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Activation of STAT5 by Lipopolysaccharide Through Granulocyte-Macrophage Colony-Stimulating Factor Production in Human Monocytes

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LPS is a potent stimulator of monocytes, inducing many of their functions. Although the details of how LPS exerts such functions remain largely unknown, transcription factors such as nuclear factor-κB, nuclear factor-IL-6, and activator protein-1 have been shown to be involved in this process. However, to date it has been thought that no known STAT molecule plays a role in the activation of monocytes by LPS. In this study we examined whether some known STAT molecule is stimulated by LPS, based on the finding that a GAS motif sequence is conserved in the promoter regions of human, mouse, and rat cyclo-oxygenase-2 (COX-2) genes. Consequently, LPS induced activation of STAT5 in human monocytes, and this STAT5 activation occurred in an indirect way via granulocyte-macrophage CSF (GM-CSF) secreted by LPS-stimulated monocytes. Expression of COX-2 protein was partially reduced by treatment of anti-human GM-CSF Ab. Activation of STAT5 was inhibited by either IL-10 or dexamethasone (Dex), but not by aspirin. IL-10 blocked activation of STAT5 indirectly by suppressing GM-CSF production, while Dex inhibited this activation both directly and indirectly. Taken together, these results suggest that in addition to other transcription factors, STAT5 plays an important role in activation of monocytes by LPS, and that STAT5 is another target for IL-10 and Dex to inhibit COX-2 expression in activated monocytes. The Journal of Immunology, 1998, 160: 838–845.

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1 Abbreviations used in this paper: NF-κB, nuclear factor-κB; AP-1, activator protein-1; LIL, lipopolysaccharide-induced and interleukin-1-induced; LILRE, lipopolysaccharide- and IL-1-responsive element; Dex, dexamethasone; COX, cyclooxygenase; GM-CSF, granulocyte-macrophage colony-stimulating factor; h, human; EMSA, electrophoretic mobility shift assay.

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LPS-induced activation of monocytes, and that IL-10 and Dex exert inhibitory effects on COX-2 expression through deactivation of STAT5.

Materials and Methods

Materials

LPS was purchased from Difco Laboratories (Detroit, MI). Dex, aspirin, and cyclohexamide were purchased from Sigma Chemical Co. (St. Louis, MO). Herbimycin A was purchased from Wako (Osaka, Japan). Anti-STAT1, -2, -3, and -6 Abs were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-STAT4 Ab was given by Dr. Koh Yamamoto (Tokyo Medical and Dental University, Tokyo, Japan). Anti-STAT5 Ab and the oligonucleotide probe, GRR (5'-GTATTTCCCAAGAAAAG GAAC-3'), were provided by Dr. Hiroshi Wakao (Tokyo University, Tokyo, Japan). Anti-COX-2 Ab was purchased from Transduction Laboratories (Lexington, KY). Anti-phosphotyrosine mAb (4G10) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Recombinant human GM-CSF (hGM-CSF) was a gift from Japan Schering-Plough Corp. (Osaka, Japan). Anti-hGM-CSF Abs were either given by Dr. Toshio Kitamura (DNAX Research Institute, Palo Alto, CA) or purchased from Genzyme (Cambridge, MA). Recombinant human IL-10 (hIL-10) was a gift from Dr. Satish Menon (DNAX Research Institute).

Isolation and culture of human monocytes

The procedures of isolation and culture of human monocytes were previously described (18). Briefly,uffy coats from healthy donors were provided by the Numazu Red Cross Center (Numazu, Japan). Mononuclear cells were separated over Ficoll-Hypaque. Subsequently, these mononuclear cells were adhered to culture dishes for 2 h with a culture medium composed of RPMI 1640 with 10% FCS, then washed three times vigorously with warmed PBS to remove nonadherent cells. The remaining adherent cells consisted of >90% nonspecific esterase positive and >99% viable, as determined by trypan blue exclusion. These cells were further cultured with the culture medium for the experiments described below.

Extraction of nuclear proteins and electrophoretic mobility shift assay (EMSA)

The procedures of extraction of nuclear proteins and EMSA were previously described (22). Human monocytes were stimulated by 1 μg/ml LPS for the indicated times, by hGM-CSF at the indicated concentrations for 15 min, or by 10 ng/ml hL-10 for 30 min and then lysed to extract nuclear proteins. In some experiments, monocytes were pretreated with 2 μg/ml herbimycin A for 2 h before the stimulation. In pretreatment experiments, 10 ng/ml hL-10 for 30 min, 1.0 μM Dex for 2 h, 50 mM aspirin for 2 h, or 5 μg/ml cyclohexamide for 1 h was added before monocyte stimulation. Some experiments were performed in the presence of 10 μg/ml anti-hGM-CSF Ab. The amounts of loaded nuclear extracts were normalized before mixing.

Nuclear extract was mixed with binding buffer (20 mM HEPES-NaOH (pH 7.9), 2 mM EDTA, 100 mM NaCl, 10% glycerol, and 0.2% Nonidet P-40), poly(dI-dC), and 32P-labeled oligonucleotide probe. The mixtures were incubated at room temperature for 30 min. To detect supershifted bands, anti-STAT Abs or anti-phosphorylated STAT Ab were added on ice for 30 min to the assay mixtures. The reaction mixtures were loaded on a 4% polyacrylamide gel and run with a running buffer of 0.25× TBE. The gel was then dried onto Whatman 3MM paper (Whatman, Clifton, NJ). The DNA/protein complexes were visualized by autoradiography.

The oligonucleotide probes used were: COX-2-sp3 (5'-TCTCTTTTCAAGAAACAAG-3'), COX-2-sp4 (5'-ATTTCTCTGTTGAAAGCA-3'), and GRR.

Results

Activation of STAT5 by LPS in human monocytes

The search for COX-2 genes has revealed that the promoter region of the human COX-2 gene has two GAS motif sequences (TTC-NNN(N)GAA; COX-2-sp3 and COX-2-sp4), and that COX-2-sp3 was well conserved in both rat and mouse COX-2 genes (23–25) (Fig. 1). This prompted us to investigate whether some STAT molecule is activated by LPS in human monocytes, leading to binding to these sequences. To test this hypothesis, we performed EMSA using two kinds of probes, COX-2-sp3 and COX-2-sp4.

When monocytes were stimulated by LPS for 15 min, no band was detected. However, formation of the complex of some activated molecule with the COX-2-sp3 probe occurred 90 min after LPS stimulation (Fig. 2A, lanes 1–3). On the other hand, the COX-2-sp4 probe did not elicit any band in our system (data not shown). The appearance of this band was inhibited by adding an excess of cold COX-2-sp3, but not cold COX-2-sp4 (Fig. 2A, lanes 4 and 5), demonstrating the specificity of the binding of this molecule to COX-2-sp3 probe. Pretreatment of monocytes with herbimycin A abrogated the band, and adding monoclonal anti-phosphotyrosine Ab to the mixture diminished the band, proving that this molecule was truly tyrosine phosphorylated (Fig. 2, B and C). The nuclear extracts of two human monocytic cell lines, THP-1 and U937, and mouse monocytic cell lines, Raw264.7 and J774.1, did not raise this band, suggesting that the formation of this band was observed only in human peripheral blood monocytes (data not shown).

It was assumed that this activated molecule belongs to the STAT family, because the GAS motif sequence is known to bind to some STAT molecule (11). To explore this possibility, the Abs of known STAT molecules were added to the binding mixtures. All these Abs has been ascertained to work for the supershift assay (data not shown). Only anti-STAT5 Ab generated a supershifted band; however, the Abs against the other STAT molecules did not influence...
the band (Fig. 3, A and B). Nonspecific bands appeared in the presence of anti-STAT4 and anti-STAT5 Ab, which were observed even in the absence of the nuclear extracts (Fig. 3A, lanes 6–9). Furthermore, the mobility of the band induced by LPS was the same as that of hGM-CSF-induced STAT5 (Figs. 4 and 5). These results showed that the molecule(s) activated by LPS in human monocytes contained STAT5.

**Activation of STAT5 through GM-CSF production**

Although activation of STAT5 was not detected 15 min after LPS stimulation, activation of STAT5 was observed 15 min after stimulation with hGM-CSF (Fig. 2A, lane 2, and Fig. 4B, lane 2). This delayed profile of STAT5 activation by LPS raised the possibility that LPS indirectly activates STAT5 by inducing some other molecules. To address this question, we assessed the effect of cyclohexamide on STAT5 activation by LPS. Treatment of LPS-stimulated monocytes with cyclohexamide resulted in complete abrogation of STAT5 activation, indicating that this activation is executed by some newly synthesized molecule (Fig. 4A).

It is known that LPS promotes generation of various kinds of cytokines, such as TNF-α, IL-1β, IL-6, IL-8, IL-10, IL-12, G-CSF, M-CSF, and GM-CSF, from human monocytes (26–28). It has been shown that among these cytokines, GM-CSF causes STAT5 activation in human monocytes (12, 29, 30). To investigate whether STAT5 is activated by LPS through GM-CSF production, we examined the effect of neutralizing anti-hGM-CSF Ab on LPS-induced STAT5 activation. When monocytes were incubated with hGM-CSF for 15 min, an activated molecule appeared, and this molecule was ascertained to be STAT5 by supershift assay (Fig. 4B, lanes 1–3). LPS stimulation caused the appearance of the same mobility band as STAT5, and this activation was attenuated by approximately 42% in the presence of anti-GM-CSF Ab as judged by image analyzer, compared with the presence of isotype control Ab (six donors were tested for the analysis; Fig. 4B, lanes 4–6). Two kinds of anti-hGM-CSF Abs equally inhibited the activation

![FIGURE 2. Formation of the complex of LPS-stimulated nuclear extract with COX2-sp3 probe. Nuclear extracts were obtained from human monocytes stimulated without (A–C, lane 1) or with 1 μg/ml LPS for 15 min (A, lane 2) or for 90 min (A, lanes 3–5; B, lane 2 and 3; C, lanes 2 and 3), and then incubated with the 32P-labeled COX2-sp3 probe. In B, lane 3, monocytes were pretreated with 2 μg/ml herbimycin A (HA) for 2 h before adding LPS. An excess of the cold COX2-sp3 probe (A, lane 4), the cold COX2-sp4 probe (A, lane 5), or monoclonal antiphosphotyrosine (PY) Ab (C, lane 3) were present in the assay mixtures. The arrow (+) indicates the position of the DNA/protein complex.](http://www.jimmunol.org/)

![FIGURE 3. Supershift of the LPS-induced band by anti-STAT Abs. Nuclear extracts were obtained from human monocytes stimulated without (A and B, lane 1) or with 1 μg/ml LPS for 90 min (A, lanes 2–7; B, lanes 2 and 3), and then incubated with the 32P-labeled COX2-sp3 probe. In A, lanes 8 and 9, the probe was incubated with either anti-STAT4 Ab or anti-STAT5 Ab without nuclear extracts. The indicated anti-STAT Abs (A, lanes 3–9; B, lane 3) were present in the assay mixtures. The arrows (+, *, and #) indicate the positions of the DNA/protein complex, the supershifted STAT5, and nonspecific bands raised by anti-STAT4 Ab and anti-STAT5 Ab, respectively.](http://www.jimmunol.org/)
These results suggest that secreted GM-CSF contributes, at least in part, to STAT5 activation in LPS-stimulated monocytes. Treatment of GM-CSF-stimulated monocytes with cy-clohexamide did not affect STAT5 activation, proving that STAT5 is activated directly by GM-CSF (Fig. 4C). Given that anti-hGM-CSF Ab did not completely prevent LPS-induced STAT5 activation, we could not exclude the possibility that some cytokine other than GM-CSF induced by LPS would also lead to STAT5 activation. Treatments of neither Abs against G-CSF or M-CSF, which have been shown to activate STAT5 in other cells, influenced LPS-induced STAT5 activation (31, 32) (data not shown).

Next, to evaluate the amount of secreted GM-CSF triggered by LPS, we compared the intensity of the STAT5 band induced by 1 µg/ml LPS with that of bands induced by various concentrations of hGM-CSF. Stimulation of monocytes with hGM-CSF at a concentration of 10 pg/ml up to 10 ng/ml showed STAT5 activation in a dose-dependent manner (Fig. 4, lanes 3–6). The intensity of the LPS-induced band was less than that of 10 pg/ml hGM-CSF, indicating that the level of secreted GM-CSF induced by LPS is <10 pg/ml (Fig. 5). This idea is further supported by our findings that tyrosine phosphorylation of STAT5 could not be detected upon stimulation of either 1 µg/ml LPS or 10 pg/ml hGM-CSF by Western blotting (data not shown). In addition, GM-CSF in the supernatant of LPS-stimulated monocytes was below the lowest level detectable by ELISA (8 pg/ml; data not shown).

**Effect of GM-CSF on expression of COX-2 protein in human monocytes**

The fact that STAT5 was activated by LPS through secretion of GM-CSF prompted us to test whether STAT5 activation by GM-CSF contributes to COX-2 expression. When monocytes were coincubated with anti-hGM-CSF Ab, LPS-induced COX-2 expression was decreased by approximately 60% compared with that after treatment with the isotype control Ab, as analyzed by the National Institutes of Health Image 1.55 program (four donors were tested for the analysis; Fig. 6). These results indicate that GM-CSF secretion contributes, at least in part, to LPS-induced COX-2 expression. Together with the results of STAT5 deactivation and COX-2 down-regulation by anti-hGM-CSF Ab, it is supposed that the regulation mechanisms of both STAT5 activation and COX-2 expression are parallel. However, stimulation of hGM-CSF alone did not cause any COX-2 expression (data not shown). Thus, these results raise the possibility that GM-CSF-stimulated STAT5, in concert with other transcription factors, such as NF-κB, NF-IL-6, and AP-1, might regulate COX-2 expression in LPS-stimulated monocytes.

**Effects of IL-10 and Dex on STAT5 activation**

It has been shown that IL-10 and Dex effectively abrogate PGE₂ production in LPS-stimulated monocytes by suppressing the transcription of COX-2 gene (18, 19, 33, 34). To investigate whether
IL-10 and Dex influence LPS-induced STAT5 activation, we pretreated monocytes with either hIL-10 or Dex before LPS stimulation. As a result, hIL-10 and Dex completely blocked STAT5 activation by LPS, whereas aspirin did not evoke any effect (Fig. 7A). In view of previous findings that both IL-10 and Dex significantly inhibited GM-CSF production in LPS-stimulated monocytes, it was assumed that abrogation of LPS-induced STAT5 activation by hIL-10 and Dex is, at least partly, ascribable to the suppression of GM-CSF production in LPS-stimulated monocytes (27, 35).

Next, to test the possibility that either hIL-10 or Dex affects STAT5 activation by secreted GM-CSF, we pretreated the cells with either hIL-10 or Dex before hGM-CSF stimulation. Interestingly, pretreatment of Dex inhibited STAT5 activation by almost 50%, as judged by image analyzer, whereas pretreatment of hIL-10 was without effect (Fig. 7B). The expression levels of STAT5 and GM-CSF receptor were invariant by pretreatment with Dex, as estimated by Western blotting and flow cytometry, respectively (Fig. 7C and data not shown). The expression level of STAT5 was constant during treatment with hIL-10, as evaluated by Western blotting (data not shown). These results indicate that IL-10 indirectly inhibits STAT5 activation by abrogating GM-CSF production, whereas Dex inhibits this activation in both direct and indirect ways.

Specificity of binding of IL-10-activated STAT1
The finding that pretreatment of hIL-10 did not cause the appearance of any band using the COX-2-sp3 probe was unexpected, because it had been previously reported that IL-10 activates both STAT1 and STAT3 using the GRR probe (12, 36). To explore the possibility that this discrepancy was due to the difference in probes, we subjected both the COX-2-sp3 probe and the GRR probe to EMSA to detect IL-10-activated STAT molecules. The hIL-10-stimulated nuclear extract did not elicit any band using the COX2-sp3 probe, whereas the same extract did elicit a clear band using GRR (Fig. 8A, lanes 1–4). This band was strongly inhibited by adding an excess of the GRR probe rather than by adding an excess of the COX-2-sp3 probe (Fig. 8A, lanes 5–8). Anti-STAT1 Ab, but not anti-STAT3 and STAT6 Abs, supershifted this IL-10-induced band (Fig. 8B). To date, it is unclear why activation of STAT3 was not detected in our system. These results implied that a GAS motif-containing sequence preferentially binds to an activated STAT molecule, with the consequence that IL-10-activated STAT1 has higher affinity for the GRR sequence than for the COX-2-sp3 sequence.

Discussion
In the present study we have shown that activation of STAT5 was induced by LPS in human peripheral monocytes through secretion of GM-CSF. We ascertained that the activated molecule that forms the complex with COX-2-sp3 was STAT5, because only anti-STAT1 Ab elicited the supershifted band, and the mobility of the band was the same as that of STAT5 induced by exogenous GM-CSF (Figs. 2–5). Our failure to detect either tyrosine phosphorylation of STAT5 by Western blotting or GM-CSF protein by ELISA suggests that for short periods of incubation (90 min), LPS-stimulated monocytes secrete very small amounts of GM-CSF. In this regard, our EMSA system is much more sensitive than the above two systems in detecting LPS-induced GM-CSF production via STAT5 activation in monocytes.

Previous studies suggested that no known STAT molecule was involved in LPS-induced monocyte activation (12, 13). Consistent with a previous report (12), we did not detect any STAT activation 15 min after LPS stimulation; however, during the longer period (90 min), STAT5 activation was clearly induced by secreted GM-CSF (Figs. 2–5). Our failure to detect either tyrosine phosphorylation of STAT5 by Western blotting or GM-CSF protein by ELISA suggests that for short periods of incubation (90 min), LPS-stimulated monocytes secrete very small amounts of GM-CSF. In this regard, our EMSA system is much more sensitive than the above two systems in detecting LPS-induced GM-CSF production via STAT5 activation in monocytes.

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stimulation, whereas STAT5 was not. 3) LIL-STAT activation was observed in a human monocytic cell line, THP-1, whereas STAT5 activation was not. 4) A G residue at position 8 of LILRE (TTCCTGAGA) was critical for binding to LIL-STAT, whereas COX-2-sp3 did not have a G residue at position 8. These results may suggest that LPS activates more than two STAT molecules, either directly or indirectly. It has been shown that LPS induces the secretion of TNF-α, IL-1β, IL-6, IL-8, IL-10, IL-12, G-CSF, and GM-CSF from monocytes, and that IL-6, IL-10, IL-12, and G-CSF cause activation of STAT1, -3, and -4 among these cytokines (11, 26, 27). In addition, i.p. injection of LPS leads to STAT3 activation in mouse hepatocytes, probably by IL-6 secretion (37). However, we could not detect any activation of these STAT molecules in the present study (Fig. 3). This may have been due to the low level of secretion of these cytokines or to inappropriate probe selection. Further studies are needed to resolve this point.

STAT5 was initially purified from sheep mammary gland as a prolactin-induced transcription factor regulating expression of the β-casein gene and was subsequently shown to be encoded by two distinct genes, STAT5A and STAT5B (29, 38). To date, STAT5 activation has been proved to be induced by IL-2, IL-3, IL-5, IL-7, IL-15, G-CSF, M-CSF, GM-CSF, erythropoietin, thrombopoietin, growth hormone, epidermal growth factor, platelet-derived growth factor, and engagement of B cell receptor (29, 31, 32, 39–43). Our present study revealed that in addition to these stimuli, LPS causes STAT5 activation indirectly. The role of STAT5 as a proliferation signal is still controversial; however, it has been verified that activation of STAT5 is essential for growth hormone-induced expression of hepatic serine protease inhibitor 2.1; oncostatin M expression stimulated by IL-2, IL-3, and erythropoietin; and IL-3-induced expression of cis, osm, and pim-1 (29, 44–47). In the present study, we demonstrated that treatment of anti-hGM-CSF Ab partially reduced LPS-induced COX-2 expression (Fig. 6). This indicates that GM-CSF secretion contributes to COX-2 expression induced by LPS, and that it may be due to STAT5 activation induced by secreted GM-CSF. It has been shown that deletion of the GAS motif sequence of the human COX-2 gene does not influence the expression level of COX-2 induced by LPS using a transient transfection assay in bovine arterial endothelial cells (48). However, it may be that GM-CSF is not secreted from endothelial cells stimulated by LPS. These results suggest that STAT5 plays some role, such as expression of COX-2 in activated human monocytes.

The role of LPS-induced GM-CSF production in monocyte activation has been well investigated to date, demonstrating that GM-CSF leads to enhancement of proliferation, Ag presentation, killing of parasites, oxidative metabolism, and antitumor immunity of monocytes (49). In these biologic activities of GM-CSF, it is not known which signal pathway is transduced through STAT5. Studies of STAT5 gene-targeting mice and analyses of the dominant negative phenotype of STAT5 would be helpful in clarifying this point. Furthermore, the studies in gene-disrupted mice of GM-CSF or IL-3/GM-CSF/IL-5 receptor have exhibited abnormal pulmonary pathologic features consisting of lymphocytic infiltration and areas resembling alveolar proteinosis in steady state, indicating that GM-CSF is essential for normal pulmonary physiology and resistance to local infection (50–52). However, it remains to be examined what LPS-induced signal pathway is triggered by secreted GM-CSF. Further studies aimed at analyses of the impaired function in monocytes of these mice in response to LPS would be useful in this regard.
on our findings, we speculate that different stimuli cause different intramolecular modification of the same STAT molecule, or association of different molecules with the same STAT molecule, resulting in different abilities to form the DNA/protein complex. Further analyses have to be conducted to resolve this point. The fact that we could not detect the binding of IL-10-induced STAT1 to the COX-2-sp3 probe might suggest that STAT5 deactivation is more important than STAT1 activation for IL-10 to exert its anti-inflammatory effect. This idea is further supported by a study of STAT1 gene-disrupted mice, in which the signal pathway of IFN-α is impaired but the inhibitory effect of IL-10 on LPS-stimulated TNF production is not affected (56). Taken together, these results may provide a clue to elucidate the anti-inflammatory mechanism of IL-10.

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