Effect of Granulocyte Colony-Stimulating Factor Treatment on Ex Vivo Blood Cytokine Response in Human Volunteers

By Thomas Hartung, Wolf-Dietrich Döcke, Florian Gantner, Gerhard Krieger, Achim Sauer, Paul Stevens, Hans-Dieter Volk, and Albrecht Wendel

We explored the ex vivo alterations in the cytokine release of stimulated blood taken from healthy volunteers treated subcutaneously with 480 μg granulocyte colony-stimulating factor (G-CSF). In a double-blind, controlled, randomized study with 21 volunteers who received G-CSF once or twice 24 hours apart, we measured lipopolysaccharide (LPS)-inducible release of various cytokines and soluble receptors at different times after treatment. At day 1 after a single dose of G-CSF, mediator release was also initiated with muramyl dipeptide, Staphylococcus aureus enterotoxin A, lipoteichoic acid, streptolysin O, complement factor C5a, phytohemagglutinin, or phorbol myristate acetate. In blood from G-CSF-treated subjects, our major findings were (1) a maximal 12-fold increase in interleukin-1 receptor antagonist (IL-1ra) release and an increase of both the p55 and p75 soluble tumor necrosis factor (TNF) receptors; (2) a reduction in TNF release when using all the various stimuli described except LPS; (3) an increase in G-CSF and, to lesser extent, in IL-6, IL-8, and IL-10 release; and (4) an attenuation of interferon-γ (IFN-γ) and granulocyte-macrophage (GM)-CSF release. Our findings demonstrate that the major effect of G-CSF treatment is a change in the responsiveness of blood towards a variety of stimuli, which we interpret as a shift toward an antiinflammatory cytokine response.

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COLONY-STIMULATING factors (CSFs) were originally identified as hematopoietic growth factors. They are now increasingly recognized as modulators of acute inflammation. During the clinical use of granulocyte (G)-CSF (Neupogen; Filgrastim, Hoffman-La Roche, Basel, Switzerland) it was noticed in neutropenic patients that the drug reduced the incidence of infections and the need for antibiotics.1 This observation has led to the consideration of G-CSF for prophylaxis or even treatment of non-neutropenic sepsis.2,3 The fact that endogenous G-CSF serum levels are increased 30-fold in patients with severe infection4 supports the interpretation that a physiologic defense mechanism may exist that can be reinforced or shifted in time by pharmacologic intervention. Some plausible mechanisms for this pharmacodynamic property of G-CSF in reducing infection have been identified, such as recruitment of granulocytes from the bone marrow,5 shortening of leukopenia initially associated with severe infection,6 reduction of emergence time,7 and priming of neutrophils for enhanced bacterial killing.8 Various complementary animal studies have been reported that demonstrate that G-CSF treatment ameliorates the outcome in different models of infection.9-12

Septic shock, recently redefined as systemic inflammatory response syndrome (SIRS),13 is a hazardous complication of infection resulting in septic organ failure caused by an overactivation of the nonspecific immune system, mainly macrophages and neutrophils. Based on this view, CSFs are anticipated to further overactivate this system in sepsis by recruiting and priming leukocytes. However, we found that G-CSF pretreatment was protective in non-neutropenic rodents against LPS-induced septic organ failure,11 ie, in an animal model where microbial killing by granulocytes is not required. It is well established that the macrophage product tumor necrosis factor (TNF)-α is a distal mediator of septic organ failure.12 In our animal study, we found that G-CSF pretreatment attenuated lipopolysaccharide (LPS)-inducible serum TNF concentrations in vivo, as well as ex vivo LPS-inducible TNF release of different macrophage populations,14 while there was no similar effect of G-CSF in vitro on macrophage cultures. These results indicate that G-CSF treatment presumably alters the septic cytokine response indirectly by undefined factors or effector cells.

It is now of interest to determine whether G-CSF treatment results in similar alterations of the cytokine response of human peripheral white blood cells to inflammatory stimuli. The approach chosen consisted of an in vivo treatment of healthy volunteers with G-CSF followed by ex vivo exposure of whole blood toward defined stimuli. To cover a broad spectrum of stimuli of host defense against infectious challenge, a variety of model compounds other than the Gram-negative cell wall component LPS was used in the in vitro experiments. These include the bacterial stimulus muramyl dipeptide (MDP), the Gram-positive stimulus lipoteichoic acid (LTA), the superantigen Staphylococcus enterotoxin A (ETA), the ion channel opener streptolysin O (SLO), the signal transduction activator phorbol ester (PMA), and general immune stimuli, such as the lectin phytohemagglutinin (PHA) and the complement component C5a. This explorative design allowed collection of a maximum of basic mechanistic information without exposing humans directly to these agents.

The results of our investigation demonstrate that in humans G-CSF induces a switch in the responsiveness of blood leukocytes toward bacterial stimuli, which is characterized by an attenuated release of some proinflammatory cytokines as well as by an increased production of antiinflammatory counterregulatory molecules, such as interleukin-1 receptor antagonist (IL-1ra) and the soluble TNF receptors.
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day -2 1 2 3 4 5 8
blood incubations

G-CSF 480 ug or vehicle s.c.

Fig 1. Schematic representation of the design of the study. Three groups of seven volunteers each were randomized according to age, weight, and sex. The volunteers were treated double-blind, 24 hours apart, twice with placebo (placebo), twice with 480 µG G-CSF subcutaneously (s.c.), or G-CSF at day 1 and placebo at day 2. Blood was withdrawn at the time points indicated, and cytokine release was initiated ex vivo.

MATERIALS AND METHODS

Study design. The study was conducted in accordance with the precepts of the Helsinki declaration. The protocol was approved by the Institutional Review Board of the University of Konstanz (Konstanz, Germany). Twenty-one volunteers (students of the University of Konstanz; 13 male and eight female) were checked by physical examination and routine clinical chemistry to exclude major diseases, especially any acute infections. Three groups of seven subjects were randomized according to age, weight, and sex. This distribution was checked for homogeneity of white blood cell (WBC) count. No subgroup differed significantly from the following overall averages of the whole group: age, 25 ± 2 years; weight, 74 ± 11 kg; sex, two to three females per group; and initial WBC count, 5.9 × 10^9/mL ± 1.2 × 10^9/mL blood. The study design is shown in Fig 1.

On day 1, pretreatment blood was withdrawn and either 480 µg Neupogen (Hoffmann-LaRoche, Basel, Switzerland) or solvent placebo was given subcutaneously immediately. This treatment was repeated on the next day at the same time. Blood was withdrawn after 8 hours (noted as day 1, pm, in the figures), at 24 hours (immediately before the second treatment), at 32 hours (noted as day 2, pm), at 48 hours, at 72 hours, at 96 hours, and at 168 hours after the first treatment.

Incubations with LPS as stimulus at days 1 to 5. WBC counts were assessed at each time point of blood collection using a Coulter STKS (Coulter, Krefeld, Germany). Without any further separation of cells, whole blood incubations were performed as described10 with 20% citrate blood in RPMI 1640 medium (Biochrom, Berlin, Germany) plus 2 IU/mL heparin (Liquemins; Hoffmann-LaRoche), ie, under recalcified conditions. Blood was stimulated immediately with 10 µg/mL endotoxin (LPS) from Salmonella abortus equi (Sigma, Deisenhofen, Germany) for 4 or 24 hours at 37°C and 5% CO2. The plateau concentration of 10 µg/mL was chosen as standard stimulus in the ex vivo incubations to overcome varying LPS-binding capacity (such as LPS-binding protein, LBP), bactericidal permeability-increasing protein (BPI), and soluble CD14 (sCD14) in blood from different donors and to maximally activate leukocyte cytokine release (ie, to measure cytokine release capacity). A parallel control incubation lacking LPS for every assay was performed. After the incubation period, blood cells were sedimented by centrifugation (2,000g, 3 minutes). Cell-free supernatants were frozen at −80°C until cytokine measurement.

Incubations with other stimuli at day 2. At day 2 (ie, 24 hours after the first G-CSF dose and before the second dose), we conducted the following in vitro experiments. Stimuli other than LPS were incubated with blood at the following fixed concentrations: MDP, 10 µg/mL; ETA, 1 µg/mL; LTA, 100 µg/mL; SLO, 2.5 hemolysing units; C5a, 100 ng/mL; PHA, 15 µg/mL; and PMA; 100 nmol/L. All stimuli were purchased from Sigma except MDP, which was from Bachem (Heidelberg, Germany). In the presence of each stimulus, blood from seven treated and seven placebo donors was incubated for 4 hours; in the case of TNF, 24-hour incubations were performed because TNF release by stimuli other than LPS was slow. Pooled samples from seven individual incubations were measured. Thus, data represent the average from seven individuals. Inhibition (TNF) was calculated as the percentage of reduction in immunoreactive free TNF (Quantikine assay; Biomerieux, Bad Nauheim, Germany); similarly, increase [soluble TNF receptors (sTNF-Rs) and IL-1ra] was calculated as the multiplication factor of release from G-CSF–treated compared with placebo-treated donor blood. The more sensitive enzyme-linked immunosorbent assay (ELISA; Quantikine) for sTNF-R p75 used in this experiment detected significant amounts of this factor also in stimulated placebo donor blood.

Kinetics of cytokine release at day 2. To test whether G-CSF treatment had any influence on the kinetics of factor release, the time course of cytokine release induced by LPS was determined in five placebo– and five G-CSF–treated donors. Blood was incubated for 1, 2, 4, 6, 8, 12, 18, and 24 hours in the presence of 10 µg/mL LPS.

In vitro incubation with G-CSF. The in vitro effect of G-CSF on LPS-inducible sTNF-R and IL-1ra release was assessed by preincubating the blood of five placebo donors for 2, 4, or 10 hours with 1, 10, or 100 ng/mL G-CSF before LPS challenge with 10 µg/mL for a further 4 hours.

Transfer experiments with granulocytes at day 2. To mimic granulocytosis, we added granulocytes from either placebo (n = 4) or treated donors (n = 4) to placebo blood (n = 4). Granulocytes were prepared using Ficoll/Hypaque (Monopoly resolving medium; ICN, Meckenheim, Germany) according to the product information. Blood smears exhibited 96% neutrophil granulocytes. Polymorphonuclear cells (PMN; 2 × 10^5) from either treatment group were resuspended in 1 mL RPMI 1640 or readded to 1 mL diluted placebo donor blood (200 µL blood plus 800 µL RPMI 1640), ie, at a concentration of 10^4 PMN per milliliter of blood and stimulated concurrently. PMN and blood donors were blood-group-matched. Each experiment was performed in quadruplicate.

Cytokine determinations. TNF was assessed by three commercial ELISA kits (Medgenix: Ratingen, Germany; Cistron: Biomar, Marburg, Germany; and Quantikine: Biermann, Germany). The Medgenix assay was previously shown to measure total TNF11,12 including the amount of TNF bound to the soluble receptor and cleavage products of TNF. In contrast, we interpret the results of the Quantikine ELISA as free TNF for the following reasons. First, supernatants from PMA-activated human neutrophils containing high amounts of shedded sTNF-Rs interfered with TNF detection by the Quantikine assay. Second, the Quantikine assay correlated perfectly (r = 0.96, n = 80) with a Cistron ELISA (Biomar) described to measure free TNF only,13 while the correlation of the Quantikine assay with the Medgenix assay was lower (r = 0.62, n = 80). Third, in whole blood, both the Quantikine and the Cistron ELISAs detected only about 40% of the amount of TNF determined with the Medgenix assay. IL-1β, IL-1ra, IL-3, IL-4, IL-6, soluble IL-6 receptor (sIL-6R), IL-8, and the sTNF-Rs p55 and p75 were also measured by ELISAs.
(QuantiKine), as well as sTNF-R p75 (Bender Systems, Serva, Heidelberg, Germany). As measured by the Bender assay, sTNF-R p75 was detectable in neither LPS-stimulated nor control cell supernatants. However, in experiments using the more sensitive ELISA (QuantiKine), we realized that ≤0.5 ng/mL sTNF-R p75 was present in unstimulated blood from control donors and that 1 ng/mL sTNF-R p75 was detectable in LPS-stimulated control blood (data not shown). Other cytokine ELISAs were purchased as follows: IL-2 and interferon-γ (IFNγ; Endogen, Biomar), soluble IL-2 receptor (sIL-2-R; Cellfree, Biermann), IFNα (GIBCO, Eggenstein, Germany), and IL-10 (Advanced Magnetics, Biermann). Transforming growth factor-β1 (TGF-β1) was determined by radioimmunoassay (RIA; NEN Dupont, Dreieich, Germany).

Endotoxin was measured using a chromogenic limulus assay (Coattest; Chromogenix Haemochrom Diagnostica GmbH, Essen, Germany); 1 ng of LPS from Escherichia coli 0111:B4 corresponds to 12 IU.

Statistical analysis. Statistical analysis was performed using the two-tailed Wilcoxon significance test (option of the GraphPad Instat program, release 1.13, Inset Systems, San Diego, CA). The level of significance was set to $P < .05$. Data are given as means ± SEM. Data were corrected for the dilution factor of 5 (20% of blood was stimulated) and were expressed per milliliter of blood.

RESULTS

Three groups of seven volunteers each were treated double-blind with placebo twice, G-CSF twice (double G-CSF), or G-CSF at day 1 and placebo at day 2 (single G-CSF). G-CSF treatment was well tolerated by the volunteers. Mild bone pain was reported by nine subjects (one placebo recipient, eight treatment recipients), four persons reported headache or fatigue (one placebo recipient, three treatment recipients), and a single case of tachycardia was observed (treatment recipient).

Leukocyte alterations. Single G-CSF treatment resulted in a significant increase in neutrophil granulocyte (PMN) counts, which reached a maximum at 24 hours after a single injection and at 36 hours after the first injection in the group receiving G-CSF twice (Fig 2A). These findings confirm the known major effect of G-CSF on human peripheral granulocytes. We observed also a significant twofold increase in circulating monocytes, with a maximum as early as 8 hours after a single G-CSF injection that was maintained until day 3 after double G-CSF treatment (Fig 2B). No significant differences between the placebo group and either treatment group were observed with respect to lymphocyte or eosinophil granulocyte numbers (data not shown).

LPS-induced mediator release on days 1 to 5. Factor release on different days after treatment was measured with LPS-challenged blood of all individuals on study. When calculated per milliliter of blood, there was no significant difference between the different treatment groups in the following parameters: free TNFα (9.6 ± 1.1 ng/mL within 4 hours), determined with a Quantikine or Cistron ELISA, or total TNF (20.2 ± 1.7 ng/mL within 4 hours), measured using a Medgenix assay. There was also no significant difference between the G-CSF- and placebo-treated groups in LPS-inducible release of IL-1α (2.0 ± 0.3 ng/mL within 4 hours).

However, with LPS stimulation, a sixfold increase in TNF-R p55 release 24 hours after single G-CSF treatment was observed, which was prolonged by 1 day in the double G-CSF-treated group (Fig 3A). Blood taken from the single G-CSF-treated group also released significant amounts of sTNF-R p75 on days 2 and 3 after treatment. This effect was again prolonged by 1 day in the double G-CSF-treated group (Fig 3B). As shown in Fig 3C, LPS also initiated a greatly enhanced release of IL-1ra in blood from G-CSF-treated donors compared with the placebo group that was most pronounced at day 3 in the double G-CSF-treated group. All parameters measured in this time course study had returned to control values by day 5 after single or double treatment.

To test whether a possible change in the kinetics of ex vivo cytokine release had occurred because of G-CSF treatment, the time courses of LPS-inducible TNF, IL-1, sTNF-R p55, sTNF-R p75, and IL-1ra were measured. Although the absolute amounts of factors released differed between the placebo and G-CSF-treated group (24 hours after single treatment) in some cases, the kinetics were not significantly different.

The role of cell numbers in ex vivo incubations. The fate of LPS added to blood during the incubation period was checked to exclude different binding or uptake of LPS caused by WBC differences. Of the initial LPS concentration of 10 μg/mL (7.7 × 10⁸ ± 0.4 × 10⁷ IU) that was added to blood after 4 hours of incubation, 5.5 μg/mL (4.2 × 10⁸ ± 0.4 × 10⁷ IU)
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Fig 3. Time course of ex vivo sTNF-R or IL-1ra release initiated with 10 μg/mL LPS in blood from volunteers treated once or twice with 480 μg G-CSF or placebo. Citrate blood was taken at the time points indicated from healthy volunteers. At days 1 and 4, 480 μg G-CSF or placebo was injected subcutaneously. Whole blood was incubated in the presence of 10 μg/mL LPS for 4 hours, and inducible mediators were measured in the supernatants by ELISA. *P ≤ .05 compared with placebo; #P ≤ .05, comparing double and single treatments.

Table 1. LPS-Stimulated Incubations With Granulocytes From Placebo- or Single G-CSF–Treated Donors Transferred to Placebo-Treated Blood

<table>
<thead>
<tr>
<th></th>
<th>sTNF-R p55</th>
<th>sTNF-R p75</th>
<th>IL-1ra</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo-treated blood</td>
<td>5 ± 1</td>
<td>9 ± 3</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>Placebo-treated PMN (107/mL)</td>
<td>4 ± 1</td>
<td>&lt;0.4</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Single G-CSF–treated PMN (107/mL)</td>
<td>24 ± 7*</td>
<td>16 ± 9*</td>
<td>13 ± 3*</td>
</tr>
<tr>
<td>Placebo-treated blood + placebo-treated PMN</td>
<td>11 ± 1</td>
<td>18 ± 5</td>
<td>33 ± 10</td>
</tr>
<tr>
<td>Placebo-treated blood + single G-CSF–treated PMN</td>
<td>25 ± 9*</td>
<td>27 ± 9</td>
<td>163 ± 41*</td>
</tr>
</tbody>
</table>

Blood was taken 24 hours after subcutaneous treatment with 480 μg G-CSF or placebo. PMN (2 × 10⁷) isolated from four individuals of both treatment groups were either resuspended in 1 mL RPMI 1640 or in 200 μL placebo-treated blood plus 800 μL RPMI 1640. All incubations were stimulated concurrently with 10 μg/mL LPS for 4 hours. Data are means ± SEM and are expressed as ng/mL blood. *P ≤ .05 compared with placebo PMN or placebo-treated blood plus placebo-treated PMN, respectively.

10⁷ IU) was found in placebo blood and 4.7 μg/mL (3.6 × 10⁷ ± 0.1 × 10⁷ IU) in blood from G-CSF–treated individuals. Also, after 24 hours of incubation, no significant difference in LPS concentrations in the two groups was observed, although the total content had dropped to 2.6 × 10⁷ ± 0.6 × 10⁷ IU. These data show that no differences in LPS concentrations during stimulation of blood might have influenced the factor release measured in our blood incubations.

Transfer experiments were then performed to examine whether or not the observed effects of G-CSF treatment were due to changes in cell numbers only. When the same number of isolated PMN from either G-CSF–treated donors (single dose, 24 hours before) or placebo-receiving donors was stimulated by LPS, the cells from G-CSF blood released greater amounts of factors compared with placebo donor cells (Table 1). These findings demonstrate that isolated PMN from placebo and G-CSF blood differ in their capacity to release cytokine antagonists after stimulation. Next we added a fixed number of 10⁷ isolated PMN prepared from either treatment group to 1 mL of undiluted placebo donor blood. The data in Table 1 show that addition of placebo PMN to placebo blood alone significantly enhanced sTNF-R and IL-1ra release in the presence of LPS. This increase is likely to represent enhanced release due to additional PMN added to the sample. If transferred to placebo donor blood, the G-CSF–elicited population of cells released significantly more IL-1ra after LPS stimulation than it had released without whole blood. Thus, PMN from G-CSF–treated donors have greater IL-1ra release capacity than those prepared from placebo donors. These findings suggest that an increase in cell count as well as the ability of an increased release capacity come together to account for the overall effect of increased ex vivo factor release in blood from G-CSF–treated donors. A similar conclusion can be drawn from the data calculated on a release capacity per cell basis (see Discussion).

In volunteers treated with 480 μg G-CSF subcutaneously, the maximum blood peak level of G-CSF that can be reached is estimated to be 50 ng/mL. To test for a direct effect of G-CSF on blood leukocytes, placebo donor blood was preincubated in vitro in the presence of 1, 10, or 100 ng/mL G-CSF for 2, 4, or 10 hours before it was incubated for a further 4 hours in the presence of LPS. Preincubation in the absence of G-CSF did not significantly affect the LPS-inducible release of sTNF-R and IL-1ra. However, all three concentrations of G-CSF used resulted in a mean 2.3-fold increase in IL-1ra at any preincubation time (2.28 ± 0.20; P
Day priming of PMN by G-CSF. Measurements of total TNFα in the samples generated essentially the same data as supernatants of each treatment group were pooled before assessing immunoreactive free TNFα. Using any of these release (Table 2). It should be noted that throughout, the amount of G-CSF recovered from the injection was subtracted from the amount determined after endogenous G-CSF in blood from G-CSF-treated sub-

Table 2. Factor Release Initiated by Various Stimuli From Whole Blood of Single G-CSF-Treated Volunteers (24 hours after G-CSF injection)

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Placebo</th>
<th>Single G-CSF</th>
<th>Inhibition (%)</th>
<th>Placebo</th>
<th>Single G-CSF</th>
<th>Increase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDP</td>
<td>16.6</td>
<td>7.8</td>
<td>53</td>
<td>7.8</td>
<td>14.4</td>
<td>185</td>
</tr>
<tr>
<td>ETA</td>
<td>10.6</td>
<td>0.6</td>
<td>95</td>
<td>10.1</td>
<td>24.6</td>
<td>224</td>
</tr>
<tr>
<td>LTA</td>
<td>25.1</td>
<td>2.0</td>
<td>92</td>
<td>9.2</td>
<td>22.7</td>
<td>247</td>
</tr>
<tr>
<td>SLO</td>
<td>7.8</td>
<td>4.8</td>
<td>38</td>
<td>8.7</td>
<td>10.7</td>
<td>193</td>
</tr>
<tr>
<td>C5a</td>
<td>3.7</td>
<td>1.8</td>
<td>53</td>
<td>8.2</td>
<td>18.5</td>
<td>228</td>
</tr>
<tr>
<td>PHA</td>
<td>11.2</td>
<td>7.0</td>
<td>38</td>
<td>8.2</td>
<td>26.7</td>
<td>327</td>
</tr>
<tr>
<td>PMA</td>
<td>10.8</td>
<td>4.9</td>
<td>55</td>
<td>9.6</td>
<td>40.1</td>
<td>420</td>
</tr>
<tr>
<td>LPS</td>
<td>13.4</td>
<td>13.0</td>
<td>3</td>
<td>11.6</td>
<td>33.0</td>
<td>285</td>
</tr>
</tbody>
</table>

Average 53 ± 11* 266 ± 27* 248 ± 16* 1,386 ± 162*

Whole blood was taken 24 hours after treatment of volunteers with placebo or 480 μg G-CSF subcutaneously (n = 7 per group), and cytokine release was initiated by addition of MDP (10 μg/mL), ETA (1 μg/mL), LTA (100 μg/mL), SLO (2.5 U/mL), C5a (0.1 μg/mL), PHA (15 μg/mL), PMA (0.1 μmol/L), or LPS (10 μg/mL). Data represent determinations performed in pooled blood samples that were incubated individually. The average calculated was the mean ± SEM of all stimuli used.

* Significant (P < .05) compared with placebo.

= .0002). In contrast, a marginal 1.2-fold increase in sTNF-R p75 release (1.20 ± 0.06; P = .01) and no significant effect on sTNF-R p55 release (1.10 ± 0.09; not significant). The ex vivo release of G-CSF on IL-1α release capacity is of a similar extent as the ex vivo increase in LPS-inducible IL-1α calculated per PMN. Thus, the observed increase in LPS-inducible IL-1α formation per cell might be due to direct priming of PMN by G-CSF.

Ex vivo mediator release induced by various stimuli on day 2. To check whether or not these phenomena were restricted to LPS only, blood was taken from either placebo– or single G-CSF–treated donors at 24 hours after injection, and TNF release was initiated by addition of a variety of host defense stimuli in vitro. After incubation for 24 hours in the presence of the different stimuli, the whole blood supernatants of each treatment group were pooled before assessing immunoreactive free TNFα. Using any of these stimuli except LPS, release of free TNFα into the supernatants was diminished in blood from G-CSF–treated subjects (Table 2). The greatest difference in TNF release between placebo and treatment groups was observed when LTA or ETA had been incubated with blood. Measurements of total TNFα in the samples generated essentially the same data as obtained for free TNFα (52% ± 10% inhibition). When IL-1β was assessed in the same samples, no significant reduction was found (6% ± 3% inhibition).

We also checked whether similar results would be obtained for the soluble receptors or the receptor antagonist. Data shown in Table 2 show that stimuli other than LPS also induced an enhanced soluble receptor release in blood from G-CSF–treated donors. This was also true for IL-1α release (Table 2). It should be noted that throughout, the enhancement of IL-1α release (Fig 3C, Table 2) was quantitatively the most pronounced alteration observed in either treatment group of the study.

Measurements of other cytokines and soluble receptors. Various additional cytokines and soluble receptors were determined in LPS-stimulated blood of all 21 volunteers at each time point of study. Data from day 1 (pretreatment) and day 2 (24 hours after first treatment) are given in Table 3. LPS-inducible ex vivo cytokine release doubled in the case of IL-6 (P = .0003) and IL-8 (P = .0064) 1 day after single G-CSF. LPS-inducible ex vivo IL-10 release doubled (P = .0013) 8 hours after G-CSF injection, but was not significantly different at any other time point. In contrast, LPS-inducible ex vivo IFN-γ release was decreased in blood from G-CSF–treated subjects (P = .02). In blood from G-CSF–treated donors, a decrease in LPS-stimulated GM-CSF release of about 50% was also noted (P = .003). We also determined possible alterations of the LPS-inducible release of endogenous G-CSF in blood from G-CSF–treated subjects. Therefore, the amount of G-CSF recovered from the injection was assessed in control incubations without stimulation and was subtracted from the amount determined after stimulation. As early as 8 hours after single G-CSF treatment, a dramatic increase in LPS-stimulated ex vivo G-CSF release capacity was observed in blood from single G-CSF–

Table 3. LPS-Inducible Release of Various Cytokines by Whole Blood Withdrawn 24 Hours After Single G-CSF Treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pretreatment</th>
<th>Posttreatment (24 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>Single G-CSF</td>
<td>Placebo</td>
</tr>
<tr>
<td>IL-6</td>
<td>4.3 ± 1.0</td>
<td>4.5 ± 0.5</td>
</tr>
<tr>
<td>IL-8</td>
<td>41 ± 10</td>
<td>40 ± 5</td>
</tr>
<tr>
<td>IL-10</td>
<td>2.9 ± 0.2</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>34.9 ± 11.4</td>
<td>28.8 ± 5.2</td>
</tr>
<tr>
<td>G-CSF</td>
<td>4.4 ± 0.5</td>
<td>4.1 ± 0.5</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>0.15 ± 0.04</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>5.4 ± 1.5</td>
<td>4.9 ± 0.8</td>
</tr>
</tbody>
</table>

Whole blood was taken from seven placebo-treated and 14 G-CSF–treated volunteers (immediately before treatment and 24 hours after subcutaneous injection) and incubated in the presence of 10 μg/mL LPS for 24 hours. Data are means of individual incubations ± SEM (ng/mL blood within 24 hours).

* Significant (P < .05) compared with placebo.
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treated volunteers (25.6 ± 2.5 ng/mL compared with 3.3 ± 0.7 ng/mL for placebo blood; P < .0001), which was no longer seen in blood at 24 hours after G-CSF injection (Table 3).

The following no-effect data were obtained. For serum TGFβ1 (2.2 ± 0.2 ng/mL), no significant differences were found between placebo- and either G-CSF–treated group at any time point investigated. There was no additional LPS-stimulated TGFβ1 release. In unstimulated blood from placebo donors, significant amounts of sIL-6-R were detected (13.4 ± 0.6 ng/mL), which were altered neither by G-CSF treatment nor by in vitro LPS stimulation. IL-2 (greater than 25 pg/mL), sIL-2-R (greater than 120 IU/mL), IL-3 (greater than 50 pg/mL blood), IL-4 (greater than 50 pg/mL) or IFN-α (greater than 125 pg/mL) release was not detectable in supernatants of LPS-stimulated 20% blood incubations from controls or from G-CSF–treated donors.

DISCUSSION

Circumstantial evidence from clinical observations as well as from animal studies makes G-CSF an attractive pharmacologic candidate for damping the overactivation of the non-specific immune response to bacterial stimulation during the initiation phase of sepsis. The major products of the immune response are cytokines, whose circulating blood levels have been shown to correlate with the severity of septic complications in patients. Measuring changes in cytokine response to obtain primary information for studying the possible anti-inflammatory potential of G-CSF, however, would require initiation of the host response in vivo, i.e., exposure of healthy humans to potentially harmful agents. To avoid such hazards, an ex vivo approach was chosen that used whole blood incubations and has the advantages of natural cell environment, few preparation artefacts, and practical performance. The ex vivo cytokine release capacity obtained by this experimental design represents a stable measure of cytokine derangements caused by in vivo treatment with the test drug and allows an estimate of the changes in host defense status of the subjects. G-CSF is considered to represent a lineage-specific factor acting on PMN and their precursors. However, our study also demonstrated a significant increase in peripheral monocytes. In general, blood leukocytes represent a rather transient reservoir of immunocompetent cells compared with the actual inflammatory tissue leukocytes. Our primary analysis of data was based on net production of cytokines or soluble receptors per milliliter blood. However, by stimulating blood leukocyte as a model of the activated tissue leukocyte actually involved in the inflammatory reaction, it seems also appropriate to calculate the mediator release capacity per cell as an estimate for the individual contribution of a cell population to a net reaction. Due to the monocytosis induced by G-CSF treatment, a reduction of cytokine release per monocyte can be deduced: for example, 8 hours after first treatment (i.e., at the time point of maximal increase in monocyte numbers after G-CSF injection), LPS-inducible free TNF release was 29 ± 4 ng/10⁶ monocytes in the placebo group and 18 ± 1 ng/10⁶ monocytes in blood from single G-CSF–treated donors (P = .03). The corollary of these calculations is that an attenuated release capacity of TNF per macrophage similar to the one caused in monocytes by G-CSF treatment could then be of importance as inflammatory foci. Similar calculations performed for the monokines IL-1β and macrophage inflammatory protein 1α (MIP1α) did not reveal such differences in factor release per cell between treatment and placebo groups at 8 hours after G-CSF injection. However, the increase in G-CSF release capacity continued to be significant when corrected for monocyte count (placebo group, 0.6 ± 1.2 ng/10⁶ monocytes, compared with G-CSF–treated donor blood, 25.4 ± 2.7 ng/10⁶ monocytes; P = .0014).

In this study, LPS was chosen as the main stimulus due to its importance in the pathogenesis of Gram-negative septic shock. Using this stimulus, no significant change in ex vivo inducible TNF in blood from G-CSF–treated donors was observed. However, free and total TNF release capacity were consistently attenuated in blood from G-CSF–treated donors when any of the other seven alternative stimuli with various different activation principles was used. In view of these data and taking into account that an LPS-inducible overshooting soluble receptor release might have also suppressed TNF bioactivity, we conclude that G-CSF treatment attenuated the general responsiveness of blood to release TNF after stimulation, with LPS representing the exception. A key finding of our study was a dramatic increase in sTNF-R and IL-1ra. Considerable evidence is available to suggest that this release of soluble cytokine antagonists has beneficial influences on the pathophysiology of inflammatory reactions: IL-1ra is known to be released by PMN,21,22 and mononuclear cells.23 Mature tissue macrophages are known to be even more efficient producers of IL-1ra,24 while releasing less IL-1.25 IL-1ra was shown to suppress LPS-inducible IL-1 and TNF release by monocytes25 and was also protective in animal sepsis models26,27,28 prompting clinical trials.28 Therefore, stimulation of the endogenous production of IL-1ra at the site of inflammation represents an antiinflammatory principle that might be initiated by G-CSF treatment and be similar to the response after IL-6 infusion.29 Based on these experimental and clinical findings, it seems feasible that either a part or, perhaps, the main principle of the efficacy of G-CSF in experimental sepsis is due to an induction of enhanced IL-1ra release.

Soluble TNF-Rs are released primarily via shedding from PMN,30 and there is an additional mobilizable pool of sTNF-R p55 in the specific granula of these cells.31 Also, sTNF-R are formed in experimental endotoxemia in humans.32 They have been shown to protect in animal sepsis models,33,34 and clinical trials are in progress.35 In vitro, at least a 10-fold excess of sTNF-R is required to inhibit TNF bioactivity.36 The ratio of sTNF-R to TNF in our incubations approximates this value. An extrapolation from data of an in vitro titration37 of TNF bioactivity or immunoreactivity in a concentration expected in septic shock (up to 1.5 ng/mL blood) to in vivo conditions suggests that the amounts of sTNF-R determined here ex vivo would suffice to neutralize a relevant portion of circulating TNF. Although this is a rough extrapolation

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between ex vivo release capacity and circulating cytokines, it shows that G-CSF pretreatment induces a systemic response to LPS that would enable it to serve as an endogenous transient buffer against overshooting TNF production in early phase shock.

The question arises whether these changes in release capacity of blood from G-CSF–treated subjects were simply caused by releasing or shedding of these components from an increased number of granulocytes. Therefore, the data were also calculated on a per-granulocyte basis, instead of only on a per-milliliter-of-blood basis. The results demonstrate that G-CSF causing enhanced IL-1α release is unlikely to be due to granulocytosis (or monocytosis) alone. At day 2, for instance, LPS-inducible IL-1α release was increased from $3.9 \pm 0.5$ ng/10⁶ PMN in the placebo group to $6.0 \pm 0.5$ ng/10⁶ PMN in the single G-CSF–treated group ($P = 0.012$). This suggests that additional mechanisms are activated by G-CSF beyond shear increase in cell numbers. However, it should be noted that the results of these corrections for neutrophil numbers are of lesser magnitude than the net effects in whole blood. There was only a small increase (1.3-fold) in sTNF-R p55 release per cell ex vivo in blood from G-CSF–treated donors. Such calculations cannot be performed in the case of sTNF-R p75 because pretreatment and placebo blood release capacity was below the detection limit. When G-CSF was added to placebo blood in vitro, there was a similar increase in LPS-inducible IL-1α release per PMN as observed ex vivo. This in vitro experiment shows that the increase in IL-1α by G-CSF was not caused by leukocyte recruitment only.

G-CSF treatment alters the cytokine release capacity of whole blood, as characterized by increased IL-1α, both sTNF-Rs, G-CSF, IL-6, IL-8, and IL-10 and reduced TNF, IFN-γ, and GM-CSF. We interpret the major changes as being caused by priming of leukocytes or recruitment of different leukocyte subpopulations from the bone marrow, whereas minor increases in IL-6, IL-8, and IL-10 release capacity can obviously be explained by changes in leukocyte counts. Thus, the general conclusion of this study is that leukocytes are switched toward an antiinflammatory state after G-CSF treatment, as characterized by two principles: hyperresponsiveness of antiinflammatory effector (sTNF-R and IL-1α) release and hyperresponsiveness of proinflammatory mediator release (TNF, IFN-γ, and GM-CSF)²⁹. This major counterregulation against overactivation of the host defense to a wide spectrum of both Gram-negative and -positive stimuli may additionally be reinforced by G-CSF increasing its own release capacity. This would be in addition to enhancement of the number and antimicrobial power of circulating neutrophils. Thus, it is conceivable that G-CSF may have an antiinflammatory role and, therefore, a potential use in humans with acute inflammatory conditions.

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