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Production of Nitric Oxide and TNF-α, and Expression of iNOS and NFκB in Peritoneal Macrophages Activated with Interferon Gamma

Abstract

Interferon gamma (IFN-γ) is a cytokine produced by cells from the immune system, such as T-cells and natural killer (NK) cells. This molecule has several effects on macrophage (Mθ) activities including stimulation of the respiratory burst and *in vitro* enhancement of antimicrobial activity of Mθs against bacterial, fungal and mycobacterial organisms. It is known that peritoneal murine Mθs, once activated with IFN-γ, exert a fungicidal effect against some pathogenic fungi, including *Paracoccidioides brasiliensis*, and that this mechanism is mediated by nitric oxide (NO). In addition, it has been demonstrated that IFN-γ is important in the *in vivo* control of certain mycotic infections. Our purpose was to determine if *in vitro,* IFN-γ-activated peritoneal murine Mθs participate in the production of both nitric oxide (NO) and TNF-α, and if the corresponding mechanisms implicated the inducible nitric oxide synthase (iNOS) and nuclear factor (NF-κB) expression. The results showed that the IFN-γ-activated Mθs had an increase in both NF-κB and iNOS expressions at 6 and 18 h, respectively, indicating a high NO production but a non-detectable TNF-α production. These data suggest that NF-κB expression preceded iNOS expression, inducing both high NO production and IFN-γ-activation. Apparently, the latter cytokine by itself is not sufficient to induce TNF-α production on peritoneal murine Mθs.

Key words: IFN-gamma, Macrophages, iNOS, Nitric oxide, NFκB.

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Interferon gamma (IFN-γ) is a cytokine produced by T-cells and natural-killer (NK) cells that exerts several effects on macrophage (Mθ) activities including stimulation of the respiratory burst and *in vitro* enhancement of Mθ antimicrobial activity against bacterial, fungal and mycobacterial organisms (Ahmed & Niederman, 2000). Thus, IFN-γ is considered one of the most important stimuli for Mθ activation and it has also been shown to play a key role in the death of intracellular parasites through the production of nitric oxide (NO) (Mühl & Dinarello, 1999).

On the other hand, monocytes/Mθs play an important role in host defense and are also implicated in an innate resistance mechanism against fungi through their direct microbicidal capacity and synthesis of certain cytokines. It also has been demonstrated that murine peritoneal Mθs activated with IFN-γ exert a potent fungicidal activity against the dimorphic pathogenic fungus, *Paracoccidioides brasiliensis*, which is mediated by NO (González *et al.*, 2000).

NO synthesis is stimulated by cytokines resulting in enhanced transcription of the inducible nitric oxide synthase (iNOS) enzyme thus leading to the conversion of L-arginine to L-citrulline with liberation of high NO levels (Hemmes & Mayer, 1999). Additionally, the nuclear transcription factor kappa B (NFκB) is the molecule responsible for boosting the transcription of the gene coding for a variety of protein syntheses, such as TNF-α and iNOS (Baeuerle & Henkel, 1994).

Cano *et al.* (1998), using a pulmonary experimental model of paracoccidioidomycosis in resistant (A/Sn) and susceptible (B10.A) mice, showed that IFN-γ plays a protective role and that this cytokine is one major mediator of resistance against *P. brasiliensis* infection in mice.

Souto *et al.* (2000) investigated the role of IFN-γ and TNF-α in resistance to *P. brasiliensis* infection. When infected with the fungus, mice with homologous disruption of the IFN-γ (GKO) or the TNF-α receptor (p55KO) genes were unable to control proliferation of yeast cells and succumbed to the infection shortly after inoculation. On the other hand, wild-type mice controlled the infection thus suggesting that both IFN-γ and TNF-α mediate resistance to *P. brasiliensis*.

In the present study, we examined whether the *in vitro* activation of murine peritoneal Mθs with IFN-γ–induced the production of both NO and TNF-α and whether iNOS and NF-κB were expressed during the early stages (first 96 h) of this Mθ-IFN-γ activation process.

Murine peritoneal Mθs were obtained from adult (8 to12 weeks old) male BALB/c mice. Groups of 10-12 animals were used. The mice were anesthetized with a mixture of 0.6 mL of ketamine (100 mg), 0.4 mL of xylazine (20 mg) in 1.0 mL of sterile distilled water. Each animal received 150 μL (i.m.) of the mixture after 10 min, when the animals were already insensitive to pain, they were killed and peritoneal lavage was done by i.p injection of 8-10 ml of RPMI-1640 medium containing antibiotics (penicillin 100 U/mL, streptomicyn 100 mg/mL). The mouse abdomen was then massaged softly, after which the accumulated peritoneal liquid was removed through punction. The content of this lavage was deposited in 15 mL conical plastic tubes that were placed immediately in an ice bath and centrifuged afterwards for 15 min (314g, 4°C). The supernatant was extracted from each tube and the pellet was smoothly resuspended in 1.0 mL of supplemented RPMI-1640 containing 10% fetal bovine serum and the antibiotics already described. The pellets, free of blood cells and cellular debris, were mixed and counted in a hemocytometer; and cell viability was determined with trypan blue. The cellular suspension was adjusted to contain 1 x 10^6 viable cells per ml (González *et al.*, 2000).

Aliquots (0.25 mL) of the peritoneal-cell suspension were deposited aseptically in each well of a Lab-Tek-chamber slide (Nalge Nunc International, catalog number 177402, Naperville, IL., USA). The slide chambers were incubated at 37°C in 5% CO_2-95% air for 2 h to allow the cell adherence process; then the nonadherent cells were removed by aspiration, and the adherent monolayers were rinsed with RPMI 1640. The number of nonadherent cells was determined and subtracted from the number of incubated peritoneal cells (González *et al.*, 2000). The Mθ monolayers thus prepared were stimulated (activated) with 50 U/ml of recombinant interferon-gamma (rIFN-γ) (PharMingen, number of catalog 19301U, San Diego, does CA, USA) and incubated for 1, 2, 3, 6, 18, 24 and 96 h. The monolayers were fixed in cold acetone.

Once the rIFN-γ-stimulated Mθ monolayer had been incubated for the indicated periods, cells were fixed for 10 min with cold acetone before proceeding to immunocytochemical analyses. Blocking of the endogenous peroxidase (3% peroxide solution) and of the non-specific immunoglobulin binding sites (1% rabbit serum and 5% bovine serum albumin–BSA) was then carried out. Mθs were then washed with PBS plus 0.01% Tween-20. These monolayers were incubated for 1 h either with polyclonal goat anti-iNOS (Santacruz Biotecnology, Inc.cat. No. 650-6) or polyclonal goat anti-NFκB (same firm cat. No. sc1192).

After this incubation, the Mθs were washed with the PBS/Tween-20 mixture and incubated for 1 h with rabbit anti-goat biotinilated secondary antibody (cat. No. B7024, Sigma chemical company). Bound rabbit antibody was detected using the ABC vectastain complex (Vector Laboratories, Inc) and diaminobenzidine (DAB) (Cat. No. 3966, DAKO) as the substrate. Monolayers were counterstained with Harris hematoxilin (Act. No. HHS-16, Sigma). The percentage of reactive cells was calculated counting 500 cells in different areas of the monolayers.

All experiments were done in duplicate and repeated at least 3 times. The results are expressed as means (± SEM) and were analyzed by one-way ANOVA.

In the monolayers containing IFN-γ-activated Mθs, initiation of NFκB expression was observed within 1 h (Figure 1), with a significant increase in the number of reactive cells at 6 h post-activation, 33.4 ± 8.9 %, in comparison with non-treated cells (8.9 ± 1.2%) ($p < 0.005$). Similarly, in the activated Mθs, iNOS expression started at 6 h post-stimulation, with a maximum value and a significant proportion of positive cells (34.2 ± 6.5 %) at 18 h when compared with control non-activated cells (0.6 ± 0.1%) (Figure 2; $p < 0.000001$).

Additionally, we also determined TNF-α levels on the Mθ culture supernatants. For this purpose, after each time period, the supernatants corresponding to each Mθ culture were collected and frozen at -70 °C until assayed. The samples were thawed only once, immediately prior to performing ELISA assays using commercial murine cytokine TNF-α (OptEIA set; San Diego, CA, USA). As shown in Table 1, during the different periods assayed, no significant increment of this protein was observed when compared with the control (supernatant from non-activated Mθs), suggesting that in this system IFN-γ-activation alone is inadequate to induce *in vitro* TNF-α production in murine peritoneal Mθs.

The *in vitro* NO production by these Mθs was also determined. The concentration of NO_2 in the culture supernatants was used as an indicator of NO generation and measured with the Griess reagent [1% sulfanilamida (Sigma S-9251), 0.1% of N-(1-naphthyl) ethylenediamine dihydrochloride (Sigma N-5889) and 2.5% of phosphoric acid (Sigma P-6560)]. Briefly, 50 mL of the supernatant was added to an equal volume of the

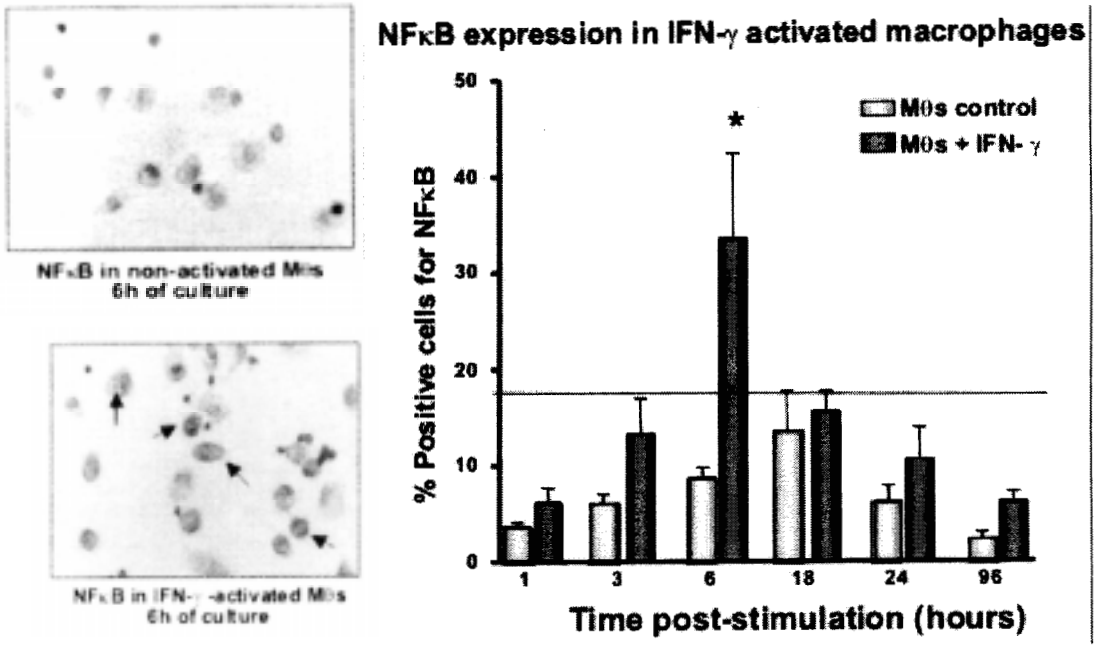

Figure 1. Expression of NF-κB in murine peritoneal Mθs activated with IFN-γ (50 U/mL) post-activation 1, 3, 6, 18, 24 and 96 h. Data expressed as average ± SEM of the three experiments (n=6). * $p < 0.005$ when comparing with non-activated cells.

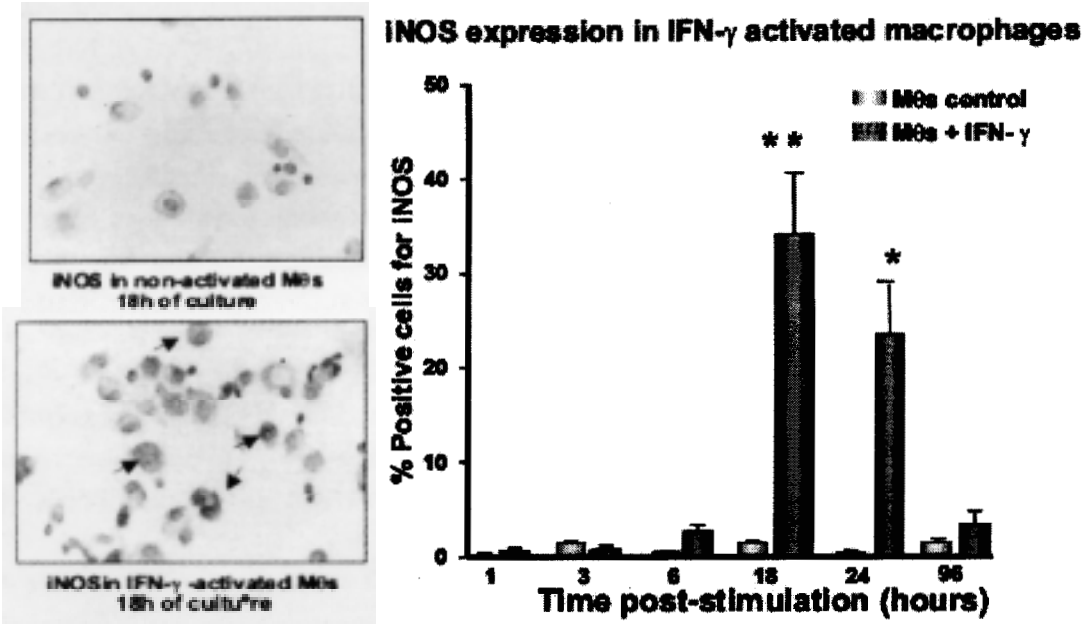

Figure 2. Expression of iNOS in murine peritoneal Mθs activated with IFN-γ (50 U/mL) 1, 3, 6, 18, 24 and 96 h post-activation. Data expressed as average ± SEM of the three experiments (n=6). * $p < 0.005$ and ** $p < 0.000001$ when comparing with non-activated cells.

Griess reagent in triplicate wells of a 96 well microplate (cat. No. 3075, Falcon, Lincoln Park, N.J.). After incubation at room temperature for 10 min, plates were read in an ELISA electronic reader set at 540 nm. NO_2 was determined by using sodium nitrite as a standard (Ding *et al.*, 1988). As observed in Figure 3, when the Mθs were activated with IFN-γ, increased production of NO was observed which, when compared with control non-activated cells, revealed the highest NO_2 levels (41.3 ± 6.6 μM, $p < 0.001$) at 96 h when

control non-activated monolayer produced only 3.01 ± 0.9 μM.

The results obtained in this study show that recombinant IFN-γ activated murine peritoneal Mθs induced NFκB and iNOS expression, with maximal expressions at 6 and 18 h, respectively. These results indicate that NFκB expression does precede iNOS expression. We also demonstrated that these cells produced high NO levels but did not express TNF-α. Thus, production of the latter cytokine maybe independent of NF-κB expression, an intermediate molecule known to operate in the IFN-γ NO-induced pathway.

Table 1. TNF-α levels detected on the IFN-γ-activated-Mθ culture supernatants 1, 3, 6, 18, 24 and 96 h post-activation.

Time of culture (hours)	TNF-α levels (pg/ml) in macrophage culture supernatants (± SEM)	
	Without IFN-γ	With IFN-γ (50 U/ml)
1	2.65 ± 0.005	2.65 ± 0.003
3	2.65 ± 0.005	2.66 ± 0.008
6	2.74 ± 0.03	2.75 ± 0.04
18	2.74 ± 0.03	2.74 ± 0.03
24	2.74 ± 0.03	2.74 ± 0.03
96	2.74 ± 0.04	2.76 ± 0.03

Dorger *et al.* (1997) studied the production of NO and iNOS expressions in both rat and hamster alveolar Mθs and found a species difference in the production of NO, specifically that when these cells were stimulated with IFN-γ, LPS or TNF-α, their iNOS protein expression changed, with rat alveolar Mθs expressing iNOS and producing NO in a dose-dependent manner after being treated with IFN-γ and LPS, but not with TNF-α. Conversely, after the same treatment, hamster alveolar Mθs neither produced NO nor expressed iNOS.

IFN-γ is a co-stimulus required for iNOS expression, through the signaling pathway and for the transcriptional elements, functions that are well documented (Kamijo *et al.*, 1994; Martin *et al.*, 1994; Meraz *et al.*, 1996; Salkowski *et al.*, 1996). IFN-γ response has been shown to be localized between positions –913 and –1029 of the 5' flanking region of the iNOS promoter. This region contains a cluster of motifs characteristic of IFN-γ-responsive individuals, including the IFN-γ-activated sequence (GAS) and two IFN-γ-stimulated response elements that bind to the transcriptional factors Stat1a and IRF-1 (Kamijo *et al.*, 1994; Martin *et al.*, 1994; Meraz *et al.*, 1996; Salkowski *et al.*, 1996). The murine iNOS promoter RAW 264.7 obtained from cells with BALB/c origin has been sequenced and various regulatory elements in the region have been identified, including several copies of IFN-γ and TNF-α response elements, NFκB and AP-1 sites, as well as other elements (Lowenstein *et al.*, 1993). On the contrary, the transcription factor NF-κB has been shown to enhance the expression of a number of proteins including iNOS (Blackwell & Christman, 1997; Xie *et al.*, 1993).

Souto *et al.* (2000) used knocked out mice for both IFN-γ (GKO) and TNF-α receptors (p55KO) and infected them with *P. brasiliensis* yeast, showing that the animals

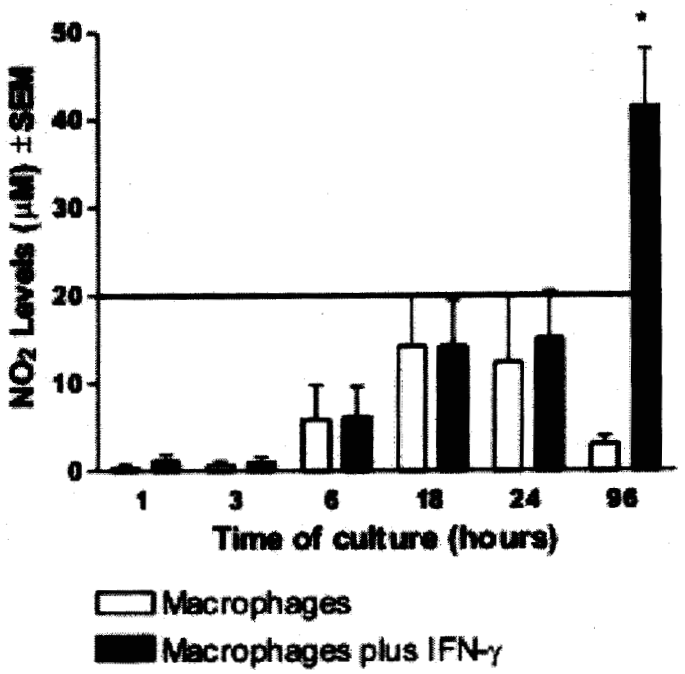

Figure 3. NO_2 levels, as markers of NO production, in supernatants of Mθs activated with recombinant IFN-γ after 1, 3, 6, 18, 24 and 96 h post-activation. Bars represent the average ± SEM. * Significant values ($p < 0.000001$).

were unable to control the infection. This suggested that both IFN-γ and TNF-α through their receptors, mediate the resistance to *P. brasiliensis* infection. On the other hand, Calich and Kashino (1998), studying resistant (A/Sn) or susceptible (B10.A) mice to *P. brasiliensis*, found that peritoneal Mθs from the latter strain secreted low levels of TNF-α in comparison with the former animals that were able to release high levels of this cytokine. These authors suggested that production of these cytokines plays a crucial role in the mechanism of resistance to *P. brasiliensis* infection.

In conclusion, the present study suggests that *in vitro* stimulation of murine peritoneal Mθs with IFN-γ induces an internal molecular signal that results in expression of a nuclear factor (NF-κB) and of iNOS, with both being probably responsible for the observed NO production. The latter mediates the fungicidal mechanism exerted against *P. brasiliensis* by activated macrophages although apparently this pathway is not the same that induces TNF-α production.

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