Activation of Monocytic Cells Through Fcγ Receptors Induces the Expression of Macrophage-Inflammatory Protein (MIP)-1α, MIP-1β, and RANTES

Nieves Fernández, Marta Renedo, Carmen García-Rodríguez and Mariano Sánchez Crespo

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Activation of Monocytic Cells Through Fcγ Receptors Induces the Expression of Macrophage-Inflammatory Protein (MIP)-1α, MIP-1β, and RANTES

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Monocytic cells were stimulated with IgG-OVA equivalence immune complexes, mAb reacting with FcγRI, FcγRIIA, and FcγRIII, LPS, TNF-α, and the combination of ionomycin and phorbol ester, to address their effects on the expression of the mRNAs encoding for chemokines. Stimulation of monocytes with immune complexes induced a rapid expression of macrophage-inflammatory protein (MIP)-1α, MIP-1β, and IL-8 mRNAs. In contrast, RANTES mRNA was already detectable in resting cells and only increased after 16 h of stimulation. A similar pattern was observed following homotypic stimulation of FcγR with mAb reacting with FcγRI and FcγRIIA, but not with a mAb reacting with FcγRIII, a subtype of receptor not expressed in THP-1 cells, thus indicating that both FcγRI and FcγRIIA are involved in the response. The pattern of chemokine induction elicited by LPS and the combination of ionomycin and PMA showed some similarities to those produced by FcγR cross-linking, although expression of IFN-γ-inducible protein 10 mRNA was also observed in response to those agonists. The production of MIP-1α, MIP-1β, and RANTES proteins encompassing the induction of their mRNAs was confirmed by specific ELISA. Experiments to address the transcription factors involved in the regulation of MIP-1α using pharmacological agents and EMSA showed the possible involvement of CCAAT/enhancer-binding protein β sites and ruled out the functional significance of both NF-AT and AP-1 sites. The Journal of Immunology, 2002, 169: 3321–3328.

*Instituto de Biología y Genética Molecular, Consejo Superior de Investigaciones Científicas, and Unidad de Investigación, Hospital Clínico Universitario, Valladolid, Spain

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N.F., M.R., and C.G.-R. contributed equally to this study.

Address correspondence and reprint requests to Dr. Mariano Sánchez Crespo, Instituto de Biología y Genética Molecular, Facultad de Medicina, 47005-Valladolid, Spain. E-mail address: msres@ibgm.uva.es

Abbreviations used in this paper: IC, immune complex; ALLN, N-acetyl-leucinyl-leucinyl-norleucinal; C/EBP, CCAAT/enhancer-binding protein; HTB, 2-hydroxy-4-trichloromethyl-5(4H)oxazolone acid; IP-10, IFN-γ-inducible protein 10; MAP, mitogen-activated protein; MCP-1, monocyte chemoattractant protein-1; MIP, macrophage-inflammatory protein. 

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well as studies of the binding activities for regulatory sites in the nuclear extracts of monocyctic cells suggest the involvement of C/EBPβ rather than NF-κB, AP-1, and NF-AT sites in their induction.

Materials and Methods

Reagents
IgG Ab were raised in rabbits by s.c. injections of OVA in CFA, followed by booster i.m. IgG Ab were purified from heat-inactivated serum by precipitation with ammonium sulfate. Solutions of both OVA and Ab were sterilized by ultracentrifugation before use. IgG-OVA equivalence IC were made according to classical procedures (24), and washed extensively to ensure the removal of remaining serum components. The purity of the IC was assayed by SDS-PAGE, which showed the presence of IgG and OVA, mAb anti-FcRI (32.2), anti-FcRIII (IV.3), and anti-FcRIII (3G8) were from Medarex (Annandale, NJ). The proteasome inhibitor N-acetyl-leucinyl-leucinyl-norleucinal (ALLN) was from Sigma-Aldrich (St. Louis, MO). The 2-hydroxy-4-trifluoromethylbenzoic acid (HTB), a salicylate derivative with potent inhibitory effects on NF-κB activation (25), was from URIACH Laboratories (Barcelona, Spain).

Cell culture
THP-1 cells were cultured in plastic dishes in RPMI 1640 medium supplemented with 10% heat-inactivated FBS. Cells were deprived of FBS for 16 h, except in the samples used for the assay of MIP-1α, MIP-1β, and RANTES proteins, in which the amount of serum in reduced to 2%, according to the manufacturer’s instructions, to prevent loss of chemokines in the culture supernatants before assay. Human monocytes were isolated from peripheral blood of healthy donors (laboratory staff) by centrifugation into Ficoll cushions and adherence to plastic dishes. When the purpose of the experiment required nonadhered monocytes, the adhered cells were detached by cold and scraping and maintained in polypropylene tubes for 18 h before the addition of the stimuli to avoid adherence.

RNA extraction and RNase protection assays
Total cellular RNA was extracted by the TRIzol method (Life Technologies, Grand Island, NY) and used to assay the level of expression of RANTES, MIP-1α, IFN-γ-inducible protein 10 (IP-10), MIP-1β, IL-8, L32, and GAPDH mRNAs by RibonQuant RNase protection assay using the hCK-5 multiprobe template set from BD PharMingen (San Diego, CA). For this purpose, riboprobes were labeled with [32P]UTP in the presence of T7 polymerase and used for overnight hybridization with 3 μg RNA. The hybridized RNA was digested with RNase and proteinase K, and the RNase-protected probes were purified and resolved on denaturing PAGE. The identification of the specific chemokine bands was conducted on the basis of their individual migration patterns in comparison with the undigested probes. Radiolabeled bands on the gel were acquired using the Personal Molecular Image FX and quantitated using Quantity One software (Bio-Rad, Hercules, CA). Sample loading was normalized by the housekeeping genes L32 and GAPDH.

Electrophoretic mobility shift assay
THP-1 cells were washed with ice-cold hypotonic lysis buffer. Unbroken cells were eliminated by centrifugation at 1,000 × g for 10 min, and the nuclei were collected by centrifugation at 15,000 × g for 1 min. The nuclear pellet was resuspended in high salt extraction buffer containing 25% glycerol and 0.5 M KCl, and the nuclear extract was obtained by pelleting for 30 min at 105,000 × g for 1 min. Double-stranded oligonucleotide probes were end labeled with [γ-32P]ATP using T4 polynucleotide kinase and separated from the unincorporated label by minicolumn chromatography. A total of 10 μg nuclear protein was incubated for 20 min on ice with radiolabeled oligonucleotide probes (2–6 × 106 cpm) in a 25 μl reaction buffer containing 2 μg poly(dI-dC), 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 8% Ficoll, and 4% glycerol. Nucleoprotein-oligonucleotide complexes were resolved by electrophoresis in a 4% nondenaturing PAGE. The gel was dried, and the radiolabeled bands were acquired with the personal imager FX. The oligonucleotide sequences used for the detection of binding activity to the AP-1 and C/EBPβ sites from the MIP-1α promoter are shown in Fig. 1. Additional experiments were conducted with an AP-1 consensus sequence, which differs from the AP-1 site sequences from the MIP-1α promoter.

ELISA of human MIP-1α, MIP-1β, and RANTES production
MIP-1α, MIP-1β, and RANTES were assayed in cell culture medium. The procedure was conducted with reagents from Endogen (Woburn, MA) in the case of MIP-1α, and Amersham Pharmacia Biotech (Piscataway, NJ) for the assay of MIP-1β and RANTES. These procedures use Ab-precotted well plates, biotinylated rabbit anti-human Abs, and streptavidin conjugated to HRP. The ELISA are developed using a peroxidase reaction, and the minimum detectable doses of this assay are 5 pg/ml for MIP-1α, and 2 pg/ml for RANTES.

Results

Occupancy of FcγRI and FcγRIIA induces chemokine mRNAs in THP-1 cells and peripheral blood monocytes
Stimulation of THP-1 cells with IC induced a strong expression of the mRNA of RANTES, MIP-1α, MIP-1β, and IL-8 in a dose-dependent manner (Fig. 2A). MIP-1α and MIP-1β showed the most marked increase, because their mRNAs were almost undetectable before the addition of the stimulus and strongly increased between 10 min and 3–8 h (Fig. 2, B and C). This temporal pattern was similar to that observed for IL-8, although in this case it was difficult to detect mRNA expression after 3 h. In contrast, the expression of RANTES mRNA showed a different pattern, because it was already detectable before addition of the stimulus, and showed a ~3-fold increase above prestimulation levels between 16 and 48 h after addition of IC (Fig. 2B), as judged from densitometric scanning of the blot area. The dose dependency of the response was studied at a fixed time of 3 h, and optimal induction was observed with concentrations above 20 μg/ml (Fig. 2A). To rule out whether some unidentified contaminant of both OVA and Ab solutions could account for the effect attributed to IC, THP-1 cells were incubated with 100 μg/ml of both OVA and Ab. As shown in the rightmost panel of Fig. 2B, these treatments did not induce the expression of chemokine mRNA, thus making it unlikely that contaminant and, e.g., LPS could account for the observed effects. To address whether the results obtained in THP-1 cells agreed with the response observed in human monocytes, adherent monocytes from peripheral blood were stimulated with IC. As shown in Fig. 2D, adhering resting monocytes showed a slight expression of RANTES, MIP-1α, and MIP-1β, and a marked expression of IL-8 mRNA; however, addition of IC significantly enhanced the expression of MIP-1α and MIP-1β. In contrast, when detached monocytes were maintained for 18 h in polypropylene tubes, there was a mild expression of RANTES, whereas IC induced a prominent induction of MIP-1α and MIP-1β (Fig. 2D, right panel), thus suggesting that purification of blood monocytes taking advantage of their adherence to plastic dishes has a prominent effect on IL-8 expression, whereas engagement of FcγR by IC impinges on the induction of both MIP-1α and MIP-1β, as it was shown in THP-1 monocytes. These findings make the THP-1 cell line a suitable model for the study of monocyctic cells, because it may grow without adhering to plastic and then circumvents the occurrence of adherence-mediated activation. Attempts to relate the expression of chemokines to the cross-linking of the specific types of FcγRII expressed in THP-1 cells were conducted by the homotypic approach by incubating THP-1 cells with 10 μg/ml mAb reacting with FcγRI, FcγRIIA, and FcγRIII for 10 min at 4°C, followed by washing to eliminate the Ab not bound to cell receptors and replacement by new medium prewarmed at 37°C before the addition of goat anti-mouse F(ab′)2 (26). The pattern of chemokine induction elicited by the mAb resembled one another, as well as that produced by the combination of mAb (Fig. 3). In contrast, stimulation of THP-1 cells with the 3G8 mAb did not induce the expression of chemokine mRNA, nor did anti-mouse F(ab′)2 alone produce any effect, thus agreeing with the absence of FcγRIII expression in THP-1 cells (26) (Fig. 3). In keeping with previous studies addressing homotypic stimulation of FcγRIII, we have shown that FcγRIII does not play a role in the induction of chemokine mRNAs.
mAb does not produce strong Fc-FcγR interactions, such as those required for optimal activation of monocytes (26, 27). Noteworthy, the pattern of induction elicited by other stimuli was somewhat different from that elicited by IC, albeit the induction of both MIP-1α and MIP-1β was again most prominent. Thus, the combination of ionomycin and PMA, which activates both Ca2+-and protein kinase C-dependent pathways, produced a strong induction of IL-8 mRNA, whereas ionomycin alone failed to produce IL-8 induction, and LPS induced IP-10 mRNA as well (Fig. 4A). Interestingly, TNF-α produced a distinct pattern of induction of chemokines characterized by a delayed induction of RANTES, IP-10, MIP-1α, MIP-1β, and MCP-1 (Fig. 4B).

**Effect of pharmacological agents on the expression of chemokines’ mRNAs**

Initial attempts to disclose the transcription factors that could be involved in the regulation of the different chemokines were conducted with pharmacological agents well known as selective inhibitors of...
transcription factors. Because the cross-linking of FcγR activates NF-κB and this has functionally been associated with the expression of MCP-1 (8), the effect of inhibiting NF-κB was addressed by using two structurally unrelated inhibitors of NF-κB. As shown in Fig. 4C, both HTB and ALLN decreased the mRNA expression of both MIP-1α and MIP-1β in response to IC. In contrast, the expression of RANTES mRNA did not show any significant change as a result of these treatments, and the expression of IL-8 mRNA was enhanced in a dose-dependent manner by ALLN, which represented an unexpected result in view of the documented presence of functional NF-κB binding sites in the IL-8 promoter (28–30). As shown in Fig. 4D, cyclosporin A did not influence the effect of IC, i.e., less than 20% inhibition in three independent experiments, thus suggesting that NF-AT sites do not exert a central role in the transcriptional regulation of MIP-1α, MIP-1β, RANTES, and IL-8.

**Cross-linking of FcγR activates AP-1 and C/EBPβ**

Incubation of THP-1 cells with IC increased AP-1-binding activity in the nuclear extracts in a time-dependent manner, as judged from the results of EMSA conducted with a probe containing the 5′-TGAGTCA-3′ core consensus sequence for AP-1. As shown in Fig. 5A, increased binding activity was already observed 5–10 min after addition of the stimulus, reached maximal intensity at ~20 min, and was completely reversed by incubation with a 100-fold excess of unlabeled oligonucleotide with the AP-1 consensus sequence. However, no binding activity was observed when the reaction was conducted with a probe designed on the basis of the AP-1 site sequences of the MIP-1α promoter (Fig. 5B), thus indicating that even though activation of FcγR activates AP-1, its involvement in the transcriptional regulation of MIP-1α is

**FIGURE 2.** Stimulation of THP-1 cells with IC induces the expression of the mRNAs of several chemokines. THP-1 monocytes were incubated with different concentrations of IC for 3 h (A), or in the presence of 100 μg/ml IC for different periods (B and C). The effect of 100 μg/ml OVA and 100 μg/ml Ab is shown in the rightmost lanes of B. The Ab solution was ultracentrifuged at 100,000 × g for 1 h at 4°C to eliminate the IgG aggregates that could form spontaneously. Total RNA was extracted at the times indicated and used for RNase protection assays. These are representative experiments of three with similar results. D, The outcome of experiments conducted on adhered human monocytes stimulated with IC for 3 h, or in detached monocytes maintained in polypropylene tubes for 18 h before the stimulation with IC to prevent adherence-induced activation.

**FIGURE 3.** Cross-linking of FcγRI and FcγRIIA with mAb activates the expression of chemokine mRNAs. THP-1 monocytes were incubated at 4°C with 10 μg/ml mAb IV.3, 32.2, and 3G8, as indicated, followed by washing to eliminate the Ab not bound to cells, and replacement by new medium prewarmed at 37°C and 20 μg/ml goat anti-mouse F(ab′)2. The effect of anti-mouse F(ab′)2 alone is shown in the rightmost lane. Total RNA was extracted at the times indicated and used for RNase protection assays. This is a representative experiment of three.
unlikely. In contrast, the nuclear extracts showed binding activity to the probe containing the –122–100 C/EBPβ sequence of the sense strand. Maximal binding activity was seen between 20 and 45 min after addition of IC (Figs. 5C and 6A), and showed a clear dose dependency, because this was already observed with concentrations of IC above 20 μg/ml (Fig. 5D). Binding to the MIP-1α/C/EBPβ wt probe was reversed by a 100-fold excess of unlabeled oligonucleotide (Fig. 5C, lane marked “competitor”). Experiments conducted with the MIP-1αwt oligonucleotide that contains both the putative AP-1 and the C/EBPβ binding sites (Fig. 6B, lanes 5–8) showed a binding pattern similar to that observed with the MIP-1α/C/EBPβwt probe, which again was reversed by the unlabeled oligonucleotide sequence (Fig. 6B, lane 8). Further attempts to assess the selectivity of the binding reactions were addressed with mutated oligonucleotides designed to disrupt the C/EBPβ site according to the TRANSFAC database. In fact, no binding activity was observed when labeled *MIP-1α/C/EBPβm and *MIP-1αm probes were used in the binding reactions (Fig. 6A, lanes 4–6 and 8, respectively), and MIP-1α/C/EBPβm did not reverse the binding of the labeled *MIP-1α/C/EBPβwt probe (Fig. 6A, lane 7).
IC lung injury have shown a role for both MIP-1 and MIP-1β in macrophage activation, because despite a wide induction of other CC chemokines, only the blockade of either MIP-1α or MIP-1β reduced the recruitment of neutrophils and the production of lung injury (16–18).

The comparison of the effect of FcγR activation with that elicited by other stimuli provides some hints as to the involvement of different chemokines in distinct pathophysiological conditions. Thus, the set of chemokines elicited by FcγR engagement shows some differences with those triggered by TNF-α, LPS, and the combination of ionomycin and PMA. In fact, LPS and ionomycin shared the feature of inducing IP-10, whereas this was not observed in response to IC. In contrast, ionomycin alone did not induce IL-8. Many different monocytic cell lines, including THP-1 cells, show constitutive nuclear κB activity (31), and unlike MIP-1α, RANTES has two κB sites near the TATA signal, thus explaining possible interactions with components of the general transcription machinery that could account for the expression of RANTES detected in resting cells. In contrast, the delayed pattern of RANTES mRNA induction by IC agrees with the characterization of RANTES as an unusual gene induced late after T lymphocyte activation, the expression of which has been related to a novel transcription factor termed RANTES factor of late activated T lymphocytes, which belongs to the Kruppel-like family of transcription factors (32), although other factors have been associated with the cell-specific pattern of transcriptional regulation of RANTES, among them NF-κB (33), C/EBPβ (34), and IFN regulatory factor (35).

The combined induction of both MIP-1α and MIP-1β fits well with the presentation of these molecules as a doublet; however, it is more difficult to address the functional significance of the different regulatory elements located to their promoter regions. Early studies have restricted the functional relevance for transcription to a proximal promoter region of 256 bp (20), in which several binding sites for members of the C/EBP family of transcription factors were characterized, as well as a GGAAA motif homologous to a highly conserved half site of a putative consensus NF-κB recognition sequence. Taking into account these early functional studies

**FIGURE 6.** The binding activity to MIP-1α promoter induced by IC is lost by mutations in the C/EBPβ site. Nuclear extracts from cells treated with 100 μg/ml IC for the times indicated were incubated with the labeled sequences *MIP-1αC/EBPβwt (A and B) , *MIP-1αC/ EBPβmut (A), and *MIP-1αwt (B), as indicated. In the lanes specified, unlabeled oligonucleotides containing both wild-type and mutated sequences were used to assess the specificity of the binding reactions.
FIGURE 7. Production of MIP-1α, MIP-1β, and RANTES proteins. THP-1 cells at the concentration of 1.5 × 10^6 cells/ml were incubated in the presence (filled symbols) or absence (open symbols) of 100 μg/ml IC. At the times indicated, the cell culture supernatant was collected and used for the assay of MIP-1α and MIP-1β (A) or RANTES (B) proteins by ELISA. The effect of different concentrations of IC, PMA, and TNF-α in cells stimulated for 24 h is shown in C. Data represent mean ± SE of four experiments.

and the quality of the matches of the matrix analysis of the promoter sequence with MatInspector software and the updated data of the TRANSFAC database (36), we have defined some binding sites that could have functional significance (Fig. 1A). These binding sites include three AP-1 sites, a GATA-1 site, and a C/EBPβ site in the sense strand; and two more AP-1 sites, two C/EBPβ sites, which also encompass a NF-AT site, and an additional NF-AT site in the antisense strand. In contrast, no κB sites have been detected using updated database. Analysis of the binding activity associated with the nuclear extracts from cells stimulated with IC has shown abundant AP-1-binding activity; however, this only could be disclosed with the AP-1 consensus sequence, and not with those found in the MIP-1α promoter region, thus suggesting that AP-1 sites are not involved in the regulation of MIP-1α expression. Moreover, pharmacological approaches using compounds with known activity on the NF-κB system have provided contradictory results. For instance, the salicylate derivative HTB, which has been found to inhibit NF-κB activation in several cell systems (25, 37, 38), inhibited the induction of MIP-1α and MIP-1β without influencing IL-8 expression. In contrast, the proteasome inhibitor ALLN showed unexpected effects, because in addition to an inhibitory effect on the expression of both MIP-1α and MIP-1β, this compound increased the expression of IL-8 mRNA in a dose-dependent manner. These results are reminiscent of those recently reported in human monocytes. Thus, triggering of β2 integrins by both Ab and soluble CD23 has shown a strong induction of both MIP-1α and MIP-1β, which was sensitive to proteasome inhibitors, whereas this treatment enhanced IL-8 expression (39). In addition, treatment of human monocytes with 15-deoxy-Δ12,14-Prostaglandin J2 has been found to produce IL-8 gene expression (40), although the function of this peroxisome proliferator-activated receptor-γ activator has been associated with an anti-inflammatory effect linked to the inhibition of the NF-κB route (41). Previous studies have suggested that NF-κB was involved in the induction of MIP-1α and MIP-1β; however, the evidence was indirect and only stemmed from the coincidental presence of binding activity to a consensus κB site in the nuclear extracts obtained under these conditions, and from the effect of pharmacological treatments. Regarding GATA-1 sites, their involvement in the transcriptional regulation of MIP-1α and RANTES is unlikely for a number of reasons. First, these sites differ somewhat from the consensus (T/A)GATA(A/G), which accounts for most of the sites in which a functional role for GATA-1 has been demonstrated. Moreover, GATA-1 expression shows a restricted level of expression among cells derived from hemopoietic progenitor cells, for instance, megakaryocytes, mast cells, basophils, and eosinophils (42). In fact, we have not detected the expression of GATA-1 mRNA by the RT-PCR approach in THP-1 cells (data not shown). In contrast, the functional relevance of the C/EBPβ site in the transcriptional regulation of MIP-1α can be supported by several arguments: 1) the presence of a high homology C/EBP site on the proximal promoter region according to rigorous criteria; 2) the appearance of binding activity to this site in the nuclear extracts displaying a strong dependency on critical bases in the core sequence, before the induction of MIP-1α expression; 3) the similar dose-response pattern of both MIP-1α expression and appearance of C/EBPβ-binding activity; 4) the parallel pattern of MAP kinase activation (26) and C/EBPβ-binding activity in the nuclear extracts elicited by FcγR cross-linking, which agrees with the reported phosphorylation at threonine-235 of C/EBPβ by MAP kinase as an essential step for C/EBPβ activation (43). Moreover, the pattern of MIP-1α induction in this system is similar to that disclosed for cyclooxygenase-2 (26), a gene the expression of which has been found to be strongly dependent on C/EBPβ in both human and mouse macrophages (44, 45). Taken together, these findings extend previous studies showing the activation of a set of transcription factors by FcγR signaling, i.e., NF-κB (6, 8), AP-1, and C/EBPβ, and suggest the coupling of C/EBPβ to the induction of some elements of the chemokine family involved in the production of immune-mediated tissue injury.

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References


