Transforming Growth Factor-β1 Inhibits Non-pathogenic Gram-negative Bacteria-induced NF-κB Recruitment to the Interleukin-6 Gene Promoter in Intestinal Epithelial Cells through Modulation of Histone Acetylation*

Dirk Haller, Lisa Holt, Sandra C. Kim, Robert F. Schwabe, R. Balfour Sartor, and Christian Jobin‡

From the Departments of Medicine, Microbiology and Immunology and the Center for Gastrointestinal Biology and Disease, University of North Carolina, Chapel Hill, North Carolina 27599

We have shown that non-pathogenic enteric Gram-negative Bacteroides vulgatus induces RelA phosphorylation, NF-κB activation, and proinflammatory gene expression in primary and intestinal epithelial cell (IEC) lines. We now demonstrate the transient induction of nuclear phospho-RelA (day 3) followed by persistent activation of phospho-Smad2 (days 3 and 7) in IEC from mucosal tissue sections of B. vulgatus-monoassociated rats, indicating that both NF-κB and transforming growth factor-β1 (TGF-β1) signaling are induced in vivo following bacterial colonization. Interestingly, TGF-β1 inhibited B. vulgatus- and lipopolysaccharide (LPS)-induced NF-κB transcriptional activity as well as interleukin-6 (IL-6) mRNA accumulation and protein secretion in IEC. The inhibitory effect of TGF-β1 is mediated independently of B. vulgatus/LPS-induced IκBα, Akt, and RelA phosphorylation as well as NF-κB DNA binding activity. Moreover, the specific histone deacetylase inhibitor trichostatin A blocked B. vulgatus/LPS-induced histone acetylation/phosphorylation (Lys-9/Ser-10) and reversed TGF-β1-mediated inhibition of IL-6 gene expression. Chromatin immunoprecipitation analysis revealed that B. vulgatus/LPS-induced RelA recruitment to the IL-6 promoter is inhibited by TGF-β1 treatment. Adenoviral delivery of Smad7 and dominant negative Smad3 (Smad3D) reversed the TGF-β1-mediated inhibition of NF-κB transcriptional activity and NF-κB recruitment to the IL-6 promoter. In addition, TGF-β1 and Ad5Smad3/4 prevent B. vulgatus/LPS-induced CBP/p300 and p65 nuclear co-association. We concluded that the TGF-β1/Smad signaling pathway helps maintain normal intestinal homeostasis to commensal luminal enteric bacteria by regulating NF-κB signaling in IEC through altered histone acetylation.

Various enteroinvasive and pathogenic bacteria induce a complex profile of proinflammatory genes in intestinal epithe-

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† To whom correspondence should be addressed: Division of Digestive Disease and Nutrition, CB 7038, Glaxo Bldg., University of North Carolina, Chapel Hill, NC 27599-7080. Tel.: 919-966-7884; Fax: 919-966-7468; E-mail: jcb@med.unc.edu.

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The abbreviations used are: IEC, intestinal epithelial cells; NF-κB, nuclear transcription factor κB; TGF-β1, transforming growth factor-β1; ChIP, chromatin immunoprecipitation; IBD, inflammatory bowel disease; MAPK, mitogen-activated protein kinases; IL, interleukin; HDAC, histone deacetylase; LPS, lipopolysaccharide; m.o.i., multiplicity of infection; HA, hemagglutinin; Ab, antibody; HAT, histone acetyltransferase; ELISA, enzyme-linked immunosorbent assay; cfu, colony-forming units; GST, glutathione S-transferase; GFP, green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TSA, trichostatin A.
it accumulates. Simultaneously, activation of nuclear signaling proteins permits access to gene promoter regions (11). In the resting state, transcriptionally inactive chromatin is tightly wrapped around histone proteins that hinder DNA binding by transcriptional factors, providing an additional complex layer of gene regulation. Histone wrapping to DNA and consequently accessibility of transcription factors to gene promoter elements is regulated by post-translational modifying mechanisms, such as histone acetylation, phosphorylation, and methylation. Signaling cascades responsible for the activation of transcription factors must act synchronously with the different pathways leading to chromatin unfolding in order to achieve coordinate gene expression. For example, signaling cascades that impact the levels of histone acetylation strongly influence chromatin remodeling and gene expression (11–13). The status of histone acetylation is dictated by the opposite action of the two enzymes histone acetyltransferase (HAT), which catalyzes acetylation of lysine residues present at the N-terminal part of the histone tail, and the histone deacetyltransferase (HDAC), which removes acetyl groups. Indeed, NF-κB binding to transcriptional active chromatin is influenced by the status of histone acetylation/phosphorylation (14–16). Whether the level of histone acetylation impacts bacteria-induced NF-κB-dependent gene expression in IEC is currently unknown.

In vitro, the response of IEC to commensal bacteria is regulated by adjacent lamina propria mononuclear cells, which mediate immunologic tolerance and maintain mucosal homeostasis (17). Among the anti-inflammatory molecules implicated in the regulation of intestinal homeostasis, transforming growth factor (TGF)-β1 has been shown to play a key role in controlling proliferation, differentiation, and function of numerous immune and non-immune cells (18–20). The biological effect of TGF-β1 is mediated through activation of various signaling cascades such as Smad and the mitogen-activated protein kinases, the latter including extracellular signal-regulated kinases, stress-activated protein kinases/c-Jun N-terminal kinases, and the p38 pathway. Upon TGF-β1 binding to the receptor complex, the Smad signaling pathway is activated through phosphorylation of the receptor activated Smad1–3 (R-Smads), which dissociate from the receptor complex and associate with the common partner Smad4 (co-Smad4). Resulting heterodimers then translocate to the nucleus and interact directly with Smad-binding elements on the DNA to induce target gene transcription (21–24). Another important aspect of TGF-β signal transduction is the induction/activity of inhibitory molecules such as Smad6 and Smad7, which act as negative regulators of TGF-β1/Smad signaling by interfering with either the activation of R-Smads or the association of R-Smads with the common partner Smad4 (co-Smad4) (25).

Although TGF-β1 regulates the host immune response to various micro-organisms and inflammatory mediators (26, 27), the molecular mechanisms remain to be elucidated. In this study, we characterized TGF-β1 signal transduction in IEC and investigated its effect on bacteria-induced NF-κB signaling. We report that TGF-β1-activated Smad signaling mediates inhibition of B. vulgatus and LPS-induced NF-κB recruitment to the IL-6 gene promoter through modulation of histone acetylation. Activation of this inhibitory pathway may be important in down-regulating IEC responses to commensal bacteria.

MATERIALS AND METHODS

Cell Culture and Bacterial Stimulation of IEC—The mouse IEC line CMT93 (passage 10–30) (ATCC CRL 223, American Type Culture Collection (ATCC), Manassas, VA) was grown to confluency in 12- or 6-well plates (Nunc). B. vulgatus derived from a guinea pig with caggasan-induced colitis (a gift from Dr. A. B. Onderdonk, Harvard University, Cambridge, MA) was anaerobically grown at 37 °C in brain-heart infusion, and B. vulgatus lysates were prepared as described previously (3). Confluent monolayers of IEC were stimulated with B. vulgatus (5 × 10⁴ cfu/ml), B. vulgatus lysate (100 µg of protein/ml), or LPS (10 µg/ml; from E. coli serotype O111:B4, Sigma). Where indicated IEC were treated with TGF-β1 (20 ng/ml, R & D Systems) and the specific histone deacetylase (HDAC) inhibitor trichostatin A (TSA) (2 h pretreatment with 1 µg/ml, Sigma).

Immunohistochemistry and Isolation of Primary Intestinal Epithelial Cells—Germ-free (sterile) Fisher F344 rats were monoassociated at 10–12 weeks of age with B. vulgatus and maintained in the Gnotobiotic Animal Core at the College of Veterinary Medicine, North Carolina State University, Raleigh. Bacterial monoassociation was confirmed by anaerobic culture of stool samples. Rats were killed at day 3 and 7 after initial bacterial colonization. Sections of the ileum, maximal and distal colon were fixed in 10% neutral buffered formalin. The fixed tissue was embedded in paraffin. Immunohistochemistry was performed using anti-phospho-NF-κB p65 (RelA), anti-phospho-Smad2, anti-phospho-c-Jun, and anti-phospho-p38 Abs (Cell Signaling, Beverly, MA) according to the protocol of the manufacturer, and sections were counterstained with hematoxylin. Histology scoring was analyzed by scoring the degree of lamina propria mononuclear cell infiltration, crypt hyperplasia, goblet cell depletion, and architectural distortion as described previously (8).

Adenoviral Infection—CMT93 cells were infected overnight with Ad5αB-LUC as described previously (3). Where indicated CMT93 were co-infected for an additional 12 h with Ad5IκBα (a generous gift from Dr. David A. Brenner, University of North Carolina, Chapel Hill, NC) or Ad5Smad3ΔC or Ad5Smad3Ad5Smad4 (a generous gift of Dr. X. F. Wang, Duke University, Durham, NC) at a multiplicity of infection (m.o.i.) of 50. The Smad3ΔC, Smad3, and Smad4 constructs were described previously by Wu et al. (28) and Zhang et al. (29). The Ad5GFP containing green fluorescent protein was used as a viral negative control. The adenoviruses were washed off, and fresh medium containing serum was added. Cells were stimulated at various time points with B. vulgatus (5 × 10⁴ cfu/ml), B. vulgatus lysate (100 µg/ml), or LPS (10 µg/ml).

Transfection and Reporter Gene Assay—CMT93 cells were transfected with plasmid expressing 3TP-LUX (1 µg, a generous gift of Dr. Richard A. Rippe, University of North Carolina, Chapel Hill) using LipofectAMINE Reagent (Invitrogen) as described previously (3). Cells were then stimulated with TGF-β1 (20 ng/ml) for 12 h. Cell extracts were prepared using enhanced luciferase assay reagents (Analytical Luminescence, San Diego). Luciferase assay were performed on a Monolight 2010 luminometer for 20 s (Analytical Luminescence, San Diego), and results were normalized for extract protein concentrations measured with the Bio-Rad protein assay kit (Bio-Rad).

Chromatin Immunoprecipitation (ChIP) Analysis—CMT93 cells were stimulated with LPS for 0–2 h. After stimulation, IEC were washed in cold phosphate-buffered saline (1x) and fixed by adding formaldehyde to a final concentration of 1%. Nuclear and chromatin immunoprecipitation were performed as described previously (16). Cells were lysed after formaldehyde fixation in L1 lysis buffer (50 mM Tris, pH 8.0, 2 mM EDTA, 0.1% Nonidet P-40, and 10% glycerol) supplemented with protease inhibitors. Nuclei were pelleted and resuspended in 300 µl of L2 lysis buffer (50 mM Tris, pH 8.0, 0.1% SDS, and 5 mM EDTA). Chromatin was sheared by sonication (4 times for 15 s at about one-fifth of maximum power), centrifuged, and diluted in dilution buffer (50 mM Tris, pH 8.0, 0.5% Nonidet P-40, 0.2 µM NaCl, and 0.5 mM EDTA). Extracts were normalized according to their DNA concentration and were pre-cleared for 3 h with salmon sperm-saturated protein A/G-agarose. Immunoprecipitation were carried out overnight with 10 µg of anti-p65 Ab (Rockland, Gilbertsville, PA). Immune complexes were collected with salmon sperm-saturated protein A/G-agarose for 30 min and washed three times in high salt buffer (20 mM Tris, pH 8.0, 0.1% SDS, 0.5 mM NaCl, 1% Nonidet P-40, 2 mM EDTA) followed by three washes with no salt buffer (TE 1x). Immune complexes were extracted 3 times with 150 µl of extraction buffer (1% sodium deoxycholate, 0.5% sodium dodecyl sulfate, 0.1% Triton X-100, and 0.1% SDS). DNA cross-links were reverted by heating for 4 h at 65 °C. After protease K (100 µg for 1 h) 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 (1 µl, input control) and immunoprecipitated DNA (1 µl) using the following IL-6 promoter-specific primers: 5′-GGACGAGATTGCACAATGTGACGTCG-3′ (position 1112–1132). The length of the amplified product was 125 bp. The PCR products (10 µl) were subjected to electrophoresis on 2% agarose gels containing gel Star
In Vitro Kinase Assays—IKK activity on B. vulgatus-induced serine RelA phosphorylation was determined by immunocomplex kinase assay as described previously (3). Briefly, CMT93 cells were lysed in Triton x-100 buffer containing protease and phosphatase inhibitors after stimulation with B. vulgatus/LPS at various times in the presence or absence of TGF-β1. 300 μg was immunoprecipitated with 2 μl of anti-IKKγ (Cell Signaling, Beverly, MA), and the kinase reaction was performed by glutathione S-transferase (GST)-p65 (1–305 and 354–551) (a generous gift of Dr. Hiroaki Sakurai, Tanabe Seiyaku, Osaka, Japan) as substrate for 30 min at 30 °C. Substrate protein was resolved by gel electrophoresis, and phosphate incorporation was assessed by autoradiography and PhosphoImager analysis (Amersham Biosciences).

RNA Extraction and Reverse Transcriptase-PCR Analysis—RNA was isolated using Trizol (Invitrogen), and 1 μg of total RNA was reverse-transcribed and amplified (reverse transcriptase PCR) using specific primers for mouse IL-6 and GAPDH as described previously (3). The following oligonucleotide primers were used: IL-6-A, (5′) 5-AAGTAAGTTCTCCTCCTGCAAGAGACT-3′ (position 35–56), and IL-6-B, (3′) 5-CACAGTTGTGGCAGTAGATGCTC-3′ (position 646–669); GAPDH-A, (5′) 5-CGGTGCTGATGTGGTCCTGGAGTCTC-3′ (position 310–334), and GAPDH-B, (3′) 5-GTTATTATGGGGGTCTGGGATGGAA-3′ (position 1140–1164). The length of the amplified product was of 638 and 854 bp, respectively.

Western Blot Analysis—CMT93 cells were stimulated for various times (0–4 h) with B. vulgatus (5×10^5 cfu/ml), B. vulgatus lysate (100 μg/ml), or LPS (10 μg/ml). The cells were lysed in 1× Laemmli buffer, and 20 μg of protein was subjected to electrophoresis on 10% SDS-polyacrylamide gels as described previously (3). Where indicated CMT93 cells were pretreated for 1 h with 20 μM of the proteasome inhibitor MG132 (100 μM, Biomol, Plymouth Meeting, PA). Anti-phosphoserine Smad2 (Ser-465/467, Cell Signaling, Beverly, MA), anti-phosphoserine Smad2/3 (Ser-433/435, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Smad4/DPC4 (Upstate Biotechnology, Lake Placid, NY), anti-phosphothreonine 3′-actin (ICN, Costa Mesa, CA), anti-phospho-Akt (S473, Cell Signaling, Beverly, MA), anti-acetyl and phosphorylated histone 3 (Lys-9/Ser-10, Cell Signaling, Beverly, MA), and anti-β-actin (ICN, Costa Mesa, CA) were used to detect immunoreactive phospho-Smad2, phospho-Smad2/3, Smad4/DPC4, and 3′-actin, respectively, using enhanced chemiluminescence light detecting kit (Amersham Biosciences).

Immunoprecipitation—CMT93 cells were stimulated with B. vulgatus/LPS for 30 min in the presence or absence of TGF-β1. Where indicated CMT93 cells were co-infected with Ad5Smad3 and Ad5Smad4. Nuclear extract were prepared as described above, and protein concentration was normalized. Immunoprecipitation were carried out overnight at 4 °C using 10 μl of anti-NF-κB p65 Ab (Rockland, Gilbertsville, PA). Immune complexes were collected with protein A/G-agarose for 30 min and washed 3 times in high salt buffer (20 mM Tris, 305 and 354–551, 500 mM NaCl, 1% Nonidet P-40, 2 mM EDTA) followed by three washes with no salt buffer (1 TE). The immune complexes were cleaved from the beads by adding 50 μl of 1× Laemmli buffer. Western blot analysis was carried out using anti-β-actin Ab (Upstate Biotechnology, Lake Placid, NY) as described above.

Nuclear Extracts and Electrophoretic Mobility Shift Assay—CMT93 cells were stimulated for various times (0–4 h) with B. vulgatus lysate (100 μg/ml), and nuclear extracts were prepared as described previously (3). E2F activator p53 (5 μg) were incubated with radiolabeled double-stranded class I major histocompatibility complex class II Ag sites (GGCTGGGGATTCCACCTC), separated by nondenaturing electrophoresis, and analyzed by autoradiography as described previously (30).

ELISA—CMT93 cells were stimulated with B. vulgatus and LPS for 16–24 h. Murine IL-6 was determined in the cell culture supernatant using ELISA kit (Pharmacia & Upjohn Systems).

Statistical Analysis—Data are expressed as means ± S.D. of triplicate experiments. Statistical significance was performed by the two-tailed Student’s t test for paired data and considered significant if p values were <0.05 and <0.01.

RESULTS

B. vulgatus Monoassociated Rats Show Nuclear Localization of Phospho-RelA and Phospho-Smad2 in IEC—We first sought to demonstrate activation of NF-κB and TGF-β signaling in vitro following B. vulgatus monoassociation of germ-free Fisher F344 rats. Mannoassociated rats were killed 3 and 7 days after initial bacterial colonization. Sections of the ileum, cecum, and proximal and distal colon were fixed in 10% neutral buffered formalin. Immunohistochemistry was performed on paraffin-embedded tissue sections using anti-RelA antibody, and sections were counterstained with hematoxylin. A, nuclear phospho-RelA staining of cecal sections from germ-free and bacterial monoassociated rats is shown at a magnification of ×20. Isotype antibodies are used as a control. B, nuclear phospho-RelA staining of sections from ileum, cecum, and proximal and distal colon of B. vulgatus monoassociated rats (3 days) is shown at a magnification of ×40. Representative sections of two different experiments are shown.
revealed signs of pathologic inflammation.

Interestingly and in contrast to RelA, nuclear phospho-Smad2 is sustained throughout days 3 and 7 after bacterial monoassociation, with no signal detected in germ-free controls (Fig. 2A). Nuclear phospho-Smad2 staining was first detected in the lower crypt region of the cecal epithelium at day 3 but was strongest in the surface region at day 7 after initial bacterial colonization of germ-free rats. Similar to the localization of phospho-RelA, phospho-Smad2 was almost absent in the ileum, the proximal and distal colon, or in lamina propria cells (Fig. 2B). In addition, bacteria-induced RelA and Smad2 phosphorylation in primary large intestinal epithelial cells isolated from B. vulgatus-monoassociated germ-free rats was confirmed by Western blot analysis (Fig. 3A). We next sought to determine whether B. vulgatus induces RelA and Smad2 phosphorylation ex vivo in primary IEC isolated from germ-free rats. Fig. 3B shows that B. vulgatus stimulation induces RelA but not Smad2 phosphorylation, suggesting that bacteria-induced Smad signaling is mediated by activation of TGF-β1 production by adjacent intestinal lamina propria mononuclear cells.

These data suggest different temporal and spatial phospho-RelA and phospho-Smad2 distribution in B. vulgatus-monoassociated rats and demonstrate that TGF-β1 and NF-κB signaling pathways are activated in the intestine in vivo after non-pathogenic bacterial colonization.

**TGF-β1 Activates the Smad Signal Transduction Pathway in IEC Lines**—Since B. vulgatus-monoassociated rats showed nuclear localization of phospho-Smad2 in IEC (Figs. 1 and 2), we...
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Ad5Smad7 and AdSmadΔ3 Reverse the Inhibitory Effect of TGF-β1 on Bacteria-induced NF-κB Transcriptional Activity—Proximal TGF-β1 signal transduction has been shown to be down-regulated by the endogenous inhibitor Smad7 (31). To study the functional role of TGF-β1/Smad in blocking B. vulgatus-induced NF-κB transcriptional activity, we used an adenoviral vector to deliver biologically active Smad7 (Ad5S

mad7). Initially, CMT93 cells were infected with Ad5x-B-LUC in combination with Ad5Smad7 and then stimulated with B. vulgatus or LPS. Interestingly, TGF-β1-mediated inhibition of B. vulgatus (Fig. 7A) and LPS-induced (Fig. 7B) NF-κB transcriptional activity was completely reversed in Ad5Smad7-but not Ad5GFP-infected cells. Infection of CMT93 cells with Ad5Smad7 was confirmed by Western blot analysis using anti-Smad7 and anti-HA Abs (Fig. 7A).

To document further the involvement of Smad signaling in TGF-β1-induced inhibitory effect on NF-κB transcriptional activity, we used a dominant negative Smad3 (Ad5SmadΔ3). Similarly to Smad7, TGF-β1-mediated suppression of B. vulgatus- and LPS-induced NF-κB transcriptional activity is inhibited in Ad5SmadΔ3-infected CMT-93 cells (Fig. 7, C and D). Infection of CMT93 cells with Ad5SmadΔ3 was confirmed by Western blot analysis using anti-Smad7 and anti-HA Abs (Fig. 7C).

TGF-β1 Inhibits Histone 3 Acetylation/Phosphorylation and Bacteria-induced Recruitment of RelA to the IL-6 Promoter—Previous reports (14–16) have demonstrated that changes in histone acetylation/phosphorylation strongly influence recruitment of NF-κB to selected target genes. Because TGF-β1-mediated inhibition of IL-6 gene expression occurs independently of the classical NF-κB signal transduction, we next investigated the effect of this immunosuppressive cytokine on histone acetylation levels. We first used the specific histone deacetylase inhibitor TSA to determine whether the inhibitory effect of TGF-β1 is dependent of the status of histone acetylation. Interestingly, B. vulgatus- and LPS-induced histone 3 acetylation/phosphorylation (Lys9/Ser10) is inhibited in TGF-β1-treated cells (Fig. 8A, top and middle panels) but reversed in the presence of TSA (Fig. 8A, lower panels). Moreover, TGF-β1-mediated inhibition of LPS-induced IL-6 secretion was reversed in TSA-treated cells (Fig. 8B). This suggests that TGF-β1-mediated inhibition of IL-6 gene expression involves changes in histone acetylation.

To address specifically the effect of TGF-β1-mediated repression of B. vulgatus/LPS-induced IL-6 gene expression, we performed ChIP using a p65 antibody. CMT93 cells were stimulated with LPS in the presence or absence of TGF-β1, and p65 recruitment to the IL-6 promoter region was determined by PCR. Fig. 8C shows that LPS strongly induced p65 recruitment to the IL-6 promoter at 2 h (Fig. 8C, upper panel), which was blocked by TGF-β1 treatment (Fig. 8C, upper panel). Similar levels of IL-6 were amplified from the total pool of genomic DNA (input control), documenting that equal amounts were used for the ChIP analysis (Fig. 8C, lower panel). To address the role of Smad signaling on TGF-β1-mediated inhibition of IL-6 gene expression, we next used Ad5Smad7 or Ad5GFP-infected cells for ChIP analysis. Ad5Smad7 but not Ad5GFP reversed TGF-β1-mediated inhibition of NF-κB recruitment to the IL-6 gene promoter (Fig. 8D). This suggests that TGF-β1-induced Smad signaling is necessary for inhibition of B. vulgatus/LPS-induced RelA recruitment to the IL-6 promoter in IEC.

**TGF-β1 Inhibits B. vulgatus-induced CBP/p300 and RelA Nuclear Co-association**—Since the nuclear co-activator CBP/p300 binds to the transcriptional co-activator p300 and RelA, we were interested in the role of this protein complex in TGF-β1-mediated repression of B. vulgatus-induced NF-κB transcriptional activity. CBP/p300 was amplified using specific primers from genomic DNA, and its recruitment to the IL-6 promoter was determined by PCR. Ad5Smad7 but not Ad5GFP reversed TGF-β1-mediated inhibition of CBP/p300 recruitment to the IL-6 promoter (Fig. 8E). This suggests that TGF-β1-induced Smad signaling is necessary for inhibition of B. vulgatus/LPS-induced RelA recruitment to the IL-6 promoter in IEC.

**Fig. 5.** A–C, TGF-β1-mediated inhibition of B. vulgatus-induced NF-κB transcriptional activity and IL-6 gene expression in IEC. A, CMT93 cells were infected with Ad5x-B-LUC (5 m.o.i.) for 12 h. Cells were washed and co-infected for additional 12 h with Ad5IxB-oAA. Finally, IEC were stimulated with B. vulgatus (5 × 10⁷ cfu/ml), B. vulgatus lysate (100 μg total protein/ml), LPS (10 μg/ml), or medium alone for additional 12 h in the presence or absence of TGF-β1 (20 ng/ml). Cell extracts and the luciferase assay were performed as described under “Materials and Methods,” and results were normalized for extract protein concentrations. B, CMT93 cells were stimulated with B. vulgatus lysate and LPS for 6 h in the presence or absence of TGF-β1 (20 ng/ml). Total RNA was extracted, reverse-transcribed, and amplified using specific IL-6 and GAPDH primers. PCR products were run on a 2% agarose gel and stained with gel star. These results are representative of two independent experiments. C, CMT93 cells were stimulated with B. vulgatus lysate and LPS, and IL-6 protein secretion was measured in the cell culture supernatants after 24 h of stimulation by ELISA. The bars represent the combined mean values (±S.D.) of triplicate experiments from two different experiments. **n.d.**, not determined.
Fig. 6. A–C, role of TGF-β1 on B. vulgatus-induced NF-κB signaling cascade. A, CMT93 cells were stimulated with B. vulgatus lysate (100 μg of protein/ml) for 0–2 h in the presence or absence of TGF-β1 (20 ng/ml). Where indicated CMT93 cells were pretreated with 20 μM MG132. Total protein was extracted, and 20 μg of protein was subjected to SDS-PAGE followed by phospho-1xβ, RelA, and Akt immunoblotting using the ECL technique. B, LPS-induced serine RelA phosphorylation in the presence or absence of TGF-β1 (20 ng/ml) was determined by an immunocomplex kinase assay as described under “Materials and Methods.” The kinase reaction was performed by incubating 25 μl of kinase buffer with either glutathione S-transferase GST-p65 (residues 354–551) as substrate for 30 min at 30 °C. Substrate protein was resolved by gel electrophoresis, and phosphate incorporation was assessed by autoradiography and PhosphorImager analysis. Coomassie Blue staining shows equal loading (lower blot). C, CMT93 cells were stimulated with B. vulgatus lysate (100 μg of protein/ml) or p300 has intrinsic HAT activity and associates with NF-κB to induce transcription (32, 33); we next investigated the effect of TGF-β1 or Ad5Smad3 and Ad5Smad4 on p65 and CBP/p300 association. Cells were treated with TGF-β1 or Ad5Smad3/Ad5Smad4 and then stimulated with LPS. Interestingly, co-immunoprecipitation experiment demonstrates that TGF-β1 treatment reversed LPS-induced nuclear CBP/p300 association with p65 (Fig. 9, compare lanes 2 with 4). Moreover, the presence of Ad5Smad3/Ad5Smad4 also reversed LPS-induced CBP/p300 association with p65 (Fig. 9, compare lanes 2 and 5), suggesting that TGF-β1-induced Smad signaling may prevent bacteria-induced p65-CBP/p300 interaction in IEC.

In conclusion, these results suggest that B. vulgatus/LPS-induced IL-6 gene expression in IEC is partially regulated by TGF-β1 through decreased histone acetylation/phosphorylation and loading of NF-κB to the gene promoter.

DISCUSSION

Numerous reports point to a critical immunoregulatory function of TGF-β1 in the maintenance of intestinal homeostasis. First, Yang et al. (34) showed that targeted disruption of the Smad3 gene caused massive T cell infiltration and pyogenic abscess formation in the stomach and intestine. Subsequently, Monteleone et al. (35) showed that Smad7, the endogenous inhibitor of the Smad signal transduction pathway, is overexpressed in mucosal T cells from patients with IBD. In vitro blocking of Smad7 restored TGF-β1-mediated Smad phosphorylation and enhanced TGF-β1-mediated inhibition of inflammatory cytokine production in lamina propria mononuclear cells isolated from IBD patients. Moreover, TGF-β1-deficient mice spontaneously develop colitis (36), and overexpression of Smad3 in lamina propria immune cells inhibited Th1-mediated experimental 2,4,6-trinitrobenzene sulfonic acid-induced colitis (37, 38). The absence of colitis and pathogenic immune responses to commensal enteric bacteria suggests that normal hosts have well developed immunosuppressive mechanisms of their mucosal immune systems.

Although the immunosuppressive effect of TGF-β1 is well established, the molecular mechanisms responsible for the immunomodulation remain to be elucidated. We now demonstrate that TGF-β1/Smad signaling inhibits B. vulgatus and LPS-induced IL-6 gene expression through reduction of histone acetylation/phosphorylation and decreased recruitment of NF-κB to the IL-6 promoter. First, we show for the first time a sequential increase of nuclear RelA (day 3) and Smad2 (days 3 and 7) phosphorylation in cecal epithelial cells of B. vulgatus-monoassociated germ-free Fisher F344 rats, suggesting that this commensal bacterial strain induced both pro-inflammatory and immunosuppressive signaling pathways in the host in vivo. Second, by using primary and colonic cell lines, we provide evidence that TGF-β1 induced Smad2 phosphorylation, association of phospho-Smad2/Smad3 with Smad4, and enhanced TGF-β1-dependent transcriptional activity, suggesting a functional TGF-β1-mediated Smad signal transduction in IEC. Third, we show that TGF-β1 significantly inhibits B. vulgatus and LPS-induced NF-κB transcriptional activity as well as IL-6 gene expression in IEC, demonstrating the biological significance of TGF-β1 signal transduction in regulating IEC responsiveness to luminal Gram-negative bacteria and bacterial products.

We have shown previously that B. vulgatus-induced NF-κB activation and pro-inflammatory gene expression require en-
TGF-β1 Inhibits NF-κB Activity in IEC

hanced phosphatidylinositol 3-kinase/Akt and RelA phosphorylation (3). However, TGF-β1 did not affect B. vulgatus-induced IkBα, RelA, and Akt phosphorylation nor alter NF-κB DNA-binding, suggesting an alternative mechanism for the TGF-β1-mediated inhibition of B. vulgatus-induced NF-κB transcriptional activity in IEC. Of interest, levels of histone acetylation/phosphorylation directly impact expression of some NF-κB-dependent genes by controlling access of the transcription to the appropriate gene promoter (39). In addition, recent studies (40, 41) demonstrate that TGF-β1-mediated transcriptional repression involves recruitment of HDAC. Interestingly, we demonstrate that the HDAC inhibitor TSA reversed the TGF-β1-mediated inhibitory effect on B. vulgatus/LPS-induced IL-6 gene expression, suggesting that TGF-β1 modulates the status of histone acetylation. More directly, we show that B. vulgatus/LPS-induced histone acetylation/phosphorylation is inhibited by TGF-β1 treatment. Similarly, we show that loading of NF-κB to the IL-6 promoter is inhibited by TGF-β1 treatment in IEC. Together, these data point to a new regulatory mechanism of TGF-β1-mediated immunosuppression which involves decreased histone acetylation and inhibition of NF-κB access to the gene promoter.

The mechanisms controlling the coordinated nuclear migration of transcription factors and access to various gene promoters are not elucidated. Recently, activation of the mitogen-activated protein kinase p38 was shown to be critical in LPS-mediated histone phosphorylation and NF-κB access to select gene promoters in dendritic cells (16). Interestingly, we failed to observe enhanced p38 phosphorylation in B. vulgatus- or LPS-stimulated CMT93 cells. Although a role for p38 in bacteria and LPS-induced histone acetylation/phosphorylation could not be totally ruled out, our data suggest that other pathways may participate in histone modification, at least in IEC.

The precise mechanism of enhanced NF-κB transcriptional activity by signal-induced RelA phosphorylation is not clearly established. Zhong et al. (32, 33) have shown that LPS-induced RelA serine 276 phosphorylation loosens its interaction with HDAC and promotes its binding to the transcriptional co-activator CBP/p300 containing HAT activity. Interestingly, association of HDAC1 and HDAC2 with unphosphorylated nuclear RelA is associated with transcriptionally inactive NF-κB (42). However, we found no correlation between TGF-β1-mediated inhibition of RelA recruitment to the IL-6 gene promoter and the level of RelA phosphorylation. This was confirmed by investigating both endogenous serine 536 phosphorylation levels by Western blot and by an in vitro kinase assay using a GST-RelA substrate (serine 526 and 529). Since LPS-induced serine 276 phosphorylation strongly modulates NF-κB transcriptional activity (32, 33), the possibility remains that TGF-β1 interferes with this phosphorylation site and prevents bacteria-induced NF-κB transcriptional activity. However, RelA serine 276 phos-

Fig. 7. A–D, TGF-β1-mediated inhibition of B. vulgatus/LPS-induced NF-κB transcriptional activity was reversed by Ad5Smad7 and Ad5Smad3Δ3. CMT93 cells were infected with Ad5x-

LUC (5 m.o.i.) for 12 h. Cells were washed and co-infected with Ad5Smad7 (A and B, 50 m.o.i.), Ad5Smad3Δ3 (C and D, 50 m.o.i.), or Ad5GFP (50 m.o.i.) for an additional 12 h. CMT93 cells were then stimulated with B. vulgatus lysate (100 μg of total protein/ml), LPS (10 μg/ml), or medium alone for an additional 12 h in the absence or presence of TGF-β1. Expression of HA-tagged Smad3Δ3 (C–D) or Smad7 (A–B) protein of representative experiments is shown in the upper panel. Cell extracts and the luciferase assay were performed as described under “Materials and Methods,” and results were normalized for extract protein concentrations. B. vulgatus/LPS-induced luciferase activity is expressed as fold increase over control determined as the mean of triplicate experiments. These results are representative of two independent experiments.
phorylation is not induced in B. vulgatus- and LPS-stimulated IEC (3), suggesting that the TGF-β1-mediated inhibition operates independently of the RelA phosphorylation status. Therefore, although we observed a decrease in RelA phosphorylation (day 7) but enhanced Smad phosphorylation (days 3–7) in B. vulgatus monoassociated rats, these two events are likely independent of each other. Decreased RelA phosphorylation may represent an intrinsic down-regulatory mechanism present in IEC.

B. vulgatus signaling to RelA phosphorylation and NF-κB DNA binding activity is intact in TGF-β1-treated cells, but RelA recruitment to the IL-6 promoter is blocked. This suggests that the immunoregulatory cytokine interferes with the activity of a co-repressor or co-activator of NF-κB. Interestingly, TGF-β1-mediated Smad signal transduction is necessary for inhibition of B. vulgatus/LPS-induced NF-κB transcriptional activity and IL-6 gene expression, as demonstrated by reversed suppression in Ad5Smad3 or Ad5Smad4-infected cells. Of note, TGF-induced nuclear Smad complex associates with the CBP/p300 transcriptional co-activator (43–45). Interestingly, we show that TGF-β1 and the presence of Ad5Smad3/Ad5Smad4 reversed LPS-induced CBP/p300 p65 nuclear interaction. This suggests that the mechanism of TGF-β1-induced inhibitory effect involved decreased p65-CBP/p300 association, possibly through negative titration by Smad proteins. Therefore, diminished CBP/p300 association with p65 in conjunction with decreased HAT activity could lead to impaired histone acetylation/phosphorylation and/or lower p65 recruitment to the IL-6 gene promoter. The formal interaction of Smad protein with CBP/p300 and the precise role of this co-activator in bacteria signaling in IEC will necessitate further investigation.

In conclusion, we demonstrate that TGF-β1 inhibits non-

Fig. 8. A–D, TGF-β1 inhibits B. vulgatus/LPS-induced recruitment of NF-κB to the IL-6 promoter through blockade of histone acetylation. A, CMT93 cells were stimulated with B. vulgatus lysate (100 µg of protein/ml) and LPS (10 µg/ml) for 0–2 h in the presence or absence of TGF-β1 (20 ng/ml). Where indicated cells were pretreated with trichostatin A (2 h pretreatment with 1 µg/ml). Total protein was extracted, and 20 µg of protein was subjected to SDS-PAGE followed by acetyl-phospho-histone 3 (Lys-9/Ser-10) and β-actin immunoblotting using the ECL technique. B, CMT93 were stimulated with LPS for 16 h in the presence or absence of TGF-β1. Where indicated cells were pretreated with trichostatin A (2 h pretreatment with 1 µg/ml). IL-6 protein was measured in the cell culture supernatant by ELISA. Bars represent the combined mean values (± S.D.) of triplicates from two independent experiments. C and D, CMT93 cells were stimulated with LPS (10 µg/ml) for 0–2 h in the presence or absence of TGF-β1 (20 ng/ml). Where indicated CMT-93 cells were infected with Ad5Smad7 (50 m.o.i.) or Ad5GFP (50 m.o.i.). ChIP analysis was performed as described under “Materials and Methods.” Anti-RelA Ab was used to immunoprecipitate equal amounts of DNA (input DNA). PCR was performed with a 1:20 dilution of ethanol-precipitated DNA using primers specific for mouse IL-6 promoter. Densitometric analysis of amplified PCR products was performed. These results are representative of three different experiments.
pathogenic commensal Gram-negative bacteria-induced histone acetylation/phosphorylation and RelA loading to the IL-6 gene promoter in IEC. We suggest that the immunoregulatory cytokine TGF-β1 participates in the maintenance of intestinal homeostasis by decreasing the ability of commensal luminal enteric bacteria to activate the NF-κB signaling pathway at the level of gene promoter accessibility.

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