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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**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. The volunteers 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 × 10^6/mL ± 1.2 × 10^6/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 described with 20% citrate blood in RPMI 1640 medium (Biochrom, Berlin, Germany) plus 2 IU/mL heparin (Liquemin; 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 performed 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 hemolyzing 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; BiErrmmer, Bad Naubiem, 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 Ficol/Hypaque (Monopoly resolving medium; ICN, Mecknenheim, Germany) according to the product information. Blood smears exhibited 96% neutrophil granulocytes. Polymorphonuclear cells (PMN; 2 × 10^6) 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 10^6 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: Ratiingen, Germany; Cistron: Biomar, Marburg, Germany; and Quantikine: Biermm, Germany). The Medgenix assay was previously shown to measure total TNF, 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 = 96, n = 80) with a Cistron ELISA (Biomar) described to measure free TNF only, while the correlation of the Quantikine assay with the Medgenix assay was lower (r = 62, n = 80). Third, in whole blood, both the Quantikine and the Cistron ELISAs detected only about 40% of the amount of TNF detected 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 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), IFNγ (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 (Coastost; 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-1β (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 \( \mu g/mL \) (7.7 \( \times \) 10\(^8\) ± 0.4 \( \times \) 10\(^8\) IU) that was added to blood after 4 hours of incubation, 5.5 \( \mu g/mL \) (4.2 \( \times \) 10\(^8\) ± 0.4 \( \times \) 10\(^8\) IU) was present after 24 hours of incubation.
G-CSF CHANGES BLOOD CYTOKINE RESPONSE

with 480 pg G-CSF. Citrate blood was taken at the time points indicated from healthy volunteers. At days 1 and 2, 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. 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 x 10^9 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 10^7 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

![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. PMN (2 x 10^7) 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 100 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.

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 (10^7/mL)</td>
<td>4 ± 1</td>
<td>&lt;0.4</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Single G-CSF–treated PMN (10^7/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 x 10^7) 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 100 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.
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>G-CSF Treated</th>
<th>Placebo</th>
<th>G-CSF Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDP</td>
<td>16.6</td>
<td>7.8</td>
<td>7.8</td>
<td>14.4</td>
</tr>
<tr>
<td>ETA</td>
<td>10.6</td>
<td>0.6</td>
<td>10.1</td>
<td>24.6</td>
</tr>
<tr>
<td>LTA</td>
<td>25.1</td>
<td>2.0</td>
<td>9.2</td>
<td>22.7</td>
</tr>
<tr>
<td>SLO</td>
<td>7.8</td>
<td>4.8</td>
<td>8.7</td>
<td>10.7</td>
</tr>
<tr>
<td>C5a</td>
<td>3.7</td>
<td>1.8</td>
<td>8.2</td>
<td>18.5</td>
</tr>
<tr>
<td>PHA</td>
<td>11.2</td>
<td>7.0</td>
<td>8.2</td>
<td>26.7</td>
</tr>
<tr>
<td>PMA</td>
<td>10.8</td>
<td>4.9</td>
<td>9.6</td>
<td>40.1</td>
</tr>
<tr>
<td>LPS</td>
<td>13.4</td>
<td>13.0</td>
<td>11.6</td>
<td>33.0</td>
</tr>
</tbody>
</table>

Average 53 ± 11*  
Placebo 266 ± 27*  
G-CSF Treated 248 ± 16*  

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 is 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 in vitro effect of G-CSF on IL-1ra release capacity is of a similar extent as the ex vivo increase in LPS-inducible IL-1ra calculated per PMN. Thus, the observed increase in LPS-inducible IL-1ra 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-1ra release (Table 2). It should be noted that throughout, the enhancement of IL-1ra 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-10 (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-
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 pla-
cele 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
tests or from G-CSF–treated donors.

**DISCUSSION**

Circumstantial evidence from clinical observations as well
as from animal studies makes G-CSF an attractive pharma-
logenic 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 complica-
tions 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, ie, exposure of healthy
humans to potentially harmful agents. To avoid such hazards,
an ex vivo approach was chosen that used whole blood incu-
bations and has the advantages of natural cell environment,
low 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 mono-
cytes. In general, blood leukocytes represent a rather tran-
sient 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, using the stimulated
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 (ie, at the time point of maximal increase in mono-
cyte 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 inflamma-
tory foes. Similar calculations performed for the monokines
IL-1β and macrophage inflammatory protein 1α (MIP1α) did
not reveal such differences in factor release per cell be-
tween treatment and placebo groups at 8 hours after G-
CSF injection. However, the increase in G-CSF release ca-
pacity continued to be significant when corrected for mono-
cyte 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 releas-
ing less IL-1.25 IL-1ra was shown to suppress LPS-inducible
IL-1 and TNF release by monocytes26 and was also protec-
tive in animal sepsis models,27 prompting clinical trials.28
Therefore, stimulation of the endogenous production of IL-
1ra at the site of inflammation represents an antiinflamma-
tory 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 effi-
cacy 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. Also, sTNF-R
are formed in experimental endotoxemia in humans.31 They
have been shown to protect in animal sepsis models,32,33 and
clinical trials are in progress.34 In vitro, at least a 10-fold
excess of sTNF-R is required to inhibit TNF bioactivity.35
The ratio of sTNF-R to TNF in our incubations approximates
this value. An extrapolation from data of an in vitro titration36
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
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 ± 0.5 ng/10⁶ PMN in the placebo group to 6.0 ± 0.5 ng/10⁶ PMN in the single G-CSF–treated group (P = .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-CSF³⁸). 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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