

Selective activation of STAT3 in human monocytes stimulated by G-CSF: implication in inhibition of LPS-induced TNF- α production

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Nishiki, Saori, Fumihiko Hato, Noriko Kamata, Erina Sakamoto, Taro Hasegawa, Akiko Kimura-Eto, Masayuki Hino, and Seiichi Kitagawa. Selective activation of STAT3 in human monocytes stimulated by G-CSF: implication in inhibition of LPS-induced TNF- α production. *Am J Physiol Cell Physiol* 286: C1302–C1311, 2004. First published January 21, 2004; 10.1152/ajpcell.00387.2003.—Lipopolysaccharide (LPS) induced tumor necrosis factor (TNF)- α production in human monocytes, which was dependent on activation of extracellular signal-regulated kinase (ERK), p38, c-Jun NH₂-terminal kinase (JNK), and nuclear factor (NF)- κ B. LPS-induced TNF- α production was inhibited by granulocyte colony-stimulating factor (G-CSF) and interleukin (IL)-10. G-CSF, like IL-10, exerted the inhibitory effect even when simultaneously added with LPS. Among the signaling pathways, signal transducer and activator of transcription 3 (STAT3) was selectively activated in monocytes stimulated by G-CSF or IL-10. G-CSF-mediated inhibition of LPS-induced TNF- α production as well as G-CSF-induced STAT3 phosphorylation and suppressor of cytokine signaling 3 mRNA expression were prevented by pretreatment of monocytes with AG-490, an inhibitor of Janus kinase 2. G-CSF did not affect LPS-induced activation of ERK, p38, JNK, and NF- κ B, indicating that G-CSF affects the pathway downstream or independently of these signaling molecules. G-CSF-induced, but not IL-10-induced, STAT3 phosphorylation was attenuated in the presence of LPS. These findings suggest that G-CSF, like IL-10, inhibits LPS-induced TNF- α production in human monocytes through selective activation of STAT3, and the immunomodulation observed in vivo by G-CSF administration may be partly ascribed to the direct effect of G-CSF on monocyte functions.

monocytes; granulocyte colony-stimulating factor; interleukin-10; lipopolysaccharide; tumor necrosis factor- α ; signal transducer and activator of transcription 3

GRANULOCYTE COLONY-STIMULATING FACTOR (G-CSF) is a hematopoietic growth factor that regulates the proliferation and differentiation of hematopoietic progenitor cells and enhances mature neutrophil functions such as superoxide anion release, phagocytosis, and antibody-dependent cell-mediated cytotoxicity (8, 19, 29). These biological effects of G-CSF clearly contribute to augmenting the host defense against invading microorganisms. In addition to these effects, it has been shown that G-CSF modulates the immune or inflammatory responses. For example, G-CSF treatment protects mice against lipopolysaccharide (LPS)-induced lethal toxicity by suppressing systemic tumor necrosis factor (TNF)- α production in vivo (13). Peripheral blood leukocytes from G-CSF-treated healthy vol-

unteers show a reduced capacity for producing proinflammatory cytokines such as TNF- α , granulocyte-macrophage CSF (GM-CSF), and interferon (IFN)- γ when stimulated with LPS ex vivo (14). Mononuclear cells from G-CSF-mobilized peripheral blood stem cell (PBSC) harvests show a decreased activity in natural killer and lymphokine-activated killer cell-mediated cytotoxicity, and G-CSF appears to promote T helper 2 (Th2) immune deviation (18, 49). These immunomodulatory effects of G-CSF are consistent with the observation that the patients undergoing PBSC transplantation do not demonstrate an increased incidence or severity of acute graft-versus-host disease compared with bone marrow transplantation, despite a markedly increased number of T cells in G-CSF-mobilized PBSC harvests used for transplantation (33). However, the precise mechanisms by which G-CSF exerts its immunomodulatory effect remain to be determined.

It has been reported that the functions of human monocytes and lymphocytes are directly affected by G-CSF itself or indirectly affected by G-CSF via activated neutrophils or other mechanisms (5, 34, 39, 41, 45). A recent study shows that G-CSF may affect human monocytes directly to inhibit LPS-induced TNF- α production (5). More recently, it has been shown that dendritic cells differentiated from monocytes treated with G-CSF lose the ability to produce interleukin (IL)-12 in response to CD40 ligand or LPS, suggesting that G-CSF directly affects monocytes (49). However, the signals provoked by the binding of G-CSF to its receptors on human monocytes as well as the mechanisms by which G-CSF modulates monocyte functions are largely unknown. We studied the signaling pathways activated in human monocytes stimulated by G-CSF and the mechanisms by which G-CSF inhibits LPS-induced TNF- α production. The results show that G-CSF, like IL-10, may inhibit LPS-induced TNF- α production in human monocytes through selective activation of signal transducer and activator of transcription 3 (STAT3).

MATERIALS AND METHODS

Reagents. Recombinant human G-CSF, IL-10, and TNF- α produced by *Escherichia coli* were provided by Kirin Brewery (Tokyo, Japan), Schering-Plough (Osaka, Japan), and Dainippon Pharmaceutical (Osaka, Japan), respectively. The specific activity of TNF- α was 3×10^6 U/mg protein. Endotoxin contamination of each preparation was <100 pg/mg protein. LPS from *Escherichia coli* was purchased from Sigma (St. Louis, MO). Ficoll and the enhanced chemiluminescence (ECL) Western blotting system were purchased from Amer-

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sham Pharmacia Biotech (Amersham, UK). Conray was purchased from Mallinckrodt (St. Louis, MO). AG-490 [Janus kinase 2 (JAK2) inhibitor], SB-203580 [p38 mitogen-activated protein kinase (MAPK) inhibitor], and SN-50 [nuclear factor (NF)- κ B inhibitor] were purchased from Calbiochem (San Diego, CA). SP-600125 [c-Jun NH₂-terminal kinase (JNK) inhibitor] was purchased from Tocris Cookson (Ellisville, MO). PD-98059 [MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitor], and rabbit polyclonal antibodies against ERK1/2, Thr²⁰²/Tyr²⁰⁴-phosphorylated ERK1/2, p38 MAPK, Thr¹⁸⁰/Tyr¹⁸²-phosphorylated p38 MAPK, Thr¹⁸³/Tyr¹⁸⁵-phosphorylated JNK/stress-activated protein kinase (SAPK), I κ B, Ser³²-phosphorylated I κ B, STAT3, Tyr⁷⁰⁵-phosphorylated STAT3, and Ser⁷²⁷-phosphorylated STAT3 were purchased from Cell Signaling Technology (Beverly, MA).

Preparation of cells. Human neutrophils and mononuclear cells were prepared from healthy adult donors as described previously (19, 29) by using dextran sedimentation, centrifugation with Conray-Ficoll, and hypotonic lysis of contaminated erythrocytes. Neutrophil fractions contained >98% neutrophils. Monocytes were further purified from mononuclear cells by centrifugal elutriation in a Hitachi SRR6Y elutriation rotor (Hitachi, Tokyo, Japan) (55). Monocyte fractions contained 90–95% monocytes and 5–10% lymphocytes. Cells were suspended in Hanks' balanced salt solution (HBSS) containing 10 mM HEPES (pH 7.4). For experiments with cell cultivation, cells were suspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS).

Determination of TNF- α production. For the experiment with TNF- α production, monocytes (3×10^6 cells/ml) were suspended in RPMI 1640 supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μ g/ml), and 1% human serum. The monocyte suspension (1 ml) was placed in a polypropylene tube (Falcon no. 2059; Falcon Labware, Becton Dickinson, NJ) and were cultivated with or without LPS (1 μ g/ml) in 5% CO₂-95% humidified air at 37°C. When required, G-CSF or IL-10 was added to the culture medium. In some experiments, monocytes were pretreated with PD-98059 (50 μ M), SB-203580 (10 μ M), SP-600125 (5 or 25 μ M), AG-490 (50 μ M), or SN-50 (100 μ g/ml) for 1 h at 37°C before stimulation with LPS. After incubation, the amount of TNF- α in the cell-free culture supernatants was determined by the TNF- α -specific ELISA kit (R&D Systems, Minneapolis, MN), which can detect >0.18 pg/ml TNF- α .

Western blotting. Human neutrophils or monocytes suspended in HBSS were prewarmed for 10 min at 37°C and were then stimulated with cytokines or LPS for 10 min at 37°C. When required, cells were pretreated with AG-490 (50 μ M) for 1 h at 37°C before stimulation with cytokines. The reactions were terminated by rapid centrifugation, and the pellets were frozen in liquid nitrogen after aspiration of the supernatant. The cell pellets were resuspended in ice-cold solution containing 50 mM HEPES (pH 7.4), 1% Triton X-100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 100 μ g/ml aprotinin, and 10 μ g/ml leupeptin and were lysed for 10 min at 4°C. After rapid centrifugation, the supernatant was mixed 1:1 with 2 \times sample buffer [4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% mercaptoethanol, and a trace amount of bromophenol blue dye in 125 mM Tris-HCl, pH 6.8], heated at 100°C for 5 min, and then frozen at -80°C until use. Samples were subjected to 10% SDS gel electrophoresis. After electrophoresis, proteins were electrophoretically transferred from the gel onto a nitrocellulose membrane in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol at 2 mA/cm² for 1.5 h at 25°C. Residual binding sites on the membrane were blocked by incubating the membrane in Tris-buffered saline (pH 7.6) containing 0.1% Tween 20 and 5% nonfat dry milk for 2 h at 25°C. The blots were washed in Tris-buffered saline containing 0.1% Tween 20 and then incubated with appropriate antibody overnight at 4°C. After washing, the membrane was incubated with anti-rabbit IgG antibody conjugated with horseradish peroxidase, and the antibody complexes were

visualized by the ECL detection system as directed by the manufacturer. Immunoreactive bands were quantified with the use of a NIH Image program on a Macintosh computer.

Reverse transcriptase-polymerase chain reaction analysis. Total RNA was isolated from monocytes and neutrophils with the RNeasy mini kit (Qiagen, Hilden, Germany). To generate cDNA, we used 500 ng of RNA for each reaction. The reaction mixtures (10 μ l) contained RNA, random primer pd(N)₆ (1 μ M), RNase inhibitor (0.5 U/ μ l; Roche Molecular Biochemicals, Mannheim, Germany), dNTP mixture (500 μ M of each dNTP), and Omniscript reverse transcriptase (0.2 U/ μ l; Qiagen), and the reaction mixtures were incubated for 60 min at 37°C. Semiquantitative RT-PCR analysis was performed by using GeneAmp PCR system model 9700 (Perkin Elmer, Norwalk, CT). PCR reaction mixtures (25 μ l) contained cDNA, dNTP mixture (200 μ M of each dNTP), MgCl₂ (1.5 mM), Taq DNA polymerase (0.02 U/ μ l; Fermentas AB, Vilnius, Lithuania), and the forward and reverse primers.

The following primer pairs were used. For each set, the forward and reverse primers as well as the accession number are listed: TNF- α , 5'-AGA GGG AAG AGT TCC CCA GGG AC-3', 5'-TGA GTC GGT CAC CCT TCT CCA G-3', M10988; suppressor of cytokine signaling 3 (SOCS3), 5'-CTC GCC ACC TAC TGA ACC CTC-3', 5'-AAG CGG GGC ATC GTA CTG GT-3', AF159854; β -actin, 5'-CCA ACC GCG AGA AGA TGA-3', 5'-GGA AGG AAG GCT GGA AGA GT-3', X00351; and β 2-microglobulin, 5'-GCT ATG TGT CTG GGT TTC-3', 5'-TAC ATG TCT CGA TCC CAC-3', V00567. For the analysis of G-CSF receptor (accession no. M59818), three sets of overlapping primers were used (4). For each set, the forward and reverse primers are listed: primer A (nt 2006–2351), 5'-GTC CTC ACC CTG ATG ACC TTG-3' and 5'-CAT AGG TCT GGA CCA GAG TGG-3'; primer B (nt 2229–2550), 5'-GCC TTG GCA CGC CAC CCA TCA-3' and 5'-GCC TGG AAC CAG AGG TTC TCA-3'; and primer C (nt 2461–2740), 5'-AGG GCA CTA TCT CCG CTG TGA-3' and 5'-TCT TCT CCA GCT AGC TCA GGC-3'.

The conditions for PCR amplification were as follows: denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and elongation for 30 s at 72°C with 26 cycles for TNF- α , SOCS3, β -actin, and β 2-microglobulin; and denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and elongation for 30 s at 72°C with 35 cycles for G-CSF receptor. The PCR products were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide. The PCR products were purified, and the nucleotide sequence was analyzed for confirmation.

Statistical analysis. An ANOVA followed by a multiple comparison test (Bonferroni method) was used to determine statistical significance.

RESULTS

G-CSF inhibits LPS-induced TNF- α production in human monocytes. Cultivation of human monocytes with LPS resulted in increased TNF- α mRNA expression and massive production of TNF- α protein. The level of TNF- α mRNA expression reached the maximal level within 2 h after stimulation and declined to the basal level at 6–8 h (Fig. 1A). When G-CSF was simultaneously added with LPS to the culture medium, LPS-induced TNF- α production was inhibited by G-CSF in a dose-dependent manner (Fig. 1B) (5). Significant inhibition was obtained at 100 ng/ml G-CSF, a relatively higher concentration compared with that required for priming of human neutrophil functions and stimulation of hematopoietic progenitor cells (8, 19, 29). LPS-induced TNF- α production was also markedly inhibited when IL-10 (10 ng/ml) was simultaneously added with LPS (9). G-CSF and IL-10 by themselves did not affect TNF- α production in monocytes cultivated in the ab-

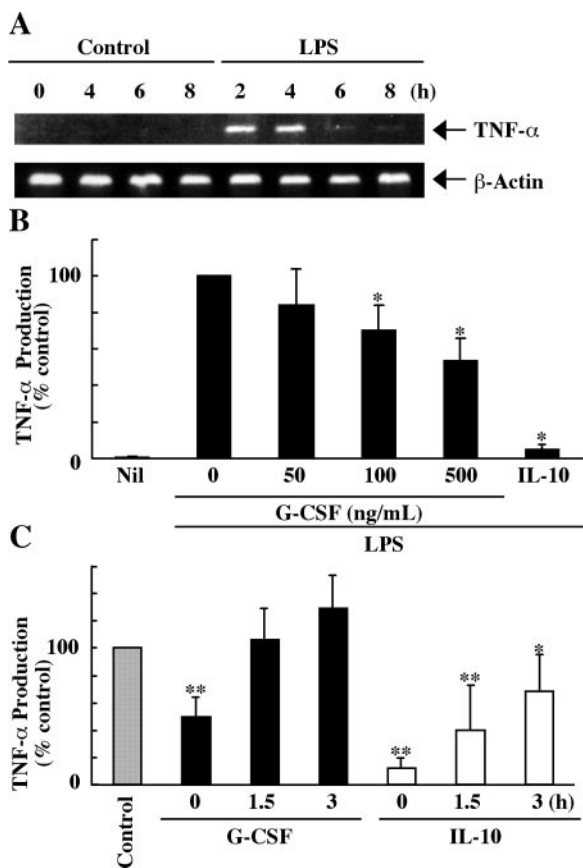


Fig. 1. LPS-induced TNF- α mRNA expression and granulocyte colony-stimulating factor (G-CSF)- and IL-10-mediated inhibition of LPS-induced TNF- α production in human monocytes. **A:** monocytes were cultivated with LPS (1 μ g/ml) for 2–8 h, and expression of TNF- α mRNA was then analyzed by RT-PCR. β -Actin was used as an internal control. Results are representative of 3 independent experiments. **B:** monocytes (3×10^6 cells/ml) were cultivated with LPS (1 μ g/ml) in the presence or absence of G-CSF (50–500 ng/ml) or IL-10 (10 ng/ml) for 24 h. After cultivation, the amount of TNF- α in the culture supernatant was determined. When required, G-CSF or IL-10 was added simultaneously with LPS. In this experiment, LPS-induced TNF- α production in control cells (no addition of G-CSF or IL-10) was 6.92 ± 3.27 ng/ 3×10^6 cells. Data are expressed as means \pm SD of 5 independent experiments. * $P < 0.01$, significant inhibition by G-CSF or IL-10. **C:** monocytes (3×10^6 cells/ml) were cultivated with LPS (1 μ g/ml) in the presence or absence of G-CSF (500 ng/ml) or IL-10 (10 ng/ml) for 24 h. After cultivation, the amount of TNF- α in the culture supernatant was determined. G-CSF or IL-10 was added 0, 1.5, or 3 h after the addition of LPS. In this experiment, LPS-induced TNF- α production in control cells was 3.33 ± 0.18 ng/ 3×10^6 cells. Data are expressed as means \pm SD of 3 independent experiments. * $P < 0.05$; ** $P < 0.01$, significant inhibition by G-CSF or IL-10.

sence of LPS (data not shown). Because simultaneous addition of G-CSF or IL-10 with LPS caused significant reduction of LPS-induced TNF- α production, the effect of delayed addition of G-CSF or IL-10 was examined. As shown in Fig. 1C, no significant inhibition was detected when G-CSF was added 1.5 h after LPS stimulation, at which time the level of TNF- α mRNA expression reached almost the maximal level. On the other hand, significant inhibition was still detected even when IL-10 was added 3 h after LPS stimulation, although the delayed addition of IL-10 resulted in the reduced inhibition.

Involvement of ERK, p38, JNK, and NF- κ B in LPS-induced TNF- α production. Stimulation of monocytes with LPS resulted in activation of ERK, p38, and JNK, as evidenced by

phosphorylation of these molecules. LPS also induced activation of NF- κ B, as evidenced by phosphorylation and degradation of I κ B (Fig. 2A). Possible involvement of these signaling pathways in LPS-induced TNF- α production was explored with the use of PD-98059 (MEK inhibitor), SB-203580 (p38 inhibitor), SP-600125 (JNK inhibitor), and SN-50 (NF- κ B inhibitor) (3, 21, 23, 42). As shown in Fig. 2B, LPS-induced TNF- α production was markedly inhibited by PD-98059, SB-203580, and SP-600125 and was significantly inhibited by SN-50. In addition, LPS-induced increase in TNF- α mRNA expression was markedly inhibited by PD-98059, SB-203580, and SP-600125 (Fig. 2C). These findings suggest that activation of ERK, p38, JNK, and NF- κ B is involved in LPS-induced TNF- α production in human monocytes (3, 7, 36, 47, 56). It has been reported that p38 is involved in LPS-induced NF- κ B activation and TNF- α production in human neutrophils (26). Possible participation of ERK and p38 in LPS-induced NF- κ B activation in monocytes was then explored using PD-98059 and SB-203580. As shown in Fig. 2D, LPS-induced I κ B degradation was unaffected by these inhibitors, indicating that ERK and p38 are unlikely to be involved in LPS-induced NF- κ B activation in human monocytes. Under these conditions, LPS-induced ERK1/2 phosphorylation was markedly inhibited by PD-98059. On the other hand, LPS-induced p38 phosphorylation was unaffected by SB-203580, as expected, because SB-203580 is an inhibitor of p38 but not an upstream kinase of p38 (Fig. 2D) (21).

G-CSF and IL-10 do not affect LPS-induced activation of ERK, p38, JNK, and NF- κ B. The results depicted in Figs. 1 and 2 raise the possibility that G-CSF and IL-10 might inhibit LPS-induced TNF- α production in monocytes by impairing the signaling pathways activated by LPS. To determine this possibility, we stimulated monocytes with LPS in the presence or absence of G-CSF or IL-10 and examined phosphorylation of ERK, p38, JNK, and I κ B. As shown in Fig. 2A, LPS-induced phosphorylation of all these molecules was unaffected by the addition of G-CSF or IL-10. LPS-induced I κ B degradation was also unaffected by the addition of G-CSF or IL-10. Pretreatment of cells with G-CSF or IL-10 for 1 h before LPS stimulation gave the same results (data not shown). The findings obtained with IL-10 were consistent with the previous report with primary human macrophages, in which IL-10 inhibits LPS-induced TNF- α production without impairing activation of p38 and NF- κ B (9). Thus G-CSF- or IL-10-mediated inhibition of LPS-induced TNF- α production is unlikely to be ascribed to impaired activation of ERK, p38, JNK, and NF- κ B.

Selective activation of STAT3 in monocytes stimulated by G-CSF. To elucidate the mechanisms by which G-CSF inhibits LPS-induced TNF- α production in monocytes, the signaling pathways activated in monocytes stimulated by G-CSF were explored. As shown in Fig. 3, STAT3, especially STAT3 α , was strongly tyrosine-phosphorylated by stimulation with G-CSF. STAT3 was also tyrosine-phosphorylated by stimulation with IL-10 and was serine-phosphorylated by stimulation with TNF- α or IL-10 but not with G-CSF (11, 15). The potency of these cytokines to induce tyrosine phosphorylation of STAT3 was IL-10 > G-CSF. ERK was phosphorylated in monocytes stimulated by TNF- α but not by G-CSF or IL-10. No significant phosphorylation of ERK was detected in G-CSF-stimulated monocytes even when the incubation time was prolonged

to 90 min or when a high concentration of G-CSF (500 ng/ml) was employed (Fig. 3C). Phosphorylation of p38 was induced by stimulation with TNF- α but not with G-CSF or IL-10 (Fig. 3, A and B). TNF- α also induced phosphorylation of JNK in

monocytes. G-CSF-induced tyrosine phosphorylation of STAT3 in monocytes was already detected at 5 min after stimulation with 50 ng/ml G-CSF, and the maximal level was obtained at 10 min, followed by a gradual decline of the level (Fig. 3C). Tyrosine phosphorylation of STAT3 was dependent on the concentration of G-CSF used as stimulus. A significant effect was detected at 5 ng/ml G-CSF, and the optimal effect was obtained at 50 ng/ml (Fig. 3C).

The activation profile of the signaling pathways observed in monocytes was markedly different from that in neutrophils. In human neutrophils, ERK and p38, but not JNK, were phosphorylated by stimulation with G-CSF or TNF- α but not with IL-10 (15, 42, 43). Phosphorylation of ERK and p38 induced by these cytokines was greater in neutrophils than in monocytes on a cell basis (Fig. 3, A and B). G-CSF also induced tyrosine phosphorylation of STAT3, especially STAT3 γ , in neutrophils (15), whereas IL-10 failed to induce any effect on neutrophils. Tyrosine phosphorylation of STAT3 induced by these cytokines was greater in monocytes than in neutrophils (Fig. 3A). Thus the results indicate that the signaling pathways are differentially activated in human neutrophils and monocytes even when the same cytokines are employed. In particular, it should be noted that G-CSF induces strong phosphorylation of STAT3 and no phosphorylation of ERK and p38 in monocytes. This profile contrasts to that in G-CSF-stimulated neutrophils and is similar to that in IL-10-stimulated monocytes (Fig. 3, A and B).

Identical G-CSF receptor is expressed on human neutrophils and monocytes. Differential activation of the signaling pathways in human neutrophils and monocytes stimulated by G-CSF might be ascribed to either a difference in G-CSF receptor isoforms expressed on these cells or the difference of the signaling system from the identical G-CSF receptor. In fact, seven isoforms were reported for G-CSF receptor (4). G-CSF receptor isoforms expressed on neutrophils and monocytes

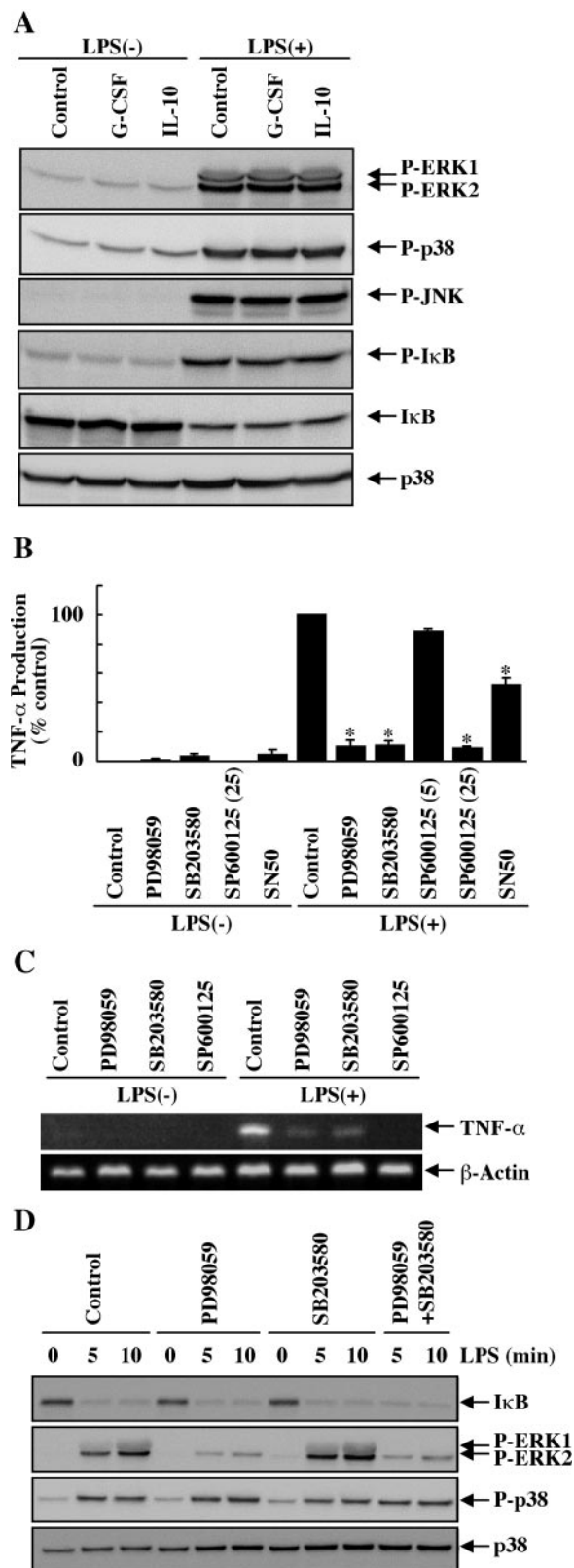
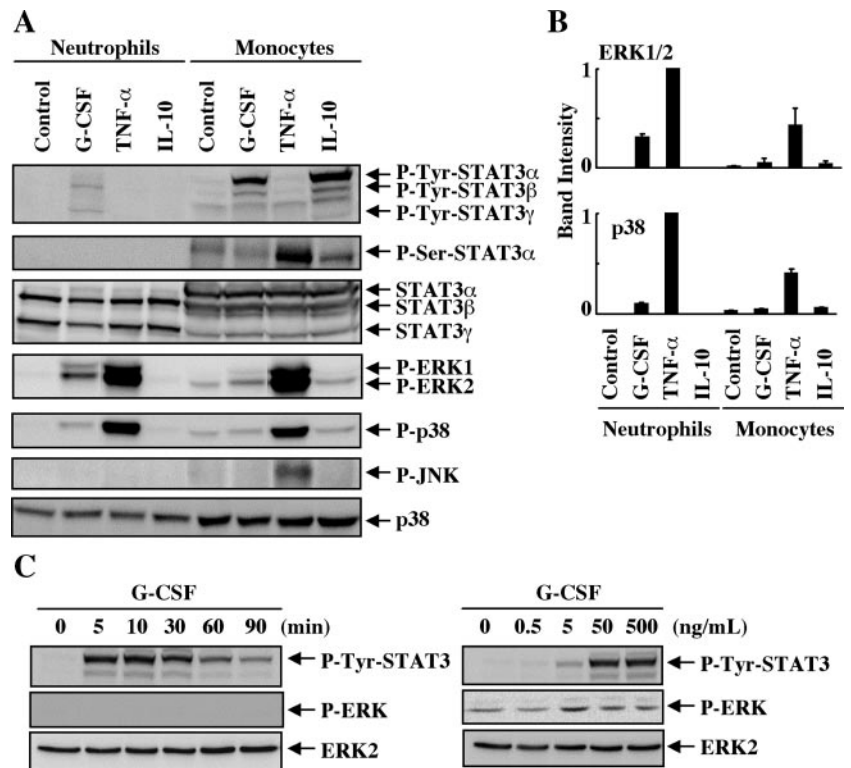


Fig. 2. LPS-induced activation of ERK, p38, JNK, and NF- κ B and involvement of these molecules in LPS-induced TNF- α production. **A**: monocytes were stimulated with LPS (1 μ g/ml) for 5 min for analysis of I κ B or 10 min for analysis of ERK1/2, p38, and JNK at 37°C. When required, G-CSF (500 ng/ml) or IL-10 (10 ng/ml) was added simultaneously with LPS. Phosphorylation of ERK1/2, p38, JNK, and I κ B was analyzed by immunoblotting, using antibody against the phosphorylated form (P) of each protein. The protein level of I κ B and p38 was analyzed by immunoblotting, using antibody against each protein. Cell lysates equivalent to 2.5×10^6 cells were loaded onto each lane. Results are representative of 3 independent experiments. **B**: monocytes (3×10^6 cells/ml) were pretreated with PD-98059 (50 μ M), SB-203580 (10 μ M), SP-600125 (5 or 25 μ M), or SN-50 (100 μ g/ml) for 1 h at 37°C and then cultivated with LPS (1 μ g/ml) for 24 h. After cultivation, the amount of TNF- α in the culture supernatant was determined. In this experiment, LPS-induced TNF- α production in control cells (no addition of any inhibitor) was 4.61 ± 2.66 ng/ 3×10^6 cells. Data are expressed as means \pm SD of 3 independent experiments. * $P < 0.01$, significant inhibition by PD-98059, SB-203580, SP-600125 (25 μ M), or SN-50. **C**: monocytes were pretreated with PD-98059 (50 μ M), SB-203580 (10 μ M), or SP-600125 (25 μ M) for 1 h at 37°C and then cultivated with LPS (1 μ g/ml) for 3 h. After cultivation, expression of TNF- α mRNA was analyzed by RT-PCR. β -Actin was used as an internal control. Results are representative of 3 independent experiments. **D**: monocytes were pretreated with PD-98059 (50 μ M) and/or SB-203580 (10 μ M) for 1 h and then cultivated with LPS (1 μ g/ml) for 5–10 min at 37°C. Phosphorylation of ERK1/2 and p38 was analyzed by immunoblotting, using antibody against the phosphorylated form of each protein. The protein level of I κ B and p38 was analyzed by immunoblotting, using antibody against each protein. Cell lysates equivalent to 2.5×10^6 cells were loaded onto each lane. Results are representative of 3 independent experiments.

Fig. 3. Differential activation of the signaling pathways in human neutrophils and monocytes stimulated by various cytokines and selective phosphorylation of signal transducer and activator of transcription 3 (STAT3) in monocytes stimulated by G-CSF. **A:** neutrophils or monocytes were stimulated with G-CSF (50 ng/ml), TNF- α (100 U/ml), or IL-10 (10 ng/ml) for 10 min at 37°C. Phosphorylation of STAT3, ERK1/2, p38, and JNK was analyzed by immunoblotting, using antibody against the phosphorylated form of each protein. Neutrophil lysates equivalent to 1.3×10^6 cells or monocyte lysates equivalent to 2.5×10^6 cells were loaded onto each lane. Results are representative of 3 independent experiments. P-Tyr, tyrosine-phosphorylated; P-Ser, serine-phosphorylated. **B:** densitometric data from 3 independent experiments, including those in **A**, are expressed as means \pm SD. Relative band intensity is shown on a cell basis for proper comparison of neutrophils and monocytes. **C:** monocytes were stimulated with G-CSF (50 ng/ml) for indicated periods at 37°C (*left*) or with indicated concentrations of G-CSF for 10 min at 37°C (*right*). Phosphorylation of STAT3 and ERK1/2 was analyzed by immunoblotting, using antibody against the phosphorylated form of each protein. Cell lysates equivalent to 2.5×10^6 cells were loaded onto each lane. Results are representative of 3 independent experiments.



were then analyzed by RT-PCR, using three sets of primers that have been shown to be able to discriminate G-CSF receptor isoforms (4). The results showed that a G-CSF receptor band with an identical size was detected for neutrophils and monocytes when each of these different primer sets was used (data not shown), in agreement with the previous report by Boneberg et al. (5). These findings indicate that an identical G-CSF receptor is primarily expressed on neutrophils and monocytes and suggest that the differential activation of the signaling pathways in these cells may reflect the difference of the signaling system from the identical G-CSF receptor.

G-CSF inhibits LPS-induced TNF- α production through activation of the JAK2-STAT3 pathway. The results depicted in Figs. 1 and 3 show that both G-CSF and IL-10 induced selective phosphorylation of STAT3 and inhibited LPS-induced TNF- α production in monocytes. IL-10 was more potent than G-CSF in both functions. In addition, it has been shown that activation of STAT3 is essential for IL-10-mediated inhibition of LPS-induced TNF- α production (28, 44). Furthermore, G-CSF and IL-10 inhibited LPS-induced TNF- α mRNA expression (Fig. 4C), and IL-10 was more potent than G-CSF in this effect. All of these findings raise the possibility that G-CSF, like IL-10, might inhibit LPS-induced TNF- α production through STAT3 activation. This possibility was explored by using AG-490, a potent inhibitor of JAK2 (15, 24). As shown in Fig. 4A, G-CSF-induced STAT3 phosphorylation in monocytes was significantly inhibited by pretreatment of cells with AG-490, indicating that G-CSF may activate the JAK2-STAT3 pathway in monocytes (15). On the other hand, IL-10-induced STAT3 phosphorylation was unaffected by AG-490. The failure of AG-490 to inhibit IL-10-induced STAT3 phosphorylation may be explained by the fact that Tyk2 and JAK1, but not JAK2, are involved in IL-10-induced STAT3 phos-

phorylation (10). Consistent with these findings, G-CSF-mediated, but not IL-10-mediated, inhibition of LPS-induced TNF- α production and mRNA expression was significantly prevented by pretreatment of monocytes with AG-490. In the absence of AG-490, G-CSF and IL-10 inhibited LPS-induced TNF- α production by 40.1 ± 7.1 and $94.2 \pm 2.2\%$, respectively, whereas in the presence of AG-490, they inhibited it by 25.5 ± 2.9 and $95.1 \pm 3.3\%$, respectively ($n = 5$). G-CSF-mediated, but not IL-10-mediated, inhibition of LPS-induced TNF- α production was significantly ($P < 0.05$) prevented by AG-490 (Fig. 4B). Similarly, the densitometric analysis showed that, in the absence of AG-490, G-CSF and IL-10 inhibited LPS-induced TNF- α mRNA expression by 43.0 ± 10.2 and $85.8 \pm 13.9\%$, respectively, whereas in the presence of AG-490, they inhibited it by 20.3 ± 4.9 and $84.2 \pm 13.9\%$, respectively ($n = 3$). G-CSF-mediated, but not IL-10-mediated, inhibition of LPS-induced TNF- α mRNA expression was significantly ($P < 0.05$) prevented by AG-490 (Fig. 4C).

To obtain additional evidence that the JAK2-STAT3 pathway functions in human neutrophils and monocytes, we studied the effect of G-CSF on SOCS3 mRNA expression, which is inducible by STAT3 activation (20). As shown in Fig. 5, RT-PCR analysis revealed that SOCS3 mRNA expression in human neutrophils and monocytes was upregulated by G-CSF stimulation. Increased expression of SOCS3 mRNA was evident as early as 10 min after stimulation. G-CSF-mediated upregulation of SOCS3 mRNA expression in monocytes was inhibited by pretreatment of cells with AG-490. These findings suggest that G-CSF induces SOCS3 mRNA expression through activation of the JAK2-STAT3 pathway.

STAT3 phosphorylation induced by G-CSF, but not IL-10, is attenuated in the presence of LPS. It was recently reported that IL-6-induced STAT3 phosphorylation in murine macrophages

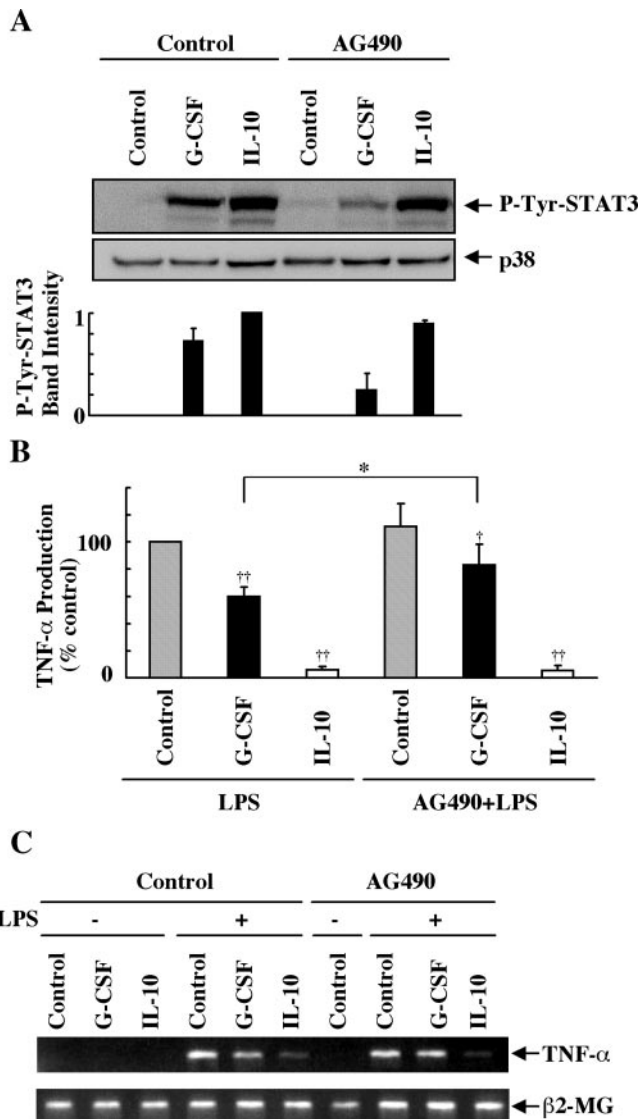


Fig. 4. G-CSF-mediated inhibition of LPS-induced TNF- α production is prevented by AG-490. **A**: monocytes were pretreated with AG-490 (50 μ M) for 1 h and then stimulated with G-CSF (500 ng/ml) or IL-10 (10 ng/ml) for 10 min at 37°C. Phosphorylation of STAT3 was analyzed by immunoblotting, using antibody against the phosphorylated form of STAT3. Results are representative of 3 independent experiments (*top*). Densitometric data from 3 independent experiments are expressed as means \pm SD (*bottom*). **B**: monocytes (3×10^6 cells/ml) were pretreated with AG-490 (50 μ M) for 1 h and then cultivated with LPS (1 μ g/ml) in the presence or absence of G-CSF (500 ng/ml) or IL-10 (10 ng/ml). After cultivation for 4 h at 37°C, the amount of TNF- α in the culture supernatant was determined. In this experiment, LPS-induced TNF- α production in control cells (no addition of G-CSF, IL-10, or AG490) was 21.10 ± 7.52 ng/ 3×10^6 cells. Data are expressed as means \pm SD of 5 independent experiments. $\dagger P < 0.05$; $\ddagger P < 0.01$, significant inhibition of LPS-induced TNF- α production by G-CSF or IL-10 regardless of the presence of AG-490. $*P < 0.05$, significant prevention of G-CSF-mediated inhibition of LPS-induced TNF- α production by AG-490. **C**: monocytes were pretreated with AG-490 (50 μ M) for 1 h and then cultivated with LPS (1 μ g/ml) in the presence or absence of G-CSF (500 ng/ml) or IL-10 (10 ng/ml). After cultivation for 3 h at 37°C, expression of TNF- α mRNA was analyzed by RT-PCR. β 2-Microglobulin (β 2-MG) was used as an internal control. Results are representative of 3 independent experiments.

is attenuated in the presence of LPS, possibly through induction of SOCS3 protein by LPS (54). A similar phenomenon might occur in human monocytes stimulated by G-CSF in the presence of LPS, because SOCS3 is recruited to activated

G-CSF receptor and can inhibit G-CSF signaling (17). To explore this possibility, we analyzed the kinetics of G-CSF- and IL-10-induced STAT3 phosphorylation in the presence or absence of LPS. As shown in Fig. 6, G-CSF-induced STAT3 phosphorylation was significantly attenuated and rapidly (within 30 min) declined in the presence of LPS. In contrast, IL-10-induced STAT3 phosphorylation was essentially unaffected and sustained at least for 90 min in the presence of LPS. The attenuation of G-CSF-induced STAT3 phosphorylation by LPS was already evident at 10 min after stimulation with G-CSF in the presence of LPS. In contrast to murine macrophages (54), SOCS3 protein in monocytes was undetectable by immunoblotting at least during the period of 10–90 min after stimulation with G-CSF, IL-10, LPS, or the combination of these agents (data not shown), despite the induced expression of SOCS3 mRNA by each of these agents (Fig. 5) (54). Thus the attenuation of G-CSF-induced STAT3 phosphorylation by LPS is unlikely to be ascribed to induction of SOCS3 protein by LPS, a finding consistent with the rapid attenuation of IL-6-induced STAT3 phosphorylation by LPS in human macrophages (1, 27).

DISCUSSION

The present experiments show that human monocytes express G-CSF receptor identical to that expressed on human neutrophils, and that, among the signaling pathways, the JAK2-STAT3 pathway is selectively activated in monocytes stimulated by G-CSF. G-CSF, like IL-10, may inhibit LPS-induced TNF- α production in monocytes through selective activation of STAT3 but not through prevention of ERK, p38, JNK, and NF- κ B activation.

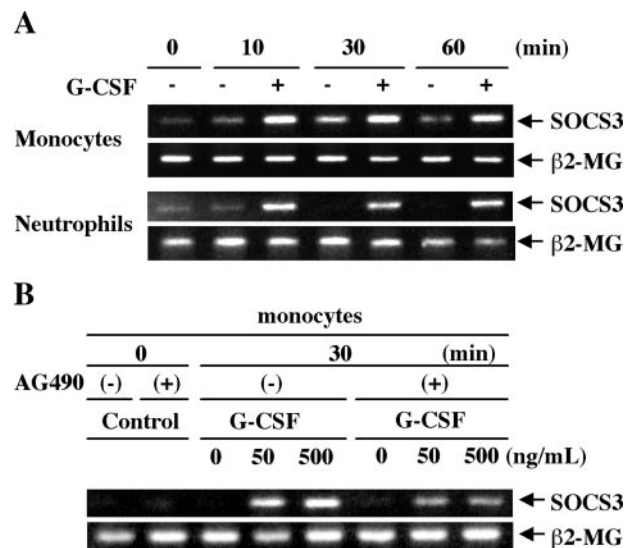
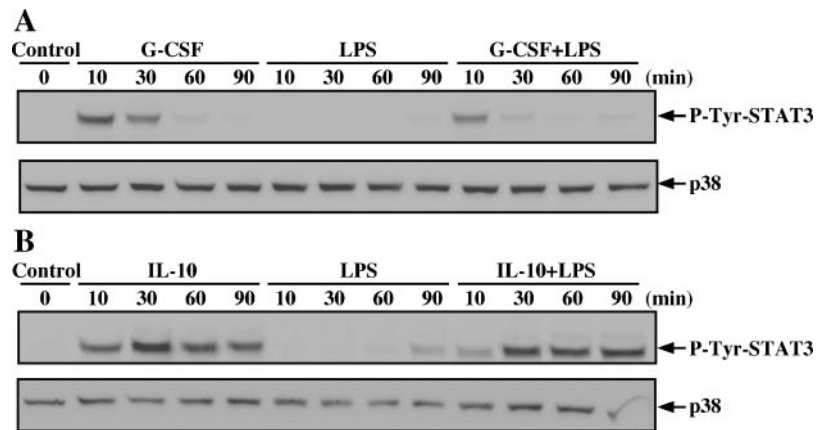


Fig. 5. Induction of suppressor of cytokine signaling 3 (SOCS3) mRNA in human neutrophils and monocytes stimulated by G-CSF. **A**: human neutrophils and monocytes were stimulated with G-CSF (50 ng/ml) for 10–60 min at 37°C. Expression of SOCS3 mRNA was analyzed by RT-PCR. β 2-MG was used as an internal control. Results are representative of 3 independent experiments. **B**: monocytes were pretreated with AG-490 (50 μ M) for 1 h and then stimulated with indicated concentrations of G-CSF for 30 min at 37°C. After cultivation, expression of SOCS3 mRNA was analyzed by RT-PCR. β 2-MG was used as an internal control. Results are representative of 3 independent experiments.

Fig. 6. STAT3 phosphorylation induced by G-CSF, but not IL-10, is attenuated in the presence of LPS. **A**: monocytes were stimulated with G-CSF (500 ng/ml), LPS (1 μ g/ml), or G-CSF (500 ng/ml) + LPS (1 μ g/ml) for indicated periods at 37°C. **B**: monocytes were stimulated with IL-10 (10 ng/ml), LPS (1 μ g/ml), or IL-10 (10 ng/ml) + LPS (1 μ g/ml) for indicated periods at 37°C. Phosphorylation of STAT3 was analyzed by immunoblotting, using antibody against the phosphorylated form of STAT3. Results are representative of 3 independent experiments.



The signaling pathways were differentially activated in neutrophils and monocytes stimulated by G-CSF despite the facts that the identical G-CSF receptor isoform is expressed on both types of cells and that the surface expression of G-CSF receptor on these cells is comparable (5). The differential activation of the signaling pathways in neutrophils and monocytes was also observed when TNF- α or IL-10 was used as stimulus. The cell-type-specific activation of a different set of distinct signaling pathways from the identical cytokine receptors may reflect the different role of these cytokines on neutrophils and monocytes (42). For example, Kitagawa and colleagues (15, 35) recently reported that the JAK2-STAT3 pathway activated in human neutrophils stimulated by G-CSF contributes to G-CSF-mediated prolongation of neutrophil survival. The present experiments suggest that the JAK2-STAT3 pathway activated in human monocytes stimulated by G-CSF contributes to G-CSF-mediated inhibition of LPS-induced TNF- α production. In hematopoietic progenitor cells, STAT3 activation has been demonstrated to be required for G-CSF-dependent proliferation and granulocytic differentiation (25, 40, 50), although a recent study suggested that STAT3 is rather a negative regulator of granulopoiesis and is not required for G-CSF-dependent differentiation (22). These findings taken together suggest that STAT3 plays a different role in G-CSF-mediated responses according to the stages of cell differentiation and the cell types.

LPS-induced TNF- α production in monocytes was highly dependent on activation of ERK, p38, JNK, and NF- κ B, because it was markedly inhibited by specific inhibitors for each signaling pathway. These signaling pathways may regulate TNF- α production at the transcriptional and/or posttranscriptional level. In fact, NF- κ B, Egr-1, Sp1, ATF-2 (a p38 or JNK substrate), c-Jun (a JNK substrate), and Ets and Elk-1 (ERK substrates) are recruited to the TNF- α promoter in response to LPS stimulation, and the binding sites for each of these transcription factors are required for LPS-stimulated TNF- α gene expression (46, 53). JNK and p38 may also regulate TNF- α production at the posttranscriptional level (3, 7). However, G-CSF and IL-10 did not affect activation of ERK, p38, JNK, and NF- κ B, which constitute the MyD88-dependent signaling pathway (2), suggesting that G-CSF and IL-10 affect the pathway downstream or independently of these signaling molecules. It was recently reported that LPS-induced TNF- α production is abolished in murine macrophages lacking

Toll/IL-1 receptor (TIR) domain-containing adaptor-inducing IFN- β (TRIF), and LPS stimulation leads to almost normal activation of JNK and NF- κ B in TRIF-deficient fibroblasts (52). The TRIF-dependent pathway is known to be independent of MyD88. These findings suggest that both the MyD88-dependent and the TRIF-dependent (MyD88 independent) pathways are required for LPS-induced TNF- α production and raise the possibility that G-CSF and IL-10 might affect the TRIF-dependent pathway to inhibit LPS-induced TNF- α production.

G-CSF, like IL-10, induced selective activation of STAT3 in monocytes. STAT3 has been demonstrated to play an essential role in IL-10-mediated inhibition of LPS-induced TNF- α production (28, 32, 44). G-CSF-induced STAT3 phosphorylation and SOCS3 mRNA expression were inhibited by AG-490 (a JAK2 inhibitor), indicating that G-CSF may activate the JAK2-STAT3 pathway in monocytes (15). G-CSF-mediated inhibition of LPS-induced TNF- α production and TNF- α mRNA expression in monocytes was also prevented by AG-490. These findings suggest that the JAK2-STAT3 pathway is involved in G-CSF-mediated inhibition of LPS-induced TNF- α production in monocytes. The specific action of AG-490 on G-CSF-mediated effects was supported by the lack of AG-490 action on IL-10-mediated effects, which may be dependent on Tyk2 and JAK1 but not on JAK2 (10). Both G-CSF and IL-10 inhibited LPS-induced TNF- α mRNA expression as well as TNF- α production in monocytes, a finding consistent with a recent report showing that IL-10 inhibits LPS-induced TNF- α production in human macrophages at the transcriptional as well as posttranscriptional levels (9). In contrast to our present results, it was reported that the level of TNF- α mRNA in whole blood stimulated with LPS is not affected by G-CSF (6). This difference might be ascribed to the difference of samples used; i.e., highly purified monocytes and whole blood. G-CSF-induced STAT3 phosphorylation was significantly attenuated and rapidly declined in the presence of LPS. The attenuation of G-CSF-induced STAT3 phosphorylation by LPS was rapid (within 10 min) and is unlikely to be ascribed to induction of SOCS3 protein by LPS. By contrast, IL-10-induced STAT3 phosphorylation was essentially unaffected and sustained in the presence of LPS (27). In addition, G-CSF was less potent than IL-10 in stimulating STAT3 phosphorylation. These characteristics of G-CSF action on monocytes may partly explain the less potent inhibitory effect of G-CSF than IL-10.

It has been reported that the JAK1-STAT3 pathway is necessary but not sufficient for IL-10-mediated inhibition of TNF- α production, and an additional signaling pathway derived from the COOH-terminal domain of IL-10 receptor may also be required for this effect (32). In this regard, it is of interest that a high concentration of G-CSF (>100 ng/ml) was required to inhibit LPS-induced TNF- α production, although an almost maximal level of STAT3 phosphorylation was obtained at the lower concentration (50 ng/ml) of G-CSF. These findings suggest that, besides STAT3 activation, an additional signaling pathway activated by a high concentration of G-CSF also may be required for efficient inhibition of LPS-induced TNF- α production by G-CSF. In fact, it has been shown that the signaling pathways could be differentially activated by G-CSF, depending on G-CSF concentration (51).

The precise mechanisms by which STAT3 activation inhibits TNF- α production remain to be elucidated. The coactivator proteins cAMP response element-binding protein (CREB)-binding protein (CBP) and p300 play an important role in integrating multiple transcription factors in various cell systems (38), and CBP/p300 is involved in LPS-induced TNF- α gene expression (46). In addition, it has been shown that CBP/p300 interacts physically with STAT3 in certain cell lines (31, 37). Furthermore, interferon regulatory factor 3 (IRF3), a transcription factor downstream of TRIF, also interacts with CBP/p300 (12). These findings raise the possibility that the inhibition of TNF- α gene expression by G-CSF or IL-10 might be caused by increased competition for limiting amounts of CBP/p300 by STAT3.

LPS-induced TNF- α production in monocytes was inhibited by G-CSF in a dose-dependent manner at a concentration range from 100 to 500 ng/ml. This finding is consistent with a recent report showing that G-CSF inhibits LPS-induced TNF- α production in whole blood in a dose-dependent manner at a concentration range from 10 to 300 ng/ml (5). These findings indicate that a higher concentration of G-CSF may be required for inhibition of LPS-induced TNF- α production compared with that required for efficient stimulation of granulopoiesis and activation of neutrophil functions (8, 19, 29). It has been reported that endogenous plasma G-CSF levels are 0.02–0.09 ng/ml in healthy persons and increase to 0.8–4 ng/ml upon bacterial infections. Therapeutic intravenous administration of G-CSF (3.45–11.5 μ g/kg) gives a plasma concentration of 20–400 ng/ml (16, 48). Our previous study shows that intravenous administration of 200 μ g/m² G-CSF gives a plasma concentration of 100 ng/ml (29). When G-CSF-mobilized PBSC were harvested, donors received much higher doses of G-CSF; i.e., subcutaneous administration of 200 μ g/m² G-CSF twice daily (15). These findings suggest that therapeutic administration of G-CSF could give a plasma concentration of G-CSF sufficient for inhibition of LPS-induced TNF- α production in monocytes. Such a high concentration of endogenous G-CSF also might be produced locally at the inflammatory sites. On the other hand, it has been reported that endogenous plasma IL-10 levels are 0.1–0.15 ng/ml upon endotoxemia (30), and IL-10 inhibited LPS-induced TNF- α production in monocytes by >90% at high concentration (10 ng/ml). These findings taken together suggest that G-CSF at the moderate concentrations potentiates the host defense against invading microorganisms through increased production of neutrophils and activation of neutrophil functions, and

G-CSF at the high concentrations exerts the anti-inflammatory or immunomodulatory effect by inhibiting the overproduction of proinflammatory cytokines from monocytes. This concentration-dependent dual action of G-CSF may be physiologically relevant and important in maintaining the host-defense mechanism. Furthermore, the results presented here indicate that the immunomodulation observed in vivo by G-CSF administration (18, 33, 49) may be partly ascribed to the direct effect of G-CSF on monocyte functions, which may be mediated through selective activation of STAT3.

GRANTS

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