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Activation of Monocytic Cells Through Fc γ Receptors Induces the Expression of Macrophage-Inflammatory Protein (MIP)-1 α , MIP-1 β , and RANTES¹

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Monocytic cells were stimulated with IgG-OVA equivalence immune complexes, mAb reacting with Fc γ RI, Fc γ RIIA, and Fc γ RIII, 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)-1 α , MIP-1 β , 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 Fc γ R with mAb reacting with Fc γ RI and Fc γ RIIA, but not with a mAb reacting with Fc γ RIII, a subtype of receptor not expressed in THP-1 cells, thus indicating that both Fc γ RI and Fc γ RIIA 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 Fc γ R cross-linking, although expression of IFN- γ -inducible protein 10 mRNA was also observed in response to those agonists. The production of MIP-1 α , MIP-1 β , 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-1 α 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.

Activation of the receptors for the Fc portion of the IgG molecule (Fc γ R) plays a central role in immune-mediated tissue injury due to the ability of these receptors to recruit effector mechanisms of inflammation. This has been highlighted by studies in murine strains with targeted disruption of the γ -chain of Fc γ R, in which it has been possible to uncouple immune complex (IC)⁴ deposition from the subsequent inflammatory response (1–3), thus depicting a general model of IC-mediated injury in which Fc γ R-mediated events rather than the complement system are critical to the tissue damage. The proinflammatory events to date associated with activation of Fc γ R include the induction of both proinflammatory and chemotactic cytokines (4–8), the activation of the mitogen-activated protein (MAP) kinase cascade (9–11), and the induction of enzymes playing a role in the inflammatory reaction, such as the inducible isoform of NO synthase and cyclooxygenase-2. Regarding the induction of chemo-

kine expression elicited by Fc γ R, most studies have focused on both monocyte chemoattractant protein-1 (MCP-1) and IL-8, whereas few studies have addressed the induction of other CC chemokines such as RANTES, macrophage-inflammatory protein (MIP)-1 α , and MIP-1 β . In this connection, RANTES has been characterized as a chemoattractant for monocytes and lymphocytes, which has been implicated in several clinical conditions such as allergic inflammation (12), endotoxemia (13), the cytotoxic activity of human HIV-specific CD8⁺ T cells (14), and ulcerative colitis (15). The role of MIP-1 α and MIP-1 β deserves detailed attention, because these chemokines form a doublet and are involved in the development of the acute lung injury induced by IC (16, 17) and endotoxin (18), as it has been shown in experimental studies by using specific Ab against these molecules. Moreover, MIP-1 α is rapidly induced by factors that activate macrophages, thus belonging to the class of rapid-response or immediate-early genes (19). Studies addressing the transcriptional regulation of both MIP-1 α (20) and MIP-1 β (21) have indicated a strong dependence on short proximal promoter sequences containing activating transcription factor/CREB, CCAAT/enhancer-binding protein β (C/EBP β), c-Ets, and κ B sites, thus explaining the rapid and cell-specific induction of these chemokines in macrophages by LPS, serum, and cycloheximide (18, 22, 23). However, these data cannot be readily extended, because some studies have been conducted on murine cells, and promoter analyses have been conducted with early databases. In this study, we have addressed the pattern of induction of several functionally relevant chemokines following activation of Fc γ R using both homotypic and heterotypic cross-linking of the subtypes of Fc γ R expressed in THP-1 cells, and compared this pattern with that elicited by other well-known activators of phagocytes. Our data show a rapid induction of MIP-1 α , MIP-1 β , and IL-8 during the first 3 h that follow the addition of the stimulus, and a more protracted induction of RANTES. Analysis of the promoter regions of both MIP-1 α and RANTES as

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⁴ Abbreviations used in this paper: IC, immune complex; ALLN, *N*-acetyl-leucine-leucine-norleucine; C/EBP, CCAAT/enhancer-binding protein; HTB, 2-hydroxy-4-trifluoromethylbenzoic acid; IP-10, IFN- γ -inducible protein 10; MAP, mitogen-activated protein; MCP-1, monocyte chemoattractant protein-1; MIP, macrophage-inflammatory protein.

well as studies of the binding activities for regulatory sites in the nuclear extracts of monocytic cells suggest the involvement of C/EBP β rather than NF- κ B, 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 ultrafiltration 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-Fc γ RI (32.2), anti-Fc γ RII (IV.3), and anti-Fc γ RIII (3G8) were from Medarex (Annandale, NJ). The proteasome inhibitor *N*-acetyl-leuciny-leucinylnorleucinal (ALLN) was from Sigma-Aldrich (St. Louis, MO). The 2-hydroxy-4-trifluoromethylbenzoic acid (HTB), a salicylate derivative with potent inhibitory effects on NF- κ B 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-1 α , MIP-1 β , and RANTES proteins, in which the amount of serum was 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 nonadherent 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-1 α , IFN- γ -inducible protein 10 (IP-10), MIP-1 β , IL-8, L32, and GAPDH mRNAs by RiboQuant RNase protection assay using the hCK-5 multiprobe template set from BD PharMingen (San Diego, CA). For this purpose, riboprobes were labeled with [α -³²P]UTP in the presence of T7 RNA polymerase and used for overnight hybridization with 3 μ g 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 Imager 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 \times *g* for 10 min, and the nuclei were collected by centrifugation at 15,000 \times *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 \times *g*. 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 μ g nuclear protein was incubated for 20 min on ice with radiolabeled oligonucleotide probes (2–6 \times 10⁴ cpm) in a 25 μ l reaction buffer containing 2 μ g 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/EBP β sites from the MIP-1 α 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-1 α promoter.

ELISA of human MIP-1 α , MIP-1 β , and RANTES production

MIP-1 α , MIP-1 β , and RANTES were assayed in cell culture medium. The procedure was conducted with reagents from Endogen (Woburn, MA) in

the case of MIP-1 α , and Amersham Pharmacia Biotech (Piscataway, NJ) for the assay of MIP-1 β and RANTES. These procedures use Ab-precoated 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-1 α , and 2 pg/ml for RANTES.

Results

Occupancy of Fc γ RI and Fc γ RIIA 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-1 α , MIP-1 β , and IL-8 in a dose-dependent manner (Fig. 2A). MIP-1 α and MIP-1 β 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 μ g/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 μ g/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 contaminants as, 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, adhered monocytes from peripheral blood were stimulated with IC. As shown in Fig. 2D, adhered resting monocytes showed a slight expression of RANTES, MIP-1 α , and MIP-1 β , and a marked expression of IL-8 mRNA; however, addition of IC significantly enhanced the expression of MIP-1 α and MIP-1 β . 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-1 α and MIP-1 β (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 Fc γ R by IC impinges on the induction of both MIP-1 α and MIP-1 β , as it was shown in THP-1 monocytes. These findings make the THP-1 cell line a suitable model for the study of monocytic 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 Fc γ R expressed in THP-1 cells were conducted by the homotypic approach by incubating THP-1 cells with 10 μ g/ml mAb reacting with Fc γ RI, Fc γ RIIA, and Fc γ RIII 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 Fc γ RIII expression in THP-1 cells (26) (Fig. 3). In keeping with previous studies addressing homotypic stimulation of Fc γ R, the responses were less robust, thus suggesting that cross-linking of Fc γ R by

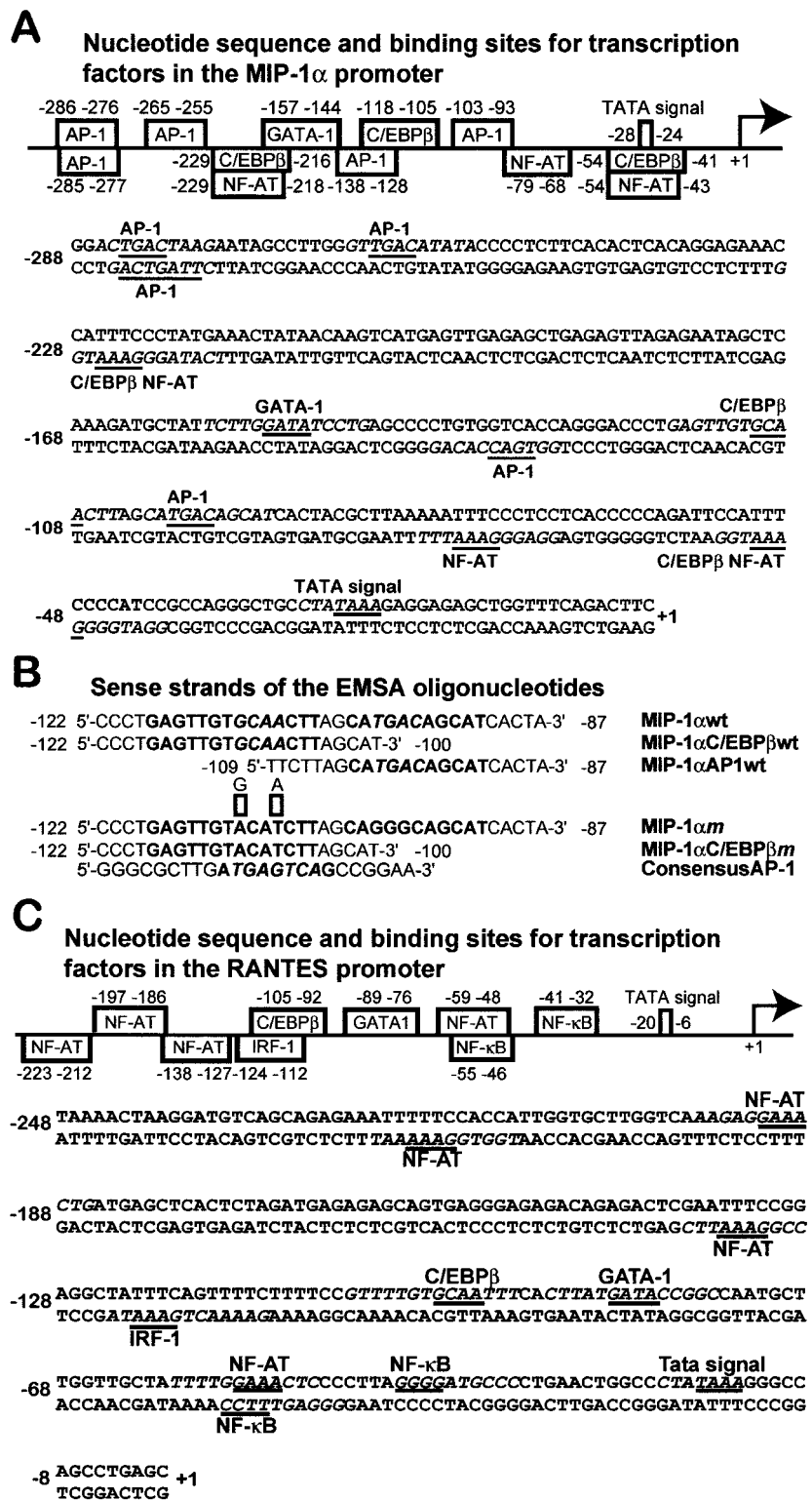


FIGURE 1. Sequence analysis of the MIP-1 α and RANTES promoters and description of the oligonucleotides used in EMSA experiments. The proximal sequence of the human MIP-1 α promoter (GenBank accession D90144; see Ref. 23) was analyzed using the MatInspector program and the TRANSFAC database (36). Binding sites for transcription factors showing the highest quality regarding both matrix and core similarity on both strands are shown. Sequences of the binding sites are marked in italics. Underlined letters indicate the core string (A). The sequences of the sense strands in the 5' to 3' orientation of the oligonucleotides, including the C/EBP β and the nearby AP-1 sites, are shown in B. Letters on the boxes on top of the MIP-1 α mut oligonucleotide indicate the bases in the wild-type sequence that have been mutated to eliminate the binding site. The nucleotide sequence of the RANTES promoter (GenBank accession no. S64885; see Ref. 46) is shown in C. IRF-1, IFN regulatory factor-1.

mAb does not produce strong Fc-Fc γ R 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-1 α and MIP-1 β 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). Inter-

estingly, TNF- α produced a distinct pattern of induction of chemokines characterized by a delayed induction of RANTES, IP-10, MIP-1 α , MIP-1 β , 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

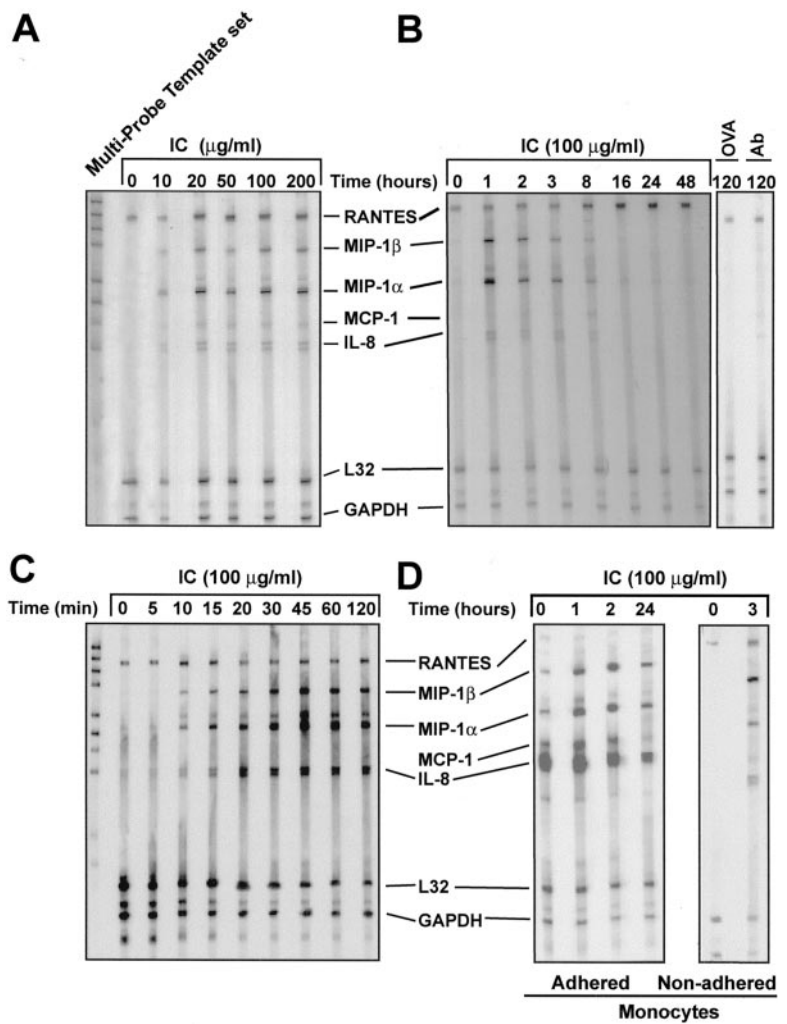


FIGURE 2. Stimulation of THP-1 cells with IC induces the expression of the mRNAs of several chemokines. THP-1 monocytes were incubated with different concentrations of IC for 3 h (A), or in the presence of 100 μ g/ml IC for different periods (B and C). The effect of 100 μ g/ml OVA and 100 μ g/ml Ab is shown in the *rightmost lanes* of B. The Ab solution was ultracentrifuged at 100,000 \times 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.

transcription factors. Because the cross-linking of Fc γ R activates NF- κ B and this has functionally been associated with the expression of MCP-1 (8), the effect of inhibiting NF- κ B was addressed by using two structurally unrelated inhibitors of NF- κ B. As shown in Fig. 4C, both HTB and ALLN decreased the mRNA expression of both MIP-1 α and MIP-1 β 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 κ B 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-1 α , MIP-1 β , RANTES, and IL-8.

Cross-linking of Fc γ R activates AP-1 and C/EBP β

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 \sim 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-1 α promoter (Fig. 5B), thus indicating that even though activation of Fc γ R activates AP-1, its involvement in the transcriptional regulation of MIP-1 α is

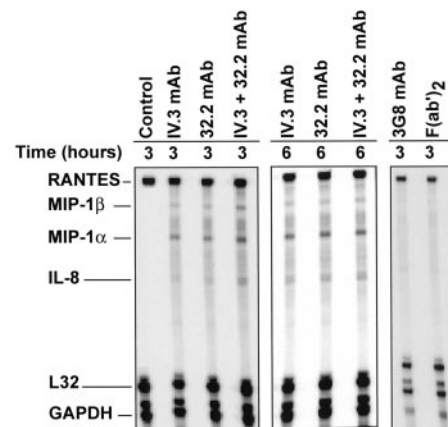


FIGURE 3. Cross-linking of Fc γ RI and Fc γ RIIA with mAb activates the expression of chemokine mRNAs. THP-1 monocytes were incubated at 4°C with 10 μ g/ml mAb IV.3, 32.2, and 3G8, as indicated, followed by washing to eliminate the Ab not bound to cells, and replacement by new medium prewarmed at 37°C and 20 μ g/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.

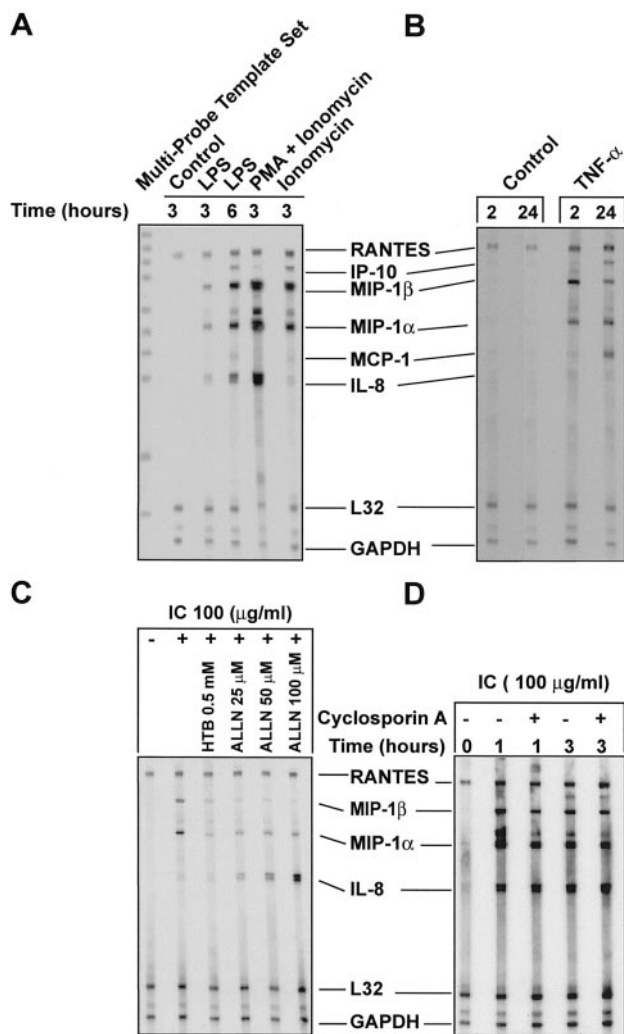


FIGURE 4. Effect of different agonists and pharmacological agents on the induction of chemokine mRNAs. THP-1 monocytes were incubated in the presence of 10 μ g/ml LPS, 1 μ M ionomycin, or the combination of ionomycin and 100 nM PMA. At the times indicated, total RNA was extracted and used for RNase protection assays. This is a representative experiment of three with similar results (A). The effect of the incubation of THP-1 cells with 100 U/ml TNF- α is shown in B. The effect of HTB and ALLN on the expression of chemokine mRNAs induced by IC is shown in C. The effect of cyclosporin A is shown in D. Unless otherwise stated, pharmacological agents were added 30 min before the stimulus and total RNA was extracted 3 h thereafter for RNase protection assays.

unlikely. In contrast, the nuclear extracts showed binding activity to the probe containing the -122-100 C/EBP β 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 μ g/ml (Fig. 5D). Binding to the MIP-1 α C/EBP β wt probe was reversed by a 100-fold excess of the unlabeled oligonucleotide (Fig. 5C, lane marked "competitor"). Experiments conducted with the MIP-1 α wt oligonucleotide that contains both the putative AP-1 and the C/EBP β binding sites (Fig. 6B, lanes 5-8) showed a binding pattern similar to that observed with the MIP-1 α C/EBP β wt 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/EBP β site according to the TRANSFAC database. In fact, no

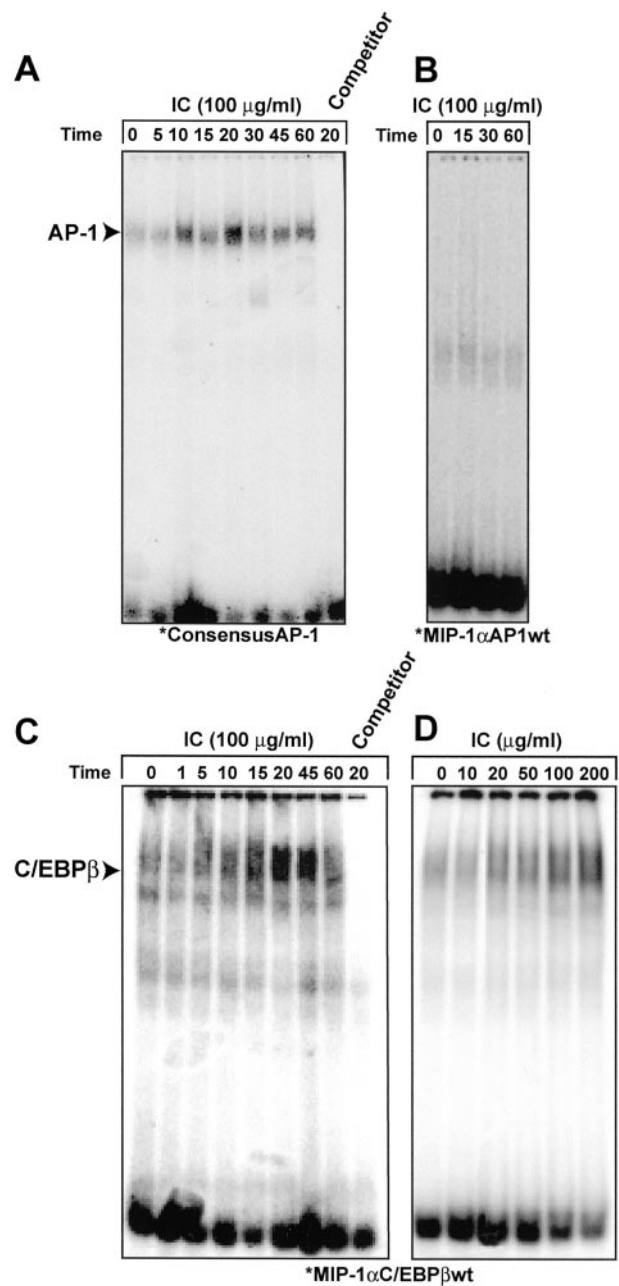


FIGURE 5. Nuclear extracts from cells incubated in the presence of IC contain binding activities to AP-1 consensus sites and to the C/EBP β site from the MIP-1 α promoter. THP-1 cells were incubated with 100 μ g/ml IC and, at the times indicated, cell lysates were collected and the nuclear extracts assayed for binding to probes containing an AP-1 consensus sequence 5'-TGAGTCA-3' (A), or to probes designed from the AP-1 (B) and C/EBP β (C) sites of the MIP-1 α promoter. Lanes marked "Competitor" indicate that the binding reaction was conducted in the presence of a 100-fold excess of unlabeled oligonucleotide probes. To address the dose dependency of the induction of binding activity to the C/EBP β site, nuclear extracts from cells stimulated for 30 min in the presence of different concentrations of IC were used (D). These are representative experiments of four with identical results.

binding activity was observed when labeled *MIP-1 α C/EBP β m and *MIP-1 α m probes were used in the binding reactions (Fig. 6A, lanes 4-6 and 8, respectively), and MIP-1 α C/EBP β m did not reverse the binding of the labeled *MIP-1 α C/EBP β wt probe (Fig. 6A, lane 7).

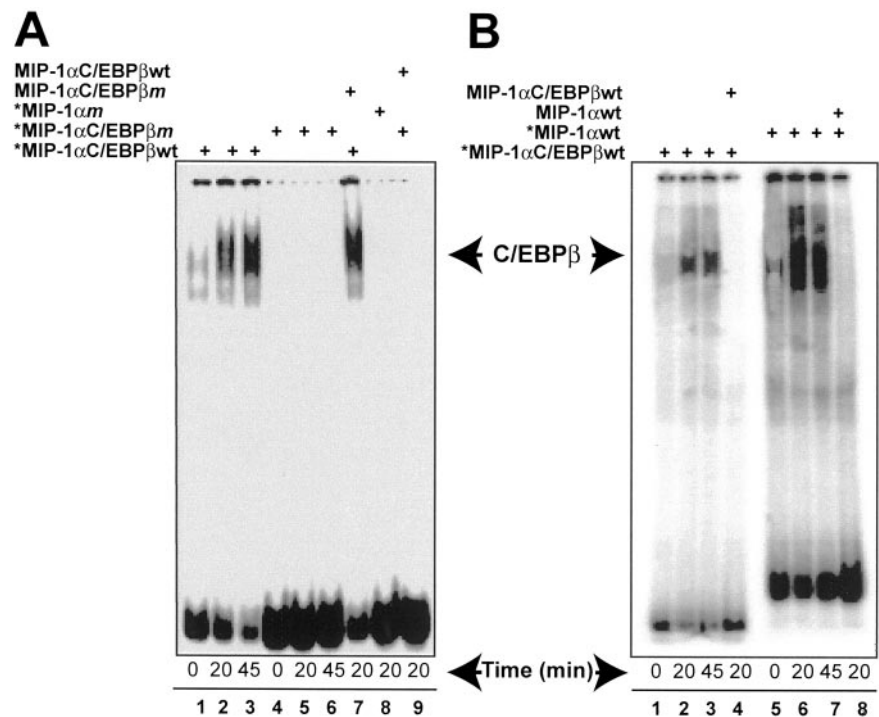


FIGURE 6. The binding activity to MIP-1 α promoter induced by IC is lost by mutations in the C/EBP β site. Nuclear extracts from cells treated with 100 μ g/ml IC for the times indicated were incubated with the labeled sequences *MIP-1 α C/EBP β wt (A and B), *MIP-1 α C/EBP β m (A), and *MIP-1 α wt (B), 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.

Engagement of Fc γ R elicits MIP-1 α , MIP-1 β , and RANTES protein production

Because stimulation of THP-1 cells by IC led to an enhanced expression of both MIP-1 α and MIP-1 β mRNAs, it was addressed whether this was accompanied by a parallel production of MIP-1 α and MIP-1 β proteins. As shown in Fig. 7A, MIP-1 α and MIP-1 β proteins were undetectable in resting cells and increased in a time-dependent manner after incubation with IC. Interestingly, MIP-1 β protein showed a higher increase than that observed for MIP-1 α , because levels of 25 ng/ml were assayed at 8 h after addition of the complexes as compared with 2–4 ng/ml for MIP-1 α , even though more prolonged incubations showed in some cases a significant reduction of the protein, thus suggesting that changes related to the stability and/or degradation of MIP-1 β might occur. RANTES protein was already detectable in cell cultures of resting THP-1 cells, but it significantly increased up to 48 h after the addition of the stimulus to reach a \sim 5-fold increase above resting levels (Fig. 7B). The production of both MIP-1 α and RANTES was dose dependent, as measurable amounts of MIP-1 α were detected with concentrations of IC above 50 μ g/ml, and the production of RANTES also increased with this concentration of stimulus above the level detected in resting cells. Interestingly, the production of MIP-1 α and RANTES elicited by IC was comparable with those elicited by PMA and TNF- α (Fig. 7C), thus indicating that it might reach concentrations similar to those elicited by other stimuli.

Discussion

The present study extends the array of proinflammatory effects resulting from the stimulation of Fc γ R in monocytic cells by showing a widespread pattern of induction of chemokines, in which the expression of MIP-1 α and MIP-1 β reaches the highest level of expression. This is somewhat surprising, because most studies have stressed the production of MCP-1 and IL-8 in response to the activation of Fc γ R. In keeping with the functional association of both MIP-1 α and MIP-1 β , studies in a rat model of IC lung injury have shown a role for both MIP-1 α and MIP-1 β in macrophage activation, because despite a wide induction of other

CC chemokines, only the blockade of either MIP-1 α or MIP-1 β reduced the recruitment of neutrophils and the production of lung injury (16–18).

The comparison of the effect of Fc γ R 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 Fc γ R 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 κ B activity (31), and unlike MIP-1 α , RANTES has two κ B 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- κ B (33), C/EBP β (34), and IFN regulatory factor (35).

The combined induction of both MIP-1 α and MIP-1 β 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- κ B recognition sequence. Taking into account these early functional studies

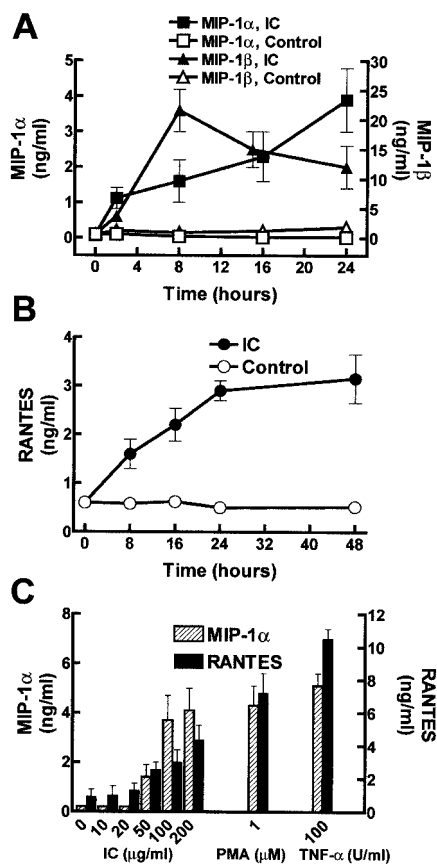


FIGURE 7. Production of MIP-1 α , MIP-1 β , and RANTES proteins. THP-1 cells at the concentration of 1.5×10^6 cells/ml were incubated in the presence (filled symbols) or absence (open symbols) of 100 μ g/ml IC. At the times indicated, the cell culture supernatant was collected and used for the assay of MIP-1 α and MIP-1 β (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 \pm 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/EBP β site in the sense strand; and two more AP-1 sites, two C/EBP β sites, which also encompass a NF-AT site, and an additional NF-AT site in the antisense strand. In contrast, no κ B 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-1 α promoter region, thus suggesting that AP-1 sites are not involved in the regulation of MIP-1 α expression. Moreover, pharmacological approaches using compounds with known activity on the NF- κ B system have provided contradictory results. For instance, the salicylate derivative HTB, which has been found to inhibit NF- κ B activation in several cell systems (25, 37, 38), inhibited the induction of MIP-1 α and MIP-1 β 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-1 α and MIP-1 β , 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

β_2 integrins by both Ab and soluble CD23 has shown a strong induction of both MIP-1 α and MIP-1 β , which was sensitive to proteasome inhibitors, whereas this treatment enhanced IL-8 expression (39). In addition, treatment of human monocytes with 15-deoxy- $\Delta^{12,14}$ PGJ $_2$ 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- κ B route (41). Previous studies have suggested that NF- κ B was involved in the induction of MIP-1 α and MIP-1 β ; however, the evidence was indirect and only stemmed from the coincidental presence of binding activity to a consensus κ B 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-1 α 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/EBP β site in the transcriptional regulation of MIP-1 α can be supported by several arguments: 1) the presence of a high homology C/EBP β 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-1 α expression; 3) the similar dose-response pattern of both MIP-1 α expression and appearance of C/EBP β -binding activity; 4) the parallel pattern of MAP kinase activation (26) and C/EBP β -binding activity in the nuclear extracts elicited by Fc γ R cross-linking, which agrees with the reported phosphorylation at threonine-235 of C/EBP β by MAP kinase as an essential step for C/EBP β activation (43). Moreover, the pattern of MIP-1 α 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/EBP β 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 Fc γ R signaling, i.e., NF- κ B (6, 8), AP-1, and C/EBP β , and suggest the coupling of C/EBP β to the induction of some elements of the chemokine family involved in the production of immune-mediated tissue injury.

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