Accumulating evidence that granulocyte colony-stimulating factor (G-CSF), the key hematopoietic growth factor of the myeloid lineage, not only represents a major component of the endogenous response to infections, but also affects adaptive immune responses, prompted us to investigate the therapeutic potential of G-CSF in autoimmune type 1 diabetes. Treatment with G-CSF protected NOD mice from developing spontaneous diabetes. G-CSF triggered marked recruitment of dendritic cells (DCs), particularly immature CD11c<sup>+</sup>B220<sup>+</sup> plasmacytoid DCs, with reduced costimulatory signal expression and higher interferon-γ release capacity than DCs in excipient-treated mice. G-CSF recipients further displayed accumulation of functional CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cells that produce transforming growth factor-β1 (TGF-β1) and actively suppressed diabetes transfer by diabetogenic effector cells in secondary NOD-SCID recipients. G-CSF’s ability to promote key tolerogenic interactions between DCs and regulatory T-cells was demonstrated by enhanced recruitment of TGF-β1-expressing CD4<sup>+</sup>CD25<sup>+</sup> cells after adoptive transfer of DCs isolated from G-CSF–relative to vehicle-treated mice into naive NOD recipients. The present results suggest that G-CSF, a promoter of tolerogenic DCs, may be evaluated for the treatment of human type 1 diabetes, possibly in association with direct inhibitors of T-cell activation. They also provide a rationale for a protective role of the endogenous G-CSF produced during infections in early diabetes. Diabetes 54:78–84, 2005

There is compelling evidence that granulocyte colony-stimulating factor (G-CSF), well known as the hematopoietic growth factor of the myeloid lineage (1) and a major response factor to infections (2), also exerts profound immunoregulatory influence in adaptive immunity. In patients treated with G-CSF, T-cell allogeneic and mitogenic reactivities are inhibited (3), corresponding to a reduced interferon (IFN)-γ production capacity (4) and a cytokine response skewed toward TH2 (5). Recipients of G-CSF–mobilized peripheral blood stem cells do not display a higher incidence of graft-versus-host disease (GVHD) than bone marrow recipients, despite the larger numbers of T-cells in unmanipulated stem cell grafts (6). In experimental models, G-CSF was shown to protect mice from GVHD by orienting the T-cell response to TH2 (7). Moreover, G-CSF was protective in experimental endotoxemia by inhibiting the inflammatory response, particularly production of tumor necrosis factor-α (8). More recently, G-CSF was reported to induce the emergence of tolerogenic dendritic cells (DCs) (i.e., pro-DC2) (9,10), as well as Tr1-like regulatory T-cells (11) in the peripheral blood of normal human recipients, thus promoting the expansion of potentially tolerogenic subsets at both poles of the antigen-presenting cell (APC)/T-cell interaction. We therefore investigated whether G-CSF, a particularly well-tolerated molecule, might also be used to promote tolerance in pathophysiological settings such as autoimmune diseases.

We recently showed that G-CSF, at 200 μg·kg<sup>−1</sup>·day<sup>−1</sup>, exhibits therapeutic potential in two different models of autoimmune diseases: systemic lupus (12), a spontaneous systemic disease characterized by immune complex deposits leading to lethal glomerulonephritis, and experimental autoimmune encephalomyelitis (EAE) (13), a T-cell–mediated demyelinating disease actively induced by immunization with myelin antigens. G-CSF has also been shown to be expressed within central nervous system lesions at the acute phase of multiple sclerosis (14), thus suggesting an endogenous role for this hematopoietic growth factor in the control of autoreactive processes. We now present evidence that G-CSF treatment prevents another T-cell–mediated disease, spontaneous autoimmune type 1 diabetes, in the NOD mouse. This protection...
correlated with marked recruitment of CD11c<sup>+</sup>B220<sup>+</sup> plasmacytoid DCs (pDCs), an immature tolerogenic DC subset that in turn recruits CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cells, which exhibit suppressive properties in secondary recipients.

**RESEARCH DESIGN AND METHODS**

NOD/Thy1.2 (K<sup>R</sup>, I-A<sup>dp</sup>, D<sup>p</sup>) mice were bred in our animal facilities under specific pathogen-free conditions. Female NOD mice (n = 12 mice per group) received five consecutive daily subcutaneous injections of recombinant human G-CSF (Neupogen; Amgen, 200 μg·kg<sup>-1</sup>·day<sup>-1</sup>), which is active in mice (7), at ages 4, 8, 12, and 16 weeks. The excipient consisted of 5% dextrose in sterile H<sub>2</sub>O. Colorimetric strips were used to monitor glycosuria twice a week (Glukotest; Boehringer-Mannheim, Mannheim, Germany) and glycerinemia (Hemoglukotest and Reflo lux F; Boehringer-Mannheim). Mice with blood glucose levels >250 mg/dl on two consecutive readings were considered diabetic. All animal experiments were conducted according to the regulations of the French Agricultural Ministry.

Pancreata were removed, embedded in Tissue-Tek OCT (optimal cutting temperature) (Sakura, Zoeterwoude, the Netherlands), and snap-frozen in liquid nitrogen. For conventional histopathology, serial 2-μm sections were stained with hematoxylin and eosin to score mononuclear cell infiltration as follows: grade 0 = normal islets; grade 1 = focal or peripheral insulitis (lymphocytes gathered at a pole or surrounding the islet, but no destruction of endocrine cells as assessed by labeling with anti-insulin antibodies); and grade 2 = invasive destructive insulitis.

**Antibodies and fluorescence-activated cell sorter analysis.** Spleen and pancreatic lymph node (PLN) cell content was analyzed at age 20 weeks. For DC analyses, spleens and PLN cells were digested with collagenase (1 mg/ml) and DNase (40 μg/ml), after which single-cell suspensions were prepared. CD4<sup>+</sup> and CD11c<sup>+</sup> cells (90 and 60–80% pure, respectively, confirmed by fluorescence-activated cell sorter [FACS] analysis) were positively enriched with anti-CD4<sup>−</sup> and anti-CD11c<sup>−</sup> labeled immunomagnetic cell sorting (MACS) microbeads (Miltenyi Biotec, Paris, France, respectively).

The cell surface phenotype of PLN cells, splenocytes, and isolated cell subsets was analyzed by flow cytometry. All cells were incubated in cold PBS supplemented with 2% FCS and 0.02% azide. Cells were preincubated in 20 μl for 15 min at room temperature, under constant shaking, with anti-FC-γ receptor I/II (clone 2.4G2) to reduce nonspecific binding, followed by the mononuclear antibodies anti-CD4 (clone GK1.5), anti-CD25 (clone 7D4), anti-CD3 (clone 145-2C11), anti-B220 (clone RA3-6B2), anti-CD11c (clone HL3), anti-CD80 (BT-1; clone 16-10A1), anti-CD86 (BT-2; clone GL-1), anti-CD40 (clone 3D3), anti-IA/IE (clone M1/70), and anti–Mac-1/CD11b (clone M1/90), which were used to prepare from PharoMingen (Becton Dickenson, Le Pont de Claix, France) and were used coupled with biotin and revealed by streptavidin-APC (PharoMingen), or with phycoerythrin (PE) or fluorescein isothiocyanate (FTIC). Using the FACScalibur flow cytometer (Becton Dickenson), 10,000 events were acquired. The analyses of acquired data were performed using CellQuest software (Becton Dickenson).

**Expression of postmature molecules and cytokine production by DCs.** CD11c<sup>+</sup> cells were enriched from spleen with MACS immunobeads (Miltenyi Biotec) and cultivated (2 × 10<sup>6</sup> cells in vehicle-treated mice), corresponded to a 2.8-fold enrichment in DC numbers per spleen. G-CSF had a 10-fold increase in CD11c<sup>+</sup> cells (90 and 60–85% pure, respectively, confirmed by FACS analysis) were positively enriched with anti-CD4<sup>−</sup> and anti-CD11c<sup>−</sup> labeled immunomagnetic cell sorting (MACS) microbeads (Miltenyi Biotec, Paris, France, respectively).

**Isolation of CD11c<sup>+</sup> DCs from PLN of G-CSF– and excipient-treated NOD mice and transfer into secondary NOD recipients.** Female NOD mice, age 4 weeks, were injected over 5 consecutive days with 200 μg/kg G-CSF or excipient (n = 5 per group). On day 10, spleen CD11c<sup>+</sup> cells were isolated with immunomagnetic microbeads (Miltenyi Biotec) and successively transferred intravenously (4 × 10<sup>6</sup> cells/100 μl) into the secondary recipients, 4-week-old female NOD mice. Then 7 days later, the percent of CD4<sup>+</sup>CD25<sup>+</sup> cells within the spleen of secondary recipients (n = 3 per group) was assessed by FACS analysis.

**Statistical analysis.** Student’s t test was used to determine the statistical significance of differences among groups. The occurrence of diabetes in the different experimental groups was plotted using the Kaplan–Meier method (i.e., nonparametric cumulated survival plot). The statistical comparison was performed using the log-rank (Mantel-Cox) test, which provided the corresponding x<sup>2</sup> values.

**RESULTS**

**G-CSF treatment prevents spontaneous diabetes and insulitis.** NOD (Thy1.2) female mice, age 4 weeks, received subcutaneous treatment with recombinant human G-CSF (200 μg·kg<sup>-1</sup>·day<sup>-1</sup>) or vehicle over 5 consecutive days; this protocol was repeated every 4 weeks thereafter (i.e., at ages 4, 8, 12, and 16 weeks). Recipients of G-CSF were significantly protected from diabetes (P = 0.002, x<sup>2</sup> test) (Fig. 1A). At age 25 weeks, vehicle-injected mice presented with advanced destruction of pancreatic islets (with very few remaining islets, heavily infiltrated, grade 2), whereas numerous pancreatic islets remained in G-CSF–protected mice, of which 70% showed peripheral and/or focal insulitis (grade 1) and 30% showed invasive insulitis (grade 2) (Fig. 1B). Insulitis scores remained essentially unchanged in the G-CSF–treated mice examined at age 30 weeks (n = 3; data not shown), and the animals remained disease free.

**Accumulation of immature DCs in G-CSF–treated mice.** Several lines of evidence support the notion that an imbalance exists in NOD mice favoring immunogenic versus tolerogenic DCs, thereby predisposing NOD mice to autoimmune diabetes development (15–19). It is important to note that G-CSF has been shown to promote the development of specific tolerogenic DC subsets in human subjects (10). We therefore analyzed DC phenotype and maturation status in vehicle- and G-CSF–treated NOD mice. Spleen CD11c<sup>+</sup> percentages were significantly increased in G-CSF– relative to vehicle-treated mice (4.8 ± 0.7 vs. 2.7 ± 0.4% of total spleen cells; data not shown), which, combined with the relative spleen enlargement due to the hematopoietic properties of G-CSF (77 ± 10 vs. 48 ± 4.2 × 10<sup>6</sup> cells in vehicle-treated mice), corresponded to a 2.8-fold enrichment in DC numbers per spleen. G-CSF had no effect on the relative percent of myeloid CD11c<sup>+</sup>CD11b<sup>+</sup> DCs (data not shown). Conversely, 15 ± 1% CD11c<sup>+</sup> cells in G-CSF recipients expressed the CD11c<sup>+</sup>B220<sup>+</sup> phenotype, which has been reported to
correspond to pDCs with tolerogenic properties (20,21), compared with 8.8 ± 0.5% of CD11c− cells in vehicle-treated mice (Fig. 2A), representing a 5.4-fold enrichment in pDC numbers per mouse spleen (5.55 vs. 1.03 × 10⁶ pDCs per spleen). Moreover, CD11c− DCs from G-CSF−treated mice displayed characteristics of immature DCs, with lower levels of expression of the MHC II complex and the costimulatory molecule CD80, but not CD86 or CD40 (Fig. 2B), than DCs from vehicle-treated mice.

DCs from G-CSF− or excipient-treated mice further displayed a distinct spontaneous cytokine production pattern, as measured in supernatants after 24-h incubation in culture medium. Thus, in keeping with their enrichment in CD11c+ B220+ pDCs, CD11c− cells enriched from G-CSF−treated mice spontaneously released in their supernatants more IFN-α, but much less IL-12 p70, than CD11c+ cells from excipient-treated diabetic mice (Fig. 2C).

**G-CSF enhances CD4+CD25+ T-cell subset.** Given the properties of pDCs to expand regulatory T-cells (22,23), we next analyzed whether this significant increase in pDC number in G-CSF recipients correlated with enhanced accumulation or preservation of the tolerogenic T-cell subset. Indeed, an imbalance between diabetogenic effectors and the immunoregulatory T-cell subset expressing the CD4+CD25+ phenotype is observed as spontaneous diabetes develops in the NOD mouse (24). Diabetogenic effectors leave the PLN to invade the pancreatic islets, as the regulatory T-cell pool declines with age and loses suppressive potential (25). In 20-week-old mice, the size of the CD4+CD25+ cell subset in the PLN was notably enhanced (3.5-fold) after G-CSF treatment (0.497 ± 0.06 [G-CSF−treated mice] vs. 0.141 ± 0.015 [excipient-treated mice] vs. 0.28 ± 0.03 [untreated 6-week-old NOD mice] × 10⁶ cells) (Fig. 3A). We observed a 50% less increase in CD4+CD25+ cell numbers (1.76 fold) in spleen cells in G-CSF− vs. excipient-treated mice (3.18 ± 0.35 vs. 1.8 ± 0.15 × 10⁶ cells) (Fig. 3A).

**CD4+CD25+ cells in PLN of G-CSF recipients express membrane-bound and secreted TGF-β1.** To examine the functional properties of the CD4+CD25+ cells accumulating in G-CSF recipients, spontaneous membrane expression of TGF-β1 (26,27) was measured on nonpermeabilized PLN cells. Half of gated CD4+CD25+ cells expressed high and intermediate levels of the suppressive cytokine in 20-week-old G-CSF recipients relative to only 34% of CD4+CD25+ cells in age-matched, excipient-treated NOD mice (Fig. 3B) and naive 6-week-old NOD mice.

The cytokine production pattern was also analyzed by FACS after PMA and ionomycin activation (Fig. 3C). In both groups of mice, ~30% of PLN cells and 50% of CD4+ cells within this compartment expressed intracellular TGF-β1, but levels of expression were remarkably higher in G-CSF− relative to excipient-treated mice. IL-10 production was observed in only 1.5% of PLN CD4+ cells (0.8% total PLN cells) in G-CSF recipients, all of which produce high levels of TGF-β1 (data not shown). Conversely, only 2.7% of CD4+ PLN cells produced IFN-γ after PMA and ionomycin activation in G-CSF recipients, relative to 5.3% in vehicle-treated mice; IL-4 was undetectable.

Adoptively transferred CD25+ cells isolated from PLN, but not from spleen of G-CSF-treated mice, protect secondary NOD-SCID recipients against diabetogenic effectors. To assess the in vivo suppressive
CD11c<sup>lo</sup>B220<sup>+</sup> cells in G-CSF recipients relative to 5.3% in vehicle recipients (not shown), were subsequently transferred (4 × 10<sup>5</sup> cells) into secondary 4-week-old NOD recipients; the percent of CD4<sup>+</sup>CD25<sup>+</sup> T-cells in recipient mice was analyzed 7 days later. In keeping with their relative enrichment in pDCs (which have the potential to recruit regulatory T-cells), adoptively transferred DCs from G-CSF recipients led to threefold increased proportions of CD4<sup>+</sup>CD25<sup>+</sup> cells in the spleen of the recipients relative to DCs isolated from vehicle-treated mice (Fig. 5A). An important finding was that CD4<sup>+</sup>CD25<sup>+</sup> T-cells accumulating in the recipients of DCs from G-CSF–treated mice expressed significantly higher levels of membrane-bound TGF-β1 (Fig. 5B). A more limited (1.2-fold) CD4<sup>+</sup>CD25<sup>+</sup> cell enrichment was also observed at days 7 and 20 in peripancreatic and mesenteric lymph nodes of DC recipients (not shown), in keeping with the splenic origin of the transferred DCs and the intravenous route of injection used. Thus, G-CSF promoted key tolerogenic interactions between DCs and regulatory T-cells.

DISCUSSION

G-CSF treatment administered to NOD mice over 5 consecutive days every 4 weeks starting from age 4 weeks provided significant protection against spontaneous diabetes, a T-cell–mediated autoimmune disease. This protection correlated with marked recruitment of two major regulatory subsets, pDCs and regulatory CD4<sup>+</sup>CD25<sup>+</sup> T-cells.

The possibility that G-CSF has a direct effect on T-cells cannot be eliminated, as the presence of G-CSF receptors on minor subsets of the T-cell lineage, yet to be identified, has been recently reported (10,28,29). However an effect via cells of the myeloid lineage is more likely, as the presence of G-CSF receptors is widely recognized on these cells (1,2) and we have previously shown that G-CSF was able to switch macrophages to a pro-Th2 chemokine balance in mice undergoing EAE (13). An imbalance favoring immunogenic rather than tolerogenic DCs is suspected to contribute to diabetes development in the
NOD mouse. Reported DC abnormalities in NOD mice include increased proportions of a myeloid DC (CD11c+CD11b+) subset within the spleen relative to diabetes-resistant, recombinant, congenic NOR mice (15), as well as elevated costimulatory properties through the increased percent of CD80- and CD86-expressing CD11c+ cells and increased expression of these molecules during T-cell stimulation (15,16). NOD DCs are also characterized by persistent hyperactivation of nuclear factor-κB, which is responsible for coordinated upregulation of MHC expression, costimulation of molecules, and cytokine synthesis (18,19). It is remarkable that G-CSF has been shown to favor accumulation of pro-DC2 in human subjects and human recipients of G-CSF–mobilized peripheral blood mononuclear cells (9). We therefore examined the effect of G-CSF treatment on DC subsets in the NOD mouse. CD11c+ cells were significantly increased in spleen of G-CSF–treated NOD recipients. Although no significant difference was found in the relative proportions of myeloid CD11c+CD11b+ DCs, G-CSF recipients displayed enhanced proportions of the CD11c+B220+ dendritic subtype, a phenotype attributed to tolerogenic pDCs, that were accordingly characterized with high IFN-α (20,30) but low IL-12p70 production capacity. In addition, they displayed features of immature DCs, with a lower expression of the MHC II complex and the costimulatory molecule CD80. It is important that specific tolerogenic or immature pDC subsets have been shown to exert a major influence on regulatory T-cell accumulation (22,31). Indeed, when CD11c+ DCs isolated from the spleen of mice treated for 5 days with G-CSF, which were enriched in CD11c+B220+ cells relative to excipient-treated mice, were transferred into secondary naive NOD recipients, they triggered recruitment of CD4+CD25+ cells at a higher level than did CD11c+ cells enriched from excipient-treated mice. This confirmed a primary role of DCs in the specific accumulation of the regulatory CD4+CD25+ T-cell subset observed in G-CSF recipients relative to excipient-treated mice.

That CD4+CD25+ T-cells accumulating in G-CSF recipients presented functional suppressive properties was demonstrated in vitro by FACS analysis, showing their capacity to produce the immunosuppressive cytokine TGF-β1 at the membrane and intracellular levels (26,27) and in vivo by their protective properties in cotransfer experiments against diabetogenic effectors in secondary NOD-SCID recipients. These observations concur with recent reports of regulatory T-cells accumulating in peripheral blood of human G-CSF recipients (11). However, in human subjects, IL-10–producing Th1-like regulatory T-cells have been described, at variance with the CD4+CD25+ cells we observed in NOD mouse PLN that produced low levels of IL-10 but high levels of TGF-1. It is noteworthy that when isolated, only the CD25+ cell fraction from PLN cells in these G-CSF recipients exerted active suppression in vivo, with as few as 2.5 × 105 of these cells significantly delaying diabetes transfer by 3 × 105 diabetogenic splenocytes in cotransfer experiments in NOD-SCID recipients. Because immunization with autoantigen(s) has been demonstrated to take place within the draining lymph nodes of the target tissue, the more pronounced enrichment of these functional regulatory T-cells within PLN supports the notion that the cellular mechanisms accounting for G-CSF’s protective effects are specifically targeted at the autoreactive response. Moreover, the present data also confirm that CD4+CD25+ regulatory T-cells from PLN display a much higher protective efficiency against diabetes than CD4+CD25+ cells from spleen or other peripheral lymph nodes (32). The present data obtained in the NOD mouse model show that G-CSF administration durably preserves the pool of functional regulatory T-cells by resetting the critical DC balance toward tolerance.

Given that G-CSF’s capacity to promote in vivo expansion of immature DCs as well as functional regulatory T-cells has also been reported in humans (9,10,33,34), this hematopoietic growth factor may have therapeutic potential in human subjects with autoimmune diseases. Indeed, treatment with the clinically well-tolerated G-CSF may represent a safer and less invasive therapeutic strategy than cell therapy with bone marrow–derived DCs. However, the therapeutic window for intervention with DCs or tolerogenic DC-inducing agents such as G-CSF remains to be ascertained. Thus far, DCs have been shown to protect NOD mice from diabetes when transferred at age 5–6 weeks (35–37). Their influence at later disease stages has not yet been reported. An exacerbation of autoimmune diseases, particularly neurological manifestations of lupus and multiple sclerosis, has been reported in patients with end-stage autoimmune disease who were treated with G-CSF in the course of mobilizing hematopoietic stem cell recipients of G-CSF.
cells for clinical trials of autologous stem cell transplantation (38). These aggravating effects of G-CSF were abrogated when G-CSF was administered together with cyclophosphamide or high-dosage corticosteroids. We could not observe any effect, either deleterious or protective, of G-CSF on diabetes incidence in the NOD mouse when treatment was started later in the disease (age 9 weeks; data not shown). One possible interpretation of these findings is that the early infiltration of islets by immunogenic DCs, which occurs at age 3–7 weeks and onwards in the NOD mouse (39) and initiates (subsequent autoimmune islet injury) may actually define a checkpoint for immunointervention with tolerogenic DC subsets as with G-CSF. Given the role of DCs to T-cell interaction in the control of immune tolerance, the possibility that a synergistic combination of G-CSF, as a DC maturation inhibitor, with T-cell–activation inhibitors such as the CD3 antibody (40,41), might prove useful in patients at risk for developing type 1 diabetes (42) warrants further investigation.

Our results may also shed some light on the possible role that infectious events that trigger endogenous production of G-CSF can play on the development of autoimmune diabetes and more generally on molecular mechanisms involved in the control of adaptive immunity by innate immunity. Indeed, there is compelling evidence that infections may influence diabetes frequency in human populations (43). Vaccination with mycobacteria products such as complete Freund adjuvant (44) can prevent the onset of type 1 diabetes in the NOD mouse. Recent reports suggest that not only might protection be achieved by mycobacteria components such as hsp65 (45), but also that endogenous response factors to infections, such as G-CSF, could be involved. For example, mycobacterial infection of IFN-γ−/− mice is characterized by enhanced extramedullary hematopoiesis and the presence of high levels of G-CSF in the serum of these mice in conjunction with IL-6. These observations have disclosed a critical balance in innate immunity between IFN-γ and G-CSF (46). Our present results demonstrate that this molecular balance may also influence the onset of autoimmune diseases and suggest that stimulation of an endogenous production of G-CSF by infections at an early phase of autoimmune diabetes may prevent disease.

In conclusion, our results disclose that G-CSF, an essential mediator in innate immune responses, may also contribute to the control of adaptive immunity and, as was shown here, particularly of autoimmune type 1 diabetes. Patients’ remarkable tolerance for G-CSF, which has been used in clinics for >10 years, should prompt the therapeutic evaluation of this molecule in autoimmune diseases.

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