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Involvement of TNF and NF-κB in the Transcriptional Control of Cyclooxygenase-2 Expression by IFN-γ in Macrophages[1]

Virginia Vila-del Sol and Manuel Fresno[2]

IFN-γ induces cyclooxygenase (COX)-2 expression and PG production in mouse macrophage cells. IFN-γ activates COX-2 promoter-driven transcription. Deletion of the IFN sequence regulatory element (ISRE) I −1541/−1522 and ISRE II −1215/−1206 sites of the mouse COX-2 promoter minimally decrease this IFN-γ induction. In contrast, deletion of the −965/−150 region from the COX-2 promoter abrogated IFN-γ induction. In this region a NF-κB site has been described and mutation of this site impairs the induction of the full COX-2 promoter by IFN-γ. Moreover, IFN-γ induction of the COX-2 promoter was also strongly reduced by transfection of plasmid encoding the NF-κB inhibitor, IκBα. Interestingly, IFN-γ induction of the COX-2 and PGE_2 synthesis was absent in macrophages from $TNF^{-/-}$ mice, and neutralizing anti-TNF Abs inhibited COX-2 promoter induction by IFN-γ in RAW 264.7 macrophages. Moreover, NF-κB activity was induced late after stimulation with IFN-γ correlating with the effect of autocrine TNF, and this NF-κB activation was absent in macrophages from $TNF^{-/-}$ mice. Taken together our results suggest a model in which IFN-γ-induced TNF activates NF-κB, which is required for full COX-2 expression. ***The Journal of Immunology,*** **2005, 174: 2825–2833.**

Macrophages play an important role in inflammatory processes. Several proinflammatory stimuli such as LPS, IFN-γ, or TNF induce activation of macrophages to produce inflammatory mediators as NO which is synthesized by inducible NO synthase (iNOS)[3] enzyme (1, 2), or PGs (3). IFN-γ, produced by activated T cells and NK cells, is a cytokine involved in antiviral, antitumor, and immune response by inducing transcription of a number of genes (4, 5). IFN-γ plays a pivotal role in macrophage activation and function in part by inducing transcription of proinflammatory mediators such as iNOS, IL-1β, and MHC proteins (4, 5).

The mechanism of activation by which IFN-γ occurs is primarily due to the activation of the Janus tyrosine kinases 1 and 2, which phosphorylate STAT1α. Once STAT1α is phosphorylated, it goes to the nucleus and binds to IFN-γ-activated sites in different promoters, inducing transcriptional activation. Nevertheless, the transcriptional regulation induced by IFN-γ is more complex and in some cases is mediated independently of STAT1α activation (6–8). IFN-γ activity is also mediated through IFN regulatory factors (IRF), which constitute a family of transcription factors (up to nine members have been characterized to date) with a characteristic helix-turn-helix motif (9). IRF-1 and IRF-2 are induced upon IFN activation in many cell types. It is thought that IRF-2 acts as a transcriptional repressor of IRF-1 activity (10–13). Another cytokine involved in the inflammatory process is TNF. TNF is a pleiotropic cytokine that can exert beneficial or detrimental effects in the host. TNF is produced by activated macrophages and it protects against infection, promotes tissue remodeling and activates inflammatory response (14, 15). Conversely, TNF mediates septic shock in chronic infections and causes inflammation in rheumatoid arthritis patients (16–18).

PGs are inflammatory mediators, which are produced by different cellular types, including activated macrophages. The rate-limiting enzyme in the synthesis of PGs is the cyclooxygenase (COX) (3). Two main isoforms of COX have been described to date, named COX-1 and COX-2. COX-1 is considered a housekeeping gene constitutively expressed in most tissues and responsible for the homeostatic production of PGs. COX-2 is induced by several stimuli including growth factors, mitogens, cytokines, and tumor promoters (3). Besides being a proinflammatory enzyme, COX-2 plays an important role in proliferation, cell transformation, and oncogenesis (19, 20). For this reason, its transcriptional regulation is of paramount importance. It has been described that many proinflammatory stimuli such as LPS, IL-1β, TNF, and recently IFN-γ, induce COX-2 expression in macrophages as well as other cellular types (21–24). This induction is the consequence of increased transcription (25–28), as well as increased mRNA stability (29, 30).

Transcriptional regulation of mouse COX-2 expression is mediated by different regulatory elements, which are distributed along the COX-2 promoter sequence. COX-2 promoter contains a classical TATA box, an E-box, and binding sites for transcription factors such as NF-κB, C/EBP, CREB, AP-1, and NFAT. These sequences have been shown to act as positive regulatory elements for COX-2 transcription in different cell types (26, 31–37, 40–42). A NF-κB site has been described at position −402/−392 and seems to be important in COX-2 induction in macrophages and other cell types (24–27). The C/EBP element located at position −138/−130 also plays an important role on COX-2 induction by LPS in macrophages and fibroblasts (38, 39). Recently, it has been reported the transcriptional up-regulation of COX-2 by IFN-γ and the involvement of two IFN sequence regulatory elements (ISRE)

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[3] Abbreviations used in this paper: INOS, inducible NO synthase; COX, cyclooxygenase; ISRE, IFN sequence regulatory element; IRF, IFN regulatory factor; PGHS, PG G/H synthase.

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(ISRE I −1541/−1522 and ISRE II −1215/−1206) (21). The transcription factor IRF-1 seems to be involved in this induction because macrophages from IRF-$1^{-/-}$ mice do not express COX-2 in response to IFN-γ stimulation (21).

In this work, we show that production of TNF is critical for IFN-γ-induced COX-2 expression because blocking of endogenous production of TNF with neutralizing Abs attenuates COX-2 expression in response to IFN-γ treatment. In addition, TNF-deficient macrophages do not show detectable levels of COX-2 protein or PGE_2 production upon IFN-γ stimulation. We further show that NF-κB activation is involved in induction of COX-2 expression by IFN-γ. Moreover, NF-κB activation induced by IFN-γ is abolished in macrophages from TNF-deficient mice, emphasizing again the important role of endogenous TNF in this response. These data indicate that IFN-γ-induced production of TNF and subsequent NF-κB activation are an integral part of COX-2 expression in macrophages.

Materials and Methods

Cell culture

The macrophage cell line RAW 264.7 (American Type Culture Collection) was grown in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 5% (v/v) FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (1000 U/ml), and glutamine (2 mM). Thioglycolate-elicited peritoneal macrophages were isolated from 7- to 8-wk male mice C57BL/B6 × 129S6 ($TNF^{-/-}$ mice) and C57BL/6J (wild-type mice; The Jackson Laboratory), as previously described (43). Cells were treated with 25 U/ml recombinant mouse IFN-γ and/or 5–10 ng/ml recombinant mouse TNF (BioSource International) for indicated times, in RPMI 1640 supplemented with 2% FCS, antibiotics, and glutamine. LPS from *Escherichia coli* serotype O26:B6 was purchased from Sigma-Aldrich and used at 1 μg/ml for indicated times. The neutralizing anti-TNF Ab (R&D Systems) was used at 1 μg/ml.

TNF and PGE_2 determination

Supernatants of control or IFN-γ-treated cells were tested for TNF production using the Quantikine M murine kit for mouse TNF (R&D Systems), following manufacturer's instructions. PGE_2 production was determined by a competitive enzyme immunoassay using the PGE_2 EIA kit (Cayman Chemicals).

Nuclear extracts

Nuclear proteins were obtained as described (44). Briefly, cells were harvested by centrifugation, washed twice with PBS, and resuspended in 500 μl of buffer A (10 mM HEPES, pH 7.6, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.75 mM spermidine, 0.15 mM spermine, 1 mM DTT, 0.5 mM PMSF, 10 mM Na_2MoO_4, and 2 μg/ml each of inhibitors leupeptin, aprotinin, and pepstatin A). After 15 min at 4°C, 5 μl of a Nonidet P-40 10% solution were added. Samples were vortexed for 10 s and centrifuged for 20 min at 3000 rpm and 4°C. The supernatants were used as cytosolic extracts. For nuclear protein extraction, 50 μl of buffer C (20 mM HEPES, pH 7.6, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 mM Na_2MoO_4, and 2 μg/ml each of inhibitors leupeptin, aprotinin, and pepstatin A) were added and nuclear pellets were incubated for 30 min at 4°C with gentle agitation. Samples were centrifuged for 10 min at 14,000 rpm and 4°C, and supernatants were used as nuclear extracts. Protein concentration was determined by the Bradford assay (Bio-Rad).

Western blot

After the indicated treatments, cells were harvested by centrifugation (1200 rpm/5 min) and washed twice with PBS. Then, 30 μl 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_3VO_4, and 2 μg/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 14,000 rpm at 4°C, and protein concentration was determined by the BCA method (Pierce). Total protein (30 μg) was separated in a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad). The membranes were blocked in TBS-0.1% Tween 20 with 5% skim milk, washed twice with TBS-0.1% Tween 20, and incubated with a mAb against mouse COX-2 (BD Transduction Laboratories), 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).

EMSA

EMSAs were performed basically as described (32) by using a probe that contains the NF-κB element from the murine COX-2 promoter (5′-GGG GAG AGG TGA GGG GAT TCC CTT AGT TAG GAC C-3′), or consensus NF-κB probe (Promega). For binding reaction 5 μg of nuclear extract were incubated with 2 μg of poly(dI-dC) with 8 mM $MgCl_2$ for 10 min at room temperature. Radiolabeled probe (0.5 ng) was added and samples were incubated for an additional 15 min at 4°C. Binding reaction was stopped by adding loading buffer, complexes were resolved by electrophoresis in a 4% SDS-polyacrylamide gel in 0.4× TBE buffer for 3 h at 200 V. For competition studies, a 50-fold molar excess of a cold probe or a consensus NF-κB probe were added to the reaction, 10 min before addition of the radiolabeled probe.

Plasmids

PG G/H synthase (PGHS)-2 medium, PGHS-2 short, and PGHS-2–43 constructions, kindly provided by Dr. S. Vogel (University of Maryland, Baltimore, MD) have been previously described (21). PGHS-2–150 and PGHS-2–88 constructs were generated by cloning *Bgl*II-*Hin*dIII-flanked PCR products derived from PGHS-2 medium into the *Bgl*II-*Hin*dIII-digested pGL2-basic vector (Promega). A unique reverse primer from pGL2-basic vector was used for PCR amplification (GL2 primer, 5′-CTT TAT GTT TTT GGC GTC TTC CA-3′). Different forward primers lying in the 5′ region of the transcription start site of the promoter were used to obtain the deletion constructs (−150 primer, 5′-ggg aga tct CCG CTG CGG TTC TTG CG-3′; −88 primer, 5′-ggg aga tct GGA AGC CTA AGC GGA AAG-3′; lowercase letters indicate Bgl*II* restriction sites). The sequence of all PCR-derived constructs was confirmed by automatic sequencing. NF-κB, C/EBP mutations in PGHS-2 short construction, and NF-κB, ISRE I and ISRE II mutations in PGHS-2 medium construction were generated by using QuikChange Site-Directed Mutagenesis kit (Stratagene). The oligonucleotides used for mutagenesis were: NF-κB 5′-GGG GAG AGG TGA GGG ccT TCC CTT AGT TAG GAC C-3′; C/EBP 5′-GCC GCT GCG GTT Ccc GCt CAA CTC ACT GAA GCA GAG AGG-3′; ISRE I 5′-CCA TGC ACT TCC CGC ATT TAA AAT Aag gCA Ggg AAC AAG AAC TAT TTA AAG GG-3′; ISRE II 5′-GGA TTC CCC CAT GTG GAT ATT CTC CCC Tcc CAT ccT TGT TTT GTT TTG TTC-3′, respectively. Mutant nucleotides are lowercase and underlined. The IκBα expression plasmid (pCMV-IκBα) was previously described (45).

Transient transfection

RAW 264.7 cells were transiently transfected by using LipofectAMINE PLUS Reagent (Invitrogen Life Technologies) following manufacturer's instructions. For the COX-2 promoter constructions, 250 ng of DNA per 10^6 cells were used. In cotransfection experiments, IκB expression vector or corresponding empty vector (pcDNA3) was used at 100 ng of DNA per 10^6 cells. At 24 h after transfection, cells were treated with LPS (1 μg/ml) or IFN-γ (25 U/ml) for 16 h. In experiments for measuring kinetic induction of COX-2 promoter activity, cells were treated 16 h after transfection with IFN-γ for the indicated time points. pRL-TK-luc (Promega) was used for determining transfection efficiency by measuring Renilla luciferase activity in the samples. Luciferase activity is represented as relative luciferase units of firefly per renilla.

RT-PCR

Total RNA was obtained by using TRIzol reagent (Invitrogen Life Technologies), following manufacturer's instructions. For RT-PCR, 1 μg of total RNA was reverse transcribed into cDNA and used for PCR amplification with specific oligonucleotides by using the two-step RT-PCR kit Gene Amp RNA PCR Core kit (PerkinElmer). The sequence of each oligonucleotide used was as follows: COX-2 sense, 5′-TTC AAA AGA AGT GCT GGA AAA GGT-3′; COX-2 antisense, 5′-GAT CAT CTC TAC CTG AGT GTC TTT-3′; β-actin sense, 5′-CTC TTT GAT GTC ACG CAC GAT TTC-3′; β-actin antisense, 5′-GTG GGC CGC TCT AGG CAG CAA-3′. Briefly, the PCR was amplified by 25 repeat denaturation cycles at 94°C for 45 s, annealing at 58°C for 45 s, and extension at 72°C for 45 s. For quantification, TaqMan gene expression assays were used (Applied Biosystems), by following manufacturer's instructions. Assays-on-Demand Gene Expression products consist of a mix of unlabeled PCR primers and TaqMan MGB probe (FAM dye-labeled). Gene expression quantification is performed in a two-step RT-PCR in which the PCR step is coupled with a 5′ fluorogenic nuclease assay.

Statistical analysis

Values in the figures were expressed as mean ± SD of duplicate samples. Student's two-tailed *t* test was used to compare means between groups, as was indicated in corresponding figures with values expressed as mean ± SD of three independent experiments in duplicate. A value of $p < 0.05$ was considered to be statistically significant.

Results

IFN-γ induces COX-2 expression in RAW 264.7 macrophages

To study the effect of IFN-γ treatment on COX-2 expression in murine macrophages, RAW 264.7 cells were stimulated with IFN-γ, and PGE_2 production and COX-2 protein and mRNA levels were analyzed. As shown in Fig. 1*A*, COX-2 protein was barely detected in those cells in the absence of stimulus, but increased in a time-dependent manner after IFN-γ stimulation. A weak expression can be observed in the first 4 h, but it was clearly detected after 8 h of stimulation. The peak of COX-2 protein expression was detected after 48 h of IFN-γ treatment. Accordingly, PGE_2 production was increased with a similar kinetics. Thus, PGE_2 increased over basal levels in the supernatants of IFN-γ-stimulated RAW 264.7 macrophages after 8 h of treatment with IFN-γ, and increased up to 24 h of stimulation (Fig. 1*B*). We next analyzed COX-2 mRNA levels by quantitative RT-PCR after IFN-γ treatment (Fig. 1*C*). COX-2 mRNA expression could be detected at low levels at 1 h of IFN-γ treatment. We clearly found two different kinetics: a fast increase between 1 and 6 h, and a slower one between 8 and 24 h of IFN-γ stimulation. At this time, we observed the highest COX-2 mRNA levels that returned approximately to basal levels at 48 h after stimulation. To investigate whether IFN-γ activates COX-2 expression at the transcriptional level, RAW 264.7 cells were transiently transfected with a reporter construction that contains the luciferase gene under control of 2.1 kb from murine COX-2 promoter (PGHS-2 medium), and were treated with IFN-γ alone or in combination with LPS, which has been reported to induce COX-2 expression at transcriptional level (27). As shown in Fig. 1*D*, IFN-γ treatment increased luciferase activity to the same level as did LPS treatment. Combination of both stimuli produced a cooperative increase of COX-2 promoter activity. These data clearly indicate that IFN-γ induces COX-2 transcription in RAW 264.7 macrophages. Next, we analyzed the kinetic of this induction. Although this assay requires the accumulation of luciferase protein, we found a similar biphasic kinetic as the one observed in Fig. 1*C*. Thus, we found a sharp increase in COX-2 promoter activity between 0 and 8 h of stimulation, and a second smaller increment up to 24 h of IFN-γ treatment (Fig. 2*A*).

IFN-γ treatment induces COX-2 promoter activity in absence of ISREs

To analyze the region of the COX-2 promoter responsible of the IFN-γ-induced transcriptional activity, different deletions of the promoter were transiently transfected on RAW 264.7 cells, which were then stimulated with IFN-γ for 16 h (Fig. 2*B*). Surprisingly, deletion of the region between positions −2100/−965, which contain the two ISREs previously described (PGHS-2 short construction) (21), only showed a partial decrease in luciferase activity induced by IFN-γ, suggesting that another region or regions from the COX-2 promoter are involved in IFN-γ response. By contrast, when the region between positions −965/−150 was deleted (PGHS-2–150 construction), IFN-γ-induced promoter activity strongly diminished, suggesting that this region is important for IFN-γ induction of COX-2 transcriptional activity in RAW 264.7 cells.

NF-κB involvement in COX-2 induction by IFN-γ

A NF-κB site is the only regulatory element that has been identified so far in the −965/−50 region from COX-2 promoter, and it participates in COX-2 induction by several proinflammatory stimuli like LPS or TNF in macrophages and other cell types (25, 26). Because IFN-γ-induced COX-2 promoter activity was strongly diminished when the −965/−150 region was deleted, we tested

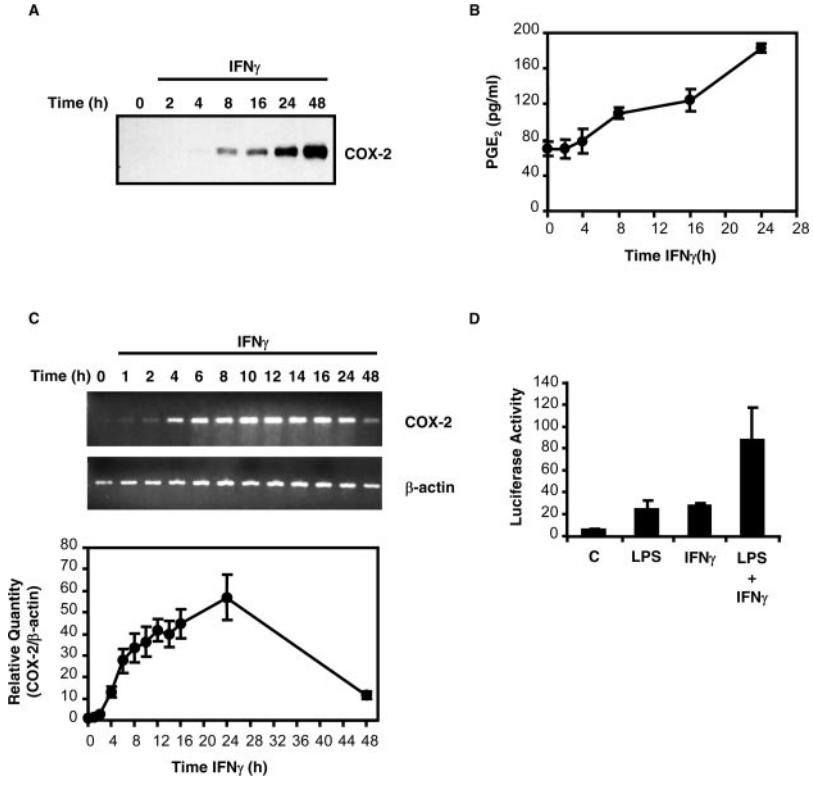

FIGURE 1. Analysis of COX-2 induction. RAW 264.7 cells were treated with IFN-γ (25 U/ml) in a time-course experiment. COX-2 protein expression detected by Western blot analysis (*A*) and PGE_2 release (*B*) were determined. *C*, RAW 264.7 cells were treated with IFN-γ (25 U/ml) at different times. Total RNA was isolated and COX-2 RNA levels (*top*) were determined by RT-PCR with specific primers for COX-2. TaqMan quantification analysis (*bottom*) is shown. *D*, RAW 264.7 cells were transiently transfected with PGHS-2 medium construction and 24 h after transfection were treated with LPS (1 μg/ml), IFN-γ (25 U/ml), or both. Luciferase activity was determined 16 h after stimulation. A representative experiment from the three independent assays performed for each type of analysis is shown.

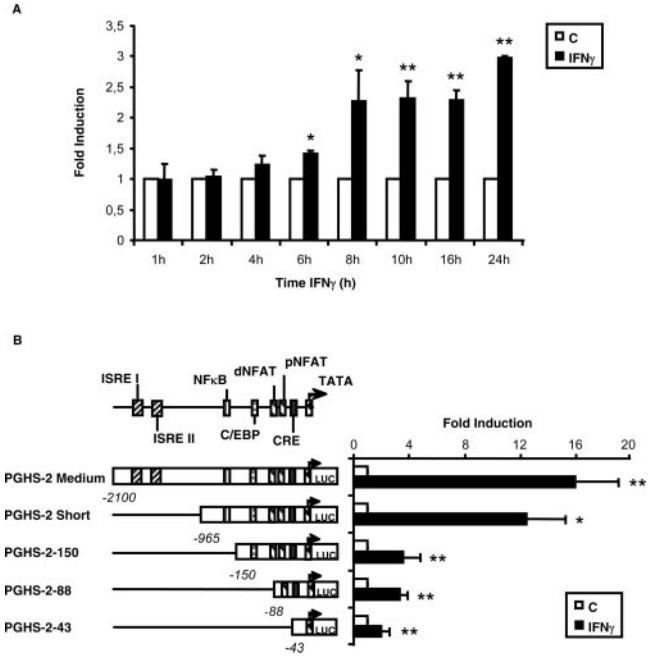

FIGURE 2. Analysis of COX-2 promoter regions involved in IFN-γ response. *A*, Time-course analysis of COX-2 promoter activity. RAW 264.7 cells were transiently transfected with PGHS-2 medium construct and 16 h after transfection were treated with IFN-γ for the indicated times. *B*, Cells were transiently transfected with the indicated constructions and treated with IFN-γ for 16 h. The results are expressed as fold induction by IFN-γ over the respective controls in absence of stimulation. The results (*A* and *B*) are the average of three independent experiments. *, $p < 0.05$ and **, $p < 0.01$. dNFAT, Distal NFAT site; pNFAT, proximal NFAT site.

whether NF-κB site was involved in IFN-γ response. For this and to determine the contribution of the different elements of the promoter to the transcriptional activity induced by IFN-γ, RAW 264.7 cells were transfected with different constructs of the promoter mutated in ISRE I, ISRE II, NF-κB, or C/EBP (NF-IL-6) sites (Fig. 3). Mutation of ISRE I or ISRE II produced a 25–30% of reduction of the transcriptional activity induced by IFN-γ, whereas NF-κB mutation in the context of PGHS-2 medium construct diminished the promoter activity in ~40% (Fig. 3*A*), indicating that ISRE I, ISRE II, and NF-κB elements are required for the IFN-γ response. Moreover, when the NF-κB site was mutated in the context of PGHS-2 short construction (Fig. 3*B*), the IFN-γ response was reduced to 50% to the same level deletion of the entire −965/−150 region and to the basal promoter. This suggests that no other site present in that region was required. Moreover, mutation of the C/EBP site had no effect on COX-2 transcriptional activity mediated by IFN-γ. Those results confirm the important involvement of the NF-κB site on COX-2 expression mediated by IFN-γ treatment.

Next, we tested whether IFN-γ activates NF-κB by performing EMSA with a specific NF-κB probe from the COX-2 promoter (Fig. 4*A*). A small amount of a specific complex could be detected in untreated cells. Interestingly, we could not detect a significant increase in the NF-κB binding to DNA at early time points (up to 4 h) of IFN-γ stimulation (Fig. 4*A*). In contrast, a significant increase in NF-κB binding was observed at 8 h poststimulation (Fig. 4*A*). Those complexes were specifically competed out by an excess of both the unlabeled probe as well as a κB consensus probe.

In the absence of stimulation, NF-κB transcription factors are bound to IκB proteins, which retain it in an inactive form in the cytoplasm. Thus, to further analyze the contribution of NF-κB to COX-2 transcriptional activity mediated by IFN-γ, IκBα was overexpressed in RAW 264.7 cells transiently transfected with PGHS-2 medium promoter construction. As shown in Fig. 4*B*, IκBα overexpression strongly inhibited IFN-γ-induced COX-2 promoter activity.

Involvement of TNF in IFN-γ-induced COX-2 expression

Because NF-κB is a rapid response transcription factor, the delayed kinetics of activation by IFN-γ suggested that the effect was

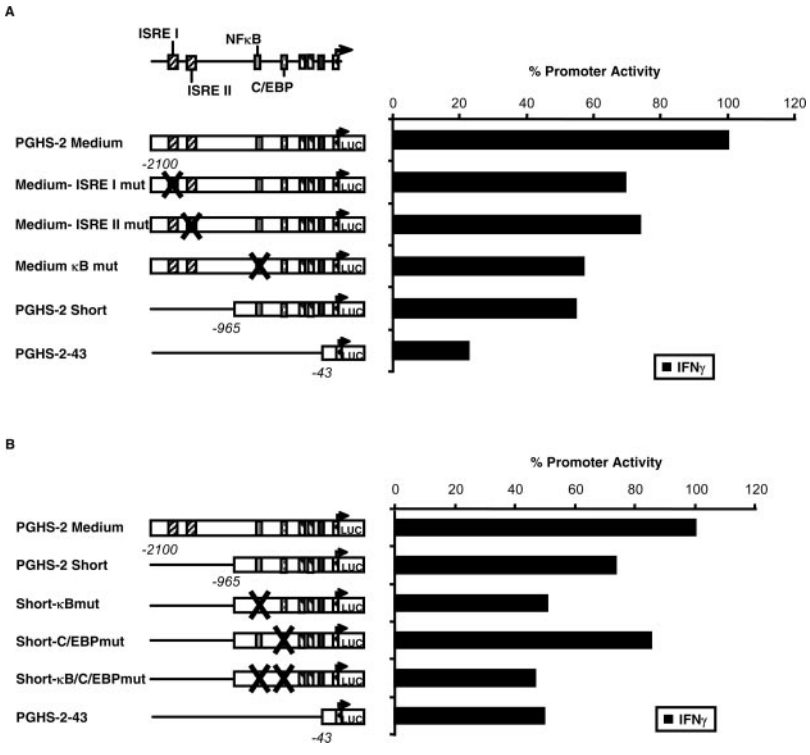

FIGURE 3. Mutation of the NF-κB site inhibits IFN-γ-induced COX-2 transcriptional activity. RAW 264.7 cells were transiently transfected with different mutations on PGHS-2 medium construction (*A*) or PGHS-2 short construction (*B*) as indicated, and stimulated with IFN-γ for 16 h. Results are expressed as a percentage of promoter activity induced by IFN-γ calculated over their respective controls in absence of stimulation. The results shown are representative experiments from three independent assays.

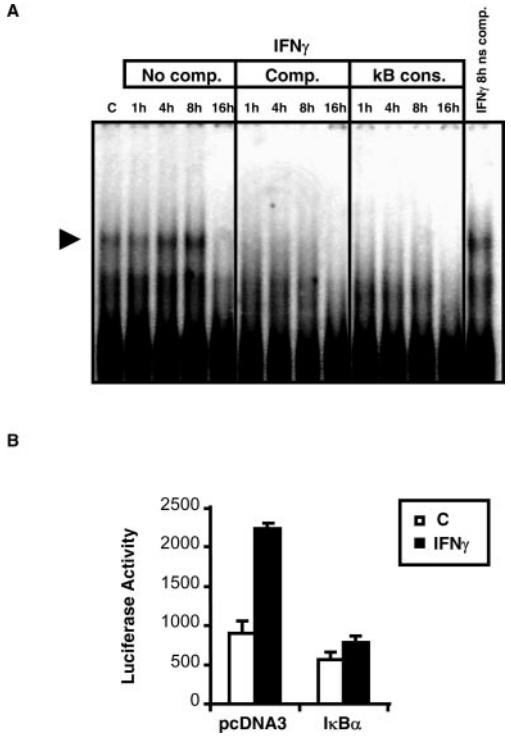

FIGURE 4. NF-κB involvement on COX-2 expression driven by IFN-γ. *A*, Nuclear extracts were obtained from RAW 264.7 cells stimulated with IFN-γ at indicated times, and were used to perform an EMSA experiment with NF-κB-specific probe from the COX-2 promoter. A 50-fold molar excess of cold-specific probe was added at the point marked as competitor (Comp). Nonspecific probe (AP-1 consensus sequence) was added at the point marked as nonspecific (ns comp). Additional competition assays were performed with a 50-fold molar excess of NF-κB consensus probe (κB cons). Closed arrowhead indicates NF-κB complexes. *B*, IκBα was overexpressed in RAW 264.7 cells transiently transfected with PGHS-2 medium construction. Cells were treated with or without IFN-γ for 16 h and luciferase activity was determined. A representative experiment from three independent assays performed is shown.

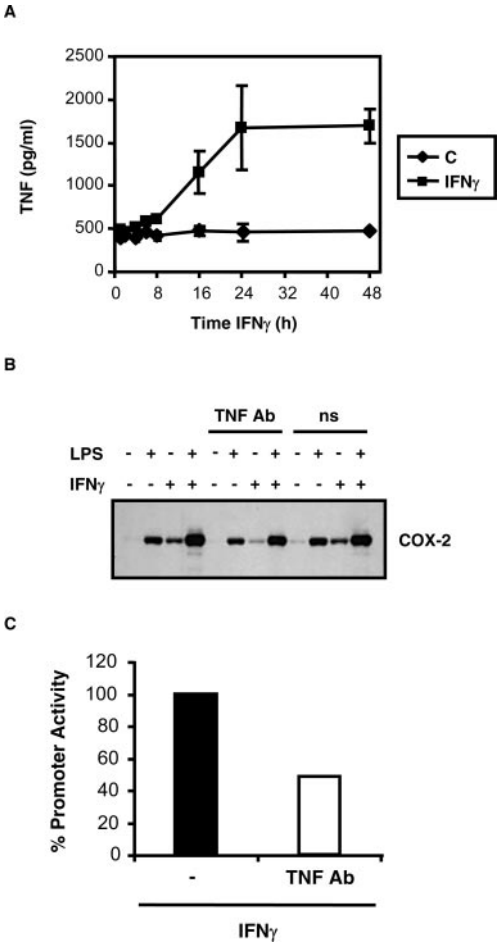

FIGURE 5. TNF production is involved in COX-2 induction by IFN-γ. *A*, TNF production by RAW 264.7 cells stimulated with medium or IFN-γ in a time-course assay. *B*, Neutralizing anti-TNF Abs abrogates IFN-γ-induced COX-2 expression. RAW 264.7 cells were stimulated with LPS, IFN-γ, or both, either alone or in presence of anti-TNF Abs (TNF Ab) or control IgG (ns) for 24 h, and COX-2 expression was analyzed by Western blot analysis. A representative experiment from three independent assays performed is shown. *C*, RAW 264.7 cells were transiently transfected with PGHS-2 medium construction and 24 h after transfection were stimulated with IFN-γ and with or without anti-TNF (TNF Ab) neutralizing Abs for an additional 16 h. Results are expressed as a percentage (%) of COX-2 promoter activity in which IFN-γ stimulation is taken as 100% of activation. A representative experiment from three independent assays performed is shown.

likely to be indirect, and could indicate the existence of an autocrine mechanism involving the action of a secreted cytokine on COX-2 expression induced by IFN-γ, which could activate NF-κB translocation and binding to DNA. To test this hypothesis, we first determined the release of several cytokines to the supernatants from RAW 264.7 cells treated with IFN-γ at different times. As shown in Fig. 5*A*, IFN-γ induced TNF release in a time-dependent manner. Other macrophage cytokines able to induce NF-κB, such as IL-1β or IL-12 (data not shown), could not be detected upon IFN-γ induction in those conditions.

TNF was reported to induce COX-2 expression in several cell types through NF-κB activation (24, 26). For those reasons, an attractive hypothesis was that IFN-γ could induce COX-2 expression through induction of TNF production. To investigate this possibility, we examined the effect of neutralizing anti-TNF Abs on COX-2 protein expression in RAW 264.7 macrophages treated with IFN-γ and/or LPS. As shown in Fig. 5*B*, TNF neutralization strongly reduced the IFN-γ-induced COX-2 protein expression, whereas it had no effect on LPS and slightly inhibited LPS plus IFN-γ-induced COX expression. Treatment with control nonspecific serum (normal goat serum) did not affect COX-2 expression induced by any stimuli. These results suggested that IFN-γ-induced TNF production contributes to IFN-γ-mediated COX-2 expression.

At this point, we investigate whether autocrine TNF affects COX-2 transcriptional activity. For this, RAW 264.7 cells were transiently transfected with PGHS-2 medium construction and treated with IFN-γ in the presence or in absence of TNF neutralizing Abs. As shown in Fig. 5*C*, the promoter activation induced by IFN-γ was significantly reduced in the presence of TNF neutralizing Abs. TNF blockade produced a partial inhibition of COX-2 promoter activity (~50%) induced by IFN-γ, indicating that IFN-γ-induced TNF exerts its effect at least in part at the transcriptional level.

TNF-deficient macrophages do not synthesize COX-2 in response to IFN-γ stimulation

To clearly ascertain the role of TNF production on IFN-γ-induced COX-2 expression, primary peritoneal macrophages were isolated from TNF-deficient mice or control littermates, and stimulated with IFN-γ. As shown in Fig. 6*A*, IFN-γ stimulation induced TNF

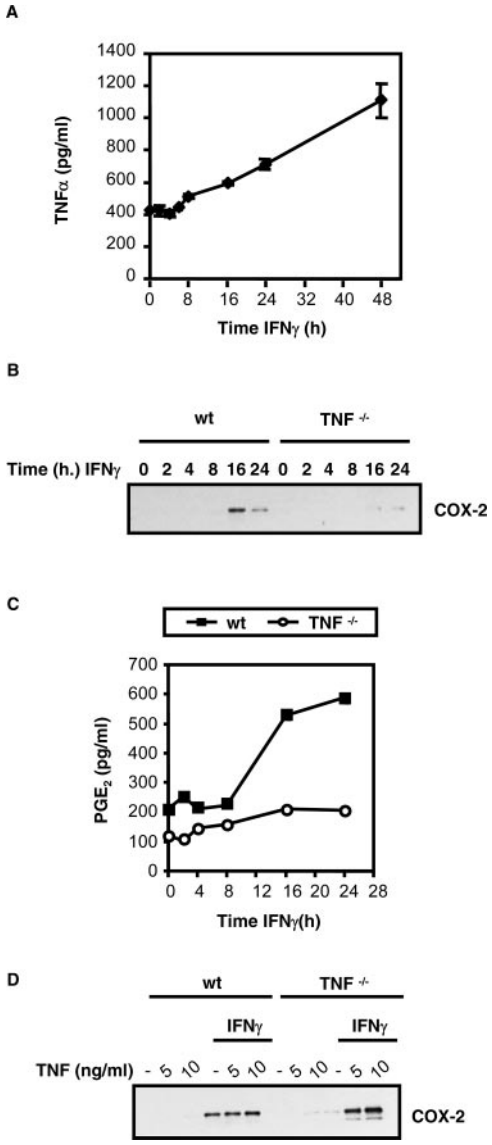

FIGURE 6. Deficient COX-2 expression in response to IFN-γ treatment in $TNF^{-/-}$ mice. Primary peritoneal macrophages were isolated from normal C57BL/6J (wt) or TNF-deficient ($TNF^{-/-}$) C57BL/B6 mice and stimulated for various time periods with IFN-γ. *A*, TNF production by peritoneal macrophages from wild-type mice stimulated with IFN-γ in a time-course assay. *B*, COX-2 expression analysis by Western blot analysis. *C*, Determination of PGE_2 release to the supernatants from cultured primary peritoneal macrophages in response to IFN-γ treatment. *D*, Effect of exogenous TNF on COX-2 expression in TNF-deficient mice. Primary peritoneal macrophages from normal C57BL/6J (wt) or TNF-α-deficient ($TNF^{-/-}$) C57BL/B6 mice, were stimulated with IFN-γ (25 U/ml) and/or TNF at indicated doses for 24 h, and COX-2 expression was analyzed by Western blot analysis. A representative experiment from various independent assays performed is shown.

release in a time-dependent manner in wild-type mice, as we previously detected in RAW 264.7 macrophages. When we analyzed COX-2 expression (Fig. 6*B*), we found that COX-2 protein was clearly detectable at 16 h of stimulation with IFN-γ in macrophages from wild-type mice, whereas in $TNF^{-/-}$ mice there was no significant COX-2 protein induction up to 24 h poststimulation. This result correlated with the PGE_2 release induced by IFN-γ (Fig. 6*C*). Thus, IFN-γ treatment resulted in an increase in PGE_2 released by peritoneal macrophages from wild-type mice, which was time-dependent and detectable only after 8 h of induction. By contrast, PGE_2 release could not be detected in the supernatants from $TNF^{-/-}$ peritoneal macrophages treated with IFN-γ at any time point tested.

These results indicate that TNF secretion in response to IFN-γ is necessary for COX-2 expression. To further corroborate this result, we investigated whether exogenous addition of TNF could restore COX-2 expression in IFN-γ-treated macrophages from $TNF^{-/-}$ mice. As shown in Fig. 6*D*, treatment with TNF alone did not induce COX-2 expression in macrophages from wild-type or $TNF^{-/-}$ mice. In wild-type macrophages, addition of TNF slightly increased COX-2 protein levels induced by IFN-γ alone. By contrast, in TNF-deficient macrophages in which there was no expression of COX-2 in response to IFN-γ, addition of exogenous TNF in combination with IFN-γ restored in a dose-response manner COX-2 protein levels comparable to wild-type mice. Together, all these results clearly indicate that the autocrine effect of TNF secretion is necessary for COX-2 expression induced by IFN-γ.

Because we showed that IFN-γ induces NF-κB binding to the κB element in the COX-2 promoter, and NF-κB is involved in IFN-γ induction of COX-2 expression, we considered whether endogenous TNF produced by IFN-γ is responsible for the observed NF-κB activation. Thus, we performed an EMSA using nuclear extracts obtained from peritoneal macrophages from wild-type mice or TNF-deficient mice treated with IFN-γ at different times. As shown in Fig. 7, IFN-γ did not induce NF-κB activation until 2 h of treatment in wild-type mice. This stimulation is maintained until 8 h of IFN-γ treatment in these mice. By contrast, there was no NF-κB activation in TNF-deficient mice induced by IFN-γ at any time tested. As a control, NF-κB activation in response to LPS treatment was similar in $TNF^{-/-}$ mice and in wild-type mice.

Discussion

IFN-γ is a pleiotropic cytokine responsible for macrophage activation and differentiation and subsequent triggering of inflammatory response (4). COX-2 is the rate-limiting enzyme of prostanoid synthesis, and its uncontrolled activity is thought to play an important role in many chronic inflammatory diseases such as rheumatoid arthritis (46). For those reasons, COX-2 expression must be tightly regulated. In this work, we have shown that IFN-γ induces COX-2 expression and PGE_2 synthesis in mouse macrophages, in agreement with previous results of Blanco et al. (21). Those authors identified two ISRE located at positions −1552/−1541 and

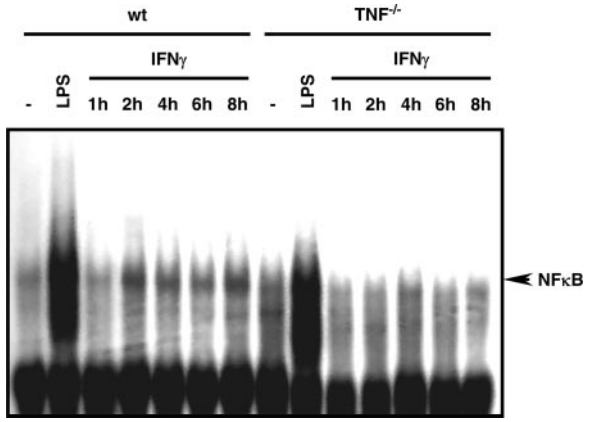

FIGURE 7. NF-κB activation in peritoneal macrophages. Nuclear extracts were obtained from peritoneal macrophages from normal C57BL/6J (wt) or TNF-deficient ($TNF^{-/-}$) C57BL/B6 mice, treated with LPS for 30 min or IFN-γ for the indicated times, and an EMSA was performed with a NF-κB consensus probe.

−1215/−1206, respectively, in the distal region of COX-2 promoter in which the transcription factor IRF-1 can bind and modulate COX-2 transcription in IFN-γ-stimulated macrophages. We have found in this report that a COX-2 promoter construction in which ISRE were deleted (PGHS-2 short) showed a small decrease in IFN-γ-induced promoter activity (20–30%), which was similar to the inhibition produced by mutation of ISRE I or ISRE II. In contrast, deletion of the −965/−150 region that contains a NF-κB element strongly inhibited IFN-γ response. In addition, NF-κB mutation diminished IFN-γ-induced COX-2 transcriptional activity in presence as in absence of ISRE (Fig. 3). All these data indicate that in addition to the role of ISRE and the IRF-1 transcription factor, there is an important role of NF-κB in IFN-γ induction of COX-2 transcription. This was corroborated by the fact that overexpression of the inhibitory protein IκBα almost completely abrogates COX-2 transcriptional activation induced by IFN-γ treatment. Because IFN-γ-induced COX-2 transcriptional activity is not completely inhibited by mutation of ISRE I, ISRE II, or NF-κB elements, it is likely that all these sites would be necessary and complementary for a correct transcriptional induction of COX-2 gene. It is tempting to speculate that the factors that are bound to ISRE I, ISRE II, and the NF-κB site could be collaborating in producing a complete IFN-γ response (see Fig. 8).

Although from the experiments we present the involvement of NF-κB activation in COX-2 induction by IFN-γ is clear, this cytokine does not seem to be a good direct inducer of NF-κB. Thus, it is clear that NF-κB binding to the κB element from the COX-2 promoter was increased after IFN-γ stimulation, but this takes place around to 2 h after the treatment, indicating a late activation of this transcription factor. Because NF-κB activation in response to all known inducers is usually rapid (10–30 min) (47), this late activation of NF-κB binding to DNA could be likely explained by the autocrine effect of another cytokine distinct from IFN-γ. We hypothesized that the cytokine responsible of this late NF-κB activation was TNF, because its production has been shown to be induced by IFN-γ (Figs. 5*A* and 6*A*) (48) and it is able to activate NF-κB (49). Our report has demonstrated this role of endogenously produced TNF in IFN-γ-induced NF-κB activation because peritoneal macrophages from TNF-deficient mice did not show NF-κB activation by IFN-γ. Moreover, we have demonstrated that TNF is absolutely required for COX-2 induction by IFN-γ, acting through an autocrine loop. This has been shown by the fact that TNF blockade with neutralizing Abs in RAW 264.7 cells, or the absence of TNF in peritoneal macrophages from TNF knockout mice, strongly reduced or abrogated COX-2 expression induced by IFN-γ, respectively. A similar autocrine role of TNF seems to be required for IFN-γ induction of other proteins like iNOS (V. Vila-del Sol, unpublished observations) (50) or high mobility group box 1 protein (51). In addition, COX-2 expression induced by IFN-γ in other cellular types, such as epidermal keranocytes or bronchial epithelial cells, is also mediated by an autocrine loop involving TGF-α or epidermal growth factor, respectively, that are produced in response to IFN-γ treatment (22, 23). Thus, as in many other cytokine systems, IFN-γ gene regulation seems to proceed by the concerted and sequential action of secreted cytokines in an autocrine fashion. Taken together, all these results suggest that IFN-γ triggers a signal for induction of distinct cytokines or growth factors depending on the cellular types that in a second phase are activating or inducing another transcription factor. In macrophages, the second signal could be provided by TNF, which is absolutely necessary for full COX-2 expression. A working model of IFN-γ induction of COX-2 transcription is shown in Fig. 8. IFN-γ signaling induces the expression of the IRF-1 transcription factor, which binds to ISRE I and ISRE II on mouse COX-2 promoter and allows early COX-2 expression at low levels. At the same time, IFN-γ stimulation induces TNF expression and secretion, which in an autocrine fashion binds to its receptors in the cell surface and triggers a signaling pathway that culminates in NF-κB activation and binding to the corresponding element in the COX-2 promoter. This simultaneous binding of IRF-1 and NF-κB allows the recruitment of transcriptional coactivators as CBP/p300 and the formation of an active transcriptional complex for enhancing transcriptional synergy. This autocrine and sequential mechanism for COX-2 regulation will fit with the observed biphasic induction of COX-2 mRNA. Thus, at early times a clear induction of mRNA but not so much of protein is observed. Around 8 h after activation a second slower COX-2 mRNA increase is detected. Moreover, NF-κB activation was not detected by EMSA up to 2–4 h post-IFN-γ treatment. So, around 8 h a transition takes place in IFN-γ-treated macrophages, consisting of an increase in mRNA levels associated to a significant TNF secretion and NF-κB activation. Those results are corroborated by the fact that TNF-deficient mice do not activate NF-κB in response to IFN-γ treatment in EMSA.

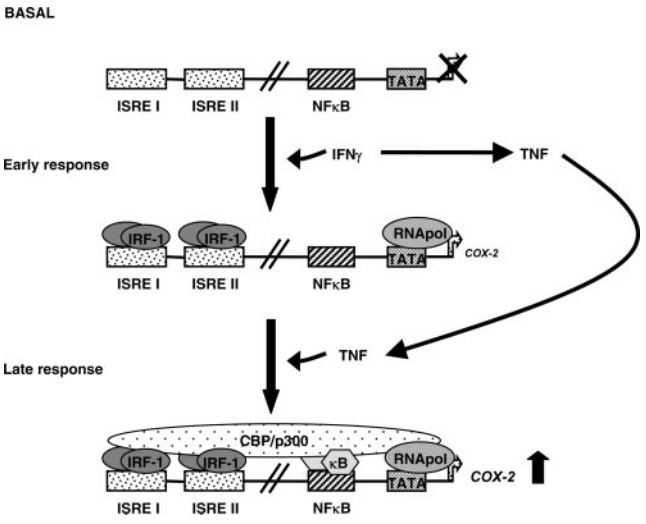

FIGURE 8. Model of COX-2 induction by IFN-γ. IFN-γ stimulation induces an early response that allows the binding of IRF-1 to ISRE I and ISRE II of the mouse COX-2 promoter. At the same time, IFN-γ stimulation induces TNF secretion, which in autocrine fashion activates NF-κB that binds to their corresponding elements of the promoter. This simultaneous binding of IRF-1 and NF-κB allows a more efficient binding of transcription coactivators as CPB/p300 and the basal transcription machinery.

Many studies have revealed the synergistic role of TNF and IFN-γ in the induction of many promoters including IRF-1 and iNOS promoters (52, 55). The basis of this synergism seems to lie in the interaction between STAT1α or IRF-1 induced by IFN-γ and NF-κB induced by TNF (52–55). Our results are in agreement with this interaction and will help to explain the general phenomenon of the synergism of IFN-γ and TNF in macrophage activation. Because COX-2 and iNOS are two proinflammatory genes coexpressed by many cell types in response to stimulation, it is likely that the synergism observed by IFN-γ and TNF treatment in COX-2 expression occurs by a similar mechanism. Thus, it is tempting to speculate that the collaboration between transcription factors induced by IFN-γ (like IRFs) and transcription factors activated or induced by TNF (such as NF-κB) is responsible for the synergistic response observed in peritoneal macrophages from TNF-deficient mice treated with both stimuli.

In addition to the transcriptional role of autocrine TNF in COX-2 induction, this cytokine may also act at the posttranscriptional level by regulating COX-2 mRNA stability. In this regard, it has been described that TNF regulates COX-2 expression by stabilizing COX-2 mRNA levels (29). Future studies are necessary for determining in detail the role of autocrine TNF induced by IFN-γ on stabilizing COX-2 mRNA. Thus, the result of transcriptional and posttranscriptional effects of autocrine TNF could explain the sustained expression of COX-2 protein induced by IFN-γ.

In conclusion, with the results shown we propose a mechanism for COX-2 regulation by IFN-γ. First, IFN-γ induces COX-2 expression at low levels by the action of distinct IRFs, like IRF-1. Furthermore, IFN-γ stimulates TNF production, and this TNF acts in an autocrine fashion cooperating with IFN-γ and continuously increasing COX-2 expression. TNF is acting by activating NF-κB binding to the COX-2 promoter, and this factor is collaborating with IFN-γ-induced IRF-1 and enhancing transcriptional expression of COX-2.

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Disclosures

The authors have no financial conflict of interest.

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