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# IFN- $\gamma$ -Induced TNF- $\alpha$ Expression Is Regulated by Interferon Regulatory Factors 1 and 8 in Mouse Macrophages<sup>1</sup>

Virginia Vila-del Sol,<sup>2</sup> Carmen Punzón, and Manuel Fresno<sup>3</sup>

We have previously described that IFN- $\gamma$  induces cyclooxygenase 2 and inducible NO synthase expression by a mechanism that involved endogenously produced TNF- $\alpha$ . In this study, we report that TNF- $\alpha$  production is induced by IFN- $\gamma$  treatment in the murine macrophage cell line RAW 264.7. TNF- $\alpha$  mRNA levels are increased in cells treated with IFN- $\gamma$  in a time-dependent manner and IFN- $\gamma$  also increased human TNF- $\alpha$  promoter-dependent transcription. Two regions in the TNF- $\alpha$  promoter seem to be responsible for the IFN- $\gamma$  response: a distal region between -1311 and -615 bp of the human TNF- $\alpha$  promoter, and a proximal region located between -95 and -36 bp upstream of the transcriptional start. In contrast, IFN- $\gamma$  stimulation induces the expression of the transcription factors IRF-1 and IRF-8. Overexpression of these transcription factors produces an increase in the transcriptional activity of the human TNF- $\alpha$  promoter. There is a correlation between the regions of the TNF- $\alpha$  promoter responsible of the transcriptional activation elicited by IRF-1 and IRF-8 and those required for IFN- $\gamma$  response. In addition, IRF-1 and IRF-8 are recruited to the TNF- $\alpha$  promoter in IFN- $\gamma$ -treated RAW 264.7 cells, as demonstrated by chromatin immunoprecipitation assays. Moreover, overexpression of IRF-1 and IRF-8 induces TNF- $\alpha$  production in unstimulated RAW 264.7 macrophages, comparable to the production of TNF- $\alpha$  elicited by IFN- $\gamma$  stimulation, and silencing of IRF-1 and/or IRF-8 with specific small interfering RNAs, decreases IFN- $\gamma$ -elicited TNF- $\alpha$  production. In summary, IFN- $\gamma$  treatment induces TNF- $\alpha$  expression at transcriptional level requiring the coordinate action of IRF-1 and IRF-8. *The Journal of Immunology*, 2008, 181: 4461–4470.

IFN- $\gamma$  which is produced by activated T cells and NK cells, is a pleiotropic cytokine responsible for macrophage activation and differentiation (1). IFN- $\gamma$  induces transcription of several proinflammatory genes, such as inducible NO synthase (iNOS),<sup>4</sup> cyclooxygenase-2 (COX-2), and IL-1 $\beta$ , as well as MHC proteins (1–3). IFN- $\gamma$  signal transduction pathway begins with the recruitment of Janus kinases, JAK1 and JAK2, to the IFN- $\gamma$  receptor inducing their phosphorylation in tyrosine. As a consequence, the transcription factor Stat1 $\alpha$  is also recruited to the IFN- $\gamma$  receptor and phosphorylated by JAKs. Phosphorylated Stat1 $\alpha$  dimerizes and translocates to the nucleus where it induces transcriptional activation of several genes by binding to the gamma-activated sites of their promoters. Among the genes induced by Stat1 $\alpha$ , IFN regulatory factor (IRF)-1, and IRF-8 are also transcription factors

which also mediate the transcriptional regulation induced by IFN- $\gamma$ .

IRFs constitute a family of transcription factors (up to nine members have been characterized to date) with a characteristic helix-turn-helix DNA binding motif (4). IRF-1 and IRF-2 were the first members of the family identified and are induced upon IFN activation in many cell types (5, 6). IRF-2 is thought to act as a transcriptional repressor of IRF-1 activity (6–8). IRF-1 has diverse functions in host defense, being involved in the regulation of the expression of several genes in response to different stimuli in various cell types, driving innate and adaptive immune responses (4). Thus, IRF-1-deficient mice present a defect in IL-12 production resulting in increased susceptibility to *Leishmania major* infection (9). Macrophages from those IRF-1 knock-out mice have no iNOS induction (10), and diminished MHC-I and MHC-II expression (11) in response to IFN- $\gamma$ . IRF-8 is another member of IRF transcription factor family, with a lymphoid and myeloid restricted expression. IRF-8 is involved in the control of myeloid lineage differentiation (12). IRF-8-deficient mice present a pathology similar to human chronic myeloid leukemia (13), and it has been found that IRF-8 has an important function in the regulation of the expression of several genes involved in immune response to pathogens (14, 15). IRF-8 knock-out mice as IRF-1-deficient mice also have a diminished iNOS expression in response to IFN- $\gamma$  treatment (16). To date, several studies have revealed an important functional cooperation between IRF-1 and IRF-8 in transcriptional regulation of different IFN- $\gamma$ -induced genes, such as IL-12, iNOS, NADPH phagocyte oxidase, or IL-1 $\beta$  (17–22).

Among the genes induced by IFN- $\gamma$  in macrophages, TNF- $\alpha$  was one of the earliest described (23, 24). TNF- $\alpha$  is an important cytokine that can exert beneficial or detrimental effects in the host (25–28). TNF- $\alpha$  is a key mediator of cellular activation of T lymphocytes (29) and macrophages (30), and has an important role in the induction of inflammatory mediators such as NO and prostaglandins (31–36). Transcriptional regulation of TNF- $\alpha$  promoter is

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<sup>4</sup> Abbreviations used in this paper: iNOS, inducible NO synthase; IRF, IFN regulatory factor; COX-2, cyclooxygenase-2; siRNA, small interfering RNA.

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very complex, and involves the assembly of a unique enhancer-complex, with the participation of different transcription factors depending of the stimuli and the cellular type studied (37–42). In this way, multiple transcription factor binding sites have been identified in the TNF- $\alpha$  promoter, including several NF- $\kappa$ B sites, a cyclic AMP response element, various NFAT elements, and several Ets binding sites (37–44).

Previously, we have shown that IFN- $\gamma$  treatment induces TNF- $\alpha$  production by macrophages, and this endogenous production is absolutely necessary for COX-2 and iNOS induction by IFN- $\gamma$  (35, 36). In this study, we have characterized the transcriptional regulation of TNF- $\alpha$  expression and identify the regions of the TNF- $\alpha$  promoter responsible of the IFN- $\gamma$  response. Also, we report that IRF-1 and IRF-8, which are induced by IFN- $\gamma$  in RAW 264.7 cells, regulate TNF- $\alpha$  transcription. IRF-1 and IRF-8 are recruited to the TNF- $\alpha$  promoter *in vivo*, and drive TNF- $\alpha$  transcription by the same promoter regions that are required for IFN- $\gamma$  response. Thus, the results presented in this study suggest a mechanism for regulation of TNF- $\alpha$  gene expression by IFN- $\gamma$ , which is mediated by the coordinate action of IRF-1 and IRF-8 transcription factors.

## Materials and Methods

### Abs and reagents

Abs against IRF-1, PU.1 (polyclonal rabbit Abs), IRF-8, actin (polyclonal goat Ab) (used at 1/1000 dilution), and normal goat and rabbit serum were purchased from Santa Cruz Biotechnology. R-PE-conjugated rat anti-mouse TNF- $\alpha$  mAb was purchased from BD Pharmingen Biosciences. Recombinant mouse IFN- $\gamma$  (activity:  $10^6$  U/mg) used at 2.5 ng/ml, and anti-TNF- $\alpha$  neutralizing Ab (1  $\mu$ g/ml) were obtained from R&D Systems. LPS from *Escherichia coli* serotype 026:B6 was purchased from Sigma-Aldrich and used at 1  $\mu$ g/ml.

### Plasmids

Different constructs of the human TNF- $\alpha$  promoter were provided by Dr. J. Economou (UCLA School of Medicine, Los Angeles, CA) (45): pTNF (-1311), pTNF (-615), pTNF (-528), pTNF (-120), pTNF (-95), and pTNF (-36). The deletion vectors pTNF (-389), pTNF (-362), and pTNF (-173) were generated by intermediate cloning of the corresponding PCR fragment in pGEM T-easy (Promega), releasing of the fragment by digestion with *Sall*-*Bgl*II, and subsequent cloning into the *Sall*-*Bgl*II-digested pXP2 vector (46). A unique reverse primer from human TNF- $\alpha$  gene was used for PCR amplification (+90 primer: 5'-AGA TCT GAG GGT TGT TTT CAG GGG GGG TCT-3'). Different forward primers lying in the 5'-region of the transcription start site of the human TNF- $\alpha$  promoter were used to obtain the deletion constructs (-389 primer: 5'-CCT GTC TGG AAG TTA GAA GGA AAC AGA CCA CAG ACC TGG-3'; -362 primer: 5'-CCA CAG ACC TGG TCC CCA AAA GAA ATG GAG GCA ATA GGT TTT GAG GGG-3'; -173 primer: 5'-CGC CCC CGC GAT GGA GAA GAA ACC GAG ACA GAA GG-3'). The sequence of all PCR-derived constructs was confirmed by automatic sequencing. The IRF-1 and IRF-2 expression vectors (pAct1 and pAct2, respectively), and the corresponding control empty vector (pActC), were provided by Dr. T. Taniguchi (University of Tokyo, Tokyo, Japan) (6). The IRF-8 expression vector (pUE5) and the corresponding empty vector (LK440), were a gift by Dr. K. Ozato (National Institutes of Health, Bethesda, MD) (47). pRL-TK-luc (Promega), which express *Renilla luciferase* was used for determining transfection efficiency.

### Cell culture

The macrophage cell line RAW 264.7 (American Type Culture Collection) was grown in RPMI 1640 medium (Sigma-Aldrich) supplemented with 5% (v/v) FCS, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), gentamicin (1000 U/ml), and glutamine (2 mM). For experiments, cells were treated with IFN- $\gamma$  (2.5 ng/ml) and/or LPS (1  $\mu$ g/ml) for the indicated times, in RPMI 1640 supplemented with 2% FCS, antibiotics, and glutamine.

### Cytokine determination

Supernatants of control or IFN- $\gamma$  treated cells were measured for TNF- $\alpha$  presence using the Quantikine M murine kit for mouse TNF- $\alpha$  (R&D Systems), following the manufacturer's instructions.

### Chromatin immunoprecipitation assay (ChIP)

ChIP assays were performed using the ChIP assay kit from Upstate Biotechnology following the manufacturer's instructions with slight modifications, as previously described (48). Six  $\times 10^6$  cells were treated with 1% of formaldehyde for 30 min at room temperature. After three washes with cold PBS, cells were lysed with 600  $\mu$ l of SDS lysis buffer for 10 min at 4°C and sonicated (10 s each, four times at 30% of maximum power) to shear genomic DNA. After centrifugation for 10 min at 14,000 rpm and 8°C, 100  $\mu$ l of supernatant was diluted with ChIP dilution buffer and pre-cleared with 50  $\mu$ l of protein A/G PLUS-agarose (Santa Cruz Biotechnology) supplemented with salmon sperm DNA (1 mg/ml; Sigma-Aldrich) for 1 h at 4°C. Pre-cleared lysate was incubated with 1  $\mu$ l of normal serum or 5  $\mu$ g of anti-IRF-1 or anti IRF-8 Ab, overnight at 4°C. Fifty microliters of protein A/G PLUS-agarose was added to the samples for 1 h at 4°C to collect the Ab-chromatin complexes. After extensive washing with each of the following buffers in order: low salt wash buffer, high salt wash buffer, LiCl wash buffer, and 1 $\times$  TE (two washes), chromatin complexes were eluted, the crosslink was reversed and proteins were digested with proteinase K. DNA was purified by using QIA Quick PCR Purification kit (Qiagen), following the manufacturer's instructions. PCR amplifications were conducted by using CERTAMP kit for Complex Amplifications (Biotools). The primers from TNF- $\alpha$  promoter used for PCR were: TNF - 533, 5'-CCT CCA AGA ACT CAA ACA GGG GGC TTT CCC-3'; TNF + 45, 5'-CTC CTG GCT AGT CCC TTG CTG TCC TCG CTG-3'.

### RT-PCR

Total RNA was obtained by using TRIzol reagent (Invitrogen Life Technologies), following the manufacturer's instructions. For quantification of TNF- $\alpha$ , IRF-1, IRF-2, and IRF-8 mRNA levels, TaqMan Gene Expression Assays were used (Applied Biosystems) by following the manufacturer's instructions. References of the assays are: TNF- $\alpha$ , Mm00443258\_m1; IRF-1, Mm00515191\_m1; IRF-2, Mm00515204\_m1; IRF-8 (ICSBP), Mm00492567\_m1.

### Western blot

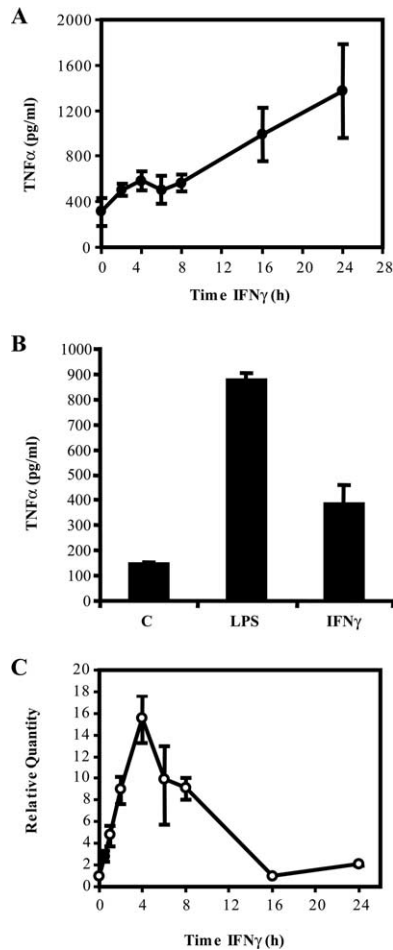
Cells were harvested by centrifugation (1200 rpm/5 min) and washed twice with PBS. Thirty microliters of lysis buffer (20 mM Tris-HCl (pH 7.5); 150 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, 10 mM NaF, 10 mM Na<sub>3</sub>VO<sub>4</sub>, and 2  $\mu$ g/ml each of inhibitors leupeptin, aprotinin, and pepstatin A) were added to the cellular pellet and incubated for 30 min in ice. Whole cell extracts were obtained by centrifugation for 10 min at 14000 rpm at 4°C, and protein concentration was determined by BCA method (Pierce). Total protein (30  $\mu$ g) was separated in a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (BioRad). The membranes were blocked in TBS-0.1% Tween 20 with 5% of skim milk, washed twice with TBS-0.1% Tween 20, and incubated with specific Abs for 1 h at room temperature. Then, membranes were washed three times and incubated with a secondary Ab for 1 h at room temperature. After extensive washing, ECL detection was performed according to the manufacturer's instructions (Amersham).

### Transient transfection

RAW 264.7 cells were transiently transfected by using LipofectAmine Plus Reagent (Invitrogen Life Technologies) following the manufacturer's instructions. For TNF- $\alpha$  promoter constructs, 250 ng of DNA per  $10^6$  cells were used. In cotransfection experiments, IRF-1 (pAct1), IRF-2 (pAct2), and IRF-8 (pUE5) expression vectors were used at 25–100 ng/ $10^6$  cells as indicated, and total DNA concentration was kept constant with the corresponding empty vectors (pActC and LK440). Sixteen hours after transfection, cells were treated with LPS (1  $\mu$ g/ml) or/and IFN- $\gamma$  (2.5 ng/ml) for additional 6 h (see Figure legends). pRL-TK-luc (Promega) was used for determining transfection efficiency by measuring *Renilla luciferase* activity in the samples. Luciferase activity is thus represented as relative luciferase units firefly/relative luciferase units renilla.

### Determination of intracellular TNF- $\alpha$ by flow cytometry

RAW 264.7 cells were nucleofected using the Cell Line Nucleofector Kit V and the program D-32, following the manufacturer's instructions (Amaxa Biosystems). The efficiency of nucleofection was around 40% as determined by analysis of GFP expression by flow cytometry. Immunofluorescence staining of intracellular TNF- $\alpha$  in nucleofected RAW 264.7 cells was performed by using BD Cytfix/BD Cytoperm Plus with BD GolgiStop (BD Biosciences). Sixteen hours after nucleofection, RAW 264.7 cells were incubated with GolgiStop for additional 4 h. Then immunofluorescence staining with phycoerythrin-conjugated anti-mouse TNF- $\alpha$  mAb (BD Pharmingen) was conducted by following the general procedure



**FIGURE 1.** IFN- $\gamma$  treatment induces TNF- $\alpha$  expression in RAW 264.7 cells. *A*, RAW 264.7 macrophages were stimulated with IFN- $\gamma$  (2.5 ng/ml) in a time-course assay and TNF- $\alpha$  release to supernatants was measured by ELISA. *B*, Analysis of TNF- $\alpha$  production in RAW 264.7 cells treated with LPS (1  $\mu$ g/ml) and IFN- $\gamma$  (2.5 ng/ml) for 16 h. *C*, TNF- $\alpha$  mRNA levels were determined by quantitative RT-PCR. See *Materials and Methods* for details.

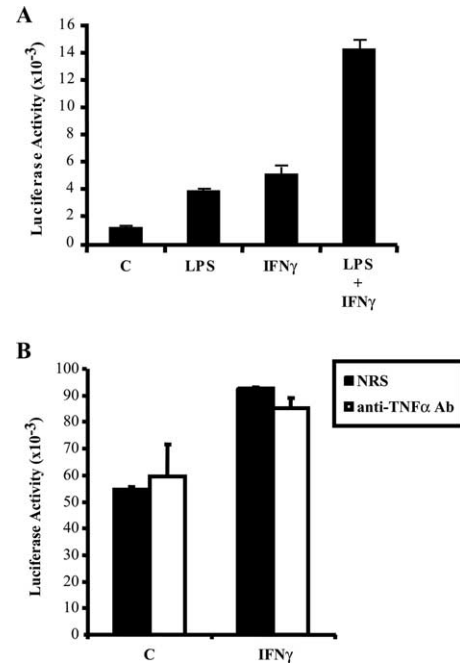
suggested by the manufacturer. Results were analyzed in a FACSCalibur flow cytometer (BD Biosciences).

#### Small interfering RNA (siRNA) transfection

RAW 264.7 cells were transiently transfected with siRNA by using Lipofectamine 2000, following the manufacturer's instructions for siRNA transfection. The day before transfection, cells were plated at 50% of confluence in RPMI 1640 without FCS and antibiotics. After transfection, the medium was replaced with OptiMEM (Invitrogen), and cells were exposed to a mixture of Lipofectamine 2000 and 100 nM of IRF-1 and/or IRF-8 siRNA, for 6 h. Then, medium was replaced with RPMI 1640 supplemented with 5% FCS and antibiotics. Forty-two hours after transfection, RAW 264.7 cells were treated with IFN- $\gamma$  (2.5 ng/ml) for additional 6 h. Whole cell extracts were obtained following the protocol described before, and IRF-1 and IRF-8 silencing was determined by Western blot. Supernatants of transfected cells were assayed for TNF- $\alpha$  production by ELISA technique. The Silencer Predesigned siRNA ID: 154907 and ID: 158209 from Ambion (Applied Biosystems) were used for silencing IRF-1 and IRF-8 expression, respectively.

#### Statistical analysis

Values in figures are expressed as mean  $\pm$  SD of at least three independent experiments in duplicate, unless otherwise specified. Student's two-tailed *t* test was used to compare means between groups. A value of *p* < 0.05 was considered to be statistically significant.



**FIGURE 2.** TNF- $\alpha$  expression is regulated at transcriptional level by IFN- $\gamma$ , independently of endogenous TNF- $\alpha$ . *A*, RAW 264.7 cells were transiently transfected with human TNF- $\alpha$  promoter. Sixteen hours after transfection, cells were treated with LPS (1  $\mu$ g/ml) and/or IFN- $\gamma$  (2.5 ng/ml) for additional 6 h, and luciferase activity was determined. Representative experiment from five experiments with similar outcomes are shown. *B*, RAW 264.7 cells were transiently transfected with pTNF (1311) vector. Sixteen hours after transfection, cells were stimulated with medium (C) or IFN- $\gamma$  (2.5 ng/ml), in presence of anti-TNF- $\alpha$  (anti-TNF- $\alpha$  Ab) neutralizing Ab (1  $\mu$ g/ml) or normal rabbit serum (NRS). Luciferase activity was determined 6 h later. A representative experiment is shown from three independent assays with similar outcomes.

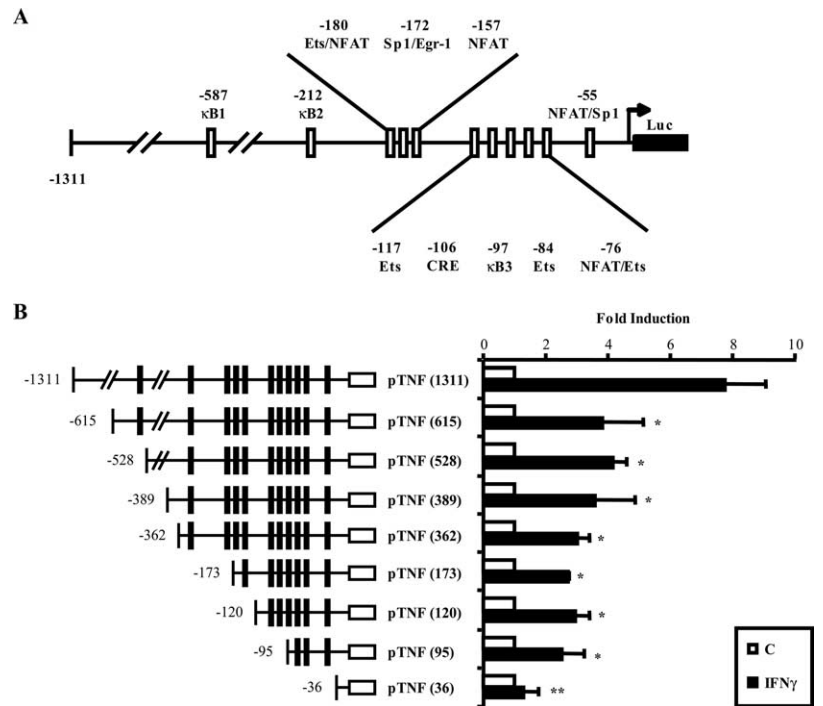
## Results

### IFN- $\gamma$ induces TNF- $\alpha$ expression in the murine macrophage cell line RAW 264.7 at transcriptional level

We have previously shown that IFN- $\gamma$  induces COX-2 and iNOS expression in macrophages (35, 36). This induction was absolutely dependent of the endogenous production of TNF- $\alpha$  elicited by IFN- $\gamma$  treatment, because primary peritoneal macrophages from TNF- $\alpha$ -deficient mice were unable to express COX-2 or iNOS by IFN- $\gamma$  stimulation (35, 36). To study in depth the regulation of TNF- $\alpha$  expression by IFN- $\gamma$  treatment, we first analyzed the TNF- $\alpha$  release to supernatants of RAW 264.7 cells treated with IFN- $\gamma$  in a time-course assay (Fig. 1*A*). RAW 264.7 cells produced some TNF- $\alpha$  in absence of stimulation. However, IFN- $\gamma$  treatment continuously increased TNF- $\alpha$  levels released to the supernatant from 8 to 24 h (last time tested). TNF- $\alpha$  production elicited by IFN- $\gamma$  was lower than that produced by LPS stimulation for 16 h (Fig. 1*B*). When TNF- $\alpha$  mRNA expression was determined by quantitative RT-PCR (Fig. 1*C*), we found that TNF- $\alpha$  mRNA quickly increases upon IFN- $\gamma$  treatment, being significantly different from the untreated cells at 30 min of stimulation. TNF- $\alpha$  mRNA increased up to 4 h of stimulation, when mRNA levels begin to decrease and reach basal level of expression after 16 h of IFN- $\gamma$  treatment.

The above results suggest that TNF- $\alpha$  was induced at the transcriptional level. To corroborate this hypothesis, RAW 264.7 cells were transiently transfected with the pTNF(-1311) vector, which contains the luciferase reporter gene under the control of human

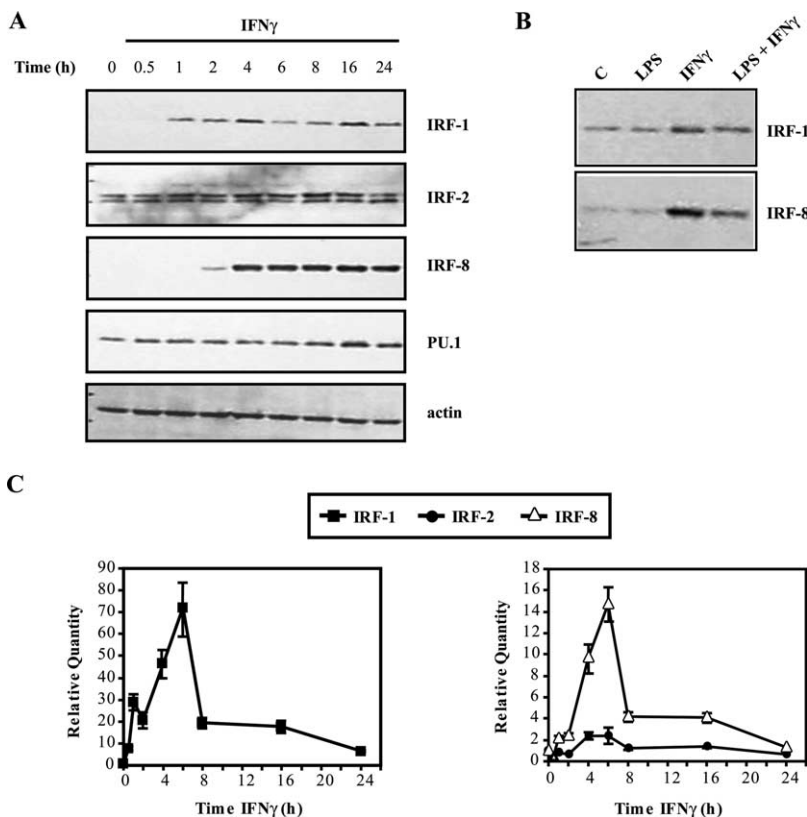
**FIGURE 3.** Two different regions are involved in TNF- $\alpha$  transcriptional regulation by IFN- $\gamma$ . *A*, Illustration for transcription factors binding sites which have been described in the TNF- $\alpha$  promoter. *B*, Transient transfection experiments with different deletion constructs of the human TNF- $\alpha$  promoter, in RAW 264.7 cells. Sixteen hours after transfection, cells were treated with IFN- $\gamma$  (2.5 ng/ml) for an additional 6 h. The result showed in figure is the mean of three different experiments. \*,  $p < 0.05$ ; \*\*,  $p \leq 0.001$ ; in respect to the IFN- $\gamma$  response showed by complete TNF- $\alpha$  promoter construction.



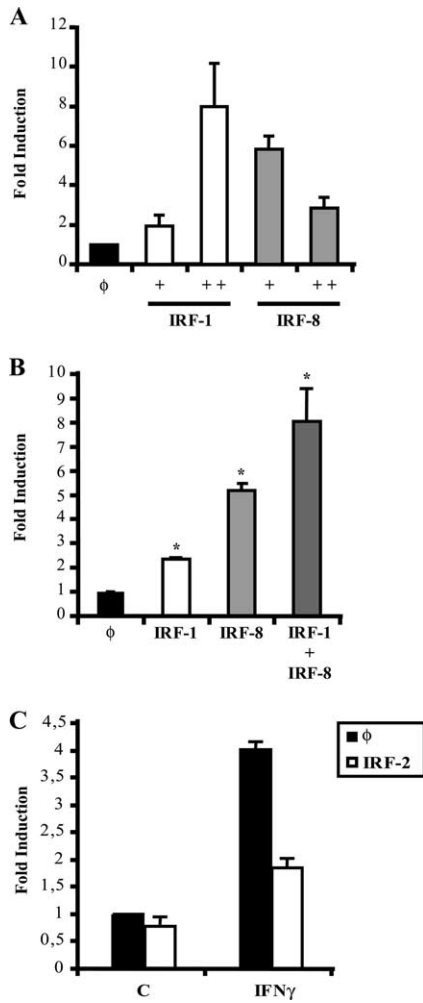
TNF- $\alpha$  promoter. Cells were stimulated with IFN- $\gamma$  and/or LPS as a control of transcriptional activation. As shown in Fig. 2A, IFN- $\gamma$  treatment produced an increase in luciferase activity similar to the one induced by LPS treatment. Combination of both stimuli resulted in a cooperative increment in luciferase activity. This result indicates that IFN- $\gamma$  is inducing TNF- $\alpha$  expression at the transcriptional level. In addition, this induction was not due to an autocrine effect of endogenously produced TNF- $\alpha$ , because cell treatment

with neutralizing anti-TNF- $\alpha$  Ab did not inhibit TNF- $\alpha$  promoter activity induced by IFN- $\gamma$  (Fig. 2B).

Different transcription factor binding sites have been described along the human TNF- $\alpha$  promoter (Fig. 3A). To elucidate the region(s) of the TNF- $\alpha$  promoter responsible for the transcriptional regulation by IFN- $\gamma$ , we tested different deletion constructs from the human TNF- $\alpha$  promoter in transient transfection assays in RAW 264.7 cells (Fig. 3B). When the region between positions



**FIGURE 4.** IFN- $\gamma$  treatment induces IRF expression in RAW 264.7 macrophages. RAW 264.7 cells were treated with IFN- $\gamma$  (2.5 ng/ml) for different timepoints. *A*, IRF-1, IRF-8, IRF-2, and PU.1 protein levels were determined by Western blot. *B*, Study of IRF-1 and IRF-8 expression induced by different stimuli. RAW 264.7 macrophages were treated with LPS (1  $\mu$ g/ml), IFN- $\gamma$  (2.5 ng/ml), or both for 6 h, and IRF-1 and IRF-8 expression was analyzed by Western blot. *C*, IRF-1 (left panel), IRF-8, and IRF-2 (right panel) mRNA levels were analyzed by quantitative RT-PCR.

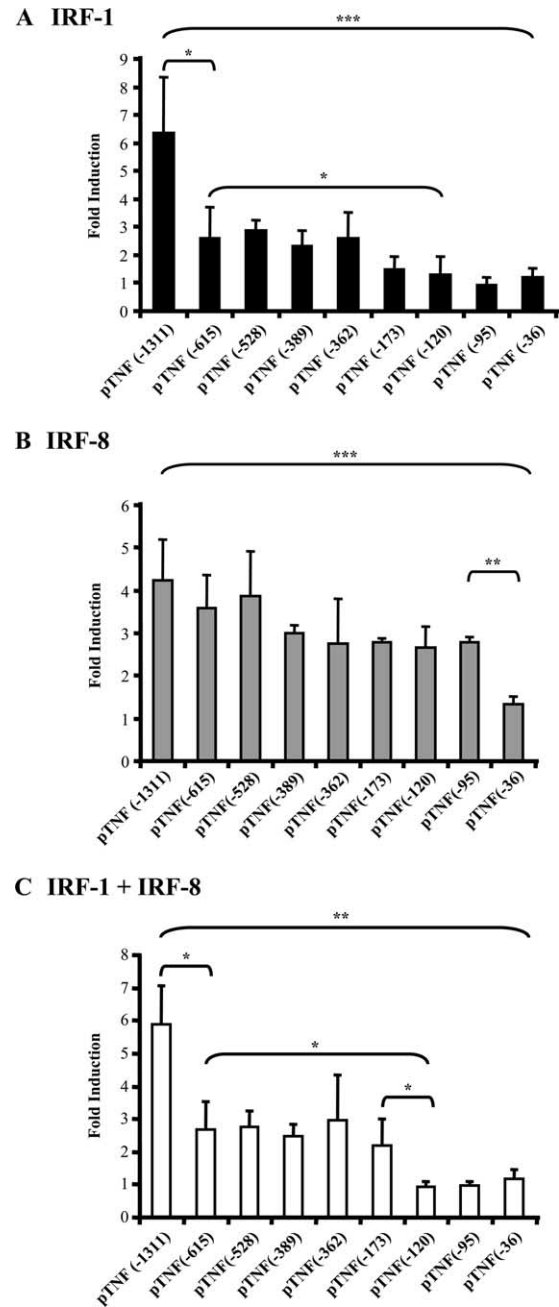


**FIGURE 5.** IRF-1 and IRF-8 overexpression induces TNF- $\alpha$  promoter activity. *A*, RAW 264.7 cells were transiently transfected with different doses of IRF-1 or IRF-8 expression vectors or their corresponding empty vectors ( $\phi$ ), and human TNF- $\alpha$  promoter construct. Luciferase activity was determined 24 h after transfection. Representative experiment from five with similar results are shown. +, 25 ng vector/ $10^6$  cells; ++, 100 ng vector/ $10^6$  cells. *B*, Analysis of the effects of IRF-1 and/or IRF-8 overexpression (25 ng vector/ $10^6$  cells, each) on TNF- $\alpha$  promoter activity. The results shown are the mean of three independent experiments. \*,  $p < 0.05$ , TNF- $\alpha$  transcriptional activity elicited by IRFs, respect to the response induced by control empty vectors ( $\phi$ ). *C*, RAW 264.7 cells were transiently transfected with IRF-2 expression vector (100 ng/ $10^6$  cells) or its corresponding empty construct ( $\phi$ ), and the human TNF- $\alpha$  promoter. Sixteen hours after transfection, cells were stimulated with IFN- $\gamma$  (2.5 ng/ml) and luciferase activity was determined 6 h later. A representative experiment is shown from three independent assay with similar outcomes.

-1311/-615 bp was deleted, IFN- $\gamma$ -induced transcriptional activity was strongly diminished (around 50%), suggesting that this region was important for TNF- $\alpha$  transcriptional regulation by IFN- $\gamma$ . Successive deletions up to -95 bp have little, and not statistically significant, effect on IFN- $\gamma$  induced transcriptional activity. However, deletion of the region between positions -95/-36 bp, returned the transcriptional activation to the basal levels. These results suggested that these two regions are important for IFN- $\gamma$ -induced TNF- $\alpha$  transcriptional activity in RAW 264.7 cells.

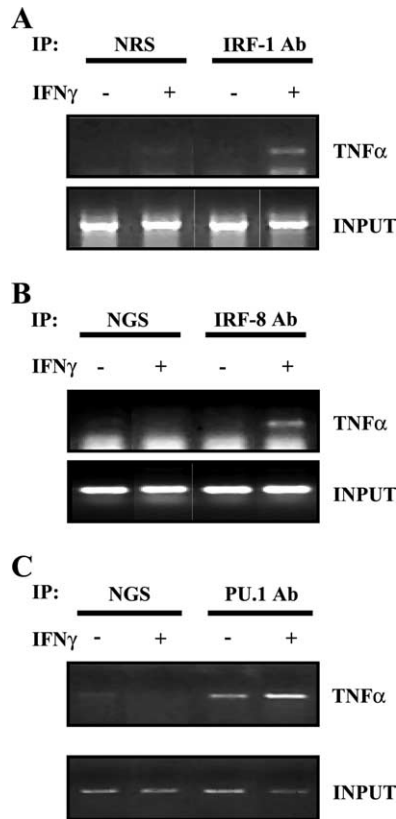
*IFN- $\gamma$  treatment induces IRF-1 and IRF-8 expression in RAW 264.7 macrophages*

IRF-1, IRF-2, and IRF-8 have been implicated in the transcriptional regulation of several genes, such as IL-12, IL-1 $\beta$  or COX-2,



**FIGURE 6.** Determination of IRF-1 and IRF-8 responsive regions of the TNF- $\alpha$  promoter. RAW 264.7 macrophages were transiently transfected with IRF-1 (*A*), IRF-8 (*B*), or both (*C*) (25 ng vector/ $10^6$  cells, each), and different deletion constructs of human TNF- $\alpha$  promoter. Luciferase activity was determined 24 h after transfection. Mean of three independent experiments performed. Transcriptional activity elicited by IRF overexpression was compared between the different deletions of TNF- $\alpha$  promoter. \*,  $p < 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ .

in response to IFN- $\gamma$  (2, 17, 22). To analyze whether IRF-1 and/or IRF-8 could be involved in TNF- $\alpha$  transcription, we first tested whether IRF-1 and IRF-8 could be induced in RAW 264.7 cells by IFN- $\gamma$  treatment. For this, we performed Western blot assays to study the expression of these transcription factors. As shown in Fig. 4*A*, IRF-1 expression was quickly induced (after 1 h of IFN- $\gamma$  stimulation), and increased in a time dependent manner up to 4 h of treatment. A small decrease in IRF-1 protein levels was observed at 6 h of treatment, but the expression was recovered at 16 h of stimulation. By contrast, IRF-2 was expressed in absence of



**FIGURE 7.** IRF-1, IRF-8, and PU.1 are recruited to the TNF- $\alpha$  promoter in vivo. RAW 264.7 macrophages were treated with medium or IFN- $\gamma$  (2.5 ng/ml) for 4 h, and after cross-linking with formaldehyde for 30 min, ChIP assay was performed (see *Materials and Methods* for details). IRF-1 (A), IRF-8 (B), and PU.1 (C) immunoprecipitation demonstrates the recruitment of these transcription factors to the TNF- $\alpha$  promoter. NRS, normal rabbit serum; NGS, normal goat serum.

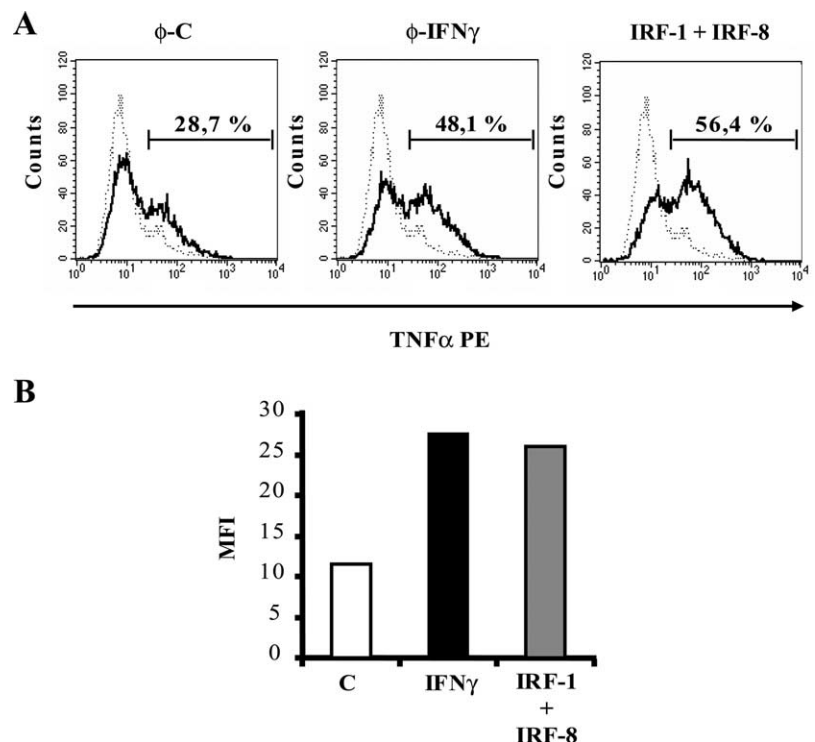
stimulation and its levels did not significantly change upon IFN- $\gamma$  treatment (Fig. 4A). IRF-8 protein expression was first detected after 2 h of IFN- $\gamma$  treatment, reaching the maximal expression at 4 h of stimulation. IRF-8 protein levels were maintained up to 24 h of IFN- $\gamma$  treatment (last time tested). Nevertheless, neither IRF-1, nor IRF-8 expression was induced by LPS treatment (Fig. 4B). PU.1 is a transcription factor of the Ets family (49), which has been described as an important partner of IRF-1 and/or IRF-8 in the transcriptional regulation of several genes, such as IL-1 $\beta$  or NADPH phagocyte oxidase (21, 22). For this reason, we analyzed the expression of PU.1 by Western blot in RAW 264.7 cells treated with IFN- $\gamma$ . As shown in Fig. 4A, PU.1 was constitutively expressed in RAW 264.7 macrophages, and IFN- $\gamma$  treatment did not induce any significant increase in PU.1 protein levels.

To confirm those data at the mRNA level, the expression of IRF-1, IRF-2, and IRF-8 mRNA was analyzed by quantitative RT-PCR. As shown in Fig. 4C, IRF-1 mRNA was rapidly expressed in response to IFN- $\gamma$  (30 min). Moreover, the expression of its mRNA was increased 70-fold approximately, at 6 h of IFN- $\gamma$  treatment (maximal expression). IRF-8 mRNA expression was increased after 2 h of treatment in agreement with protein data, and also reached the maximum level at 6 h (15-fold increase) (Fig. 4C). After 8 h of treatment, IRF-1 and IRF-8 mRNA levels strongly decreased and returned to basal expression after 24 h of IFN- $\gamma$  stimulation. IRF-2 mRNA was already detected in unstimulated RAW 264.7 cells and its level remained unchanged along the time tested (Fig. 4C). These results clearly demonstrate that IFN- $\gamma$  treatment induces IRF-1 and IRF-8 expression in RAW 264.7 macrophages, in agreement with previous reports (6, 50, 51).

#### *IRF-1 and IRF-8 induce transcriptional activity of TNF- $\alpha$ promoter*

Next, we addressed whether IRF-1 and IRF-8 transcription factors could be involved in the transcriptional regulation of TNF- $\alpha$  expression by IFN- $\gamma$ . First, RAW 264.7 cells were transiently co-transfected with pTNF (1311) promoter vector and increasing

**FIGURE 8.** IRF-1 and IRF-8 overexpression increases TNF- $\alpha$  synthesis by RAW 264.7 macrophages. RAW 264.7 cells were nucleofected with IRF-1 and IRF-8 expression vectors (100 ng/10<sup>6</sup> cells, each) or with the corresponding empty vectors ( $\phi$ ). Sixteen hours after nucleofection, cells were treated with or without IFN- $\gamma$  (2.5 ng/ml), and with GolgiStop to block intracellular trafficking, for an additional 4 h. TNF- $\alpha$  production was analyzed by flow cytometry. A, Percentage of RAW 264.7 cells which are producing TNF- $\alpha$ .  $\phi$ , cells nucleofected with empty vectors and unstimulated (C) or stimulated with IFN- $\gamma$ . IRF-1 plus IRF-8 indicates RAW 264.7 cells nucleofected with IRF-1 and IRF-8 expression vectors in absence of stimulation. B, Changes in mean fluorescence intensity (MFI) of TNF- $\alpha$ -producing cells, elicited by IFN- $\gamma$  stimulation or IRF-1 and IRF-8 overexpression.

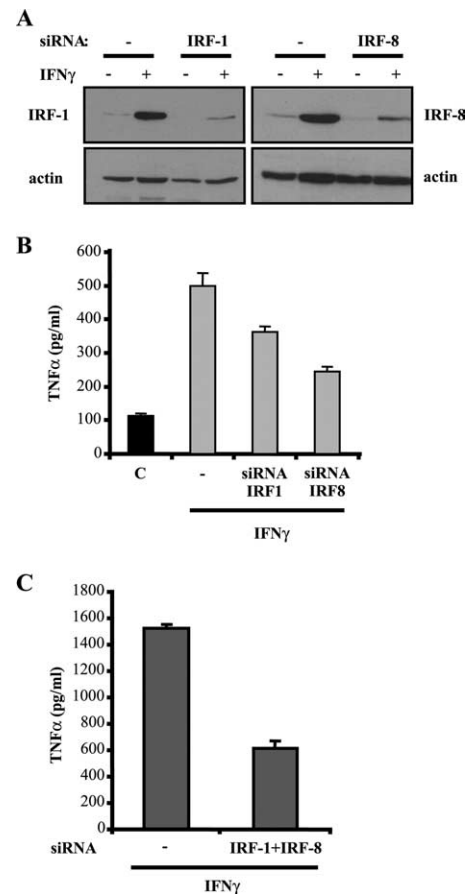


amounts of either IRF-1 or IRF-8 expression vectors or their control empty vectors. As shown in Fig. 5A, TNF- $\alpha$  transcriptional activity was induced by IRF-1 expression in a dose-dependent manner, and reached the maximal transactivation with the highest concentration of IRF-1 used. Although IRF-8 induced TNF- $\alpha$  promoter activity in the same way that IRF-1, a dose response effect in TNF- $\alpha$  promoter transactivation was not observed. To analyze whether IRF-1 and IRF-8 exert a coordinate action on TNF- $\alpha$  transcriptional activity, we cotransfected both factors, at the lowest doses, in RAW 264.7 macrophages, and determined their effect on TNF- $\alpha$  promoter. As shown in Fig. 5B, cotransfection of IRF-1 and IRF-8 produced an additive increase in luciferase activity. In contrast, IRF-2 is a repressor of transcription elicited by active members of the IRFs family (6). IRF-2 overexpression has no effect by itself on basal promoter activity, but diminished in  $\sim 60\%$  the transactivation of TNF- $\alpha$  promoter induced by IFN- $\gamma$  treatment Fig. 5C. This result indirectly confirms the involvement of IRFs in IFN- $\gamma$ -induced TNF- $\alpha$  transcriptional activity.

To investigate the region(s) of the TNF- $\alpha$  promoter responsible for the activation by IRF-1 and/or IRF-8, different deletion constructs of the TNF- $\alpha$  promoter were cotransfected with IRF-1 and/or IRF-8 expression vectors in RAW 264.7 macrophages. Deletion of the first region significantly decreased IRF-1 inducibility and further deletion of the region between  $-173/-120$  bp, completely abrogated IRF-1 inducibility (Fig. 6A). Thus, there were two main regions responsive to IRF-1 overexpression: the region between positions  $-1311/-615$  bp, and the region between positions  $-173/-120$  bp. In contrast, IRF-8 binding site must be present in the 60 nucleotides upstream of the minimal TNF- $\alpha$  promoter, because the induction by IRF-8 remained essentially similar up to deletion of position  $-95$ , disappearing in deletion from  $-95$  to  $-36$  bp (Fig. 6B). When IRF-1 and IRF-8 were coexpressed, the induction of the different deletion constructs was closely similar to the one observed with IRF-1. Thus, there was a strong decrease in transcriptional activity when the region between  $-1311$  and  $-615$  bp was eliminated, and the deletion between  $-173$  and  $-120$  bp completely abrogated TNF- $\alpha$  promoter activity (Fig. 6C). These results suggest that the important role of IRF-8 in the regulation of TNF- $\alpha$  transcription likely results from its cooperation with IRF-1.

#### IRF-1 and IRF-8 are recruited to the TNF- $\alpha$ promoter *in vivo*

From the results presented above, it is likely that IRF-1 and IRF-8 may drive TNF- $\alpha$  transcription in response to IFN- $\gamma$  treatment. Because those results were obtained from overexpression experiments, we next investigate whether IRF-1 and IRF-8 were bound to the TNF- $\alpha$  promoter in cells treated with IFN- $\gamma$ . For this, RAW 264.7 macrophages were stimulated with IFN- $\gamma$  for 4 h and binding of IRF-1 or IRF-8 to the TNF- $\alpha$  promoter was determined by ChIP assay. In absence of stimulation, neither IRF-1 (Fig. 7A) nor IRF-8 (Fig. 7B) binds to the TNF- $\alpha$  promoter, whereas the treatment with IFN- $\gamma$  induced the recruitment of IRF-1 and IRF-8 to the promoter (Fig. 7, A and B, respectively). Control normal rabbit serum or normal goat serum did not immunoprecipitate any factor bound to the TNF- $\alpha$  promoter, indicating that the IRF-1 and IRF-8 recruitment was specific and took place *in vivo*. Because PU.1 acts as a partner of IRF-1 and IRF-8 in the transcriptional activation of several genes, such as IL-1 $\beta$  or NADPH oxidase (21, 22), and there are several Ets response elements along the TNF- $\alpha$  promoter, we studied whether PU.1 was recruited to the TNF- $\alpha$  promoter *in vivo*. As before, RAW 264.7 cells were treated with IFN- $\gamma$  for 4 h, and PU.1 binding to the TNF- $\alpha$  promoter was analyzed by ChIP assay. As shown in Fig. 7C, PU.1 was bound to the TNF- $\alpha$  promoter in absence of stimulation, although treatment with IFN- $\gamma$  increased the amount of PU.1 recruited to the promoter, suggesting



**FIGURE 9.** IRF-1 and IRF-8 inhibition by siRNA transfection decreases TNF- $\alpha$  production. RAW 264.7 cells were transfected with siRNAs (100 nM) for IRF-1 and IRF-8. Forty-two hours after transfection, cells were stimulated with IFN- $\gamma$  for additional 6 h. A, IRF-1 and IRF-8 expression was analyzed by Western blot (B and C). Effect of IRF-1 or IRF-8 silencing on TNF- $\alpha$  production in RAW 264.7 cells. TNF- $\alpha$  release to supernatants from RAW 264.7 cells transiently transfected with IRF-1 and IRF-8 siRNAs or both. Representative experiments from three independent assays with similar outcomes are shown.

that this transcription factor plays a role in TNF- $\alpha$  transcription induced by IFN- $\gamma$ , likely due to its known ability to cooperate with IRF-1 and IRF-8.

#### IRF-1 and IRF-8 regulate TNF- $\alpha$ production

To demonstrate that IRF-1 and IRF-8 were involved in TNF- $\alpha$  production, RAW 264.7 cells were nucleofected with IRF-1 and IRF-8 expression vectors or its corresponding control empty vectors, and intracellular TNF- $\alpha$  was measured by flow cytometry. As shown in Fig. 8, 28.7% of unstimulated RAW 264.7 cells were expressing TNF- $\alpha$ . When cells were treated with IFN- $\gamma$ , an increase of 20% in TNF- $\alpha$  expressing cells was observed. In addition, when RAW 264.7 cells were nucleofected with IRF-1 and IRF-8, TNF- $\alpha$  expressing cells increased in  $\sim 28\%$ , even in absence of stimulation, closely resembling the 30–40% of nucleofected cells in those cultures (data not shown). Moreover, overexpression of IRF-1 and IRF-8 produced an increase in the mean fluorescence intensity, to the same extent as the one induced by IFN- $\gamma$  treatment (Fig. 8B). Those results suggest that IRF-1 and IRF-8 were sufficient for triggering TNF- $\alpha$  synthesis in macrophages.

Next, we analyzed whether IRF-1 and IRF-8 inhibition affected TNF- $\alpha$  production. For this, we used siRNAs to decrease IRF-1



and IRF-8 expression in RAW 264.7 cells. As shown in Fig. 9A, IFN- $\gamma$  induced the synthesis of IRF-1 and IRF-8, and the transfection of siRNA specific for IRF-1 or IRF-8 diminished the expression levels of these transcription factors in response to IFN- $\gamma$ . The inhibition of IRF-1 expression with siRNAs decreased IFN- $\gamma$ -induced TNF- $\alpha$  production, analyzed by ELISA, around ~30%, whereas, IRF-8 silencing diminished TNF- $\alpha$  release by IFN- $\gamma$  in ~50% (Fig. 9B). When RAW 264.7 cells were cotransfected with both IRF-1 and IRF-8 siRNAs, TNF- $\alpha$  production induced by IFN- $\gamma$  was diminished in ~60% (Fig. 9C). Altogether, the above results corroborate that IRF-1 and IRF-8 play an important role in regulating TNF- $\alpha$  expression in response to IFN- $\gamma$ .

## Discussion

IFN- $\gamma$  is a pleiotropic cytokine involved in promoting the inflammatory response, as a consequence of tissue damage or infection by microorganisms. In macrophages, this cytokine induces the expression of a wide variety of genes coding for proinflammatory mediators, cytokines, or molecules involved in Ag presentation, such as iNOS, COX-2, NADPH phagocyte oxidase, IL-12, IL-1 $\beta$ , CD40, etc. (2, 17, 23, 35, 52). In several of those cases, it has been reported that IFN- $\gamma$  exerts this effect indirectly, through a mechanism that involves the synthesis of another cytokine, which acts in an autocrine manner to produce optimal gene expression. That is the case of COX-2 and iNOS expression induced by IFN- $\gamma$ . We have previously reported that IFN- $\gamma$ -induced TNF- $\alpha$  is absolutely required for COX-2 and iNOS induction by IFN- $\gamma$  (35, 36). This mechanism also takes place in the regulation of other genes in response to IFN- $\gamma$ , such as CD40 or high mobility group B 1 (53, 54). Because endogenous TNF- $\alpha$  seems to be a very important molecule for the optimal expression of proinflammatory genes in response of IFN- $\gamma$ , the aim of this work was to elucidate the molecular mechanism by which IFN- $\gamma$  is regulating TNF- $\alpha$  expression.

A previous report described that IFN- $\gamma$  treatment induces TNF- $\alpha$  expression in the murine macrophage cell line RAW 264.7 at the transcriptional level, as demonstrated by run-on assays (24). However, to our knowledge, there is no report in which the transcriptional regulation of TNF- $\alpha$  by IFN- $\gamma$  has been studied. In this study, we have found that IFN- $\gamma$  treatment induced TNF- $\alpha$  expression at protein and mRNA levels. Transcriptional studies with human TNF- $\alpha$  promoter, revealed that IFN- $\gamma$  induced transcriptional activation as well as LPS stimulation, which is thought to be a main inducer of TNF- $\alpha$  transcription in macrophages. By deletion analysis of the promoter, we have identified two regions that seem to be mainly responsible for IFN- $\gamma$ -induced transactivation. The first region (distal region) was delimited between positions -1311 and -615 bp, because its deletion causes a decrease of 40–50% approximately in IFN- $\gamma$ -induced TNF- $\alpha$  promoter activity. The remaining transcriptional activity was completely abolished when region comprised between -95 and -36 bp (proximal region) was deleted. These results suggest that distal region may act as an enhancer region necessary for the optimal expression of the TNF- $\alpha$  gene by IFN- $\gamma$ . This is a common feature of IFN- $\gamma$ -regulated genes that contain enhancer regions located upstream of the transcriptional initiation site regulating the transcriptional responses (2, 55). Commonly, response elements for IFN- $\gamma$ -regulated transcription factors can be found in these enhancer regions, including binding sites for IRFs.

Among the genes induced by IFN- $\gamma$ , IRF-1 and IRF-8 have been described as responsible for the control of the expression of several cytokines including IL-12, IL-1 $\beta$ , or IL-18 (17, 22, 56). Another example of genes regulated by IRF-1 and/or IRF-8 are iNOS, COX-2, or NADPH phagocyte oxidase (gp91<sup>phox</sup>, p67<sup>phox</sup>), proin-

flammatory genes involved in the inflammatory response elicited by IFN- $\gamma$  (2, 10, 19–21). Transcriptional regulation of those genes in response to IFN- $\gamma$  treatment has been found to be complex and involves the participation of other transcription factors like PU.1, a myeloid-specific transcription factor that regulates gene expression by interacting with IRF-4/IRF-8 and IRF-1 transcription factors.

We have found in this study that in RAW 264.7 macrophages, IRF-1 and IRF-8 synthesis is induced by IFN- $\gamma$  in a time-dependent manner, whereas IRF-2 expression does not change with IFN- $\gamma$  treatment. In addition, PU.1 is constitutively expressed in RAW 264.7 cells, and its expression is not regulated by IFN- $\gamma$ . Our results clearly demonstrate that IRF-1 and IRF-8 regulate TNF- $\alpha$  gene expression induced by IFN- $\gamma$ : first, overexpression of IRF-1 and IRF-8 increase transcriptional activity of TNF- $\alpha$  promoter. Second, IRF-2 (described as the main repressor factor of the IRF family) inhibits IFN- $\gamma$ -induced TNF- $\alpha$  transcriptional activity. Third, IRF-1 and IRF-8 are both recruited to the TNF- $\alpha$  promoter in vivo, as demonstrated by ChIP experiments. Fourth, IRF-1 and IRF-8 nucleofection causes the induction of endogenous TNF- $\alpha$  production, measured by flow cytometry. And last, the inhibition of IRF-1 and IRF-8 expression by using specific siRNAs, produces an important decrease in TNF- $\alpha$  synthesis induced by IFN- $\gamma$  treatment.

Our results suggest that the effect of IFN- $\gamma$  on TNF- $\alpha$  transcription seems to require the combined action of IRF-1/IRF-8, as well as PU.1, because recruitment of this factor to the TNF- $\alpha$  promoter in vivo was increased by IFN- $\gamma$  treatment. IRF-1 probably has two binding sites: one located in the region between -1311 and -615 bp, and another one located in the region between -173 and -120 bp. In contrast, IRF-8 seems to act on the region between -95 and -36 bp. Taken together, these data suggest that the IFN- $\gamma$  response may be driven by IRF-1 bound to the distal region acting as an enhancer, and the cooperation between IRF-1 bound to the intermediate region (-173/-120 bp), and IRF-8 bound to the proximal region (-95/-36 bp). In the last case, PU.1 might play an important role by increasing the transactivation of TNF- $\alpha$  promoter, through the cooperation of IRF-1 with IRF-8. Combined action of IRF-1 and IRF-8 (as well as PU.1) have been previously described in the induction of RANTES (57) and iNOS (19, 58) by IFN- $\gamma$ . Interestingly, TNF- $\alpha$  transcriptional activity induced by IRF-1 and IRF-8 is inhibited when the region between -173 and -120 bp was deleted. This result suggests that IRF-1 binding to the TNF- $\alpha$  promoter is absolutely necessary for the response elicited by cooperation between IRF-1 and IRF-8.

IRF-1-deficient mice are resistant to LPS-lethal dose, as a consequence of a defect in TNF- $\alpha$  production and serum and hepatic TNF- $\alpha$  and IFN- $\gamma$  levels were diminished in IRF-1 deficient mice, compared with the wild-type mice (59). By contrast, TNF- $\alpha$  production by splenocytes from the same mice was not affected by IRF-1 absence. The same results were observed regarding IL-12 p40 expression, which is also regulated by the coordinated action of IRF-1 and IRF-8 (17). Senaldi et al. concluded that IRF-1 deficiency protects against LPS-induced mortality by impairing the production of the proinflammatory cytokines TNF- $\alpha$  and IFN- $\gamma$  (59). However, discrepancies between results obtained in serum TNF- $\alpha$  levels and spleen-produced TNF- $\alpha$  in the above mentioned work may be explained by the effect of IRF-8 on TNF- $\alpha$  expression that we describe in this study, because IRF-8 has a myeloid-restricted expression and may be responsible of the TNF- $\alpha$  production in spleen in absence of IRF-1.

In contrast, studies with IRF-8-deficient mice also support the involvement of this transcription factor on TNF- $\alpha$  regulation. Thus, J. Zhao et al. (60) described that IRF-8 knock-out mice

show defects in the production of several cytokines in response to LPS and IFN- $\gamma$  treatment. In addition, protection against infection with *Listeria monocytogenes* is highly dependent of TNF- $\alpha$  and IFN- $\gamma$  synthesis (15). In this regard, it has been shown that IRF-1 and IRF-8 knock-out mice are unable to control the *L. monocytogenes* infection, suggesting an important role of these transcription factors in the mechanisms involved in clearing up this infection (9, 15).

In the TNF- $\alpha$  promoter, several binding sites for different transcription factors such as AP-1, NF $\kappa$ B, or NFAT have been described (37–43). However, we have been unable to detect activation of these transcription factors in RAW 264.7 cells by IFN- $\gamma$  at the time at which we have detected TNF- $\alpha$  induction by IFN- $\gamma$  (data not shown) (36). However, additional studies are necessary to determine the role of PU.1 and its cooperation with IRF-1 and IRF-8 on IFN- $\gamma$ -induced TNF- $\alpha$  expression.

In summary, our work demonstrates that TNF- $\alpha$  expression induced by IFN- $\gamma$  is regulated at the transcriptional level. This transcriptional regulation by IFN- $\gamma$  is the result of the coordinated recruitment of the transcription factors IRF-1 and IRF-8, and possibly PU.1, on the TNF- $\alpha$  promoter, in a similar way as it has been described for another inflammatory cytokines or chemokines, such as IL-12, IL-1 $\beta$ , or RANTES. It is plausible that it may represent a common mechanism of regulation of the IFN- $\gamma$  response, in which the cooperation between IRF-1 and IRF-8 is essential for promoting gene expression.

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## Disclosures

The authors have no financial conflict of interest.

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