

## Interleukin-10 Signaling Blocks Inhibitor of $\kappa$ B Kinase Activity and Nuclear Factor $\kappa$ B DNA Binding\*

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**The transcription factor nuclear factor  $\kappa$ B (NF- $\kappa$ B) coordinates the activation of numerous genes in response to pathogens and proinflammatory cytokines and is, therefore, pivotal in the development of acute and chronic inflammatory diseases. In its inactive state, NF- $\kappa$ B is constitutively present in the cytoplasm as a p50-p65 heterodimer bound to its inhibitory protein I $\kappa$ B. Proinflammatory cytokines, such as tumor necrosis factor (TNF), activate NF- $\kappa$ B by stimulating the activity of the I $\kappa$ B kinases (IKKs) which phosphorylate I $\kappa$ B $\alpha$  on serine residues 32 and 36, targeting it for rapid degradation by the 26 S proteasome. This enables the release and nuclear translocation of the NF- $\kappa$ B complex and activation of gene transcription. Interleukin-10 (IL-10) is a pleiotropic cytokine that controls inflammatory processes by suppressing the production of proinflammatory cytokines which are known to be transcriptionally controlled by NF- $\kappa$ B. Conflicting data exists on the effects of IL-10 on TNF- and LPS-induced NF- $\kappa$ B activity in human monocytes and the molecular mechanisms involved have not been elucidated. In this study, we show that IL-10 functions to block NF- $\kappa$ B activity at two levels: 1) through the suppression of IKK activity and 2) through the inhibition of NF- $\kappa$ B DNA binding activity. This is the first evidence of an anti-inflammatory protein inhibiting IKK activity and demonstrates that IKK is a logical target for blocking inflammatory diseases.**

Interleukin-10 (IL-10)<sup>1</sup> is a pleiotropic cytokine produced by many cell types including monocytes/macrophages, cells that play a critical role in the inflammatory process (1–3). The anti-inflammatory effect of IL-10 is achieved through the suppressed production of macrophage inflammatory proteins such as IL-1, IL-6, IL-8, IL-12, TNF, granulocyte-macrophage colony stimulating factor, granulocyte colony stimulating factor, MHC class II molecules, B7, and intercellular adhesion molecule-1 (3–10) and through diminishing Th1 cell activity by suppres-

sion of IL-2 and interferon- $\gamma$  (11). Evidence for the *in vivo* role of IL-10 as an important immunoregulator with potent anti-inflammatory and immunosuppressive activities comes from the observation that IL-10-deficient mice develop chronic enterocolitis with similarities to inflammatory bowel disease (12). IL-10 treatment has shown benefits in models of induced colitis (13–15) and arthritis (14, 16), as well as in models of experimental autoimmune encephalomyelitis, pancreatitis, diabetes mellitus, and experimental endotoxemia *in vivo* (17–20). Moreover, patients suffering from Crohn's disease display clinical improvement following IL-10 treatment (21, 22).

Some molecular mechanisms of monocyte deactivation by IL-10 have been described. IL-10 was found to inhibit protein-tyrosine kinase activation induced by LPS binding to the CD14 receptor and to consequently block the downstream Ras signaling pathway (23). Moreover, IL-10 has been shown to interfere with protein-tyrosine kinase-dependent CD40 signaling controlling IL-1 $\beta$  synthesis in monocytes (24).

Many of the proinflammatory cytokines and costimulatory proteins demonstrated to be suppressed by IL-10 are known to be regulated by the transcription factor NF- $\kappa$ B (25). Furthermore, NF- $\kappa$ B also has a role in IL-10 gene expression (26). Classical NF- $\kappa$ B, a heterodimer composed of p50 and p65 subunits, is a potent activator of gene expression (25, 27, 28). NF- $\kappa$ B resides in the cytoplasm as an inactive complex bound to the inhibitor protein I $\kappa$ B (27). In response to a variety of extracellular stimuli, such as IL-1 $\beta$ , TNF, LPS, or phorbol esters, I $\kappa$ B proteins are rapidly phosphorylated by the recently identified I $\kappa$ B kinase (IKK) complex. IKK-induced phosphorylation of I $\kappa$ B occurs on residues Ser<sup>32</sup> and Ser<sup>36</sup> for I $\kappa$ B $\alpha$  and on Ser<sup>19</sup> and Ser<sup>23</sup> for I $\kappa$ B $\beta$  (25, 29, 30) which targets these inhibitory proteins for rapid polyubiquitination and degradation through the 26 S proteasome (31). This results in liberation of NF- $\kappa$ B from I $\kappa$ B and subsequent translocation of NF- $\kappa$ B to the nucleus where it regulates gene transcription. The recent identification of the kinases responsible for I $\kappa$ B phosphorylation is a critical step for understanding the mechanisms of NF- $\kappa$ B activation. The cytokine-responsive I $\kappa$ B kinase complex is composed of stoichiometric amounts of IKK- $\alpha$  and IKK- $\beta$  and the recently discovered IKK $\gamma$ /NEMO (32). IKK $\gamma$  preferentially interacts with IKK $\beta$  and is required for the activation of the IKK complex (29, 32–36). Further evidence that IKK $\alpha$  and IKK $\beta$  are required for the functional IKK complex is supported by experiments which demonstrate that the overexpression of IKK $\alpha$  or IKK $\beta$  activated an NF- $\kappa$ B-dependent reporter, whereas dominant negative mutants of IKK $\alpha$  or IKK $\beta$  inhibited TNF- or IL-1-induced NF- $\kappa$ B activation (29, 33–36).

Although IL-10 has been found to inhibit the activity of NF- $\kappa$ B in monocytes/macrophages and T cells, results from different groups have been variable as to whether the block occurs at the level of I $\kappa$ B (37–40, 44). Furthermore, no molecular mechanisms have been elucidated which may control

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<sup>1</sup> The abbreviations used are: IL, interleukin; TNF, tumor necrosis factor; MHC, major histocompatibility complex; LPS, lipopolysaccharide; NF- $\kappa$ B, nuclear factor  $\kappa$ B; I $\kappa$ B, inhibitor of  $\kappa$ B; IKK, I  $\kappa$ B kinase; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis.

NF- $\kappa$ B activity in response to IL-10. Our data provides evidence that IL-10 inhibits TNF-induced NF- $\kappa$ B activity by blocking TNF-induced IKK activity, thus inhibiting degradation of I $\kappa$ B and the subsequent NF- $\kappa$ B nuclear translocation. Additionally, a second mechanism appears to be functional: IL-10 signaling blocks the ability of translocated NF- $\kappa$ B to bind to DNA.

#### EXPERIMENTAL PROCEDURES

**Cell Culture and Treatments**—The human monocytic cell lines THP-1 (TIB202) and U937 (CRL-1593) were obtained from the American Type Culture Collection (ATCC) and were cultured at 37 °C in RPMI 1640 containing L-glutamine (Life Technologies, Inc., Gaithersburg, MD), supplemented with 10% fetal bovine serum and antibiotics (Life Technologies). Media for THP-1 cells was additionally supplemented with HEPES (pH 7.8) to a final concentration of 20 mM and with 2-mercaptoethanol (Life Technologies, Inc.) to a final concentration of  $5.5 \times 10^{-5}$  M. The human intestinal epithelial cell line HT-29 (HTB 38) was obtained from ATCC and was cultured at 37 °C in McCoy's 5A Media supplemented with 10% fetal bovine serum and antibiotics.

Recombinant human TNF was purchased from Promega (Madison, WI). Recombinant human IL-10 was purchased from R&D Systems (Minneapolis, MN). Stock solutions were found to have less than 0.1 ng of endotoxin/1  $\mu$ g of cytokine as determined by the LAL method. LPS (from *Escherichia coli* Serotype 055:B5) was purchased from Sigma. Cells were grown to a density of  $1 \times 10^6$  cells/ml and stimulated with TNF or LPS, in the presence or absence of IL-10. After the indicated time periods, cells were harvested and processed for electrophoretic mobility shift assays, Western blot analysis, or I $\kappa$ B kinase assays.

**Electrophoretic Mobility Shift Assays (EMSA)**—Nuclear extracts for EMSAs were prepared as described previously (41). 0.05% of Nonidet P-40 was used to extract nuclei. 8  $\mu$ g of extract were incubated for 20 min in a total of 20  $\mu$ l containing 1  $\mu$ g of poly(dI-dC) with 4–6  $\times 10^4$  cpm of a  $^{32}$ P-labeled oligonucleotide probe containing a  $\kappa$ B site from the class I MHC promoter (5'-CAGGGCTGGGGATTCCCATCTCCACAGTTTCACTTC-3'). The final buffer concentration was 10 mM Tris-HCl (pH 7.7), 50 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol. Complexes were resolved on a 5% polyacrylamide gel in Tris glycine buffer (25 mM Tris, 190 mM glycine, 1 mM EDTA) at 25 mA for 2–3 h at room temperature. Dried gels were exposed on film for 15–48 h. For supershift analysis, antibodies against specific NF- $\kappa$ B subunits were added to the nuclear extract and incubated for 15 min prior to the addition of poly(dI-dC) and labeled oligonucleotide probe. The following antibodies were used for supershift analysis: p65 (Rockland, Boyertown, PA), p50 (NLS, Santa Cruz Biotechnology), and c-Rel (C, Santa Cruz Biotechnology).

**Transfections and Luciferase Assays**—For transient transfections of THP-1 cells, cells were divided the day prior to transfection. THP-1 cells were transiently transfected with the 3 $\kappa$ B luciferase reporter plasmid, containing three copies of the MHC class I NF- $\kappa$ B consensus site or were transfected with a mutant 3 $\kappa$ B (mut3 $\kappa$ B) luciferase reporter, which is no longer transcriptionally activated by NF- $\kappa$ B. On the day of transfection, cells were collected and washed with  $\times 1$  phosphate-buffered saline. A total of 5  $\mu$ g of plasmid DNA was incubated in a total volume of 500  $\mu$ l of suspension Tris-buffered saline containing 25 mM Tris-Cl (pH 7.4), 137 mM NaCl, 5 mM KCl, 0.6 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.7 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>. 500  $\mu$ l of suspension Tris-buffered saline were combined with 500  $\mu$ l of diethylaminoethyl-dextran (DEAE-dextran) (Sigma) to a final concentration of 100 mg/ml. This mixture was added to the pelleted cells that were carefully resuspended and incubated for 60 min at 37 °C. Cells were then washed with suspension Tris-buffered saline and medium and were resuspended in 10 ml of complete medium. After a period of 48 h, IL-10 was added 60 min prior to stimulation with TNF for an additional 5 h. Cell extracts were prepared and luciferase activity was monitored as described previously (41). HT-29 cells were transfected with SuperFect Transfection Reagent (Qiagen GmbH, Hilden, Germany) as recommended by the manufacturer.

**Northern Blot Analysis**—Total RNA was isolated using the RNeasy Mini Kit as recommended by the manufacturer (Qiagen Inc., Valencia, CA). RNA samples were fractionated on an agarose gel and transferred overnight onto a nylon filter. The next day RNA was cross-linked with a UV cross-linker (Stratagene, La Jolla, Ca). For detection of I $\kappa$ B $\alpha$  and IL-8 mRNAs, blots were hybridized in QuickHyb buffer supplemented with 100  $\mu$ g of salmon sperm DNA as recommended by the manufacturer (Stratagene, La Jolla, Ca). All probes were generated with a random primed labeling kit (Amersham Pharmacia Biotech) in the presence of [ $\alpha$ - $^{32}$ P]dCTP (NEN Life Science Products Inc.). DNA prod-

ucts were purified over micro-Sephadex G-50 columns (Life Technologies), boiled, and added to the hybridization mixture. Washes were performed twice in  $2 \times$  SSC, 0.1% SDS for 10 min at room temperature, followed by two washes in  $0.1 \times$  SSC, 0.1% SDS for 20 min at 65 °C. Membranes were then exposed to film overnight.

**Western Blot Analysis**—Nuclear and cytoplasmic extracts were prepared as described previously (41). Equal amounts of extracts were subjected to SDS-PAGE electrophoresis and transferred to nitrocellulose membranes (Schleicher & Schuell). Blocking was performed in 5% nonfat dry milk,  $1 \times$  TBST (25 mM Tris-HCl, pH 8.0, 125 mM NaCl, 0.1% Tween 20). Primary and secondary antibodies were diluted in 0.25% bovine serum albumin,  $1 \times$  TBST and incubations proceeded for 30 min at room temperature. Washes were performed in  $1 \times$  TBST for 5 min and repeated 3 times. Specific proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech). Antibodies for I $\kappa$ B $\alpha$  (C-21), I $\kappa$ B $\beta$  (C-20), I $\kappa$ B $\epsilon$  (M-121), and p50 (NLS) were obtained from Santa Cruz Biotechnology. The antibody for p65 was obtained from Rockland.

**I $\kappa$ B $\alpha$  Kinase Assay**—Cells were treated with TNF for various times without or with prior incubation with IL-10. Whole cell extracts were immunoprecipitated with an antibody against IKK- $\beta$  (gift of Dr. F. Mercurio, Signal Pharmaceuticals) and the immunoprecipitates subjected to an IKK activity assay (36), using GST-I $\kappa$ B $\alpha$ (1–54) WT (4  $\mu$ g) or a mutated form of I $\kappa$ B $\alpha$ (S32T,S36T) as substrates. Samples were subjected to SDS-PAGE and gels were dyed with Gelcode Blue stain (Pierce Inc.) for equal loading control. Bands were quantitated on a PhosphorImager System (Storm 840; Molecular Dynamics Inc., Sunnydale, CA).

#### RESULTS

**IL-10 Inhibits TNF-induced Proinflammatory IL-8 Cytokine Production and NF- $\kappa$ B-dependent Transcription in Human Monocytic and Intestinal Epithelial Cells**—To investigate whether the anti-inflammatory effects of IL-10 are mediated through the ability of this cytokine to inhibit NF- $\kappa$ B, we chose to use the human monocytic cell lines THP-1 and U937 and the human intestinal epithelial cell line HT-29 as *in vitro* models. To elucidate whether IL-10 induced a similar effect in THP-1, U937, and HT-29 cells as previously demonstrated in peripheral blood mononuclear cells (7), cells were stimulated with TNF or LPS in either the presence or absence of human recombinant IL-10. Stimulation with TNF or LPS for 8 h led to an increase in IL-8 production in THP-1, U937, and HT-29 cells, which was inhibited by the addition of IL-10 (data not shown).

Since it has been well established that TNF up-regulates IL-8 transcription through an NF- $\kappa$ B-dependent mechanism (42), we wanted to determine whether IL-10 inhibited transcriptional activation of NF- $\kappa$ B. THP-1 and HT-29 cells were transiently transfected with the 3 $\kappa$ B luciferase reporter plasmid, containing three copies of the MHC class I NF- $\kappa$ B consensus site. To ensure that luciferase activities were specific for NF- $\kappa$ B-dependent transcription, experiments were also performed in parallel using the mutant 3 $\kappa$ B (mut 3 $\kappa$ B) luciferase reporter, which is not transcriptionally activated by NF- $\kappa$ B (43). Forty-eight hours after transfection, cells were treated with TNF in either the presence or absence of IL-10, harvested 5 h following addition of TNF, and assayed for NF- $\kappa$ B-dependent transcription. As shown in Fig. 1A, TNF induced the transcriptional activity of NF- $\kappa$ B approximately 20-fold in THP-1 cells (*left panel*) and approximately 35-fold in HT-29 cells (*right panel*). However, pretreatment with IL-10 strongly inhibited the TNF-induced NF- $\kappa$ B activity in both THP-1 and HT-29 cells (Fig. 1A). The ability of IL-10 to modulate NF- $\kappa$ B transcriptional activity was specific, since transfection experiments were normalized to the mut 3 $\kappa$ B luciferase reporter (Fig. 1A, *left and right panels*).

Since IL-10 repressed an NF- $\kappa$ B responsive reporter in transient transfection assays, we wanted to determine whether IL-10 would also block the ability of TNF to up-regulate NF- $\kappa$ B responsive gene expression. To experimentally address this question, Northern blot analysis for I $\kappa$ B $\alpha$  and IL-8 (both NF-

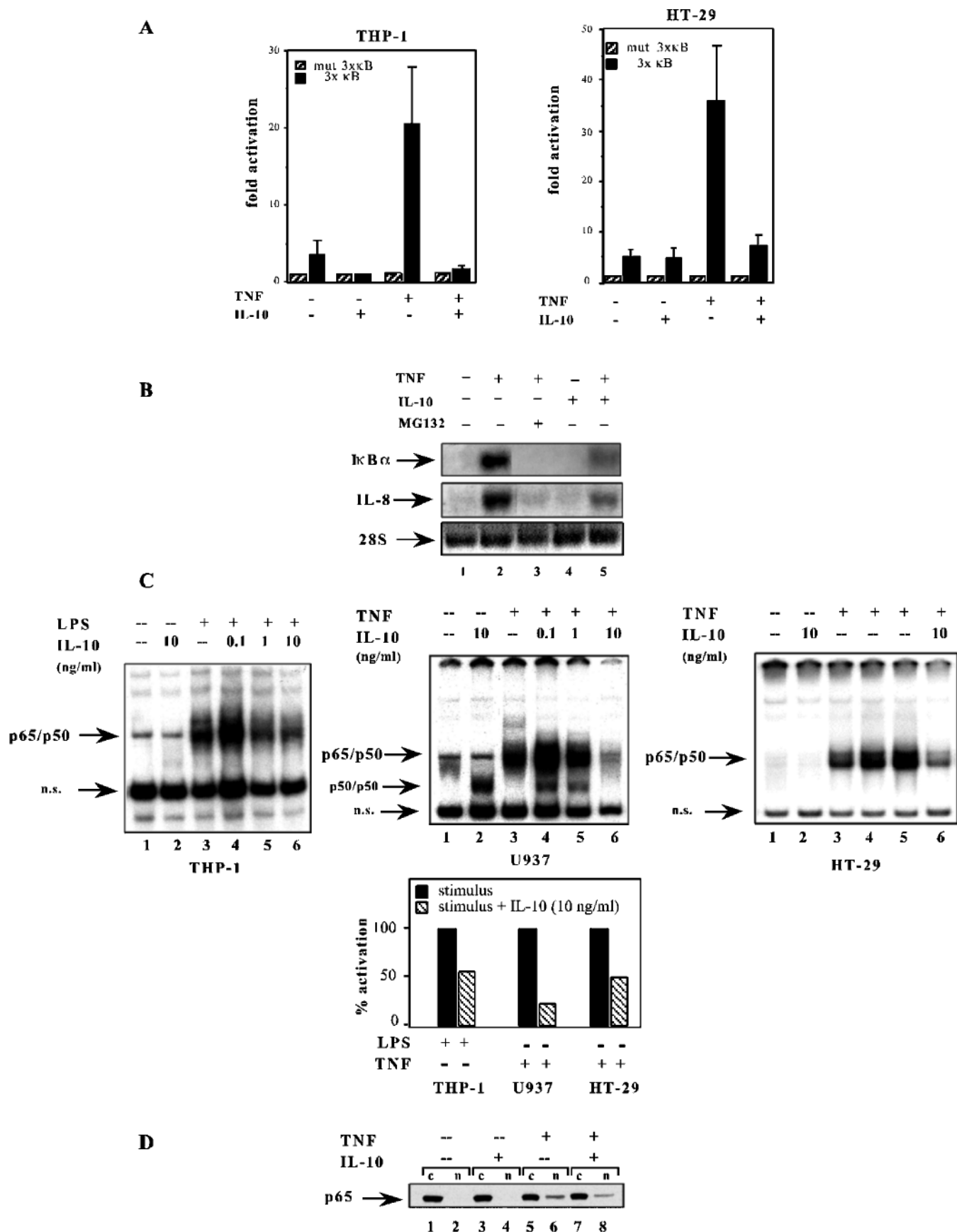


FIG. 1. A, IL-10 inhibits NF- $\kappa$ B dependent transcription in human monocytic and intestinal epithelial cells. THP-1 and HT-29 cells were transfected with the 3 $\kappa$ B-Luc or mut 3 $\kappa$ B-Luc construct by the DEAE-dextran method (THP-1) or with SuperFect (HT-29). 48 h after transfection cells were exposed to TNF (2 ng/ml) in the presence or absence of IL-10 (100 ng/ml) for 5 h. Cells were then lysed and the cytosolic extracts (100  $\mu$ g) were used in luciferase assays to determine activity. Fold induction is relative to luciferase activity in cells transfected with mut 3 $\kappa$ B-Luc. Data is expressed as the mean of two independent experiments  $\pm$  S.E. B, IL-10 suppresses TNF-induced steady state I $\kappa$ B $\alpha$  mRNA levels. THP-1



$\kappa$ B-regulated genes) was performed. Total RNA was prepared from THP-1 cells that had been treated with TNF alone, with TNF plus IL-10, or with TNF plus the NF- $\kappa$ B inhibitor MG 132 (55). TNF-treated cells displayed a strong increase in both I $\kappa$ B $\alpha$  and IL-8 gene expression (Fig. 1B, lanes 1 and 2, upper and middle panels). However, I $\kappa$ B $\alpha$  and IL-8 mRNA levels were suppressed approximately 60% in cells that had been pretreated by IL-10 and subsequently stimulated with TNF (Fig. 1B, lane 5, upper and middle panels). As predicted, pretreatment with the NF- $\kappa$ B inhibitor MG 132 suppressed TNF-induced I $\kappa$ B $\alpha$  and IL-8 mRNA levels almost completely (Fig. 1B, lanes 2 and 3, upper and middle panels). These results indicate that IL-10 is capable of inhibiting TNF-induced expression of two endogenous, NF- $\kappa$ B-regulated genes.

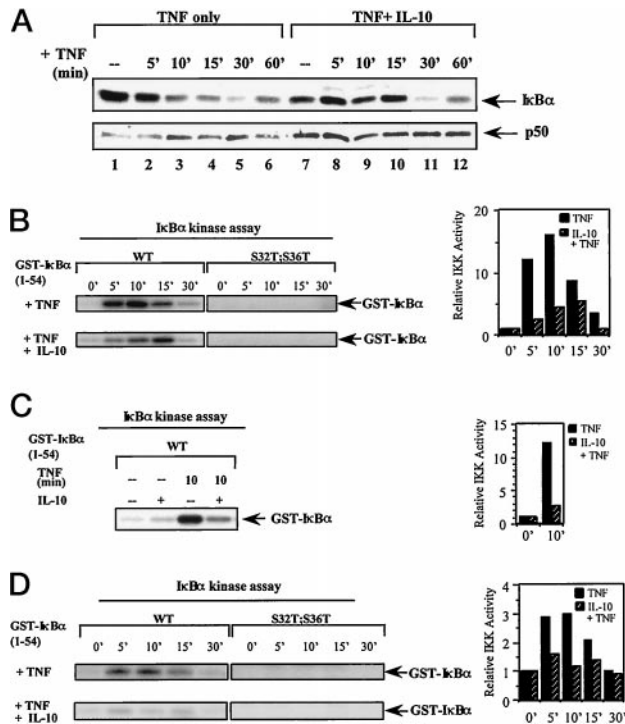
**Inhibition of NF- $\kappa$ B DNA Binding Activity by IL-10**—To assess whether IL-10 inhibited NF- $\kappa$ B-dependent transcription through the suppression of DNA binding, EMSAs were performed. Nuclear extracts were prepared from cells treated with TNF or LPS in the presence or absence of IL-10 and nuclear proteins were analyzed for their ability to recognize a <sup>32</sup>P-labeled NF- $\kappa$ B consensus site. Analysis of nuclear extracts from TNF- or LPS-stimulated cells demonstrated an increase in NF- $\kappa$ B DNA binding activity as compared with nuclear extracts from unstimulated cells (Fig. 1C, compare lane 1 to 3, left, middle, and right panels). Pretreatment with IL-10 inhibited TNF- and LPS-induced DNA binding in a dose-dependent manner (Fig. 1C, lanes 4–6, left and middle panels). IL-10 induced its effects on NF- $\kappa$ B DNA binding by specifically affecting the p65/p50 heterodimer complex, as determined by antibody supershift experiments (data not shown). Even though IL-10 blocked both TNF- and LPS-induced NF- $\kappa$ B DNA binding, the ability of IL-10 to inhibit NF- $\kappa$ B was greater in response to TNF than in response to LPS. Importantly, IL-10 inhibited TNF-induced NF- $\kappa$ B DNA binding activity to a similar level in both THP-1 and U937 cells (Fig. 1C and data not shown). Although we observed a similar inhibitory effect on NF- $\kappa$ B DNA binding activity in HT-29 cells following TNF stimulation, this effect was not as dramatic as observed for monocytic cells (Fig. 1C).

**IL-10 Inhibits NF- $\kappa$ B Nuclear Translocation by Preventing TNF-induced Degradation of I $\kappa$ B $\alpha$** —The primary level of control of NF- $\kappa$ B is through its interaction with the inhibitor protein I $\kappa$ B. Thus, one mechanism to explain the ability of IL-10 to inhibit NF- $\kappa$ B activity is by the ability of this anti-inflammatory cytokine to inhibit nuclear translocation of NF- $\kappa$ B by blocking I $\kappa$ B $\alpha$  degradation in response to TNF stimulation. To experimentally address this question, THP-1 cells were stimulated with TNF for 15 min in either the presence or absence of IL-10, cytoplasmic and nuclear proteins were isolated, and Western blot analysis was performed to determine whether IL-10 addition effected nuclear translocation of the p65 protein. As shown in Fig. 1D, p65 was predominantly cytoplasmically localized in unstimulated cells (Fig. 1D, lanes 1

and 2) and in cells treated with IL-10 alone (lanes 3 and 4). Cells stimulated with TNF (15 min) demonstrated an increase in nuclear translocation of p65 (lanes 5 and 6). In contrast, cells pretreated with IL-10 and then stimulated with TNF for 15 min demonstrated a reduction in nuclear p65 (Fig. 1D, compare lanes 7 and 8 with lanes 5 and 6). To further elucidate if the IL-10-induced block of p65 nuclear translocation was caused by the ability of this cytokine to interfere with TNF-induced degradation of I $\kappa$ B $\alpha$ , cytoplasmic extracts of cells stimulated with TNF for various times in the absence or presence of IL-10 were subjected to Western blot analysis. Immunoblot analysis for I $\kappa$ B $\alpha$  demonstrated that treatment with TNF resulted in a time-dependent degradation of I $\kappa$ B $\alpha$ . In contrast, pretreatment with IL-10 prevented I $\kappa$ B $\alpha$  degradation up to 15 min following the addition of TNF (Fig. 2A). However, IL-10 failed to prevent TNF-induced degradation of I $\kappa$ B $\alpha$  30 min post-stimulation (Fig. 2A, lane 11). The fact that I $\kappa$ B $\alpha$  reaccumulates following degradation is not explained by the inability of IL-10 to block NF- $\kappa$ B activation since IL-10 blocks the induction of I $\kappa$ B $\alpha$  mRNA (see Fig. 1B). Differences in I $\kappa$ B $\alpha$  protein levels observed in TNF and IL-10 treated cells were not due to differences in protein loading since immunoblot analysis demonstrated similar levels of p50 expression (Fig. 2A). These results indicate that single dose pretreatment with IL-10 transiently blocks I $\kappa$ B $\alpha$  degradation in response to TNF stimulation (see “Discussion”).

**IL-10 Blocks the TNF Induced Activation of the I $\kappa$ B Kinase**—Since TNF-mediated activation of NF- $\kappa$ B has been shown to require the IKK-induced phosphorylation of I $\kappa$ B $\alpha$  (29, 33–36), we wanted to determine if IL-10 inhibited NF- $\kappa$ B by targeting the IKK complex. In order to measure TNF-induced IKK activity, IKK was immunoprecipitated from whole cell extracts and used in an *in vitro* kinase assay to measure the ability of IKK to phosphorylate a GST-I $\kappa$ B $\alpha$  substrate containing serine residues 32 and 36. The specificity of the IKK assay was determined by the inability to phosphorylate a GST-I $\kappa$ B $\alpha$  S32T/S36T mutant in which serine residues 32 and 36 have been converted to threonine residues. As shown in Fig. 2B, TNF addition induced IKK activity in a time-dependent fashion in U937 cells. Maximum IKK activity in response to TNF addition was observed within 10 min, and was greatly diminished within 30 min post-TNF stimulation. However, TNF-induced IKK activity was inhibited by IL-10, with peak effects at 5 and 10 min post-TNF stimulation (Fig. 2B). Note that the quantification of relative IKK activity shown in Fig. 2B is derived from comparison to time T<sub>0</sub> and is not a direct comparison of IKK activity between the two experimental conditions at a particular time point. The ability of TNF to induce IKK activity was specific for serine residues 32 and 36, since the GST-I $\kappa$ B $\alpha$  S32T/S36T mutant could not be phosphorylated in the kinase assays (Fig. 2B). Although similar kinetics of TNF-induced IKK activity were seen in both THP-1 and U937 cells (Fig. 2, B, C, and D), the inhibitory effects of IL-10 in THP-1 cells (Fig. 2C) were much more pronounced in comparison to U937 cells (com-

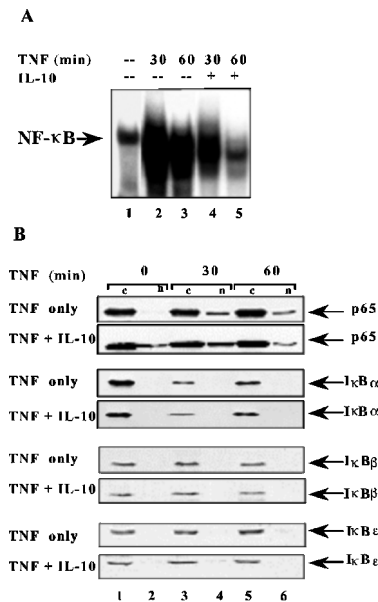
cells were left untreated (lane 1), treated with IL-10 (10 ng/ml) alone (lane 4), treated with TNF (10 ng/ml) for 2 h without (lane 2) or with a 1-h pretreatment with MG 132 (100  $\mu$ M) (lane 3) or IL-10 (10 ng/ml) (lane 5). Total RNA was extracted and subjected to Northern blot analysis and probed for I $\kappa$ B $\alpha$  and IL-8. Equal loading of RNA was confirmed by visualizing the 28 S RNA. The data is representative of two independent experiments. C, IL-10 inhibits NF- $\kappa$ B DNA binding activity in monocytic and intestinal epithelial cells. THP-1 cells (left panel) were stimulated with LPS (10  $\mu$ g/ml) for 60 min without (lane 3) or with a 5-min pretreatment with various doses of IL-10 (0.1–10 ng/ml) (lanes 4–6). U937 cells (middle panel) were stimulated with TNF (10 ng/ml) for 15 min without (lane 3) or with a 5-min pretreatment with various doses of IL-10 (0.1–10 ng/ml) (lanes 4–6). HT-29 cells (right panel) were stimulated with varying doses of TNF (2 ng/ml, lane 3; 10 ng/ml, lane 4; 50 ng/ml, lane 5) for 30 min without (lanes 3–5) or with a 5-min pretreatment with IL-10 (10 ng/ml) (lane 6). Nuclear proteins were extracted and assayed for NF- $\kappa$ B DNA binding activity in EMSA. The ability of IL-10 to inhibit NF- $\kappa$ B DNA binding activity was greater in response to TNF (80% inhibition in U937 cells; 50% inhibition in HT-29 cells) than in response to LPS (45% in THP-1 cells) (lower panel). D, inhibition of NF- $\kappa$ B DNA binding activity by IL-10 under conditions of TNF treatment up to 15 min is caused by a block in p65 nuclear translocation. Nuclear (n) and cytoplasmic (c) extracts of THP-1 cells that were left untreated (lanes 1 and 2), treated with IL-10 (10 ng/ml) alone (lanes 3 and 4), or treated with TNF (10 ng/ml) without (lanes 5 and 6) or with a 5-min pretreatment with IL-10 (10 ng/ml) (lanes 7 and 8) were loaded adjacent and subjected to SDS-PAGE followed by p65 immunoblotting and compared for the induction or inhibition of p65 nuclear translocation.



**FIG. 2. IL-10 prevents TNF-induced degradation of I $\kappa$ B $\alpha$  by blocking I $\kappa$ B kinase activity.** *A*, THP-1 cells were left untreated (lane 1), treated with IL-10 (10 ng/ml) alone (lane 7), or treated with TNF (10 ng/ml) without (lanes 2–6) or with a 5-min IL-10 pretreatment (10 ng/ml) (lanes 8–12) for the times indicated. Equivalent amounts of cytoplasmic extracts were analyzed by Western blot using antibodies against I $\kappa$ B $\alpha$ . Immunoblotting for p50 demonstrated equal loading of protein (lower panel). One representative experiment of three experiments is shown. *B*, U937 cells were treated with TNF (10 ng/ml) for various times. Whole cell extracts were immunoprecipitated with an antibody against IKK $\beta$  and the immunoprecipitates subjected to an IKK activity assay. GST-I $\kappa$ B $\alpha$ (1–54) WT (4  $\mu$ g) or a mutated form of GST-I $\kappa$ B $\alpha$ (S32T,S36T) was used as substrate. Western blotting for IKK was performed as a loading control (not shown). Coomassie Blue staining of gels showed equal loading of the GST-I $\kappa$ B $\alpha$  substrate (not shown). IKK activity as quantitated by PhosphorImager analysis (Molecular Dynamics) was normalized to activity of untreated cells and expressed as fold induction. The data is representative of three independent experiments. *C*, anti-IKK $\beta$  immunoprecipitates from whole cell extracts of THP-1 cells were left untreated, treated with IL-10 alone (10 ng/ml), or were stimulated with TNF (10 ng/ml) for 10 min without or with a 5-min pretreatment with IL-10 (10 ng/ml) and examined for IKK activity. WT GST-I $\kappa$ B $\alpha$  was used as substrate. TNF-induced IKK activity in THP-1 cells is expressed as fold induction after normalization to activity of untreated cells. The data is representative of three independent experiments. *D*, I $\kappa$ B $\alpha$  kinase assay with anti-IKK $\beta$  immunoprecipitates from whole cell extracts of THP-1 cells that were treated with low dose TNF (2 ng/ml) without or with an IL-10 pretreatment.

pare with Fig. 2*B*). IL-10-mediated inhibition of IKK activity was also observed when cells were treated with lower doses of TNF (2 ng/ml) (Fig. 2*D*). Although the kinetics of the IKK activity occurs quite rapidly and persists for only 30 min following TNF stimulation, our results clearly demonstrate that IL-10 controls NF- $\kappa$ B activity in part through the regulation of the IKK complex.

**IL-10 Inhibits NF- $\kappa$ B DNA Binding After Prolonged TNF Stimulation**—Since IL-10 inhibited NF- $\kappa$ B-dependent transcription in transient transfection experiments up to 5 h post-TNF stimulation (Fig. 1*A*), we wanted to determine whether IL-10 could also inhibit NF- $\kappa$ B activity at later time points of TNF stimulation. Interestingly, IL-10 significantly blocked NF- $\kappa$ B DNA binding following longer TNF treatment (Fig. 3*A*). Inhibition of DNA binding at these later time points of TNF did not coincide with the same time frame in which IL-10 inhibited IKK activity (Fig. 2, *B* and *D*, and data not shown) and inhib-



**FIG. 3. IL-10-mediated inhibition of NF- $\kappa$ B DNA binding upon longer TNF treatment is independent of blocking p65 nuclear translocation.** *A*,  $10^7$  THP-1 cells were stimulated with TNF (10 ng/ml) for 30 and 60 min without (lanes 2 and 3) or with a 5-min IL-10 pretreatment (10 ng/ml) (lanes 4 and 5). Nuclear proteins were extracted and assayed for NF- $\kappa$ B DNA binding activity by EMSA. *B*, equal amounts of cytoplasmic (c) and nuclear (n) proteins of THP-1 cells of the same experiment as in *A* were loaded adjacent and subjected to SDS-PAGE followed by immunoblotting for p65, I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , and I $\kappa$ B $\epsilon$ . Extracts of cells that were left untreated (lanes 1 and 2, first panel) or treated with TNF (10 ng/ml) for 30 and 60 min (lanes 3–6, first panel) were compared with extracts of cells treated with IL-10 alone (10 ng/ml) (lanes 1 and 2, second panel) or cells pretreated with IL-10 (10 ng/ml) before a 30- and 60-min treatment with TNF (lanes 3–6, second panel) for the induction or inhibition of p65 nuclear translocation and for the cellular distribution of I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , and I $\kappa$ B $\epsilon$ .

ited I $\kappa$ B $\alpha$  degradation (Fig. 2*A*). In order to determine whether IL-10 was inhibiting DNA binding activity of NF- $\kappa$ B at these later time points through mechanisms involving nuclear translocation of p65, Western blot analysis was performed. Western blot analysis for p65 of cytoplasmic and nuclear extracts of TNF-treated cells with or without the pretreatment of IL-10 revealed that the inhibition of NF- $\kappa$ B DNA binding under these conditions was not due to a block of NF- $\kappa$ B nuclear translocation (Fig. 3*B*). Similar amounts of nuclear p65 were detected in cells treated with TNF for 30 and 60 min regardless of the addition of IL-10 (Fig. 3*B*, upper panels, compare nuclear levels of p65 at the 30- and 60-min time points). These data indicate that even under situations where p65 is nuclear there is an inhibition of NF- $\kappa$ B DNA binding activity by IL-10. To determine whether the I $\kappa$ B proteins, I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , and I $\kappa$ B $\epsilon$ , play a role in inhibiting NF- $\kappa$ B DNA binding in response to IL-10, cytoplasmic and nuclear proteins were assayed for I $\kappa$ B proteins by Western blot analysis. Analysis of nuclear extracts failed to demonstrate an accumulation of I $\kappa$ B proteins ( $\alpha$ ,  $\beta$ , and  $\epsilon$ ) following IL-10 treatment, suggesting that the inhibitory action of IL-10 on NF- $\kappa$ B DNA binding is not mediated by increased nuclear levels of these inhibitory molecules.

## DISCUSSION

Interleukin-10 controls inflammatory processes by suppressing the production of proinflammatory cytokines which are known to be transcriptionally controlled by NF- $\kappa$ B. Although IL-10 has been found to inhibit the activity of NF- $\kappa$ B in various cell types, results from different groups have been variable and no molecular mechanisms have been elucidated which may control NF- $\kappa$ B activity in response to IL-10. We wanted to

investigate whether the anti-inflammatory effects of this cytokine are mediated through its ability to inhibit NF- $\kappa$ B. In agreement with previous studies (7), stimulation with TNF or LPS led to an increase in IL-8 production, which was inhibited by IL-10 addition. TNF-induced IL-8 production is regulated through an NF- $\kappa$ B-dependent mechanism (42). In transient transfection experiments we show that IL-10 was able to specifically inhibit NF- $\kappa$ B-dependent transcription (Fig. 1A). These results indicate that IL-10 not only inhibits TNF-induced IL-8 cytokine production, as previously reported for human neutrophils (7), but also inhibits the transcriptional activity of NF- $\kappa$ B in monocytic and intestinal epithelial cells. Consistent with previous reports (40), our data indicate that NF- $\kappa$ B-dependent transcriptional regulation is a target for the anti-inflammatory actions of IL-10. This finding is further supported by the observation that IL-10 pretreatment was able to suppress TNF-induced mRNA levels for I $\kappa$ B $\alpha$  and IL-8, which are both positively up-regulated by the NF- $\kappa$ B transcription factor (Fig. 1B).

Although it has been previously established that IL-10 inhibits NF- $\kappa$ B activity (37, 39, 40), the mechanisms governing this process have not been fully elucidated. Here we demonstrate that pretreatment with IL-10 inhibits TNF-induced and LPS-induced DNA binding of NF- $\kappa$ B in a dose-dependent manner and specifically affects the p65/p50 heterodimer complex (Fig. 1C). Importantly, the ability of IL-10 to block DNA binding activity of NF- $\kappa$ B was not limited to one type of inflammatory stimulus, nor was it a cell-specific phenomenon, since IL-10 inhibited TNF and LPS-induced NF- $\kappa$ B activity in both U937 and THP-1 monocytic cells. This is further supported by our observation that IL-10 also inhibited TNF-induced NF- $\kappa$ B activity in HT-29 intestinal epithelial cells, although to a lesser extent than in monocytic cells. Interestingly, the extent of IL-10's inhibitory effects was also dependent on the activating stimulus, since IL-10 inhibited TNF-induced NF- $\kappa$ B activity to a greater degree than it inhibited LPS-stimulated NF- $\kappa$ B activity in monocytic cells. These results indicate that IL-10 functions to block the signal transduction pathway required for induction of NF- $\kappa$ B DNA binding activity. Our data is supported by studies in human peripheral blood mononuclear cells (37) and murine macrophages (40) that have demonstrated the ability of IL-10 to block LPS-induced NF- $\kappa$ B DNA binding, but is in contrast to other studies in human monocytes (38) in which IL-10 was not able to block LPS-induced activation of NF- $\kappa$ B DNA binding. These differences might arise from different cell types used in the studies (peripheral blood mononuclear cells *versus* monocytes) or the concentrations of LPS that were used. In agreement with our results, Wang *et al.* (37) also demonstrated the ability of IL-10 to block TNF-induced NF- $\kappa$ B DNA binding in human peripheral blood mononuclear cells using similar doses of TNF and IL-10.

The potential inhibition of I $\kappa$ B degradation in response to cytokine stimulation would be one mechanism to explain the ability of IL-10 to inhibit NF- $\kappa$ B. We report here that IL-10 inhibits NF- $\kappa$ B nuclear translocation by preventing TNF-induced degradation of I $\kappa$ B $\alpha$  (Fig. 2A). Pretreatment with IL-10 prevented I $\kappa$ B $\alpha$  degradation up to 15 min following the addition of TNF but failed to prevent TNF-induced degradation of I $\kappa$ B $\alpha$  30 min post-stimulation. These results indicate that IL-10 can induce its inhibitory effects on NF- $\kappa$ B, at least in part, by delaying TNF-induced degradation of I $\kappa$ B $\alpha$  (also see below).

It has recently been established that IKK phosphorylates I $\kappa$ B $\alpha$  on serine residues 32 and 36, and that this phosphorylation is the prerequisite for ubiquitination and proteasome-dependent degradation of I $\kappa$ B, thus liberating NF- $\kappa$ B and allowing it to translocate to the nucleus (25, 27). Here we demonstrate that IL-10 blocks the TNF-induced activation of

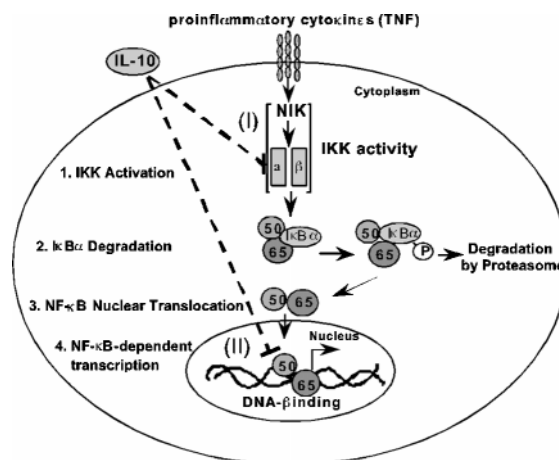


FIG. 4. **Scheme explaining mechanisms of IL-10-mediated inhibition of NF- $\kappa$ B activity.** *I*, in the absence of IL-10, TNF activates the I $\kappa$ B kinase (IKK), which phosphorylates the inhibitory protein I $\kappa$ B $\alpha$  on serine residues 32 and 36. *2*, phosphorylated I $\kappa$ B $\alpha$ , which functions to sequester the inactive p65/p50 heterodimer in the cytoplasm, is then ubiquitinated and degraded by the 26 S proteasome. *3*, degradation of I $\kappa$ B $\alpha$  frees the p65/p50 heterodimer, which then can translocate to the nucleus, where it transcriptionally regulates NF- $\kappa$ B-dependent genes (*4*). In the presence of IL-10, NF- $\kappa$ B is regulated by dual mechanisms. (*I*), upon short term exposure to TNF, pretreatment with IL-10 blocks TNF-induced IKK activity, thus inhibiting phosphorylation and degradation of I $\kappa$ B $\alpha$ . The preserved I $\kappa$ B $\alpha$  continues to bind NF- $\kappa$ B in the cytoplasm, which prohibits NF- $\kappa$ B nuclear translocation and NF- $\kappa$ B dependent transcription. (*II*), upon longer exposure to TNF, pretreatment with IL-10 can directly block NF- $\kappa$ B DNA binding by a mechanism that is independent of NF- $\kappa$ B nuclear translocation.

the I $\kappa$ B kinase up to 80% with peak effects at 5 and 10 min post-TNF stimulation (Fig. 2, B-D). To our knowledge, this is the first demonstration of a cytokine-mediated inhibition of IKK activity and identifies, in part, the molecular target through which IL-10 inhibits NF- $\kappa$ B activation. The specific blockade of IKK has recently been identified as the molecular target of aspirin- and sodium salicylate-mediated inhibition of NF- $\kappa$ B activity (45) and further emphasizes IKK as a crucial target of anti-inflammatory drugs. It is important to point out that even though IL-10 has dramatic inhibitory effects in both THP-1 and U937 cells with respect to NF- $\kappa$ B activity, we found that the inhibitory effects of IL-10 on IKK activity were more pronounced on THP-1 cells (Fig. 2, B, C, and D). Currently we are investigating whether the level of IL-10 receptor expression in these cell lines could account for the differences in IKK inhibition mediated by IL-10.

We demonstrate that IL-10 is able to block NF- $\kappa$ B DNA binding after prolonged TNF stimulation, however, under these conditions the inhibition of NF- $\kappa$ B activity was not caused by a block of NF- $\kappa$ B nuclear translocation (Fig. 3, A and B). These results indicate that IL-10 is capable of blocking NF- $\kappa$ B activity by initially inhibiting IKK activity, I $\kappa$ B $\alpha$  phosphorylation, and NF- $\kappa$ B nuclear translocation, but that prolonged exposure of cells to TNF and IL-10 results in an inhibition of NF- $\kappa$ B DNA binding through a yet unknown mechanism. We failed to detect nuclear accumulation of I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , and I $\kappa$ B $\epsilon$  following TNF and IL-10 treatment. This strongly suggests that these molecules do not play a role in the IL-10-mediated inhibition of NF- $\kappa$ B DNA binding in the nucleus. Since it has been reported that binding of NF- $\kappa$ B to DNA is controlled by the phosphorylation status of p65 (27, 46), it is possible that IL-10 may negatively regulate NF- $\kappa$ B activity by modulating its phosphorylation status. Alternatively, IL-10 signaling could lead to the interaction of NF- $\kappa$ B with a nuclear protein that is capable of blocking DNA recognition.

NF- $\kappa$ B is known to positively regulate the promoter region of



many different proinflammatory cytokine genes, including TNF, IL-1 $\beta$ , IL-8, and IL-6 (25, 42, 47). Moreover, exposure of cells to these cytokines in turn is known to stimulate NF- $\kappa$ B transcriptional activity. Thus, the dysregulation of autocrine and paracrine modulating factors has been proposed to contribute to chronic NF- $\kappa$ B activation, which is commonly associated with autoimmune disorders, rheumatoid arthritis, and inflammatory bowel disease (48–54). One of the body's natural defenses against chronic NF- $\kappa$ B activation involves the production of anti-inflammatory cytokines, such as IL-10. Although IL-10 has been demonstrated to block NF- $\kappa$ B activation, the molecular target for IL-10-induced inhibition of NF- $\kappa$ B had not been established. In this study we provide evidence that IL-10 regulates NF- $\kappa$ B by dual mechanisms. First, within 15 min of exposure to TNF, IL-10 inhibits TNF-induced activation of the IKK complex (Fig. 4, *D*). The ability of IL-10 to inhibit IKK activity is consistent with the observation that IL-10 delays TNF-mediated degradation of I $\kappa$ B protein. Although IL-10 inhibits TNF-induced IKK activity, this effect accounts only for the immediate inhibitory action of IL-10 on NF- $\kappa$ B activity. Interestingly, in agreement with previous reports (37, 40), under conditions of longer exposure of monocytic cells to TNF, IL-10 blocked TNF-induced NF- $\kappa$ B DNA binding activity by an alternative mechanism which was not associated with the inhibition of IKK activity or the inhibition of NF- $\kappa$ B nuclear translocation (Fig. 3, *A* and *B*, and Fig. 4, *II*). Therefore, in addition to blocking the activity of the IKK complex, IL-10 appears to have a secondary mechanism of inhibiting NF- $\kappa$ B DNA binding activity. This dual type control would allow inhibition of NF- $\kappa$ B nuclear translocation through the inhibition of IKK-dependent mechanisms, as well as blocking DNA binding of NF- $\kappa$ B that has successfully translocated to the nucleus after longer exposure to TNF. Thus we provide evidence that IL-10 strongly blocks TNF-induced NF- $\kappa$ B transcriptional activity up to 5 h post-TNF stimulation suggesting that both mechanisms, IL-10-mediated inhibition of IKK activity and inhibition of DNA-binding, contribute to the ability of IL-10 to block NF- $\kappa$ B-dependent transcription. Our study has elucidated the anti-inflammatory mechanisms of IL-10 through inhibition of NF- $\kappa$ B in monocytic and intestinal epithelial cell lines *in vitro*, which may show differences when compared with the multiple, complex biological interactions of IL-10 with interacting regulatory pathways *in vivo*. In our *in vitro* system single IL-10 treatment induced acute and time-limited responses. However, it would be predicted that the inhibitory action of this cytokine *in vivo* would be continuous due to constant exposure and may lead to prolonged anti-inflammatory effects by the repetitive IL-10-mediated inhibition of IKK and of NF- $\kappa$ B DNA binding. Future experiments will further explore the signal transduction pathways required to inhibit the IKK complex. Moreover, additional experiments will elucidate the mechanisms by which IL-10 inhibits DNA binding of NF- $\kappa$ B. Our data presented here underscores the importance of the inhibitory action of IL-10 on IKK activity making the I $\kappa$ B kinase complex an attractive target for anti-inflammatory intervention.

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