Cell Type- and Developmental Stage-specific Activation of NF-κB by fMet-Leu-Phe in Myeloid Cells*

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Chemoattractants induce a variety of phagocytic functions including transendothelial migration, degranulation, and the generation of superoxide anions. We report here that the prototypic chemotactic peptide fMet-Leu-Phe (fMLF) stimulates the activation of nuclear factor-κB (NF-κB), a transcription factor that is central to the regulation of proinflammatory immediate-early gene expression. In freshly prepared peripheral blood mononuclear cells, fMLF (1–100 nM) induced a κB binding activity that was receptor-dependent and involved the p50 and p65 subunits of the NF-κB/Rel family of proteins. The activation of NF-κB by fMLF appeared to be cell-specific and different from the activation of NF-κB by tumor necrosis factor-α (TNFα). Neutrophil preparations that responded to fMLF, TNFα, and lipopolysaccharides with interleukin-8 secretion did not show NF-κB activation, whereas N-formyl peptide receptor (FPR)-transfected HL-60 cells were responsive to TNFα but not fMLF for NF-κB activation. Differentiation of FPR-transfected HL-60 cells with dimethyl sulfoxide for 3–5 days conferred the capability of the cells to activate NF-κB in response to fMLF without a significant increase in the amount of FPR. These results identify NF-κB as a transcription factor that can be activated by the prototypic chemotactic peptide and demonstrate that this function is both highly regulated and dependent on signaling components specifically expressed during myeloid differentiation.

The coordinated regulation of gene expression by phagocytic leukocytes is generally considered to be essential to the early inflammatory response in humans. The production of cytokines, chemokines, cell-surface receptors/adhesion proteins, and other molecules by activated monocytes, macrophages, and neutrophils can serve to orchestrate immune cell development and is essential to host defense against invading microorganisms. The regulation of gene expression in these cells is governed by the activities of transcription factors such as nuclear factor-kappa B (NF-κB),1 NF-IL-6, and AP-1, which are them-
or in the promyelocytic HL-60 cells. Differentiation of HL-60 cells with dimethyl sulfoxide (Me2SO) resulted in the acquisition of FMLF-stimulated activation of NF-κB that was independent of receptor up-regulation. In addition, transfected HL-60 cells expressing functional FPR were responsive to TNFa but not to FMLF for NF-κB activation, suggesting the involvement of separable signaling pathways leading to transcription factor activation. These findings indicate that activation of NF-κB may be a general function of chemoattractants but is limited to specific classes of terminally differentiated myeloid cells. Furthermore, this work highlights the utility of HL-60 cells as a model for a more detailed investigation of the signaling mechanisms leading to NF-κB activation by different ligands.

**EXPERIMENTAL PROCEDURES**

**Materials**—LPS isolated from *Salmonella minnesota* Re595 was a gift from R. Ulevitch (Scripps Research Institute). Recombinant murine TNFα was kindly provided by V. Kravchenko (Scripps Research Institute). The recombinant TNFα contains less than 0.277 ng/mg LPS as measured by the Limulus amebocyte lysate assay (Cap Cod Associate, Woods Hole, MA). Pyrroldinedithiocarbamate (PDTC), purchased from Sigma, was kept as a 1× stock in H2O and was added to cultures directly. FITC-FMLF ( Molecular Probes, Eugene, OR) and unlabeled FMLF (Sigma), were diluted with phosphate-buffered saline from concentrated stocks maintained at −20 °C in Me2SO. A double-stranded oligonucleotide probe containing a decamer κB sequence (underlined) 5′-AGTTGAGGGGACTTTCCCAGG-3′ was labeled using polynucleotide T4-kinase (Life Technologies, Inc.) and [γ-32P]ATP (10 μCi/ml, Amersham Corp.). Rabbit polyclonal antibodies against a panel of NF-κB/Rel family of proteins were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CD14 monoclonal antibody was from Coulter Immunology (Miami, FL), and phycoerythrin conjugated goat anti-mouse IgG was purchased from Sigma. Detection of IL-8 in culture supernatants was carried out using a commercially available enzyme-linked immunosorbent assay kit according to the procedures suggested by the manufacturer (Biosource International, Camarillo, CA).

**Preparation of Cells**—Human peripheral blood leukocytes were fractionated using Percoll based on the method of Ulmer and Flad (17). Briefly, blood was collected from healthy donors using acid citrate buffer containing 2% dextrose as an anticoagulant. Erythrocytes were removed by sedimentation with 6% hetastarch (Baxter). Peripheral blood mononuclear cells (PBMC) and neutrophils in the supernatant were further separated by centrifugation at 450 × g at 10 °C for 40 min through Percoll step gradients (70 and 55%). Viability of cells in a routine preparation was approximately 98% as determined by trypan blue exclusion. Following a wash in fresh RPMI 1640 medium (serum-free), cells (5 × 106) were stimulated with various ligands in 1.5-ml microcentrifuge tubes at 37 °C.

PBMCs prepared from several healthy donors were found to contain three populations of cells as identified by flow cytometry on forward and side scatter dot plots: platelets, lymphocytes and monocytes (Fig. 1). Using CD14 as a cell-surface marker for the monocytes, we observed that only these cells, but not platelets or lymphocytes, express the FPR as detected by FITC-FMLF direct binding (Fig. 1). Thus, PBMC was used without further fractionation, and any responses to FMLF could be attributed to binding and stimulation of monocytes in these preparations. The transfection of HL-60 cells with the FPR has been detailed elsewhere (18). Both transfected and untransfected HL-60 cells were maintained at 37 °C with 5% CO2 in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES (pH 7.0), penicillin (100 IU/ml), and streptomycin (50 μg/ml). HL-60 cells (5 × 105 cells/ml) were differentiated with Me2SO (1.3% v/v) at 37 °C in 5% CO2. Cells were incubated at 37 °C for 7 days in a humidified CO2 incubator.
A saturating concentration of fMLF (100 nM) to freshly isolated PBMC resulted in a rapid and transient activation of kB binding activity as measured by EMSA (Fig. 2A). The DNA-protein complexes were detected 30 min following addition of fMLF, peaked in amount after 60 min, and subsequently decreased to less than half of the maximum in 4 h. The extent of the induced DNA binding activity was dependent on the dose of fMLF as the response was detectable with nanomolar concentrations of fMLF and reached a maximum with 50 nM fMLF (not shown). The effectiveness of nanomolar concentrations of fMLF to induce kB binding activity suggested that it is a primary response to fMLF stimulation mediated by a high affinity receptor. This point was confirmed by blocking fMLF-induced kB binding activity with N-cinnamoyl-FLFLF (Fig. 2A), a newly identified fMLF receptor antagonist. ² Further fractionation of PBMC by centrifugation through additional Percoll gradients revealed that the purified monocytes displayed essentially the same kB binding activity in response to fMLF stimulation (data not shown).

Because NF-kB activation by fMLF has not been previously reported, we examined the specificity and composition of the resultant kB binding activity in the stimulated PBMC. Competition with unlabeled kB probe significantly reduced the major species identified on these gels thus indicating that the DNA binding activity was specific to the prototypic kB sequence (Fig. 2B). PDTC, an antioxidant inhibitor of NF-kB that functions by stabilization of the inhibitory protein IxB (21), was found to block the appearance of the DNA-protein complexes in fMLF-stimulated cells (Fig. 2B). Gel mobility supershift with specific antibodies is widely used as supportive evidence for the identification of specific proteins in DNA-protein complexes detected by EMSA. In order to confirm the identity of the putative NF-κB species observed in fMLF-treated PBMC, nuclear extracts were preincubated with equal amounts of antibodies directed against well documented subunits of the NF-kB/Rel proteins. Gel retardation assays revealed upward shifting of the DNA-protein complexes only by antibodies against the p50 and p65 proteins (Fig. 2C), similar to the supershift pattern observed in other systems (22). These results indicate the presence of p50 and p65 subunits of the NF-κB/Rel protein family, possibly forming a p50/p65 homodimer and a p50/p65 heterodimer in the DNA-protein complex induced by fMLF. Preincubation of nuclear extracts with antibodies specific to p52, c-Rel, and RelB did not cause a supershift of the DNA-protein complex (Fig. 2C), but this experiment alone could not completely rule out the possibility that fMLF may activate other NF-κB/Rel proteins in these cells. Taken together, results derived from competition and supershift experiments confirmed fMLF induction of NF-κB activation in monocytes. The finding that PDTC inhibits the response suggested that fMLF-stimulated activation of NF-κB involves IxB degradation similar to the responses reported for other inducers of NF-κB (4, 21).

It has been well established that monocytes are a major source of proinflammatory cytokines. More recently it has been reported that neutrophils also have transcriptional activity and can secrete cytokines including IL-1β, IL-6, and IL-8 following activation with either cytokines such as TNFα or chemotactants such as C5a and fMLF (15, 23). Since the expression of IL-1β and IL-8 is regulated to a great extent by NF-κB at the level of transcription, we reasoned that stimulation with fMLF might activate NF-κB in these cells. This notion was examined by comparing the ability of PBMC and neutrophils to respond to fMLF as well as TNFα and LPS (Fig. 3A). The magnitude of the NF-κB response to fMLF in PBMC was similar to that of the better characterized inducers such as LPS and TNFα. Surprisingly, none of these agonists were able to induce detectable NF-κB activation in neutrophils stimulated for 1 h (Fig. 3A) or

² H. Solomon, unpublished observations.
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For several different time periods from 15 min to 2 h (data not shown).

In order to confirm that the neutrophil preparations were capable of de novo synthesis and secretion of cytokines, the cells were treated with fMLF, and the IL-8 protein secreted into the medium was measured. Stimulation of neutrophils with 100 nM fMLF caused a 4–5-fold increase in the levels of IL-8 in the supernatant was measured. The readings ± S.E. from three independent experiments are shown.

As a preliminary effort directed at the elucidation of the mechanism of NF-κB activation by fMLF, our attention focused on HL-60 cells as a potential model. The HL-60 cell is a promyelocytic cell line that can be induced to differentiate into both monocytic and granulocytic lineages (24). Treatment of HL-60 cells with Me2SO has been shown to induce granulocytic differentiation of the promyelocytic cells (25). Although other reagents such as phorbol myristate acetate and dibutyryl cyclic AMP can also initiate differentiation of HL-60 cells, they are capable of activating NF-κB (26) and therefore were not chosen for our studies. Following Me2SO (1.3% v/v) treatment, HL-60 cells attained the ability to respond to fMLF with NF-κB activation, while the undifferentiated cells were unresponsive to saturating concentrations of fMLF (Fig. 5A). As early as 3 days following Me2SO treatment, fMLF-dependent NF-κB activation could be detected, and this response was maximal in cells allowed to differentiate for 5 days. Me2SO treatment induced very little spontaneous NF-κB activation as the background remained low. Subunit analysis of fMLF-induced NF-κB composition by supershift assay indicated the presence of both p50 and p65 proteins similar to that observed in fMLF-stimulated monocytes (Fig. 5A; compare with Fig. 2C). Thus, Me2SO-differentiated HL-60 cells are fully capable of activating NF-κB in response to fMLF.

A number of granulocytic functions are acquired by HL-60 cells upon treatment with Me2SO, including chemotactic and phagocytic competence, inducible superoxide generation, and the increased expression of several chemoattractant receptors. As measured by FITC-fMLF binding, the expression of FPR on the cell surface increased dramatically in Me2SO-treated HL-60 cells after the 3rd day (Fig. 5B). In order to determine whether expression of FPR was the essential component responsible for fMLF-inducible NF-κB activation in differentiated cells, we tested FPR cDNA-transfected HL-60 cells with or without additional Me2SO treatment. As reported previously, the stably transfected HL-60 cells express functional FPR without differentiation (18). Stimulation of the transfected cells with fMLF induces several G protein-coupled functions including calcium mobilization and actin polymerization (18). How-
however, in our experiments the transfected HL-60 cells did not exhibit significant levels of NF-κB activation when stimulated with FMLF (Fig. 6A). Similarly, only a weak NF-κB response was observed in HL-60 cells stably transfected to express the C5a receptor, which responded well to C5a with mobilization of Ca²⁺ (Fig. 6A). Notably, we observed that TNFα was able to stimulate a potent NF-κB activity in the transfected cells (Fig. 6A) as well as in untransfected HL-60 cells (see below), indicating that the signaling pathways utilized by TNFα for NF-κB activation were fully functional in these cells. This finding is in line with the Western blot data indicating that p50 and p65 subunits are present in the undifferentiated HL-60 cells (Fig. 4). These results suggest that TNFα stimulation of NF-κB activation utilizes signaling mechanisms that may be different from the ones employed by the G protein-coupled FPR in HL-60 cells, which apparently lack the response to FMLF despite the expression of the FPR by transfection.

The above data indicate that untransfected HL-60 cells lack not only the FPR but also a component that is necessary for efficient NF-κB activation in response to FMLF. This component may be acquired by differentiation of the cells with Me₂SO, which at the same time also induces the expression of the FPR. To test this hypothesis, we treated the FPR-transfected HL-60 cells with Me₂SO for 5 days and measured the capability of the differentiated cells to activate NF-κB in response to FMLF. As shown in Fig. 7, Me₂SO-treated cells displayed marked NF-κB activation following FMLF stimulation while the background kB binding activity remained low. A similar result was obtained in C5a receptor-transfected HL-60 cells, which responded after Me₂SO differentiation (data not shown). To examine whether Me₂SO induced a significant increase in the FPR expression level that contributed to the NF-κB response to FMLF, the cells were subjected to flow cytometry analysis for quantitation of receptor expression. Without Me₂SO treatment, FPR-transfected HL-60 cells expressed on average 148,000 receptors/cell based on mean fluorescence measurement against the FITC standard. Following differentiation with Me₂SO for 5 days, the cells expressed on average 170,000 receptors/cell (n = 3) (Fig. 7). This is comparable with untransfected HL-60 cells, which expressed approximately 151,000 receptors/cells after a similar induction with Me₂SO. Thus, the small increase (15%) in the numbers of FPR following Me₂SO treatment cannot explain the marked gain in responsiveness to FMLF stimulation.

In order to determine whether the increase in responsiveness of NF-κB to FMLF in HL-60 cells after differentiation was specific to the chemoattractant but not a result of a general increase in activable NF-κB, HL-60 cells at different stages following treatment with Me₂SO were analyzed for p50 and p65 content by Western blot and for responsiveness to TNFα by EMSA (Fig. 8). In these experiments NF-κB activated by TNFα did not increase after differentiation but appeared to decrease in HL-60 cells treated with Me₂SO for 3 and 5 days. The relative amounts of p50 and p65 proteins visible on Western blots also did not increase in HL-60 cells following Me₂SO induction (Fig. 8). In fact, there appeared to be a slight decrease in the levels of the p65 protein. Thus, our data suggest the presence of a myeloid differentiation-associated factor that is
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Results from this study provide direct evidence for the activation of NF-κB by fMLF at concentrations comparable with those that stimulate other phagocyte cellular responses. This function of the well-characterized chemoattractant may be of physiological relevance since chemoattractants are among the first and major activating factors that stimulate a migrating phagocyte. The physiological consequences of NF-κB activation as relevant to gene transcription were not the focus of this study. However, owing to both qualitative and quantitative similarities in the fMLF-induced NF-κB response to those elicited by either TNFα or LPS in monocytes, it is suggested that gene expression would result as has been well documented for these other ligands. Studies from this and other laboratories indicate that the chemoattractants LTβ and PAF may also stimulate NF-κB-dependent gene expression suggesting that this might be a general function of leukocyte chemoattractants (9–12).

Leukocyte chemoattractants bind receptors with 7 transmembrane domains that signal by activating heterotrimeric G proteins. Proximal signaling events mediated by G proteins differ from those triggered by cytokines suggesting that chemoattractants might utilize signaling mechanisms different from those of TNFα and IL-1β for activating NF-κB. It is predicted that both cytokine- and chemoattractant-initiated pathways converge at some point. As shown here TNFα could activate NF-κB in undifferentiated HL-60 whereas fMLF could not. The requirement of differentiation for fMLF responsiveness could thus be explained by the up-regulation of signaling components that function upstream of the point of convergence. It is also possible that chemoattractant-initiated pathways might involve one of the several signaling mechanisms utilized by TNFα for NF-κB activation; however, the slight decrease in TNFα-responsive NF-κB during HL-60 differentiation does not support this idea. Thus, results shown here indicate the presence of specific signaling components that may be unique to the G protein-mediated pathways leading to NF-κB activation, reflecting a potentially novel mechanism for activating this transcription factor. Further study is necessary to identify the myeloid differentiation-associated factor and to determine whether myeloid differentiation is required for other chemoattractants to activate NF-κB.

Neutrophils are the primary effector cells found at sites of acute inflammation. In spite of the relative scarcity of ribosomes and endoplasmic reticulum, neutrophils are capable of mRNA synthesis and protein production (27). Neutrophils respond to a large number of proinflammatory stimuli including fMLF and other classical chemoattractants. It has been demonstrated that the chemoattractants fMLF, C5a, and LTβ1 could synergize the effect of LPS on IL-8 synthesis by neutrophils, although the study did not demonstrate that these chemoattractants themselves could induce IL-8 production (28). Cassatella et al. (15) independently reported release of IL-8 by neutrophils after stimulation with fMLF alone. They observed de novo IL-8 protein synthesis, accompanied by an accumulation of the IL-8 mRNA. Furthermore, only nanomolar concentrations of fMLF were required for stimulation of IL-8 release, in agreement with our findings. These studies provided direct evidence for chemoattractant stimulation of cytokine production in neutrophils, but by themselves did not elucidate the transcriptional mechanisms for chemoattractant-induced cytokine production. Although NF-κB is a primary regulator of IL-8 gene expression in other cell types (29), the lack of κB binding activity in neutrophils as demonstrated by this work suggests that NF-κB is unlikely to play a major role in the transcription of cytokine genes in these terminally differentiated granulocytes. Thus, future investigations of gene transcription in neutrophils should be focused on the activation of other transcription factors such as NF-IL-6 and AP-1. Activation of the mitogen-activated protein kinases has been demonstrated by others to occur in neutrophils in response to fMLF (30–32). Since the activation of AP-1 can be regulated by the mitogen-activated protein kinase pathways (33), this transcription factor may have a role in neutrophil gene expression.

Recently Miyamasu et al. (16) reported that fMLF and C5a could cause secretion of cytokines by eosinophils. Although this response required pretreatment of the cells with cytochalasin B, it was dependent on transcription. Based on the finding that the antioxidant PDTC could block IL-8 generation, the authors postulated the involvement of NF-κB in fMLF- and C5a-stimulated response. However, no direct evidence was shown that supports NF-κB activation in the stimulated eosinophils. In light of our finding that neutrophils contain very little p50 and p65 and respond poorly to a number of agonists that induce NF-κB activation, additional work is necessary to determine whether eosinophils, a type of terminally differentiated gran-
ulocyte, differ from neutrophils in terms of NF-κB protein content and responsiveness to chemoattractants.

The finding that activation of NF-κB by fMLF in HL-60 cells requires granulocytic differentiation suggests that the final differentiation product more closely resembles a transitory state between promyelocyte and neutrophil. This notion is supported by the recent finding that certain granulocytic functions are enhanced in HL-60 cells when retinoic acid is added to the culture medium in the last day of differentiation with Me2SO (34). Furthermore, our results indicate that there is no increase in the content of the p50 protein and a visible decrease of the p65 protein in Me2SO-treated HL-60 cells undergoing granulocytic differentiation (Fig. 8). Thus, granulocytic differentiation may eventually result in the depletion of the NF-κB subunits as was observed here in neutrophils. Recent studies indicate a function for NF-κB in preventing apoptosis (35–37), raising the possibility that the loss of NF-κB and the commitment of neutrophils to cell death may not be just incidental.

Whether chemoattractant-activated NF-κB occurs transiently during granulocytic differentiation in vivo is presently unknown, but the idea that NF-κB might serve a functional role during the differentiation of granulocytes is intriguing and warrants further study.

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