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# G-CSF treatment prevents cyclophosphamide acceleration of autoimmune diabetes in the NOD mouse

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**Abstract**

Cyclophosphamide (CY) accelerates autoimmune diabetes in the NOD mouse at different levels, including critical targeting of a regulatory T cell subset, exacerbation of pro-Th1 IFN-γ production and promotion of inflammation in pancreatic islets. Here we evaluated the ability of G-CSF to antagonize the acceleration of the disease induced by CY. Human recombinant G-CSF, administered daily at 200 μg/kg by s.c. injection, protected NOD mice from CY-accelerated onset of glycosuria and insulitis. G-CSF accelerated the recovery of the T cell compartment after the depletion of the lymphoid compartment triggered by CY injection. It selectively prevented the loss of the immunoregulatory T cells expressing the $CD4^+CD25^+$ phenotype that also stained $CD62L^+$ in peripancreatic lymph nodes and promoted their expansion in the spleen. In addition to this, it abrogated the robust cytokine - particularly IFN-γ- and chemokine burst triggered in immune cells by CY. G-CSF promoted only slight changes in the inflammatory effects of CY at the target tissue site, assessed by chemokine induction within the pancreas. Thus the immunoregulatory properties of G-CSF were critical in the early control of the accelerating effects of CY on autoimmune diabetes in the NOD mouse.


*Keywords:* Chemokines; Cytokines; G-CSF; Regulatory T cells; Type 1 diabetes

## 1. Introduction

There is compelling evidence that G-CSF not only acts as the hematopoietic factor of the myeloid lineage, but also exerts profound immunoregulatory influence in adaptive immunity. Patients treated with G-CSF display reduced IFN-γ production capacity [1] and a cytokine response skewed toward TH2 [2] correlating with inhibited T cell allogeneic and mitogenic reactivities [3]. Recently, regulatory Tr-1-like cells have been described in human G-CSF recipients [4]. In experimental models, G-CSF was shown to protect mice from GVHD by orienting the T cell response to TH2 [5]. Moreover, G-CSF was protective in experimental endotoxemia by inhibiting the inflammatory response [6].

We have already shown that these immunoregulatory properties of G-CSF conferred a therapeutic potential to this molecule in two different models of autoimmune diseases. In systemic lupus [7], a systemic disease

*Abbreviations:* CY, cyclophosphamide; AID, autoimmune disease; RPA, RNAse Protection Assay; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1 alpha; MIP-1β, macrophage inflammatory protein-1 beta; IP-10, IFN-γ-inducible protein-10; GVHD, graft-versus-host-disease; EAE, experimental autoimmune encephalomyelitis; MLN, mesenteric lymph nodes; PLN, peripancreatic lymph nodes.

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characterized with immune complex deposits leading to lethal glomerulonephritis, the protective effect of G-CSF was mainly based on its anti-inflammatory properties. In experimental autoimmune encephalomyelitis (EAE) [8], a T-cell mediated demyelinating disease actively induced by immunization with myelin antigens, G-CSF treatment not only prevented, but also reversed, ongoing disease in SJL/J mice immunized with myelin basic protein (MBP). In this model, G-CSF exerted not only an anti-inflammatory effect (reducing TNF-α levels, thereby limiting early CNS infiltration and affecting macrophage chemokine production with a decreased MIP-1α (CCL3) over MCP-1 (CCL2) ratio), but also triggered an immune deviation of the autoreactive T-cell response characterized by reduced IFN-γ and increased IL-4 and TGF-β1 production. Interestingly, Lock et al. [9] recently confirmed the protective effect of G-CSF in the myelin-oligodendrocyte glycoprotein (MOG)-induced model of EAE in the C57/Bl6 mouse, and observed an increased expression of G-CSF gene transcript in multiple sclerosis lesions at the acute phase of the disease, suggesting a possible regulatory role for endogenous G-CSF.

It was therefore interesting to evaluate the therapeutic potential of G-CSF in another model of cell-mediated autoimmune disease. Having concentrated on an acute model of autoimmune type 1 diabetes accelerated by cyclophosphamide, we present evidence that G-CSF treatment protects the NOD mouse from the accelerating effects of CY. This protection required early administration of G-CSF and was mainly based on its immunoregulatory properties as it correlated with the accelerated recovery of the regulatory T cell subset within the peripancreatic lymph nodes (PLN), abrogation of the cytokine and chemokine burst triggered by CY, but only slight changes in the production of inflammatory chemokines within the pancreatic tissue.

## 2. Materials and methods

### 2.1. Mice and CY treatment

NOD/Thy1.2 ($K^d$, $I\text{-}A^{g7}$, $D^b$) mice were bred in our animal facilities under specific pathogen-free conditions. Seven-eight week-old male mice ($n=8$ or 10 mice per group) received one single injection of CY (Endoxan-Asta, Laboratoires Lucie, Colombes, France) i.p. at 200 mg/kg (1 mg/ml in saline). Colorimeric strips were used to monitor glycosuria (Glukotest, Boehringer-Mannheim, Mannheim, Germany) and glycemia (Haemoglukotest and Reflolux F, Boehringer-Mannheim).

### 2.2. G-CSF treatment

Recombinant human G-CSF (a gift from Amgen, Thousands Oaks, CA), which is active in mice [5], was injected s.c. at 200 μg/kg/day, a dose efficient in lupus and EAE experimental models [7–9]. Excipient consisted of 5% dextrose in sterile $H_20$.

### 2.3. Antibodies and FACS analysis

The cell surface phenotype of splenocytes was analyzed by flow cytometry. All cells were incubated in cold PBS supplemented with 2% FCS and 0.02% azide. One million cells per sample were pre-incubated in 20 μl for 15 min at room temperature, under constant shaking, with anti-Fc gamma receptors II/III (clone 2.4G2) to reduce non-specific binding, followed by mAbs: anti-CD4 (clone GK1.5), anti-CD8 (clone 53–6.7), anti-CD3 (clone 145-2C11), anti-αβTCR (clone H57–597), anti-B220 (clone RA3–6B2), anti-CD19 (clone 1D3), anti-Mac-1/CD11b (clone M1/70), anti-Gr-1 (clone RA3–8C5), anti-CD11c (clone HL3). They were produced in our laboratory from hybridomas or purchased from Pharmingen (BD Biosciences, Le Pont de Claix, France) and used coupled either to biotin and revealed by streptavidin-cychrome, or to PE or FITC. Ten thousand events were acquired using the FACScan flow cytometer (BD Biosciences). Analysis of the acquired data was performed using CellQuest software (BD Biosciences).

### 2.4. Histopathology analysis

Pancreases were removed and frozen in OCT. For conventional histopathology, serial 2 μm sections were stained with haematoxylin 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); grade 2 = invasive destructive insulitis.

### 2.5. Splenocyte subpopulation enrichment

$CD4^+$ cells were isolated (98% pure, confirmed by FACS analysis) with anti-CD4-labeled immunomagnetic cell sorting (MACS) microbeads (Miltenyi Biotec, Paris, France).

### 2.6. Cytokine and chemokine production analysis

$CD4^+$ cells isolated from the spleen, mesenteric or peri-pancreatic lymph nodes at day 10 after CY injection and treatment with either G-CSF or excipient, were activated ($2\times10^5$/well) in the presence of the anti-CD3 antibody (2.5 μg/ml final concentration) or PBS, in RPMI Glutamax medium supplemented with 5% fetal bovine serum (InVitrogen, Cergy Pontoise, France), 1% penicillin and streptomycin, and β-mercaptoethanol $5\times10^{-5}$ M, in 96-well culture plates. Supernatants for