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# IL-6 Induces NF- $\kappa$ B Activation in the Intestinal Epithelia<sup>1</sup>

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**IL-6 is a potent proinflammatory cytokine that has been shown to play an important role in the pathogenesis of inflammatory bowel disease (IBD). It is classically known to activate gene expression via the STAT-3 pathway. Given the crucial role of IL-6 in the pathogenesis of chronic intestinal inflammation, it is not known whether IL-6 activates NF- $\kappa$ B, a central mediator of intestinal inflammation. The model intestinal epithelial cell line, Caco2-BBE, was used to study IL-6 signaling and to analyze whether suppressor of cytokine signaling 3 (SOCS-3) proteins play a role in the negative regulation of IL-6 signaling. We show that IL-6 receptors are present in intestinal epithelia in a polarized fashion. Basolateral IL-6 and, to a lesser extent, apical IL-6 induces the activation of the NF- $\kappa$ B pathway. Basolateral IL-6 stimulation results in a maximal induction of NF- $\kappa$ B activation and NF- $\kappa$ B nuclear translocation at 2 h. IL-6 induces polarized expression of ICAM-1, an adhesion molecule shown to be important in the neutrophil-epithelial interactions in IBD. Using various deletion constructs of ICAM-1 promoter, we show that ICAM-1 induction by IL-6 requires the activation of NF- $\kappa$ B. We also demonstrate that overexpression of SOCS-3, a protein known to inhibit STAT activation in response to IL-6, down-regulates IL-6-induced NF- $\kappa$ B activation and ICAM-1 expression. In summary, we demonstrate the activation of NF- $\kappa$ B by IL-6 in intestinal epithelia and the down-regulation of NF- $\kappa$ B induction by SOCS-3. These data may have mechanistic and therapeutic implications in diseases such as IBD and rheumatoid arthritis in which IL-6 plays an important role in the pathogenesis. *The Journal of Immunology*, 2003, 171: 3194–3201.**

Interleukin-6 is a pleotropic cytokine whose expression, under physiological conditions, is important for the host response to a number of infections, exerts Ag-specific immune responses, and has both pro- as well as anti-inflammatory effects (1, 2). Under pathological states, excessive secretion and dysregulation of IL-6 and its signaling pathway may play a major role in the pathogenesis of many diseases, including rheumatoid arthritis (1) and inflammatory bowel disease (IBD)<sup>4</sup> (3–5). In IBD, IL-6 has been shown to be required for the development of Th1 cell-mediated colitis (3). IL-6 is one of the major cytokines that is produced in substantially higher amount in both serum and tissue of patients with active IBD where its levels have been shown to correlate with the severity of disease and is predictive of a flare (6–8). Intestinal epithelial cells and lamina propria mononuclear cells have been shown to be the major source of IL-6 seen in IBD (8–11).

IL-6 exerts its biological effects through a receptor complex composed of the IL-6 binding subunit gp80 and dimer of the signal-transducing receptor subunit gp130 (12). After ligand binding and disulfide-linked dimerization of gp130, constitutively associ-

ated kinases of the Janus family (Janus kinase (JAK)) become activated by autophosphorylation of gp130, subsequently tyrosine phosphorylated on its cytoplasmic tail and recruit the transcription factors of the family of STAT (STAT-3 predominantly). Tyrosine-phosphorylated STAT in turn forms homo- and heterodimers that translocate to the nucleus and activate transcription of IL-6-inducible genes. Recently, STAT-3 but not other STATs was shown to be activated in the epithelia and lamina propria of patients with ulcerative colitis and Crohn's disease and STAT-3 activation was shown to be involved in the perpetuation of experimental colitis (5). IL-6 receptors are expressed on a variety of cells as monocyte, macrophage, lymphocyte, neutrophil, and epithelial cells including intestinal epithelial cells (13–16).

Cytokine signals transduced by the JAK/STAT pathway are regulated, in part, by a recently cloned family of endogenous JAK inhibitor proteins referred to as suppressors of cytokine signaling (SOCS) (17, 18). SOCS proteins bind to the positive regulatory tyrosine in the activation loop of JAK through their respective Src homology 2 domains, thereby occluding the accessibility of the active site to the substrate. Interestingly, several cytokines including IL-1 $\beta$ , IFN- $\gamma$ , TNF- $\alpha$ , and IL-6 induce SOCS proteins which serve as a feedback mechanism to down-regulate cytokine signaling in a variety of cells. Dysregulation of SOCS-3 expression has been associated with active inflammation in the intestine and joints in mice models of colitis and arthritis, respectively (5, 19). SOCS-3 is highly expressed in active ulcerative colitis and Crohn's disease in human as well as in the colon of DSS-treated mice and several other T cell-dependent colitis models (5). Moreover, expression of a dominant-negative form of SOCS-3 in mice rendered them more susceptible to dextran sodium sulfate-induced colitis (5). Thus, IL-6, by activating the STAT pathway, acts as a potent proinflammatory cytokine while it is able to suppress its signaling by activating the anti-inflammatory signaling through SOCS-3 and an imbalance in the pro- and anti-inflammatory effects of IL-6 is thought to contribute to inflammation.

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<sup>4</sup> Abbreviations used in this paper: IBD, inflammatory bowel disease; JAK, Janus kinase; SOCS, suppressor of cytokine signaling; CAT, chloramphenicol acetyltransferase; pIRE, palindromic IL-6/IFN binding element; PI-3, phosphatidylinositol 3.

NF- $\kappa$ B is a potent proinflammatory nuclear transcription factor and is considered to be a central mediator of immune and inflammatory response (20). Over 100 genes, mostly proinflammatory, are activated by this transcription factor (20). Increased NF- $\kappa$ B activity is found in inflamed intestinal mucosa, and factors that are implicated in IBD, such as TNF- $\alpha$ , LPS, and IL-1, are potent activators of NF- $\kappa$ B (21). Additionally, many therapies for IBD act at least in part through the inhibition of NF- $\kappa$ B or through inhibition of signals that activate NF- $\kappa$ B (22). One of the proinflammatory genes regulated by NF- $\kappa$ B is ICAM-1, which is a cell surface glycoprotein that serves as a counter receptor for the  $\beta_2$  integrins, LFA-1 (CD11a/CD18), and MAC-1 (CD11b/CD18) (23, 24). ICAM-1 plays a critical role in mediating leukocyte-endothelial and some forms of leukocyte-epithelial adhesion and is a marker of active inflammation (25). ICAM-1 expression is up-regulated in the intestinal epithelia by various cytokines such as IFN- $\gamma$ , TNF- $\alpha$ , and IL-1 $\beta$  (21, 23, 25, 26) and blockade of ICAM-1 seems to benefit T cell-mediated mouse colitis (27) and patients with steroid-refractory Crohn's disease (28). Interestingly, ICAM-1 is one of the acute phase response genes induced by IL-6 in a variety of tissues (21, 29, 30–33). However, the mechanism of ICAM-1 induction by IL-6 in the intestinal epithelia is not known. Since IL-6 is a potent proinflammatory cytokine, we investigated whether IL-6 activates the NF- $\kappa$ B pathway and, if so, whether NF- $\kappa$ B plays a role in ICAM-1 induction by IL-6. Additionally, since SOCS-3 is a major protein that is involved in the termination of STAT-3 signaling by IL-6, we examined whether SOCS-3 plays a role in the down-regulation of IL-6 induced NF- $\kappa$ B and ICAM-1 expression.

## Materials and Methods

### Reagents

All tissue culture supplies were obtained from Life Technologies (Grand Island, NY). Reagents for SDS-PAGE and nitrocellulose membranes (0.45- $\mu$ m pores) were from Bio-Rad (Hercules, CA). MG-132 was obtained from Calbiochem-Novabiochem (San Diego, CA) and was used at 20  $\mu$ M in DMSO, and cells were pretreated for 1 h (34). Anti-STAT-3 and anti-phospho-tyrosine 705 STAT-3 Abs were purchased from Cell Signaling Technology (Beverly, MA); anti-SOCS-3 (M-20), anti-I $\kappa$ B $\alpha$ , anti-gp130, and anti-ICAM-1 (M-19) Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-p65 was obtained from BD Transduction Laboratories (Lexington, KY). Agarose-conjugated anti-FLAG was purchased from Sigma-Aldrich (St. Louis, MO). Recombinant IL-6 and anti-gp-80 were obtained from R&D Systems (Minneapolis, MN).

### Cell culture

Caco2-BBE cells (35) were grown as confluent monolayers in a 1:1 mixture of Dulbecco's Vogt modified Eagle's medium and Ham's F12 medium supplemented with 15 mM HEPES (pH 7.5), 14 mM NaHCO<sub>3</sub>, and 10% newborn calf serum. Monolayers were subcultured every 7 days by trypsinization with 0.1% trypsin and 0.9 mM EDTA in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free PBS as previously described (36). Experiments were done on cells plated for 8–10 days on collagen-coated permeable supports (area = 0.33 cm<sup>2</sup> or 5 cm<sup>2</sup>, pore size = 0.4- $\mu$ m inserts).

### Northern blot

Northern blot was performed as described previously (37). Briefly, total RNA was extracted from cells with Tri-reagent (Molecular Research Center, Cincinnati, OH) according to manufacturer's protocol. Total RNA (20  $\mu$ g) was separated on 1% formaldehyde agarose gel and transferred to Gene Screen Plus membranes (NEN Life Science Products, Boston, MA). After fixation under calibrated UV light, the membranes were hybridized with  $\alpha$ -<sup>32</sup>P-labeled SOCS-3 cDNA and visualized by autoradiography. The probe for SOCS-3 cDNA was generated by RT-PCR using SOCS-3-specific primers 5'-cagatggctactggggaagt-3' and 5'-tgacctgtccgtttac-3' (284-bp product) and sequence was verified by automated DNA sequencer (Ambion, Austin, TX). GAPDH cDNA was used as a control.

### Plasmids and transient transfection

Mammalian expression vectors for SOCS-3 tagged with FLAG were described previously and were obtained from Dr. D. Hilton (Victoria, Australia) (38). NF- $\kappa$ B reporter assay was performed with NF- $\kappa$ B-dependent chloramphenicol acetyltransferase (CAT) vector (Clontech Laboratories, Palo Alto, CA). Full-length ICAM-1 promoter and various deletion constructs linked to luciferase was obtained from Dr. C. Stratowa (Boehringer Institute, Vienna, Austria) (39). Based on the data that 5' and 3' flanking regions (–199 to –170) of the NF- $\kappa$ B site in the ICAM-1 promoter is essential for the full function of the NF- $\kappa$ B site in the ICAM-1 promoter (40), we deleted these sequences from the full-length ICAM-1 promoter linked to luciferase. The deletion was performed using PCR-mediated overlap extension to facilitate fusion of cDNA sequence (primer 1, GGGT CATCGCCCTGCCACCGGGAGGATGACCTCTCGGCC and primer 2, GGCCGAGAGGGTCACTCTCCCGTGGCAGGGCGATGACC) using a Quick Change site-directed mutagenesis kit (Stratagene, Cedar Creek, TX). The constructed plasmid cDNA sequences were verified by sequencing. Plasmids were purified using a Qiagen Maxiprep kit (Valencia, CA). A transdominant-negative I $\kappa$ B $\alpha$  construct was obtained from Dr. P. Khavari (Stanford University, CA) (41, 42). Subconfluent Caco2-BBE cells grown on six-well plates as described above were transfected with appropriate vectors using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Seventy-two hours after transfection, cells were washed with HBSS, equilibrated at 37°C for 10 min, and then stimulated with IL-6 (100 ng/ml) or TNF- $\alpha$  (10 ng/ml) for the indicated times. Samples were analyzed for the expression of SOCS-3 protein by immunoprecipitation with anti-FLAG (Sigma-Aldrich) and Western blot using anti-SOCS-3 Ab (Santa Cruz Biotechnology). CAT assays were performed according to the manufacturer's protocol (CAT assay kit; Promega, Madison, WI). For luciferase assay, cells were cotransfected with pRL-null vector linked to *Renilla* luciferase reporter. After 72 h, cells were stimulated with IL-6 and the luciferase assay was done according to the manufacturer's protocol (Dual Luciferase Reporter Assay; Promega). Cells were lysed and protein quantitation was done using a Lowry assay kit (Bio-Rad).

### EMSA

Caco2-BBE cells were grown to confluency on 5-cm<sup>2</sup> collagen-coated filters. The monolayers were washed in HBSS and incubated with apical or basolateral IL-6 (100 ng/ml) for various time points. The monolayers were rinsed in ice-cold HBSS and harvested by scraping. Nuclear extracts were prepared as described elsewhere (43) with additional wash steps of isolated nuclei to remove potential contamination with cytoplasmic proteins. Complementary oligonucleotides representing the NF- $\kappa$ B consensus site (upper strand, 5'-CCC CAGAGG GGA CTT TCC GAG AGG CTC-3'; lower strand, 5'-GGG GAG CCT CTC GGA AAG TCC CCT CTG-3'; Promega) were annealed and 3' end-labeled with [ $\gamma$ -<sup>32</sup>P]dCTP with Klenow polymerase using standard procedures. Binding reactions were performed by preincubating 5  $\mu$ g of nuclear extract protein in 20 mM HEPES (pH 7.9), 60 mM KCl, 1 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 10% glycerol, 0.5 mM DTT, and 2  $\mu$ g of poly(dI-dC) on ice for 10 min, followed by the addition of double-stranded <sup>32</sup>P-labeled probe and a second incubation at room temperature for 20 min. Samples were loaded directly onto nondenaturing 6% polyacrylamide gels prepared in 45 mM Tris borate/45 mM boric acid/0.1 mM EDTA (0.5 $\times$  TBE). Competitions were performed by using unlabeled double-stranded oligonucleotides. The specific NF- $\kappa$ B site competitor contained the sequence listed above. The nonspecific competitor consisted of a portion of the human haptoglobin promoter containing a C/EBP binding site (5'-CCC CAG AGG CGA CTT TCC GAG AGG CTC-3') and (5'-GGG GAG CCT CTC GGA AAG TCG CCT CTG-3'). Electrophoresis was performed at room temperature for 2–2.5 h at 170 V. The gels were then dried and exposed to Kodak MS film with appropriate intensifying screens. The band intensity was quantitated using a gel documentation system (Alpha Innotech, San Leandro, CA).

### Confocal microscopy

Monolayers of Caco2-BBE cells were washed in HBSS, fixed in buffered 3.7% paraformaldehyde for 20 min, incubated overnight with respective primary Abs in a humidity chamber, washed with HBSS, and subsequently incubated with fluoresceinated secondary Abs (Jackson ImmunoResearch Laboratories, West Grove, PA). Monolayers were also counterstained with rhodamine-phalloidin to visualize actin. Monolayers, mounted in *p*-phenylenediamine glycerol (1:1) were analyzed by confocal microscopy (Zeiss dual laser confocal microscope; Zeiss, Oberkochen, Germany) as described previously (44). Using actin staining, the apical most surface of the cell was marked as 0  $\mu$ m and basolateral surface was marked at the level of

actin stress fiber ( $\sim 18.7 \mu\text{m}$  from the top of the cell).  $xy$  sections were taken at  $\sim 1.2 \mu\text{m}$  from the top (above the level of tight junction) and at the level of actin stress fiber.

#### SDS-PAGE and Western blot

Cells were lysed with PBS containing 1% Triton X-100 and 1% Nonidet P-40 (v/v), protease inhibitor mixture (Boehringer Mannheim, Indianapolis, IN), EDTA, SDS, sodium orthovanadate, and sodium fluoride. SDS-PAGE was performed according to the Laemmli procedure using 4–20% acrylamide gel. Proteins were electrotransferred to nitrocellulose membranes and probed with primary Ab (anti-phospho-STAT-3, 1/1000; anti-ICAM-1, 1/1000; anti-SOCS-3, 1/100; and anti-STAT-3, 1/1000). The membranes were incubated with corresponding peroxidase-linked secondary Ab diluted 1/2000, washed, and subsequently incubated with ECL reagents (Amersham Pharmacia Biotech, Piscataway, NJ) before exposure to high-performance chemiluminescence films (Amersham Pharmacia Biotech). For  $M_r$  determination, polyacrylamide gels were calibrated using standard proteins (Bio-Rad) with  $M_r$  markers within the range 7,700–214,000.

#### Cell surface biotinylation and immunoprecipitation

Apical or basolateral sides of the filter-grown monolayers were biotinylated using sulfo-succinimidobiotin (s-NHS-biotin; Pierce, Rockford, IL) as previously described (44). The cell lysate was then incubated with streptavidin-agarose (Pierce) to bind biotinylated proteins. Proteins were separated by SDS-PAGE and Western blotting was done as described previously (44).

#### Data analysis

Results were analyzed using Student's  $t$  test. Differences were considered significant at the  $p < 0.05$  level.

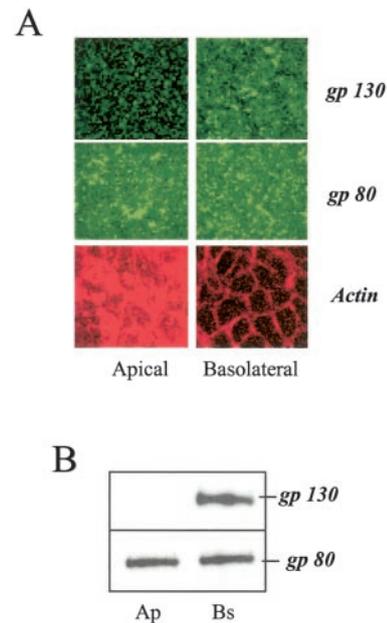
## Results

### IL-6 receptors are present on intestinal epithelial cells

We and others have previously shown that intestinal epithelia secrete IL-6 and IL-6 is elevated in the luminal fluid of patients with IBD (8–11). In this study, we sought to see whether IL-6 receptors are present in model intestinal epithelia and, if so, whether they are polarized. Although there is indirect evidence for the presence of IL-6 receptors in Caco2 cells and in intact human intestinal epithelia (14, 15), the polarity is not known. As seen in Fig. 1A, using confocal imaging of intact Caco2-BBE monolayer, we show that the gp80 subunit of the IL-6R is present in both apical and basolateral membranes. The regulatory subunit, gp130, is expressed predominantly at the basolateral surface of polarized model intestinal epithelial cells. To verify this data biochemically, we biotinylated Caco2-BBE monolayers selectively on the apical or basolateral surface. Biotinylated proteins were immunoprecipitated with streptavidin and immunoprecipitated IL-6 receptors were detected with IL-6R-specific Ab by Western blotting. As seen in Fig. 1B, IL-6 receptors were expressed in both apical and basolateral membranes of Caco2-BBE cells as evidenced by the appearance of an 80-kDa band consistent with the IL-6R. However, gp130, the signal-transducing subunit of the IL-6 receptors are expressed predominantly on the basolateral surface. These results indicate that IL-6 receptors are present on intestinal epithelial cells and may be poised to respond to IL-6 present in the lumen during inflammatory conditions.

### IL-6 induces the activation of NF- $\kappa$ B

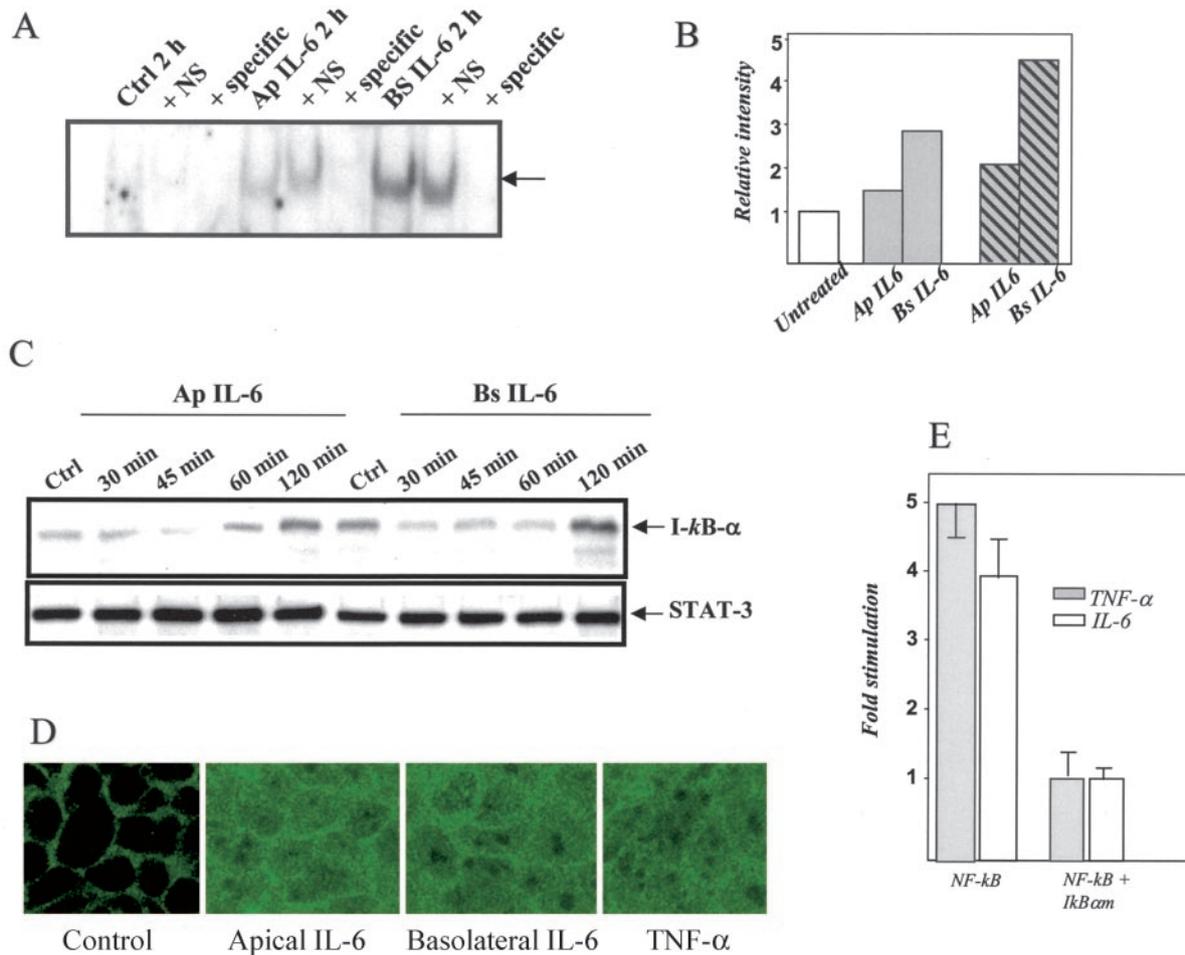
Since IL-6 is a potent proinflammatory cytokine and NF- $\kappa$ B activation is considered sine quo non of active inflammation, we investigated whether IL-6 induces activation of the NF- $\kappa$ B pathway. We used EMSA to determine whether interaction of IL-6 with Caco2-BBE monolayers could induce NF- $\kappa$ B translocation and DNA binding. For these experiments, a double-stranded oligonucleotide NF- $\kappa$ B consensus motif was used to probe nuclear extracts derived from Caco2-BBE monolayers treated with apical or basolateral IL-6. Resting cells showed no NF- $\kappa$ B-binding activity



**FIGURE 1.** IL-6 receptors are present in model intestinal epithelial cells. *A*, Monolayers were fixed and stained with anti-gp80 Ab (1/500) or anti-gp130 (1/100) Ab followed by FITC-conjugated secondary Ab (green). Monolayers were also stained with rhodamine-phalloidin (actin-red). Membrane staining was determined by immunofluorescence labeling and confocal microscopy as described in *Materials and Methods*. Vertical sections were taken off the monolayers to define the top (set at  $0 \mu\text{m}$ ) and bottom of the monolayer. En face sections ( $x$ - $y$ ) were then generated from apical and basolateral planes in the vertical section. Shown here are en face ( $xy$ ) images taken at the level of the apical (at  $1.2 \mu\text{m}$ , above the level of tight junction) and basolateral (at  $16.0 \mu\text{m}$ ) poles of the epithelial monolayer. The en face images document the presence of IL-6R in both the apical and basolateral membranes of Caco2-BBE cells while gp130 is present predominantly at the basolateral membrane. *B*, Caco2-BBE monolayers grown on semipermeable filters were subjected to domain-specific biotinylation (Ap, apical domain and Bs, basolateral domain) for 30 min followed by Western blot analysis of total cell lysate. Biotinylation experiments confirm the presence of IL-6R in both the apical and basolateral membranes as evidenced by an 80-kDa band and gp130 present predominantly at the basolateral membrane.

while Caco2-BBE exposed to apical or basolateral IL-6 showed an induction of nucleoprotein complex within 1 h (blot not shown) with a maximal induction seen within 2 h (Fig. 2A). Using quantitative scanning densitometry, we showed that basolateral IL-6 was more potent than apical IL-6 to induce the NF- $\kappa$ B nucleoprotein complex (relative density compared with control: 1 h, apical IL-6 = 1.5 and basolateral IL-6 = 2.7; 2 h, apical IL-6 = 2; and basolateral IL-6 = 4.5; Fig. 2B).

In response to many stimuli, the DNA-binding activity of NF- $\kappa$ B is preceded by inducible phosphorylation of I $\kappa$ B $\alpha$  at two conserved serine residues (serines 32 and 36) with subsequent proteolytic degradation of an I $\kappa$ B $\alpha$  followed by release and translocation of NF- $\kappa$ B to the nucleus. We thus measured the degradation of I $\kappa$ B $\alpha$  and subsequent nuclear translocation of NF- $\kappa$ B. Immunoblots from unstimulated cells revealed a single 37-kDa band, corresponding to the observed molecular mass of I $\kappa$ B, detected with I $\kappa$ B-specific Ab (Fig. 2C). Cells treated with apical or basolateral IL-6 showed degradation of I $\kappa$ B $\alpha$  in a time-dependent fashion. Degradation of I $\kappa$ B $\alpha$  was seen at 30 min after basolateral stimulation with resynthesis observed within 2 h (Fig. 2C). The time course of I $\kappa$ B $\alpha$  degradation was seen at 45 min after apical



**FIGURE 2.** IL-6 induces the activation of NF- $\kappa$ B. Model intestinal cells Caco2-BBE were stimulated with IL-6 (100 ng/ml) apical or basolateral for various times. **A**, EMSA: nuclear extracts from untreated or IL-6-treated monolayers were mixed with  $^{32}$ P-labeled NF- $\kappa$ B. Competition studies were performed by adding unlabeled double-stranded NF- $\kappa$ B sequence (specific) or random double-stranded oligonucleotide (nonspecific, NS) 10 min before the addition of labeled sequences. The NF- $\kappa$ B complex is indicated with an arrow. **B**, Quantitative scanning densitometry of the EMSA data.  $\square$ , unstimulated cells;  $\blacksquare$ , 1-h IL-6 stimulation;  $\text{▨}$ , 2-h stimulation. Data represent the responses observed in two separate experiments. **C**, Caco2-BBE monolayers were treated with apical (Ap) or basolateral (Bs) IL-6 (100 ng/ml) for the indicated times. Whole-cell detergent lysates ( $\sim 15 \mu\text{g}$  protein/lane) were resolved by SDS-PAGE and immunoblotted for total I- $\kappa$ B. The blot was stripped and probed with anti-STAT-3 (bottom panel). **D**, Caco2-BBE monolayers were incubated with apical or basolateral IL-6 (100 ng/ml) or TNF- $\alpha$  (10 ng/ml) for 1 h. Nuclear staining was determined by immunofluorescence labeling and confocal microscopy. The en face images document the presence of NF- $\kappa$ B staining in the nucleus in cells treated with apical or basolateral IL-6 or TNF- $\alpha$ . Control cells show NF- $\kappa$ B staining localized to the cytosol. **E**, Subconfluent Caco2-BBE cells were cotransfected with NF- $\kappa$ B reporter linked to CAT and a transdominant-negative I $\kappa$ B $\alpha$  mutant vector. Seventy-two hours after transfection, cells were stimulated with IL-6 (100 ng/ml) or TNF- $\alpha$  (10 ng/ml) for 6 h. CAT activity was determined by ELISA. Data represent fold increase in CAT activity compared with unstimulated transfected cells observed in two separate experiments from duplicate determination of two samples per group,  $n = 4$ .

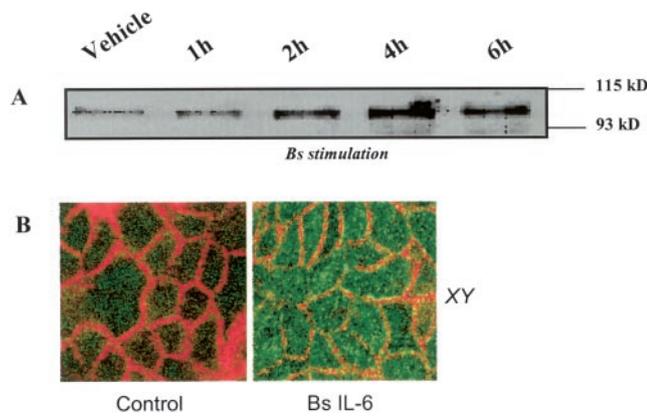
stimulation with resynthesis at 1 h. Although we see I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B nuclear translocation within 1 h after stimulation with IL-6, the NF- $\kappa$ B DNA-binding activity is seen maximally at 2 h. This discrepancy may be due to the sensitivity of Western blot in detecting intermediates in the NF- $\kappa$ B signaling pathway compared with EMSA. As a control for loading, we stripped and reprobed the blot with anti-STAT-3 (Fig. 2C, bottom panel).

NF- $\kappa$ B nuclear translocation was next determined by confocal microscopy using NF- $\kappa$ B specific Ab. As seen in Fig. 2D, unstimulated cells showed NF- $\kappa$ B staining distributed in the cytosol while apical or basolateral stimulation with IL-6 induced NF- $\kappa$ B staining in the nuclei at 60 min after stimulation. To further substantiate our data on the induction of NF- $\kappa$ B by IL-6, we used NF- $\kappa$ B reporter plasmid linked to CAT. Subconfluent Caco2-BBE cells were cotransfected with NF- $\kappa$ B reporter plasmid with vector alone or with transdominant-negative I $\kappa$ B $\alpha$  mutant as described in *Materials and Methods* and stimulated with vehicle, IL-6, or

TNF- $\alpha$ . As shown in Fig. 2E, TNF- $\alpha$  or IL-6 induced a 5- and 4-fold increase, respectively, in CAT activity compared with control cells. Cotransfection with transdominant-negative I $\kappa$ B $\alpha$  mutant attenuated the CAT activity induced by IL-6 and TNF- $\alpha$ . The foregoing data collectively indicate that apical or basolateral IL-6 induces NF- $\kappa$ B DNA-binding activity via activation of I $\kappa$ B kinase and subsequent proteosomal degradation of I $\kappa$ B and nuclear translocation of NF- $\kappa$ B. In addition, IL-6 induces NF- $\kappa$ B-dependent reporter activity. The data also show that basolateral stimulation was more potent than apical stimulation to activate the pathway.

#### IL-6 induces expression of ICAM-1

As seen in Fig. 2, basolateral stimulation was significantly more potent than apical stimulation to induce NF- $\kappa$ B activation. Hence, in subsequent studies we only used basolateral IL-6 stimulation. We next examined the effect of IL-6 on the expression of ICAM-1, an adhesion molecule known to be induced by STAT-3 or NF- $\kappa$ B

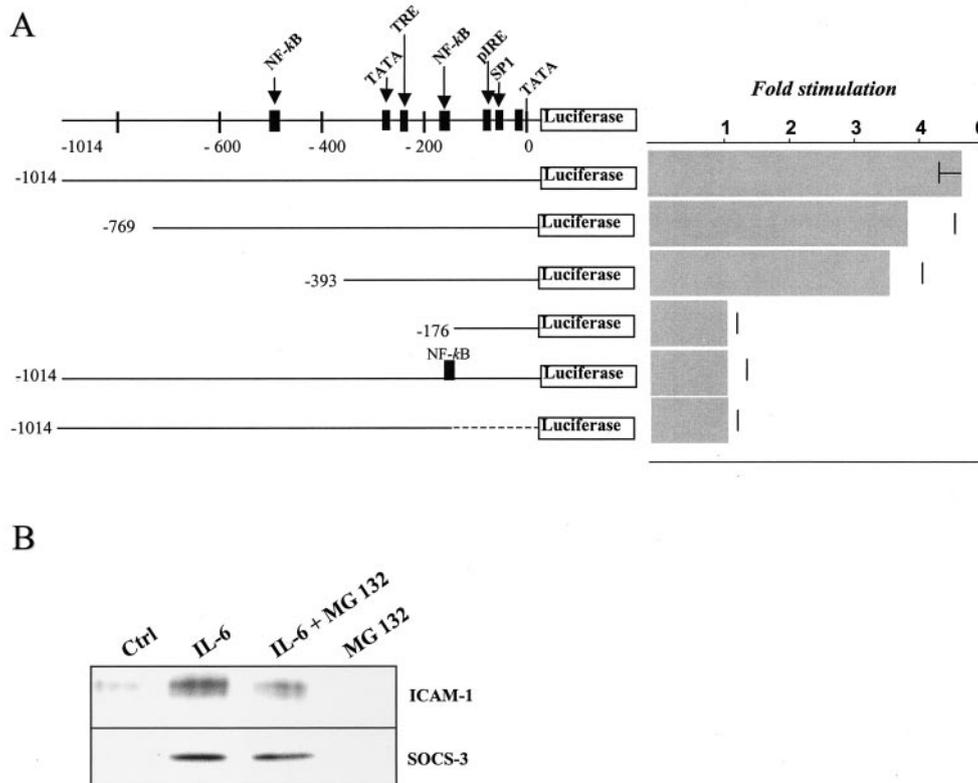


**FIGURE 3.** IL-6 induces the expression of ICAM-1. *A*, Whole-cell lysates (30  $\mu$ g protein/lane) were prepared from Caco2-BBE monolayers treated with IL-6 (100 ng/ml) for various times indicated. Samples were resolved by SDS-PAGE and immunoblotted for ICAM-1. *B*, Monolayers were treated with IL-6 (100 ng/ml) or vehicle for 2 h, after which the monolayers were fixed and stained with anti-ICAM-1 Ab followed by FITC-conjugated secondary Ab. Monolayers were also stained with rhodamine-phalloidin (actin-red). En face sections (*x-y*) were then generated from apical planes in the vertical section. Shown here are en face (*xy*) images taken at the level of the apical pole of the epithelial monolayer. Data represent the responses observed in three separate experiments. Bs, Basolateral.

activation. As shown in Fig. 3A, ICAM-1 was detectable in small amounts in monolayers treated with vehicle alone (*lane 1*). IL-6 added to the basolateral compartment induced a significant increase in the expression of ICAM-1 at 2 h (Fig. 3A, *lane 3*) and was maximal at 4 h. ICAM-1 expression was also visualized by confocal imaging. As seen in Fig. 3B, monolayers stimulated with IL-6 showed an expression of ICAM-1 at the apical pole.

#### IL-6-induced ICAM-1 expression requires NF- $\kappa$ B DNA binding element

We next examined the mechanism by which IL-6 induces the expression of ICAM-1. ICAM-1 promoter has been localized to  $-41$  to  $-600$  upstream of the transcriptional start site (39). The ICAM-1 promoter region contains several consensus sequences known to bind AP-1, NF- $\kappa$ B, STAT, palindromic IL-6/IFN binding element (pIRE), or retinoic acid responsive element. The expression of IL-6-mediated ICAM-1 is known to be regulated by STAT and NF-IL-6 localized to  $-116$  to  $-106$  bp upstream of the translational start site in various cells. To test the relative contribution of the NF- $\kappa$ B binding site in the induction of ICAM-1 by IL-6, plasmids with deletions of NF- $\kappa$ B and pIRE sites in the ICAM-1 promoter linked to firefly luciferase were cotransfected with the *Renilla* luciferase vector (Fig. 4) and stimulated with IL-6. IL-6 induced an  $\sim 5$ -fold increase in relative firefly/*Renilla* luciferase activity compared with unstimulated cells. Deletion of the



**FIGURE 4.** Effect of IL-6 on ICAM-1 promoter activity and analysis of IL-6-mediated regulation of IL-6 promoter constructs. *A*, Schematic representation of ICAM-1 promoter. Transcription binding sites (relative to transcriptional start site): pIRE ( $-106$  to  $-116$ ), NF- $\kappa$ B ( $-176$  to  $-187$ ), SP1 ( $-96$  to  $199$ ), atypical NF- $\kappa$ B ( $-540$ ). Caco2-BBE cells were cotransfected with the indicated reporter plasmids (5  $\mu$ g) and *Renilla* luciferase control plasmid (5 ng) as described in *Materials and Methods*. Transfectants were treated with IL-6 (100 ng/ml) or vehicle for 6 h and luciferase activity (firefly:*Renilla*) was determined as described in *Materials and Methods*. Data represent fluorescence ratio relative to unstimulated transfected cells  $\pm$  SD observed in two separate experiments plotted as mean  $\pm$  SD from duplicate determination of two samples per group,  $n = 2$ . *B*, Monolayers were washed with HBSS, equilibrated at  $37^{\circ}\text{C}$  for 20 min, and then were pretreated with MG-132 (20  $\mu$ M) 1 h before the addition of basolateral IL-6 (100 ng/ml). Cells were lysed 4 h after IL-6 treatment and lysates were immunoblotted for ICAM-1. Blots were stripped and then reprobed for SOCS-3 using anti-SOCS Ab (1/100). Data are representative of two independent experiments. Ctrl, Control.

distal atypical NF- $\kappa$ B (-393) had no effect on the relative luciferase activity compared with IL-6 stimulation alone. However, truncation at the NF- $\kappa$ B site (-176) dramatically reduced the relative luciferase activity induced by IL-6. In addition, specific deletion of the NF- $\kappa$ B site with its flanking sequences decreased the luciferase activity induced by IL-6. The construct that contained only the pIRE (STAT binding site) was not sufficient for the induction of the ICAM-1 promoter by IL-6. Consistent with this data is our observation that MG-132, a proteasomal inhibitor, inhibits IL-6-induced ICAM-1 expression. As seen in Fig. 4B, basolateral IL-6 induced the expression of ICAM-1 and pretreatment with MG-132 for 1 h inhibited this increase. MG-132 alone seems to decrease the baseline expression of ICAM-1. However, MG-132 did not affect the expression of SOCS-3 induced by IL-6. Taken together, these results suggest that the NF- $\kappa$ B binding site is necessary for the activation of the ICAM-1 promoter by IL-6.

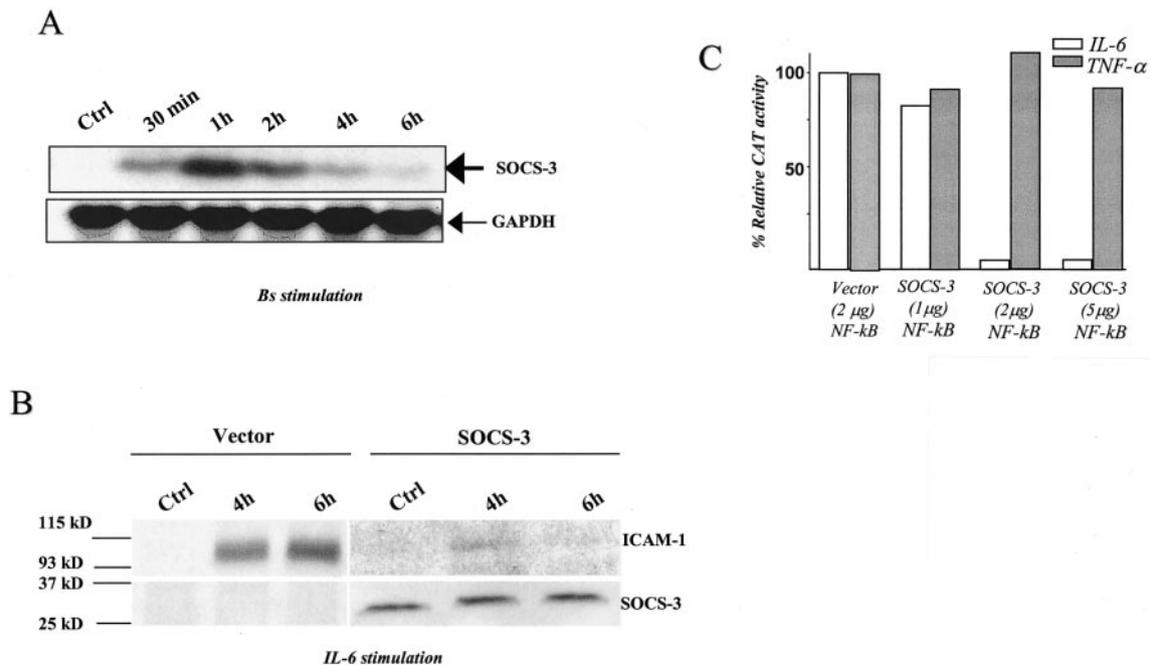
#### SOCS-3 down-regulates IL-6-mediated ICAM-1 expression and NF- $\kappa$ B activation

Our data show that the induction of the NF- $\kappa$ B pathway is transient despite the continued presence of IL-6. Since it is known that SOCS-3 is an integral part of the IL-6 signal transduction pathway and plays an important role in the termination of IL-6 signaling in intestine and other tissues, we next studied whether IL-6 induces SOCS-3 expression in model intestinal epithelia and whether SOCS-3 plays a role in the down-regulation of IL-6-induced NF- $\kappa$ B activation. As shown in Fig. 5, IL-6 increases SOCS-3 mRNA levels in a time-dependent fashion. Basolateral IL-6 increases SOCS-3 mRNA which is seen at 30 min, maximal at 60 min, and is undetectable at 6 h after stimulation (Fig. 5A). Un-

stimulated monolayers had no detectable expression of SOCS-3 mRNA.

To study the effect of SOCS-3 on the down-regulation of ICAM-1 expression, Caco2-BBE monolayers were transfected with FLAG-tagged SOCS-3 mammalian expression vector or the corresponding empty vector. In the vector-transfected cells, IL-6 induced the expression of ICAM-1 at 4 and 6 h after stimulation (Fig. 5B, lanes 2 and 3, respectively). In cells transfected with SOCS-3, there was a significant decrease in the expression of ICAM-1 at 4 and 6 h after IL-6 stimulation (Fig. 5B, lanes 3–6). SOCS-3 expression was verified in transfected cells (Fig. 5B, lanes 3–6, bottom panel) by immunoprecipitation with anti-FLAG Ab followed by immunoblot using anti-SOCS-3 Ab. These data demonstrate that SOCS-3 suppresses the induction of ICAM-1.

We next addressed whether SOCS-3 is able to down-regulate NF- $\kappa$ B activation induced by IL-6. Caco2-BBE cells were cotransfected with NF- $\kappa$ B reporter construct linked to CAT and FLAG-tagged SOCS-3 and then stimulated with IL-6 or TNF- $\alpha$ . IL-6 and TNF- $\alpha$  stimulation for 6 h induced 5.3- and 6-fold increases in CAT activity, respectively (represented in Fig. 5C as maximal response) compared with unstimulated cells. As shown in Fig. 5C, in cells cotransfected with NF- $\kappa$ B reporter construct and SOCS-3, IL-6-induced NF- $\kappa$ B reporter activation was completely abolished in a dose-dependent fashion. SOCS-3 overexpression had no effect on TNF- $\alpha$ -induced NF- $\kappa$ B reporter activity. In cells overexpressing SOCS-3, TNF- $\alpha$  induced 5- to 6-fold increases in NF- $\kappa$ B reporter activity compared with unstimulated cells. Collectively, the above data demonstrate that IL-6 induces the expression of SOCS-3 in Caco2-BBE cells and overexpression of SOCS-3



**FIGURE 5.** SOCS-3 down-regulates ICAM-1 expression and NF- $\kappa$ B activation induced by IL-6. *A*, Caco2-BBE monolayers were washed with HBSS. After equilibration of 20 min at 37°C, cells were stimulated with basolateral (Bs) IL-6 (100 ng/ml, respectively). Total RNA was prepared and probed for SOCS-3 and GAPDH at the indicated times as described in *Materials and Methods*. *A*, Northern blot from basolateral IL-6 stimulation. *B*, Caco2-BBE monolayers were transfected with empty vector or SOCS-3 expression vector (see *Materials and Methods*). Cells were stimulated with IL-6 (100 ng/ml) 72 h posttransfection and whole-cell lysates were prepared 4 and 6 h after stimulation. Whole-cell lysates (30  $\mu$ g protein/lane) were resolved by SDS-PAGE and immunoblotted for ICAM-1 (*B*). Data represent observations made in two separate experiments. Ctrl, Control. *C*, Caco2-BBE cells were cotransfected with NF- $\kappa$ B reporter linked to CAT and FLAG-SOCS-3 vector. Seventy-two hours after transfection, cells were stimulated with IL-6 (100 ng/ml) or TNF- $\alpha$  (10 ng/ml) for 6 h. Cells were lysed and CAT activity was determined by ELISA. Data represent percent relative CAT activity compared with unstimulated transfected cells observed in two separate experiments from duplicate determination of two samples per group,  $n = 4$ .

inhibits NF- $\kappa$ B activation induced by IL-6. NF- $\kappa$ B activation induced by TNF- $\alpha$  was not affected by SOCS-3 overexpression.

## Discussion

In this study, we have provided evidence that IL-6 induces the activation of NF- $\kappa$ B, an important proinflammatory pathway in intestinal inflammation. NF- $\kappa$ B activation is required for the induction of ICAM-1 expression by IL-6. We show that SOCS-3, a classic inhibitor of the IL-6-induced phospho-STAT-3 pathway, abolishes the activation of the NF- $\kappa$ B pathway by IL-6. Thus, SOCS-3 not only suppresses cytokine-mediated JAK/STAT signaling, but also inhibits other pathways (NF- $\kappa$ B) that are triggered by the same receptor, and SOCS proteins may therefore modulate signaling in ways that were previously unforeseen.

Using the polarized model human intestinal epithelia, we show that gp80, the ligand-binding subunit of the IL-6R is present at both the apical and basolateral surfaces while the signal-transducing subunit, gp130, as demonstrated by others, is predominantly expressed at the basolateral surface (45). Basolateral IL-6 results in a rapid and robust induction of phospho-STAT-3 (data not shown) and NF- $\kappa$ B compared with apical stimulation. The apparent potent basolateral response to both STAT-3 and NF- $\kappa$ B is likely related to the higher density of gp130, the signal-transducing subunit of the receptor, at the basolateral surface. This would enable an efficient coupling of IL-6R to JAK resulting in rapid and robust signal transduction at the basolateral surface. The presence of the gp80 subunit at the apical surface is somewhat puzzling. One possible explanation is that under the pathological state, gp130 may be aberrantly expressed at the apical surface such that signal transduction occurs in the presence of luminal IL-6. Indeed, we have previously shown that intestinal epithelia secrete IL-6 polarized to the lumen in response to various inflammatory stimuli and that IL-6 is present in significantly high levels in the intestinal luminal fluid of patients with active IBD (10).

We have provided evidence that epithelial cells respond to IL-6 by the activation of NF- $\kappa$ B and up-regulation of ICAM-1. The activation of the NF- $\kappa$ B pathway by IL-6 is novel and has not been reported. Our data demonstrate that the NF- $\kappa$ B activation is required for the induction of ICAM-1 in model intestinal epithelial cells. MG-132, a proteasomal inhibitor, inhibits ICAM-1 induction by IL-6. Interestingly, MG-132 also inhibited the baseline expression of ICAM-1 in Caco2-BBE cells, suggesting that NF- $\kappa$ B may play a role in the expression of ICAM-1 in resting cells. Given that the MG-132 data may not be direct evidence for the requirement of NF- $\kappa$ B in the IL-6-induced ICAM-1 expression, we used various deletion constructs of the ICAM-1 promoter to further examine the role of NF- $\kappa$ B in the ICAM-1 expression induced by IL-6. In various cell lines, IL-6 has been shown to induce ICAM-1 expression via phospho-STAT-3 which binds to the NF-IL-6 site and/or to STAT-responsive elements in the ICAM-1 promoter (33). Our data indicate that the NF- $\kappa$ B binding site located at -186 to -177 is required for ICAM-1 expression induced by IL-6. Based on our results, IL-6-mediated ICAM-1 induction requires both NF- $\kappa$ B and STAT-3 binding elements. The mechanism of NF- $\kappa$ B activation by IL-6 is not known. The rapid induction of NF- $\kappa$ B activation suggests that it is not likely to be mediated by the synthesis of another proinflammatory cytokine induced by IL-6. One possible mechanism by which IL-6 induces NF- $\kappa$ B activation may be related to the activation of phosphatidylinositol 3 (PI-3) kinase. In the case of the multifunctional cytokine IFN- $\alpha$ , which induces both the classical STAT pathway and the NF- $\kappa$ B pathway, it has been shown that PI-3 plays a crucial role in the activation of NF- $\kappa$ B (46). Activation of PI-3 kinase and its downstream target PKB/Akt (a serine threonine kinase) by IFN results in the activation of I $\kappa$ B

kinase, leading to the phosphorylation and degradation of I $\kappa$ B and subsequent release of the NF- $\kappa$ B subunit (47). The IFN-dependent recruitment of PI-3 kinase to the IFNAR1 chain of the type I IFNR requires the tyrosine phosphorylation of the STAT-3 docking site on the intracellular domain of IFNAR1. Interestingly, IL-6 is known to activate PI-3 kinase in some cell lines (46–48) and we are currently exploring the involvement of PI-3 kinase in the NF- $\kappa$ B activation by IL-6.

Our data demonstrate that IL-6 induces a time-dependent activation of SOCS-3, a negative regulator of IL-6 signaling. SOCS-3 overexpression not only suppresses STAT-3 activation but also inhibits NF- $\kappa$ B activation induced by IL-6. Interestingly, SOCS-3 does not inhibit TNF- $\alpha$ -induced NF- $\kappa$ B activation. These data are consistent with the mechanism of action of SOCS-3 in down-regulating IL-6 signaling: SOCS-3 binds to the phosphorylated gp130 signal-transducing domain and prevents further phosphorylation of the receptor by JAK and possibly directs the IL-6R to the degradation pathways.

In conclusion, we provide evidence that IL-6-induced NF- $\kappa$ B activation is critical for the biological responses to IL-6. Furthermore, our data demonstrate the mechanism of ICAM-1 induction by IL-6 and the indispensable role of NF- $\kappa$ B in the induction of ICAM-1. IL-6 is classically known to activate early acute phase response genes such as C-reactive protein via the STAT-3 pathway. Given the crucial role of IL-6 in the pathogenesis of chronic intestinal inflammation, our data on the activation of the NF- $\kappa$ B pathway provides further insight into the role of IL-6 in the initiation and/or propagation of chronic inflammation. Understanding the molecular basis of IL-6 action is important when one considers the therapeutic potential of targeting the IL-6 signaling pathway in diseases such as rheumatoid arthritis and IBD as well as its role as a model for understanding the function of many cytokines.

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