Granulocyte–Colony-Stimulating Factor Treatment Enhances Foxp3⁺ T Lymphocytes and Modifies the Proinflammatory Response in Experimental Autoimmune Neuritis

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Despite the evolution of treatments in the past decade, acute inflammatory autoimmune diseases of the peripheral nervous system are still a concern due to their considerable morbidity and mortality [1]. Guillain-Barré syndrome (GBS) is an acute inflammatory disorder. The study of experimental autoimmune neuritis (EAN), the experimental model of GBS, has provided most of the knowledge regarding GBS disease mechanisms. Inflammatory lesions are primarily located in the sciatic nerves and the cauda equina of EAN-affected Lewis rats [2]. Lesions are characterized by an accumulation of inflammatory cells, primarily T cells, and macrophages. The inflammatory response during EAN is characterized mainly by the migration of neuritogenic cells into the peripheral nerves and by the production and secretion of proinflammatory cytokines, such as IFN γ , TNF α , IL-6, and IL-17 [3].

During the last years, hematopoietic factors, such as erythropoietin, granulocyte–macrophage colony-stimulating factor and granulocyte–colony-stimulating factor (G-CSF), have been successfully used to modify experimental autoimmune diseases [4,5]. Moreover, G-CSF has an important and strong tolerogenic function. This cytokine recruits immature dendritic cells (DCs), which induce tolerance through the enhancement of regulatory T cells [5].

In this study, we investigated the effects of short-term G-CSF treatment on the evolution of EAN. EAN was induced using the neuritogenic peptide P2₅₃₋₇₈ as previously described [6]. The clinical signs of EAN were significantly reduced by the *in vivo* adminis-

tration of G-CSF in a short-term treatment regimen (Figure 1A). The development of EAN is characterized by the sensitization and activation of neuritogenic T cells in the peripheral immune organs. These cells migrate into peripheral nerves and release inflammatory mediators, which may contribute directly or indirectly to the destruction of the myelin sheath. We observed a significant reduction in the specific proliferative response of T cells from Lewis rats with EAN treated with G-CSF compared with the cells from the control animals (Figure 1B). Moreover, G-CSF treatment reduced the mononuclear cell infiltration into the sciatic nerve (Figure 1C).

The EAN autoaggressive response is characterized by the expression and release of Th1 and/or Th17 cytokines. Therefore, we evaluated the expression, by real-time PCR (Figure S1), of Th1 and Th17 cytokines and transcription factors, in the lymph nodes 10 days after immunization, prior to the migration of the neuritogenic cells from the periphery to the target tissue. We observed a consistent downregulation in the expression of the Th1 (T-bet) and Th17 (ROR α and ROR γ t) transcription factors in the G-CSF-treated group compared with control group (Figure 1D). Corroborating the changes in the transcription factors expression profile, the expression of proinflammatory cytokines (IFN γ , IL-17A, IL-17E, and IL-6) was reduced in the G-CSF-treated group compared with the control group. G-CSF treatment also led to an increase in the expression of anti-inflammatory cytokines, such as IL-10 and

Figure 1 Clinical evolution of EAN in Lewis rats, which were intraperitoneally administered G-CSF at doses of 200 µg/kg/day (black circle) and 100 µg/kg/day (white square) for 5 consecutive days, starting on day 0. The control group (black squares) received the same volume of sterile PBS for the same period (A). Proliferative response of T lymphocytes (CD3⁺CD4⁺), stained with CFSE (after 96h of culture), from the lymph nodes of Lewis rats with EAN treated with saline or G-CSF at a dose of 200 µg/kg/day and stimulated with the P2₅₃₋₇₈ peptide, 5 µM (The result is representative of 3 independent experiments; Fig. S1) (B). Histological sections of the sciatic nerve of a naïve rat and rats from the saline and G-CSF-treated groups (15 days after immunization) stained with Luxol Fast Blue and cresyl violet (arrows indicate inflammatory foci). Transverse sections imaged using an 8× (left column) or 40× objective (right column) (C). Expression of transcription factors (T-bet, ROR α , ROR γ t and STAT-1), pro-inflammatory (IFN γ , IL-17A, IL-17F and IL-6), and anti-inflammatory (IL-10 and TGF β) cytokines and BDNF in the lymph node cells of rats treated with saline (black bars) or G-CSF (white bars) (D). **P* < 0.05; ***P* < 0.01 (Mann–Whitney *U*-test).

Figure 2 Flow cytometric quantification of CD4⁺Foxp3⁺ T lymphocytes from the lymph nodes (A, representative dot plot; B, mean of 5 independent experiments). Quantification of CD8⁺Foxp3⁺ T lymphocytes from the lymph nodes (C, representative dot plot; D, mean of 5 independent experiments). Expression of Foxp3 mRNA in the lymph nodes of rats treated with saline (black bar) or G-CSF (white bar) (E). Proliferative response of neuritogenic T lymphocytes. Cells (4×10^6 , initial input) were cultured in medium only (black bar) or medium supplemented with 10 ng/mL of G-CSF (white bar) and pulsed with the specific antigen. After 60 h of culture, the cells were stained with trypan blue and counted in a TC10 automated cell counter (BioRad, USA) (F). Real-time PCR analysis of transcription factors (T-bet, ROR α and ROR γ t) after 60 h of culture with (black bars) or without (white bars) G-CSF (G). After 60 h of culture, the cells (5×10^6) cultured or not in the presence of G-CSF were adoptively transferred through the intravenous route into a naïve Lewis rat. The clinical scores were then evaluated daily (H). * $P < 0.05$; ** $P < 0.01$ (Mann–Whitney test)

TGF β_1 , which are the main cytokines produced by regulatory T cells [7].

Regulatory T cells suppress the activation of the immune system and thereby prevent excessive inflammation and/or autoimmunity [7]. Indeed, flow cytometer analysis showed that G-CSF-treated

animals had a higher percentage of CD4⁺Foxp3⁺ cells in the lymph nodes compared with the control group (Figure 2A,B). Moreover, treatment with G-CSF also increased the expression of Foxp3 by CD8⁺ T cells (Figure 2C–D). These results indicate that G-CSF treatment enhances, directly or indirectly, through tolerogenic

DCs, the expression of Foxp3 in T lymphocytes (Figure 2E). The increase in the proportion of regulatory T cells seems to be responsible, at least in part, for the suppression of the inflammatory response observed in the treated animals. In fact, other authors and we have shown that the enhancement of regulatory T cells ameliorates autoimmune diseases [7,8].

Interestingly, we also detected an augmentation in the expression of BDNF, a neuroprotective factor, in the lymph nodes of G-CSF-treated group compared with the control group. Indeed, inflammatory cells also secrete this growth factor [9]. Thus, our data suggest that G-CSF acts simultaneously as an anti-inflammatory and a neuroprotective cytokine in the EAN model.

G-CSF is thought to affect the inflammatory response by recruiting immature DCs, which have a tolerogenic profile and enhance the regulatory status of naïve T lymphocytes [5]. To investigate the effect of G-CSF on activated T cells, which may express G-CSFR [9], and/or on mature APCs, we generated neuritogenic P2₅₃₋₇₈-specific T-cell clones from the immunized animals (Figure S1). We observed a reduction in the proliferative response of neuritogenic T cells cultured in the presence of G-CSF compared with neuritogenic T cells cultured in the absence of G-CSF (Figure 2F). Given the reduction in the proliferative response verified, we evaluated the expression of Th1 and Th17 transcription factors by these cells. The neuritogenic cells cultured in the presence of G-CSF presented a significant decrease in the expression of T-bet, ROR α , and ROR γ t. Interestingly, the expression of Foxp3 was not detected in the neuritogenic T cells, cultured with or without G-CSF, *in vitro*. These data suggest that, in addition to the effect on the proliferative response, G-CSF also acts on the activation status of neuritogenic T cells. In order to confirm these

results, we have adoptively transferred an equal number (5×10^6) of neuritogenic T cells cultured or not in the presence of G-CSF. The adoptive transfer of these autoreactive T cells normally induces EAN in naïve Lewis rats. We observed a reduction of the disease severity in the animals that received G-CSF-cultured T cells compared with the animals that received the control T cells (Figure 2H). These results indicate that G-CSF may act either on the mature APCs and/or on activated T lymphocytes.

Our results clearly demonstrate that short-term treatment with G-CSF significantly reduces the clinical manifestation of EAN, with concomitant reduction in the activation of neuritogenic T cells both *in vitro* and *in vivo*. The reduction in the function of autoreactive T cells may be explained by the increase in the proportion of Foxp3⁺ T cells and anti-inflammatory cytokines in the draining lymph nodes of treated animals and by the direct effect of the G-CSF on the neuritogenic T-cell activation.

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

The following supplementary material is available for this article:

Figure S1. Flow cytometry analysis of CFSE dilution from CD3⁺CD4⁺ T lymphocytes (mean of 3 independent experiments)

from the lymph nodes of control and G-CSF-treated group. Seven generations were detected in the control group and five in the G-CSF-treated group. **P* < 0.05; ***P* < 0.01 ****P* < 0.001 (Mann–Whitney *U*-test).