Critical Role of Tumor Necrosis Factor-α and NF-κB in Interferon-γ-induced CD40 Expression in Microglia/Macrophages*

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CD40 is a member of the tumor necrosis factor (TNF) receptor superfamily. CD40 expression on antigen-presenting cells (including macrophages and microglia) is crucial for T-cell activation. Aberrant expression of CD40 has been associated with autoimmune inflammatory diseases such as multiple sclerosis and rheumatoid arthritis. We have recently shown that the cytokine interferon (IFN)-γ is the most potent inducer of CD40 expression in macrophages and microglia, and this induction is mediated by the IFN-γ-activated transcription factor STAT-1α and constitutively expressed PU.1 and/or Spi-B. In this study, we have discovered that a major component of IFN-γ-induced CD40 expression involves the endogenous production of the cytokine TNF-α. The inclusion of anti-TNF-α-neutralizing antibody significantly inhibits IFN-γ-induced CD40 mRNA and CD40 promoter activity. IFN-γ-induced CD40 protein expression is attenuated in TNF-α-deficient microglia and can be restored with exogenous TNF-α. Site-directed mutagenesis studies demonstrate that three of the four NF-κB elements in the CD40 promoter are required for IFN-γ-induced CD40 promoter activity. IFN-γ treatment leads to the activation of NF-κB in a time-dependent manner, which is inhibited in the presence of anti-TNF-α-neutralizing antibody. These results indicate that IFN-γ-induced TNF-α production and subsequent NF-κB activation are integral parts of the mechanism of IFN-γ-induced CD40 expression.

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The interaction between CD40 and its cognate ligand, CD40L (CD154), is critical for a productive immune response (for review see Ref. 1). X-linked hyper IgM syndrome individuals have defects in CD154-CD40 interactions between their T-cells and antigen-presenting B-cells, exhibit elevated levels of IgM with the virtual absence of other antibody isotypes, and are extremely susceptible to bacterial, viral, and opportunistic infections (for review see Ref. 2). CD154-CD40 interactions promote B-cell growth, differentiation, and immunoglobulin class switching. Also, up-regulation of various co-stimulatory molecules (ICAM-1, VCAM-1, E-selectin, LFA-3, B7.1, B7.2, and CD40) occurs upon CD40-CD154 contact, as does production of numerous cytokines and chemokines (IL-1, IL-6, IL-8, IL-10, IL-12, TNF-α, and MIP-1α) and cytotoxic radicals (for review see Ref. 1). The production of IL-12 is particularly important for promoting T-cell maturation toward the Th1 pathway (3–5). CD40 has been implicated in participating in many human diseases, particularly autoimmune diseases (for review see Ref. 6). Aberrant expression of CD40 and CD154 has been described in rheumatoid arthritis, multiple sclerosis, and other diseases that involve a hyperactive immune system (7–9). Because CD40 is functionally critical and nonredundant for the activation of immune responses, blocking the interaction between CD40-CD154 with anti-CD154 or CD40-Ig has been shown to be beneficial in animal models of autoimmune diseases (10–14). These findings illustrate the importance of CD40-CD154 interactions for homeostasis of immune responses. Despite the importance of CD40 in regulating the immune system, little is known about the regulation of CD40 expression.

We have previously shown that microglia/macrophages constitutively express CD40 at a low level, which is enhanced by IFN-γ (15, 16). In these cells, IFN-γ-activated STAT-1α cooperates with the constitutive transcription factors PU.1 and/or Spi-B that directly bind to the CD40 promoter to activate CD40 gene expression (16). In addition to the binding sites for STAT-1α, PU.1, and Spi-B, the CD40 promoter also contains four potential NF-κB-binding sites (16). We have shown previously that TNF-α alone is a very weak inducer of CD40 expression and modestly enhances IFN-γ-induced CD40 expression (15). Based on these observations, we attempted to unravel the potential role of TNF-α in IFN-γ-induced CD40 expression.

In this study, we demonstrate that production of TNF-α is critical for IFN-γ-induced CD40 expression, because blocking endogenous production of TNF-α significantly attenuates the ability of IFN-γ to induce CD40 expression. TNF-α-deficient microglia express low levels of CD40 in comparison with wild type cells upon treatment with IFN-γ, emphasizing the importance of endogenous TNF-α in this response. We further show that blocking NF-κB activation by the use of dominant-negative IκB kinase (IKK) constructs reduces IFN-γ-induced CD40 promoter activity. IFN-γ-activated NF-κB, both the p65 and p50 members, binds to the distal NF-κB site (dNBS) at −530,

CD40 is a 50-kDa type I member of the TNF receptor superfamily. CD40 is expressed by a wide variety of cells such as B-cells, macrophages, dendritic cells, keratinocytes, endothelial cells, thymic epithelial cells, fibroblasts, and tumor cells.
the medial NF-xB site (mNBS) at −494, and another medial NF-xB site (mNBS2) at −442, in the human CD40 promoter. Inclusion of anti-TNF-α-neutralizing antibody prevents IFN-γ activation of NF-xB. Targeted disruption of these NF-xB elements significantly decreases IFN-γ-induced CD40 promoter activity. These data indicate that IFN-γ-induced production of TNF-α and subsequent NF-xB activation are an integral part of CD40 gene expression in microglia/macrophages.

**EXPERIMENTAL PROCEDURES**

**Recombinant Proteins and Reagents—**Recombinant murine IFN-γ was purchased from Genzyme (Boston, MA), and murine TNF-α and neutralizing antibody was purchased from Endogen (Woburn, MA). Rat IgG2a-α mouse anti-TNF-α antibody (clone 3/23), biotinylated mouse anti-rat IgG2a, and phycoerythrin-conjugated streptavidin were purchased from PharMingen (San Diego, CA). Anti-human NF-xB p50, p65, p52, c-Rel, and RelB antisera were a generous gift from Dr. Nancy Rice (National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD). The expression vectors for wild type and dominant-negatives of IKK-α and -β (17) were kindly provided by Dr. Rudolph Noelle (Dartmouth Medical School, Lebanon, NH).

**Cells—**The microglial cell line EOC13 was derived from C57Bl6/Hj CH-2k mice using a nonviral immunization procedure as described previously (15). These colony stimulating factor-1-dependent lines are maintenance Center, Frederick, MD). The expression vectors for wild type and dominant-negatives of IKK-α and -β (17) were kindly provided by Dr. Rudolph Noelle (Dartmouth Medical School, Lebanon, NH).

**RNA Isolation, Riboprobes, and Ribonuclease Protection Assay—**Total cellular RNA was isolated from confluent monolayers of EOC13 and RAW264.7 cells. The riboprobes for murine CD40, IRF-1, and GAPDH mRNA were prepared as described previously (16). 20 mg of the hCD40 promoter was cloned into Bluescript SK(−) and 270 nucleotides, respectively. 20 μg of total RNA from RAW264.7 or EOC13 cells was hybridized with CD40, IRF-1, and GAPDH riboprobes (25 x 106 cpm) at 42 °C overnight in 20 μl of 40 mM PIPES (pH 6.4), 80% deionized formamide, 200 μM NaOAc, 2.0 μM EdTA. The mixture was then treated with RNase ATⅠ (1:200 dilution in 200 μl of the RNase digestion buffer) at room temperature for 1 h and analyzed by 70% denaturing (8 μm urea) polyacrylamide gel electrophoresis, and the gels were exposed to x-ray film for varying periods of time. The protected fragments of the CD40, IRF-1, and GAPDH riboprobes are 419, 314, and 212 nucleotides in length, respectively. Quantification of the protected RNA fragments was performed by scanning with the PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The values for CD40 and IRF-1 mRNA expression were normalized to GAPDH mRNA levels for each experimental condition. GAPDH mRNA was utilized as a "housekeeping gene," because its levels are not affected by cytokine treatment.

**Nuclear Extracts and Electrophoretic Mobility Shift Assays (EMSA)—**The cells were incubated with medium or IFN-γ (5 ng/ml) for various time periods (0–24 h), and nuclear extracts were prepared. EMSA was performed with 5–10 μg of nuclear extract in a total volume of 15 μl of binding buffer (50 mM NaCl, 1 mM MgCl2, 0.1 mM EDTA, 4% glycerol, 0.5 mM dithiothreitol, 4 mM Tris-Cl, pH 7.5, 1 μg of poly(dI-dC)-oligo(dI-dC) probe) and incubated on ice for 5 min. Bound and free DNA were then resolved by electrophoresis through a 6% polyacrylamide gel in 0.5 x TBE buffer at 250 V for 1 h. For supershift analysis, 1 μg of indicated antibody was added, or for competition analysis, a 100-fold molar excess of the indicated cold oligonucleotide was added to the nuclear extracts and incubated on ice for 30 min, followed by an additional incubation for 15 min with the labeled probe.

**Transient Transfection and Analysis—**0.2 μg of the hCD40 promoter constructs were co-transfected with 0.02 μg of the pCMV-β-galactosidase construct into 2 x 106 RAW264.7 cells in 12-well plates using the LipofectAMINE Plus method as described previously (16). βG3-Base was used as a negative (background) control in all experiments. After 3 h of transfection, the cells were allowed to recover for 6 h prior to transfection with IFN-γ (5 ng/ml) for 12 h, which was determined to be optimal for IFN-γ-induced activation of the hCD40p0.7 construct (16). The cells were washed with phosphate-buffered saline and lysed with 250 μl of lysis buffer (25 mM trisphosphate, pH 7.8, 2 mM dithiothreitol, 2 mM diaminocyclohexane tetraacetic acid, 10% glycerol, and 1% Triton X-100). The extracts were assayed in triplicate for luciferase activity in a total volume of 130 μl (30 μl of cell extract, 20 μl Tricine, 0.1 mM EDTA, 1 mM MgCO3, 2.67 mM MgSO4, 33.3 mM dithiothreitol, 0.27 mM coenzyme A, 0.47 mM luciferin, and 0.53 mm ATP), and light intensity was measured using a luminometer (Promega, Madison, WI). Luciferase activity was determined over a 10-s time period. The extracts were also assayed in triplicate for β-galactosidase enzyme activity as described previously (16). The luciferase activity of each sample was normalized to β-galactosidase activity to yield relative luciferase activity. Fold induction of mutated constructs to that of the full-length promoter, which was set at 100%. For transfections that include the IKK-α and IKK-β expression constructs, differences in the amount of DNA were adjusted with appropriate empty vector.

**Measurement of TNF-α Activity—**TNF-α activity in culture supernatants was determined in a biologic assay by using the WEHI 164 clone 13 mouse fibrosarcoma cells as described previously (18). TNF-α activity was expressed as TNF-α pg/μg protein of total cell lysate. The absolute amount of TNF-α was determined by extrapolation from the standard curve that was generated by using known amounts of mouse recombinant TNF-α. All samples were tested in triplicate.

**RESULTS**

**Involvement of TNF-α in IFN-γ-induced CD40 Gene Expression—**We have previously shown that IFN-γ is the most potent inducer of CD40 expression in microglia and macrophages (15, 16). Curiously, TNF-α stimulation had a minimal influence on CD40 expression (Fig. 1A). Furthermore, mRNA, isolated as the ratio of fold induction of mutated constructs to that of the full-length promoter, which was set at 100%. For transfections that include the IKK-α and IKK-β expression constructs, differences in the amount of DNA were adjusted with appropriate empty vector. The absolute amount of TNF-α was determined by extrapolation from the standard curve that was generated by using known amounts of mouse recombinant TNF-α. All samples were tested in triplicate.

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Because IFN-γ is known to induce TNF-α in a variety of cell types, including microglia and macrophages (19, 20), we assessed whether the inclusion of neutralizing antibody against TNF-α would influence IFN-γ-induced CD40 expression. The murine microglial cell line EOC13 and the macrophage cell line RAW264.7 were incubated with medium or IFN-γ in the presence of 10 μg/ml of TNF-α-neutralizing antibody or isotype control antibody, and then total RNA was harvested and analyzed for CD40, IRF-1, and GAPDH mRNA expression using ribonuclease protection assay (Fig. 1A). Low levels of CD40 mRNA were expressed constitutively (lanes 1 and 5), and IFN-γ enhanced CD40 mRNA expression by ~22-fold in RAW264.7 cells and ~37-fold in EOC13 cells (lanes 2 and 6). The inclusion of isotype antibody had no effect on IFN-γ-induced CD40 mRNA expression (lanes 3 and 7), whereas incubation with anti-TNF-α-neutralizing antibody inhibited IFN-γ-induced CD40 mRNA expression by ~73 and ~85% in RAW264.7 and EOC13 cells, respectively (lanes 4 and 8). Anti-TNF-α antibody treatment had a minimal effect on the ability of IFN-γ to induce IRF-1 expression, suggesting specific suppression of IFN-γ-induced CD40 mRNA expression (Fig. 1A). Comparable results were obtained when examining the effect of anti-TNF-α-neutralizing antibody on IFN-γ-induced CD40 promoter activity in RAW264.7 cells (~66% inhibition) (Fig. 1B). These results indicate that endogenously produced

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Involvement of TNF-α in IFN-γ-induced CD40 Expression
TNF-α contributes to IFN-γ-induced CD40 expression and that the effect of TNF-α is on CD40 gene transcription.

To formally demonstrate that IFN-γ stimulation led to the production of TNF-α, cells were incubated with medium or IFN-γ for 24 h, and then the supernatants were harvested and analyzed for biologically active TNF-α (Table I). RAW264.7 cells constitutively produced TNF-α protein, which was enhanced upon IFN-γ treatment. Also, EOC13 cells constitutively expressed low levels of TNF-α that were elevated with IFN-γ stimulation. IFN-γ enhancement of TNF-α production was observed as early as 4 h and persisted for 48 h (data not shown).

**TNF-α Is Required for Optimal IFN-γ-induced CD40 Expression**—To further confirm the importance of TNF-α in IFN-γ-induced CD40 expression, primary microglia from TNF-α-deficient mice were examined. Wild type or TNF-α-deficient primary microglia were incubated with medium or IFN-γ for 48 h, and then CD40 surface protein expression was assessed by fluorescence-activated cell sorter analysis. IFN-γ induced expression of CD40 in wild type primary microglia, whereas only a modest induction of CD40 expression was seen in TNF-α-deficient cells (Fig. 2). The inclusion of exogenous TNF-α modestly augmented IFN-γ-induced CD40 expression in wild type microglia, whereas in TNF-α-deficient cells, the addition of TNF-α plus IFN-γ-induced CD40 levels comparable with wild type microglia (Fig. 2). These results illustrate that optimal expression of CD40 in response to IFN-γ requires TNF-α.

**IFN-γ-induced CD40 Promoter Activity Requires NF-κB Activation**—We have shown that an autocrine response to IFN-γ-induced TNF-α is important for IFN-γ-induced CD40 mRNA expression and CD40 promoter activity (Fig. 1). Because the activation of NF-κB is one of the major signaling pathways that TNF-α initiates (for review see Ref. 21), we wished to determine the involvement of NF-κB in IFN-γ-induced CD40 expression. Dominant-negative (DN) expression constructs of IKK-α or -β (17) that contain substitutions of alanine for an essential lysine in the ATP-binding site, rendering the proteins catalytically inactive, were utilized. The DN constructs, as well as wild type IKK-α and IKK-β constructs, were co-transfected with hCD40p0.7 into RAW264.7 cells, treated with medium or IFN-γ, and then assayed for luciferase and β-galactosidase activities. Fold induction was calculated as described under "Experimental Procedures" (B). The data shown are the mean ± S.E. of three experiments.
Involvement of TNF-α in IFN-γ-induced CD40 Expression

Three NF-κB-binding Sites in the Human CD40 Promoter Are Important for IFN-γ-induced CD40 Promoter Activity—Within the human CD40 promoter, we identified at least four potential NBSs. They are named according to their position with respect to the transcription initiation sites: dNBS, mNBS, m2NBS, and pNBS (Fig. 4A). To ascertain the functional roles of these NF-κB cis-regulatory elements, site-directed mutagenesis was performed either individually or in combination (see “Experimental Procedures”). Mutation of the dNBS led to a partial inhibitory effect on IFN-γ-induced CD40 promoter activity (−60%) compared with the full-length promoter construct (Fig. 4B). Also, mutation of the mNBS and m2NBS caused a 40% inhibition in IFN-γ-induced CD40 promoter activation. Curiously, mutation of the pNBS led to a reproducible increase in IFN-γ-induced CD40 promoter activity. Combining mutations of the dNBS with the mNBS, m2NBS, or both did not significantly affect CD40 promoter activity relative to that of the dNBS mutation alone (Fig. 4B), whereas the combination mutation of mNBS and m2NBS led to a −50% inhibition of IFN-γ-induced CD40 promoter activity. These results suggest that the dNBS, mNBS, and m2NBS elements are involved in IFN-γ induction of CD40 promoter activity.

To confirm that NF-κB actually binds to the NBS identified functionally as important for CD40 promoter activity, EOC13 cells were treated with IFN-γ for 30 min to 24 h, nuclear extracts were prepared, and EMSA were performed using dNBS oligonucleotides as probes and/or competitors. Fig. 5A demonstrates a low basal binding activity using nuclear extracts from untreated cells (lane 1), which increases in intensity upon IFN-γ stimulation (lanes 2–8). Optimal complex formation over the dNBS probe was detected after 6–12 h of IFN-γ stimulation (lanes 6 and 7). The complexes were competed by a 100-fold molar excess of unlabeled dNBS oligonucleotide (data not shown). The identity of the IFN-γ-induced complex was confirmed by supershifting with antibodies to NF-κB family members. Anti-p50 antibody partially supershifted the upper complex (Fig. 5B, lane 2), whereas anti-p65 antibody supershifted both the upper and lower complexes (lane 3). Incubation of nuclear extracts with antibodies against p50 and p65 led to a complete supershift of both complexes (lane 4). Normal rabbit serum (lane 1) and antibodies against p52, c-Rel, or RelB (lanes 5–7) did not affect complex formation. These data suggest that the IFN-γ-induced protein complexes bound to the dNBS of the CD40 promoter are composed of p65 homodimers and p65/p50 heterodimers. Similar binding patterns and composition of the complexes were observed using mNBS and m2NBS oligonucleotides as probes (data not shown). Similar results were obtained using nuclear extracts from RAW264.7 cells (data not shown).

To demonstrate the involvement of IFN-γ-induced TNF-α production in NF-κB activation, nuclear extracts were prepared from cells treated with IFN-γ alone, IFN-γ plus isotype control antibody, or IFN-γ plus neutralizing TNF-α antibody for 6 h. As shown in Fig. 5C, the inclusion of anti-TNF-α antibody prevents IFN-γ induction of NF-κB (lane 4), whereas the isotype-matched control has no effect (lane 3). These findings indicate that IFN-γ treatment leads to TNF-α production, which is subsequently responsible for activation of NF-κB.

As another measure of the involvement of NF-κB in this response, we utilized broad spectrum pharmacological inhibitors of NF-κB such as TPCK and PTDTC. RAW264.7 cells were incubated with medium or IFN-γ in the absence or presence of TPCK (30 μM) or PTDTC (30 μM) for 8 h, and then CD40 mRNA expression was examined. As shown in Fig. 5D, low levels of CD40 mRNA were expressed constitutively (lane 1) and were not influenced by the inclusion of TPCK or PTDTC (lanes 2 and 3). Stimulation with IFN-γ enhanced CD40 mRNA levels (lane 4), and addition of TPCK and PTDTC strongly inhibited expression by −74% and −63%, respectively (lanes 5 and 6). These findings confirm the importance of NF-κB in IFN-γ-induced CD40 expression.

DISCUSSION

CD40 has been implicated as a proinflammatory molecule that is involved in a variety of critical immunologic functions, yet its regulation remains an enigma. This study is an extension of our previous report describing the involvement of IFN-γ-activated STAT-1α and constitutively expressed PU.1/Spi-B transcription factors in IFN-γ-induced CD40 expression in macrophages and microglia (16). In this study, we present data that IFN-γ induction of CD40 gene expression is critically dependent on the endogenous production of TNF-α, which then leads to NF-κB activation and binding to NF-κB regulatory
elements within the CD40 promoter. Our results indicate that co-treatment of macrophages and microglia with IFN-\(\gamma\) and neutralizing antibodies to TNF-\(\alpha\) attenuates the induction of IFN-\(\gamma\)-induced CD40 mRNA expression and CD40 promoter activity (Fig. 1). To substantiate the importance of endogenous TNF-\(\alpha\) production in this response, microglia from TNF-\(\alpha\)-deficient mice were examined for IFN-\(\gamma\) induction of CD40 protein expression. Such cells were refractive to IFN-\(\gamma\) stimulation; however, the inclusion of exogenous TNF-\(\alpha\) plus IFN-\(\gamma\) induced CD40 protein levels comparable with wild type primary microglia (Fig. 2). These results suggest that the autocrine response to IFN-\(\gamma\)-induced TNF-\(\alpha\) production in macrophages and microglia is critical for optimal CD40 expression. Indeed, we demonstrated that IFN-\(\gamma\) treatment of RAW264.7 and EOC13 cell lines induced the production of TNF-\(\alpha\) (Table 1). The levels of TNF-\(\alpha\) produced are clearly sufficient for subsequent CD40 expression, because the addition of exogenous TNF-\(\alpha\) (50 ng/ml) only modestly enhanced IFN-\(\gamma\)-induced CD40 expression (15). Synergistic interactions between IFN-\(\gamma\) and TNF-\(\alpha\) are known for many genes such as class I MHC, IP-10, IRF-1 and ICAM-1 (22–26). However, these synergistic responses are achieved upon inclusion of exogenous sources of both TNF-\(\alpha\) and IFN-\(\gamma\). In contrast, for IFN-\(\gamma\)-induced CD40 expression in macrophages and microglia, the autocrine response to IFN-\(\gamma\)-induced endogenous TNF-\(\alpha\) production is sufficient for optimal CD40 expression.

Signaling through TNF receptors leads to the activation of the NF-\(\kappa\)B pathway and subsequent expression of a wide array of genes (for review see Refs. 27 and 28). NF-\(\kappa\)B is sequestered in an inactive form in the cytoplasm through interaction with inhibitory proteins, the I\(\kappa\)Bs. Exposure to TNF-\(\alpha\) leads to the rapid phosphorylation, ubiquitination, and proteolytic degradation of I\(\kappa\)B, allowing NF-\(\kappa\)B to translocate to the nucleus to regulate gene transcription. The multi-subunit IKK, which is responsible for inducible I\(\kappa\)B phosphorylation, contains several catalytic subunits; among them are IKK-\(\beta\) and IKK-\(\gamma\) (27). We employed a variety of strategies to assess the importance of NF-\(\kappa\)B in IFN-\(\gamma\)-induced CD40 gene expression. First, blocking the activation of NF-\(\kappa\)B by transfection of DN constructs of IKK-\(\beta\) and IKK-\(\gamma\) inhibited IFN-\(\gamma\)-induced CD40 promoter activity (Fig. 3). Differences were observed using the two constructs; the IKK-\(\beta\) DN partially inhibited CD40 promoter activity, whereas the IKK-\(\gamma\) DN construct abolished IFN-\(\gamma\)-induced CD40 promoter activation. DN versions of IKK have been shown to inhibit TNF-\(\alpha\) activation of VCAM-1 promoter activity (29). In this system, similar to ours, DN IKK-\(\gamma\) was a more potent inhibitor than IKK-\(\beta\). These findings may reflect the fact that IKK-\(\beta\) may be the IKK isoform critical for inflammatory responses. Broad spectrum pharmacological inhibitors of NF-\(\kappa\)B such as TPCK and PDTC also suppressed IFN-\(\gamma\)-induced CD40 mRNA expression (Fig. 5D). Collectively, these results suggest that the pathways leading to NF-\(\kappa\)B
Involvement of TNF-α in IFN-γ-induced CD40 Expression

**A.** IFN-γ (h)

0 0.5 1 2 3 6 12 24

- NF-κB
- NF-κB

**B.** IFN-γ (6 h)

NRS p50 p65 p70 p65 p50 crel RelB

- Supershift
- NF-κB
- NF-κB

**C.** IFN-γ (6 h)

Medium Ant-TNF-α

| Medium | No Ab | Isotype Ab | Anti-TNF-α |
|--------|-------|-----------|------------|
| IFN-γ  | +     | +         | +          |

| Medium | IFN-γ (8 h) |
|--------|-------------|
| PTC (30 μM) | - + + + - + - |

**D.** Medium IFN-γ (8 h)

- CD40
- IRF-1
- GAPDH

IFN-γ-activated NF-κB binds to the human CD40 distal NF-κB-binding site. EOC13 cells were incubated with medium (lane 1) or IFN-γ (5 ng/ml) for 30 min to 24 h (lanes 2–8), and then nuclear extracts were prepared. EMSA was performed using the human CD40 dNBS as a probe (A). Nuclear extracts from EOC13 cells stimulated with IFN-γ for 6 h were incubated with normal rabbit serum (lane 1) or with 1 μl of the indicated antisera (lanes 2–7) for 30 min before the addition of labeled dNBS probe (B). The data shown are representative of three experiments. EOC13 cells were incubated with medium (lane 1) or IFN-γ (lane 2) in the presence of 10 μg/ml of isotype antibody (lane 3) or TNF-α-neutralizing antibody (lane 4) for 6 h. Nuclear extracts were prepared, and EMSA were performed using the human CD40 dNBS as a probe (C). RAW264.7 cells were incubated with medium (lane 1), TPCK (30 μM, lane 2), PDTC (30 μM, lane 3), IFN-γ (5 ng/ml, lane 4), IFN-γ plus TPCK (lane 5), or IFN-γ plus PDTC (lane 6) for 8 h. Total RNA was harvested and analyzed by ribonuclease protection assay for CD40, IRF-1, and GAPDH mRNA expression (D). The data shown are representative of two experiments.

**Fig. 5.** IFN-γ-activated NF-κB binds to the human CD40 distal NF-κB-binding site. EOC13 cells were incubated with medium (lane 1) or IFN-γ (5 ng/ml) for 30 min to 24 h (lanes 2–8), and then nuclear extracts were prepared. EMSA was performed using the human CD40 dNBS as a probe (A). The data shown are representative of three experiments. EOC13 cells were incubated with medium (lane 1) or IFN-γ (lane 2) in the presence of 10 μg/ml of isotype antibody (lane 3) or TNF-α-neutralizing antibody (lane 4) for 6 h. Nuclear extracts were prepared, and EMSA were performed using the human CD40 dNBS as a probe (C). RAW264.7 cells were incubated with medium (lane 1), TPCK (30 μM, lane 2), PDTC (30 μM, lane 3), IFN-γ (5 ng/ml, lane 4), IFN-γ plus TPCK (lane 5), or IFN-γ plus PDTC (lane 6) for 8 h. Total RNA was harvested and analyzed by ribonuclease protection assay for CD40, IRF-1, and GAPDH mRNA expression (D). The data shown are representative of two experiments.

Within the human CD40 promoter, there are four potential NBS. Mutation of each NBS individually or in combination suggested that the dNBS, mNBS, and m2NBS are involved in IFN-γ-induced CD40 promoter activity, with the dNBS being most important for IFN-γ-induced CD40 promoter activity (Fig. 4). Nuclear extracts from IFN-γ-stimulated cells formed a complex over the dNBS probe (Fig. 5). This complex contains both p50 and p65 NF-κB family members as determined by gel shift analysis. The importance of TNF-α in this response was demonstrated by the inclusion of anti-TNF-α neutralizing antibody, which inhibited IFN-γ induction of NF-κB activation (Fig. 5C). The kinetics of IFN-γ-induced NF-κB activation are delayed (optimal response after 6–12 h of IFN-γ stimulation); this reflects the need for TNF-α synthesis in response to IFN-γ treatment. We have previously observed that inclusion of the protein synthesis inhibitor puromycin partially inhibits (~50%) IFN-γ induction of CD40 mRNA expression in EOC13 cells (15). This effect may be due in part to inhibition of TNF-α production.

Interestingly, we consistently observed an enhancement of IFN-γ-induced CD40 promoter activity when the pNBS was mutated, suggesting that negative regulatory element(s) may reside in this region. Curiously, IFN-γ-induced NF-κB binding to the pNBS (data not shown) similar to what was observed with the dNBS, mNBS, and m2NBS. The importance of this region in regulating CD40 expression is currently under investigation.

The results from this study suggest the importance of autocrine responsiveness to IFN-γ-induced TNF-α and the subsequent activation of NF-κB in IFN-γ-induced CD40 expression. The combination of the data from this study and our previous reports promoted us to provide a revised model of IFN-γ-induced CD40 expression (Fig. 6). In this model, seven cis-regulatory elements are involved in IFN-γ-induced CD40 promoter activation; two Ets elements, two γ activated sequence elements, and three NF-κB elements. Constitutively expressed PU.1/Spi-B binds to EtsA and EtsB sites, IFN-γ-activated STAT-1α binds to the medial and distal γ activated sequence elements, and IFN-γ-induced TNF-α-activated NF-κB binds to the dNBS, mNBS, and m2NBS. Together, these transcription factors recruit and coordinate a complex that we tentatively call “integrator,” which mediates CD40 gene transcription. The
cAMP response element-binding protein-binding protein is a likely candidate given its ability to interact with PU.1, STAT-1α, and NF-κB (for review see Ref. 30). In this regard, we have preliminary data that inclusion of a cAMP response element-binding protein-binding protein expression construct enhances IFN-γ-induced CD40 promoter activity (data not shown). The involvement and components of this integrator are currently being investigated in our laboratory.

The model proposed above has only been tested thus far in macrophages and microglial cells. CD40 gene expression occurs in a cell type-specific manner, depending on the stimulus utilized. For example, in cultured rat vascular smooth muscle cells, IFN-γ and TNF-α alone induce only a modest increase in CD40 expression, whereas combined stimulation leads to a significant increase in CD40 (31). Interestingly, IL-1β alone induced significant levels of CD40 in these cells (31). In human endothelial cells and thymic epithelial cells, IFN-γ, IL-1β, and TNF-α individually enhance CD40 expression (32, 33). Fibroblasts do not express CD40 in response to IFN-γ alone and are weakly inducible by TNF-α, and IFN-γ plus TNF-α synergistically induce CD40 mRNA expression (34). Interestingly, the induction by IFN-γ plus TNF-α was abrogated in fibroblasts from p65-deficient mice, demonstrating a role for NF-κB in this response (34). The inability of IFN-γ alone to induce CD40 in fibroblasts may reflect the fact that IFN-γ alone has no influence on NF-κB binding activity, likely because of the inability to induce NF-κB production (24, 35). Macrophages/microglia are one of the few cell types that can produce TNF-α in response to IFN-γ alone; most cell types need a combination of stimuli such as lipopolysaccharide plus IFN-γ or IL-1β plus IFN-γ (for review see Ref. 36). Thus, the molecular mechanisms underlying CD40 gene expression are complex and will reflect the availability of transcription factors such as PU.1/Spi-B, NF-κB, and STAT-1α and possibly others.

CD40 expression by resident cells of the central nervous system, most likely microglia, is critical for the infiltration/retention of inflammatory cells in the central nervous system, leading to the disease of experimental allergic encephalomyelitis (10). Given the important role of CD40 in inflammatory events in the central nervous system as well as other organ systems (for review see Refs. 6 and 37), it is imperative to understand the molecular mechanisms contributing to both CD40 induction and repression in various cell types. We have previously shown that IL-4 inhibits IFN-γ-induced CD40 expression in microglia in a STAT-6-dependent manner (38). Several recent studies have shown that IL-4 inhibits E-selectin gene transcription and osteoclastogenesis through STAT-6-dependent inhibition of NF-κB (39, 40). Given our findings of the importance of NF-κB in IFN-γ-induced CD40 expression, future studies will assess whether IL-4/STAT-6 inhibition is operative by antagonism of NF-κB binding. Other inhibitors of CD40 expression on microglia include neurotrophins, IL-10, and IL-11 (41). It will be of interest to elucidate the molecular basis of their inhibitory actions.

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