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In vitro activation of mouse neutrophils by recombinant human interferon-gamma: Increased phagocytosis and release of reactive oxygen species and pro-inflammatory cytokines

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ABSTRACT

Here, we investigated the ability of IFN-γ to modulate the functions of mouse neutrophils in vitro. Neutrophils incubated in the presence of IFN-γ showed enhanced phagocytosis in response to zymosan, opsonized zymosan or precipitated immune complexes of IgG and ovalbumin. The effect of IFN-γ was dose-dependent with an initial response at 10 U/ml and a maximal response at 150 U/ml; 2 h of incubation were required to reach the optimal response level. These stimuli can also induce IFN-γ-pretreated neutrophils to release reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide and hypochlorous acid, as well as granule lysosomal enzymes and the pro-inflammatory cytokines TNF-α and IL-6. We found that increased expression of FcγR, dectin-1 and complement receptors (CRs) correlated with these effects in these cells. The enhancing effect of IFN-γ on the respiratory burst was found to be associated with up-regulation of the $gp91^{phox}$ and $p47^{phox}$ subunits of NADPH oxidase, as measured by their mRNA levels. The enhancing effect of IFN-γ on phagocytosis and ROS release may not only be relevant for the efficient killing of invading microorganisms, but may also produce oxidative stress on adjacent cells, resulting in a possible inflammatory role that could also be favored by the liberation of the pro-inflammatory cytokines TNF-α and IL-6.

1. Introduction

Neutrophils are the most abundant nucleated cells in the blood and constitute the first line of defense protecting the host against microbial pathogens. The antimicrobial function of phagocytes partially depends on the generation of superoxide anion (O_2^-) and other highly reactive oxidants derived during the respiratory burst [1]. Formation of O_2^- is catalyzed by a membrane-associated enzyme system, NADPH oxidase, that is dormant in resting cells and becomes activated during phagocytosis or upon interaction of the cells with suitable soluble stimuli [2–4].

Production of O_2^- in response to a stimulant is potentiated by prior treatment of the phagocytes with activating or priming agents, such as interferon-γ (IFN-γ) [3,5]; lipopolysaccharide (LPS); chemotactic molecules, such as formylated peptides; and cytokines, such as granulocyte-macrophage colony-stimulating factor [6]. IFN-γ has recently been produced by recombinant DNA techniques, and its role as a lymphokine participating in immunological responses is being investigated extensively [7–10]. This recombinant IFN-γ provided a new opportunity to characterize the immunomodulatory properties of IFN-γ, because it is free from other lymphokines, including macrophage-activating factor [11–13]. However, the effects of recombinant IFN-γ on neutrophil function are only recently becoming appreciated [14–18]. Cruz et al. [19] suggested that excessive release of IL-17 during repeated mycobacterial exposure leads to neutrophil recruitment and neutrophil-mediated tissue damage in mice. This response appears to be attenuated by IFN-gamma production during a normal antimycobacterial immune response [20], indicating that neutrophil accumulation and the resulting tissue damage may be caused by the failure of the immune system to contain the infectious agents. Schurgers et al. [21] have highlighted the role of IFN-γ in pathogenesis of human Rheumatoid Arthritis (RA) and collagen-induced arthritis (CIA) in mice.

Previous work in human neutrophils showed that a few hours of IFN-γ treatment induces an enhancement of respiratory burst capability that is dependent on mRNA and protein synthesis; furthermore, IFN-γ-treated neutrophils exhibited no alterations in the amount of cytochrome b_{558} or enhanced expression of receptors [22]. In contrast, Steinbeck et al. [23] were unable to show an effect of recombinant bovine IFN-γ on the respiratory burst of bovine neutrophils. Lieser et al. [24] showed that IFN-γ up-regulates human neutrophil oxidative responses to N-formyl-methionyl-leucyl-phenylalanine (FMLP), but not to phorbol myristate acetate (PMA).

Despite the large number of studies on the regulatory activity of IFN-γ on neutrophil functions [25,26], some conflicting results have been reported regarding phagocytosis and the production of oxygen reactive species when using different stimuli [27–29].

In view of the physiological and pathophysiological importance of the regulatory activity of recombinant IFN-γ on neutrophil functions

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and the diverse, sometimes conflicting results reported, we decided to investigate the effects of this human recombinant cytokine on phagocytosis, the production of reactive oxygen species and the release of lysosome enzymes mediated by different types of immune receptors in mouse neutrophils.

2. Experimental procedure

2.1. Chemicals

Superoxide dismutase (SOD), ferricytrochrome C, Percoll, zymosan, horseradish peroxidase (HRP), bovine albumin serum (BSA), sodium pyruvate, penicillin, streptomycin sulfate, RPMI-1640 tissue culture medium, fluorescein isothiocyanate (FITC) and PKH26 Red Fluorescent Cell Linker were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum was acquired from Life technologies (New York, NY, USA), sodium bicarbonate and ethanol from Merck (Darmstadt, Germany) and the gelatin (microbiological grade) was acquired from Difco Laboratories (Detroit, MN, USA). Phosphate buffered saline (PBS) containing 0.9% NaCl and 0.007 M phosphate buffer, pH 7.2 was used. All the other chemicals and solvents used in this work were of analytical grade and purchased from commercial sources.

2.2. Animals

Male 6- to 8-week-old BALB/c mice were obtained from Ribeirão Preto Medical School, University of São Paulo animal center and maintained under a 12 h light:dark cycle with food and water available ad libitum. The mice were sacrificed by decapitation, and blood was collected in heparin tubes. The animal protocol utilized was approved by the ethics committee of the Faculty of Medicine of Ribeirão-USP (Protocol No. 053/2009).

2.3. Preparation of mouse neutrophils

Neutrophil isolation was performed as described by Boxio et al. [30]. Mouse blood was collected in heparin tubes and added to HBSS-EDTA (without calcium, with magnesium, phenol red and sodium bicarbonate, pH 7.2, 15 mM EDTA, 1% bovine serum albumin (BSA)). After centrifugation (400 *g*, 10 min, 4 °C), cells were resuspended in 1 ml HBSS-EDTA. Cells were then layered onto a three-layer Percoll gradient of 78%, 69% and 52% Percoll diluted in HBSS (100% Percoll = nine parts Percoll and one part 10× HBSS) and centrifuged at 1500 *g* for 30 min at room temperature. The density of each Percoll layer was determined according to the manufacturer's instructions (52%, $\delta = 1.083$ g/ml; 69%, $\delta = 1.090$ g/ml; 78%, $\delta = 1.110$ g/ml). Neutrophils were harvested from the 69/78% interface and the upper part of the 78% layer into 1% BSA-coated tubes after careful removal of cells from the upper phases. After one wash with 2 ml HBSS-EDTA + 1% BSA, the remaining red blood cells were eliminated by lysis with 0.83% (w/v) NH_4Cl at pH 7.2 for 5 min. After a final wash with 2 ml HEPES buffer, the cells were suspended in 1 ml of HBSS and used within 4 h.

2.4. Priming neutrophils

Mouse neutrophils were activated with 150 U/ml recombinant human IFN-γ, produced in *Escherichia coli* (Sigma, St Louis MO, USA) for 2 h prior to all experiments.

2.5. Phagocytosis assay

Immune complexes (ICs) were produced by staining goat red blood cells with (GRBCs) PKH26 according to the manufacturer's instructions and incubating them for 30 min at 37 °C with mouse anti-GRBC antibody. The antibody was produced and purified as previously described by Mantovani [31] and incubated at 37 °C for 15 min with complement (IC-C) to promote opsonization. Zymosan (Zy) was resuspended in carbonate buffer with 25 μg/ml FITC for 30 min at 37 °C and incubated with complement (Opzy). Neutrophils (2×10^6 cells/ml) were incubated at 37 °C for 45 min with 500 μl RPMI-1640 (Sigma St Louis MO, USA) medium containing 10% fetal bovine serum and different phagocytic stimuli, including an immune complex of IgG bound to red blood cell-PKH26 (4×10^6), an immune complex of IgG bound to red blood cell-PKH26 opsonized with complement, Zy-FITC (50 μg), or Zy-FITC opsonized with complement (Opzy-50 μg). The neutrophils were incubated with 150 U/ml IFN-γ for 2 h, and the cells were washed with PBS after incubation. Red blood cells from the immune complex bound to neutrophils were lysed by hypotonic shock as described by Mantovani [31]. The fluorescence of internalized particles was measured by flow cytometry (FACSCanto, BD Biosciences) after fluorescence quenching with trypan blue (2 μg/ml) of the Zy-FITC particles bound to the surface of the neutrophils. The results were analyzed using FlowJo® (Tree Star) software and represented as the mean fluorescence intensity (MFI) per neutrophil.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Cytokines in culture supernatants were measured by a sandwich ELISA using DuoSet (R&D Systems, Minneapolis, MN, USA—TNF-α, DY410 and IL-6, DY406); the procedure was carried out according to the manufacturer's instructions. The tetramethylbenzidine (TMB) reagent set (BD Biosciences) was used as the horseradish peroxidase (HRP) substrate, and absorbance was measured at 450 nm.

2.7. Liberation of granules assay

The β-glucuronidase assay was performed according to methods described by Fishman et al. [32]. Neutrophils (2×10^6) were rested or pre-treated with IFN-γ- (150 U/ml for 2 h) and suspended in RPMI-1640 medium incubated for 1 h at 37 °C with 500 mM cytochalasin B. The supernatant was collected after incubation and centrifuged at 730 *g* for 10 min at 4 °C. This supernatant was kept on ice for subsequent enzymatic assays. The assays used were β-glucuronidase and lactate dehydrogenase. For the β-glucuronidase assay, culture supernatants were incubated with sodium acetate buffer (0.12 M; pH 4.5) and 100 μl of phenolphthalein glucuronate (714 mM). This mixture was incubated at 39 °C for 17 h, and glycine buffer (0.48 M; pH 10.4) was subsequently added. This reaction produces a red colored compound that was measured by a spectrophotometer at 540 nm. The alkaline phosphatase assay was performed as described by Linhardt et al. [33]; for this test, culture supernatants were incubated with 0.25 M Ampol solution (2-amino-2-methyl-1-propanol), 100 mM p-nitrophenylphosphate and 1 M magnesium chloride, pH 10. This mixture was incubated at 37 °C for 30 min with shaking. The reaction was stopped by adding 0.8 N NaOH and was measured by a spectrophotometer at 410 nm.

2.8. Release of superoxide by neutrophils

Extracellular O_2^- release by neutrophils was measured using the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome c assay as previously described by Johnston et al. [34]. Neutrophils, either rested or pretreated with IFN-γ (150 U/ml at 2 h), were suspended in Hanks' containing 1% gelatin to prevent the adhesion of neutrophils to the tubes. A mixture of 800 mM ferricytochrome c and Hanks 15 mM HEPES medium, with or without SOD (15 mg/ml), were incubated with 2×10^6 cells/ml for 5 min at 37 °C. An immune complex of IgG and OVA was prepared by incubating 1 mg/ml OVA for 1 h at 37 °C with anti-OVA antibody, which was prepared and purified as described by Lucisano and Mantovani [35]. Zy or Zy opsonized with complement (Opzy) was prepared as described for the phagocytosis

assay. Stimuli were added at a final concentration of 200 μg/ml. The suspension was incubated for 60 min at 37 °C, and the reaction was stopped by placing the tubes in an ice bath. The cells were immediately centrifuged at 730 g for 10 min, and absorbance was measured by a spectrophotometer at a wavelength of 550 nm (Hitachi Instruments, Inc., Minato-Ku, Tokyo, Japan). Tubes containing neutrophils incubated with ferricytochrome c in Hanks HEPES medium plus SOD served as blanks. Duplicate or triplicate measurements were averaged and converted to nanomoles of ferricytochrome c reduced using $\Delta E_{550\ nm} = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$ [36]. Superoxide release was evaluated using rates of oxidant production during stimulation (expressed in nmol/60 min per 2×10^6 cells).

2.9. Hydrogen peroxide determination

Production of H_2O_2 was determined by the oxidation of phenol red by H_2O_2-mediated horseradish peroxidase type II (HRPO) as described by Pick and Keisari [37]. Briefly, neutrophils (2×10^6 cells/ml; rested or pre-treated with IFN-γ- (150 U/ml at 2 h)), were resuspended in assay solution containing 0.56 mM of phenol red and 20 U/ml HRPO in HBSS with phenol red and seeded in tissue culture plates at a final volume of 100 μl per well. The neutrophils were stimulated with the same stimuli used in the assay for release of superoxide. After a 2-h incubation, the reaction was stopped by adding 10 μl of 1 N NaOH per well, and absorbance was measured in a spectrophotometer at a wavelength of 620 nm. Wells with NaOH were used as blanks. The results were expressed as nmol $H_2O_2/2 \times 10^6$ cells, according to the standard curve of H_2O_2 (5–60 μM) established for each experiment.

2.10. Release of hypochlorous acid by neutrophils

The production of HOCl was determined by the oxidation of taurine for HOCl. This reaction produces taurine chloramine (TauCl), which in the presence of iodide oxidizes 3,3′,5,5′-tetramethylbenzidine (TMB) to form a blue compound that is quantified by a spectrophotometer at a wavelength of 630 nm [38]. Neutrophils (2×10^6 cell/ml) that were pre-treated with IFN-γ were incubated with an immune complex IgG and OVA, zymosan or zymosan opsonized with mouse serum and 5 mM taurine for 60 min at 37 °C. The final volume of the reaction was 1 ml. The plate was transferred after the incubation to an ice bath, and the reaction was terminated by addition of 2 mg/ml catalase. The supernatant was collected and centrifuged at 730 *g* for 10 min at 4 °C. Accumulation of TauCl was measured in a 96-well microplate by adding 200 μl of culture supernatant to 50 μl of 2 mM TMB [dissolved in 10% DMF (dimethylformamide), 100 μM NaI and 400 mm acetic acid]. The results were expressed as mM HOCl/2×10^6 cells according to the standard curve TauCl (5–120 μM) established in each experiment. This standard curve was generated by the addition of known concentrations of HOCl to a solution of 5 mM taurine.

2.11. Neutrophil receptor expression

The expression of FcγRII/CD32 and FcγRIII/CD16 receptors, CR3 and dectin-1 was assessed by flow cytometry using the following monoclonal antibodies: anti-CD32/CD16 conjugated with FITC, anti-CD11b conjugated with PE (both 2 μg/ml, BD Biosciences) and anti-dectin-1-rat Alexa Fluor 488 (1:200 dilution, Molecular Probes, Invitrogen, Eugene OR, USA). Staining was performed according to the manufacturer's instructions. Neutrophils (2×10^6 cells/ml) pre-treated with IFN-γ were fixed with 2% paraformaldehyde for 20 min at room temperature. Data acquisition was performed with a FACSCanto, and data were analyzed using FACSDiva software. The results were expressed as the mean fluorescence intensity (MFI).

2.12. RNA isolation

RNA was extracted from mouse neutrophils (2×10^6 cells/ml) using Trizol-LS Reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). RNA concentration was assessed using a BioPhotometer (Eppendorf, Hamburg, Germany), with the acceptable 260/280 absorbance ratio set to 2.0. A ratio of 2.0 indicates that the RNA is pure for all standard molecular biology applications. To ensure that samples were not contaminated with DNA, samples were treated with DNAse Amp Grade (1 U/μl – Invitrogen, Carlsbad CA – USA) according to the manufacturer's protocol.

2.12.1. Analysis of mRNA expression by real time PCR

Total RNA (1.2 mg) from each individual sample was reverse transcribed using the Superscript III Reverse Transcriptase kit (Invitrogen) to generate first-strand cDNA, and cDNA samples were amplified by real-time PCR with SYBR Green PCR Master Mix (AB Applied Biosystems, Warrington, WA, UK). The specific primer sequences are presented in Table 1. Real-time PCR amplification was performed using an Applied Biosystems 7500 Sequence Detection system (Applied Biosystems). The cycling conditions were 95 °C for 5 min followed by 40 cycles of amplification consisting of 95 °C for 12 s, 60 °C for 30 s, and 72 °C for 30 s. The real-time PCR was performed in triplicate for each sample. Relative expression levels were calculated by ΔΔCt (= ΔCt sample − ΔCt of the calibrator) [39]. The data were normalized to the housekeeping gene β-actin, and the values were compared to the neutrophils primed with IFN-γ.

3. Results

3.1. Incubation time and concentration dependence of the effects of IFN-γ on Fc-gamma receptor-mediated phagocytosis

We initially chose an incubation time of 2 h for priming neutrophils based on previous reports in the literature examining the various effects of IFN-γ. Using this condition, we tested the effect of increasing concentrations of IFN-γ on phagocytosis of red cells coated with IgG antibodies (IC). As shown in Fig. 1A, the maximum stimulation of phagocytosis was obtained with 150 U/ml IFN-γ; the threshold concentration for an appreciable effect was 10 U/ml IFN-γ.

Fig. 1B shows the time-course of stimulation with 150 U/ml IFN-γ. Stimulation of phagocytosis was detectable after 30 min at 37 °C and peaked at 2 h. The IFN-γ concentrations and incubation times that give maximum stimulation were used in all subsequent experiments.

3.2. Interferon-γ enhances Fc-γ and dectin-1 receptor-mediated phagocytosis by mouse neutrophils

To study the effects of IFN-γ on neutrophil function, we first investigated the phagocytic capacity of resting and IFN-γ-activated mouse neutrophils. We found that IFN-γ (150 U/ml for 2 h) enhanced the phagocytic capacity of neutrophils when stimulated with IC or IC-C as well as Zy or Opzy (Fig. 2). These data indicate a stimulatory effect of IFN-γ on a fundamental function of neutrophils.

Table 1
Primers used in PCR reactions.

Target	Forward primer	Reverse primer
$p47^{phox}$	CGTACCCAGCCAGCACTATGT	GCTGCCCGTCAAACCACTT
$gp91^{phox}$	TTAGTGGGAGCAGGGATTGG	CCGGCATTGTTCCTTTCCT
β-actin	AGGGAAATCGTGCGTGACA	GAACCGCTCATTGCCGATA

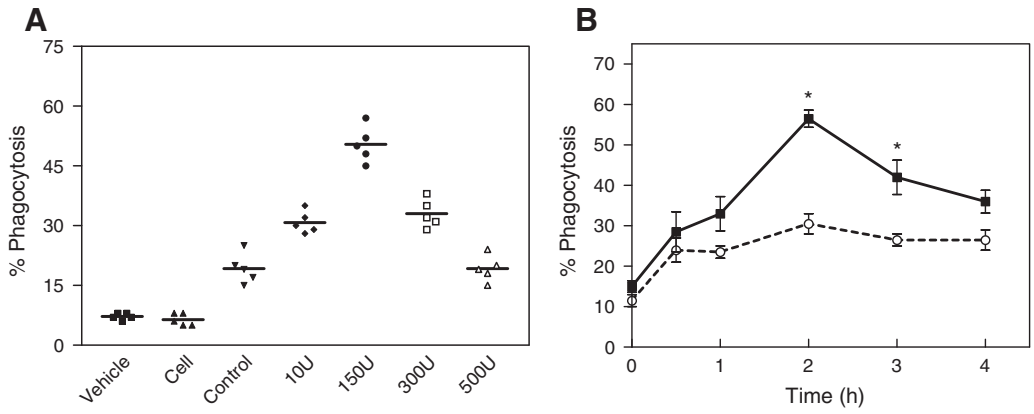

Fig. 1. (A) Effect of increasing concentrations of IFN-γ on phagocytosis of IgG-coated red blood cells by mouse neutrophils. Data were plotted as percentage phagocytosis, which means the percent of neutrophils that ingested at least one red blood cell. Control: neutrophils incubated with the immune complex without IFN-γ treatment. Bars represent median values (n = 5). (B) Time-course curves for neutrophils treated without (dotted line) and with (solid line) IFN-γ. Neutrophils were pretreated with medium alone or 150 U/ml IFN-γ for the indicated times and phagocytosis was subsequently measured. For each data point n = 4. The IFN-γ curve and control curve show a statistically significant dependence on pretreatment time. *p < 0.01 solid line vs. dotted line; Student's *t*-test.

3.3. Effect of IFN-γ pretreatment on the production of reactive oxygen species (ROS) by mouse neutrophils

We observed that pretreatment of neutrophils with IFN-γ significantly increases the release of O_2^-, H_2O_2 and HOCl (Fig. 3A, B and C, respectively), regardless of the stimuli used. O_2^- production by neutrophils treated with IFN-γ and stimulated with Zy or Opzy was significantly higher ($p < 0.001$ and $p < 0.01$, respectively) compared to stimulation with immune complexes of IgG. This result supports the hypothesis that IFN-γ pretreatment causes changes in the intracellular signaling mechanisms regulating ROS production.

3.4. Interferon-gamma enhances the release of enzymes from granules and the production cytokines by mouse neutrophils

Fig. 4A and B shows that mouse neutrophils stimulated with four types of stimuli exhibited enhanced release of β-glucuronidase, a marker for azurophilic granules, and alkaline phosphatase, a marker for specific granules, after a 2 h incubation with 150 U/ml IFN-γ. Extracellular release of the cytoplasmic marker lactate dehydrogenase (LDH) was also measured as an indicator of possible cell injury or death during the process; no extracellular release of LDH was found, confirming that IFN-γ stimulation was not toxic to the neutrophils (data not shown).

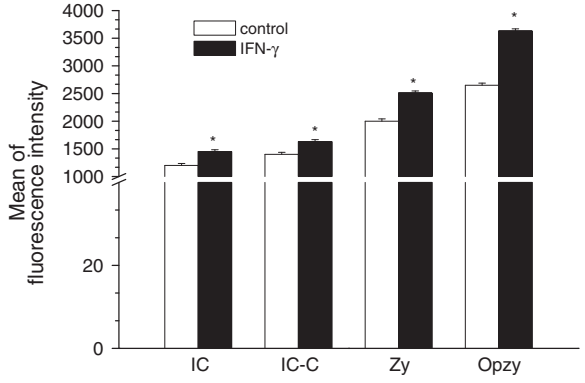

Fig. 2. Interferon-gamma enhances phagocytosis by mouse neutrophils. Resting neutrophils and neutrophils activated by IFN-γ were incubated with zymosan (Zy), zymosan opsonized with complement (Opzy) or immune complexes of IgG bound to red blood cells (IC) or red blood cells plus complement (IC-C) for 45 min at 37 °C. The fluorescence present in the neutrophils was assessed by flow cytometry, and data were plotted as the mean fluorescence intensity (n = 6–8 per group). *p < 0.05 vs. control (Student's *t*-test).

In addition, cytokine production was also affected by IFN-γ pretreatment. We found increased TNF-α and IL-6 production by IFN-γ-activated neutrophils (Fig. 4C and D).

3.5. Neutrophil FcγR and CR expression

The expression of FcγRs (CD32 and CD16), CR3 (CD11b) and dectin-1 was determined in mouse neutrophils after IFN-γ pretreatment. Dectin-1 is important in the recognition of β-glucans present in zymosan; CD32 and CD16 are critical in binding the Fcγ portion of IgG in immune complexes and CD11b is the α subunit of complement receptor CR3, which is involved in the internalization of opsonized particles.

As shown in Fig. 5, there is an increase in the expression of all immunes receptors ($p < 0.05$, Student's *t*-test) after treatment of mouse neutrophils with IFN-γ. This result suggests that the increased phagocytosis and release of enzymatic content of azurophils in neutrophils activated with IFN-γ for 2 h may be a consequence of increased receptor expression.

3.6. Effect of IFN-γ pretreatment on mRNA expression of the NADPH oxidase subunits $p47^{phox}$ and $gp91^{phox}$

To investigate whether the increased release of ROS is due to a change in the assembly of the NADPH oxidase complex, we examined the mRNA expression of the $p47^{phox}$ and $gp91^{phox}$ subunits of the NADPH oxidase complex by real-time PCR. Subunit $p47^{phox}$ was shown to be essential for the assembly and activity of the NADPH complex [40]. We showed that pretreatment of mouse neutrophils with 150 U/ml of IFN-γ for 2 h increased the expression of both the NADPH oxidase $p47^{phox}$ and $gp91^{phox}$ subunits (Fig. 6).

4. Discussion

Interferon-γ is a 17-kDa glycoprotein produced by activated T lymphocytes that was initially characterized for its antiviral and T lymphocyte proliferative activity. Investigation has focused on its proinflammatory role, which appears to be mediated via interaction with two target phagocytic cells: macrophages and neutrophils [7,8,22]. Interferon-γ has been shown to modulate many aspects of in vitro neutrophil functions [41–43]. Williams et al. [44] showed that IFN-γ protects against the development of structural damage in experimental arthritis by regulating neutrophil influx into diseases joints and in vitro studies using fibroblast-like synoviocytes, IFN-γ modulated both IL-1β and TNF-α, resulting in the down-regulation of chemokine CXCL-8. In another study, de Bruin et al. [45] have demonstrated with

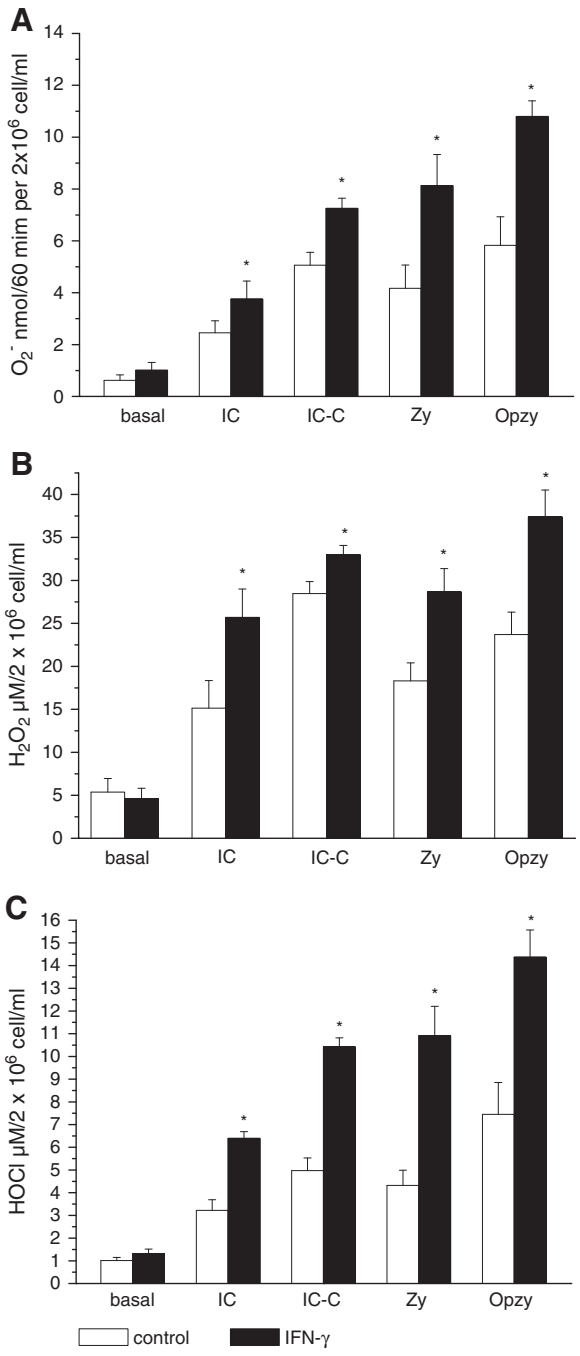

Fig. 3. Effect of IFN-γ pretreatment on the release of superoxide anion (A), hydrogen peroxide (B) and hypochlorous acid (C) by mouse neutrophils treated with 150 U/ml IFN-γ for 2 h and incubated with zymosan (Zy), zymosan opsonized with complement (Opzy), immune complex of IgG and ovalbumim (IC) or immune complex of IgG and OVA plus complement (IC-C) for 60 min at 37 °C. Data represent the mean ± standard error (n = 6–8 per group). *$p < 0.05$ vs. control group (Student's *t*-test).

in vitro experiments that IFN-γ is an important cytokine in directing myelopoiesis during acute viral infection in mice.

Despite the large number of studies on the regulatory action of IFN-γ on neutrophil functions [26,41,46,47], some conflicting results have been reported regarding phagocytosis and the production of oxygen reactive species in response to different stimuli. Some reports indicated decreased phagocytic ability and ROS production after IFN-γ pretreatment [47], whereas others have shown an increase [48]. The differences between these studies could be explained by differences in the time of exposure to IFN-γ, the ideal concentration of IFN-γ, animal species, cell type, different stimuli, the types of assay used to measure ROS release (e.g., extracellular or intracellular), or other conditions employed. Our goal was to further characterize the ability of recombinant human IFN-γ to stimulate mouse neutrophil oxidative responses as well as the release of lysosome enzymes mediated by different types of immune receptors. In this investigation we have assed recombinant human IFN-γ. We have also tested the effect of recombinant murine IFN-γ and observed that it was able to stimulated phagocytosis of zymosan particles as well as the human cytokine (25–30%, data not shown). Some studies have also been published using recombinant human IFN-γ in different species, including canine cells [12], bovine neutrophils [13] and porcine neutrophils [49].

Some studies have shown that IFN-γ can modify phagocytosis in immune cells and that this modification is dependent on the stimulus used [50–52]. Our results showed that phagocytosis by mouse neutrophils stimulated with IC or IC-C, as well as Zy or Opzy, was detectable after 30-min incubation with IFN-γ, and peaks at 2 h. Thus, IFN-γ enhances phagocytosis in neutrophils in response to these different stimuli.

In this study, we showed that IFN-γ stimulates the release of O_2^-, H_2O_2 and HOCl by mouse neutrophils. This effect was dose-dependent and peaked at 150 U/ml IFN-γ. This concentration may indicate a possible inflammatory role because it is within the range found in serum and synovial fluid of rheumatoid arthritis patients [14]. These results are also in agreement with results from Cassatella [53] who showed that human neutrophils in the presence of IFN-γ showed enhanced O_2^- and H_2O_2 production in response to FMLP, PMA or ICs of IgG and ovalbumin.

We also examined the effect of IFN-γ on the release of the enzymatic content of granules (β-glucuronidase and alkaline phosphatase) using mouse neutrophils stimulated by Zy, Opzy, IC or IC-C. Our results show that the release of both enzymes was significantly increased in the presence of IFN-γ. The effects on degranulation were not due to cell death because LDH release was the same in the presence or absence of IFN-γ. These results indicate that IFN-γ stimulates release of the contents of specific and azuriphilic granules. In contrast to our findings, Kowanko and Ferrante [54] were unable to show β-glucuronidase release in human neutrophils stimulated with opsonized zymosan treated with 200 U/ml of IFN-γ.

Some studies have shown that neutrophils are able to produce different pro-inflammatory cytokines and chemokines, including IL-8, IL12, IL1β and TNF-α, after various types of stimulation [55]. Evidence has been obtained that IL-6 plays a crucial role in the pathogenesis of many chronic inflammatory and autoimmune diseases ranging from rheumatoid arthritis [56,57], multiple sclerosis [58], diabetes [59] and asthma [60]. Furthermore, IL-6 appears to play a predominant role in linking chronic inflammation and tumor growth [61]. Our results show that zymosan, opsonized zymosan and immune complexes lead to enhanced production of TNF-α and IL-6 by IFN-γ treated neutrophils, in agreement with Gasperini et al. [62]. Their study shows that human neutrophils also have the ability to produce IL-6, TNF-α, IL-8 and inducible protein of 10 kDa (IP-10) in response to IFN-γ.

Our finding that treatment of murine neutrophils with IFN-γ could release an inflammatory cytokine (IL-6) is on line with the observations of Collota et al. [63] who found that IFN-γ could extend the life span of human granulocytes after 48 h treatment, and that this might have implications for inflammatory diseases. IL-6 stimulates the production of neutrophils by bone marrow progenitors, usually acting in concert with colony stimulating factors. The prolonged survival of neutrophils engaged in inflammatory responses, while likely to be beneficial in those circumstances in which the accumulation of effector cells is required, can, however, be potentially harmful when the prolonged presence of neutrophil may contribute to the pathogenesis of certain inflammatory diseases.

The activation of neutrophil functions also involves the modulation of cell surface molecules, a number of which have been well characterized. We assessed alterations in the surface expression of FcγRs and CR receptors in neutrophils in response to stimulation by IFN-γ. Human

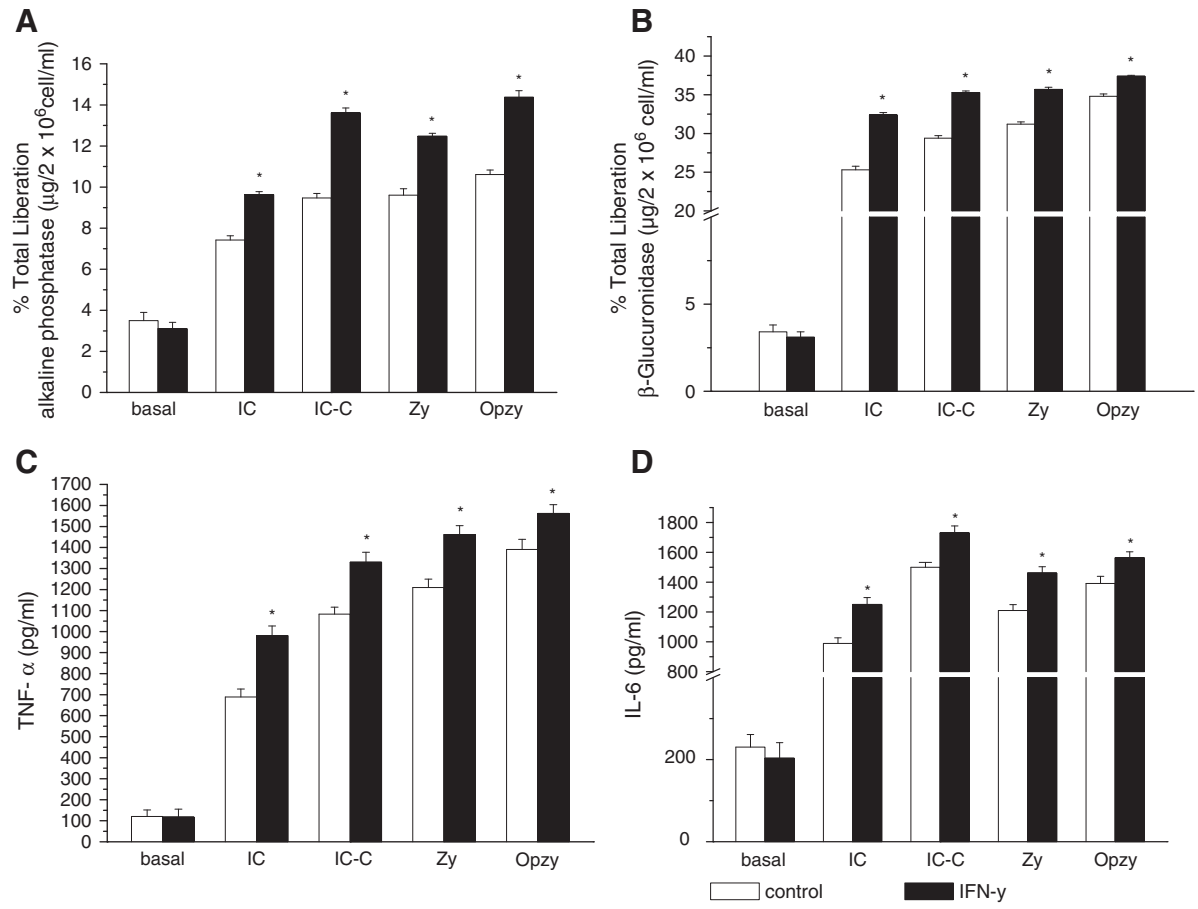

Fig. 4. Interferon-gamma enhances the release of the enzymatic content of granules and the pro-inflammatory cytokines IL-6 and TNF-α. Mouse neutrophils were isolated and pretreated with 150 U/ml IFN-γ for 2 h to induce release of (A) alkaline phosphatase, (B) β-glucuronidase and pro-inflammatory cytokines TNF-α and IL-6 (C and D). (A and B) Resting neutrophils and neutrophils activated by IFN-γ were incubated with medium alone (basal), zymosan (Zy), zymosan opsonized with complement (Opzy), immune complex of IgG and OVA (IC) or immune complex of IgG and OVA opsonized with complement (IC-C) for 45 min at 37 °C. The release of enzymatic content of granules was then determined. Data were plotted as percentage of total release of β-glucuronidase (n = 7–10 per group) and alkaline phosphatase (5 per group in duplicate). (C and D) Resting neutrophils and neutrophils activated by IFN-γ were incubated with the same stimuli described above for 24 h at 37 °C. After culture, the supernatant was collected and levels of IL-6 and TNF-α were determined by ELISA. The graphs represent mean ± standard error (n = 6–8 per group). *p < 0.05 vs. control (Student's *t*-test).

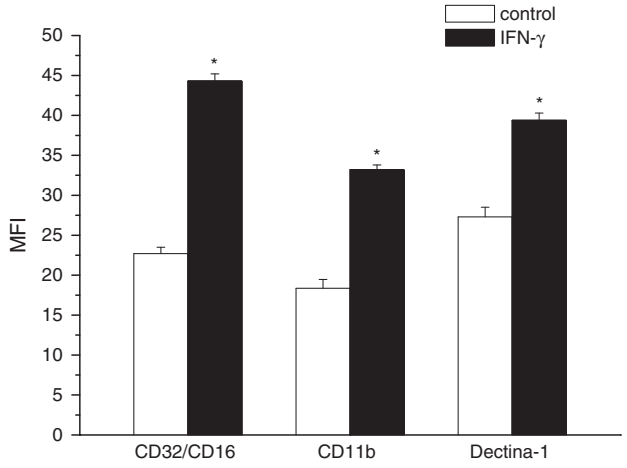

Fig. 5. Effect of IFN-γ pretreatment on the expression of CD32, CD16, CR3 and dectin-1 on the surface of mouse neutrophils. Neutrophils activated by IFN-γ (■) and resting neutrophils (□) were incubated with primary anti-dectin-1 followed by incubation with secondary antibody conjugated, antibody-conjugated CD32/CD16-FITC for measuring FcγRII/III and CD11b-PE to measure complement receptor, CR3. Data are plotted as mean fluorescence intensity (MFI) measured by flow cytometry. The graph represents the mean ± standard error (n = 6 per group). *p < 0.05 vs. control (Student's *t*-test).

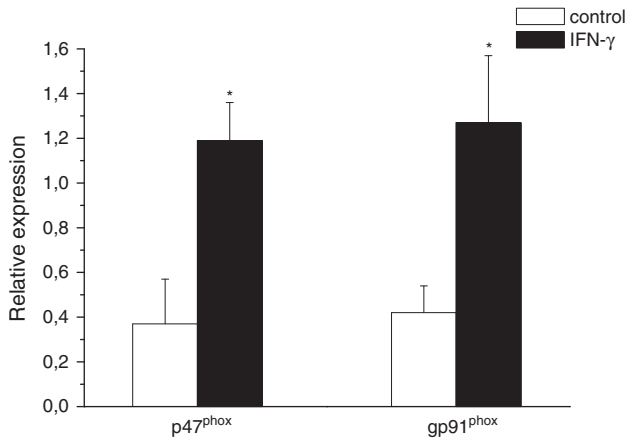

Fig. 6. Expression of the NADPH oxidase subunits p47^{phox} and gp91^{phox} is elevated in neutrophils pretreated with IFN-γ. RNA was extracted from resting and activated neutrophils by Trizol ®, and mRNA encoding subunits of the NADPH oxidase complex were amplified by RT-PCR. The amounts of gp91^{phox} and p47^{phox} mRNA were normalized to the amounts of endogenous mRNA of the control, β actin, which is constitutively expressed by cells. The graphs represent the mean ± standard error (n = 6–12 in triplicate). *p < 0.05 vs. control (Student's *t*-test).

neutrophils express three types of Fc receptors which are involved in phagocytosis, ROS production and other cellular processes, namely FcγRI, FcγRII and FcγRIII [64,65]. FcγRI, a high affinity receptor is expressed only when neutrophils are treated with some cytokines, including IFN-γ [26]. Some observations show that IFN-γ is responsible for the regulation of genes encoding FcγRI (CD 64) and $p47^{phox}$ in human neutrophils. This gene transcription, however, is undetectable in untreated cells [66,67]. Also, treatment with IFN-γ had effect on the expression of FcγRII and FcγRIII. It is possible that the expression of FcγRI could explain the increased phagocytosis and ROS production with the different stimuli (immune complexes) we have observed. Nevertheless, one cannot exclude the participation of the other two receptors in these effects. Our results shown that CD16, CD32, CR3 and dectin-1 receptors are present on the surface of mouse neutrophils and that, in agreement with other studies [68], expression can be enhanced by treatment with IFN-γ.

Because the production of superoxide is a reflection of the activity of the NADPH oxidase complex, we investigated whether IFN-γ treatment could alter the gene expression of components of this enzymatic complex in neutrophils. Our results showed that IFN-γ enhanced the mRNA expression of the gene that encodes for the $p47^{phox}$ and $gp91^{phox}$ subunits. The $p47^{phox}$ subunit plays a key role in the translocation of other cytosolic subunits and the assembly of the NADPH oxidase complex, while $gp91^{phox}$ is the central component of this complex responsible for chain formation of electron transport [69]. This is only suggestive evidence because it is difficult to estimate whether a high level of mRNA would result in a high level of the encoded protein, and the turnover rates are not known. It is known that the cytokine IFN-γ can induce transcription and translation of specific genes and proteins. Newburger et al. [70], Cassatella et al. [71], Dusi et al. [72], and Casbon et al. [73] found that enhanced capacity for ROS production in murine macrophages is partially a result of increased protein expression of $gp91^{phox}$ and $p22^{phox}$, but they also demonstrate that IFN-γ induced a shift in the predominant localization of $gp91^{phox}$ and $p22^{phox}$ from intracellular membrane compartments to the plasma membrane. Further investigation is required to determine the expression of several cytosolic and membrane-bound components in mouse neutrophils induced by IFN-γ.

Other important function of neutrophils has been reported as regulated by IFN-γ, such as antibody-dependent cell-mediated cytotoxicity [42,62]. Current research has indicated an important role of neutrophils in the interplay between the innate and acquired immune response [26]. It has been suggested that during inflammation or specific immune responses, mature myeloid cells may respond to humoral factors in a manner analogous to macrophages [42].

In our experiments we observed the effects of IFN-γ on mouse neutrophils using different agents such as complement opsonized zymosan which is typical occurrence o innate immune response as well as IgG immune complexes that in vivo would be most expected in the secondary immune response. Thus, IFN-γ has been shown to be a pleiotropic cytokine and its effects on neutrophils show the participation of these cells in the innate and acquired immune response.

5. Conclusion

This study clarified the effect of recombinant human IFN-γ on the phagocytic capacity of mouse neutrophils, which is accompanied by the up-regulation of FcγR, CR and dectin-1 receptors. The increase in ROS production mediated by these receptors with activation of the NADPH oxidase is correlated with the increased expression of $p47^{phox}$ and $gp91^{phox}$ mRNA, two important components of this enzyme complex. In addition to enhanced phagocytosis, we also observed the release of some lysosomal enzymes. The increased production of reactive oxygen species may be important to the enhanced capacity for killing phagocytosed microorganisms, but may also favor the induction of oxidative stress in adjacent cells. In agreement with this potential inflammatory role, we also demonstrated increased release of the inflammatory cytokines TNF-α and IL-6 from neutrophils treated with recombinant IFN-γ.

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