Granulocyte Colony-Stimulating Factor Downregulates Allogeneic Immune Responses by Posttranscriptional Inhibition of Tumor Necrosis Factor-α Production

By Atsushi Kitabayashi, Makoto Hirokawa, Yoshiaki Hatano, Muneyasu Lee, Jun Kuroki, Hidetaka Niitsu, and Akira B. Miura

We report downregulatory effects of granulocyte colony-stimulating factor (G-CSF) on allogeneic immune responses in vitro. G-CSF did not affect the proliferative response of peripheral blood mononuclear cells (PBMC) against allogeneic Daudi cells but did inhibit tumor necrosis factor (TNF-α) secretion. In contrast with G-CSF, granulocyte-macrophage (GM)-CSF and interleukin (IL)-3 enhanced allogeneic TNF-α production. G-CSF-mediated suppression of TNF-α production was not affected by fixation of stimulators. G-CSF did not inhibit TNF-α mRNA expression or accelerate mRNA degradation, whereas pentoxifylline inhibited the expression of TNF-α mRNA. These results indicate that G-CSF acts directly on responder cells and modulates TNF-α production at posttranscriptional levels. Suppression of TNF-α secretion was accompanied by an increase of intracellular cyclic adenosine monophosphate (cAMP) concentration in alloactivated PBMC. The cell-permeable cAMP analogue, dibutyryl cAMP, suppressed TNF-α secretion without affecting TNF-α mRNA expression. G-CSF showed an inhibitory effect on the development of cytotoxic effector cells against allogeneic Daudi cells. Anti-TNF-α monoclonal antibody (MoAb) also inhibited the induction of cytolytic activity, and the inhibitory effects of G-CSF and anti-TNF-α MoAb on killer activity generation were overcome by adding exogenous TNF-α. Hence, impaired generation of cytolytic effector cells by G-CSF is believed to be the result of reduced TNF-α production. Collectively, the results described above suggest that G-CSF downregulates allogeneic immune responses by posttranscriptionally inhibiting TNF-α production.

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MATERIALS AND METHODS

Cytokines and reagents. Human recombinant CSFs used in this study were as follows: G-CSF (gift from Kirin-Sankyo, Tokyo, Japan), macrophage (M)-CSF (gift from Midori-Juji, Osaka, Japan), granulocyte-macrophage (GM)-CSF (gift from Schering-Plough, Osaka, Japan), interleukin (IL)-3 (gift from Kirin-Sankyo). Human recombinant TNF-α and neutralizing mouse antimouse TNF-α monoclonal antibody (MoAb) were provided by Otsuka Pharmaceutical Co (Osaka, Japan). Pentoxifylline was provided by Hoechst Pharmaceuticals (Tokyo, Japan). Actinomycin D, cycloheximide, and dibutyryl cyclic adenosine monophosphate (cAMP) were purchased from Sigma Chemical Co (St. Louis, MO).

Culture medium. RPMI 1640 medium (Nissui Seiyaku Co, Tokyo, Japan) was supplemented with heat-inactivated 10% fetal calf serum (FCS; Hyclone, Logan, UT), 50 U/mL penicillin, 50 µg/mL streptomycin, and 2 mM/L L-glutamine (Nissui Seiyaku) (complete medium).

Cell preparation. PBMC were isolated from heparinized venous blood of healthy adult volunteers by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation.

Allogeneic mixed lymphocyte reaction (MLR). One-way allogeneic MLR was performed using mitomycin C (MMC)-treated Daudi cells (human Burkitt’s lymphoma cell line; American Type Culture Collection [ATCC], Rockville, MD) as stimulator cells according to the methods previously described.5,10,11 MMC treatment was performed by incubating the cells with 50 µg/mL of MMC (Kyowa Hakko Co, Tokyo, Japan) for 45 minutes at 37°C. In some experiments, stimulator cells were fixed by incubation with 1.0% paraformaldehyde/phosphate-buffered saline (PBS) for 20 minutes at room temperature.16 PBMC (5 × 10⁶) were cocultured with 1 × 10⁶ stimulator cells in 2 mL of complete medium in 24-well, flat-bottomed plates at 37°C in 5% CO₂ atmosphere. Cell-free supernatants were harvested after 4 hours of incubation, when TNF-α secretion reaches a maximal level,1 and were stored at −80°C until the assay for TNF-α. We have previously shown that Daudi cells do not secrete TNF-α against back-stimulation by allogeneic T cells.1

To determine the proliferative response of PBMC, 2 × 10⁵ cells were cocultured with 1 × 10⁵ stimulator cells in 200 µL of complete medium in 96-well, flat-bottomed plates for 4 days. The cultures were pulsed with 0.5 µCi per well of [³H]-thymidine (Amersham, Buckinghamshire, UK) for the final 18 hours. [³H]-thymidine incorporation was measured by liquid scintillation counting method.

Cytokine determination. Commercially available enzyme-linked
**Figure 1.** Inhibitory effect of G-CSF on alloactivation-induced TNF-α secretion. The percent control response was calculated by comparing the data with those from the cultures in the absence of G-CSF in each series of the experiment. Data are expressed as the mean ± SEM of four separate experiments using different PBMC donors.

**Table 1.** Effect of G-CSF on Proliferative Response of PBMC and TNF-α Production Against Stimulation With Allogeneic B Lymphoblastoid Cells

<table>
<thead>
<tr>
<th>CSF Added*</th>
<th>³H-TdR Uptake (x10⁶ cpm)†</th>
<th>Pentoxifylline (0 μg/mL)</th>
<th>Pentoxifylline (100 μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>34.7 ± 3.8</td>
<td>1,844</td>
<td>683</td>
</tr>
<tr>
<td>G-CSF</td>
<td>35.9 ± 3.1</td>
<td>933</td>
<td>474</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>34.6 ± 1.0</td>
<td>2,202</td>
<td>987</td>
</tr>
<tr>
<td>M-CSF</td>
<td>34.9 ± 1.3</td>
<td>1,784</td>
<td>772</td>
</tr>
<tr>
<td>IL-3</td>
<td>36.0 ± 0.5</td>
<td>2,366</td>
<td>1,079</td>
</tr>
</tbody>
</table>

Abbreviation: ³H-TdR, ³H-thymidine.

* Each cytokine was added to the cultures at the final concentration of 10 ng/mL.
† Data for ³H-TdR incorporation are expressed as the mean ± SEM of triplicate cultures.
‡ Cultures for the determination of TNF-α secretion were performed in the absence or presence of pentoxifylline. Culture supernatants were pooled from triplicate cultures and were assayed for TNF-α concentration.

Northern blot analysis. Total cellular RNA was prepared according to the method described by Chomczynski and Sacchi and quantified by absorbance at 260 nm. Northern blot was performed as previously described. Total RNA (10 μg for each sample) was loaded onto the formaldehyde/1% agarose gel and size-fractionated by electrophoresis. Resolved RNA was transferred to nylon membranes (Hybond-N+: Amersham) in the presence of 20× saline sodium citrate (SSC). The membranes were crosslinked by ultraviolet radiation and treated overnight at 42°C with prehybridization buffer (50% formamide, 0.1% Denhardt's solution, 0.1 mmol/L EDTA, 0.18 sodium dodecyl sulfate (SDS). 0.65 mol/L NaCl) containing 0.1 mg/mL of salmon sperm DNA. This solution was replaced with the hybridization solution containing 10⁶ cpm/mL of heat-denatured, ³²P-labeled, random-primed cDNA probe for TNF-α (Avai-HindIII fragment; provided by Asahi Chemical Industry Co, Tokyo, Japan). The membranes were hybridized at 42°C overnight. After washing with 2× SSC/0.1% SDS at 55°C, the blots were exposed to Kodak X-OMAT AR film at −70°C (Eastman-Kodak, Rochester, NY). Equivalent amounts of total RNA for each lane were assessed by monitoring 28S and 18S ribosomal RNA.

Assay for intracellular cAMP concentration. PBMC (1 × 10⁷) and 2 × 10⁷ paraformaldehyde-fixed Daudi cells were incubated in...
allogeneic stimulation. Although neither G-CSF nor the other three CSFs tested affected proliferative response of PBMC against allogeneic B lymphoblastoid Daudi cells, G-CSF inhibited TNF-α production (Table 1). This inhibitory effect of G-CSF on alloactivation-induced TNF-α production was dose-dependent (Fig 1) but rarely suppressed more than 50% of control response, even at a higher concentration of G-CSF (100 ng/mL). In contrast with G-CSF, both GM-CSF and IL-3 significantly enhanced TNF-α production (Fig 2). As previously reported, pentoxifylline by itself inhibited TNF-α production. In the presence of G-CSF, pentoxifylline showed a more striking inhibitory effect on TNF-α production (Table 1 and Fig 3). To explore the mechanisms of this inhibitory effect of G-CSF, the following experiments were undertaken.

**Mechanisms of inhibitory action of G-CSF on TNF-α production.** To determine with which cells G-CSF interferes, stimulator cells were metabolically fixed with paraformaldehyde. We have previously reported that paraformaldehyde-fixed Daudi cells are able to stimulate PBMC to produce TNF-α. Even after the fixation of stimulator cells, inhibitory effect of G-CSF on alloactivation-induced TNF-α secretion was observed with a comparable magnitude to that when stimulators were not fixed. This result suggests that G-CSF directly acts on responder cells, resulting in a decrease in TNF-α production (Fig 4).

Pentoxifylline has been shown to inhibit lipopolysaccharide (LPS)-induced TNF-α production at a transcriptional level. We confirmed that pentoxifylline decreased the expression of TNF-α mRNA induced by alloactivation, whereas G-CSF did not show significant effect on TNF-α mRNA expression (Fig 5A and B). We also examined the effect of G-CSF on the stability of TNF-α mRNA induced by alloactivation. After induction of TNF-α mRNA expression, new synthesis of RNA was blocked with actinomycin D. G-CSF did not accelerate the decay of induced TNF-α mRNA (Fig 6). These results suggest that G-CSF inhibits TNF-α production at posttranscriptional levels. As predicted by the data in Fig 3, GM-CSF increased the expression of TNF-α mRNA (Fig 5A).
It has been demonstrated that cAMP functions as a crucial second messenger generated by the activation of guanosine triphosphate (GTP)-binding proteins/adenylate cyclase system, and G-CSF activates G-proteins/adenylate cyclase system in certain cell types. Moreover, agents that cause an increase in intracellular cAMP levels, including prostaglandins, theophylline, isobutylmethylxanthine, dibutyryl cAMP, or cholera toxin, have been reported to reduce monocyte-derived cytokine production. Hence, we looked for the effect of G-CSF on intracellular cAMP levels in responder cells during allogeneic response. As shown in Table 2, G-CSF caused an increase in intracellular cAMP levels in a dose-dependent fashion. As stimulator cells were fixed with paraformaldehyde, an increase in cAMP by adding exogenous G-CSF reflects an elevation of intracellular cAMP in responder cells. With the use of this culture system, dibutyryl cAMP, a cell-permeable cAMP analogue, was added to the cultures in place of G-CSF. Dibutyryl cAMP suppressed TNF-α secretion but did not alter the TNF-α mRNA expression (Fig 7), which suggests the posttranscriptional inhibition of TNF-α production.

**G-CSF inhibits the development of cytotoxic effector cells against allogeneic Daudi cells.** TNF-α has been shown to be involved in allogeneic immune responses, the development of cytotoxic T lymphocytes, and enhancement of natural killer (NK) cells. Therefore, we examined the effect of G-CSF on the generation of cytotoxic effector cells. Addition of G-CSF into the mixed lymphocyte cultures containing PBMC and allogeneic Daudi cells inhibited the generation of cytolytic activity against stimulator cells (Fig 8A). Primed PBMC secreted less IFN-γ against restimulation with allogeneic Daudi cells when PBMC were precultured with stimulators in the presence of G-CSF (Table 3). This fact appears to be consistent with the finding that G-CSF inhibits the development of killer cells, as CD8+ T cells and NK cells have been reported to be the main sources of IFN-γ. We also observed that anti-TNF-α MoAb prevented the appearance of cytolytic activity against Daudi cells (Fig 8B). Exogenous TNF-α overcame the inhibitory effect of G-CSF and anti-TNF-α MoAb. These results suggest that G-CSF inhibits the development of killer cells by suppressing TNF-α secretion.

**DISCUSSION**

The role for allogeneic marrow transplantation in the treatment of hematologic malignancies has been established. Because of the limited availability of HLA-identical sibling donors, the number of transplants from HLA-mismatched related donors or matched unrelated donors has been increasing. Moreover, allogeneic marrow transplant tends to be
Fig 6. Effect of G-CSF on the stability of induced TNF-α mRNA in the presence of actinomycin D. PBMC were first stimulated with Daudi cells. After 2 hours of incubation, designated as time 0, the cells were incubated with actinomycin D (5 ng/mL) for varying times in the absence or presence of G-CSF (100 ng/mL). After further incubation, the cells were harvested for RNA isolation and Northern blot analysis for TNF-α mRNA expression. Onto each lane, 10 μg of total RNA was loaded.

Table 2. G-CSF Increases Intracellular cAMP Levels in Responder Cells

<table>
<thead>
<tr>
<th>Exp No.</th>
<th>G-CSF Added (ng/mL)</th>
<th>cAMP Concentration (pmol/10^6 cells)</th>
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<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>10</td>
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</tr>
<tr>
<td></td>
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<td>227</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>77</td>
</tr>
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<td></td>
<td>1</td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>10</td>
<td>74</td>
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</tbody>
</table>

PBMC were stimulated with paraformaldehyde-fixed Daudi cells in the presence of G-CSF at the indicated concentrations. The cells were harvested after 20 minutes of incubation.

Abbreviation: Exp, experiment.
MODULATION OF ALLOGENEIC RESPONSE OF G-CSF

Fig 7. Effect of dibutyryl cAMP on TNF-α protein release and mRNA expression. PBMC and MMC-treated Daudi cells were cultured with dibutyryl cAMP at the indicated concentrations. Supernatants were harvested after 4 hours of incubation and assayed for TNF-α by ELISA. For Northern blot analysis, the cells were harvested after 2 hours of incubation for RNA extraction, and 10 μg of total RNA was loaded onto each lane.

Fig 8. Inhibition of cytotoxic effector cell generation by G-CSF. PBMC were cocultured with MMC-treated Daudi cells for 7 days in the presence of the indicated reagents. (A) G-CSF and TNF-α were added at 100 ng/mL and 10 ng/mL, respectively: ○, control; ●, G-CSF; ■, TNF-α; and △, G-CSF + TNF-α (B) Mouse anti-human TNF-α MoAb (●) and control mouse IgG (□) were added at 10 μg/mL. After incubation, viable cells were isolated by Ficoll-Hypaque density gradient centrifugation method and were assayed for cytotoxicity against Daudi cells. These experiments were repeated twice with similar results.

<table>
<thead>
<tr>
<th>Exp No.</th>
<th>Cells</th>
<th>G-CSF Added (ng/mL)</th>
<th>IFN-γ Production (pg/mL)</th>
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<td>1</td>
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<td>—</td>
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</tr>
<tr>
<td></td>
<td>Primed PBMC</td>
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<td></td>
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<td>1</td>
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<td>10</td>
<td>809</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>284</td>
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PBMC were cocultured with MMC-treated Daudi cells for 7 days in the absence or presence of G-CSF at the indicated concentrations. After incubation, viable cells were isolated by Ficoll-Hypaque density gradient centrifugation. Then, primed PBMC (5 × 10^6/mL) were restimulated with MMC-treated Daudi cells (1 × 10^6/mL). Cell-free supernatants were collected after 24 hours of incubation and were assayed for IFN-γ.
	ylate both kinases and phosphatases, some of which may modulate translation of TNF-α mRNA or processing and release of TNF-α protein. A cell-permeable cAMP analogue was shown to inhibit TNF-α secretion by alloactivated PBMC without affecting mRNA expression. A G-CSF–induced increase in cAMP levels may interfere with translation or posttranslational processing of TNF-α protein.

We have also demonstrated that G-CSF causes partial inhibition of the generation of cytotoxic effector cells. This
effect seems to result from the block in the production of TNF-α, because TNF-α has been shown to be involved in the allogeneic MLR, the development of T killer cells, and augmentation of NK cell functions.\(^5\)\(^6\)\(^7\)\(^8\)\(^9\)\(^10\) This was confirmed in our experiments, with the results that anti-TNF-α MoAb inhibited the induction of cytolytic activity. It remains to be clarified whether G-CSF–induced suppression of cytotoxic effector cell generation results from reduction in the activity of lymphokine-activated NK cells or that of cytotoxic T cells.

Although the physiologic relevance of our in vitro findings remains unknown, G-CSF may regulate TNF-α secretion to protect the host from overreaction to noxious insults against the immune system. For instance, it has been recently reported that TNF-α and IL-6 secretion by human macrophages is downregulated during LPS adaptation, while IL-1β and G-CSF expression is upregulated.\(^11\) G-CSF has been widely used in a variety of clinical settings. An extended clinical study in allogeneic marrow transplantation may provide evidence for the effects of this cytokine on allogeneic immune responses in vivo.

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