Granulocyte colony-stimulating factor decreases tumor necrosis factor production in whole blood: role of interleukin-10 and prostaglandin E_2

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ABSTRACT. Previous reports have indicated that the administration of granulocyte colony-stimulating factor (G-CSF) decreases *ex vivo* tumor necrosis factor (TNF) production in humans. In this study, we report that daily pretreatment of mice with G-CSF for three days decreases *ex vivo* lipopolysaccharide (LPS)-induced TNF production in whole blood. Conversely, production of interleukin-10 (IL-10) and prostaglandin E_2 (PGE_2) is increased. The inhibitory effect of G-CSF pretreatment on TNF production is partially reversed by addition of an anti-IL-10 antibody, and completely reversed by combined addition of anti-IL-10 antibody and the cyclooxygenase (COX) inhibitor, ketoprofen. These results suggest that G-CSF decreases TNF production in this experimental model by increasing production of IL-10 and PGE_2, which are both known inhibitors of TNF production.

Keywords: G-CSF / IL-10 / endotoxin / PGE_2 / TNF

INTRODUCTION

There have been reports that granulocyte-colony stimulating factor (G-CSF) has anti-inflammatory properties in various animal models, including those for experimental colitis [1] and superantigen-induced shock [2]. More importantly, it has been reported that G-CSF-mobilized peripheral stem cell transplantation reduces the severity of acute graft-versus-host disease (GvHD) [3-5]. However, treatment of recipients with G-CSF after bone marrow transplantation has been associated with an increased incidence of GvDH [6, 7].

Administration of G-CSF to humans decreases inflammatory cytokine production, particularly tumor necrosis factor (TNF), by whole blood or dendritic cells *ex vivo* [5, 8-11], while it increases the production of cytokine antagonists such as interleukin-1 receptor antagonist (IL-1ra) and soluble TNF receptors [8, 9].

The aim of this work was to investigate the mechanism for the G-CSF-induced decrease of TNF production, *ex vivo*, using mice. In particular, we focused our attention on the role of endogenous mediators known to inhibit TNF production, interleukin-10 (IL-10) and prostaglandins. Our results indicate that G-CSF increases the production of IL-10 and prostaglandin E_2 (PGE_2), which in turn mediate the inhibition of lipopolysaccharide (LPS)-induced TNF production.

METHODS

Animals and treatments

Male CD-1 mice (20-25 g body weight) were purchased from Charles River (Calco, Italy). Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n° 116, G.U., suppl. 40, 18 Febbraio 1992, Circolare n° 8, G.U., 14 Luglio 1994) and international (EEC Council Directive 86/609, OJL 358, 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996) laws and policies. Mice were given 100 µg/kg s.c. of recombinant murine G-CSF (Amgen, Thousand Oaks, CA, USA) dissolved in saline, either once, one hour before bleeding, or daily for three consecutive days, and blood was withdrawn 24 hours after the last treatment. Control mice were given saline alone. Mice were bled by puncture of the retro-orbital plexus under ether anesthesia, and blood was collected over heparin (14 U/mL; Liquemin, Roche, Milan, Italy).

LPS-induced cytokine production in murine whole blood

Blood was then plated onto 96-well tissue culture plates (100 µL/well) and incubated for four hours at 37 °C in a 5% CO_2, humidified atmosphere, with or without 1 µg/mL of LPS (*Escherichia coli* 055:B5, Sigma, St. Louis, MO, USA). In some experiments, blood was stimulated in the

presence of 1 µM of ketoprofen (lysine salt, Dompé, L'Aquila, Italy) and/or 200 µg/mL of JES5-2A5 monoclonal antibody, a rat IgG1 neutralizing mouse IL-10 (a kind gift from T. Mosmann, University of Alberta, Edmonton, Canada), which were added to culture plates 15 min before LPS. At the end of the incubation, blood was diluted 1:1 (v/v) with ice-cold saline, centrifuged, and the supernatants were collected for TNF, IL-10 and PGE_2 determination.

TNF activity was measured by the degree of cytotoxicity on L929 cells, in the presence of 1 µg/mL actinomycin D, as previously described [12], using recombinant TNF as standard. The sensitivity of this assay is approximately 0.33 U/mL. PGE_2 and IL-10 were measured by EIA using commercially available kits from Amersham (Little Chalfont, UK) and BioSource International (Camarillo, CA, USA), respectively.

RESULTS

As reported in *table 1*, a three-day G-CSF pretreatment led to a marked increase in neutrophil (27-fold), monocyte (five-fold) and lymphocyte (two-fold) counts in peripheral blood 24 hours after the last treatment. In contrast, a single G-CSF pretreatment, one hour before bleeding, brought about only a small increase in the neutrophil count (G-CSF, 2.0±1.3 x 10^6/mL versus saline, 1.0±0.5 x 10^6/mL), with no difference in monocyte and lymphocyte counts.

We measured the production of TNF in whole blood obtained from G-CSF-treated mice after *ex vivo* stimulation with LPS. As shown in *figure 1* (left), TNF production was markedly reduced (by about 95%) in blood from mice pretreated with G-CSF daily for three days in comparison with saline-treated control mice. However, when mice were given a single dose of G-CSF, one hour before blood sampling, there was no significant change in TNF production (*figure 1*, right). No TNF was detectable in unstimulated blood (data not shown).

We then evaluated the production of two well-known inhibitors of TNF synthesis, IL-10 and PGE_2, in the blood of mice pretreated with G-CSF for three days. As shown in *figure 2*, the levels of both PGE_2 and IL-10 were similar in blood from both G-CSF and saline-treated mice in the absence of stimulation. However, LPS stimulation induced a two-fold increase in PGE_2 concentration and a 10-fold increase in IL-10 concentration in blood from G-CSF-treated mice in comparison with unstimulated blood. Nevertheless, we did not find any significant increase in either PGE_2 or in IL-10 levels in blood from saline-treated mice after LPS stimulation *(figure 2)*.

Table 1
Changes in peripheral blood leukocyte counts after a three-day G-CSF pretreatment

	Saline	G-CSF
Neutrophils (X 10^6/mL)	1.1 ± 0.4	29.8 ± 6.3 **
Monocytes (X 10^6/mL)	0.2 ± 0.05	0.8 ± 0.4 **
Lymphocytes (X 10^6/mL)	5.7 ± 1.3	14.0 ± 2.1 **

Mice were given rmG-CSF (100 µg/kg, s.c.) or saline for three consecutive days. Leukocytes were counted in blood samples taken twenty four hours after the last treatment. Data are mean ±SD (nine mice/group). ** p<0.01 *versus* saline by Duncan's test.

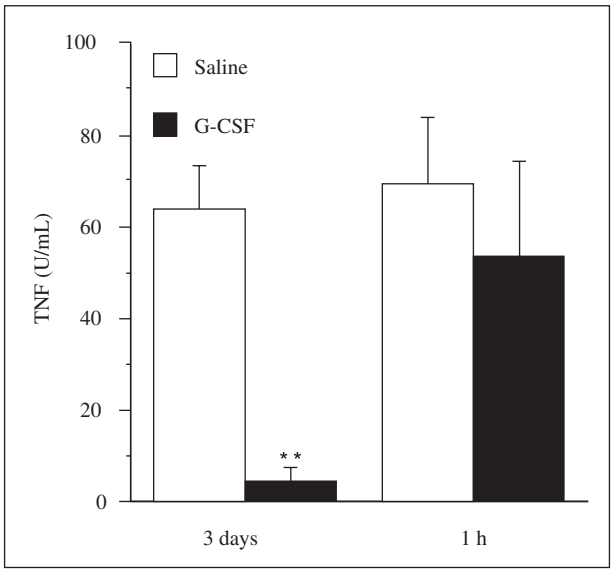

Figure 1
Effect of G-CSF pretreatment on LPS-induced TNF production *ex vivo* in mouse blood. Mice were pretreated with rmG-CSF (100 µg/kg, s.c.) or saline daily for three consecutive days (3 days, left) or once (1 h, right). Twenty four hours later, for the three day schedule, or one hour later, for the single administration, blood was taken and stimulated *in vitro* with LPS (1 µg/mL). TNF was measured in the supernatants after four hours of culture. No TNF activity was detectable in unstimulated blood (data not shown).
Data are mean ±SD (five mice/group). * p<0.05, ** p<0.01 *versus* saline by Duncan's test.

We also investigated whether G-CSF, added *in vitro* to whole blood from untreated mice at times up to one hour before addition of LPS and at concentrations ranging from 10 ng/mL to 10 µg/mL, could modulate LPS-induced TNF or IL-10 production, however we did not observe any significant effect (data not shown).

In order to investigate the role of prostaglandins and/or IL-10 in the decrease in TNF production induced by G-CSF pretreatment, we performed the blood stimulation in the presence of the cyclooxygenase (COX) inhibitor, ketoprofen, and a neutralizing anti-IL-10 antibody. We found that the IL-10 produced by whole blood under our experimental conditions was completely neutralized by the addition of the monoclonal antibody (data not shown); likewise, ketoprofen completely inhibited LPS-induced PGE_2 production in blood from G-CSF-treated mice (data not shown). As shown in *figure 3*, ketoprofen alone did not reverse the inhibition of TNF production by G-CSF, whereas the anti-IL-10 antibody significantly reversed this inhibition (from 94 % to 47 %). Notably, combined addition of ketoprofen and anti-IL-10 antibody completely restored TNF production. Similar results were obtained when indomethacin was used as the COX inhibitor instead of ketoprofen (data not shown).

DISCUSSION

The results reported here demonstrate that G-CSF inhibits the ability of blood cells to produce TNF by increasing the production of IL-10 and PGE_2. It is important to note that these are not direct effects of G-CSF. In fact, neither short-term pretreatment with G-CSF for one hour nor addition of G-CSF *in vitro* to whole blood in culture, had

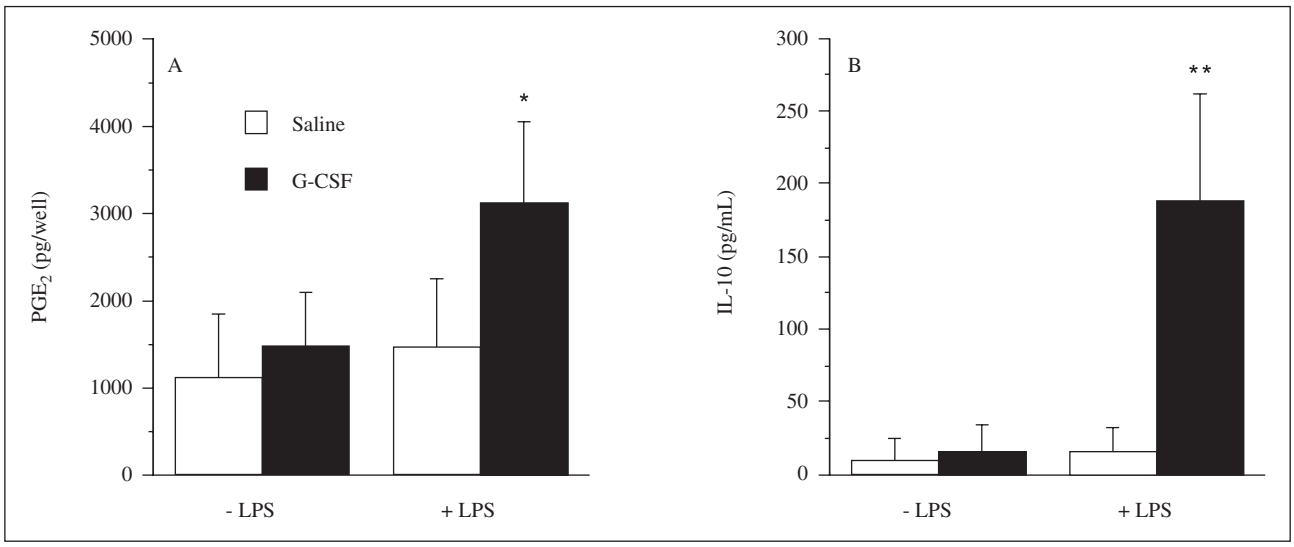

Figure 2

Effect of G-CSF administration on *ex vivo* PGE_2 (A) and IL-10 (B) production in mouse blood. Mice were pretreated with rmG-CSF (100 µg/kg, s.c.) or saline for three consecutive days. Twenty four hours after the last G-CSF administration, blood was taken and was either stimulated *in vitro* with 1 µg/mL LPS (+LPS) or left unstimulated (-LPS). IL-10 and PGE_2 were measured in the supernatants four hours later.
Data are mean ±SD (five mice/group). ** $p<0.01$ *versus* saline by Duncan's test.

any significant effect on TNF production. Since one hour pretreatment impacted leukocyte counts only minimally, while three days pretreatment had a marked effect, it is possible that the increased production of IL-10 and PGE_2 induced by G-CSF is a consequence of the changes in cell populations brought about the three-day G-CSF pretreatment.

Similar studies performed in humans reported that G-CSF-mobilized peripheral blood mononuclear cells (PBMC) display a decreased cell proliferation in response to alloantigen, in comparison with control PBMC [13]. This hyporesponsiveness has been ascribed to an increased production of IL-10 by $CD14^+$ monocytes in G-CSF-treated patients [14].

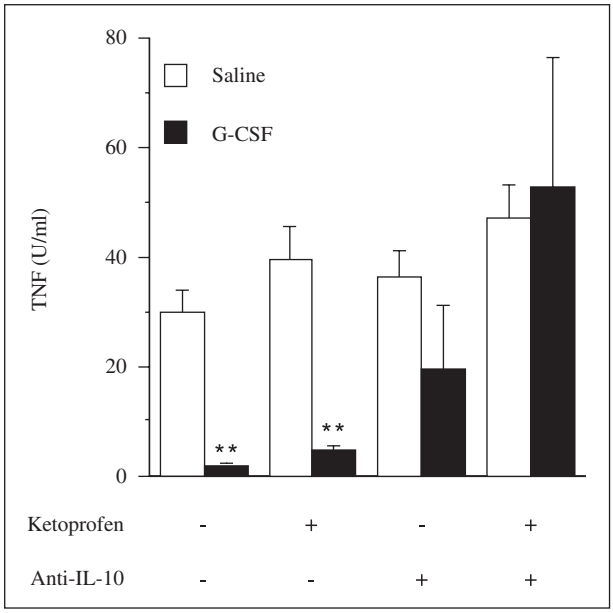

Figure 3

Reversal of G-CSF-induced inhibition of TNF production by ketoprofen and anti-IL-10 antibody. Mice were pretreated with rmG-CSF (100 µg/kg, s.c.) or saline for three consecutive days. Blood was taken twenty four hours after the last treatment. Blood samples from each animal were treated *in vitro* with ketoprofen (1 µM) or neutralizing anti-IL-10 antibody (200 µg/mL) or both. After 15 min, samples were stimulated with 1 µg/mL LPS, and TNF production was measured after four hours of culture.
Data are mean ±SD (five mice/group). ** $p<0.01$ *versus* saline by Duncan's test.

The importance of IL-10 and PGE_2 as negative feedback regulators of TNF production has been widely documented in reports showing that TNF production, both *in vivo* and *in vitro*, is increased by anti-inflammatory drugs acting as COX inhibitors [15, 16] and by inhibition of IL-10 using antibodies or knock-out mice [17-19].

At first it may seem surprising to consider PGE_2 as an anti-TNF mediator, since prostaglandins are well known inflammatory molecules. On the other hand, the paradoxical increase of TNF production by non-steroidal anti-inflammatory drugs has been extensively documented in animals [20, 21] and in human volunteers [22]. Furthermore, while evaluating the inflammatory versus anti-inflammatory (anti-TNF) activities of PGE_2, it is important to consider the relative contribution of PGE_2 and IL-10 to the TNF inhibitory effect of G-CSF. In fact, in our experimental model, IL-10 seems to be far more important than PGE_2 as an inhibitor of TNF production, on two counts: 1) G-CSF pretreatment increased IL-10 production by a factor of 10, while the production of PGE_2 was increased only by a factor of two; and 2) the anti-IL-10 antibody alone markedly reversed inhibition of TNF production by G-CSF, while ketoprofen had no effect alone and only showed effects in combination with the anti-IL-10 antibody.

In conclusion, our data provide additional information for the interactions between IL-10 and PGE_2, which should not be overlooked when considering the role of these mediators as feedback inhibitors. In particular, it is known that while IL-10 inhibits prostaglandin production, which contributes to the antiinflammatory action of IL-10, [23, 24], PGE_2 induces IL-10 production [24, 25], an observation that fits well with the negative feedback described in the present paper.

If one considers that increased IL-10 production was observed in G-CSF-mobilized mononuclear cells [14], it is possible that the mechanism elucidated in our animal model might explain the inhibitory action of G-CSF on TNF production in humans.

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