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Gene Expression and Production of the Monokine Induced by IFN-γ (MIG), IFN-Inducible T Cell α Chemoattractant (I-TAC), and IFN-γ-Inducible Protein-10 (IP-10) Chemokines by Human Neutrophils[1]

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Monokine induced by IFN-γ (MIG), IFN-inducible T cell α chemoattractant (I-TAC), and IFN-γ-inducible protein of 10 kDa (IP-10) are related members of the CXC chemokine subfamily that bind to a common receptor, CXCR3, and that are produced by different cell types in response to IFN-γ. We have recently reported that human polymorphonuclear neutrophils (PMN) have the capacity to release IP-10. Herein, we show that PMN also have the ability to produce MIG and to express I-TAC mRNA in response to IFN-γ in combination with either TNF-α or LPS. While IFN-γ, alone or in association with agonists such as fMLP, IL-8, granulocyte (G)-CSF and granulocyte-macrophage (GM)-CSF, failed to influence MIG, IP-10, and I-TAC gene expression, IFN-α, in combination with TNF-α, LPS, or IL-1β, resulted in a considerable induction of IP-10 release by neutrophils. Furthermore, IL-10 and IL-4 significantly suppressed the expression of MIG, IP-10, and I-TAC mRNA and the extracellular production of MIG and IP-10 in neutrophils stimulated with IFN-γ plus either LPS or TNF-α. Finally, supernatants harvested from stimulated PMN induced migration and rapid integrin-dependent adhesion of CXCR3-expressing lymphocytes; these activities were significantly reduced by neutralizing anti-MIG and anti-IP-10 Abs, suggesting that they were mediated by MIG and IP-10 present in the supernatants. Since MIG, IP-10, and I-TAC are potent chemoattractants for NK cells and Th1 lymphocytes, the ability of neutrophils to produce these chemokines might contribute not only to the progression and evolution of the inflammatory response, but also to the regulation of the immune response. *The Journal of Immunology*, **1999, 162: 4928–4937.**

Chemokines regulate leukocyte trafficking, and their importance in inflammatory processes is best illustrated by their ability to specifically recruit discrete leukocyte populations (1–3). Members of this large family of cytokines typically are 8- to 12-kDa proteins sharing 20 to 70% homology in amino acid sequences (1–3). Chemokines have been classified into four closely related subfamilies on the basis of the relative positions of the first two cysteine residues, but only two of these subfamilies have been extensively characterized: the "C-X-C" and the "C-C" chemokines (1–3). The C-X-C chemokines can be further subdivided into two classes depending on the presence of the glutamate-leucine-arginine (ELR) motif preceding the first two cysteines (1–3). IL-8, macrophage inflammatory protein-2 (MIP-2)[3], growth-related gene product (GRO), and other members express this motif and predominantly exert stimulatory and chemotactic activities toward neutrophils (1–3). In contrast, IFN-γ-inducible protein of 10 kDa (IP-10), monokine induced by IFN-γ (MIG), and the recently cloned IFN-inducible T cell α chemoattractant (I-TAC) (4) lack the ELR sequence and fail to attract polymorphonuclear neutrophils (PMN) (1–5). Interestingly, based on their structure and function, MIG, IP-10, and I-TAC constitute a group of three related chemokines that all act upon T lymphocytes (4–5). While the receptors for the C-X-C chemokines containing the ELR motif are expressed on different types of leukocytes, there is thus far only a single receptor, CXCR3, that is known to bind MIG, I-TAC, and IP-10 (4–6). Importantly, CXCR3 (and CCR5) seem to be preferentially expressed on activated T lymphocytes of the Th1 phenotype (7–10).

Neutrophils are usually the first cells to arrive at a site of inflammation and are essential for nonspecific host defense via the release of a variety of proteases, reactive oxygen intermediates, and arachidonic acid metabolites (11). In addition to their defensive functions, it has become well established, both in vitro and in vivo, that neutrophils can synthesize and secrete several cytokines and chemokines (12). Among the various chemokines, IL-8 (13, 14), MIP-1$\alpha\beta$ (15), MIP-2 (16), GROα (17, 18), and cytokine-induced neutrophil chemoattractant (CINC) (19) have been reported to be secreted following activation of PMN. More recently, we have demonstrated that human neutrophils possess the capacity to release IP-10 (20), but whether they can also express MIG and I-TAC has yet to be determined.

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[3] Abbreviations used in this paper: MIP, macrophage inflammatory protein; G-CSF, granulocyte-CSF; PMN, polymorphonuclear neutrophil; MIG, monokine induced by IFN-γ; IP-10, IFN-γ-inducible protein-10; I-TAC, IFN-inducible T cell α chemoattractant; GRO, growth-related gene product; GM-CSF, granulocyte-macrophage CSF; Y-IgG, yeast particles opsonized with IgG; IL-1ra, IL-1 receptor antagonist.

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In this study, we show that, following stimulation with IFN-γ in combination with either TNF-α or LPS, neutrophils synthesize and release MIG and express I-TAC mRNA. In addition, we show that IFN-α together with TNF-α, LPS, and IL-1β, may represent another potent stimulus for the generation of IP-10 by neutrophils and that IL-10, and to a lesser extent IL-4, can negatively modulate the expression of MIG, I-TAC, and IP-10 in human neutrophils. Finally, we provide evidence of the functional significance of neutrophil-derived MIG and IP-10 in acting on CXCR3-expressing lymphocytes.

Materials and Methods

Cell purification and culture

Highly purified granulocytes (>99.5%) and PBMC were isolated under endotoxin-free conditions from buffy coats of healthy donors, as previously described (21). The granulocyte populations contained usually <4% eosinophils (n = 23), as revealed by May-Grunwald-Giemsa staining. Eosinophils (purity >95%) were isolated by immunomagnetic beads, according to the method described by Hansel et al. (22). Immediately after purification, cells were suspended in RPMI 1640 medium supplemented with 10% low-endotoxin FCS (<0.009 ng/ml; Seromed, Biochrom, Berlin, Germany) and usually treated with 100 U/ml IFN-γ (21) (Hoffmann-La Roche, Basel, Switzerland), 100 U/ml IL-10 (kindly provided by Dr. K. Moore, DNAX and Schering-Plough, Palo Alto, CA) (23), 1000 U/ml IFN-α (Roferon-A; Hoffmann-La Roche), or 10 ng/ml IL-4 (Peprotech, Rocky Hill, NJ) in different combinations. Cells were then stimulated, plated at 5×10^6/ml either in 24-well tissue culture plates (Nunc, Roskilde, Denmark) or in polystyrene flasks (Greiner, Nurtingen, Germany), and cultured at 37°C, 5% CO_2 atmosphere. Cell agonists used were the following: 1 μg/ml LPS (from *Escherichia coli*, serotype 026:B6, purchased from Sigma, St. Louis, MO), 5 ng/ml TNF-α (Peprotech), heat-killed yeast particles opsonized with IgG (Y-IgG) at a particle/cell ratio of 2:1, 10 nM fMLP (Sigma), 10 ng/ml GM-CSF (Genetics Institute, Boston, MA), 1000 U/ml G-CSF (Granulokine; Hoffmann-La Roche), 20 ng/ml IL-1β (Hazleton Laboratories, Vienna, VA), and 50 ng/ml IL-8 (Sandoz, Vienna, Austria). At the indicated times, cell-free supernatants were harvested and stored at −20°C, whereas cell pellets were extracted for total RNA. All reagents used were of the highest available grade and were dissolved in pyrogen-free water for clinical use (20, 21, 23).

RNA isolation and Northern blot analysis

Total RNA from PMN and PBMC was extracted by the guanidinium isothiocyanate method, usually from $6–7 \times 10^7$ PMN and $2–3 \times 10^7$ PBMC per condition, and analyzed as already described (21). Filters were hybridized using MIG, I-TAC, IP-10, IL-1 receptor antagonist (IL-1ra), IL-8, glyceraldehyde 3-phosphate dehydrogenase (GAPD), and actin cDNA fragments, ^{32}P-labeled using a Ready-to-go DNA labeling kit (Pharmacia, Uppsala, Sweden).

MIG, IP-10, and IL-8 antigenic determination

MIG protein was measured in the cell-free supernatants by using a specific double-determinant RIA, developed in our laboratory. Briefly, flat-bottom 96-well plates (MaxiSorp, No. 439454; Nunc) were coated with 50 μl/well of protein-G-purified rabbit serum (No. 5092; Ref. 24) (40 μg/ml in 0.1 M carbonate buffer, pH 9.5) for 24 h at 4°C, and then extensively washed with PBS, pH 7.5, 0.05% Tween 20 (washing buffer). Fifty microliters per well of either MIG standards (Peprotech) or cell-derived culture supernatants were then added, followed by an incubation for 6 h at 20°C. Plates were rinsed with washing buffer before addition of 50 μl/well of ^{125}I-labeled rabbit anti-human MIG (No. 500-P50; Peprotech) (0.5 μg/ml in PBS-Tween with 50% FCS) and incubated overnight at 4°C. After extensive washings of the plates, 60 μl of 1 N NaOH was added into each well, harvested after 30 min, and read in a gamma counter. This RIA had a detection limit of 30–50 pg/ml and did not cross-react with 100 ng/ml IP-10, 100 ng/ml IL-8, 1 ng/ml MIP-1α, 10 ng/ml GROα, 10 ng/ml IFN-γ, 5 ng/ml IL-10, 10 ng/ml IL-1β, 10 ng/ml TNF-α, or 10 ng/ml GM-CSF. IP-10 and IL-8 were measured in the cell-free supernatants by, respectively, RIA (detection limit 30 pg/ml) and ELISA (detection limit 20 pg/ml), according to the procedures previously published (13, 20).

Estimation of apoptotic cells

Flow cytometric analysis of apoptosis was performed by using the method described by Nicoletti et al. (25). In brief, harvested cells were washed twice with PBS and then suspended in 1.5 ml hypotonic fluorochrome solution (propidium iodide 50 μg/ml in 0.1% sodium citrate and 0.1% Triton X-100). The mixture was placed in the dark overnight at 4°C. The fluorescence of each individual nucleus was measured using a FACScan flow cytometer (XL-Coulter, Hialeah, FL).

In vitro chemotaxis assay

To evaluate the chemotactic activities of neutrophil- and PBMC-derived supernatants, we used, as target cells, either the previously described 300-19 mouse pre-B cell clones stably transfected with human CXCR3 cDNA (6) or PHA-stimulated peripheral T lymphocytes cultured in the presence of IL-2 for 8–14 days. CXCR3 receptor expression on transfected cells or activated T cells was checked by FACScan analysis by using 1C6, an anti-hCXCR3 mAb (7), kindly provided by Dr. Carlo Agostini (University of Padova, Italy). Migration of CXCR3 transfectants was assessed in a 48-well modified Boyden chamber (NeuroProbe, Cabin John, MD) using polyvinylpyrrolidone-free polycarbonate membranes (Nucleo pore) with 5-μm pores, as we previously described (26). In brief, leukocyte-derived supernatants (28 μl) or 10 nM recombinant MIG or IP-10 (diluted in RPMI medium containing 1% FBS) were added to the bottom wells of the chemotaxis chamber. PBMC-derived supernatants from resting or IFN-γ-treated cells were usually used undiluted, whereas supernatants from resting or stimulated PMN were used either undiluted or after (approximately) a 100-fold concentration (by the Centricon Plus 20 device; Amicon, Beverly, MA). The parental cell line and CXCR3 transfectants were suspended at 5×10^6/ml in RPMI and added to the top wells of the chamber in a volume of 50 μl. Chambers were then incubated for 120 min in a 37°C, 5% CO_2 atmosphere. After the incubation period, the filters were removed, washed with PBS on the upper side, fixed, and stained with Dif-Quik (Baxter, Deerfield, IL). The number of cells migrating to the lower surface was microscopically counted in six randomly high powered fields. All assays were performed in triplicate. Spontaneous migration was determined in the absence of samples. In selected experiments, leukocyte-derived supernatants or recombinant chemokines were preincubated at 37°C for 30 min with 30 μg/ml of rabbit anti-human MIG (No. 500-P50, Peprotech), goat anti-human IP-10 (No. AF-266-NA; R&D Systems, Abingdon, U.K.), goat anti-human IL-8 (kindly provided by Dr. M. Ceska, Sandoz, Vienna), mouse anti-human TNF-α and IFN-γ (kindly provided by Dr. Giorgio Trinchieri, Wistar Institute, Philadelphia, PA), and isotype-matched Abs, to neutralize their chemotactic activities.

Chemotaxis was also assessed using a 24-well transwell chamber (6.5-mm diameter, 5-μm pore size, Costar 3421; Corning Costar, Rochester, NY), essentially as described by Gosling et al. (27). In brief, the parental cell line and CXCR3 transfectants or activated T lymphocytes were suspended at 2×10^6/ml in RPMI 1640, containing 10% FCS and 20 mM HEPES, pH 7.2. One hundred microliters of cell suspension were added to the top chamber, whereas 600 μl of leukocyte-derived supernatants or 10 nM recombinant chemokines were added to the bottom well. The chambers were then incubated for 2 h at 37° in an atmosphere containing 5% CO_2. Cells that passed through the membrane were harvested from the lower well and counted by using the CyQuant Cell proliferation assay kit (Molecular Probes, Eugene, OR), according to the manufacturer's instructions.

Adhesion assay

Human T lymphocytes expressing CXCR3 were used in these assays. T cells were first purified from PBMC by E-rosetting with neuraminidase-treated sheep RBC and then incubated (2×10^6/ml) at 37°C for 3 days in complete medium containing 500 U/ml IL-2 (Proleukin; Chiron Diagnostic, Cassina de' Pecchi, Italy) and 5 μg/ml PHA. Subsequently, T cells were maintained at a density of $3–5 \times 10^6$/ml in fresh media containing 250 U/ml IL-2 to optimize the induction of CXCR3 expression and their responsiveness to IP-10 and MIG, as recently reported by Cole et al. (4) and Loetscher et al. (28). At the days indicated in the results, activated T lymphocytes were washed and resuspended at 5×10^6/ml in PBS, 1 mM Ca^{2+}/Mg^{2+}, 10% heat-inactivated FCS (pH 7.2). Twenty microliters of cell suspension (10^5 cells) were allowed to settle for 10 min at 37°C on 18-well glass slides precoated for 16 h at 4°C with 20 μl of purified human fibronectin (20 μg/ml in PBS; Sigma, F 6277). After the cells were allowed to settle, lymphocyte adhesion was stimulated for 2 min with 5 μl of leukocyte-derived supernatants or with 5 μl of recombinant chemokines (100 nM final concentration), added at the 12 o'clock position of the well. At the appropriate time point, the slide was dipped twice in ice-cold HBSS/10 mM HEPES to remove nonadherent cells, and bound cells were then fixed in ice cold PBS containing 1.5% gluteraldehyde. Adherent cells in 0.2 mm^2 were calculated by computer-assisted enumeration. In selected experiments, specific Abs were used to neutralize the biological effects of recombinant chemokines or leukocyte-derived supernatants (see above).

Table I. *Extracellular release of MIG by human PMN (pg/ml)*[a]

	21 h	42 h
Medium	nd ($n = 12$)	nd ($n = 12$)
LPS	nd ($n = 10$)	nd ($n = 10$)
TNF-α	nd ($n = 10$)	nd ($n = 10$)
IL-1β	nd ($n = 4$)	nd ($n = 4$)
IL-8	nd ($n = 2$)	nd ($n = 2$)
fMLP	nd ($n = 2$)	nd ($n = 2$)
G-CSF	nd ($n = 3$)	nd ($n = 3$)
GM-CSF	nd ($n = 3$)	nd ($n = 3$)
IFN-γ	nd ($n = 12$)	nd ($n = 12$)
IFN-γ + LPS	23.1 ± 11.2 ($n = 12$)	159.4 ± 93.4 ($n = 12$)
IFN-γ + TNF-α	70.3 ± 45 ($n = 12$)	642.7 ± 262.5 ($n = 12$)
IFN-γ + IL-1β	nd ($n = 4$)	nd ($n = 4$)
IFN-γ + IL-8	nd ($n = 2$)	nd ($n = 2$)
IFN-γ + fMLP	nd ($n = 2$)	nd ($n = 2$)
IFN-γ + G-CSF	nd ($n = 3$)	nd ($n = 3$)
IFN-γ + GM-CSF	nd ($n = 3$)	nd ($n = 3$)

[a] PMN (5×10^6/ml) were stimulated as indicated in *Materials and Methods* for 21 and 42 h. MIG was determined in the cell-free supernatants by a specific RIA. Values are means ± SD, obtained from the number of experiments indicated in parentheses, nd, not detectable.

Each experiment was performed in quadruplicate for each condition, and the SD was calculated. Results are representative from at least two independent donors.

Statistical analysis

Data are expressed as means ± SD.

Results

MIG release by stimulated neutrophils

To investigate whether PMN produce MIG, cells were incubated for up to 42 h with a series of neutrophil agonists, including LPS, TNF-α, IL-1β, IL-8, fMLP, G-CSF, and GM-CSF, used either as single stimuli or in combination with IFN-γ. As shown in Table I, only IFN-γ plus either LPS or TNF-α stimulated a detectable extracellular release of MIG. Surprisingly, up to 50,000 U/ml IFN-γ did not induce any significant extracellular production of MIG from PMN (data not shown). Dose-response experiments further established that, in IFN-γ-treated neutrophils, 5 ng/ml TNF-α represented an optimal concentration for MIG release, whereas the stimulatory effect of LPS did not substantially differ over a wide concentration range, i.e., from 0.01 to 10 μg/ml (Fig. 1). Accurate time course analyses (not shown) revealed that, in IFN-γ plus TNF-α- or LPS-stimulated PMN, antigenic MIG started to be released only after 18–21 h of stimulation and progressively accumulated into the supernatants for up to 42 h (Table I). In contrast, more than 80% or even 100% of the total antigenic IP-10 was already released within 21 h in response to IFN-γ plus LPS and IFN-γ plus TNF-α, respectively (Table II) (20). Moreover, stimulation of neutrophils for 42 h with IFN-γ plus TNF-α consistently (in 12 independent experiments) led to the release of higher amounts of MIG than when the cells were stimulated with IFN-γ plus LPS, a pattern that was not always observed in the case of IP-10. Finally, considerable amounts of IP-10 were released by neutrophils even when they were cultured in the presence of IFN-γ in association with IL-1β, but not with GM-CSF (Table II).

MIG and IP-10 release by stimulated eosinophils and PBMC

To exclude a role for contaminating cells present in our neutrophil preparations, we determined the ability of eosinophils and PBMC to release MIG and IP-10. For this purpose, in selected experiments, eosinophils and PBMC were purified (in addition to neutrophils) and stimulated for 21 h with IFN-γ plus either LPS or

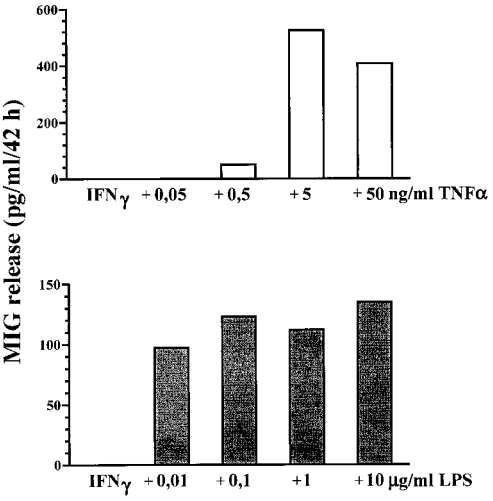

FIGURE 1. Dose-response studies for the extracellular production of MIG by stimulated neutrophils. PMN (5×10^6/ml) were stimulated with increasing doses of TNF-α or LPS, in the presence of 100 U/ml IFN-γ. Cell-free supernatants were collected after 42 h, and the levels of MIG protein were measured by RIA. Each panel shows the mean values of duplicate assays for each condition, obtained from one experiment representative of four.

TNF-α. As illustrated in Fig. 2, eosinophils did not produce MIG, but they did produce IP-10. However, substantial release of IP-10 by eosinophils occurred only in response to IFN-γ plus TNF-α. In contrast to neutrophils and eosinophils, PBMC treated with IFN-γ released amounts of MIG higher than those detected in IFN-γ plus LPS-treated cells (Fig. 3). However, the highest levels of antigenic MIG detected in PBMC culture supernatants were observed in IFN-γ plus TNF-α-treated cells (Fig. 3).

Expression of MIG and I-TAC mRNAs in neutrophils and PBMC

To gain further insights into the molecular mechanisms regulating MIG production by PMN, neutrophils were stimulated with various combinations of agonists, and total RNA was analyzed by Northern blot analysis. In the same experiments, we also investigated whether neutrophils might express I-TAC mRNA. As shown in Fig. 4*A*, resting neutrophils, as well IFN-γ-treated PMN, do not express detectable MIG or I-TAC transcripts. LPS or TNF-α, or other stimuli such as Y-IgG, fMLP, G-CSF, and GM-CSF, used alone or in the the presence of IFN-γ, also failed to induce MIG or I-TAC gene expression (data not shown). In contrast, a considerable accumulation of MIG and I-TAC mRNA was observed in

Table II. *Extracellular release of IP-10 by human PMN (pg/ml)*[a]

	21 h	42 h
Medium	nd ($n = 12$)	nd ($n = 12$)
LPS	nd ($n = 10$)	nd ($n = 10$)
TNF-α	nd ($n = 10$)	nd ($n = 10$)
IL-1β	nd ($n = 4$)	nd ($n = 4$)
GM-CSF	nd ($n = 3$)	nd ($n = 3$)
IFN-γ	51.2 ± 29 ($n = 12$)	62.8 ± 44 ($n = 12$)
IFN-γ + LPS	2289 ± 1353 ($n = 12$)	2808 ± 1340 ($n = 12$)
IFN-γ + TNF-α	3215 ± 145.3 ($n = 12$)	3194 ± 1523 ($n = 12$)
IFN-γ + IL-1β	262 ± 99 ($n = 4$)	373 ± 135 ($n = 4$)
IFN-γ + GM-CSF	48.2 ± 12 ($n = 3$)	36 ± 14 ($n = 3$)

[a] PMN (5×10^6/ml) were stimulated as indicated in *Materials and Methods* for 21 and 42 h. IP-10 was determined in the cell-free supernatants by a specific RIA. Values are means ± SD, obtained from the number of experiments indicated in parentheses. nd, not detectable.

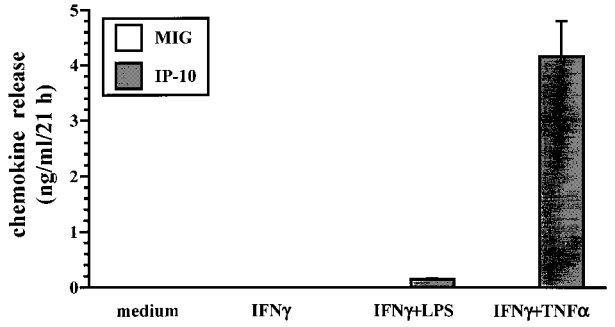

FIGURE 2. Ability of eosinophils to release MIG and IP-10. Purified populations of eosinophils were cultured with IFN-γ, alone or in the presence of either 1 μg/ml LPS or 5 ng/ml TNF-α. Cell-free supernatants were collected after 21 h, and the levels of MIG and IP-10 protein were measured by RIA. Values represent the mean ± SD calculated from three independent experiments.

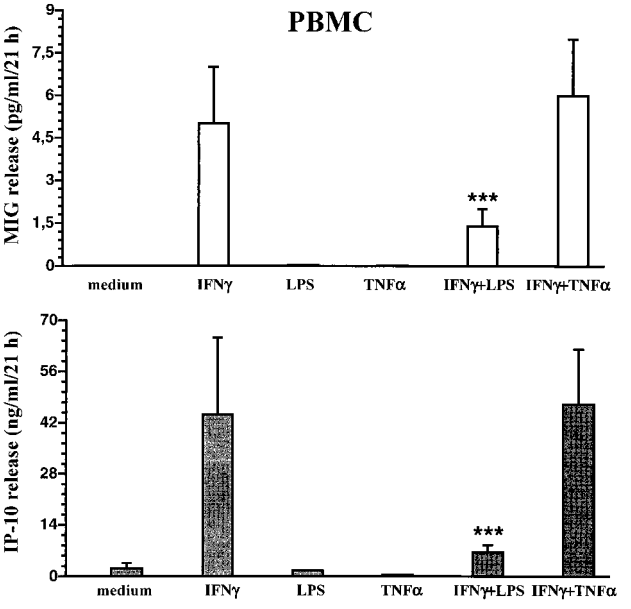

FIGURE 3. Extracellular production of MIG by stimulated PBMC. PBMC were stimulated either with 1 μg/ml LPS, or with 5 ng/ml TNF-α, in the presence or absence of 100 U/ml IFN-γ. Cell-free supernatants were collected after 21 h, and the levels of MIG and IP-10 proteins were measured by RIA. Values represent the mean ± SD calculated from seven independent experiments. The asterisks represent significant difference between IFN-γ- and IFN-γ plus LPS-treated PBMC. ***, $p < 0.005$.

PMN stimulated with IFN-γ, plus either TNF-α or LPS, with maximal expression levels being reached by 21 h, especially in the case of IFN-γ plus TNF-α-treated cells (Fig. 4*A*). IP-10 mRNA transcripts were also highly expressed under those stimulatory conditions, in agreement with our previous data (20). However, kinetics of IP-10 mRNA expression in IFN-γ plus LPS-treated neutrophils differed greatly from those of IFN-γ plus TNF-α cells; as in the former condition, IP-10 transcripts reached a maximum at 3 h and then declined, whereas in the latter maximal levels of IP-10 mRNA were detected at 21 h (Fig. 4*A*).

Fig. 4*B* shows a representative Northern blot analysis of MIG, I-TAC, and IP-10 mRNA accumulation in PBMC, stimulated as neutrophils. It is evident that IFN-γ alone represents already an optimal stimulus to induce the mRNA expression for all three chemokines and that LPS (but not TNF-α) down-modulates the up-regulatory effect of IFN-γ (Fig. 4*B*), in accord with extracellular chemokine detection (Fig. 3).

Effects of IL-10 and IL-4

Because IL-10 and IL-4 are negative modulators of chemokine production by neutrophils (29), we investigated whether they might also influence the inducible expression of MIG, I-TAC, and IP-10. IL-10 greatly reduced the mRNA expression for MIG, I-TAC, and IP-10 induced by IFN-γ plus either LPS or TNF-α, with this inhibitory effect being much more pronounced in PMN stimulated with IFN-γ plus LPS (Fig. 5*A*), as opposed to IFN-γ plus TNF-α (Fig. 5*B*). However, IL-10 potentiated the stimulatory effect of IFN-γ plus either LPS or TNF-α on IL-1ra transcripts, confirming and extending previous findings (30, 31). Consistent with the Northern blot data, a potent inhibitory action of IL-10 was observed at the level of MIG and IP-10 extracellular release, both at 21 (not shown) and 42 h poststimulation (Fig. 6), especially on IFN-γ plus LPS-treated PMN. Relative to IL-10, IL-4 only moderately suppressed MIG, I-TAC, and IP-10 mRNA accumulation (not shown), as well as MIG and IP-10 release in neutrophils. In

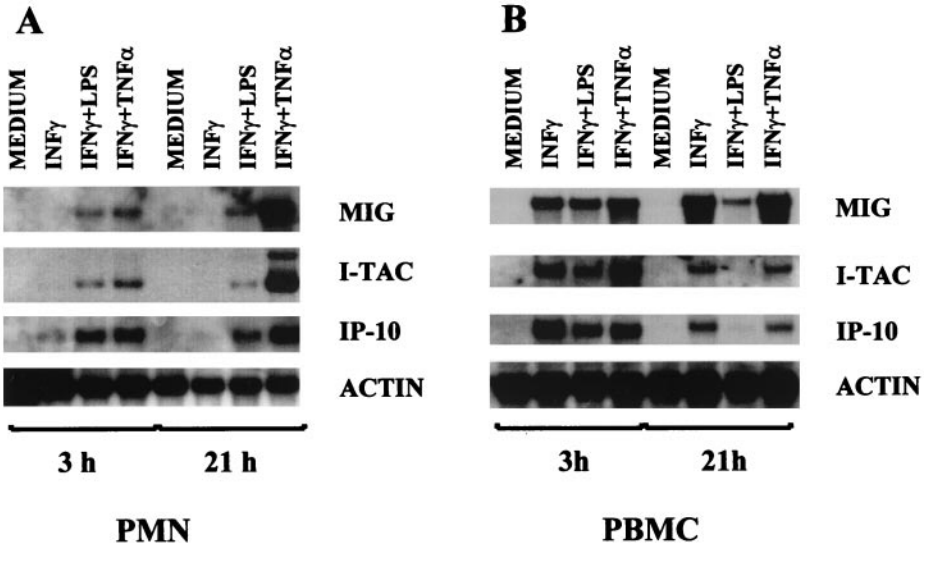

FIGURE 4. Comparative ability of neutrophils and PBMC to express MIG, I-TAC, and IP-10 mRNA. Purified populations of PMN (*A*) and PBMC (*B*) isolated from the same donor were cultured with IFN-γ, alone or in the presence of either LPS or TNF-α. After 3 and 21 h, total RNA was extracted, and Northern blot analysis of MIG, I-TAC, IP-10, and actin mRNA was performed. Ten micrograms total RNA were loaded on each gel lane. The experiment depicted in this is representative of four.

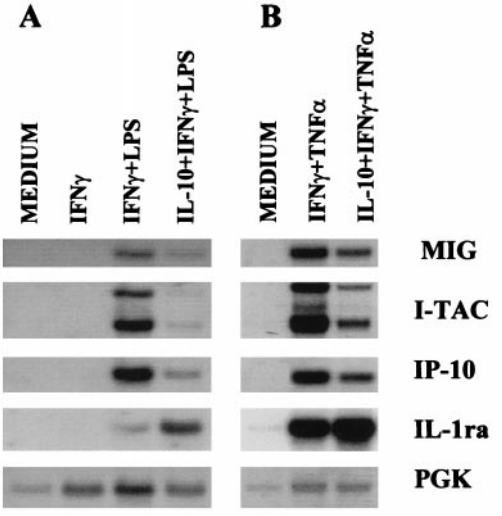

FIGURE 5. Effect of IL-10 on MIG, I-TAC, and IP-10 chemokine mRNA expression in stimulated neutrophils. Total RNA was extracted from PMN cultured for 21 h with IFN-γ plus LPS (*A*), or with IFN-γ plus TNF-α (*B*), in the presence or absence of IL-10 (100 U/ml). MIG, I-TAC, IP-10, and IL-1ra mRNA accumulation was evaluated by Northern blot analysis. This experiment is representative of three.

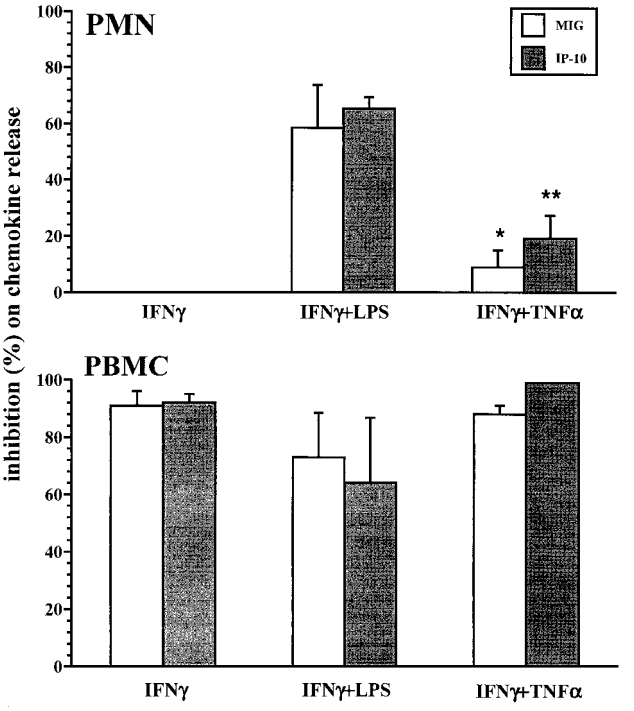

FIGURE 6. Effect of IL-10 on MIG and IP-10 release by stimulated neutrophils and PBMC. PMN and PBMC were cultured for 42 h, respectively, with IFN-γ plus either LPS or TNF-α, in the presence or absence of IL-10 (100 U/ml). The cell-free supernatants were collected, and the levels of MIG and IP-10 protein were determined by RIA. Values are the mean ± SD of the percentage of IL-10 inhibition, calculated from six (for PMN) and three (for PBMC) independent experiments. The asterisks represent significant difference vs agonist-stimulated cells in the absence of IL-10. *, $p < 0.05$; **, $p < 0.01$.

four independent experiments, IL-4-mediated inhibition of MIG and IP-10 secretion amounted to 34.5 ± 16.2% and to 24 ± 9%, respectively, in PMN stimulated for 42 h with IFN-γ plus LPS, and to 19.5 ± 12% and 15.4 ± 10%, respectively, in cells treated with IFN-γ plus TNF-α.

Fig. 6 also illustrates that IL-10 markedly inhibits MIG and IP-10 release also in PBMC cultured for 21 h. In three independent experiments, suppression of MIG and IP-10 release by IL-10 in cells treated with IFN-γ amounted to 91 ± 7 and 92 ± 3%, whereas, in those stimulated with IFN-γ plus LPS and with IFN-γ plus TNF-α, inhibition mediated by IL-10 was higher than in neutrophils (Fig. 6).

Assessment of neutrophil apoptosis

Although neutrophils undergo constitutive apoptosis when aged in vitro, there is considerable evidence to suggest that this process is substantially delayed if cells are cultured in the presence of agonists such as LPS, IFN-γ, G-CSF, or GM-CSF (32). In this context, it was recently reported that IL-10 seems to block the protective effects of LPS and other cytokines, including IFN-γ and TNF-α, on the survival of cultured neutrophils (33). As a result, we sought to determine whether the reduced chemokine expression by stimulated PMN cultured in the presence of IL-10 might be the consequence of an accelerated apoptotic process. To verify this possibility, we measured the rates of neutrophil apoptosis under our experimental conditions. Table III shows that, after a 21 and 42 h

Table III. *Effect of IL-10 on the rate of neutrophil apoptosis in vitro*[a]

	−IL-10		+IL-10	
	21 h	42 h	21 h	42 h
Medium	31.3 ± 15.0 ($n = 8$)	61.5 ± 13 ($n = 5$)	29.4 ± 12.7 ($n = 7$)	62 ± 11 ($n = 5$)
LPS	17.7 ± 11.8** ($n = 6$)	51.2 ± 16 ($n = 5$)	19.0 ± 12.0$^{\#\#}$ ($n = 5$)	48 ± 9$^{\#}$ ($n = 5$)
IFN-γ	17.0 ± 9.4** ($n = 8$)	34.2 ± 12** ($n = 5$)	19.5 ± 14.5$^{\#\#}$ ($n = 4$)	not done
IFN-γ + LPS	15.3 ± 8.0** ($n = 5$)	32.7 ± 11** ($n = 4$)	15.1 ± 7.0$^{\#\#}$ ($n = 5$)	32.5 ± 8$^{\#\#}$ ($n = 5$)
TNF-α	29.4 ± 13.0 ($n = 4$)	50.3 ± 18 (n = 4)	not done	not done
IFN-γ + TNF-α	13.1 ± 7.5** ($n = 4$)	27.3 ± 9** ($n = 4$)	13.4 ± 7.1$^{\#\#}$ ($n = 4$)	22 ± 5$^{\#\#}$ ($n = 4$)

[a] Data are reported as the percentage of fragmented nuclei reflecting the relative proportion of apoptotic cells after culture in the presence or absence of the indicated factors. Values indicate the means ± SD of the number of experiments indicated by the parentheses.

∗ Statistical evaluation between agonist and medium, in the absence of IL-10, and #, statistical evaluation between agonist and medium in the presence of IL-10, where ∗ and # = $p < 0.05$ and ∗∗ and ## = $p < 0.001$.

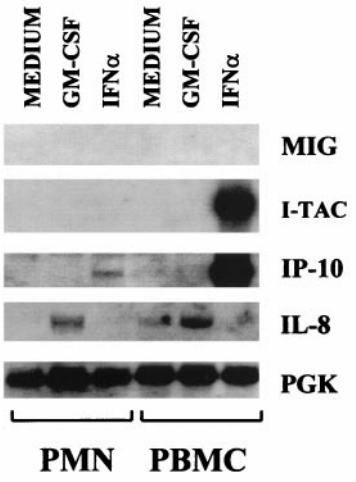

FIGURE 7. Effect of IFN-α on MIG, I-TAC, and IP-10 mRNA expression in human neutrophils and PBMC. Neutrophils and PBMC isolated from the same donor were incubated with 1000 U/ml IFN-α. After 3 h, total RNA was extracted and processed for Northern blot analysis of MIG, I-TAC, IP-10, IL-8, and actin transcripts. Ten micrograms total RNA were loaded on each gel lane. The experiment depicted in this is representative of three.

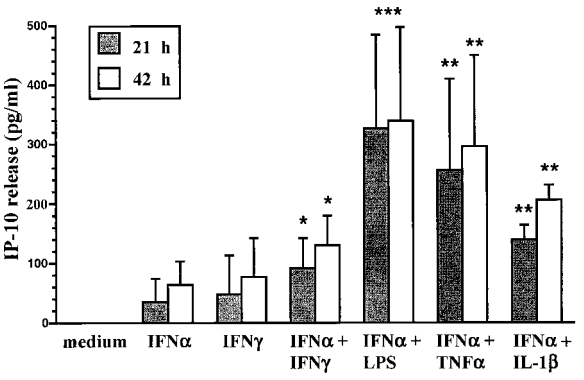

FIGURE 8. Effect of IFN-α on the extracellular production of IP-10 by stimulated neutrophils. PMN were stimulated with 100 U/ml IFN-γ, 1 μg/ml LPS, 5 ng/ml TNF-α, or 20 ng/ml IL-1β, in the presence or absence of 1000 U/ml IFN-α. Cell-free supernatants were collected after 21 and 42 h, and the levels of IP-10 protein were measured by RIA. Values represent the mean $\pm$ SD calculated from three to seven independent experiments. The asterisks represent significant difference between agonist-stimulated PMN and unstimulated controls. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

culture period, recovery of live neutrophils was 69 $\pm$ 15% and 48.5 $\pm$ 13%, respectively; IFN-γ, LPS, and, more effectively, IFN-γ plus either LPS or TNF-α were found to exert significant protective effects on neutrophil apoptosis. Under those conditions, IL-10 influenced neither the spontaneous nor the cytokine-regulated apoptosis rate of neutrophils, yet maintaining its suppressive effect on IL-8 release (not shown) (23).

Effect of IFN-α on MIG, I-TAC, and IP-10 mRNA expression and IP-10 production by neutrophils

In the attempt to identify further stimulatory conditions able to induce expression of MIG, I-TAC, or IP-10 in neutrophils, we examined the effect of IFN-α, used either alone or in association with other neutrophil agonists. As shown in Fig. 7, IFN-α alone induced only IP-10 mRNA in neutrophils, while in PBMC it induced both IP-10 and I-TAC mRNA. MIG mRNA was not up-regulated by IFN-α in either cell type, as expected (34). In both cell types, GM-CSF up-regulated IL-8 mRNA expression (29), while IFN-α down-regulated the constitutive IL-8 transcripts in PBMC, as previously reported (35). The effect of IFN-α on IP-10 mRNA expression in neutrophils was synergistically potentiated by the simultaneous addition of LPS and TNF-α (not shown). Subsequent assays on IP-10 protein release demonstrated that treatment of neutrophils for 42 h with IFN-α in combination with IFN-γ, LPS, TNF-α, or IL-1β, but not alone, resulted in a considerable release of antigenic IP-10 into the supernatants (Fig. 8). Preincubation of neutrophils for 5 h with IFN-α or IFN-γ before stimulation with LPS, TNF, IL-1β, IL-8, fMLP, and GM-CSF did not augment the release of IP-10 or MIG, as compared with the chemokine release observed without preincubation (not shown).

Biological activities of neutrophil- and PBMC-derived supernatants

To ascertain whether neutrophil-derived MIG and/or IP-10 were biologically active, we initially tested the ability of neutrophil-derived supernatants to recruit CXCR3-positive cells in a chemotaxis assay in vitro. For this purpose, we used the mouse pre-B cell line, 300-19, stably transfected with CXCR3 cDNA. These transfectants represent a reliable experimental model to determine the chemotactic activities of IP-10, MIG, and I-TAC and have been widely used by many investigators (4, 6, 28). As shown in Fig. 9, CXCR3-transfected cells readily migrated toward 10 nM MIG or IP-10, while cells from the nontransfected, parental cell line did not respond. Supernatants harvested from neutrophils stimulated with IFN-γ plus TNF-α (or, to a lesser extent, with IFN-γ plus LPS) and from IFN-γ-treated PBMC exerted significant chemotactic activities on CXCR3-transfected, but not on parental, cells (Fig. 9). By contrast, supernatants harvested from resting leukocytes or from IFN-γ-stimulated PMN were ineffective. Of note, neutralizing anti-MIG and anti-IP-10 Abs partially abrogated the chemotactic activities exerted by supernatants from both stimulated neutrophils and PBMC, indicating that MIG and IP-10 were, at least in part, responsible for these chemotactic effects (Fig. 9).

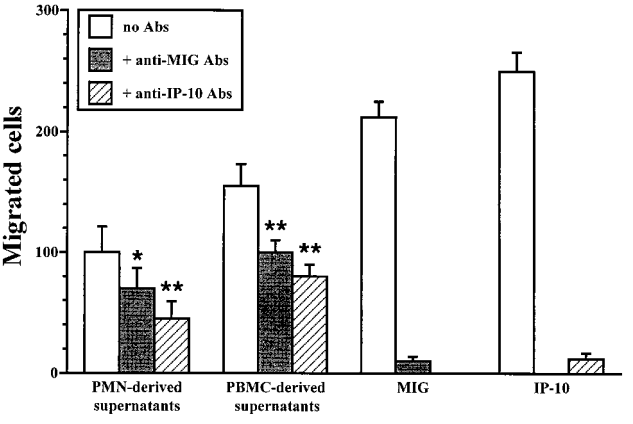

FIGURE 9. Effect of neutrophil- and PBMC-derived supernatants on migration of stable CXCR3 transfectants. Mouse 300-19 transfectants expressing CXCR3 were challenged with neutrophil- and PBMC-derived supernatants and 10 nM recombinant chemokines in the presence or absence of the indicated Abs. Chemotaxis was performed in microBoyden chambers as described in *Material and Methods*. The incubation time for chemotaxis was 2 h. Values represent the mean $\pm$ SD of the number of cells migrating in five high powered fields (HPF), calculated from three independent experiments. The asterisks represent significant differences. *, $p < 0.05$; **, $p < 0.01$.

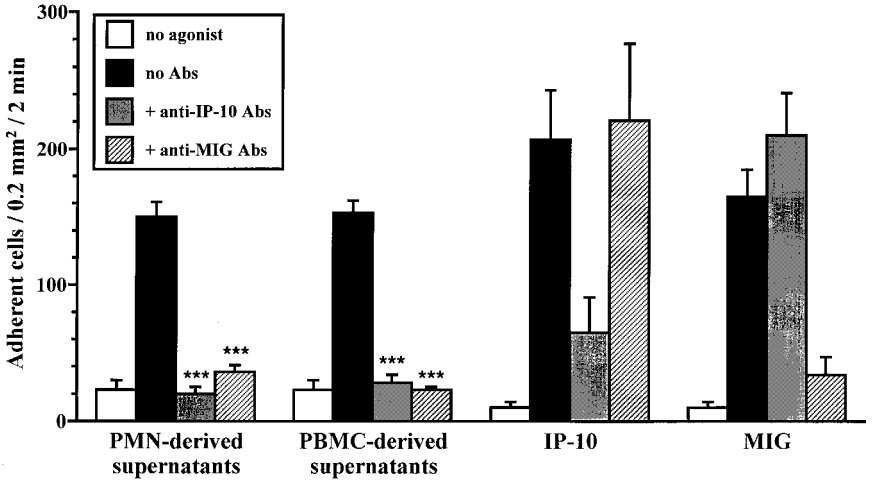

FIGURE 10. Neutrophil- and PBMC-derived supernatants induce rapid adhesion of activated T cells to fibronectin. Nine days IL-2-treated T lymphocytes were allowed to adhere in the presence of supernatants or recombinant chemokines (100 nM) to slides coated with fibronectin, as described in *Materials and Methods*. Data (mean ± SD values of triplicate wells) are expressed as absolute number of adherent T cells and represent one of three independent experiments. The asterisks represent significant differences. ***, $p < 0.005$.

No additive effects were found if neutralizing anti-MIG and anti-IP-10 Abs were used in combination (not shown). It must be pointed out that, to detect biological effects of the supernatants harvested from cultured PMN, it was necessary to concentrate them (approximately 100-fold) before assaying. Indeed, preliminary tests revealed that crude undiluted supernatants from agonist-stimulated neutrophils failed to exert chemotactic activities on CXCR3-transfected cells. Moreover, supernatants from IFN-γ-stimulated PBMC displayed chemoattraction on either CXCR3-transfected cells or activated T lymphocytes (again, partially neutralized by anti-MIG and anti-IP-10 Abs) also in the transwell assay (not shown). Unfortunately, we could not test neutrophil-derived supernatants in the transwell system because it was not feasible to obtain sufficient volumes (more than 4 ml) of concentrated samples that were necessary to perform experiments with all appropriate controls.

Subsequently, we determined whether neutrophil-derived MIG or IP-10 could trigger rapid lymphocyte adhesion to purifed integrin ligands, a recently reported biological function of some chemokines (36, 37). For this purpose, we used PHA-activated T lymphocytes cultured in the presence of IL-2 for 6–14 days, which show an optimal CXCR3 expression and responsiveness to IP-10, MIG, and I-TAC (Refs. 4 and 28, and our unpublished data). Because CXCR3-transfected mouse cell lines displayed little binding to fibronectin in response to recombinant MIG or IP-10, they could not be used in these assays. As shown in Fig. 10, both MIG and IP-10 triggered a rapid adhesion of activated T lymphocytes to fibronectin that was completely inhibited by the respective neutralizing Abs. Preliminary experiments confirmed that IP-10- and MIG-induced rapid and firm adhesion of T cells was a transient phenomenon and was maximal after 2 min, exactly as recently reported by Piali et al. (37). No binding was induced in the absence of agonists.

Concentrated supernatants from IFN-γ plus TNF-α-stimulated PMN and, to a lesser extent, from IFN-γ-stimulated PBMC also induced the rapid adhesion of activated T lymphocytes to fibronectin (Fig. 10). In contrast, no detectable binding was triggered by supernatants from resting cells or from IFN-γ-stimulated neutrophils (not shown). Importantly, adhesion triggering by supernatants was almost completely blocked by Abs to either MIG or IP-10 (Fig. 10), whereas isotype-matched and IFN-γ-, TNF-α-, and IL-8-neutralizing Abs were completely ineffective (not shown), suggesting that MIG and IP-10 present in neutrophil-derived supernatants were responsible for these biological effects.

Discussion

MIG, I-TAC, and IP-10 are three structurally related C-X-C chemokines that mainly act as chemotactic factors for stimulated T cells and NK cells (1–6, 38, 39). MIG, I-TAC, and IP-10 exert their effects through a shared receptor, called CXCR3 (4, 6). Of note, the CXCR3-expressing T cells have been shown to produce predominantly classical Th1 cytokines, supporting the concept that MIG, I-TAC, and IP-10 selectively mobilize Th1 lymphocytes (7–10). Studies in vitro have shown that MIG and IP-10 are also active as inhibitors of colony formation by hemopoietic cells (40, 41). In addition, in vivo, MIG and IP-10 inhibit neovascularization and exert antitumor effects in mouse models (42–46). MIG, I-TAC, and IP-10 are strongly induced by IFN-γ in a range of cell types, including monocytes, keratinocytes, endothelial cells, and astrocytes (4, 5).

We have recently shown that human neutrophils produce IP-10 (20). In this work, we demonstrate for the first time that neutrophils are also able to release MIG and to express I-TAC mRNA, but only under specific conditions. Indeed, extracellular release of MIG in substantial amounts was found to require costimulation of PMN with IFN-γ along with either LPS or TNF-α. IFN-γ plus TNF-α reproducibly represented the most effective combination for this response. Similar data were obtained with endothelial cells (37, 47). Strikingly, IFN-γ alone, which represents the classical trigger of MIG production in other cell types (5), proved to be ineffective toward the extracellular release of MIG by neutrophils. Other mediators, including GM-CSF, IL-1β, IL-8, and fMLP all failed to induce MIG gene expression and extracellular release by neutrophils, regardless of the presence of IFN-γ or IFN-α in the culture medium. Opsonized particles (Y-IgG) used in combination with IFN-γ, which are known to induce high amounts of TNF-α production (29), had no effect on the induction of MIG release (our unpublished observations), thus making it unlikely that the effects observed with IFN-γ plus LPS were mediated by the endogenous TNF-α (29).

We could only investigate the gene expression of I-TAC, due to the availability of only the cDNA. Our experiments showed that I-TAC mRNA is expressed in neutrophils if they are cultured with IFN-γ in association with either LPS or TNF-α. No other stimulatory conditions were found effective in inducing I-TAC mRNA expression in neutrophils. Kinetic studies of I-TAC mRNA expression in neutrophils treated with IFN-γ plus LPS or TNF-α

revealed that, similarly to MIG transcripts, maximal levels of I-TAC transcripts occurred at 21 h after stimulation. However, differently from MIG and IP-10 mRNA, I-TAC gene expression was not induced if neutrophils were stimulated under serum-free medium conditions and cultured in FBS-precoated flasks (our unpublished observations).

We have also greatly extended our previous findings on the production of IP-10 by neutrophils (20). We now provide evidence that IL-1β and GM-CSF represent effective costimuli for IP-10 release by IFN-γ-treated neutrophils and that a significant release of IP-10 by neutrophils can be obtained even if cells are stimulated with IFN-α in association with LPS, TNF-α, or IL-1β. The latter data support the ability of type I IFNs to selectively up-regulate IP-10, which was previously reported to occur in monocytes and other cells (34, 48–50). The distinctive patterns of MIG and IP-10 production in response to IFN-γ and IFN-α are understandable considering that the regulatory sequences identified in the MIG and IP-10 promoters are quite different (51, 52). Since also IFN-α, in addition to IL-12, IFN-γ, TGFβ, and hormones, has been reported to regulate Th1 development (53), production of IP-10 by IFN-α-stimulated neutrophils may represent one of the mechanisms that contributes to Th1 responses essential for clearance of those pathogens such as viruses.

Interestingly, the kinetics of IP-10 mRNA expression in, and release by, IFN-γ or IFN-α plus LPS- or TNF-α-treated PMN were faster than those of MIG mRNA expression and release, or I-TAC mRNA expression. Furthermore, neutrophils released much higher amounts of IP-10 than MIG under those conditions. The significance of a sequential regulated production of IP-10 and MIG (and likely I-TAC) by neutrophils is only a matter of speculation, but a similar pattern has been found in other cell types, for instance, endothelial cells and mouse macrophages (37, 47, 54).

Another novel observation that we report in this study is that also eosinophils have the capacity to produce IP-10 but not MIG. However, differently from neutrophils, eosinophils were observed to release significant levels of IP-10 only in response to IFN-γ plus TNF-α. The failure of IFN-γ plus LPS to induce IP-10 release in eosinophils is consistent with the poor ability of LPS to affect selected eosinophil functions, likely due to the lack of CD14 expression on these cells (55). Furthermore, we highlighted striking differences between PBMC and PMN with respect to their ability to express or produce MIG, I-TAC, or IP-10. In fact, although in PBMC the most potent stimulatory combination for both mRNA accumulation and extracellular production of MIG, I-TAC, and IP-10 consisted of IFN-γ plus TNF-α, IFN-γ alone represented a highly effective stimulus. IFN-γ was so powerful that its association with LPS led to a diminished mRNA expression of MIG, I-TAC, and IP-10 mRNA, and MIG and IP-10 production, relative to PBMC treated with IFN-γ alone. Taken together, the latter findings clearly indicate that the mechanisms governing the expression of MIG, I-TAC, and IP-10 in neutrophils, eosinophils, and PBMC are specific and subjected to distinct regulatory pathways. Furthermore, and not of lesser importance, they demonstrate that the results obtained in neutrophils with respect to MIG, I-TAC, and IP-10 expression cannot be attributed to a contamination of the PMN populations with eosinophils or mononuclear cells.

Numerous studies have established that the production of proinflammatory cytokines and chemokines by neutrophils can be markedly modulated by immunoregulatory polypeptides such as IL-10 (23, 29) or IL-4 (31, 56). Herein, we show that both IL-10 and IL-4 negatively control MIG, I-TAC, and IP-10 mRNA expression and release in neutrophils, IL-10 being much more suppressive than IL-4. Inhibition of chemokine expression and release by IL-10 was more pronounced on IFN-γ plus LPS-treated neutrophils than on IFN-γ plus TNF-α-treated cells, in agreement with the powerful capacity of IL-10 to negatively influence the effects of endotoxin (57). We did not investigate further the molecular mechanisms whereby IL-10 down-regulates chemokine production in neutrophils, but we clearly established that IL-10 does not affect the strong protective effects of IFN-γ plus either LPS or TNF-α on the rates of neutrophil apoptosis. These findings completely exclude the possibility that the suppression of MIG, IP-10, and other cytokine release (29) depends on an induced decrease of neutrophil survival by IL-10. On the other hand, our observations are in contrast with those by Keel and colleagues (33), who reported that the inhibitory effect of LPS, TNF-α, IFN-γ, G-CSF, and GM-CSF on the spontaneous apoptosis of cultured neutrophils was significantly decreased by coincubation with IL-10. At present, we have no explanation for the discrepancies between our results and those of Keel and coworkers. Although the assay performed to quantify neutrophil apoptosis was the same in both laboratories, it cannot be excluded that other different experimental conditions used to isolate, to lyse, and to culture neutrophils might affect their responsiveness to IL-10.

In addition to the numerous descriptive findings outlined above, we were also able to provide clear evidence for a functional significance of neutrophil-derived IP-10 and MIG. Indeed, we show that supernatants harvested from stimulated PMN, as well as from stimulated PBMC, exert chemotactic activities toward CXCR3-expressing lymphocytes. These effects were partially neutralized by anti-MIG and anti-IP-10 Abs, consistent with the presence of IP-10 and MIG in leukocyte-derived supernatants, and in agreement with their capability to induce migration of CXCR3-positive cells (4–6, 28). However, since neutralization of MIG and IP-10 did not completely abolish cell migration, other CXCR3 ligands, probably present in leukocyte-derived supernatants, are likely to contribute to the observed chemotactic activities. According to our gene expression data, one obvious candidate may be I-TAC. We also demonstrate that supernatants from stimulated leukocytes trigger rapid integrin-dependent adhesion of PHA/IL-2-activated T lymphocytes to fibronectin. Rapid integrin triggering is a fundamental step in the overall regulation of leukocyte diapedesis, under physiological as well as pathological conditions. Chemokines are likely to be physiological activators of integrin-dependent adhesion under flow and to direct lymphocyte extravasation and specific microenvironmental targeting in lymphoid tissues. In agreement with very recent findings (37), recombinant IP-10 and MIG were able by themselves to trigger maximal lymphocyte adhesion to immobilized integrin ligands. Interestingly, neutralizing Abs toward either MIG or IP-10 reduced by more than 90% lymphocyte adhesion triggered by supernatants from stimulated leukocytes. This could suggest a threshold effect whereby MIG, IP-10, and likely other CXCR3 ligands (I-TAC?) can cooperate to trigger the required signal leading to integrin activation. Whatever is the case, to our knowledge, these are the first demonstrations of relevant physiological processes attributed to neutrophil-derived chemokines. It may therefore be postulated that neutrophil-derived MIG and IP-10 are important in diapedesis and recruitment of immunocompetent T lymphocytes to sites of inflammation and disease.

In conclusion, the ability of PMN to produce MIG, IP-10, and likely I-TAC might be significant considering the various biological functions that these three chemokines possess. Generation of MIG, IP-10, and TAC by PMN may, for instance, contribute to recruit Th1 lymphocytes to sites of inflammation. Therefore, along with the well-known capacity of neutrophils to release IL-8, MIP-1$\alpha\beta$, MIP-2, GROα, and cytokine-induced neutrophil chemoattractants (CINCs) (29), our data reinforce the concept that neutrophils have the potential to regulate the migration of various

leukocytic cellular types into inflammatory sites. Alternatively, neutrophil-derived MIG, IP-10, or I-TAC might negatively regulate hemopoiesis (40, 41). Production of MIG, IP-10, and I-TAC by neutrophils may also represent one of the mechanisms whereby these cells exert antitumor effects (58), in light of the well-established angiostatic properties of IP-10 and MIG (42–46). Granulocytes can mediate tumor cell killing through direct or bystander effects and can participate in the cross-talk with CD8 T cells, which has been demonstrated to be instrumental in the rejection of specific cytokine-transduced tumors (59, 60). Interestingly, such crosstalk was sustained by CD8 cell-produced IFN-γ (59). Although it is still too early to speculate on an eventual in vivo role of neutrophil-derived MIG, I-TAC, or IP-10, the initial question that must be answered is under which circumstances neutrophils uniquely produce these factors, considering that their in vitro activities are so overlapping if not identical. In any case, the selective activity of MIG, I-TAC, and IP-10 on activated T cells and probably NK cells is consistent with a role in regulating the trafficking and/or function of effector cells during an immune response.

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