STAT3 regulates NF-κB recruitment to the IL-12p40 promoter in dendritic cells

Frank Hoentjen, R. Balfour Sartor, Michitaka Ozaki, and Christian Jobin

Interleukin-10–deficient (IL-10$^{-/-}$) mice develop an IL-12–mediated intestinal inflammation in the absence of endogenous IL-10. The molecular mechanisms of the dysregulated IL-12 responses in IL-10$^{-/-}$ mice are poorly understood. In this study, we investigated the role of nuclear factor–κ B (NF-κB) and signal transducers and activators of transcription 3 (STAT3) in lipopolysaccharide (LPS)–induced IL-12p40 gene expression in bone marrow derived–dendritic cells (BMDCs) isolated from wild-type (WT) and IL-10$^{-/-}$ mice. We report higher IL-12p40 mRNA accumulation and protein secretion in LPS-stimulated BMDCs isolated from IL-10$^{-/-}$ compared with WT mice. LPS-induced NF-κB signaling is similar in IL-10$^{-/-}$ and WT BMDCs as measured by IκBα phosphorylation and degradation, RelA phosphorylation and nuclear translocation, and NF-κB transcriptional activity, with no down-regulatory effects of exogenous IL-10. Chromatin immunoprecipitation demonstrated enhanced NF-κB (cRel, RelA) binding to the IL-12p40 promoter in IL-10$^{-/-}$ but not WT BMDCs. Interestingly, LPS induced STAT3 phosphorylation in WT but not IL-10$^{-/-}$ BMDCs, a process blocked by IL-10 receptor blocking antibody. Adenoviral gene delivery of a constitutively active STAT3 but not control green fluorescence protein (GFP) virus blocked LPS-induced IL-12p40 gene expression and cRel recruitment to the IL-12p40 promoter. In conclusion, dysregulated LPS-induced IL-12p40 gene expression in IL-10$^{-/-}$ mice is due to enhanced NF-κB recruitment to the IL-12p40 promoter in the absence of activated STAT3. (Blood. 2005;105:689-696)

Introduction

Interleukin-10–deficient (IL-10$^{-/-}$) mice develop spontaneous T-helper 1 (Th-1)–mediated colitis when housed under specific pathogen-free (SPF) conditions.[1-3] In this experimental model, enhanced IL-12p40 production by immune cells represents a key feature of intestinal inflammation, as demonstrated by the prevention and partial treatment of colitis by anti–IL-12 antibodies.[4,5] This suggests that in absence of IL-10, the host mounts a dysregulated innate response to the commensal intestinal microflora. Dendritic cells (DCs) are at the interface of innate and adaptive immunity by virtue of their ability to secrete various cytokines including tumor necrosis factor α (TNFα), IL-12p40, and IL-23.[6,7] For instance, IL-12p40–producing DCs skewed T-cell differentiation toward a Th-1 profile, a hallmark in the IL-10$^{-/-}$ experimental mouse model.[8,9] However, the molecular mechanisms of dysregulated IL-12p40 gene expression in IL-10$^{-/-}$ mice following SPF transfer are still poorly understood.

IL-10 is a potent immunoregulatory cytokine with numerous effects, such as down-regulation of proinflammatory cytokines, chemokines, and costimulatory molecules.[10] Several mechanisms have been proposed for the IL-10–mediated inhibition of lipopolysaccharide (LPS)–induced proinflammatory gene expression, including activation of the heme oxygenase (HO)/carbon monoxide pathway,[11] inhibition of the nuclear factor–κ B (NF-κB) pathway[12-14] and mitogen-activated protein (MAP) kinase activity,[15] mRNA stability,[16] signal transducers and activators of transcription 3 (STAT3) activation,[17] and induction of B-cell/lymphoma 3 (Bcl3).[18] However, the molecular mechanisms for dysregulated host innate responses in the IL-10$^{-/-}$ mouse model are still unknown. IL-10 mediates its inhibitory effects through binding to its receptor complex, which induces activation of the cytoplasmic receptor–associated Janus kinase 1 (JAK1) and tyrosine kinase-2 (Tyk2).[10] This is followed by STAT3 phosphorylation, homodimerization, and translocation to the nucleus where it binds to STAT-binding elements in the promoters of various IL-10–inducible genes, including suppressor of cytokine signaling 3 (SOCS3) and Bcl3.[10,18] The pivotal role of STAT3 in maintaining host homeostasis is clearly demonstrated by studies using genetic deletion. For example, STAT3 deletion is embryogenically lethal,[19] and myeloid cell–specific STAT3-deficient mice develop severe enterocolitis.[20] STAT3 deletion in bone marrow cells leads to overly activated innate immune responses[21] and interferes with the adaptive immune system by inhibiting the induction of antigen-specific T-cell tolerance.[22] Moreover, STAT3 gene inactivation leads to an aggressive and fatal form of enterocolitis mediated by IL-12.[23] These data highlight the pivotal role of STAT3 in controlling innate immunity. The absence of endogenous IL-10 in IL-10$^{-/-}$ mice provides a powerful means to investigate the immunoregulatory mechanisms of this cytokine. To date, the

From the Center for Gastrointestinal Biology and Diseases, University of North Carolina at Chapel Hill, NC; Department of Gastroenterology, Free University Medical Center, Amsterdam, The Netherlands; Department of Innovative Surgery, National Research Institute for Child Health and Development, Tokyo, Japan.

Submitted April 6, 2004; accepted June 30, 2004. Prepublished online as *Blood* First Edition Paper, July 13, 2004; DOI 10.1182/blood-2004-04-1309.

Supported by National Institutes of Health (NIH) ROI grant DK 47700, Crohn's and Colitis Foundation of America (CCFA) grants to C.J., and by NIH Center Grant P30 DK34937. R.B.S. and AGIKO (assistent geneeskundige in Opleiding tot Klinisch onderzoeker) grant 920-03-300 to F.H. from The Netherlands Organization for Health Research and Development, The Netherlands.

Reprints: Christian Jobin, Department of Medicine, Division of Gastroenterology and Hepatology, Medical Biomolecular Research Building, Rm 7341B, CB 7032, UNC-Chapel Hill, Chapel Hill, NC 27599-7032; e-mail: job@med.unc.edu.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

intracellular mechanisms of dysregulated IL-12 responses in IL-10$^{-/-}$ mice have not been revealed. In this study, we demonstrate that the increased IL-12p40 gene expression in IL-10$^{-/-}$ mice is due to enhanced NF-κB recruitment to the gene promoter caused by defective STAT3 activation. This data indicates that STAT3 plays a critical role in the resolution of LPS-induced proinflammatory gene expression and may represent a potential target for the treatment of inflammatory bowel diseases (IBDs).

Materials and methods

Cell isolation and stimulation

Wild-type (WT) and IL-10$^{-/-}$ mice (129 SvEv background) between 6 and 10 weeks of age were used to isolate bone marrow cells from femora and tibiae. Red blood cells (RBCs) were lysed using RBC lysing buffer (Sigma, St Louis, MO), and cells were cultured in 24-well low-adherence plates (Costar, Corning, NY) in complete medium containing RPMI 1640 plus 10% heat-inactivated fetal calf serum (HyClone, Logan, UT), 2 mM L-glutamine, 1 mM sodium pyruvate, 5×10^{-5} M 2-mecaptoethanol, and 50 μg/mL gentamicin in the presence of recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 (both 10 ng/mL; Peprotech, Rocky Hill, NJ). Floating cells were gently removed and medium was refreshed at day 3, and cells were collected at day 6. The cells were then washed twice and incubated overnight in regular medium without IL-4/GM-CSF. Flow cytometry analysis demonstrated a homogenous cell population with greater than 85% CD11c^{+} cells and less than 2% T cells. Additionally, major histocompatibility complex (MHC) class II, CD80, CD86, and OX40-L cell surface expression were similar between WT and IL-10$^{-/-}$ bone marrow–derived DCs (BMDCs), ruling against a possible phenotypic difference between the cells (data not shown). For cell stimulation, 1×10^5 cells (cytokine measurement) or 2×10^6 to 4×10^6 cells (proteins, RNA) were plated in a 96-well or 12-well plate, respectively. The cells were then stimulated with LPS (5 μg/mL, serotype 055:B5; Sigma) in the presence or absence of recombinant murine IL-10 (12-h preincubation, 10 ng/mL; Peprotech).

Reagents

Purified rat anti–mouse IL-10 receptor antibody and purified rat immunoglobulin G (IgG) isotype control antibody were purchased from BD Pharmingen (San Diego, CA). The JAK2 inhibitor AG490 was purchased from Upstate Biotechnology (Lake Placid, NY).

Adenoviral vectors and viral infections

The constitutively active STAT3 adenovirus (Ad5STAT3C) was engineered by substituting the cysteine residues for A661 and N663, allowing STAT3 dimerization and activation without phosphorylation at Y705.[24] The STAT3C contained an extra 24–base pair DNA nucleotide encoding for the FLAG peptide (DYLDDDDL). The NF-κB superrepressor (Ad5IκBAA)[25] and the κB-luciferase adenoviral vector (Ad5κBLUC)[26] have been characterized and described previously. The Ad5GFP virus containing the green fluorescent protein (GFP) was used as a control viral vector throughout the study.[27] The optimal multiplicity of infection (MOI) for maximal infection rate was determined by flow cytometry of Ad5GFP-infected BMDCs, showing an infection rate of more than 80% at an MOI of 50 after 2 days of infection. Viability of adenoviral-infected BMDCs was comparable to uninfected cells as measured by 7-amino-actinomycin D (7-AAD) labeling and flow cytometry analysis.

Western immunoblots

BMDCs were stimulated with LPS (5 μg/mL) at various time points in 12-well plates (Costar), collected, and lysed in 1 × Laemmli buffer, and the protein concentration was measured using Bio-Rad quantification assay (Bio-Rad Laboratories, Hercules, CA). Fifteen micrograms of protein extracts was subjected to electrophoresis on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels and transferred to nitrocellulose membranes. Antibodies against phospho-IκB, phospho-RelA, phospho-p38, p38, phospho-STAT3, STAT3 (Cell Signaling, Beverly, MA); FLAG (Sigma); and IκB, RelA, and cRel (Santa Cruz Biotechnology, Santa Cruz, CA) were all used at a 1:1000 dilution. The specific immunoreactive proteins were detected using the enhanced chemoluminescence (ECL) kit (Perkin Elmer, Shelton, CT) as described previously.[28]

RNA extraction and reverse transcriptase–polymerase chain reaction (RT-PCR) analysis

RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA), reverse transcribed (1 μg RNA), and amplified as previously described.[28] The PCR products (7 μL) were subjected to electrophoresis on 2% agarose gels containing gel Star fluorescent dye (FMC, Philadelphia, PA). Fluorescence staining was captured using an Alpha Imager 2000 (AlphaInnotech, San Leandro, CA). Sequences of primers used for IL-12p40 were 5′-GGAAGCACGGCAGCAGAATA-3′ sense, 5′-AACTTGAGGGAG-AAGTAGGAATGG-3′ antisense; IL-10, 5′-CTCTTACTGACTGGCAT-GAGGATC-3′ sense, 5′-CTATGCAGTTGATGAAGATGTCAAATT-3′ antisense; and TNFα, 5′-ATGAGCACAGAAAGCATGATC-3′ sense, 5′-TACAGGCTTGTCACTCGAATT-3′ antisense. The size of the amplified products is 180 base pair (bp) (IL-12p40), 475 bp (IL-10), and 175 bp (TNFα).

Immunofluorescence

For FLAG expression, WT and IL-10$^{-/-}$ BMDCs were infected for 48 hours with Ad5STAT3C or left uninfected. For cRel nuclear translocation, cells were stimulated with LPS (5 μg/mL) for 30 minutes. Subsequently, cells were fixed with 100% ice-cold methanol and permeabilized with 0.3% saponin for 10 minutes. Cells were blocked with 10% nonimmune goat serum (NGS; Sigma) for 30 minutes, then probed with rabbit anti-cRel antibody (Santa Cruz Biotechnology) diluted 1:200 in 10% NGS for 30 minutes, followed by rhodamine isothiocyanate–conjugated goat anti–rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:100 in 10% NGS for 30 minutes. FLAG expression was detected using mouse anti-FLAG antibody (Ab; Sigma) 1:250 diluted in 10% NGS for 30 minutes, followed by a fluorescein isothiocyanate (FITC)–conjugated goat anti–mouse IgG antibody (Jackson ImmunoResearch) diluted 1:100 in 10% NGS for 30 minutes. Slides were mounted in glycerol-based mounting medium before microscopic analysis. FLAG-STAT3C- and cRel expression were imaged using an Olympus IX70 inverted microscope (Olympus, Melville, NY) fitted with Rhodamine or FITC specific filters. Images were captured using a digital SPOTM camera (Diagnostic Instruments, McHenry, IL) and processed with the SPOT advance software. Identical exposure times were used for each data point within an individual experiment. Original magnification set at 400×.

Chromatin immunoprecipitation (ChIP) analysis

WT and IL-10$^{-/-}$ BMDCs were stimulated with LPS (5 μg/mL) for 0, 4, and 8 hours, and ChIP assays were performed using a ChIP assay Kit (Upstate) according to the manufacturer's specification according to the Upstate protocol. Briefly, proteins/DNA were cross-linked with 1% formaldehyde for 10 minutes and then cross-linking was blocked with 125 mM glycine. Cells were then lysed in L1 lysis buffer (50 mM Tris [tris(hydroxymethyl)aminomethane], pH 8.0; 2 mM EDTA [ethylene dinitrilo tetraacetic acid]; 0.1% Nonidet P-40; and 10% glycerol) supplemented with protease inhibitors, and chromatin was sheared by sonication (3 times for 35 seconds, 1.0 seconds on, 0.8 seconds off). The extracts were precleared for 1 hour with salmon sperm–saturated protein A/G-agarose (Upstate Biotechnology). Immunoprecipitation was carried out overnight at 4°C using 3 μg of anti-RelA or cRel Ab (Santa Cruz Biotechnology). Immune complexes were collected with salmon sperm–saturated protein A/G-agarose for 2 hours and washed twice in high-salt buffer (20 mM Tris, pH 8.0; 0.1% SDS; 0.5 M NaCl; 1% Nonidet P-40; 2 mM EDTA), followed by 2 washes with no-salt buffer (Tris-EDTA 1 ×), and 2 washes in 0.5M LiCL buffer. Samples were rotated for 5 minutes at 4°C in between every washing step. Immune complexes were extracted 2 times with 250 μL of freshly prepared extraction buffer (1% SDS, 0.1 M $NaHCO_2$). DNA cross-links were

reverted by heating for 8 hours at 65°C. After proteinase K (100 μg for 1 h at 45°C) digestion, DNA was extracted with phenol/chloroform and precipitated in ethanol. DNA isolated from an aliquot of the total nuclear extract was used as a loading control for the PCR (input control). PCR was performed with total DNA (2 μL, input control) and immunoprecipitated DNA (2 μL) using IL-12p40 promoter–specific primers as described previously.[29] The PCR products (8 μL) were subjected to electrophoresis on 2% agarose gels containing gel Star fluorescent dye (FMC). Fluorescent staining was captured using an AlphaImager 2000 (AlphaInnotech).

Cytokine measurement

Cells were stimulated for 7 to 24 hours with LPS (5 μg/mL), and supernatants were collected and cytokine levels were measured using commercially available kits specific for TNFα (R&D Systems, Minneapolis, MN) and IL-12p40 (Pharmingen/BD Biosciences), according to the manufacturers' instructions. Cytokine levels were determined in triplicate culture supernatants in each separate experiment.

Statistical analysis

Statistical significance was evaluated by the 2-tailed Student *t* test for paired data. A *P* value of less than .05 was considered statistically significant.

Results

Enhanced IL-12p40 and TNFα gene expression in LPS-stimulated IL-10$^{-/-}$ BMDCs

Since IL-10$^{-/-}$ mice develop spontaneous colitis accompanied by increased IL-12p40 production when housed under SPF conditions,[1,5] we first sought to evaluate the profile of Th-1 cytokine production in BMDCs isolated from both IL-10$^{-/-}$ and WT mice. As seen in Figure 1A-B, LPS induced higher IL-12p40 and TNFα secretion (approximately 2 fold) in LPS-stimulated IL-10$^{-/-}$ compared with WT BMDCs. Similarly, IL-12p40 mRNA accumulation was higher in LPS-stimulated IL-10$^{-/-}$ compared with WT BMDCs (Figure 1C). This indicates that lack of endogenous IL-10 is associated with enhanced IL-12p40 gene expression in BMDCs. Interestingly, LPS induced IL-10 mRNA accumulation (Figure 1C)

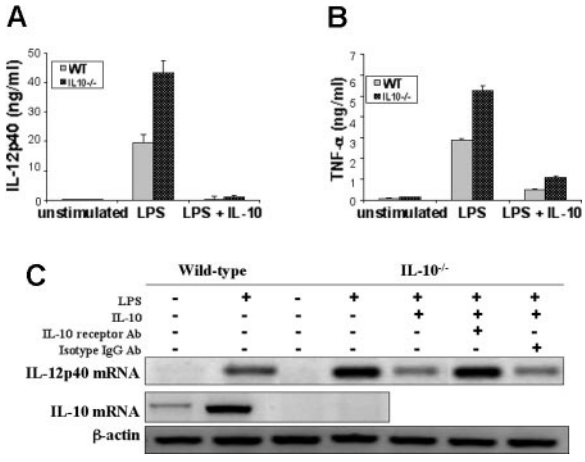


Figure 1. IL-10–mediated inhibition of increased IL-12p40 and TNFα mRNA accumulation and protein secretion in BMDCs. BMDCs from WT and IL-10$^{-/-}$ mice were stimulated with LPS (5 μg/mL) in the presence or absence of IL-10 (10 ng/mL). (A) IL-12p40 secretion at 24 hours, (B) TNFα secretion at 24 hours as measured in triplicate supernatants by enzyme-linked immunosorbent assay (ELISA). Error bars represent standard error of the mean (SEM). (C) IL-12p40 and IL-10 mRNA levels as determined by RT-PCR in RNA isolated from 12-hour LPS-stimulated BMDCs. In some samples, IL-10 receptor antibody or isotype control IgG (30 μg/mL) was used for blockade of the IL-10 receptor. The results are representative of 3 independent experiments.

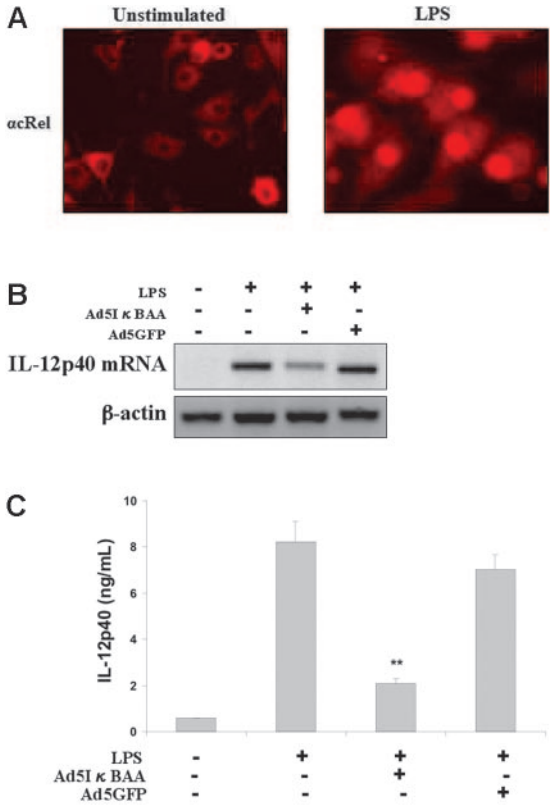


Figure 2. IL-12p40 mRNA accumulation and protein secretion in LPS-stimulated BMDCs is NF-κB dependent. (A) IL-10$^{-/-}$ BMDCs were stimulated with LPS (5 μg/mL) for 30 minutes, and immunofluorescence detecting cRel was performed as described in "Materials and methods." (B) BMDCs from IL-10$^{-/-}$ mice were infected with the IκB super repressor (Ad5IκBAA) or Ad5GFP (control) and subsequently stimulated with LPS (5 μg/mL). Cells were collected, RNA was isolated, and IL-12p40 mRNA was determined by RT-PCR. (C) IL-12p40 secretion by Ad5IκBAA- and Ad5GFP-infected IL-10$^{-/-}$ BMDCs, stimulated by LPS (5 μg/mL) for 7 hours, as measured in triplicate supernatants by ELISA. All results are representative of 3 independent experiments. Error bars represent SEM; $^{**}P < .1$.

in WT BMDCs. As expected, IL-10 mRNA remained undetectable in IL-10$^{-/-}$ BMDCs (Figure 1C). Addition of exogenous IL-10 efficiently down-regulated LPS-induced IL-12p40 mRNA accumulation and TNFα protein secretion (Figure 1A-C). This effect was blocked by IL-10 receptor blockade but not by the isotype control IgG Ab (Figure 1C). This clearly indicates that lack of IL-10–mediated signaling leads to dysregulated IL-12p40 and TNFα gene expression.

IL-12p40 secretion in IL-10$^{-/-}$ BMDCs is NF-κB mediated

LPS signals to the Toll-like receptor 4 (TLR4) to activate numerous signaling cascades including the NF-κB pathway and its subunits. For example, cRel is important for LPS-induced IL-12p40 transcription in macrophages and in BMDCs derived from cRel$^{-/-}$ p50$^{-/-}$ mice.[30,31] However, this subunit is not required for IL-12p40 gene expression in splenic dendritic cells.[32] We next determined the role and function of cRel in driving LPS-induced IL-12p40 expression in IL-10$^{-/-}$ BMDCs.

Immunofluorescence analysis demonstrated that cRel distribution is mainly cytoplasmic in unstimulated BMDCs but clearly translocates to the nucleus in LPS-stimulated cells (Figure 2A). To investigate the role of NF-κB in LPS-induced IL-12p40 gene expression, we then used adenoviral vector–mediated gene delivery to selectively block this signaling pathway using the IκB superrepressor (Ad5IκBAA). As seen in Figure 2, LPS-induced

IL-12p40 mRNA accumulation (Figure 2B) and protein secretion (Figure 2C) was blocked in Ad5IκBAA-infected but not control Ad5GFP-infected IL-10$^{-/-}$ BMDCs. Therefore, we conclude that NF-κB activation is essential for LPS-induced IL-12p40 gene expression in IL-10$^{-/-}$ BMDCs.

IL-10 has been identified as a negative regulator of NF-κB activities in different cell systems.[12-14] Thus, we hypothesized that in the absence of endogenous IL-10, NF-κB signaling is enhanced and leads to increased LPS-induced IL-12p40 gene induction. To test this hypothesis, we compared NF-κB signaling between WT and IL-10$^{-/-}$ BMDCs. As shown in Figure 3A, LPS-induced IκB degradation and phosphorylation were similar between WT and IL-10$^{-/-}$ BMDCs. Interestingly, although exogenous IL-10 blocked LPS-induced IL-12p40 gene expression, no effect was noticed on IκB degradation or phosphorylation (Figure 3A). We recently showed that LPS-induced RelA phosphorylation at serine residue 536 is critical for IL-6 gene expression.[26] Similarly, LPS-induced RelA S536 phosphorylation is comparable between WT and IL-10$^{-/-}$ BMDCs and is not inhibited by exogenous IL-10 (Figure 3A). Additionally, LPS-induced κB-luciferase activity is similar between WT and IL-10$^{-/-}$ BMDCs (data not shown). In summary, activation of the proximal NF-κB signaling pathway is similar in WT and IL-10$^{-/-}$ BMDCs stimulated with LPS.

TLR4 activates numerous signaling cascades including the MAP pathways, which also contribute to the regulation of downstream gene targets.[33] Since IL-10 modulates p38 activity that impacted gene expression,[11,15] we investigated whether other TLR4-mediated p38 and c-Jun N-terminal kinase (JNK) signaling events are affected in IL-10$^{-/-}$ cells. Therefore, we evaluated phosphorylation of the MAP kinase (MAPK) p38 and JNK in LPS-stimulated BMDCs. As seen in Figure 3B, p38 and JNK phosphorylation is induced in LPS-stimulated cells with no difference between WT and IL-10$^{-/-}$ BMDCs. IL-10 preincubation failed to block LPS-induced p38 and JNK phosphorylation in BMDCs, showing that this immunosuppressive cytokine is not targeting these MAPKs. However, blocking p38 activity with SB203580 slightly impaired LPS-induced IL-12p40 secretion (data not shown). Together, these findings suggest that although the MAPK pathway may participate with NF-κB in regulating IL-12p40 gene expression, lack of endogenous IL-10 does not lead to dysregulated MAPK signaling. Additionally, although NF-κB signaling is critical for LPS-induced IL-12p40 gene expression, enhanced IL-12p40 gene expression is not accompanied by increased proximal NF-κB signaling in IL-10$^{-/-}$ BMDCs.

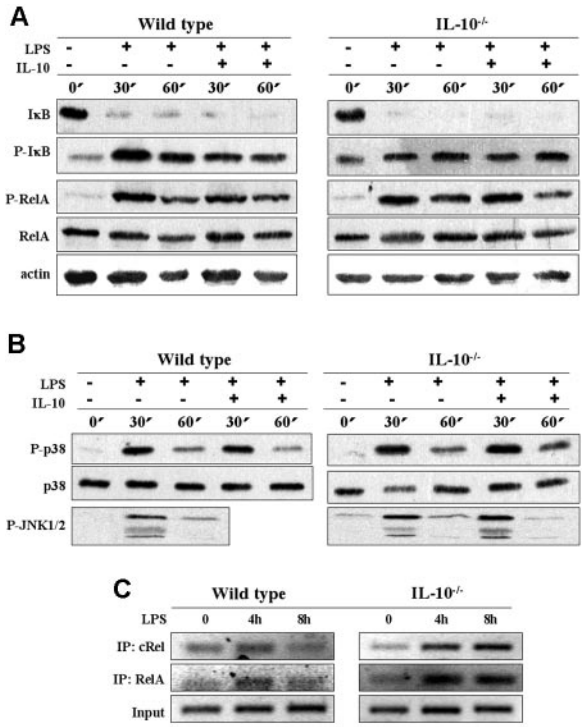


Figure 3. NF-κB signaling and MAP kinase activation in LPS-stimulated BMDCs from WT and IL-10$^{-/-}$ mice. WT and IL-10$^{-/-}$ BMDCs were stimulated with LPS (5 μg/mL) in the presence or absence of IL-10 (10 ng/mL); harvested at 0, 30, and 60 minutes; and Western blot analysis was performed for (A) phospho-IκB (P-IκB) and IκB, phospho-RelA and RelA, and (B) phospho-p38, p38, and phospho-JNK. The results are representative of 4 independent experiments. (C) WT and IL-10$^{-/-}$ BMDCs were stimulated with LPS (5 μg/mL) for 0, 4, and 8 hours. ChIP analysis was performed as described in "Materials and methods." Briefly, DNA was immunoprecipitated (IP) with cRel or RelA antibody, and PCR was performed with primers specific for the IL-12p40 gene promoter. Input samples show equal loading. Results shown are representative of 3 independent experiments.

Enhanced recruitment of cRel to the IL-12p40 promoter in IL-10$^{-/-}$ BMDCs

The recruitment of transcription factors as well as duration of binding to various gene promoters profoundly affects transcriptional activity.[34] cRel played a pivotal role in LPS-induced IL-12p40 transcription in macrophages[31] and in BMDCs derived from cRel$^{-/-}$ p50$^{-/-}$ mice[30] but is dispensable in spleen-derived dendritic cells.[32] Since we observed enhanced cRel nuclear translocation (Figure 2A) in LPS-stimulated BMDCs, we next compared cRel recruitment to the IL-12p40 gene promoter in WT and IL-10$^{-/-}$ BMDCs. As seen in Figure 3C, cRel is strongly recruited to the IL-12p40 promoter in LPS-stimulated IL-10$^{-/-}$ BMDCs, whereas minimal loading is observed in WT cells. Moreover, cRel is still loaded on the IL-12p40 promoter 8 hours after LPS stimulation, whereas no such recruitment is observed in WT BMDCs (Figure 3C). Interestingly, the NF-κB transcriptional subunit RelA is also strongly recruited to the IL-12p40 gene promoter in LPS-stimulated IL-10$^{-/-}$ BMDCs (Figure 3C middle panels). This suggests that enhanced IL-12p40 gene expression in the absence of endogenous IL-10 is likely due to both enhanced binding of NF-κB (cRel/RelA) to the IL-12p40 promoter as well as a defect in terminating gene expression rather than increased proximal NF-κB signaling.

STAT3 is activated by LPS-induced IL-10 in WT but not in IL-10$^{-/-}$ BMDCs

The lack of suppressive effect of IL-10 on LPS-mediated proximal NF-κB signaling, in conjunction with a similar activation profile for various signaling events, suggests that defective negative signaling pathways may be associated with enhanced IL-12p40 gene expression. The STAT3 pathway is required for IL-10–mediated down-regulation of LPS-induced gene expression in monocytes.[35,36] Thus, we investigated STAT3 phosphorylation in LPS-stimulated WT and IL-10$^{-/-}$ BMDCs. Interestingly, STAT3 is clearly phosphorylated between 4 and 14 hours in LPS-stimulated WT but not in IL-10$^{-/-}$ BMDCs (Figure 4A). The absence of phospho-STAT3 in LPS-stimulated IL-10$^{-/-}$ BMDCs is not due to an absence of STAT3 protein, as shown in the right panel of Figure 4A. Moreover, LPS-induced STAT3 phosphorylation in WT BMDCs is blocked by IL-10 receptor Ab but not by control IgG Ab (Figure 4B). This suggests that STAT3 activation is likely due to LPS-induced IL-10 secretion in WT BMDCs.

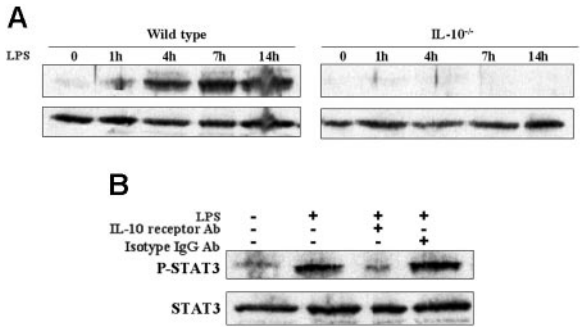
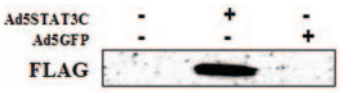


Figure 4. IL-10–dependent LPS-induced STAT3 activation in WT but not IL-10$^{-/-}$ BMDCs. BMDCs from WT and IL-10$^{-/-}$ mice were stimulated with LPS (5 μg/mL) in the presence or absence of IL-10 receptor antibody (30 μg/mL) or an isotype control Ab (30 μg/mL). Subsequently, cells were harvested at various time points and Western blot analysis was performed for (A) phospho-STAT3 and STAT3, (B) phospho-STAT3 and STAT 3 with or without IL-10 receptor blockade. The results are representative of 3 independent experiments.

STAT3 overexpression reduced cRel recruitment to the IL-12p40 gene promoter in IL-10$^{-/-}$ BMDCs

To investigate the impact of STAT3 on LPS-induced IL-12p40 gene expression in IL-10$^{-/-}$ BMDCs, we artificially activated this pathway by delivering a constitutively active STAT3 through adenoviral vector gene delivery (Ad5STAT3C). This adenoviral vector has been shown to trigger STAT3 signaling independently of an exogenous ligand.[24] As seen in Figure 5A, FLAG-tagged STAT3C is highly expressed in Ad5STAT3C-infected BMDCs compared with uninfected or control Ad5GFP-infected cells. Moreover, immunofluorescence staining clearly demonstrated STAT3C expression in more than 75% of Ad5STAT3C-infected but not in uninfected cells (Figure 5B). The pattern of staining revealed a partial nuclear STAT3 localization in infected cells (Figure 5B).

Interestingly, LPS-induced IL-12p40 mRNA accumulation is strongly blocked in Ad5STAT3C-infected but not in Ad5GFP-infected IL-10$^{-/-}$ BMDCs (Figure 6A). Additionally, LPS-induced TNFα mRNA accumulation was unaffected in Ad5STAT3C-infected cells, suggesting a selective effect of the STAT3 signaling pathway on IL-12p40 gene expression. Similarly, Ad5STAT3C but not control Ad5GFP strongly prevented LPS-induced IL-12p40 secretion in BMDCs (Figure 6B). Conversely, we used the selective JAK2 inhibitor AG490 to inhibit the JAK/STAT pathway in WT BMDCs. This blockade increased IL-12p40 secretion by 64% ± 2% ($P < .01$), confirming the importance of STAT3 in regulating IL-12p40 responses (data not shown).



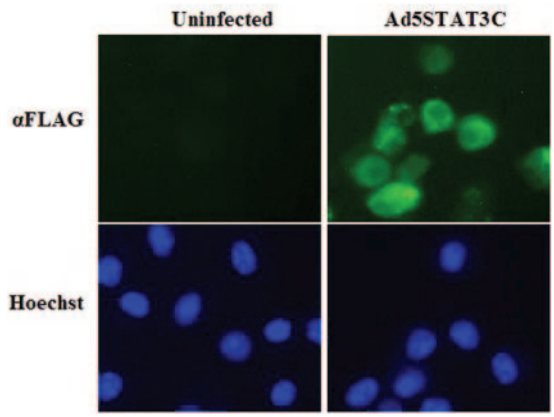


Figure 5. Overexpression of constitutively active STAT3 in IL-10$^{-/-}$ BMDCs. IL-10$^{-/-}$ BMDCs were infected with the constitutively active, FLAG-tagged STAT3 adenovirus (Ad5STAT3C) or Ad5GFP as a negative control. (A) After infection, BMDCs were collected and protein extracts from these cells were subjected to Western blot analysis using FLAG antibody. (B) After infection, cells were fixed with 100% methanol and FLAG expression was detected by immunofluorescence using FLAG antibody, followed by FITC-conjugated goat anti–mouse IgG antibody. The results are representative of 3 independent experiments.

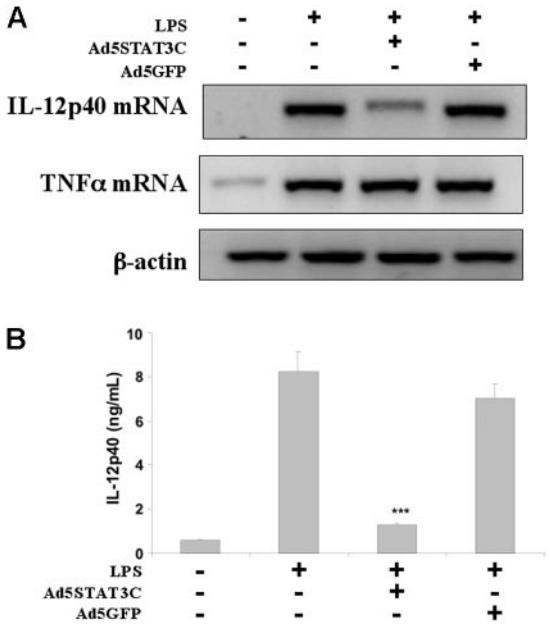


Figure 6. Adenoviral delivery of constitutively active STAT3 in IL-10$^{-/-}$ BMDCs inhibits IL-12p40 mRNA accumulation and protein secretion. IL-10$^{-/-}$ BMDCs were infected with the constitutively active STAT3 adenovirus (Ad5STAT3C) or the control Ad5GFP and subsequently stimulated with LPS (5 μg/mL). (A) Cells were collected at 4 hours, RNA was isolated, and IL-12p40 and TNFα mRNA accumulation was analyzed by RT-PCR. (B) BMDCs were infected as described in Figure 6, stimulated with LPS (5 μg/mL) for 7 hours, and IL-12p40 secretion was measured by ELISA in triplicate supernatants. ***$P < .005$. Results are representative of 2 independent experiments. Error bars represent SEM.

We next investigated whether STAT3 interferes with LPS-induced NF-κB signaling and thus IL-12p40 gene expression. LPS-induced IκB degradation and RelA phosphorylation were not blocked in Ad5STAT3C-infected cells (Figure 7A). This suggests that STAT3 blocks LPS-induced IL-12p40 gene expression independently of the proximal NF-κB signaling cascade. Because NF-κB recruitment to the IL-12p40 gene promoter is increased in IL-10$^{-/-}$ BMDCs (Figure 3C), we hypothesized that STAT3 signaling interferes with recruitment of cRel to this gene promoter. To investigate this possibility, IL-10$^{-/-}$ BMDCs were infected with Ad5STAT3C or Ad5GFP virus, stimulated with LPS for 4 hours, and cRel recruitment to the IL-12p40 gene promoter was analyzed by ChIP assays. As seen in Figure 7B, Ad5STAT3C, but not Ad5GFP, blocked cRel recruitment to the IL-12p40 gene promoter (Figure 7B). Moreover, IL-10 pretreatment inhibited LPS-induced cRel loading to the IL-12p40 gene promoter (Figure 7B). Altogether, these findings indicate that STAT3 controls LPS-induced IL-12p40 gene expression by inhibiting recruitment of NF-κB subunits to the gene promoter, a process absent in IL-10$^{-/-}$ BMDCs.

Discussion

Innate immune responses are induced by the presence of pathogenic and nonpathogenic microorganisms and lead to the activation

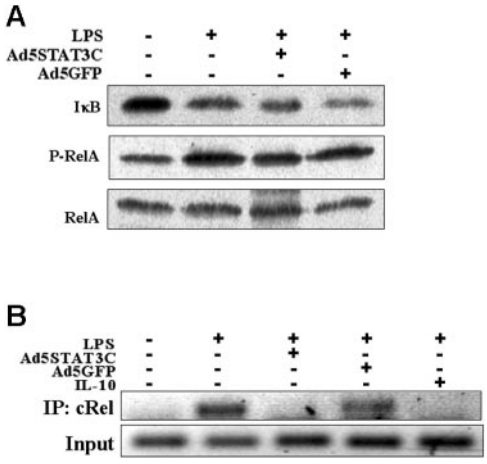


Figure 7. Adenoviral delivery of constitutively active STAT3 in IL-10$^{-/-}$ BMDCs inhibits cRel recruitment to the IL-12p40 gene promoter without affecting the NF-κB pathway. IL-10$^{-/-}$ BMDCs were infected with either Ad5STAT3C or Ad5GFP, or preincubated with IL-10 (10 ng/mL) for 12 hours, and then stimulated with LPS (5 μg/mL) for 1 or 4 hours. (A) Protein extracts from infected BMDCs were collected after 1 hour, and Western blot analysis was performed using antibodies for IκB, phospho-RelA, and RelA. (B) Cells were treated for 4 hours as described and cRel recruitment to the IL-12p40 promoter was analyzed by ChIP, as described in "Materials and methods." The results are representative of 2 independent experiments.

of a complex gene program aimed at re-establishing host homeostasis. Although initiation of innate immunity is a critical feature of host homeostasis, failure to regulate and/or terminate this response can have deleterious consequences for the host. For example, IBDs, which include Crohn disease and ulcerative colitis, are associated with dysregulated innate and adaptive immune responses to luminal nonpathogenic bacteria.[37-40]

The immunosuppressive cytokine IL-10 exerts numerous immunoregulatory functions and plays a pivotal role in maintaining intestinal homeostasis and controlling innate responses.[39] This is shown in IL-10$^{-/-}$ mice that develop spontaneous intestinal inflammation when housed under SPF conditions.[1,3] However, these mice remain healthy and disease free when born and raised under gnotobiotic conditions, suggesting that IL-10 is involved in regulating innate host responses to the luminal intestinal flora.[1] Despite numerous attempts, the molecular mechanisms of IL-10–mediated regulation of innate immune responses have not been clearly elucidated.

In this study, we investigated the molecular mechanism of dysregulated innate responses in BMDCs isolated from IL-10$^{-/-}$ mice. We report that LPS induced a stronger IL-12p40 and TNFα gene expression in BMDCs derived from IL-10$^{-/-}$ compared with WT mice. This indicates that in the absence of endogenous IL-10, LPS responsiveness is enhanced in BMDCs, leading to higher IL-12p40 and TNFα gene expression. Importantly, administration of neutralizing IL-12 antibody prevents the early onset of colitis in IL-10$^{-/-}$ mice.[5] Thus, our finding that LPS induced higher IL-12p40 gene expression in BMDCs isolated from IL-10$^{-/-}$ mice correlates with the key role for IL-12p40 in this model of experimental colitis.

LPS-induced IL-12p40 gene expression is regulated by various signaling cascades and transcription factors.[31,41,42] Among them, the NF-κB transcriptional system has been shown to play a preponderant role in IL-12p40 gene expression. In an effort to understand the dysregulated IL-12p40 gene expression in IL-10$^{-/-}$ BMDCs, we carefully investigated LPS signal transduction in both WT and IL-10$^{-/-}$ mice. We found that LPS-induced IL-12p40 gene expression is strongly inhibited in BMDCs expressing an IκB superrepressor, showing the critical role for NF-κB in regulating this cytokine in BMDCs. The negative effect of IL-10 on NF-κB signaling is controversial and may reflect cell type specificity. Interestingly, a new report showed that IL-10 blocked LPS-induced I κB kinase (IKK) activity and RelA phosphorylation in BMDCs.[14] Surprisingly, using similar DCs, we found that LPS-induced proximal NF-κB signaling is similar between WT and IL-10$^{-/-}$ BMDCs, whereas IL-12p40 gene expression is stronger in IL-10$^{-/-}$ cells. Indeed, levels of IκBα degradation/phosphorylation, RelA phosphorylation, and NF-κB transcriptional activity were comparable between LPS-stimulated WT and IL-10$^{-/-}$ cells in our study. Thus, although NF-κB activity is essential for LPS-induced IL-12p40 gene expression, this signaling cascade is not excessively activated in IL-10$^{-/-}$ BMDCs. The discrepancy between results in these studies may be related to the supraphysiologic dose of IL-10 used (50 ng/mL) by Bhattacharyya et al.[14] For example, we found that low amounts of exogenous IL-10 in the physiologic range (1 ng/mL compared with 0.7 ng/mL secreted by LPS-induced BMDCs; Figure 1B) totally blocked LPS-induced IL-12p40 and TNF secretion without inhibiting NF-κB activity (not shown in present study). In addition, our study used cells lacking endogenous IL-10, which allowed physiologic analysis of gene expression in the absence of the endogenous immunoregulatory IL-10 molecule. Also LPS-induced JNK and p38 phosphorylation is similar in WT and IL-10$^{-/-}$ BMDCs, suggesting that LPS-induced TLR4 signal transduction is not impaired in either WT or IL-10$^{-/-}$ BMDCs.

IL-10 has been shown to directly inhibit NF-κB activity through transient blockade of IκB degradation and IKK activity as well as impaired NF-κB DNA-binding activity in LPS-stimulated monocyte cell lines.[13] Interestingly, we found no evidence of IL-10–mediated inhibition of IκB degradation and phosphorylation, suggesting that this immunosuppressive cytokine acts through a different mechanism in murine BMDCs. This is in agreement with previous studies showing no effect of IL-10 on NF-κB activation[43] or MAP kinases[44] in human macrophages. This data suggests that the positive signaling cascade leading to increased IL-12p40 gene expression is not dysregulated in IL-10$^{-/-}$ BMDCs. Thus, impaired activation of inhibitory signaling cascades in IL-10$^{-/-}$ cells may lead to dysregulated innate host responses in these mice.

Persistent and sustained recruitment of transcription factors to selective gene promoters is responsible for prolonged gene expression. Indeed, kinetics of NF-κB–dependent gene transcription directly correlates with the extent and duration of recruitment of various subunits to gene promoters.[34] Of considerable interest, we showed for the first time enhanced recruitment of cRel to the IL-12p40 promoter in LPS-stimulated IL-10$^{-/-}$ BMDCs but not WT cells. Moreover, cRel was still associated with the IL-12p40 promoter at 8 hours following LPS stimulation in IL-10$^{-/-}$ BMDCs, whereas no such binding was observed in WT BMDCs. Thus, although proximal NF-κB signaling is similar between IL-10$^{-/-}$ and WT BMDCs, recruitment of NF-κB to the IL-12p40 gene promoter is clearly different. Therefore, both enhanced initial binding of cRel and failure to remove NF-κB from the IL-12p40 promoter rather than excessive proximal signaling are responsible for enhanced gene expression.

Negative regulators of LPS signaling play a pivotal role in controlling innate responses in numerous immune cells.[39] Interestingly, we found that STAT3, a negative regulator of LPS signaling, is strongly phosphorylated in endotoxin-stimulated WT but not in

IL-10$^{-/-}$ BMDCs. This prolonged and strong STAT3 phosphorylation in WT BMDCs is likely due to increased IL-10 production. First, LPS induced both IL-10 gene expression and strong STAT3 phosphorylation in WT but not in IL-10$^{-/-}$ BMDCs. Second, IL-10 receptor blocking antibody prevented LPS-induced STAT3 phosphorylation in WT BMDCs. Thus, STAT3 phosphorylation is mediated by LPS-induced IL-10 in WT BMDCs. The lack of STAT3 phosphorylation in IL-10$^{-/-}$ cells is functionally linked to enhanced IL-12p40 gene expression. This is clearly illustrated in the experiment where we delivered a constitutively active STAT3 in IL-10$^{-/-}$ BMDCs using an adenoviral vector. Using this approach, we demonstrated that LPS-induced IL-12p40 but not TNFα gene expression is strongly inhibited in IL-10$^{-/-}$ BMDCs. Conversely, blocking JAK/STAT signaling in WT BMDCs enhanced LPS-induced IL-12p40 protein secretion. Thus, STAT3 has a critical role in down-regulating LPS-induced IL-12p40 gene expression. This is a selective inhibition so this mechanism is not responsible for suppressing other proinflammatory cytokines induced by LPS, such as TNFα.

In this study, we provide clear evidence that the STAT3 pathway controlled cRel recruitment to the IL-12p40 gene promoter, thereby providing a mean to selectively terminate gene transcription. First, we showed that Ad5STAT3C prevents cRel loading to the IL-12p40 gene promoter without interfering with LPS-induced proximal NF-κB signaling. Second, IL-10–mediated blockade of LPS-induced IL-12p40 gene expression occurs independently of proximal NF-κB signaling but rather involves decreased cRel recruitment to the gene promoter. How can STAT3 activation prevent NF-κB recruitment to the IL-12p40 gene promoter independently of proximal signal transduction? One possible scenario is that activated STAT3 migrates to the nucleus and interferes with a molecule involved in the regulation of IL-12p40 gene expression. This would be consistent with the partial nuclear localization of activated STAT3 in Ad5STAT3C-infected IL-10$^{-/-}$ BMDCs. Interestingly, STAT3 directly interacted with RelA and suppressed IL-1β and LPS/interferon-γ–induced inducible nitric oxide synthase (iNOS) gene expression in mesangial cells.[45] However, using coimmunoprecipitation analysis, we were unable to detect STAT3/NF-κB interaction in WT or IL-10$^{-/-}$ BMDCs. Another potential mechanism could involve STAT3 signaling interference with chromatin remodeling through alteration of histone acetylation and/or phosphorylation. Nucleosome remodeling of nucleosome 1, which contains the Rel and CCAAT/enhancer-binding protein (C/EBP) *cis*-elements, is a critical event for signal-induced IL-12p40 gene expression.[46] We have previously reported that the transforming growth factor β (TGFβ)/Sma- and Mad-related protein (Smad) pathway blocked LPS-induced histone phosphorylation/acetylation in intestinal epithelial cells.[26] It is interesting to speculate that IL-10 activates an event through STAT3 that leads to impaired chromatin remodeling and decreased recruitment of essential transcription factors such as cRel and C/EPB. However, Zhou et al[47] demonstrated that IL-10 induced only slight changes in chromatin remodeling and C/EPB recruitment to the IL-12p40 promoter in LPS-stimulated peritoneal macrophages. Moreover, IL-10 failed to block LPS-induced histone-3 phosphorylation (Ser10) in the present study (data not shown). Whether other histone modifications are affected by IL-10 is currently under investigation. The impact of STAT3 signaling events on chromatin remodeling in BMDCs remains to be determined. Nevertheless, activation of the IL-10/STAT3 pathway likely controls the initial recruitment of transcription factors (cRel, RelA, and probably others) and/or stability of protein/DNA interaction, which ultimately dictate the amount of IL-12 being transcribed by BMDCs.

It is interesting to note that STAT3 blocks LPS-induced IL-12p40 but not TNFα gene expression. Such a specific effect has been previously reported for Bcl3 that is required for inhibition of LPS-induced TNFα but not IL-6 secretion in macrophages.[18] Moreover, IL-10–induced NF-κB p50 DNA-binding activity is responsible for blockade of TNFα-induced IL-6 and macrophage inflammatory protein 2α (MIP2α) gene expression[48] in peritoneal macrophages. Thus, the molecular mechanism of IL-10–mediated gene inhibition is diverse and cell type specific. This also indicates that STAT3 selectively altered a critical component of the transcriptional machinery involved in IL-12p40 expression.

The recently discovered cytokine IL-23 consists of the IL-12p40 and p19 subunits and is secreted by immune cells including DCs.[6] This cytokine has the ability to skew T-cell differentiation toward a Th-1 profile. Interestingly, IL-23p19 mRNA levels are increased in experimental colitis[49] and in Crohn disease patients.[50] Moreover, mice overexpressing IL-23p19 develop multiple organ failure and systemic inflammation and die before 3 months of age.[51] Since LPS-induced IL-12p40 gene expression is reduced by Ad5STAT3C, the decreased production of this subunit may not only impair IL-12 secretion but also reduce the production of IL-23 in BMDCs. Further studies will be necessary to understand the impact of STAT3 and IL-23 expression on inflammatory diseases.

Our study highlights the complex network of regulatory cascades involved in controlling LPS-induced signaling in immune cells. Clearly, malfunction in these regulatory pathways impairs host homeostasis. First, tissue-specific disruption of STAT3 in bone marrow cells during hematopoiesis led to overly activated innate immune responses and Crohn disease–like intestinal inflammation.[21] The in vivo relevance is shown by the severe intestinal inflammation caused by deletion of STAT3[20,21] or IL-10.[2] Conversely, intestinal bacteria[52] and regulatory T cells[53] engineered to produce IL-10 prevent and treat inflammation in murine models of experimental colitis.

In conclusion, we show that increased LPS-induced IL-12p40 gene expression in IL-10$^{-/-}$ BMDCs involves dysregulated recruitment of cRel to the IL-12p40 promoter due to defective STAT3 activation. We propose a model where LPS-induced IL-10 production leads to the activation of STAT3 and inhibition of IL-12p40 gene expression by preventing NF-κB access to the gene promoter. This negative feedback mechanism is essential for the attenuation of LPS-induced innate responses in immune cells. These data provide a potential molecular mechanism for inflammation in IL-10$^{-/-}$ mice. Therefore, enhancing STAT3 signaling may represent a potential therapeutic means to manipulating the innate immune system and treating intestinal inflammation.

Acknowledgments

The authors wish to thank Brigitte Allard for technical expertise, and Charlotte Walters of the ImmunoTechnology Core of the Center for Gastrointestinal Biology and Disease, University of North Carolina at Chapel Hill, for cytokine measurements, and Desmond McDonald, Lisa Wiltron and Sue Tonkonogy of the College of Veterinary Medicine, North Carolina State University, Raleigh, for their expert technical support. We also thank Vasiliki Anest for technical advice on the ChIP assay, and Dr Kaikobad Irani for providing the STAT3C adenovirus.

References

1. Sellon RK, Tonkonogy S, Schultz M, et al. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. Infect Immun. 1998;66:5224-5231.
2. Berg DJ, Davidson N, Kuhn R, et al. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. J Clin Invest. 1996;98:1010-1020.
3. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. Cell. 1993;75:263-274.
4. Davidson NJ, Hudak SA, Lesley RE, Menon S, Leach MW, Rennick DM. IL-12, but not IFN-gamma, plays a major role in sustaining the chronic phase of colitis in IL-10-deficient mice. J Immunol. 1998;161:3143-3149.
5. Spencer DM, Veldman GM, Banerjee S, Willis J, Levine AD. Distinct inflammatory mechanisms mediate early versus late colitis in mice. Gastroenterology. 2002;122:94-105.
6. Becker C, Wirtz S, Blessing M, et al. Constitutive p40 promoter activation and IL-23 production in the terminal ileum mediated by dendritic cells. J Clin Invest. 2003;112:693-706.
7. Trinchieri G, Pflanz S, Kastelein RA. The IL-12 family of heterodimeric cytokines: new players in the regulation of T cell responses. Immunity. 2003;19:641-644.
8. Bouma G, Strober W. The immunological and genetic basis of inflammatory bowel disease. Nat Rev Immunol. 2003;3:521-533.
9. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol. 2003;3:133-146
10. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol. 2001;19:683-765.
11. Lee TS, Chau LY. Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. Nat Med. 2002;8:240-246.
12. Wang P, Wu P, Siegel MI, Egan RW, Billah MM. Interleukin (IL)-10 inhibits nuclear factor kappa B (NF kappa B) activation in human monocytes: IL-10 and IL-4 suppress cytokine synthesis by different mechanisms. J Biol Chem. 1995;270:9558-9563.
13. Schottelius AJ, Mayo MW, Sartor RB, Baldwin AS Jr. Interleukin-10 signaling blocks inhibitor of kappaB kinase activity and nuclear factor kappaB DNA binding. J Biol Chem. 1999;274:31868-31874.
14. Bhattacharyya S, Sen P, Wallet M, Long B, Baldwin AS, Tisch R. Immunoregulation of dendritic cells by IL-10 is mediated through suppression of the PI3K/Akt pathway and IκB kinase activity. Blood. 2004;104:1100-1109.
15. Kontoyiannis D, Kotlyarov A, Carballo E, et al. Interleukin-10 targets p38 MAPK to modulate ARE-dependent TNF mRNA translation and limit intestinal pathology. EMBO J. 2001;20:3760-3770.
16. Brown CY, Lagnado CA, Vadas MA, Goodall GJ. Differential regulation of the stability of cytokine mRNAs in lipopolysaccharide-activated blood monocytes in response to interleukin-10. J Biol Chem. 1996;271:20108-20112.
17. Williams L, Bradley L, Smith A, Foxwell B. Signal transducer and activator of transcription 3 is the dominant mediator of the anti-inflammatory effects of IL-10 in human macrophages. J Immunol. 2004;172:567-576.
18. Kuwata H, Watanabe Y, Miyoshi H, et al. IL-10-inducible Bcl-3 negatively regulates LPS-induced TNF-alpha production in macrophages. Blood. 2003;102:4123-4129.
19. Takeda K, Noguchi K, Shi W, et al. Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. Proc Natl Acad Sci U S A. 1997;94:3801-3804.
20. Kobayashi M, Kweon MN, Kuwata H, et al. Toll-like receptor-dependent production of IL-12p40 causes chronic enterocolitis in myeloid cell-specific Stat3-deficient mice. J Clin Invest. 2003;111:1297-1308.
21. Welte T, Zhang SS, Wang T, et al. STAT3 deletion during hematopoiesis causes Crohn's disease-like pathogenesis and lethality: a critical role of STAT3 in innate immunity. Proc Natl Acad Sci U S A. 2003;100:1879-1884.
22. Cheng F, Wang HW, Cuenca A, et al. A critical role for Stat3 signaling in immune tolerance. Immunity. 2003;19:425-436.
23. Alonzi T, Newton IP, Bryce PJ, et al. Induced somatic inactivation of STAT3 in mice triggers the development of a fulminant form of enterocolitis. Cytokine. 2004;26:45-56.
24. Haga S, Terui K, Zhang HQ, et al. Stat3 protects against Fas-induced liver injury by redox-dependent and -independent mechanisms. J Clin Invest. 2003;112:989-998.
25. Jobin C, Panja A, Hellerbrand C, et al. Inhibition of proinflammatory molecule production by adenovirus-mediated expression of a nuclear factor kappaB super-repressor in human intestinal epithelial cells. J Immunol. 1998;160:410-418.
26. Haller D, Holt L, Kim SC, Schwabe RF, Sartor RB, Jobin C. Transforming growth factor-beta 1 inhibits non-pathogenic Gram negative bacteria-induced NF-kappa B recruitment to the interleukin-6 gene promoter in intestinal epithelial cells through modulation of histone acetylation. J Biol Chem. 2003;278:23851-23860.
27. Haller D, Russo MP, Sartor RB, Jobin C. IKK beta and phosphatidylinositol 3-kinase/Akt participate in non-pathogenic Gram-negative enteric bacteria-induced RelA phosphorylation and NF-kappa B activation in both primary and intestinal epithelial cell lines. J Biol Chem. 2002;277:38168-38178.
28. Jobin C, Haskill S, Mayer L, Panja A, Sartor RB. Evidence for altered regulation of I kappa B alpha degradation in human colonic epithelial cells. J Immunol. 1997;158:226-234.
29. Liu J, Beller DI. Distinct pathways for NF-kappa B regulation are associated with aberrant macrophage IL-12 production in lupus- and diabetes-prone mouse strains. J Immunol. 2003;170:4489-4496.
30. Ouaaz F, Arron J, Zheng Y, Choi Y, Beg AA. Dendritic cell development and survival require distinct NF-kappaB subunits. Immunity. 2002;16:257-270.
31. Sanjabi S, Hoffmann A, Liou HC, Baltimore D, Smale ST. Selective requirement for c-Rel during IL-12 P40 gene induction in macrophages. Proc Natl Acad Sci U S A. 2000;97:12705-12710.
32. Grumont R, Hochrein H, O'Keeffe M, et al. c-Rel regulates interleukin 12 p70 expression in CD8(+) dendritic cells by specifically inducing p35 gene transcription. J Exp Med. 2001;194:1021-1032.
33. Guha M, Mackman N. LPS induction of gene expression in human monocytes. Cell Signal. 2001;13:85-94.
34. Saccani S, Pantano S, Natoli G. Modulation of NF-kappaB activity by exchange of dimers. Mol Cell. 2003;11:1563-1574.
35. Takeda K, Clausen BE, Kaisho T, et al. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. Immunity. 1999;10:39-49.
36. Riley JK, Takeda K, Akira S, Schreiber RD. Interleukin-10 receptor signaling through the JAK-STAT pathway: requirement for two distinct receptor-derived signals for anti-inflammatory action. J Biol Chem. 1999;274:16513-16521.
37. Sartor RB. Innate immunity in the pathogenesis and therapy of IBD. J Gastroenterol. 2003;38(suppl 15):43-47.
38. Sartor RB. Animal models of intestinal inflammation. In: Kirsner's Inflammatory Bowel Diseases. 6th ed. New York, NY: Elsevier; 2004:120-137.
39. Haller D, Jobin C. Interaction between resident luminal bacteria and the host: can a healthy relationship turn sour? J Pediatr Gastroenterol Nutr. 2004;38:123-136.
40. Strober W, Fuss IJ, Blumberg RS. The immunology of mucosal models of inflammation. Annu Rev Immunol. 2002;20:495-549.
41. Murphy TL, Cleveland MG, Kulesza P, Magram J, Murphy KM. Regulation of interleukin 12 p40 expression through an NF-kappa B half-site. Mol Cell Biol. 1995;15:5258-5267.
42. Plevy SE, Gemberling JH, Hsu S, Dorner AJ, Smale ST. Multiple control elements mediate activation of the murine and human interleukin 12 p40 promoters: evidence of functional synergy between C/EBP and Rel proteins. Mol Cell Biol. 1997;17:4572-4588.
43. Denys A, Udalova IA, Smith C, et al. Evidence for a dual mechanism for IL-10 suppression of TNF-alpha production that does not involve inhibition of p38 mitogen-activated protein kinase or NF-kappa B in primary human macrophages. J Immunol. 2002;168:4837-4845.
44. Donnelly RP, Dickensheets H, Finbloom DS. The interleukin-10 signal transduction pathway and regulation of gene expression in mononuclear phagocytes. J Interferon Cytokine Res. 1999;19:563-573.
45. Yu Z, Zhang W, Kone BC. Signal transducers and activators of transcription 3 (STAT3) inhibits transcription of the inducible nitric oxide synthase gene by interacting with nuclear factor kappaB. Biochem J. 2002;367:97-105.
46. Weinmann AS, Mitchell DM, Sanjabi S, et al. Nucleosome remodeling at the IL-12 p40 promoter is a TLR-dependent, Rel-independent event. Nat Immunol. 2001;2:51-57.
47. Zhou L, Nazarian AA, Smale ST. Interleukin-10 inhibits interleukin-12 p40 gene transcription by targeting a late event in the activation pathway. Mol Cell Biol. 2004;24:2385-2396.
48. Driessler F, Venstrom K, Sabat R, Asadullah K, Schottelius AJ. Molecular mechanisms of interleukin-10-mediated inhibition of NF-kappaB activity: a role for p50. Clin Exp Immunol. 2004;135:64-73.
49. Krajina T, Leithauser F, Moller P, Trobonjaca Z, Reimann J. Colonic lamina propria dendritic cells in mice with CD4+ T cell-induced colitis. Eur J Immunol. 2003;33:1073-1083.
50. Stallmach A, Giese T, Schmidt C, Ludwig B, Mueller-Molaian I, Meuer SC. Cytokine/chemokine transcript profiles reflect mucosal inflammation in Crohn's disease. Int J Colorectal Dis. 2003;19:308-315.
51. Wiekowski MT, Leach MW, Evans EW, et al. Ubiquitous transgenic expression of the IL-23 subunit p19 induces multiorgan inflammation, runting, infertility, and premature death. J Immunol. 2001;166:7563-7570.
52. Steidler L, Hans W, Schotte L, et al. Treatment of murine colitis by Lactococcus lactis secreting interleukin-10. Science. 2000;289:1352-1355.
53. Van Montfrans C, Rodriguez Pena MS, Pronk I, Ten Kate FJ, Te Velde AA, Van Deventer SJ. Prevention of colitis by interleukin 10-transduced T lymphocytes in the SCID mice transfer model. Gastroenterology. 2002;123:1865-1876.