice, as previously reported (48). Interestingly, a
recent study has shown that IL-2 is essential to convert naïve CD4+CD25− T cells to CD4+CD25+Foxp3+ Tregs and that IL-4, IL-7, and IL-15 could sustain Foxp3 expression (49). These observations are consistent with the fact that more CD4+CD25− T cells from GM-CSF-protected mice express Foxp3 and continue to produce IL-2 and IL-4, two events that contribute to their conversion to CD4+CD25+Foxp3+ Treg and the maintain of Foxp3 expression.

Taking in account the fact that CD4+CD25+ Tregs delayed the onset of diabetes in transfer experiments of diabeticogenic spleen cells, we investigated whether long-term protection from diabetes required a continual support of tolerogenic DCs. Therefore it is attracting to suggest that protection from diabetes in GM-CSF-treated NOD mice by CD4+CD25+ Tregs requires the presence of tolerogenic-resting DCs to sustain their long-term suppressive function. Our results showed that diabetes resistance induced by GM-CSF was associated with an increase in the pool of splenic CD4+CD25+Foxp3+ Tregs. Furthermore, CD4+CD25+ Tregs from GM-CSF- and PBS-treated mice displayed similar suppressive ability, suggesting that the inhibitory functions of Tregs were not affected by GM-CSF treatment. In this respect, it has been reported that a low number of CD4+CD25+ Tregs in B7−/− and CD28−/− NOD mice correlated with exacerbation and acceleration of diabetes (50). It is therefore possible that tolerogenic DCs induced by GM-CSF act indirectly through the induction/activation of Tregs, a mechanism that would promote long-term tolerance, thereby ensuring persistent islet-specific T cell hyporesponsiveness.

CD4+CD25+ Tregs are anergic and require cognate interactions with DCs for their activation in vivo (51, 52). We determined the contribution of DCs to the tolerance induced by GM-CSF treatment using adoptive transfer experiments and found that splenocytes from GM-CSF-treated mice did not transfer diabetes into NOD.SCID recipients. However, NOD.SCID hosts injected with CD-depleted splenocytes from GM-CSF-treated mice were no longer protected and developed delayed diabetes when compared with NOD.SCID injected with CD-depleted splenocytes from PBS-treated mice. This delay was likely due to the loss of Tregs initially increased in the spleen of GM-CSF-treated mice. Keeping in line with our data, a recent study has reported that a defect in splenic APCs of NOD mice could be responsible for a suppressive defect of CD4+CD25+ Tregs (53). However, the nature, phenotype and function of APCs were not investigated in this study. Our results confirmed the previous described defect of DCs in NOD mice and presented evidence that GM-CSF reestablished tolerogenic DCs required for maintaining Treg efficiency. Yamazaki et al. (52) have shown that GM-CSF-activated DCs induced a robust proliferation of Tregs in vitro and in vivo. In addition, two recent studies have reported that CD4+CD25+ Tregs interact directly with DCs and that persistent cell-cell interactions led to sustained immunosuppressive function of CD4+CD25+ Tregs (54) thereby decreasing the time of contact between DCs and effector cells (55). Our data showed that long-term tolerance induced by GM-CSF treatment in NOD mice can be attributed to re-establishment of sufficient amount of Tregs that are supported by tolerogenic GM-CSF-induced DCs.

The protective effect of GM-CSF from diabetes we reported is corroborated with recent data showing that GM-CSF/IL-3-deficient mice developed insulitis and diabetes with age, suggesting that functional deficiencies of GM-CSF and IL-3 contribute to diabetes (56). In addition, other groups have shown that G-CSF affords diabetes protection from several autoimmune diseases including diabetes (30, 57, 58). The beneficial effect of G-CSF therapy in diabetes was shown to be mediated through promotion of tolerogenic DCs and the expansion of Tregs (30). In agreement with these observations, our data showed that GM-CSF-treated (500 ng/mouse) NOD mice were protected from diabetes over 30 wk using a protocol similar to the G-CSF study (30). Of note, GM-CSF appeared to be more effective than G-CSF because a dose of GM-CSF of 500 ng/mouse that is equivalent to 25–30 μg/kg/day brought a protective effect similar to G-CSF used at a dose of 200 μg/kg/day (30).

In summary, our data showed that GM-CSF modulates the immunoregulatory function of DCs, and strongly suggest that DCs act as guardians of the induction and maintenance of long-term T cell tolerance to islet Ags in the periphery through CD4+CD25+ Tregs.

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Disclosures

The authors have no financial conflict of interest.

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