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The Interleukin-22/STAT3 Pathway Potentiates Expression of Inducible Nitric-oxide Synthase in Human Colon Carcinoma Cells*

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Elisabeth Ziesché[‡], Malte Bachmann[‡], Hartmut Kleinert[§], Josef Pfeilschifter[‡], and Heiko Mühl[‡1]

From the [‡]Pharmazentrum Frankfurt/ZAFES, Klinikum der Johann Wolfgang Goethe-Universität Frankfurt am Main, 60590 Frankfurt am Main and the [§]Department of Pharmacology, Johannes Gutenberg Universität, 55101 Mainz, Germany

Inducible nitric-oxide synthase (iNOS) has been identified as a marker and mediator of disease in human colonic inflammation and carcinogenesis. Accordingly, identification of mediators that trigger iNOS in colon carcinoma/epithelial cells is an important topic of current research. Here we demonstrate that interleukin (IL)-22, a newly described member of the IL-10 cytokine family, potently synergizes with interferon (IFN)-γ for iNOS expression in human DLD-1 colon carcinoma cells. Detection of both IL-22 receptor chains and STAT3 phosphorylation proved robust IL-22 responsiveness of these cells. Short interfering RNA technology identified STAT3 as being crucial for up-regulation of iNOS. Compared with IFNγ, STAT1 phosphorylation by IL-22 was insufficient. IL-22 did not stabilize IL-1β/tumor necrosis factor-α/IFNγ-induced iNOS mRNA. IL-22 also failed to amplify expression of the prototypic IFNγ-inducible parameters IL-18-binding protein and CXCL-10, indicating that IL-22 is not a general amplifier of IFNγ functions. This assumption is furthermore supported by the observation that IL-22 was unable to enhance cellular activation of the pro-inflammatory transcription factor nuclear factor-κB. In contrast, IL-22 increased iNOS promoter activation as detected by using DLD-1 cells stably transfected with a corresponding 16-kb promoter construct (pNOS2(16)-Luc). IL-22 likewise enhanced iNOS in Caco-2 colon carcinoma cells. With IL-22 we introduce a novel potent determinant of iNOS expression in human colon carcinoma/epithelial cells. Considering the eminent functions of STAT3 and iNOS in inflammation and carcinogenesis, IL-22 may represent a novel target for immunotherapeutic intervention.

Interleukin (IL)[2]-22 is a newly described member of the IL-10 family of cytokines that is produced by T and NK cells under conditions of immunoactivation. Initiation of the Jak1/Tyk2/signal transducer and activator of transcription (STAT) 3 pathway appears to be the major mode of IL-22 signal transduction (1–4), although activation of STAT1 (5, 6), mitogen-activated protein kinases (5–7), nuclear factor κB (NF-κB) (8), activator protein-1 (8), and protein kinase B (6) has been related to this cytokine under specific conditions. IL-22 signaling is established by binding of the cytokine to its heterodimeric receptor complex consisting of IL-22R1 and IL-10R2 (2, 3). Because IL-10R2 is a ubiquitous protein, cellular IL-22 responsiveness is mainly determined by expression of the IL-22R1 receptor chain. Interestingly, IL-22R1 expression is restricted to nonleukocytic cells (9–11). Therefore, IL-22 appears to be unique among a vast array of cytokines in that this protein is incapable of mediating autocrine or paracrine functions between leukocytes but is rather specialized to transmit information between leukocytes and the nonleukocytic cell compartment. This distinctive biological characteristic essentially discriminates IL-22 from another major activator of the STAT3 signaling system, namely IL-6 (12). Cell types identified to be responsive to IL-22 include synoviocytes (7), pancreatic acinar cells (11), hepatocytes (5, 13, 14), colonic epithelial myofibroblasts (8), and in particular cells of epithelial origin such as keratinocytes (10, 15), lung carcinoma cells (16), and colon carcinoma cells (6, 17). Proteins that have been reported to be inducible by IL-22 include pro-inflammatory and pro-angiogenic mediators such as IL-8 and enzymes that are involved in cell migration and tissue remodeling such as matrix metalloprotease-1 and -3 (6, 8), effector molecules of innate immunity such as β-defensins (10), and immunosuppressive modulators such as IL-10 (17) and SOCS proteins (17, 18). IL-22-induced STAT3 has been associated with induction of the acute phase response (13), with proliferation, and with protection from cell death (6, 14). Interestingly, constitutive activation of the STAT3 pathway is characteristic for numerous human malignancies. Based on the capabilities of this transcription factor to inhibit apoptosis and to promote cell proliferation, STAT3 is actually considered an oncogenic protein (12, 19, 20).

Inducible nitric-oxide synthase (iNOS) and its volatile enzymatic product nitric oxide (NO) have been identified as potential promoters of tumor growth in a variety of human neoplasia, among other colorectal cancers (20–24). The ability of iNOS to

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[1] To whom correspondence should be addressed: Pharmazentrum Frankfurt, Klinikum der Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany. Tel.: 49 69 6301 6955; Fax: 49 69 6301 7942; E-mail: H.Muehl@em.uni-frankfurt.de.

[2] The abbreviations used are: IL, interleukin; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFNγ, interferon-γ; IL-18BPa, interleukin-18-binding protein a; iNOS, inducible NO synthase; NF-κB, nuclear factor-κB; NO, nitric oxide; RT, reverse transcription; TNFα, tumor necrosis factor-α; siRNA, short interfering RNA; STAT, signal transducer and activator of transcription.

promote carcinogenesis is likely associated with pro-inflammatory as well as pro-angiogenic properties of NO (22, 25–29). Because iNOS and STAT3 (23, 30–32) are both activated in cancerous tissues of the colon, we sought to investigate herein whether IL-22 has the potential to regulate iNOS expression in colon carcinoma/epithelial cells.

MATERIALS AND METHODS

Reagents—Human IFNγ, IL-6, and IL-22 were from PeproTech Inc. (Frankfurt, Germany). IL-1β was from BIOSOURCE. TNFα was kindly provided by the Knoll AG (Ludwigshafen, Germany). Actinomycin D was purchased from Sigma.

Cultivation of Human DLD-1 and Caco-2 Colon Carcinoma Cells—Human DLD-1 and Caco-2 colon carcinoma/epithelial cells were obtained from the Centre for Applied Microbiology and Research (Salisbury, UK) and the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), respectively. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum (Invitrogen). For experiments with DLD-1 cells, confluent cells grown on polystyrene plates (Greiner, Frickenhausen, Germany) were washed with phosphate-buffered saline and incubated with the indicated agents in the aforementioned medium. DLD-1 cells stably transfected with a 16-kb iNOS promoter construct (pNOS2(16)-Luc) (33) were cultivated in the aforementioned culture medium with the addition of 0.5 mg/ml G418 (Invitrogen). For experiments, confluent cells grown on polystyrene plates (Greiner) were washed with phosphate-buffered saline and incubated using this same culture medium without the addition of G418. For experiments with Caco-2 cells, stimulations were performed in the state of postconfluency. It has been reported previously that postconfluent Caco-2 cells gain responsiveness toward IFNγ (34). For that purpose, already confluent cells were further grown on polystyrene plates for an additional 14-day period. Thereafter, experiments were performed as indicated.

Detection of Human IL-22RA1, IL-10R2, and Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) mRNA by Standard PCR—After RNA isolation using peqGold TriFast (Peqlab, Erlangen, Germany), 1 μg of total RNA was transcribed using random hexameric primers and Moloney virus reverse transcriptase (RT) (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions. The following sequence was performed for each PCR: GAPDH, 94 °C for 10 min (1 cycle) followed by 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min (25 cycles); IL-22RA1 and IL-10R2, 95 °C for 10 min (1 cycle) followed by 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min (35 cycles); followed by a final extension phase at 72 °C for 7 min. The following primers were used: IL-22R1, forward 5′-GTATAAGACGTACGGAGA-3′ and reverse 5′-TCCAAGGTGCATTTGGTA-3′; IL-10R2, forward 5′-CATTGGGAATGGTACCAC-3′ and reverse 5′-CCAATAATGGTGTCATCCAC-3′; and GAPDH, forward 5′-ACCACAGTCCATGCCATCAC-3′ and reverse 5′-TCCACCACCCTGTTGCTGTA-3′. The possibility of amplification of contaminating genomic DNA was eliminated by selecting amplicons that cross exon/intron boundaries. PCR products (IL-22RA1, 372 bp; IL-10R2, 292 bp; GAPDH, 452 bp) were run on a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide. The identity of amplicons was confirmed by sequencing (AbiPrism 310 Genetic Analyzer, Applied Biosystems).

Evaluation of Human iNOS mRNA by RNase Protection Assay—Total RNAs (20 μg) were used for RNase protection assay, performed as described previously (35). Briefly, DNA probes were cloned into the transcription vector pBluescript II KS(+) (Stratagene, Heidelberg, Germany). After linearization, an antisense transcript was synthesized *in vitro* with T7 RNA polymerase (Roche Diagnostics) and [α-^{32}P]UTP (800 Ci/mmol; Amersham Biosciences). RNA samples were hybridized at 42 °C overnight with 100,000 cpm of the labeled antisense transcript. Hybrids were digested with RNase A (Roche Diagnostics) and T1 (Roche Diagnostics) for 1 h at 30 °C. Under these conditions every single mismatch was recognized by the RNases. Protected fragments were separated on 5% polyacrylamide, 8 M urea gels and analyzed using a PhosphorImager (Fuji, Straubenhardt, Germany). The individual gene expression of iNOS was evaluated on the basis of the GAPDH housekeeping gene expression. The cDNAs correspond to nucleotides 3724–3469 and 3607–3352, respectively (iNOS; transcript variant 1, GenBank™ accession number NM000625; transcript variant 2, GenBank™ accession number NM153292) and nucleotides 961–1071 (human GAPDH; GenBank™ accession number AC M33197) of the published sequences.

Determination of human IL-18-binding Protein a (IL-18BPa) mRNA by Quantitative Real Time PCR—Real time PCR was performed to assess expression of IL-18BPa and GAPDH. Changes in fluorescence are caused by the *Taq* polymerase degrading the probe that contains a fluorescent dye (6-carboxyfluorescein for IL-18BPa, VIC for GAPDH) and a quencher (6-carboxytetramethylrhodamine). Primers and probe for IL-18BPa were designed using Primer Express (Applied Biosystems) according to the published sequence (GenBank™ accession number XM035063.1): forward 5′-ACCTCCCAGGCCGACTG-3′ and reverse 5′-CCTTGCACAGCTGCGTACC-3′; probe 5′-CACCAGCCGGGAACGTGGGA-3′. The possibility of amplification of contaminating genomic DNA was eliminated by selecting an amplicon that crosses an exon/intron boundary. For GAPDH, pre-developed assay reagents were used (Applied Biosystems). Specificity of PCR products was tested by classic PCR using the aforementioned primers. 1 μg of total RNA was transcribed using random hexameric primers and Moloney virus RT (Applied Biosystems) according to the manufacturer's instructions. Real time PCR was performed on the AbiPrism 7700 sequence detector (Applied Biosystems) as follows: one initial step at 50 °C for 2 min and 95 °C for 2 min was followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Detection of the dequenched probe, calculation of threshold cycles (*Ct* values), and further analysis of these data were performed by the Sequence Detector software. mRNA expression was quantified by use of cloned cDNA standards for IL-18BPa and GAPDH. All results for IL-18BPa expression were normalized to that of GAPDH.

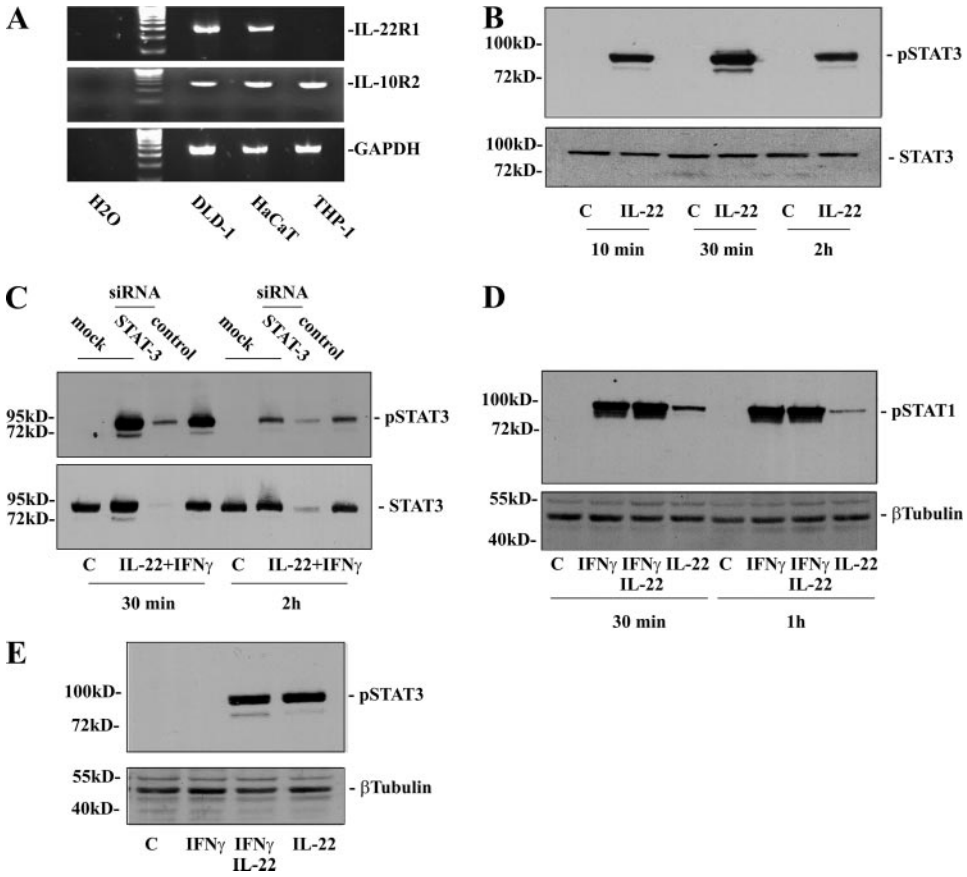

FIGURE 1. **DLD-1 cells express both IL-22 receptor chains and are activated by this cytokine.** *A*, total RNA from unstimulated DLD-1 cells, HaCaT keratinocytes, and monocytic THP-1 cells was analyzed for expression of both IL-22 receptor chains, IL-22R1 and IL-10R2, by semi-quantitative RT-PCR. *B*, DLD-1 cells were either kept as unstimulated control (*lane C*) or stimulated with IL-22 (20 ng/ml) for the indicated times. Thereafter, cellular phospho-STAT3 content was evaluated by Western blot analysis. After stripping, this same blot was stained using an antibody specific for total STAT3. One representative of four independently performed experiments is shown. *C*, DLD-1 cells were transfected as outlined under "Materials and Methods" with either siRNA directed against STAT3 or with control-siRNA. In addition, cells were mock-transfected for control conditions or IL-22 (20 ng/ml)/IFNγ (10 ng/ml) stimulations that were performed in the absence of STAT3-siRNA or control-siRNA, respectively. After the indicated incubation periods (30 min or 2 h), cellular phospho-STAT3 content was evaluated by Western blot analysis. After stripping, this same blot was stained using an antibody specific for total STAT3. One representative of three independently performed experiments is shown. *D* and *E*, DLD-1 cells were either kept as unstimulated control or stimulated with IFNγ (10 ng/ml), IL-22 (20 ng/ml), and IFNγ (10 ng/ml) plus IL-22 (20 ng/ml). After the indicated times (*D*) or a 1-h incubation period (*E*), cellular phospho-STAT1 (*D*) or phospho-STAT3 (*E*) content was evaluated by Western blot analysis. β-Tubulin was assessed on the same blots by cutting the blots in half. One representative of three independently performed experiments per experimental setup is shown.

Detection of Human iNOS, pSTAT3/STAT3, pSTAT1, IL-18BPa, and β-Tubulin by Immunoblot Analysis—For detection of intracellular proteins, cells were treated with lysis buffer (150 mM NaCl, 1 mM $CaCl_2$, 25 mM Tris-Cl, pH 7.4, 1% Triton X-100, supplemented with protease inhibitor mixture (Roche Diagnostics) and dithiothreitol, Na_3VO_4, phenylmethylsulfonyl fluoride (each 1 mM), and NaF (20 mM). Routinely, 50 μg of total protein/lane were used. For detection of total STAT3 (Fig. 1, *B* and *C*) blots were stripped and reprobed. For detection of pSTAT1 or pSTAT3 and β-tubulin in Fig. 1, *D* and *E*, blots were cut in half. To detect iNOS, STAT3, and β-tubulin on the same blot, the blot was cut in three parts as shown in Fig. 4. Antibodies and SDS-PAGE conditions were as follows: iNOS (8/10% SDS-PAGE; mouse monoclonal antibody; BD Biosciences), pSTAT3 (10% SDS-PAGE; Y705; 58E12; rabbit monoclonal antibody; Cell Signaling, Frankfurt, Germany), STAT3 (10% SDS-PAGE; mouse monoclonal antibody; Cell Signaling), pSTAT1 (10% SDS-PAGE; Y701; rabbit polyclonal antibody; Cell Signaling), and β-tubulin (10% SDS-PAGE; mouse monoclonal antibody; Santa Cruz Biotechnology). For detection of IL-18BPa, cell-free supernatants (5 ml/PS-10 plate) were trichloroacetic acid-precipitated, as described previously (35). Briefly, 1/10 volume of 70% trichloroacetic acid was added to cell-free supernatants. After 30 min on ice and a 30-min centrifugation step at 16,000 × *g*, pellets were washed in acetone and resuspended in Laemmli buffer. Trichloroacetic acid-precipitated IL-18BPa was separated by 10% SDS-PAGE and detected using a goat polyclonal antibody (R&D Systems, Wiesbaden, Germany).

Determination of CXCL-10 (IP-10) in Cell Culture Supernatants by Enzyme-linked Immunosorbent Assay (ELISA)—Levels of CXCL-10 in cell-free culture supernatants obtained from DLD-1 cultures were determined by ELISA according to the manufacturer's instruction (BD Biosciences).

Suppression of STAT3 by siRNA Technology—For experiments, DLD-1 cells were seeded at a density of 2 × 10^5 cells 24 h prior to transfection in 6-well polystyrene plates (Greiner) using the aforementioned medium. 50 nM of either STAT3-directed siRNA (number 51320, Ambion, Cambridgeshire, UK or control siRNA (Silencer®Negative Control siRNA, number 4611, Ambion) were transfected using Oligofectamine (Invitrogen) according to the manufacturer's instruction. All cultures without siRNA or control siRNA were mock-transfected under the same conditions. After 72 h of incubation in culture medium, cells were stimulated as indicated and harvested thereafter.

Analysis of the Human iNOS Promoter Activity in DLD-1/pXP$_2$-16-kb Cells Stably Overexpressing a 16-kb iNOS Promoter and the Firefly Luciferase Gene—DLD-1/pXP$_2$-16-kb cells that stably overexpress a 16-kb iNOS promoter and the firefly luciferase gene (33) were seeded in 6-well polystyrene plates (Greiner) using the aforementioned medium. Stimulation was performed as indicated. Six independent experiments were performed in triplicate. Protein content of the extracts was used for normalization of the luciferase activity. Luciferase activities were measured with the luciferase assay system (Promega

Corp., Madison, WI) according to the manufacturer's instruction using an automated chemiluminescence detector (Berthold, Bad Wildbad, Germany).

Electrophoretic Mobility Shift Assay (EMSA)—Preparation of nuclear extracts from DLD-1 cells was performed as described previously (36). Consensus oligonucleotides used in the binding reactions were obtained from Santa Cruz Biotechnology (Santa Cruz, Heidelberg, Germany). Sequences of the double-stranded oligonucleotides are as follows: NF-κB, WT 5′-AGTTGAGG-GGACTTTCCCAGGC-3′. Complementary oligonucleotides were end-labeled by T4 polynucleotide kinase (MBI Fermentas, St. Leon-Rot, Germany) using [γ-^{32}P]ATP (6000 Ci/mmol; Amersham Biosciences). Binding reactions were performed for 45 min on ice with 7.5 μg of protein in 20 μl of binding buffer containing 4% Ficoll, 20 mM HEPES, pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.25 mg/ml bovine serum albumin, 1.25 μg of poly(dI-dC), and 50,000 cpm of ^{32}P-labeled oligonucleotide. For NF-κB supershift analysis, nuclear proteins were preincubated for 30 min at room temperature with a polyclonal anti-p65 antibody (Santa Cruz Biotechnology). DNA-protein complexes were separated from unbound oligonucleotide by electrophoresis through a 4.5% polyacrylamide gel using 0.5× TBE buffer. Thereafter, gels were fixed and analyzed by PhosphorImager analysis (Fuji).

Analysis of Nitrite Production—To verify NO production, nitrite, a stable end product of NO metabolism, was measured in cell-free supernatant using the Griess reagent (Merck). Briefly, DLD-1 cells were seeded in 24-well polystyrene plates (Greiner) using the aforementioned medium and stimulated as indicated. After 48 h, cell-free supernatants were obtained and mixed with equal volume of Griess reagent. The absorbance was measured at 540 nm using a microplate reader, and nitrite concentrations were calculated using a calibration curve with sodium nitrite standards.

Statistics—Data are shown as mean ± S.D. and are presented as micromolar or nanograms/ml, as fold induction compared with unstimulated control, as % of IL-1β/TNFα/IFNγ, as % of mock-transfected control, as % of mock-transfected IL-22/IFNγ stimulated, or as % of unstimulated control. Data were analyzed by unpaired Student's *t* test on raw data using Sigma Plot (Jandel Scientific).

RESULTS

DLD-1 Colon Carcinoma Cells Are Responsive to IL-22—To demonstrate that DLD-1 cells have the capability to respond to IL-22, mRNA expression of both IL-22 receptor chains was investigated. RT-PCR analysis proved that IL-22R1 and IL-10R2 are readily detectable in these cells (Fig. 1*A*). HaCaT keratinocytes served as positive control for expression of both IL-22 receptor chains (15). Monocytic THP-1 cells were analyzed as positive control for expression of IL-10R2 and as negative control for IL-22R1 (9, 10, 17). These data agree with reports on expression of IL-22 receptors in various colon carcinoma cell lines (6, 17). Moreover, we were able to demonstrate IL-22 biological activity in DLD-1 cells by detection of substantial STAT3 phosphorylation under the influence of this cytokine (Fig. 1*B*). Accordingly, transfection of DLD-1 cells with siRNA targeting STAT3 potently suppressed STAT3 expression as well as phosphorylation in DLD-1 cells exposed to IL-22 (Fig. 1*C*). These data also proved the efficacy of the siRNA targeting STAT3 that was used in subsequent experiments. Although IL-22 could also activate the STAT1 pathway, direct comparison with IFNγ revealed that STAT1 phosphorylation by IL-22 is rather modest and a minor factor, particularly under conditions where IFNγ is present (Fig. 1*D*). In contrast, IFNγ was unable to activate STAT3 in DLD-1 cells (Fig. 1*E*).

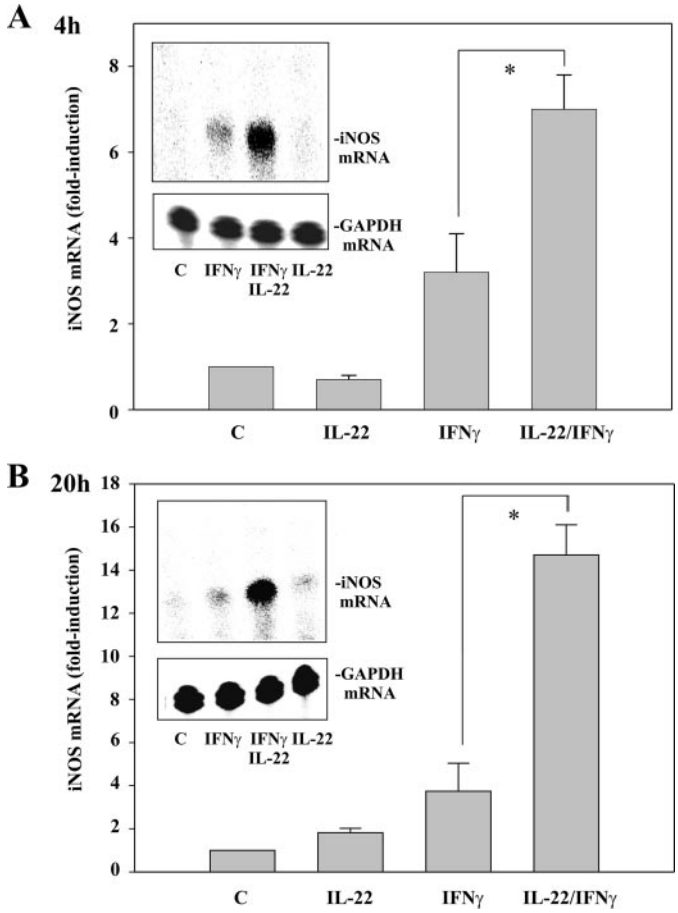

FIGURE 2. **IL-22 synergizes with IFNγ for iNOS mRNA expression in DLD-1 cells.** DLD-1 cells were either kept as unstimulated control (*lane C*) or stimulated with IFNγ (10 ng/ml), IL-22 (20 ng/ml), and IFNγ (10 ng/ml) plus IL-22 (20 ng/ml). After 4 h (*A*) or 20 h (*B*), iNOS and GAPDH mRNA expression was evaluated by RNase protection assay. iNOS mRNA was quantified and normalized to that of GAPDH by using a PhosphorImager device. Data are expressed as fold induction compared with unstimulated control ± S.D. Quantification was performed using three independently performed experiments for each condition and time point; *, $p < 0.05$. One representative RNase protection assay for each time point is shown.

IL-22 Synergizes with IFNγ for iNOS mRNA Expression in DLD-1 Cells—iNOS mRNA was evaluated by RNase protection assay. In accord with previous data (37), only suboptimal expression of iNOS was detectable after incubation of DLD-1 cells with IFNγ as a single stimulus. Whereas IL-22 alone was not capable of mediating iNOS induction, a strong synergism was observed by coincubation of cells with the combination IFNγ plus IL-22 for either 4 h (Fig. 2*A*) or 20 h (Fig. 2*B*), respectively. Western blot analysis revealed that induction of iNOS mRNA by the combination IL-22/IFNγ translated into protein expression. Neither IL-22 nor IFNγ alone was able to induce

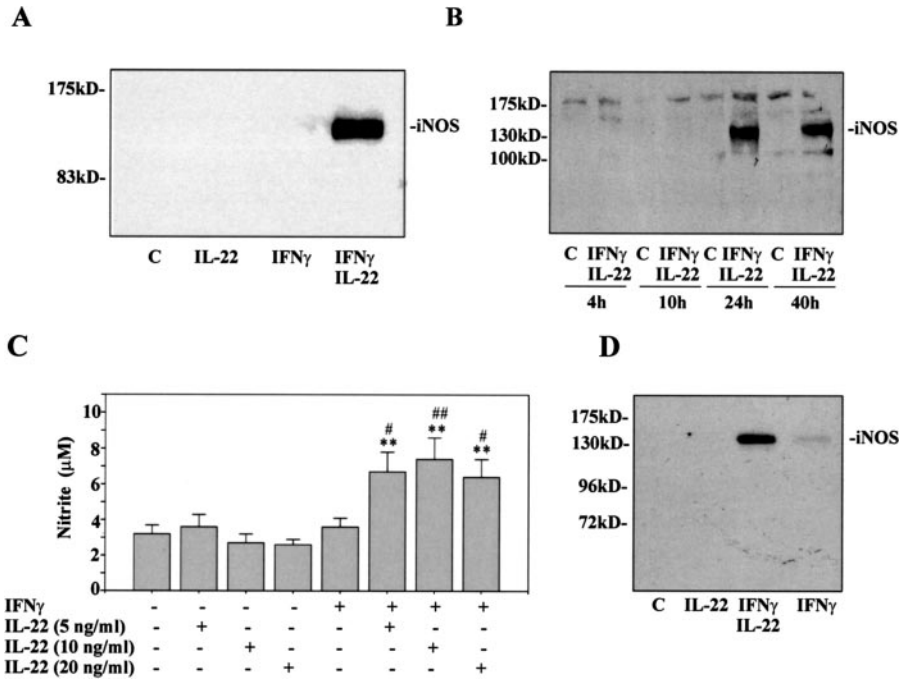

FIGURE 3. **iNOS mRNA induced by IL-22/IFNγ translates into protein expression and increases iNOS biological activity.** *A*, DLD-1 cells were either kept as unstimulated control (*lane C*) or stimulated with IFNγ (10 ng/ml), IL-22 (20 ng/ml), and with IFNγ (10 ng/ml) plus IL-22 (20 ng/ml). After 24 h iNOS protein expression was evaluated by Western blot analysis. One representative of five independently performed experiments is shown. *B*, DLD-1 cells were either kept as unstimulated control or stimulated with IFNγ (10 ng/ml) plus IL-22 (20 ng/ml). After the indicated incubation periods, iNOS protein expression was evaluated by Western blot analysis. One representative of three independently performed experiments is shown. *C*, DLD-1 cells were either kept as unstimulated control or stimulated with IFNγ (10 ng/ml), with the indicated concentrations of IL-22, and with IFNγ (10 ng/ml) plus the indicated concentrations of IL-22. After 48 h nitrite production was assessed by using the Griess assay. Data from three independently performed experiments are expressed as means ± S.D.; **, $p < 0.01$ compared with unstimulated control; #, $p < 0.05$ compared with IFNγ alone; ##, $p < 0.01$ compared with IFNγ alone. *D*, postconfluent Caco-2 cells were either kept as unstimulated control or stimulated with IFNγ (10 ng/ml), IL-22 (20 ng/ml), and with IFNγ (10 ng/ml) plus IL-22 (20 ng/ml). After 24 h, iNOS protein expression was evaluated by Western blot analysis. One representative of three independently performed experiments is shown.

significant iNOS protein (Fig. 3*A*). Interestingly, iNOS activated by IL-22/IFNγ was still detectable after 40 h of incubation, demonstrating persistent activation of iNOS expression under these conditions (Fig. 3*B*). Accordingly, nitrite levels in cell culture supernatants were determined after 48 h of incubation. Up-regulation of iNOS was associated with a significant increase of nitrite levels detectable in cell culture supernatants, indicative of increased cellular iNOS enzymatic activity (Fig. 3*C*). The observed 2–3-fold increase of nitrite concentrations in cell culture supernatants concurs with previous reports on cytokine-induced iNOS expression in human cells of epithelial origin such as DLD-1 cells or HaCaT keratinocytes (38–41). By use of Caco-2 cells an additional colon carcinoma cell line was investigated with regard to effects of IL-22 on iNOS expression. In accord with the observations made in DLD-1 cells, IL-22 and IFNγ also synergized for expression of iNOS in Caco-2 cells (Fig. 3*D*).

Silencing of STAT3 in DLD-1 Cells by Using siRNA Technology Impairs iNOS Expression Associated with IL-22—To study the impact of STAT3 on the expression of iNOS in response to IL-22/IFNγ, induction of this transcription factor was silenced by use of the siRNA approach. One representative of four independently performed experiments is shown in Fig. 4*A*. Silencing of STAT3 was in all cases associated with reduction of iNOS expression in the same experiment, indicating that in fact STAT3 plays a crucial role for iNOS induction under these conditions. Densitometric quantification of this experiment is shown in Fig. 4*B*. Data of all four experiments are depicted in the scatter plot shown in Fig. 4*C*. A close correlation was observed between suppression of STAT3 protein and iNOS expression by using STAT3 siRNA ($r = 0.9921$). Specifically, in one of these experiments STAT3 siRNA reduced STAT3 protein only to 41.5% compared with mock-transfected IL-22/IFNγ-stimulated cells. Notably, insufficient silencing of STAT3 in this particular experiment was associated with poor reduction of iNOS protein by only 24.4%. Overall, STAT3 siRNA was able to reduce STAT3 protein to 23.5 ± 12.8% of the levels observed in mock-transfected IL-22/IFNγ-stimulated cells ($n = 4$). Data for control siRNA are as follows: 74.8 ± 14.1% ($n = 4$). Reduction of STAT3 levels by STAT3 siRNA was associated with impaired induction of iNOS in these same experiments. Compared with mock-transfected IL-22/IFNγ-stimulated cells, iNOS expression was 42.8 ± 23.9% ($n = 4$). iNOS data for control siRNA are as follows: 151.8 ± 42.9% of the levels observed in mock-transfected IL-22/IFNγ-stimulated cells ($n = 4$).

IL-22 Enhances Activation of the Human iNOS Promoter as Detected in Luciferase Reporter Assays but Leaves iNOS mRNA Half-life Unaffected—Fig. 5, *A* and *B*, demonstrates that modulation of iNOS mRNA stability by IL-22 was not observed in this study. For determination of mRNA stability, iNOS was induced by a 10-h incubation period with the cytokine combination IL-1β/TNFα/IFNγ. The use of this cytokine mixture in those experiments was necessary because IFNγ alone is too weak a stimulus to provide a reliable iNOS mRNA induction that is strong enough to be the basis of further actinomycin D experiments. After 10 h of IL-1β/TNFα/IFNγ, IL-22 was either given to the cultures simultaneously with the inhibitor of transcription actinomycin D (Fig. 5*A*). In an alternative protocol actinomycin D was given 4 h subsequent to the addition of IL-22 (Fig. 5*B*). The latter protocol allowed 4 h for potential indirect effects of IL-22 that might mediate stabilization of iNOS mRNA. After the indicated incubation periods in the presence of actinomycin D, iNOS mRNA expression relative to that of GAPDH was determined by RNase protection assay. An iNOS mRNA half-life of ~4 h as detected for both protocols agrees with previous observations (42). Altogether, Fig. 5, *A* and

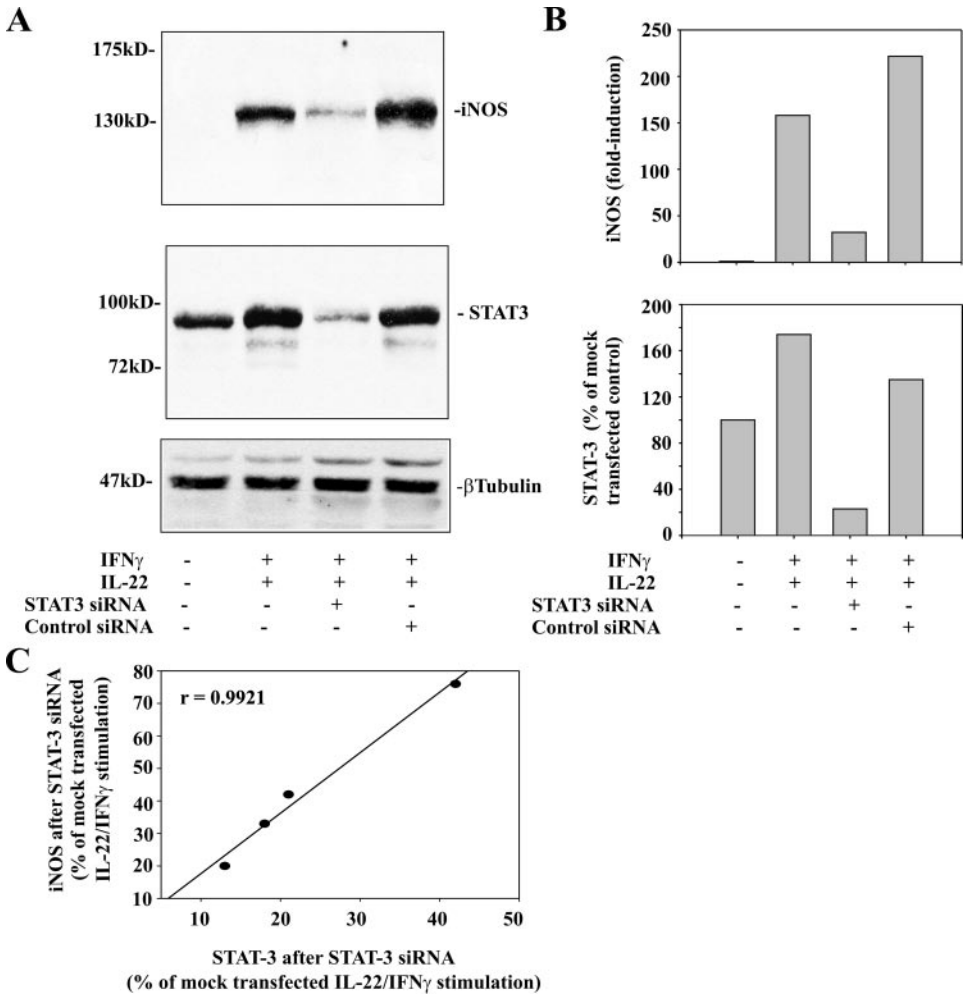

FIGURE 4. **Suppression of STAT3 by siRNA impairs induction of iNOS by IL-22/IFNγ.** DLD-1 cells were transfected as outlined under "Materials and Methods" with either siRNA targeting STAT3 or with control-siRNA. In addition, cells were mock-transfected for control conditions or IL-22 (20 ng/ml)/IFNγ (10 ng/ml) stimulations that were performed in the absence of STAT3-siRNA or control siRNA, respectively. After a stimulation period of 24 h, cells were harvested, and expression of iNOS, STAT3, and β-tubulin was evaluated by Western blot analysis. To ensure detection of these proteins on the same blot, blots were cut in three parts. One representative of four independently performed experiments is displayed (*A*). *B* shows a densitometric quantification of iNOS and STAT3 protein expression (relative to that of β-tubulin) in this particular experiment. *C*, quantified data on iNOS and STAT3 protein expression from these four independent experiments are depicted in a scatter plot, and a linear regression was performed; *r* denotes regression coefficient.

B, demonstrates that IL-22 was unable to affect iNOS mRNA stability under both experimental conditions.

siRNA experiments demonstrated that effects of IL-22 on iNOS expression are dependent on STAT3 and thus likely include a transcriptional mode of action. To analyze to what extent activation of the human iNOS promoter potentially contributes to effects of IL-22, DLD-1 cells were investigated that had been stably transfected with a pNOS2 (16)-Luc promoter construct (33). Luciferase reporter assays revealed that IL-22 was able to enhance basal iNOS promoter activity, particularly in combination with IFNγ (Fig. 5*C*).

IL-22 Is Not a General Amplifier of IFNγ Functions—To investigate whether IL-22 amplifies IFNγ actions in general, additional prototypic IFNγ-regulated genes were investigated besides iNOS. The two genes selected were IL-18BPa (35) and CXCL-10 (43). For IL-18BPa a slight tendency toward increased expression after stimulation of cells with IL-22 was noted. However, thorough investigation revealed that this effect is modest and negligible (Fig. 6*A*). Similarly, IL-22 was not able to up-regulate secretion of CXCL-10 by IFNγ-activated DLD-1 cells (Fig. 6*B*). IL-22 furthermore did not enhance the NF-κB activation status of DLD-1 cells as detected by EMSA analysis (Fig. 6*C*). This observation concurs with the inability of IL-22 to up-regulate CXCL-10 production in this cellular system. A basal level of constitutive NF-κB activation as detected herein agrees with previous reports on this cell type (44, 45). As a positive control for NF-κB activation DLD-1 cells were stimulated with the combination IL-1β plus TNFα. The retarded complex, marked by the *arrowhead* in Fig. 6*C*, seen under basal conditions and after stimulation with IL-1β plus TNFα, disappears in the presence of the anti-p65 antibody identifying p65 as a constituent of this complex.

DISCUSSION

The family of nitric-oxide synthases includes three isoforms, namely endothelial NOS, neuronal NOS, and iNOS. Among these isoforms particularly iNOS has been related to pathological processes associated with immuno-activation and inflammation. In contrast to the other isoforms, iNOS function is primarily regulated on the expression level by action of transcriptional and post-transcriptional mechanisms. Notably, processes that determine expression and function of iNOS are not only cell type-specific but are barely conserved between the human and the rodent system. In general, iNOS expression and activity are more tightly and stringently controlled in human cells. Moreover, it appears that in the human system hepatocytes and cells of epithelial origin but specifically not monocytes/macrophages are prime sources of iNOS-derived NO (46–48).

In accord with its role in pathophysiology, it became apparent that iNOS is up-regulated in various human malignancies. In colorectal cancer augmented expression of iNOS is evident in infiltrating mononuclear cells but characteristically also in tumor epithelial cells (23, 25). Notably, increased levels of nitrotyrosine residues can be detected in the colonic tumor microenvironment indicative of enhanced NOS activity (23, 30). Although the role of iNOS in tumor biology may depend on the type of cancer and on the amount of NO being produced in an

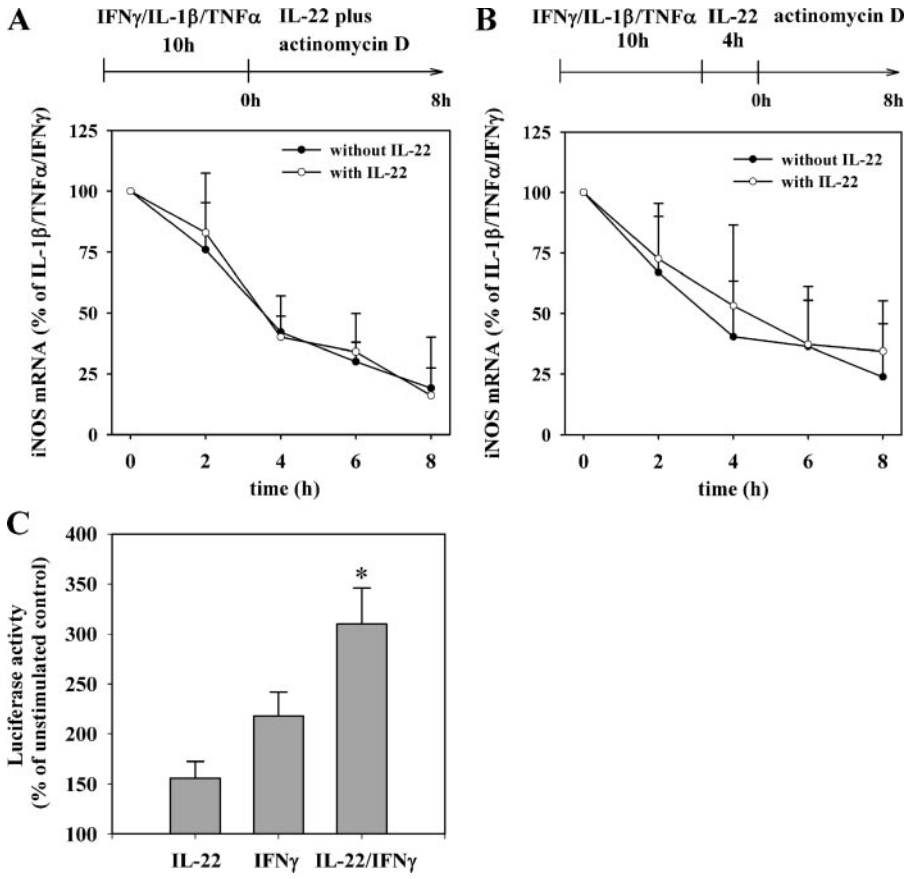

FIGURE 5. **IL-22 leaves iNOS mRNA stability unaffected but enhances iNOS promoter activity.** *A* and *B*, to analyze effects of IL-22 on iNOS mRNA stability, DLD-1 cells were activated by incubation with the combination IFNγ (10 ng/ml), TNFα (50 ng/ml), and IL-1β (50 ng/ml). *A*, after 10 h, IL-22 (20 ng/ml) was added simultaneously with actinomycin D (10 μg/ml). The latter agent blocks all further transcriptional activation. *B*, in an alternative protocol, IL-22 was likewise added after 10 h of activation by IFNγ/TNFα/IL-1β. However, this time actinomycin D was given to the cultures 4 h subsequent to IL-22. After the indicated time periods of incubation in the presence of actinomycin D, iNOS mRNA expression relative to that of GAPDH was determined by RNase protection assay and by using a PhosphorImager device. iNOS mRNA expression is shown as % of IFNγ/TNFα/IL-1β ± S.D. at time point 0, which is the time point of actinomycin D addition. Four independent experiments were performed for each data point for protocol A and three independent experiments for each data point for protocol B. *C*, to investigate effects of IL-22 on activation of the human iNOS promoter, DLD-1/pXP$_2$-16-kb cells were kept as unstimulated control or stimulated with IL-22 (20 ng/ml), IFNγ (10 ng/ml), or with the combination IL-22 (20 ng/ml) plus IFNγ (10 ng/ml). After 6 h, cells were harvested, and luciferase activity relative to the total protein content was determined. Data from six independently performed experiments are expressed as % of unstimulated control ± S.D.; *, $p < 0.05$ compared with unstimulated control.

individual patient, the bulk of evidence suggests that the iNOS/NO pathway can be considered a parameter that promotes tumor progression in human colon carcinogenesis (28, 29). Different mechanisms may account for those tumor-promoting effects of NO. Given the strong association between inflammation and colon carcinogenesis (49), pro-inflammatory actions of NO may play an important role. A mode of action that became a major focus of research is the regulation of tumor angiogenesis (50). In fact, expression of iNOS in tissues of patients with colorectal cancer correlates with intratumor microvessel density and with abundance of the pro-angiogenic mediator vascular endothelial growth factor (23, 25). Likewise, studies on cultured DLD-1 colon carcinoma cells revealed that production of pro-angiogenic mediators like vascular endothelial growth factor and CXCL-8 (IL-8) is enhanced whereas anti-angiogenic mediators like CXCL-9 (MIG) and CXCL-10 (IP-10) are suppressed under the influence of NO (51). Besides angiogenesis, NO may increase the blood supply via vasodilation at the tumor site (52). Further tumor-promoting effects of NO in colon cancer likely include up-regulation of metastases (25, 53) and p53 tumor suppressor mutation frequency (24, 28). The latter process should be highly relevant for tumor growth because as a result a selection pressure is established favoring the development of further mutated cancer cells that are resistant toward cytotoxic actions of NO. Data obtained from the human system have been complemented by animal studies demonstrating that specific inhibition of iNOS curbs rodent colon carcinogenesis (54, 55). Therefore, understanding mechanisms that direct iNOS expression in colon carcinoma/epithelial cells not only will be of pathophysiological interest but may give new leads for therapeutic intervention.

DLD-1 colon carcinoma cells are regarded as a prototypic and a most well characterized cell culture model for human iNOS regulation in a pro-inflammatory cytokine context. Actually, one of the very first reports on human iNOS induction was based on studies using this cell type almost 15 years ago (38, 56, 57). Therefore, we chose to focus on DLD-1 cells in this study. Here we demonstrate for the first time that IL-22 synergizes with IFNγ for induction of iNOS in human DLD-1 cells. Neither cytokine was able to significantly activate the iNOS pathway as a single stimulus. To complement those data on DLD-1 cells, we sought to investigate the effects of IL-22 on iNOS in an alternative colon carcinoma cell line. We selected Caco-2 cells that have been characterized in a recent study as IL-22R1-positive and thus IL-22-responsive (6). Whereas DLD-1 cells are regarded as poorly differentiated cells with a high invasive potential, Caco-2 cells are described as moderately well differentiated and are characterized by a diminished invasive potential (58). Notably, amplification of iNOS expression by IL-22 was well observed in Caco-2 cells, indicating that this regulatory pathway is independent on the differentiation status of the colon carcinoma cell line under investigation.

Interestingly, IL-22/IFNγ-induced iNOS expression was not associated with an up-regulation of the cellular NF-κB activation status. Significant gene induction of iNOS in this cell type has been exclusively linked previously to stimulatory conditions that at least coincide with increased NF-κB activity. Thus, until now, NF-κB, activated either by IL-1β and TNFα or by

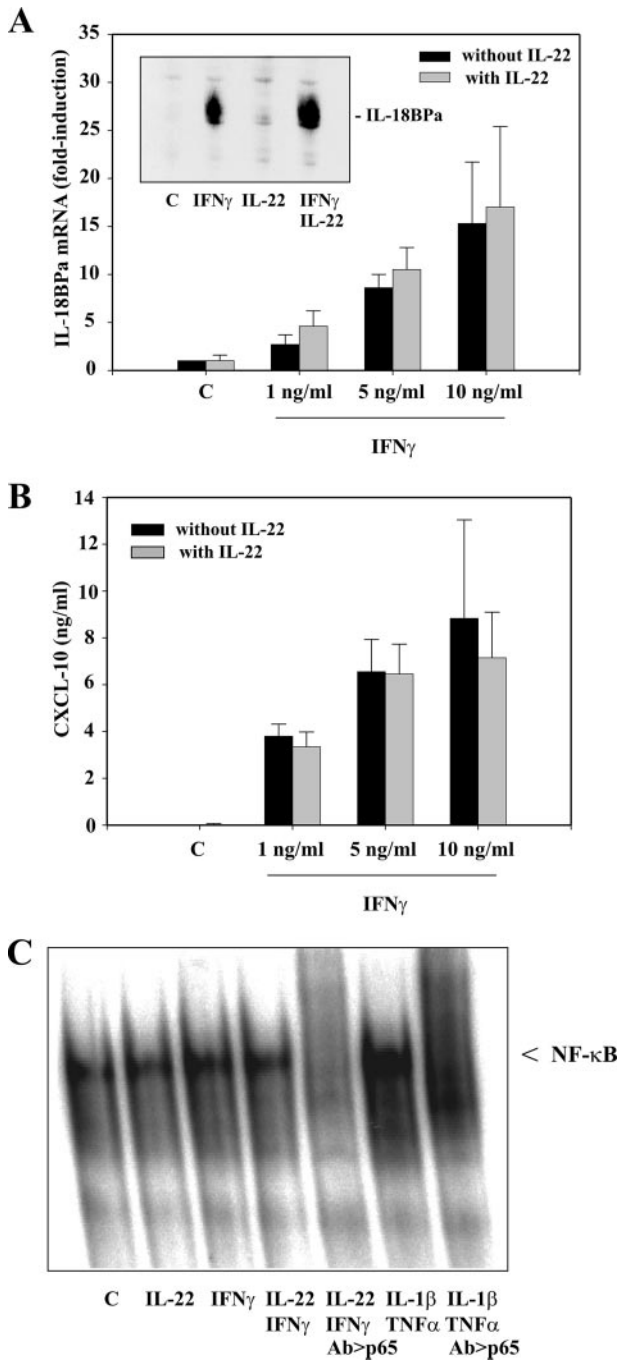

FIGURE 6. **IL-22 is not a general amplifier of IFNγ functions, analysis of IL-18BPa, and CXCL-10 expression.** *A,* DLD-1 cells were either kept as control or stimulated with the indicated concentrations of IFNγ in the presence or absence of IL-22 (20 ng/ml). After a 20-h incubation period, cells were harvested, and mRNA expression of IL-18BPa was evaluated by quantitative real time PCR. IL-18BPa mRNA expression as analyzed in three independently performed experiments was normalized to that of GAPDH and is shown as fold induction compared with unstimulated control ± S.D. *A, inset*, cells were either kept as unstimulated control or stimulated with IFNγ (10 ng/ml), IL-22 (20 ng/ml), and with IFNγ (10 ng/ml) plus IL-22 (20 ng/ml). After an incubation period of 24 h, trichloroacetic acid-precipitated supernatants were analyzed for IL-18BPa secretion by Western blot analysis. *Lane C*, control. *B,* DLD-1 cells were either kept as unstimulated control or stimulated with the indicated concentrations of IFNγ in the presence or absence of IL-22 (20 ng/ml). After a 20-h incubation period, CXCL-10 concentrations in culture supernatants were determined by ELISA analysis. Data from five independently performed experiments are expressed as means ± S.D. *C,* DLD-1 cell were either kept as unstimulated control or were stimulated with IL-22 (20 ng/ml), IFNγ (10 ng/ml), IL-22 (20 ng/ml)/IFNγ (10 ng/ml), or with IL-1β (50 ng/ml)/TNFα (50 ng/ml). After 2 h, nuclear lysates were prepared, and NF-κB activation was assessed by EMSA analysis as described under "Materials and Methods." Where indicated, an anti-p65 antibody was added to the lysates to characterize the retarded complex (marked by the *arrowhead*). One representative of three independently performed experiments is shown.

toll-like receptor agonists such as flagellin, and STAT1, activated by IFNγ, attracted much attention as the pivotal and most consistent transcription factors mediating gene induction of human iNOS (46, 59, 60). The present data indicate that induction of NF-κB activity may not be mandatory in DLD-1 cells. However, a basal level of constitutive NF-κB activity that is detectable in this cell type may actually contribute to this phenomenon (see Fig. 6*C* and see Refs. 44, 45). Instead, siRNA experiments revealed that STAT3 is essential for iNOS induction under the influence of IL-22. This observation suggests that a transcriptional mechanism is responsible for regulation of iNOS by this cytokine. Luciferase reporter assays actually confirmed that IL-22 has the potential to increase human iNOS promoter activity. A direct regulatory axis linking IL-22/STAT3 to iNOS promoter activity is furthermore supported by the fact that iNOS expression was significantly enhanced after only 4 h of incubation with IL-22/IFNγ. Recently, modulation of mRNA stability turned out to be another major regulatory mechanism that determines expression of human iNOS (46). Specifically, the KH-type splicing regulatory protein (KSRP) is able to destabilize iNOS mRNA in DLD-1 cells. In fact, down-regulation of KSRP increased iNOS mRNA stability in DLD-1 cells activated by IFNγ/IL-1β/TNFα from 4.2 to 7.7 h (42, 61). In contrast, overexpression of polypyrimidine tract-binding protein in DLD-1 cells increases (stabilization by polypyrimidine tract-binding protein) the iNOS mRNA half-life in the presence of IL-1β/TNFα/IFNγ from 3.3 to 5.9 h (62). However, the present observation that iNOS mRNA was not stabilized by IL-22 further agrees with a mode of IL-22 action that targets the iNOS promoter. Activation of STAT1 by IL-22 was modest and irrelevant in the context of IFNγ biological activity.

Current knowledge on the role of STAT3 concerning regulation of the human iNOS gene is fragmentary. Here, we demonstrate that cytokine-induced STAT3 is a key determinant of human iNOS expression in DLD-1 and Caco-2 colon carcinoma cells. In accord with these data, it was shown recently that overexpression of a biologically active STAT3 mutant is able to trigger induction of the human iNOS promoter in HeLa cells in the absence of other stimuli (63). Previously, it has been reported that IL-6 is able to enhance IL-1β/IFNγ-induced nitrite release by DLD-1 cells (56). However, the molecular basis of this IL-6 action and a possible involvement of STAT3 was not investigated. In this study we were able to confirm that IL-6, like IL-22, is a costimulus of iNOS expression (data not shown). Because IL-6, like IL-22, is a potent activator of the STAT3 signaling pathway, we propose that amplification of iNOS by IL-6 is likewise mediated by activation of this transcription factor.

Two additional prototypic IFNγ-inducible genes were investigated here, namely CXCL-10 (43) and IL-18BPa (35). Expression of CXCL-10 in human epithelial cells is primarily con-

trolled by STAT-1 and NF-κB (64), whereas IL-18BPa induction is dependent on STAT-1 and STAT-1-induced IRF-1.[3] Expression of both mediators, CXCL-10 and IL-18BPa, was not significantly affected by IL-22, neither alone nor in combination with IFNγ. This observation in fact concurs with a lack of effect of IL-22 on NF-κB and STAT1 in DLD-1 cells. Altogether these data imply that IL-22 is not a general amplifier of IFNγ responses but rather is specifically aimed at the iNOS gene.

Overexpression of iNOS and STAT3 has been reported in Crohn disease patients (65, 66) and in human colorectal cancer (23, 25, 31, 32). Because colonic inflammation is regarded a pre-cancerogenic condition and blockage of iNOS is protective in rodent colitis (67) as well as colon cancer (54, 55), the present data suggest IL-22 as a novel target for therapeutic intervention aimed at expression of the STAT3-dependent genes, in particular iNOS.

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The Interleukin-22/STAT3 Pathway Potentiates Expression of Inducible Nitric-oxide Synthase in Human Colon Carcinoma Cells

Elisabeth Ziesché, Malte Bachmann, Hartmut Kleinert, Josef Pfeilschifter and Heiko Mühl

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