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Human neutrophils produce interferon gamma upon stimulation by interleukin-12

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Interferon γ (IFNγ) is a Th1 cytokine mainly produced by T cells, NK cells and macrophages in response to interleukin (IL)-12. As polymorphonuclear neutrophils (PMN) have been shown to produce and to release numerous cytokines, in particular upon IL-12 stimulation, we investigated the ability of highly purified PMN to secrete IFNγ. We found that PMN contained a small store of IFNγ, and that this store was rapidly secreted upon stimulation by degranulating agents such as formyl peptides. Moreover, after a few hours of stimulation with appropriate agents, PMN synthesized IFNγ. The effect of IL-12 was time- and concentration-dependent, and IL-12 combinations with IL-2, IL-15, IL-18 or LPS were highly synergistic. Cycloheximide inhibited IFNγ release in such optimal conditions, confirming the ability of PMN to synthesize IFNγ. IFNγ synthesis was associated with an increase in specific mRNA content, pointing to a transcriptional mechanism. The IFNγ produced by PMN was biologically active, as demonstrated by its ability to induce TNFα synthesis by PMN themselves or to induce IL-10 synthesis by peripheral blood mononuclear cells. These findings reveal a novel pathway of autocrine and paracrine PMN activation. They also identified a new role for IFNγ, bridging innate and adaptive immune responses.
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During the last decade, numerous studies have evidenced that polymorphonuclear neutrophils (PMN) can release a wide array of cytokines bringing about the definition of new roles of PMN in pathophysiology. PMN are major cytokine sources in humans.[1,2] Through a variety of mechanisms, PMN are able to release granular stores of preformed cytokines such as oncostatin M (OSM) and hepatocyte growth factor (HGF)[3,4] and to synthesize *de novo* other cytokines such as interleukin (IL)-8, IL-12, tumor necrosis factor (TNF)α and IL-1 receptor antagonist (IL-1RA).[1] Thus, in addition to their phagocytic and killer functions, PMN can participate in anti-inflammatory responses via IL-RA, angiogenesis and tissue repair via HGF and vascular endothelial growth factor (VEGF)[1,5] B-cell homeostasis via BLys,[6] and Th2 pathway regulation via IL-4.[7] Relatively little information is available on PMN secretion of Th1 cytokines. Here, we investigated whether PMN can synthesize and release interferon γ (IFNγ).

IFNγ is the principal cytokine produced during Th1-type immune responses, in response to IL-12. The cells involved in IFNγ production include NK cells,[8] T cells,[9] macrophages,[10] B cells[11] and eosinophils.[12] It has also been suggested that PMN may synthesize IFNγ. Indeed, IFNγ-positive PMN have been observed in human endometrium,[13] and also in lung and spleen tissue of two different murine models of infection.[14,15] Yeaman *et al*[13] found that isolated PMN released IFNγ after IL-12 + TNFα stimulation *in vitro*, while other investigators failed to detect IFNγ after lipopolysaccharide (LPS) stimulation.[13]

We have previously obtained some evidence that IL-12-induced IL-8 production by PMN is dependent on endogenously produced IFNγ.[16] Here, we examined whether PMN contain preformed stores of IFNγ, and whether they are able to synthesize IFNγ *de novo*. We found that highly purified PMN contain a small preformed stock of IFNγ, and that they can also synthesize and release bioactive IFNγ after stimulation with IL-12 alone or, in a highly

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synergistic manner, in combination with LPS, IL-12, IL-15 or IL-18. This *de novo* synthesis appears to be regulated at least in part by a transcriptional mechanism. These data point to the existence of a new autocrine regulation loop in PMN, involving IL-12 and IFNγ, suggesting that PMN can influence the immune response towards a Th1 phenotype.

Materials and methods

Isolation and Purification of Human Blood PMN

PMN were purified from venous blood of healthy volunteers using a three-step procedure developed in our laboratory.[4,5,16–18] Briefly, leukocytes were isolated in endotoxin-free conditions by sedimentation on a separating medium containing 9% Dextran T-500® (Pharmacia, Uppsala, Sweden) and 38% Radioselectan® (Schering, Lys-lez-Lannoy, France). After red cell sedimentation, the leukocyte-rich suspension was centrifuged on a Ficoll-Paque® density gradient (Sigma, St Louis, MO, USA). Contaminating erythrocytes were removed by hypotonic lysis. To further purify PMN, monocytes, B lymphocytes and activated T lymphocytes were removed by 30-min incubation with pan-anti-human HLA class II-coated magnetic beads (Dynabeads M-450, Dynal AS, Oslo, Norway). As previously described,[4] CD3+ and CD19+ cells were undetectable by flow cytometry (FACScan, Becton-Dickinson, San Jose, CA, USA); we also showed the absence of CD56+ cells, confirming the recovery of highly purified PMN free of NK cells and T lymphocytes.

PMN Culture

Purified PMN were resuspended in RPMI 1640 culture medium (Bio Whittaker, Gagny, France) supplemented with 10% heat-inactivated fetal calf serum (FCS, Bio Whittaker), L-glutamine (2 mmol/ml), penicillin (100 IU/ml) and streptomycin (100 μg/ml), and 2×10^6 cells/ml were cultured for up to 48 h at 37°C with 5% CO_2 and increasing concentrations of IL-12 (1–100 ng/ml, R&D Systems Abingdon-Oxon, UK) alone or combined with 100 ng/ml LPS derived from *Escherichia coli* (055:B5, Sigma, St Louis, MO, USA). The effect of IL-12 (10 ng/ml) was compared with that of other stimulating agents, including IL-2 (10 ng/ml), IL-15 (20 ng/ml), IL-18 (20 ng/ml), GM-CSF (5 ng/ml) and TNFα (10 ng/ml) (R&D Systems), alone or combined, in the presence or absence of LPS (100 ng/ml). In some experiments, PMN were preincubated with 1 μg/ml cycloheximide (CHX, Sigma) for 30 min at 37°C and then incubated with LPS (100 ng/ml) in the presence of IL-12 (10 ng/ml) and IL-15 (20 ng/ml) for 24 h at 37°C. At the end of the culture period, cell-free supernatants were stored at −70°C until IFNγ assay. Cell viability was confirmed by trypan blue exclusion as previously described.[5]

Enzyme-Linked Immunospot (ELISpot) Assay

ELISpot assay (R&D) was used to confirm the ability of PMN to produce IFNγ. We adapted the method recently described by Chen *et al*,[19] allowing the visualization of IFNγ-secreting cells. Briefly, highly purified PMN (5×10^6/ml) were cultured for 24 h in microplates coated with a monoclonal capture antibody specific for human IFNγ. Autologous peripheral blood mononuclear cells (PBMC) (0.5×10^6/ml) obtained by Ficoll-Paque® density-gradient separation served as positive controls. Both cell preparations (PMN and PBMC) were stimulated as described above, with LPS, IL-12, IL-2, IL-15 and IL-18, alone or in combination. During incubation, IFNγ released by individual cells binds to the coating antibody. The plates are then washed and incubated at 4°C overnight with a biotinylated polyclonal antibody specific for human IFNγ. After washing, alkaline phosphatase-conjugated streptavidin is added for 2 h at room temperature before adding the substrate solution (BCIP/NBT). Blue-black spots of precipitate, representing individual IFNγ-secreting cells, are counted using an inverted microscope. All experiments were performed in triplicate.

Degranulation Experiments

Purified PMN (10^7/ml) were resuspended in Hanks' balanced salt solution (HBSS with Ca^{2+}/Mg^{2+}; Life Technologies, Cergy-Pontoise, France). Part of the cell suspension (unstimulated control PMN) was immediately centrifuged for 10 min at 4°C. Another part was kept for 10 min at 37°C, then preincubated at 37°C for 5 min with 5 μg/ml cytochalasin B (Sigma) to ensure total degranulation, prior to stimulated with 10^{-6} M *N*-formyl methionyl-leucyl-phenylalanine (fMLP; Sigma) for 10 min. In other experiments, PMN were preincubated for 15 min with LPS + IL-15, then stimulated with IL-12 for 10 min at 37°C. Cell-free supernatants were collected and the cell pellets were sonicated for 30 s to measure cell-associated IFNγ. Supernatants and cell pellets were stored at −70°C until IFNγ assay.

Western Blot Analysis

Total homogenates of LPS + IL-12-treated and -untreated PMN or recombinant human IFNγ (rh IFNγ, R&D) were added to $2 \times$ Laemmli sample buffer. Proteins were resolved by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were immunoblotted after transfer to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Nonspecific sites were blocked

by incubation for 1 h in 5% nonfat dry milk, and the membranes were then probed with a mixture (1:500) of a monoclonal anti-IFNγ antibody and then a horseradish-peroxide-labelled goat anti-mouse antibody (1:5000). The immunoblots were developed with an enhanced chemiluminescence method (Amersham, Pharmacia Biotech), following the manufacturer's instructions.

IFNγ mRNA Expression

Highly purified PMN (7×10^7) were incubated for 1 h in culture medium with LPS (100 ng/ml), IL-12 (10 ng/ml) and IL-15 (20 ng/ml). In some experiments, PMN were preincubated for 15 min with 5 μg/ml actinomycin D (Sigma) to block transcription. Total cellular RNA was isolated with RNA-B® (Bioprobe systems, Montreuil-sous-Bois, France) according to the manufacturer's instructions. Briefly, cells were lysed in guanidium thiocyanate and RNA was extracted with chloroform then precipitated with isopropanol and washed with 75% ethanol. The precipitate was solubilized in water and the RNA concentration was determined spectrophotometrically at 260 nm. An amount of 1 μg of total RNA was analyzed by electrophoresis on 1% agarose–formaldehyde gel to check RNA purity and integrity. A measure of 1 μg of total RNA was reverse transcribed in a thermocycler (Uno II, Biometra, Voisins le Bretonneux, France) 1 h at 42°C with superscript II reverse transcriptase (Invitrogen), 20 mM of each desoxyribonucleoside triphosphate (dNTP) and 50 U random hexanucleotides primers (Invitrogen). Specific amplifications of IFNγ and β-actin as a housekeeping gene, were performed in a final volume of 25 μl containing $MgCl_2$ 0.5 μM for IFNγ and 1 μM for β-actin; 200 μM of each dNTP; 12 pmol of each specific oligonucleotide primer pair. The sequence of primer pairs used for amplification of complementary DNAs (cDNAs) is as follows:

IFNγ: 5′ CGA GAT GAC TTC GAA AAG CTG ACT
       5′CCT TTT TCG CTT CCC TGT TTT A
β-Actin: 5′GGA CTT CGA GCA AGA GAT GG
          5′AGC ACT GTG TTG GCG TAC AG

The mixture was heated at 94°C for 5 min, then 2.5 U *Taq* Polymerase (Roche Diagnostics, Meylan, France) were added. polymerase chain reaction (PCR) was performed as follows: denaturation, 94°C for 30 s; annealing, 30 s at 55°C for IFNγ and 60°C for β-actin; extension, 72°C for 40 s. Amplification was stopped after 35 and 30 cycles for IFNγ and β-actin respectively. The expected PCR products of 130 and 234 bp for IFNγ and β-actin, respectively, were detected by electrophoresis in 2% agarose containing ethidium bromide, along with molecular weight standards, positive and negative controls of PCR. The specificity for IFNγ of the amplified sequence was checked using the restriction enzymes

*Fnu*4HI (Ozyme Biolabs, Saint Quentin en Yvelines, France). Signal intensity was quantified under ultraviolet light with charge-coupled device (CCD) camera using an image analyser (Gel-Analyst, Iconix, Santa Monica, CA, USA) and the expression of IFNγ mRNA was expressed as the ratio of the β-actin gene.

Biological Activity of PMN-Derived IFNγ

The biological activity of PMN-derived IFNγ was tested on two different cell type targets. In the first autocrine model, IFNγ was tested by its capacity to induce TNFα production by LPS-stimulated PMN.[16] In the second model, IFNγ bioactivity was tested by its ability to induce IL-10 production by LPS-stimulated PBMC.[20,21] PMN (10^7/ml) or PBMC (0.5×10^6/ml) were cultured with 100 ng/ml LPS, with or without IFNγ-containing PMN culture supernatant from previous experiments. Recombinant human IFNγ (rhIFNγ 250 IU/ml) was used as positive control. Cells were cultured in the presence or absence of anti-IFNγ-neutralizing antibody (1 μg/ml, R&D). After 24 h of culture at 37°C with 5% CO_2, cell-free supernatants were stored at −70°C until TNFα or IL-10 assay.

Cytokine Assays in Cell-Free Supernatants

IFNγ, TNFα and IL-10 were quantified by using enzyme-linked immunosorbent assays (ELISA) (R&D Systems) with respective detection limits of 8, 5 and 5 pg/ml.

Statistical Analysis

Results are expressed as means ± s.e.m. The various conditions of stimulation were compared by using ANOVA, followed by multiple comparison of means with Fisher's least-significance procedure. Paired comparisons were based on Wilcoxon's paired test. P-values < 0.05 were considered statistically significant.

Results

IL-12-Induced IFNγ Production by PMN

After 24 h of culture, IFNγ was not detected in the supernatants of unstimulated PMN or of PMN stimulated with LPS, TNFα, GM-CSF, IL-2, IL-15 or IL-18 alone (data not shown). By contrast, as shown in Figure 1, IL-12 alone stimulated IFNγ production, in a concentration-dependent manner, reaching a plateau after 50 ng/ml. LPS further enhanced IFNγ release induced by IL-12. As shown in Figure 2, IFNγ release was also enhanced by IL-2, IL-15 and IL-18, the IL-12 + IL-15 combination being most synergistic. LPS further enhanced the effect of IL-2.
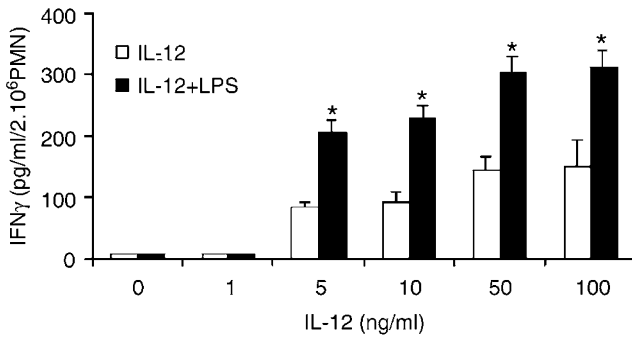
Figure 1 Concentration–response effect of IL-12 on IFNγ production by PMN. PMN (2×10^6/ml) were incubated for 24 h with increasing concentrations of IL-12, either alone or with LPS (100 ng/ml). After 24 h, IFNγ was assayed by ELISA in cell-free supernatants. Results are means±s.e.m. of three independent experiments. *$P < 0.05$ vs cells incubated with IL-12 alone.
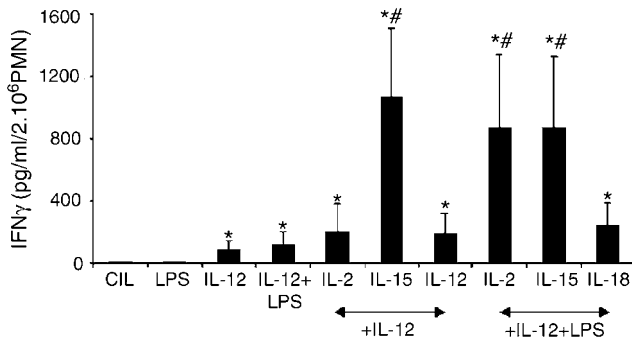

Figure 2 Comparative effects of IL-2, IL-15 and IL-18 on IL-12-induced IFNγ production by PMN. PMN (2×10^6/ml) were incubated for 24 h with various stimuli or in complete medium alone (CTL). IFNγ was assayed by ELISA in cell-free supernatants. Results are means±s.e.m. of 10 independent experiments. *$P < 0.05$ vs LPS or control. #$P < 0.05$ vs IL-12 alone.

Regulation of IFNγ Production by Stimulated PMN

To investigate the mechanism of PMN IFNγ release during 24-h culture, cells were preincubated with CHX prior to optimal stimulation (LPS+

Table 1 Time course of IFNγ release by PMN (pg/ml/ 2×10^6 PMN)

Duration of stimulation (h)	IL-12	LPS+IL-12+IL-15
2	10±7	17±9
6	39±14	187±62
12	95±36	535±181
24	127±49	872±423
48	148±38	1005±340

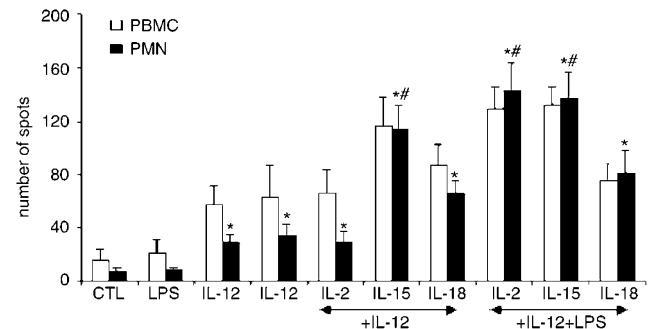Results are expressed as the means±s.e.m. of three experiments.

Figure 3 IFNγ release by individual PMN in various conditions of stimulation. IFNγ-secreting cells were visualized by ELISpot in three independent experiments. Autologous PBMC served as positive controls. Cells were stimulated with the indicated agonists. Triplicates were measured in each case and means±s.e.m. were calculated. *$P < 0.05$ vs resting cells. #$P < 0.05$ vs IL-12 alone.

Table 2 Effect of CHX on stimulus-induced IFNγ secretion by PMN

	Without CHX pretreatment	With CHX pretreatment
Control PMN	8±5	8±4
LPS+IL-12+IL-15	872±423	10±5*

PMN (2×10^6/ml) were preincubated with or without CHX and then stimulated for 24 h at 37°C in the presence of LPS+IL-12+IL-15 or in medium alone. IFNγ was assayed in the cell-free supernatants. Results are expressed as the means±s.e.m. of three experiments. *$P < 0.05$ as compared with cells incubated without CHX pretreatment.

IL-12+IL-15), in order to block protein synthesis. As shown in Table 2, the weak IFNγ release by unstimulated PMN was not affected by CHX pretreatment. In contrast, after LPS+IL-12+IL-15 stimulation, IFNγ release was significantly reduced by CHX pretreatment as compared with untreated cells, confirming the ability of PMN to synthesize IFNγ de novo upon stimulation.

The regulation of PMN IFNγ production was also studied at the mRNA level. As shown in Figure 4, IFNγ mRNA was low after 1 h in control PMN, whereas LPS+IL-12+IL-15-stimultated PMN

Time course study of IFNγ release by PMN showed that IFNγ was similarly detectable as soon as 2 h of culture upon stimulation with IL-12 alone or associated with LPS and IL-15 (Table 1). These IFNγ amounts reached a plateau by 24 h, and gradually accumulated for up to 48 h of culture (Table 1).

The central role of IL-12 in IFNγ production by PMN was confirmed by single-cell ELISpot assay. Indeed, as shown in Figure 3, the number of spots was optimal using the various associations of IL-12, IL-15, IL-18 and LPS. Positive control cells consisted of autologous PBMC cultured in similar conditions; as expected, IL-12 combined with IL-15 or IL-2 was also the most potent stimulus for IFNγ release by PBMC (Figure 3).
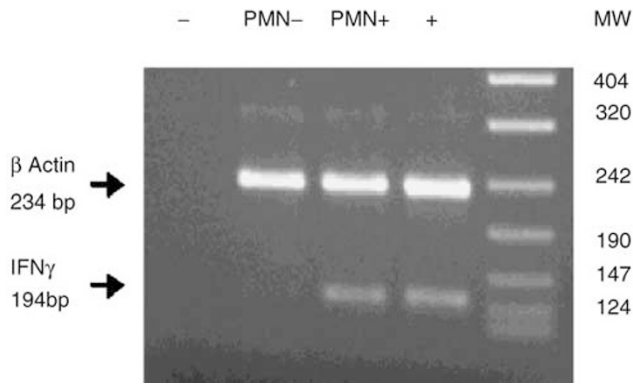
Figure 4 RT-PCR analysis of IFNγ mRNA expression in PMN. Total RNA was extracted from resting (PMN−) and activated PMN (LPS + IL-12 + IL-15) (PMN +). After reverse transcription, PCR was carried out with specific pairs of primers designed for IFNγ and β-actin. Total lung tissue served as positive control (+). The figure indicates the size of amplification products relative to molecular weight standards run in parallel (MW) and the negative control (−) (PCR buffer without cDNA).

Table 3 Effect of degranulating agents on IFNγ secretion by human PMN

	Cell-associated	Secreted
Control 4°C	33 ± 17	8 ± 5
Control 37°C	19 ± 4	11 ± 9
Cyt B+fMLP	$8 \pm 5*$	18 ± 7
LPS+IL-12+IL-15	$10 \pm 4*$	20 ± 7

Secreted IFNγ was measured in cell lysates and supernatants of of PMN (10^7/ml) maintained at 4°C or incubated for 15 min at 37°C with or without inducers of degranulation. Results are expressed as the mean ± s.e.m. of six independent experiments. *$P < 0.05$ *vs* control PMN at 4°C.

exhibited a 450% increase in IFNγ mRNA expression measured by densitometric analysis and expressed as a ratio to β-actin.

Taken together, these data suggest that regulation of the PMN IFNγ gene, in optimal conditions of stimulation, might take place, at least in part, at the transcriptional level.

Human PMN Contain a Small Intracellular Pool of IFNγ

Two complementary techniques were used to determine whether IFNγ is constitutively present in resting human blood PMN, namely degranulation, and Western blotting. Degranulation experiments were conducted with purified PMN maintained at 4°C, and with or without inducers of degranulation, for 15 min at 37°C. Released and cell-associated IFNγ were measured separately. As shown in Table 3, the amount of cell-associated IFNγ was 33 ± 17 pg/10^7 PMN in basal conditions. Incubation at 37°C in both
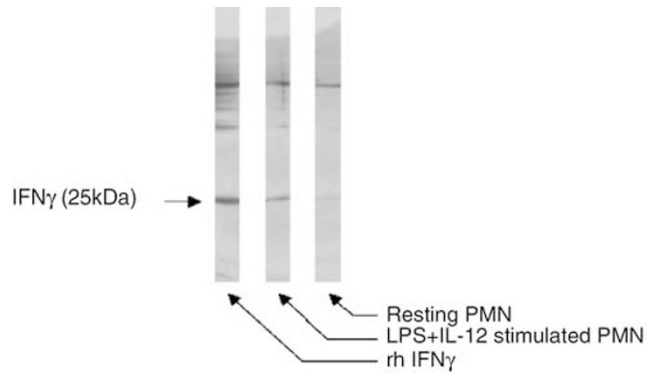

Figure 5 Western blot analysis of IFNγ expression by human PMN. Total homogenates of resting purified PMN or LPS + IL-12-treated PMN were added to $2 \times$ Laemmli sample buffer, and proteins were resolved by SDS-PAGE. Proteins were immuno-blotted after transfer to nitrocellulose membranes. Recombinant human IFNγ (rh IFNγ) served as positive control.

degranulating conditions led to a reduction in cell-associated IFNγ, with a parallel increase in extra-cellular IFNγ. These results suggested that a small pre-existing pool of IFNγ was rapidly released. Western blot analysis of total PMN homogenates both stimulated and unstimulated revealed a clear 25-kDa band migrating at the same level as recombinant human IFNγ (Figure 5).

IFNγ Bioactivity

The autocrine and paracrine regulation loops of cytokine production are of major importance. We chose to test the PMN-derived IFNγ bioactivity on two different models, using PMN or PBMC as target cells. As IFNγ is necessary to induce TNFα production by PMN, we investigated in the first model the ability of culture supernatants of LPS + IL-12 + IL-15-stimulated PMN to induce TNFα release by PMN treated with LPS alone. We selected three healthy donors, whose PMN culture supernatants contained 1.4, 1.5 and 1.6 ng/ml IFNγ, as measured by ELISA. In the second model, we investigated the ability of these same three supernatants to potentiate IL-10 release by LPS-stimulated PBMC. As shown in Figure 6a and b, rhIFNγ (1.5 ng/ml) and all three PMN culture supernatants stimulated TNFα release by PMN and IL-10 release by PBMC as compared to LPS alone. Neutralizing antibodies against IFNγ partially inhibited both cytokine productions, suggesting that PMN-derived IFNγ was biologically active.

Discussion

Our results suggest that a small pre-existing pool of IFNγ is present in resting PMN, and that it is rapidly released in degranulating conditions. IFNγ was also synthesized after PMN stimulation by various
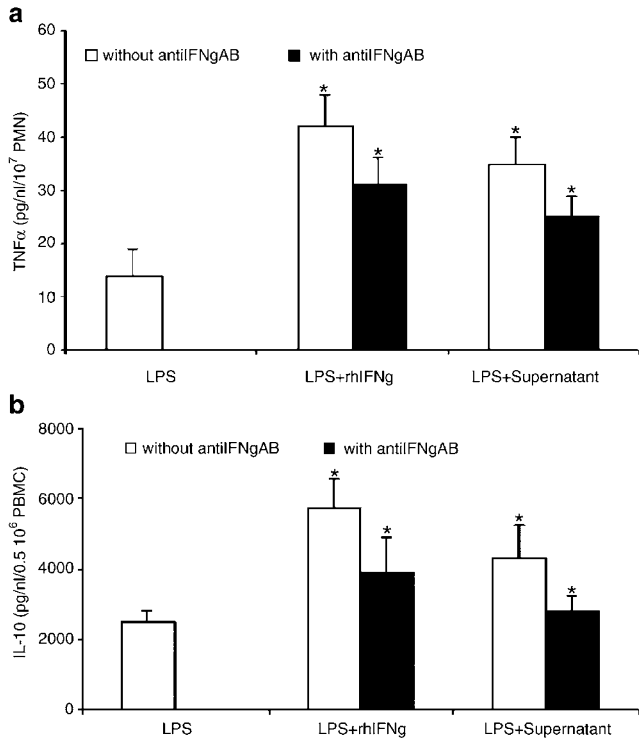
a

b

Figure 6 Biological activity of neutrophil-derived IFNγ. PMN (10^7/ml) (**a**) or PBMC (0.5×10^6/ml) (**b**) were cultured in the presence of 100 ng/ml LPS, with or without PMN culture supernatants containing IFNγ (obtained in previous experiments). Recombinant human IFNγ (rhIFNγ 250 IU/ml) was used as a positive control. Each culture was done in the presence or absence of anti-IFNγ neutralizing antibody (1 μg/ml). Cell-free supernatants were harvested after 24 h of culture at 37°C in 5% CO_2, and were stored at $-70°C$ until TNFα and IL-10 assays. *$P < 0.05$ vs LPS.

agonists, the combination of IL-12 and IL-15 being the most efficient. A transcriptional regulation of the IFNγ gene in PMN was suggested. PMN-derived IFNγ could orient adaptive immune responses, particular at sites of inflammation.

We have developed a three-step isolation procedure to rule out PMN contamination by other cell types, based on Dextran-Radioselectan sedimentation, Ficoll centrifugation, and immunomagnetic depletion of HLA class II-positive cells (particularly monocytes and activated T lymphocytes, which can release IFNγ). As previously described by our group, the purity of the PMN preparations was confirmed by several controls: flow cytometry showed neither CD3+ nor CD56+ cells (T cells and NK cells, respectively);[4] nonspecific esterase staining always evidenced less than 0.1% of monocytes;[17] neither IL-10 or IL-13 protein or mRNA could be induced.[18] Eosinophils were not involved in the observed IFNγ release, as these cells require CD28 ligation to release IFNγ.[12]

Western blot analysis revealed a band corresponding to IFNγ in resting PMN. Immunocytochemistry was also performed but did not allow to visualize

significant level of intracellular IFNγ in resting cells (data not shown). The existence of a small intracellular store of IFNγ in PMN was confirmed by IFNγ release into the extracellular medium after 15 min of incubation with degranulating agents. Although this IFNγ pool was small compared with the amount of synthesized after 24 h of culture, it may have an important role, as rapid IFNγ secretion by the numerous PMN infiltrating inflammatory tissues could orient the local immune response at an early stage. Other preformed cytokines released rapidly by PMN after exposure to degranulating agents include HGF, OSM and VEGF.[3,4,22] Our results are in keeping with previous flow cytometry-based studies showing that IFNγ is barely detectable in unstimulated PMN from human blood,[13] and from normal mouse spleen and lung.[14,15]

Our findings also show that PMN can synthesize significant amounts of IFNγ as soon as 2 h of appropriate ex vivo stimulation. We used two complementary techniques to visualize and quantify newly produced IFNγ. First, IFNγ was detected by ELISA in PMN culture supernatants after exposure to appropriate stimuli. Second, the number of ELISPOT spots increased markedly after stimulation. Several stimuli classically described to upregulate cytokine production by PMN, such as LPS, TNFα and GM-CSF[1] were ineffective when used alone. Similar findings were obtained by Keel et al[23] with LPS alone, whereas Yeaman et al[13] detected low concentrations of IFNγ after TNFα or LPS stimulation; this discrepancy could be related to differences in cell preparation and purification.

IL-12 was the most efficient stimulus after 24 h of culture, upregulating IFNγ production in a time- and concentration-dependent manner. Interestingly, IL-12 was synergistic with IL-2, IL-15 or IL-18; in particular, IL-15 potentiated IL-12-induced IFNγ release by a factor of 10. These four cytokines have already been shown to influence other PMN functions.[24] IL-12 is chemotactic for PMN, and also activates IL-8 and TNFα synthesis.[25,16] IL-15 plays a role in maintaining inflammatory processes, by increasing phagocytosis, inducing cytoskeleton changes, delaying apoptosis and increasing chemokine production.[26–28] IL-2 shares with IL-15 many biological effects on PMN;[27] the underlying mechanisms include association of lyn protein tyrosine kinase with IL-2Rβ, and direct binding of MAPK/ERK1 to lyn and a proteolytically processed full-length STAT5 protein.[29,30] IL-18 can activate PMN, by priming NADPH-oxidase, increasing β2 integrin expression, activating p38-MAPK, and driving the production of leukotriene B4.[31,32] IL-12 in combination with IL-18 or IL-15 has been reported to increase synergistically IFNγ production by various cells. For example, IL-12 and IL-15 synergize to induce murine NK cell IFNγ release.[8] IL-12 and IL-18 also exhibit marked synergism for IFNγ induction

by T cells,[9] macrophages,[10] dendritic cells and B cells;[11] the STAT4 and p38 MAPK pathways are both involved in these effect.[33] Our study demonstrates for the first time that, upon combined stimulation with IL-12, IL-15, IL-18 or IL-2, human PMN can also participate in IFNγ production. Our *in vitro* findings confirm the results of two recent *in vivo* studies[14,15] showing the involvement of lung- and spleen-infiltrating PMN in IFNγ production in two mouse models of infection (*Nocardia asteroides* and *Salmonella typhimurium*).

To elucidate the mechanism of IFNγ release by PMN, we pretreated cells with CHX prior to optimal stimulation. IFNγ release fell markedly, confirming that *de novo* protein synthesis was the main source of PMN-derived IFNγ. Moreover, specific mRNA content correlated with IFNγ protein release. RT-PCR studies showed that IFNγ mRNA expression by stimulated PMN was largely increased as early as 1 h. A similar regulatory mechanism has already been demonstrated by us and others for several cytokines such as IL-8[1,5] or OSM.[17]

The IFNγ produced by PMN was biologically active as tested by two different models. Indeed, stimulated PMN supernatants upregulated TNFα production by LPS-stimulated PMN, which is known to be IFNγ-dependent,[1,5] and IL-10 production by LPS-stimulated PBMC.[20,21] Anti-IFNγ antibodies partly inhibited this TNFα or IL-10 production since other mediators are involved in their synthesis. We chose these two models because they represent some of the major biological and functional implications for these results. The ability of PMN to produce the powerful activating cytokine IFNγ points to a new role for these cells during innate immune responses. Specifically, they suggest the existence of a new autocrine modulation loop, in which IFNγ-derived PMN might play a key role. Indeed, at sites of local inflammation, IFNγ-derived PMN could regulate several neutrophil functions, such as the oxidative burst, migration, apoptosis and cytokine production as recently reviewed.[1,33–36] Moreover, PMN-derived IFNγ and IL-12 could influence the adaptive immune response towards T-helper polarization, both locally and in the systemic circulation.[37] This would offers a new explanation for the crucial importance of IFNγ in antimicrobial immunity. Inherited disorders of IFNγ- and IL-12-mediated immunity described in recent years (defects in IFNγ receptors 1 and 2, STAT1, IL-12β receptor 1 and IL-12p40) predispose patients to severe infections by intracellular pathogens, and especially mycobacteria.[38,39] Very recently, neutralizing anti-IFNγ autoantibodies were found in a patient with severe *Mycobacteria cheloneae* infection.[40] IFNγ therapy has a beneficial effect on phagocyte functions both *in vivo* and *ex vivo*. IFNγ prophylaxis improved the PMN oxidative burst in two patients with chronic granulomatous disease, by partially correcting the abnormal splicing of NADPH-oxidase CYBB

gene transcripts.[41,42] Also, inhaled IFNγ restored normal pulmonary immune status in patients with severe trauma and immune paralysis.[43] Finally, our data may explain the intriguing role of PMN in antitumor reactions, as reviewed by Di Carlo *et al*.[44] Intratumoral PMN-derived IFNγ might be a key mediator in the cross talk between tumor cells, phagocytes and T cells, thereby orchestrating tumor rejection, particularly after recombinant IL-12 therapy.

In conclusion, our *in vitro* findings confirm recently published observations, in particular in mouse models of infection, by demonstrating that human PMN can secrete IFNγ by a two-step mechanism. First, release of a small preformed stock of IFNγ can be triggered by appropriate stimuli, and this is followed by *de novo* IFNγ synthesis. These stimuli include IL-12, both alone and combined with IL-2, IL-15 or IL-18. The potential new autocrine and paracrine regulatory loop identified in this study points to a novel pathway for cross talk between PMN and other immune cells, particularly at local sites of inflammation. PMN IFNγ release may play a pivotal role, not only during early innate immune responses and antitumoral reactions, but also during the regulation and orientation of adaptive immunity.

Acknowledgement

Duality of Interest

The authors declare no duality of interest.

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