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IKKβ and Phosphatidylinositol 3-Kinase/Akt Participate in Non-pathogenic Gram-negative Enteric Bacteria-induced RelA Phosphorylation and NF-κB Activation in Both Primary and Intestinal Epithelial Cell Lines*

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Dirk Haller, Maria P. Russo, R. Balfour Sartor, and Christian Jobin‡

From the Department of Medicine and the Center for Gastrointestinal Biology and Disease, University of North Carolina, Chapel Hill, North Carolina 27599-7080

Pathogenic and enteroinvasive bacteria have been shown to trigger the IκB/NF-κB transcriptional system and proinflammatory gene expression in epithelial cells. In this study, we investigated the molecular mechanism of the commensal Gram-negative *Bacteroides vulgatus*-induced NF-κB signal transduction in intestinal epithelial cells (IEC). We report that *B. vulgatus* induced interleukin-1 receptor-associated kinase-1 degradation, IκBα phosphorylation/degradation, RelA and Akt phosphorylation, as well as NF-κB DNA binding and NF-κB transcriptional activity in rat non-transformed IEC-6 cells. *B. vulgatus*- but not interleukin-1β-mediated NF-κB transcriptional activity was inhibited by dominant negative (dn) toll-like receptor 4. Of importance, *B. vulgatus* induced IκBα phosphorylation/degradation and IKKα/β and RelA phosphorylation in primary IEC derived from germ-free or mono-associated HLA-B27 transgenic and wild type rats, demonstrating the physiological relevance of non-pathogenic bacterial signaling in IEC. Adenoviral delivery of dn IKKβ or treatment with wortmannin inhibited *B. vulgatus*-induced endogenous RelA Ser-536 and GST-p65TAD (Ser-529/Ser-536) phosphorylation as well as NF-κB transcriptional activity in IEC-6 cells, suggesting a critical role of IKKβ and phosphatidylinositol 3-kinase/Akt in bacteria-induced RelA phosphorylation and NF-κB activation. Interestingly, *B. vulgatus*-induced IκBα degradation and NF-κB transcriptional activity in IEC transwell cultures were inhibited in the presence of lymphocytes. We propose that non-pathogenic *B. vulgatus* activates the NF-κB signaling pathway through both IκB degradation and RelA phosphorylation but that immune cells mediate tolerance of IEC to this commensal bacteria.

Inflammatory bowel diseases, including human ulcerative colitis and Crohn's disease, are chronic immune-mediated diseases of the distal intestinal tract with unknown etiologies (1, 2). Various pathogenic mechanisms have been proposed, including inflammatory responses to a persistent luminal pathogen or abnormal luminal constituent, autoimmunity, or an overly aggressive immune response to normal luminal constituents such as commensal enteric bacteria. The hypothesis that aberrant immune responses to non-pathogenic commensal luminal bacteria can cause colitis in genetically predisposed individuals is supported by several rodent models of experimental colitis (3–5). For example, HLA-B27/β_2-microglobulin transgenic rats raised under specific pathogen-free conditions spontaneously develop colitis, whereas germ-free (sterile) conditions prevent the development of chronic inflammation (6, 7). Reconstitution studies of gnotobiotic HLA-B27 transgenic rats (6, 8) and carrageenan-induced colitis in guinea pigs (9) implicate *Bacteroides vulgatus* as particularly important to the induction of colitis in these models. Of note, this *B. vulgatus* strain induced no colitis in wild type rats, documenting its wild type nature (8). Despite these observations suggesting a central role of non-pathogenic resident luminal bacteria in the regulation of intestinal inflammation, the mechanisms by which bacteria influence the mucosal immune response responsible for inducing and perpetuating chronic colitis remain unclear.

A single layer of intestinal epithelial cells (IEC)[1] isolate the host from the gut luminal environment. These cells are considered to be an integral and essential component of the innate mucosal immune system of the host (10). IEC constitutively express, or can be induced to express, co-stimulatory molecules and components of the human major histocompatibility complex including class II and classical I and non-classical class Ib human major histocompatibility complex molecules (11–13). Moreover, proinflammatory stimuli (*e.g.* TNF and IL-1) as well as certain enteric pathogens (*e.g. Salmonella* species, *Yersinia enterocolitica*, and enteropathogenic *Escherichia coli*) induce the expression and secretion of a wide range of inflammatory and chemoattractive cytokines in IEC including TNF, IL-8, MCP-1, IP-10, GROα, inducible nitric-oxide synthase, and

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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: Job@med.unc.edu.

[1] The abbreviations used are: IEC, intestinal epithelial cells; NF-κB, nuclear transcription factor κB; TLR4, Toll-like receptor 4; EMSA, electrophoretic mobility shift assay; RT, reverse transcription; IL-1β, interleukin 1β; TNF, tumor necrosis factor; IRAK, interleukin 1 receptor-associated kinase; ICAM-1, intercellular adhesion molecule 1; TAK1, transforming growth factor-β-activated kinase 1; IKK, IκB kinase complex; Ad, adenoviral; PI3K, phosphatidylinositol 3-kinase; cfu, colony-forming units; dn, dominant negative; mAb, monoclonal antibody; FACS, fluorescence-activated cell sorter; GST, glutathione *S*-transferase; HA, hemagglutinin; FCS, fetal calf serum; m.o.i., multiplicity of infection; PBMC, peripheral blood mononuclear cells; PBL, peripheral blood lymphocytes; LPS, lipopolysaccharide; TRAF, TNF receptor-associated factor; TAK, transforming growth factor-β-activated kinase; mAb, monoclonal antibody; MFI, mean fluorescent intensity.

COX-2 as well as the adhesion molecule ICAM-1 (14–19). As shown in multiple cell systems including IEC, most of these proinflammatory molecules are in part regulated at the transcriptional level by the transcription factor NF-κB (20).

Activation of the IκB/NF-κB system is a complex process that involves the participation of multiple adapter proteins and kinases acting in a coordinated fashion to give specificity to the cell surface stimuli. Although IL-1 and TNF signaling events leading to NF-κB activation has been well studied (21), the molecular mechanisms of bacterial signaling to the IκB/NF-κB transcriptional system in IEC are still largely unknown. The major molecular determinant of Gram-negative bacteria responsible for NF-κB activation is the glycolipid lipopolysaccharide (LPS). This bacterial product signals to the IκB/NF-κB system by using the cell surface toll-like receptor (TLR) 4 and its co-receptor MD-2 which then utilize downstream components of the IL-1 signaling cascade (22–24). For example, TLR4 engagement by LPS leads to the sequential recruitment/activation of the myeloid differentiation protein (MyD88), the IL-1 receptor-associated kinase (IRAK), the TNF receptor-associated factor (TRAF) 6, and the transforming growth factor-β-activated kinase (TAK) 1. Although controversial, the signal may converge on the NF-κB-inducing kinase, which then activates the IκB kinase (IKK) complex. The activated IKK complex phosphorylates IκBα at serine residues 32 and 36, which triggers its ubiquination/degradation and subsequent release of NF-κB, which then translocates to the nucleus and activates the transcription of κB-dependent genes (25, 26). In addition to nuclear translocation, modification of NF-κB transcriptional activity by phosphorylation of the RelA subunit has been shown to be an important regulatory element of the pathway (27–32). TNF and IL-1β induce phosphorylation of serine 529 and/or 536 of the RelA transactivating domain 1 (TAD1), which increased NF-κB transcriptional activity (28, 33, 34). Potential kinases involved in signal-induced RelA phosphorylation are the casein kinase II, Akt, and IKK (27, 28, 33, 35). The precise mechanism for increased transcriptional activity is not clear but may involve enhanced recruitment of transcriptional co-activator such as cAMP-response element-binding protein-binding protein (CBP/p300) to the promoter site of some specific genes. Although IEC lines were shown to express TLR4 (36–38), the functional role of this receptor and the downstream signal transduction, including RelA phosphorylation triggered by commensal non-pathogenic bacteria in IEC, is still unknown.

In this study, we characterized the molecular mechanisms of NF-κB activation triggered by the Gram-negative, commensal bacterial strain *B. vulgatus* in the rat intestinal epithelial cell line IEC-6, the human colonic cell line CaCO-2, and primary IEC derived from germ-free or *B. vulgatus* mono-associated HLA-B27/β_2-microglobulin transgenic as well as wild type rats. We report that *B. vulgatus* induces NF-κB activation in IEC through components of the IL-1R/TLR4 pathway and the induction of RelA phosphorylation. The presence of lymphocytes negatively regulates bacteria-induced NF-κB activity, suggesting a potential role of immune cells in controlling IEC responsiveness to commensal bacteria.

MATERIALS AND METHODS

Bacteria and Culture Conditions—*B. vulgatus* derived from a guinea pig with carrageenan-induced colitis (a gift from A. B. Onderdonk, Harvard University, Cambridge, MA) was anaerobically grown at 37 °C in brain heart infusion broth supplemented with cysteine (0.05%), hemin (5 mg/liter), and resazurin. *E. coli* derived from a patient with active Crohn's disease (provided by the Clinical Microbiology Laboratory of the University of North Carolina Hospitals, Chapel Hill) and the human oral isolate *Enterococcus faecalis* (a gift from M. Huycki, University of Oklahoma State), which has been shown to induce experimental colitis in mono-associated IL-$10^{-/-}$ mice (39), were aerobically grown in Luria broth containing tryptone (1%), yeast extract (0.5%), and NaCl (0.5%). The probiotic strain *Lactobacillus paracasei* strain GG of human intestinal origin (ConAgra, Lincoln, NE) was anaerobically grown in MRS broth 37 °C. All bacteria were harvested by centrifugation (3,000 × *g*, 15 min) at stationary growth phase, washed three times with phosphate-buffered saline (1×, pH 7.2, Invitrogen), and subsequently diluted to obtain final cell densities of 5×10^7 cfu/ml in Dulbecco's modified Eagle's medium (Invitrogen). Bacterial lysates were prepared as described previously (40).

Cell Culture and Stimulation of IEC—The rat nontransformed small intestinal epithelial cell line IEC-6 (passage 5–0) (ATCC CRL 1592, American Type Culture Collection (ATCC), Manassas, VA) and the human adenocarcinoma intestinal epithelial cell line CaCO-2 (passage 30–50) (ATCC HTB 38) were grown to confluency in 12- or 6-well plates (Nunc) as described previously (41). CaCO-2 cells were used in liposome-based transfections, because IEC-6 cells are not permissive to transfections. Confluent cell monolayers were stimulated with 5×10^7 cfu/ml bacteria, bacterial lysate (200 or 50 μg of protein/ml), LPS (5 or 1 μg/ml; from *E. coli* serotype O111:B4, Sigma), TNF (5 ng/ml), and IL-1β (10 ng/ml) (both from R & D Systems, Minneapolis, MN) for various times. To prevent bacterial growth, gentamicin (100 μg/ml) was added to the cultures after 2 h. Where indicated, cells were pretreated with cycloheximide (50 μg/ml, Sigma), triptolide (100 ng/ml, Biomol, Plymouth Meeting, PA), or wortmannin (100 nM, Sigma).

Intestinal Epithelial Cell/Leukocyte Co-cultures—Transwell leukocyte co-cultures with confluent CaCO-2 monolayers were established as described previously (42, 43). Briefly, human peripheral blood mononuclear cells (PBMC) derived from healthy volunteers were isolated using Ficoll-Paque 1077 (Amersham Biosciences) gradient centrifugation (500 × *g*, 30 min). To purify leukocyte subpopulations, PBMC were incubated for 2 h at 37 °C and 5% CO_2 on 225-cm^2 tissue culture plates (Costar) to allow adherence. Non-adherent peripheral blood lymphocytes (PBL) were separated from adherent cells by aspiration. Adherent peripheral blood monocytes were washed 3 times with phosphate-buffered saline (1 time) and harvested by cell scraping. PBMC, PBL, or monocytes were added to the basolateral compartment of 6-well transwell inserts (0.4-mm pore size) at cell densities of 2×10^6/ml. IEC/leukocyte co-cultures were stimulated with bacteria by adding 5×10^7 cfu/ml *B. vulgatus* to the apical surface of IEC monolayers and incubated for 4 h at 5% CO_2 and 37 °C.

Isolation of Primary Rat Small Intestinal Epithelial Cells—Germ-free (sterile) HLA-B27 transgenic and wild type Fisher F_{344} rats were euthanized, and the entire small intestine as well as the large intestine were removed and placed in calcium/magnesium-free Hanks' buffered saline solution (Invitrogen) containing 5% FCS. The small intestine was cut longitudinally, washed 3 times in calcium/magnesium-free Hanks' buffered saline solution (Invitrogen), cut into pieces 0.5 cm long, and incubated at 37 °C in 40 ml of RPMI 1640 containing 5% FCS and 1 mM dithiothreitol for 30 min in an orbital shaker. The supernatant was filtered and centrifuged for 5 min at 400 × *g*, and the cell pellet was resuspended in RPMI 1640 containing 5% FCS. The remaining tissue was incubated in 40 ml of phosphate-buffered saline (1×) containing 0.5 mM dithiothreitol and 1.5 mM EDTA for an additional 15 min. The supernatant was filtered and centrifuged for 5 min at 400 × *g*, and the cell pellet was resuspended in RPMI 1640 containing 5% FCS. Finally, primary IEC were collected by centrifugation through a 25–40% discontinuous Percoll gradient at 600 × *g* for 20 min. Cell viability and purity was assessed by trypan blue exclusion and FACS analysis using rat anti-CD3 mAb (BD Biosciences, clone G4.18). Cells were >85% viable and >90% pure. Primary rat IEC at a concentration of 2×10^6 cells/ml were incubated for 2 h in 5% CO_2 at 37 °C and then stimulated with 5×10^7 cfu/ml *B. vulgatus*, *L. paracasei*, or medium alone for 4 h.

B. vulgatus Mono-association of Germ-free Fisher Wild Type Rats—Fisher F_{344} rats raised under germ-free conditions were transferred to *B. vulgatus* isolators at the age of 10 weeks. The animals were removed from the gnotobiotic isolator after 3 days and euthanized by CO_2 asphyxiation within the next 3 h. Bacterial colonization was documented by fecal culture. Primary IEC were isolated from the small intestine, cecum, and colon as described above. Cells were prepared for Western blot as well as FACS analysis.

Adenoviral Infection—IEC-6 cells were infected overnight with adenoviral dominant negative (dn) IKKβ (Ad5dnIKKβ), dnTAK1 (Ad5dnTAK1), dnTRAF-2 (Ad5dnTRAF-2), and Ad5IκBαAA in serum-free media (Opti-MEM, Invitrogen) at different multiplicity of infection (m.o.i., 0, 25, 50, and 100). The Ad5IκBαAA, Ad5TRAF-2, Ad5dnIKKβ, and Ad5TAK1 were described previously (41, 44–49). Ad5κB-LUC consisting of three consensus NF-κB-binding sites was linked to luciferase.

Ad5GFP containing green fluorescent protein and Ad5LacZ containing the *E. coli* β-galactosidase were used as viral negative control (41). The adenoviruses were washed off, and fresh medium containing serum without antibiotics was added. Cells were stimulated at various time points with *B. vulgatus* (5×10^7 cfu/ml), TNF (5 ng/ml), and IL-1β (10 ng/ml). Dominant negative TAK1 and the super-repressor IκBαAA contained an extra 27-bp DNA nucleotides coding for a peptide derived from hemagglutinin (HA) gene (YPYDVPDYA). Dominant negative IKKβ and dnTRAF-2 contained an extra 24-bp DNA nucleotides coding for the FLAG peptide (DYLDDDDL). Expression of HA- and FLAG-tagged mutant molecules in IEC-6 cells was controlled by immunofluorescent microscopy and Western blot analysis using mouse anti-HA (Roche Molecular Biochemicals) and mouse anti-FLAG M2 (Eastman Kodak Co.) mAb.

Transfection and Reporter Gene Assay—CaCO-2 cells were transfected using LipofectAMINE Reagent (Invitrogen) as described previously (41). The $(\kappa B)_3$-luciferase motif consists of three consensus NF-κB sites linked to luciferase (47). Plasmids expressing dominant negative TLR4 (0.8 μg; generous gift of Dr. Bruce Beutler, Scripps Research Institute) or $(\kappa B)_3$-luciferase (1 μg) were transfected in combination or alone as described as under "Results," and the total amount of DNA was equalized with empty vector. Transfected cells were incubated overnight after which the DNA/LipofectAMINE was replaced with serum-containing media. Cells were then stimulated with *B. vulgatus* (5×10^7 cfu/ml), TNF (5 ng/ml), IL-1β (10 ng/ml), or medium for 12 h. Cell extracts were prepared using enhanced luciferase assay reagents (Analytical Luminescence, San Diego, CA). Luciferase assay were performed on a Monolight 2010 luminometer for 20 s (Analytical Luminescence, San Diego, CA), and results were normalized for extract protein concentrations measured with the Bio-Rad protein assay kit.

RNA Extraction and RT-PCR Analysis—RNA was isolated using Trizol (Invitrogen), and 1 μg of total RNA was reverse-transcribed and amplified (RT-PCR) using specific primers for rat ICAM-1, COX-2, and β-actin as described previously (50). The oligonucleotide TLR4 primers used are as follows: TLR4-A (5′), 5-TGTCCCTGAACCCTATGAAC-3 (positions 795–812); TLR4-B (3′), 5-ACTCAAATCTCTCAAAAGGC-3 (positions 1211–1230). The oligonucleotide MD-2 primers used are as follows: MD-2A (5′), 5-GAAGCTCAGAAGCAGTATTGGGTC-3 (positions 174–197); MD-2B (3′), 5-GGAGTTTGTCATCCTACACCAACC-3 (positions 572–596). The length of the amplified product was 438 and 422 bp, respectively. The PCR products (5 μl) were subjected to electrophoresis on 2% agarose gels containing GelStar fluorescent dye (FMC, Philadelphia, PA). Fluorescent staining was captured using an AlphaImager 2000 (AlphaInnotech, San Leandro, CA).

Western Blot Analysis—IEC were stimulated for various times (0–4 h) with bacteria, bacterial products, or cytokines. The cells were lysed in 1× Laemmli buffer, and 20 μg of protein was subjected to electrophoresis on 10% SDS-polyacrylamide gels. Where indicated IEC-6 cells were pretreated for 1 h with 20 μM of the proteasome inhibitor MG132 (Peptide Institute, Japan). Anti-phosphoserine IκBα (Cell Signaling, Beverly, MA), anti-IκBα (Santa Cruz Biotechnology, Santa Cruz, CA), anti-IRAK-1 (a generous gift from D. K. Miller, Merck), anti-phosphoserine IKKα/β (Cell Signaling, Beverly, MA), anti-phosphoserine RelA (Ser-536, Cell Signaling, Beverly, MA), anti-phosphoserine Akt (Ser-473, Cell Signaling, Beverly, MA), and anti-β-actin (ICN, Costa Mesa, CA) were used to detect immunoreactive phospho-IκBα, total IκBα, IRAK-1, phospho-IKKα/β, phospho-RelA, and β-actin, respectively, using enhanced chemiluminescence light-detecting kit (Amersham Biosciences) as described previously (41).

Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)—IEC-6 cells were stimulated for various times (0–4 h) with *B. vulgatus* (5×10^7 cfu/ml), and nuclear extracts were prepared as described previously (41). Extracts (5 μg) were incubated with radiolabeled double-stranded class I major histocompatibility complex κB sites (GGCTGGGGATTCCCCATCT), separated by nondenaturing electrophoresis, and analyzed by autoradiography as described previously (41).

In Vitro Kinase Assays—IKK activity on *B. vulgatus*-induced serine RelA phosphorylation was determined by immunocomplex kinase assay as described previously (51). Briefly, IEC-6 cells were lysed in Triton lysis buffer containing protease and phosphatase inhibitors after stimulation with *B. vulgatus* at various times. Where indicated IEC-6 cells were pretreated for 45 min with triptolide (100 ng/ml) or wortmannin (100 nM) or infected for 16 h with Ad5dnIKKβ, Ad5IκBαAA, or Ad5LacZ. 300 μg was immunoprecipitated with 2 μl of anti-IKKγ (Cell Signaling, Beverly, MA), and the kinase reaction was performed by incubating 25 ml of kinase buffer containing 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, 50 mM ATP, and 0.5 mCi of [^{32}P]ATP with either glutathione *S*-transferase (GST)-p65-(1–305) or GST-p65-(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 PhosphorImager analysis (Amersham Biosciences).

Flow Cytometry—IEC were stimulated with bacteria, bacterial products, or cytokines. ICAM-1 cell surface expression was analyzed after 24 h. Cell staining was performed for 20 min at 4 °C with saturating concentrations of the fluorescein isothiocyanate-conjugated mouse anti-rat ICAM-1 mAb (IgG1, 1A29, Pharmingen). Fluorescein isothiocyanate-conjugated mouse IgG1 (G155–228, MOPC-21, G155–178, Pharmingen) was used as isotype control. The samples were analyzed using a FACSCalibur® (BD Biosciences).

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

RESULTS

Commensal Non-pathogenic Gram-negative Bacteria and LPS Induce ICAM-1 and COX-2 mRNA Expression in IEC-6 Cells—The commensal enteric Gram-negative species *B. vulgatus* and *E. coli* as well as the Gram-positive *E. faecalis* and *L. paracasei* were used at a concentration of 5×10^7 cfu/ml to stimulate the nontransformed rat intestinal cell line IEC-6. As shown in Fig. 1*A*, the Gram-negative bacteria *B. vulgatus* and *E. coli* but not the Gram-positive *E. faecalis* and *L. paracasei* induced ICAM-1 and COX-2 mRNA expression in IEC-6 cells after 6 h of stimulation, suggesting a specific effect of Gram-negative bacterial strains on expression of these genes.

We next sought to elucidate the mechanism of *B. vulgatus*-induced gene expression in IEC-6 cells. *B. vulgatus* lysate (50 or 200 μg/ml) and LPS (1 or 5 μg/ml) were used to stimulate IEC-6 cells for 6 h, and ICAM-1 gene expression was then analyzed by RT-PCR. Bacterial lysate (Fig. 1*B*) and LPS (Fig. 1*C*) induced ICAM-1 mRNA expression in IEC-6 cells. The inductive effect of the bacterial lysates and LPS was inhibited in the presence of 150 μg/ml polymyxin B sulfate, suggesting that the *B. vulgatus*-induced ICAM-1 mRNA expression is LPS-mediated (compare *lanes 5* and *6* with *lanes 3* and *4*). Additionally, heat (100 °C for 30 min) or gentamicin (150 μg/ml) treatment of bacteria before stimulation as well as cycloheximide (50 μg/ml) treatment of IEC-6 cells failed to prevent *B. vulgatus*-induced ICAM-1 mRNA expression (data not shown). Thus, these data demonstrate that soluble bacterial cell wall components, primarily LPS, rather than bacterial attachment, invasion, or secreted proteins mediate epithelial cell activation and that induction of ICAM-1 expression does not involve production of intermediate proteins.

B. vulgatus Stimulates IRAK-1 Degradation, IκBα Phosphorylation/Degradation, RelA Phosphorylation, and NF-κB Activation in IEC-6 Cells—We have demonstrated previously (52) that IRAK-1, a signaling molecule involved in the IL-1R/TLR pathway, is rapidly degraded in CaCO-2 cells upon IL-1 stimulation. To investigate the role of IRAK-1 in *B. vulgatus* signal transduction, IEC-6 cells were stimulated with bacteria for various time points, and IRAK-1 protein levels were determined by Western blot analysis. Fig. 2 shows that IRAK-1 protein levels consistently decreased in IEC-6 cells after at least 1 h of *B. vulgatus* stimulation (*1st panel*), whereas β-actin levels remained constant (*5th panel*). IRAK-1 degradation was not affected by the proteasome inhibitor MG132 (20 μM; Fig. 2*B*). This suggests that IRAK-1 is utilized by *B. vulgatus* to signal to IEC-6 cells.

IκBα, the cytoplasmic inhibitor of NF-κB, is a downstream target of the TLR4 pathway. We next sought to determine the level of IκBα phosphorylation and degradation in *B. vulgatus*-stimulated IEC-6 cells using Western blot analysis. As shown in Fig. 2*A* (*3rd panel*), a weak phosphorylated immunoreactive

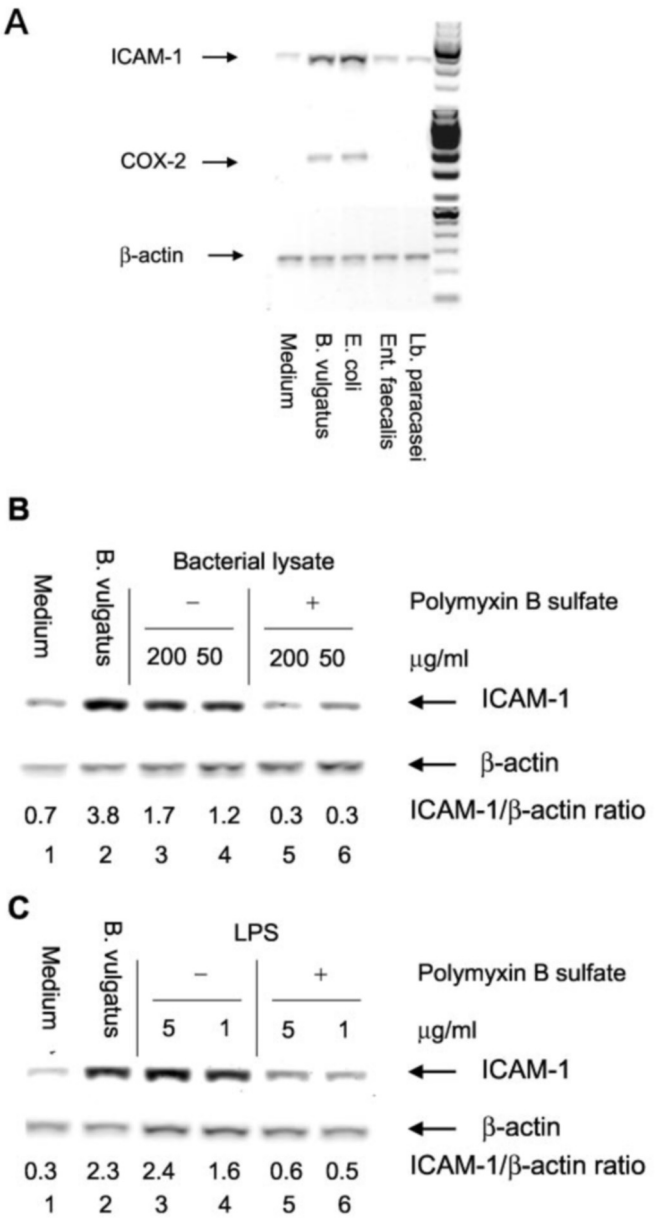

FIG. 1. ***A–C*, ICAM-1 and COX-2 mRNA expression in bacteria-stimulated IEC-6 cells as determined by RT-PCR.** IEC-6 cells were stimulated for 6 h with Gram-negative non-pathogenic *B. vulgatus* and *E. coli* as well as Gram-positive *E. faecalis* and *L. paracasei* (5×10^7 cfu/ml) (*A*), *B. vulgatus* (5×10^7/ml) and *B. vulgatus* lysate (200 or 50 μg total protein/ml) (*B*), and *B. vulgatus* (5×10^7/ml) and LPS (5 or 1 μg/ml) in the presence or absence of polymyxin B sulfate (150 μg/ml) (*C*). Total RNA was extracted, reverse-transcribed, and amplified using specific ICAM-1 and COX-2 primers. PCR products were run on a 2% agarose gel and stained with GelStar. No bacteria were present in the medium control. Results are representative of three independent experiments. Values are given as ICAM-1/β-actin ratios after densitometric analysis.

IκBα band is detected, which correlates with increased IκBα degradation after 2 h in *B. vulgatus*-stimulated IEC-6 cells, which is then slowly resynthesized at 3–4 h (*2nd panel*). The addition of the proteasome inhibitor MG132 (20 μM) completely blocked *B. vulgatus*-induced IκBα degradation (Fig. 2*B*, *2nd panel*) and allowed the accumulation of the ordinarily unstable phosphorylated IκBα (*3rd panel*).

RelA phosphorylation has been shown to increase NF-κB transcriptional activity (35). To evaluate the potential of non-pathogenic bacteria to induce RelA phosphorylation in IEC-6

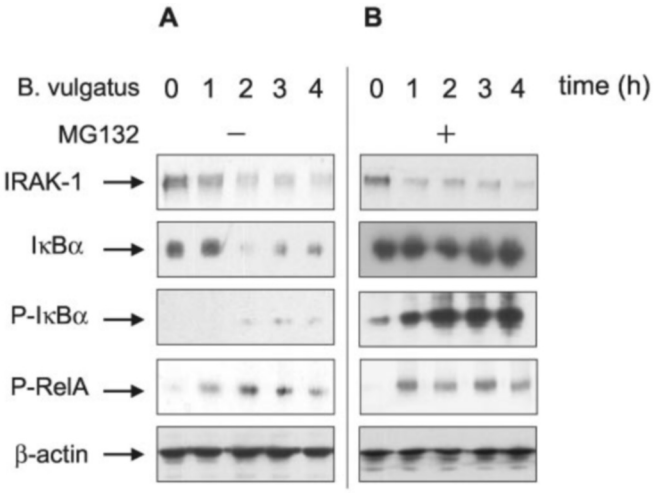

FIG. 2. ***B. vulgatus*-induced IRAK-1 degradation, IκBα degradation/phosphorylation, and RelA phosphorylation in IEC-6 cells.** IEC-6 cells were stimulated with *B. vulgatus* (5×10^7 cfu/ml) or medium alone for 0–4 h. Where indicated IEC-6 cells were pretreated for 1 h with the proteasome inhibitor MG132 (20 μM). Total protein was extracted, and 20 μg of protein was subjected to SDS-PAGE followed by IRAK-1, IκBα, phospho-IκBα, phospho-RelA, and β-actin immunoblotting using the ECL technique. These results are representative for two independent experiments.

cells, Western blot analysis was performed with RelA antibody detecting phosphorylated serine 536 residue. As shown in Fig. 2 (*4th panel*), *B. vulgatus* induced RelA phosphorylation after 1 h of stimulation in the presence or absence of MG132 (20 μM).

Following IκBα degradation, the RelA subunit of NF-κB is liberated and translocates to the nucleus. We then used EMSA to determine the NF-κB DNA binding activity in *B. vulgatus*-stimulated IEC-6 cells. As shown in Fig. 3*A*, NF-κB binding activity was induced following 3–4 h of bacterial stimulation, which correlates with nuclear RelA localization (data not shown). The presence of the p50/p65 NF-κB subunits in the binding complex was then demonstrated using monoclonal antibodies (Fig. 3*B*). Incubation of stimulated nuclear extracts with anti-p50 or anti-p65 but not with c-Jun or c-Fos antibodies abolished the binding activity. In summary, these data demonstrate that *B. vulgatus* signals through the IκB/NF-κB pathway by using proximal and distal components of the IL-1 receptor/TLR signaling cascade.

Inhibition of B. vulgatus-induced NF-κB Transcriptional Activity and Gene Expression by Ad5IκBαAA and Ad5dnIKKβ—To dissect further the signal transduction involved in *B. vulgatus*-induced NF-κB activation, we utilized adenoviral gene delivery of various signaling molecules involved in NF-κB activation. We have demonstrated previously that cytokine-induced NF-κB activation and IEC gene expression is modulated by adenoviral delivery (Ad5) of the IκBα super-repressor (Ad5IκBαAA) (45), the dominant negative (dn) IKKβ (Ad5dnIKKβ) (53), and the Ad5dnTRAF-2 (47). Immunofluorescent staining of HA- or FLAG-tagged molecules showed an infection efficiency of >85% at an m.o.i. of 50, in accordance with previous reports (45, 47), and Western blot analysis of protein extracts showed expression for all exogenous mutant molecules at an m.o.i. of 50–100 (data not shown). To determine the role of various signaling molecules in *B. vulgatus*-induced NF-κB transcriptional activity, we used a κB-luciferase reporter gene delivered by adenoviral vector (AdκBLUC). IEC-6 cells were infected for 12 h with Ad5κBLUC alone or in combination with Ad5IκBαAA, Ad5dnIKKβ, Ad5dnTAK1, Ad5dnTRAF-2, or Ad5GFP and then stimulated with *B. vulgatus* for an additional 12h. Fig. 4*A* shows that *B.*

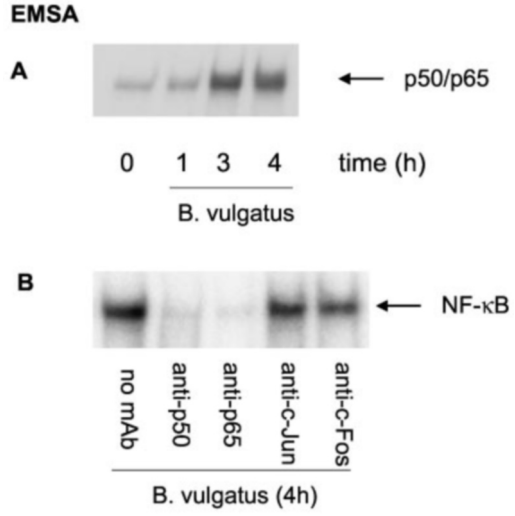

FIG. 3. ***A* and *B*, *B. vulgatus*-induced NF-κB DNA binding activity in IEC-6 cells.** *A*, IEC-6 cells were stimulated with *B. vulgatus* (5×10^7 cfu/ml) or medium alone for 0–4 h, and nuclear extracts (5 μg) were tested for NF-κB DNA binding activity by EMSA. *B*, incubation of stimulated nuclear extracts with anti-p50 and anti-p65 mAb, but not with anti-c-Jun and anti-c-Fos mAbs abolished NF-κB DNA binding activity. These results are representative for two different experiments.

vulgatus induced a 7.5-fold increase in luciferase activity in IEC-6 cells, which is significantly inhibited by Ad5IκBαAA (83%) and Ad5dnIKKβ (75%). Although Ad5dnTAK1 reduced luciferase activity by 42%, a statistically significant decrease was not observed ($p = 0.05$), suggesting a partial involvement of TAK1 in *B. vulgatus*-induced NF-κB transcriptional activity. Expression of HA- or FLAG-tagged dominant negative molecules in adenoviral infected IEC-6 cells is shown in the *upper panel*. Consistently with the data obtained by immunofluorescent nuclear RelA staining (data not shown), dnTRAF-2 failed to inhibit the *B. vulgatus*-mediated NF-κB induction. To demonstrate the functional activity of dnTRAF-2 and dnTAK1, IEC- 6 cells were infected with Ad5κBLUC and then stimulated with TNF (5 ng/ml) and IL-1β (10 ng/ml) for additional 12 h. As shown in Fig. 4*B*, TNF- and IL-1β-induced luciferase activity was significantly reduced by Ad5dnTRAF-2 (88%; $p < 0.01$) and Ad5dnTAK1 (43%; $p = 0.04$) respectively, compared with non-infected cells. No significant blockade was observed in Ad5GFP-infected cells.

To determine the role of TLR4 in *B. vulgatus*-induced NF-κB activation, CaCO-2 cells were used in liposome-based transfection studies because IEC-6 cells are not permissive to transfections. TLR4 has been shown to be expressed in IEC at the mRNA and at the protein levels (36, 37). Accordingly, our CaCO-2 cells expressed TLR4 and MD-2 mRNA (Fig. 5*A*), even though the expression levels were significantly lower compared with the highly LPS-responsive polymorphonuclear cells. We next investigated the role of TLR4 in the *B. vulgatus*-induced NF-κB transcriptional activity using κB-luciferase reporter gene activity. CaCO-2 cells were transfected for 12 h with $(\kappa B)_3$-LUC alone or in combination with dnTLR4 and then stimulated with *B. vulgatus* for 12 h. Fig. 5*B* shows that *B. vulgatus* induced a 2.6-fold increase in luciferase activity compared with the medium control, which was significantly inhibited by dnTLR4 (fold increase of 1.2; $p < 0.05$). Specificity for the inhibition of *B. vulgatus*-induced NF-κB transcriptional activity by dnTLR4 was shown in Fig. 5*C*. The stimulation of CaCO-2 cells with 50, 75, or 100 pg/ml of IL-1β induced similar low levels of NF-κB transcriptional activity as *B. vulgatus,* and transfection of CaCO-2 cells with dnTLR4 did not inhibit IL-1β-induced luciferase activity.

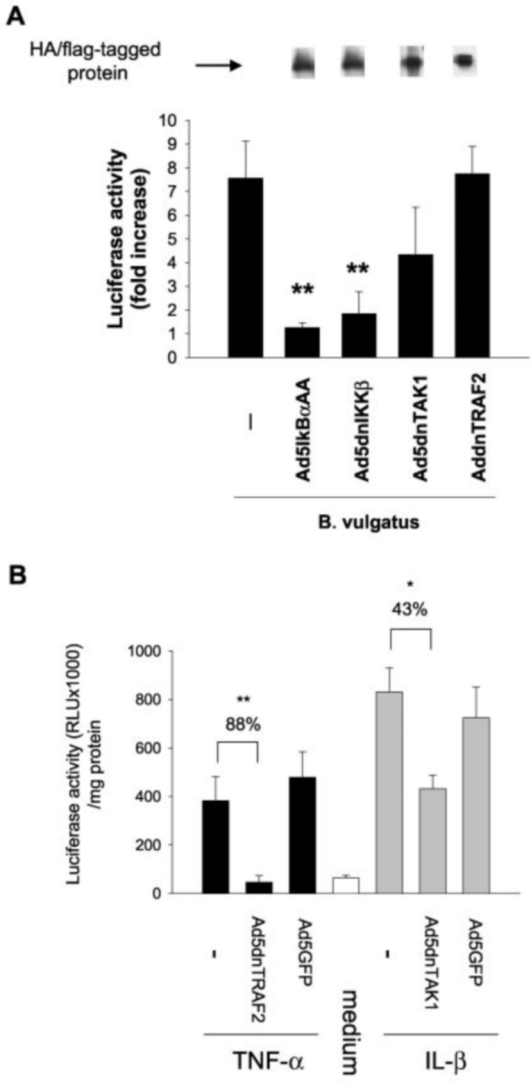

FIG. 4. ***A* and *B*, effect of adenoviral dominant negative molecules on *B. vulgatus*-mediated κB-luciferase activity in IEC-6.** IEC-6 cell were infected with Ad5NF-κB-LUC (m.o.i. of 5). Cells were washed and co-infected for an additional 12 h with Ad5IκBαAA, Ad5dnIKKβ, Ad5dnTAK1, Ad5dnTRAF-2, and control Ad5GFP (m.o.i. of 50). Finally, IEC-6 cells were stimulated for 12 h with *B. vulgatus* (5×10^7 cfu/ml), TNF (5 ng/ml), IL-1β (10 ng/ml), or medium alone. Cell extracts and the luciferase assay were performed as described under "Materials and Methods," and results were normalized for extract protein concentrations. *A*, *B. vulgatus*-induced luciferase activity is expressed as fold increase over control determined as the mean of three independent experiments measured in triplicate. Western blot analysis shows expression of dominant negative mutant molecules of representative experiments in the *upper panel*. *B*, effect of Ad5dnTRAF-2 and Ad5dnTAK1 on TNF- and IL-1β-mediated reporter gene activity, respectively. Statistical analysis was performed by using Student's *t* test, and significance is indicated as follows: *, $p < 0.05$; **, $p < 0.01$.

We next investigated the role of Ad5IκBαAA and Ad5dnIKKβ in *B. vulgatus*-induced ICAM-1 gene expression in IEC-6 cells. Fig. 6*A* shows that *B. vulgatus* (MFI of 125 ± 4.8), *B. vulgatus* lysate (200 μg/ml) (MFI of 64–96 ± 0.6), and IL-1β (MFI of 143 ± 1.8) stimulation increased ICAM-1 protein expression on the surface of IEC-6 cells by 55, 50, and 78%, respectively, when compared with the unstimulated control. *L. paracasei* did not induce ICAM-1 protein expression suggesting specificity for *B. vulgatus*-induced ICAM-1 protein expression. RT-PCR analysis showed that the bacteria-induced ICAM-1 mRNA expression was blocked by IκBαAA super-repressor and dnIKKβ, confirming the absolute requirement of

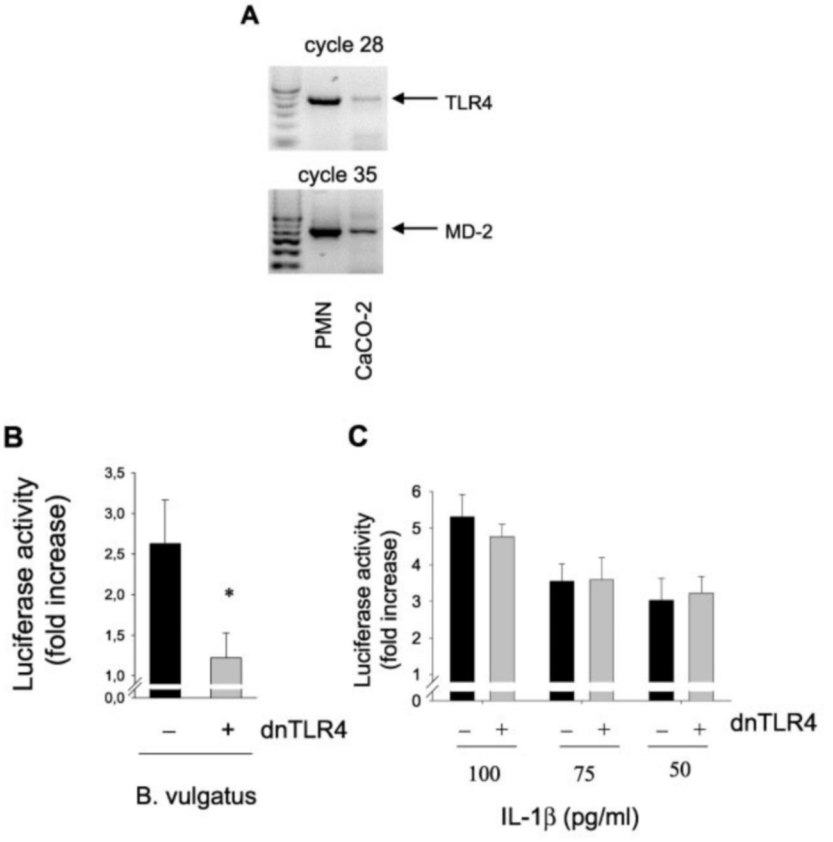

FIG. 5. ***A–C*, transfection of dominant negative TLR4 in CaCO-2 cells inhibited *B. vulgatus*-induced NF-κB transcriptional activity.** *A*, CaCO-2 total RNA was extracted, reverse-transcribed, and amplified using specific TLR4 and MD-2 primers. PCR products were run on a 2% agarose gel and stained with GelStar. *B* and *C*, CaCO-2 cells were transfected with dominant negative TLR4 (0.8 μg) or κB_3-luciferase (1 μg) alone or in combination for 12 h. Cells were then stimulated with *B. vulgatus* (5×10^7 cfu/ml) (*B*) or IL-1β at 50, 75, and 100 pg/ml (*C*) for 12 h, and luciferase activity was determined as described under "Materials and Methods." These results are representative of two different experiments.

these signaling molecules in *B. vulgatus*-induced ICAM-1 gene expression as well as NF-κB activation (Fig. 6*B*). In order to confirm the role of NF-κB on ICAM-1 gene expression, we investigated the effect of Ad5IκBαAA and Ad5dnIKKβ on *B. vulgatus*-induced ICAM-1 protein synthesis using FACS analysis. ICAM-1 protein expression in *B. vulgatus*-stimulated IEC-6 cells was significantly inhibited by Ad5IκBαAA (49.7%; MFI of 65 ± 1.7) and Ad5dnIKKβ (15.5%; 109 ± 4.2) (Fig. 6*C*) but not with the control Ad5LacZ (MFI of 129 ± 1.7) (data not shown). Thus, we showed that non-pathogenic enteric *B. vulgatus* activates the NF-κB signaling pathway in IEC by using components of the IL-1R/TLR4 signaling pathway.

Inhibition of B. vulgatus-induced RelA Phosphorylation and NF-κB Transcriptional Activity by Ad5dnIKKβ and Wortmannin—B. vulgatus induced RelA serine phosphorylation in IEC-6 cells (Fig. 2), CaCO-2 cells (Fig. 9*A*), and primary IEC (Fig. 8, *A* and *C*); this post-translational modification is associated with increased NF-κB transcriptional activity. To dissect the signaling pathway involved in *B. vulgatus*-induced RelA phosphorylation, we used the pharmacological inhibitor triptolide (100 ng/ml), an inhibitor of NF-κB transcriptional activity (54), and wortmannin (100 nM), an inhibitor of the PI3K. In addition, we used Ad5dnIKKβ and Ad5IκBαAA to selectively block IKK activity and IκBα degradation, respectively. As seen in Fig. 7*A*, *B. vulgatus* rapidly (30 min) induced RelA serine 536 and Akt serine 473 phosphorylation in IEC-6 cells (1 h), which is still detected at 2 h of treatment. Interestingly, inhibition of PI3K or IKKβ totally blocks *B. vulgatus*-induced RelA Ser-536 phosphorylation. Wortmannin but not Ad5dnIKKβ blocked Akt phosphorylation, demonstrating the key role of PI3K in *B. vulgatus*-induced Akt activation (Fig. 7*B*). As opposed, Ad5IκBαAA or triptolide failed to prevent bacteria-induced RelA phosphorylation. To document further the effect of *B. vulgatus* on RelA phosphorylation, we immunoprecipitated IKKγ and performed a kinase assay using a GST fusion protein containing the p65 transactivating domain (p65TAD; amino acids 1–305 and 354–551) as a substrate. Fig. 7*B* demonstrates that GST-p65TAD-(354–551) but not GST-p65TAD-(1–305) is phosphorylated in *B. vulgatus*-stimulated IEC-6 with a kinetic similar to endogenous RelA Ser-536 phosphorylation (Fig. 7*C*). Interestingly, *B. vulgatus*-stimulated GST-p65TAD-(354–551) phosphorylation is inhibited in both Ad5dnIKKβ-infected and wortmannin-treated but not in triptolide-exposed IEC-6 cells (Fig. 7*D*), in accordance with the data presented in Fig. 7*A*. *B. vulgatus*-induced NF-κB transcriptional activity is blocked by both triptolide and wortmannin treatment (Fig. 7*E*), suggesting that triptolide prevents NF-κB activity independently of RelA phosphorylation. These data demonstrate that RelA phosphorylation mediated by the PI3K/Akt and IKKβ is a critical step for *B. vulgatus*-induced NF-κB transcriptional activity in IEC-6 cells.

B. vulgatus-induced IκBα/RelA Phosphorylation and ICAM-1 Gene Expression in Primary Rat IEC—The physiological relevance of *B. vulgatus*-induced NF-κB signaling in the nontransformed small intestinal epithelial cell line IEC-6 was validated using primary rat IEC. To exclude the effect of prior bacterial interaction with the epithelium *in vivo*, we isolated IEC from germ-free HLA-B27 transgenic and wild type rats. Although primary IEC rapidly undergo apoptosis when detached from their matrix, 70–80% of the cells remained viable during the experiment. Freshly isolated IEC from the small and large intestine of wild type rats were stimulated for 4 h with *B. vulgatus*, *L. paracasei*, and medium alone. IκBα phosphorylation/degradation and IKKα/β as well as RelA phosphorylation were measured as an early read-out for NF-κB signaling. As shown in Fig. 8*A*, IκBα degradation and IKKα/β and RelA phosphorylation in primary small intestinal epithelial cells were induced after 1 h of stimulation with *B. vulgatus* but not with medium control, confirming the potential of certain non-pathogenic Gram-negative bacteria to induce NF-κB acti-

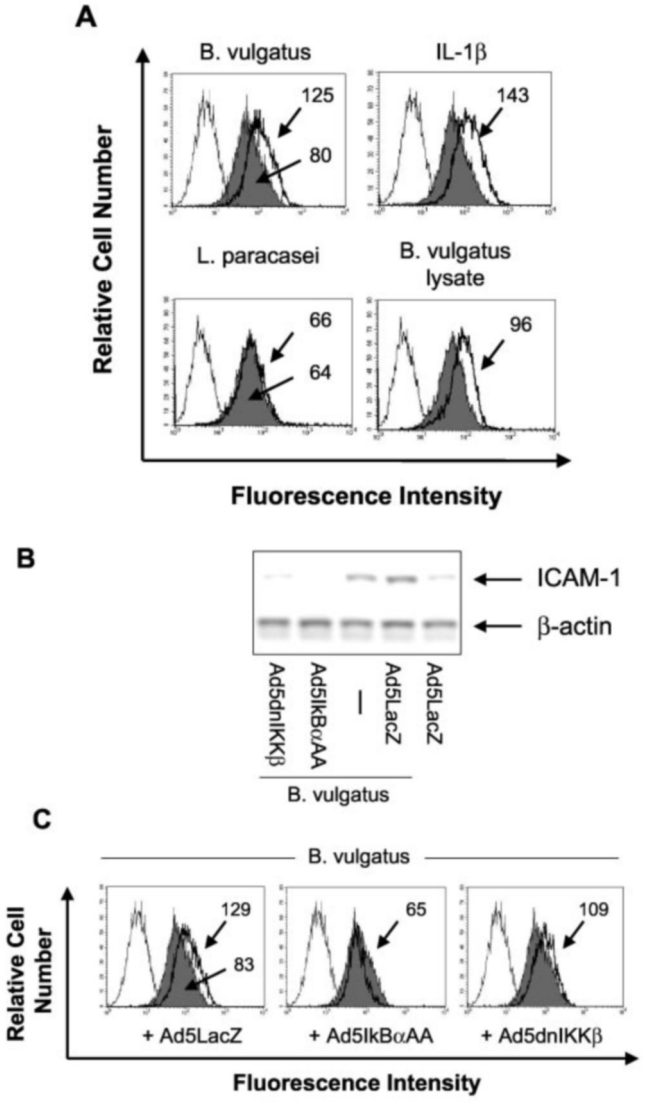

FIG. 6. ***A–C*, adenoviral dominant negative IKK*β* and IκB*α*AA inhibit *B. vulgatus*-induced ICAM-1 gene expression in IEC-6.** *A*, uninfected IEC-6 cells were stimulated for 24 h with *B. vulgatus* (10^7 cfu/ml), *L. paracasei* (5×10^7 cfu/ml), *B. vulgatus* lysate (200 μg/ml), and IL-1β (10 ng/ml). FACS analysis was used to determine ICAM-1 protein expression. *B* and *C*, IEC-6 cells were infected with Ad5IκBαAA, Ad5dnIKKβ, and control Ad5LacZ (m.o.i. of 50) for 12 h. *B*, cells were washed and stimulated with *B. vulgatus* (5×10^7 cfu/ml) or medium alone for 6 h. Total RNA was extracted, reverse-transcribed, and amplified using specific ICAM-1. PCR products were run on a 2% agarose gel and stained with GelStar. *C*, cells were washed and stimulated with *B. vulgatus* (5×10^7 cfu/ml) or medium alone for 24 h. FACS analysis was used to determine ICAM-1 protein expression. The *shaded* area in each panel represents ICAM-1 staining for the medium control. The *open area outlined* by the *darker lines* represents ICAM-1 staining of bacteria/cytokine-stimulated cells. Isotype control antibodies were used to determine unspecific staining (*open area* with *thin lines*). *Numbers* represent the mean fluorescent intensity of triplicates. Results are representative for three different experiments.

vation in "naive" IEC. Fig. 8*B* shows similar levels of phosphorylated IκBα in primary IEC derived from small or large intestine of germ-free HLA-B27 transgenic as well as wild type rats in the presence of the proteasome inhibitor MG132 after stimulation for 4 h with *B. vulgatus* but not *L. paracasei* or medium. This suggests that primary IEC from germ-free HLA-B27 transgenic as well as wild type rats have similar potential to respond to the Gram-negative but not Gram-positive bacterial challenge.

To confirm *B. vulgatus*-induced signaling to the NF-κB pathway *in vivo*, we measured RelA phosphorylation and ICAM-1 protein expression in freshly isolated IEC from *B. vulgatus* mono-associated wild type rats in comparison to the germ-free control. As shown in Fig. 8*C*, RelA phosphorylation was clearly induced in IEC derived from cecum and colon but not small intestine of *B. vulgatus* mono-associated wild type rats after 3 days in the gnotobiotic isolator. Phosphorylated RelA was not detectable in IEC from germ-free wild type rats. In addition, FACS analysis showed that the percentage of ICAM-1-positive IEC was significantly increased in the cecum (7–45%) and colon (44–60%) of *B. vulgatus* mono-associated wild type rats compared with the germ-free controls (Fig. 8*D*). The percentage of ICAM-1-positive IEC derived from the small intestine was similar in *B. vulgatus* mono-associated and germ-free wild type rats; however, the density of ICAM-1 protein expression was increased by 27% in IEC from *B. vulgatus* mono-associated wild type rats (MFI 33 to MFI 42). In conclusion, these results demonstrate that the NF-κB signaling pathway is activated in IEC from *B. vulgatus* mono-associated wild type rats shortly after colonization. RelA phosphorylation and ICAM-1 protein expression were induced in IEC derived from cecum and colon but not small intestine, which correlates with the distribution and concentration of the bacteria in the intestine.

Immune-mediated Inhibition of B. vulgatus-induced NF-κB Transcriptional Activation in CaCO-2 Cells—IEC need to retain hyporesponsiveness to luminal enteric bacteria in order to tolerate the constant antigenic drive of the intestinal microflora. Previous results (42, 43) have shown that bacteria-mediated activations of IEC are modulated in the presence of PBMC using transwell co-cultures. To investigate the question of whether *B. vulgatus*-activated IEC initiate tolerance mechanisms, CaCO-2 cells were co-cultured with PBMC. Interestingly, the presence of PBMC inhibited *B. vulgatus*-induced activation of the NF-κB signaling pathway in CaCO-2 cells at the level of IκBα degradation but allowed IRAK-1 degradation and RelA and IκBα phosphorylation (Fig. 9*A*). In addition, *B. vulgatus* induced NF-κB transcriptional activity was significantly inhibited in the presence of PBMC and PBL but not monocytes (Fig. 9*B*). IL-1β mediated signal transduction in CaCO-2 cells was not affected by co-cultured immune cells (Fig. 9*C*). This suggests that cross-talk between IEC and distinct populations of immune cells may influence non-pathogenic bacteria signal transduction to IEC.

DISCUSSION

In this study, we demonstrate that a non-pathogenic enteric Gram-negative *B. vulgatus* bacteria strain has the ability to signal to the NF-κB system in IEC lines by inducing IRAK-1 degradation, IκBα phosphorylation and degradation, NF-κB DNA binding activity, and NF-κB transcriptional activity. Interestingly, *B. vulgatus* also stimulates endogenous RelA serine 536 and GST-p65TAD phosphorylation (Ser-529/Ser-536). Although previous studies using pathogenic strains of invasive *Salmonella* species (55), enteroadherent *E. coli* (18), and *Cryptosporidium* (56) show NF-κB activation and chemokine expression in IEC lines, this is the first demonstration that non-pathogenic bacteria induce gene expression through NF-κB translocation and RelA phosphorylation. *B. vulgatus*-induced RelA phosphorylation is IKKβ- and PI3K/Akt-dependent. The physiological relevance of *B. vulgatus*-induced NF-κB signal transduction was demonstrated in primary rat IEC. First, we showed that the potential of primary naive IEC to initiate IκBα phosphorylation/degradation and IKKα/β and RelA phosphorylation in response to *B. vulgatus* stimulation. Second, and most importantly, we showed that RelA phosphorylation and ICAM-1 protein expression are induced in primary IEC derived from *B. vulgatus* mono-associated wild type rats shortly after

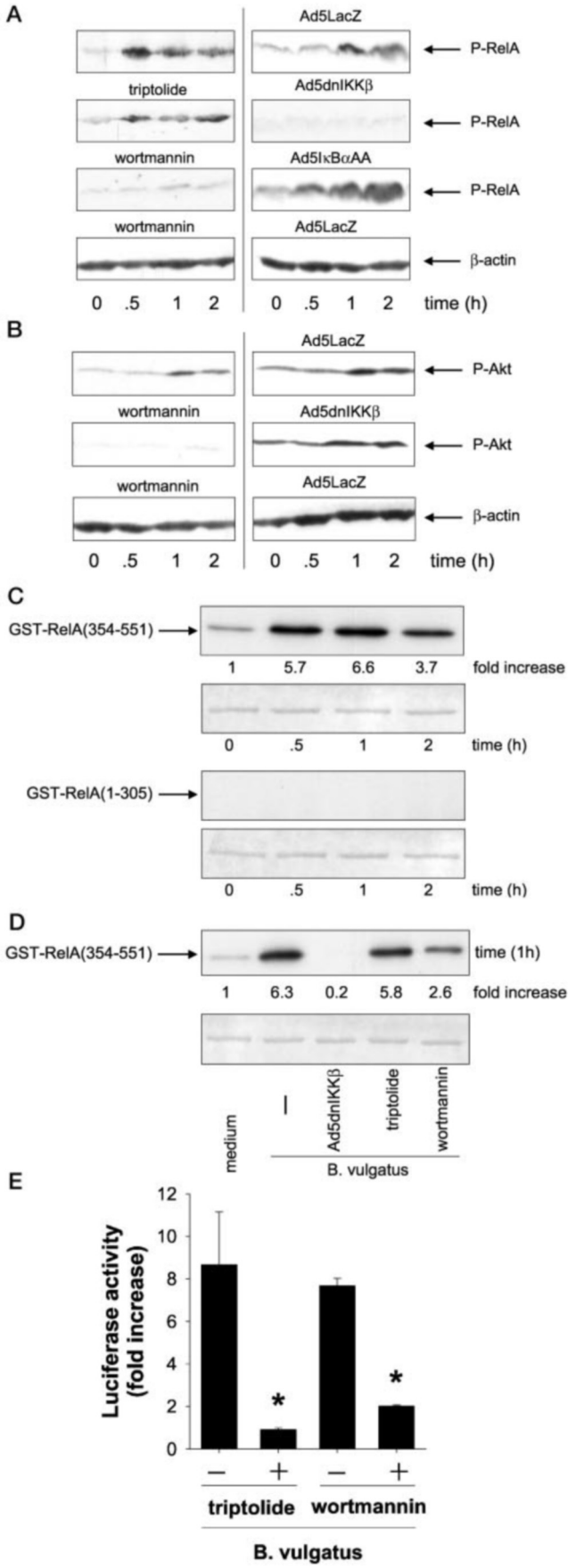

FIG. 7. ***A–E*, IKKβ and PI3K/Akt contribute to *B. vulgatus*-induced RelA phosphorylation and NF-κB transcriptional activity.** IEC-6 cells were stimulated for 30 min, 1 or 2 h with *B. vulgatus* (5×10^7 cfu/ml) or medium alone in the presence or absence of triptolide (100 ng/ml) or wortmannin (100 nM). Where indicated IEC-6 cells were infected with Ad5dnIKKβ, Ad5IκBαAA, or Ad5LacZ (50 m.o.i.) for 12 h. *A* and *B*, total protein was extracted, and 20 μg of protein was subjected to SDS-PAGE followed by phospho-RelA, phospho-Akt, and β-actin immunoblotting using the ECL technique. *C* and *D*, *B. vulgatus*-induced serine RelA phosphorylation was determined by 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-(1–305) or GST-p65-(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*). *E*, IEC-6 cells were infected with Ad5κB-LUC (5 m.o.i.) for 12 h and then were stimulated with *B. vulgatus* (5×10^7 cfu/ml) in the presence or absence of triptolide (100 ng/ml) or wortmannin (100 nM). Cell extracts and the luciferase assay were performed as described under "Materials and Methods," and results were normalized for extract protein concentrations. These results are representative for two different experiments.

bacterial colonization (3 days). The pivotal role of the IκB/NF-κB transcriptional system in *B. vulgatus* signaling to IEC was clearly demonstrated using adenoviral delivery of Ad5IκBαAA and Ad5dnIKKβ, which completely blocked bacteria-induced NF-κB transcriptional activity and ICAM-1 mRNA and protein expression in IEC-6 cells. Bacterial activation of IEC was specific for Gram-negative bacteria but was not dependent on bacterial adherence or invasion, as demonstrated by the use of Gram-negative bacterial lysates and LPS for IEC stimulation. This shows that non-pathogenic as well as pathogenic bacteria utilize the NF-κB signaling cassette to induce gene expression in IEC.

It has been shown that LPS signals through the pattern recognition receptor TLR4 to activate multiple signaling cascades including the IκB/NF-κB system (25, 57). TLR4 protein was detected in tissue sections from patients with ulcerative colitis and Crohn's disease, suggesting the potential ability of intestinal epithelial cells to respond to luminal Gram-negative bacteria or LPS (36). Cario *et al.* (36, 37) showed that human IEC lines express TLR4 and CD14 and respond to high doses (5–10 μg/ml) of LPS by inducing NF-κB DNA binding activity. However, the exact molecular mechanisms of Gram-negative bacteria or LPS signal transduction in IEC are still unclear.

We provide evidence that TLR4 and several IL-1 receptor/TLR4 signaling proteins participate in *B. vulgatus*-mediated NF-κB activation. First, we showed that the mRNA of TLR4 and its co-receptor MD-2 were expressed in CaCO-2 and dnTLR4 blocked *B. vulgatus* but not IL-1β-stimulated NF-κB transcriptional activity, demonstrating a selective role of TLR4 in *B. vulgatus* signaling to NF-κB in IEC. The relatively low expression of TLR4 and MD-2 may explain the weak induction of NF-κB transcriptional activity following *B. vulgatus* stimulation in CaCO-2 cells. Interestingly, similar findings were reported for the stimulation of CaCO-2 cells with LPS (58). Of importance, these authors were able to enhance colonic IEC responsiveness to LPS by transfecting expression vectors encoding for MD-2 and TLR4, suggesting that both receptors are important in conveying LPS signaling to NF-κB. In addition, we showed that IRAK-1 protein, a signaling molecule involved in the IL-1R/TLR pathway, is degraded in *B. vulgatus*-stimulated IEC-6 cells. Previous studies have shown that LPS-induced NF-κB activation is partially impaired in macrophages isolated from IRAK-$1^{-/-}$ mice (59), whereas macrophages isolated from IRAK-$4^{-/-}$ are strongly unresponsive (60). It remains to be seen whether *B. vulgatus* utilizes IRAK-4 to signal to NF-κB in IEC.

Although TAK1 has been linked to LPS-mediated NF-κB signaling in macrophages (61), Ad5dnTAK1 only partially inhibited *B. vulgatus*-induced RelA nuclear translocation (data

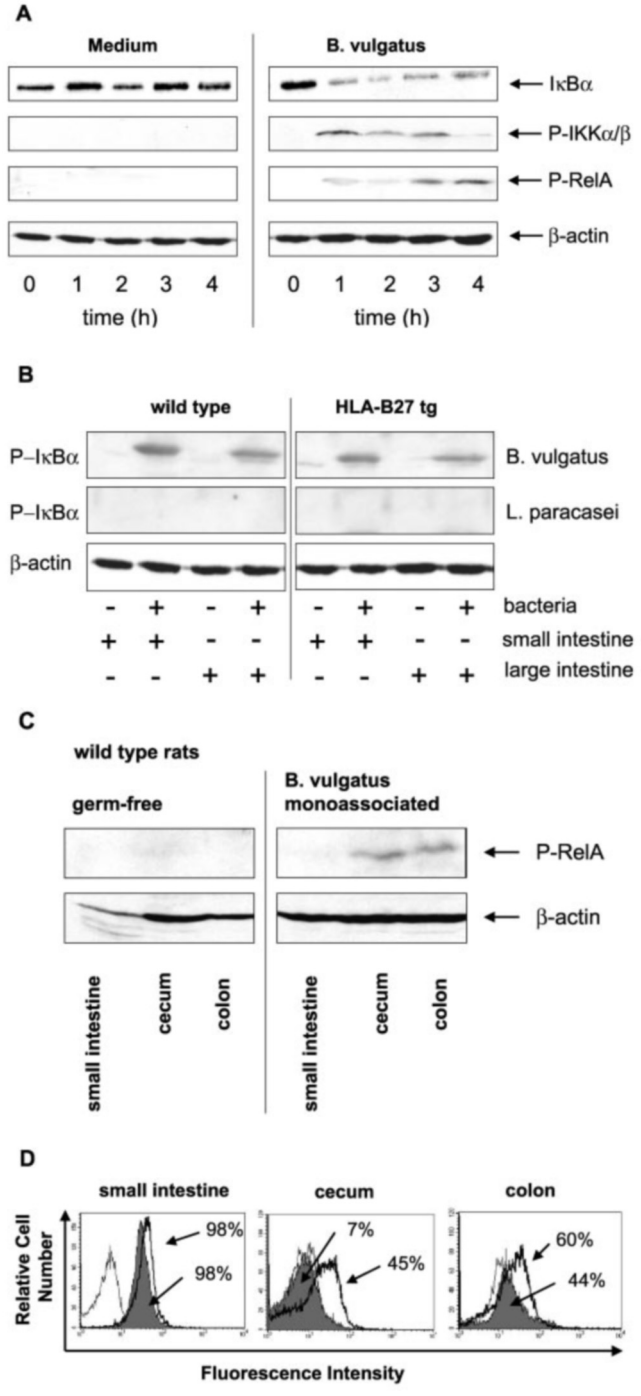

FIG. 8. ***A–D, B. vulgatus*-induced NF-κB activation in primary IEC.** *A*, detection of IκBα degradation and IKKα/β and RelA phosphorylation in primary small intestinal epithelial cells isolated from germ-free wild type rats. Cells were seeded in 12-well tissue culture plates at a concentration of 2×10^6/ml and stimulated with 5×10^7 cfu/ml *B. vulgatus*, *L. paracasei*, or medium alone for 0–4 h. *B*, detection of phospho-IκBα in primary IEC isolated from small and large intestine of germ-free HLA-B27 transgenic and wild type rats. Cells (2×10^6/ml) were pretreated for 30 min with the proteasome inhibitor MG132 (20 μM) and stimulated with *B. vulgatus*, *L. paracasei*, and medium for 4 h. *C*, detection of phospho-RelA in primary IEC derived from small intestine, cecum, and colon from *B. vulgatus* mono-associated (3 days) wild type rats or germ-free controls. Total protein was extracted, and 20 μg of protein was subjected to SDS-PAGE followed by IκBα, phospho-IκBα, phospho-IKKα/β, phospho-RelA, and β-actin immunoblotting using the ECL technique. *D*, detection of ICAM-1 protein expression on primary IEC derived from small intestine, cecum, and colon from *B. vulgatus* mono-associated (3 days) wild type rats (*dark line*) or germ-free controls (*shaded area*). The numbers represent the percentage of ICAM-1 positive cells relative to the isotype control (*thin line*).

not shown) and transcriptional activity. Interestingly, *B. vulgatus*-induced NF-κB transcriptional activity and ICAM-1 gene expression is completely blocked by Ad5dnIKKβ or Ad5IκBAA. This suggests that the signaling proteins IKKβ and IκBα constitute critical control points of Gram-negative bacterial signaling to NF-κB and gene expression in IEC. Together, these results suggest that NF-κB activation by *B. vulgatus* likely requires TLR4, IRAK-1, IKK, and IκBα in IEC.

An emerging control point in NF-κB activation is signal-induced RelA serine phosphorylation, which enhances its transcriptional activity (35). Interestingly, we found that *B. vulgatus* stimulation induced endogenous RelA 536 phosphorylation in both primary and IEC lines. In addition, immunoprecipitated IKKγ from *B. vulgatus*-stimulated cells phosphorylates GST-p65TAD-(354–551), which contains the important Ser-529 and Ser-536 residues. It has been shown that the PI3K/Akt and IKK are critical kinases for cytokine-induced RelA phosphorylation and NF-κB transcriptional activity (27, 33, 34, 62). The functional consequence of RelA phosphorylation is highlighted by the blockade of *B. vulgatus*-induced NF-κB transcriptional activity by Ad5dnIKKβ and by the pharmacological inhibitor wortmannin, both of which prevent RelA phosphorylation. The kinase as well as the signaling pathway responsible for *B. vulgatus*-induced RelA phosphorylation is still unknown. Our data demonstrate for the first time that both IKK and PI3K/Akt participate in *B. vulgatus*-mediated NF-κB transcriptional activity. Further studies are required to define the exact signaling cascade involved in bacteria-induced RelA phosphorylation and NF-κB activity.

Interestingly, although the Chinese herb derivative triptolide has been shown to inhibit NF-κB transcriptional activity (54), it failed to prevent bacteria-induced Ser-536 and Ser-529 phosphorylation. RelA is phosphorylated at various serine residues (Ser-276, Ser-529, and Ser-536) in response to either IL-1 or TNF stimulation (35, 63). Therefore, the possibility remains that triptolide interferes with the phosphorylation of serine residue 276. However, *B. vulgatus* failed to induce phosphorylation of GST-p65TAD-(1–305) which includes the Ser-276 phosphorylation site. Therefore, although triptolide blocks *B. vulgatus*-induced NF-κB transcriptional activity, its mechanism of action is unlikely to involve inhibition of RelA phosphorylation. Recruitment of transcriptional co-activator/co-repressor played an important role in the regulation of NF-κB transcriptional activity (63, 65). In addition, chromatin remodeling through modulation of histone acetylation defines access of transcription factors to gene promoter elements and therefore transcriptional activity (63–66). A possible mechanism of triptolide interference with NF-κB transcriptional activity may include blockade of transcriptional co-activator or modulation of the histone acetylation status.

The finding that commensal bacteria normally present in high concentration in the distal intestine are able to trigger NF-κB activation in IEC *in vitro* contrasts with the absence of chronic inflammation seen in the normal host. Multiple mechanisms ensure *in vivo* hyporesponsiveness of native IEC to bacteria including low expression of TLR4 and MD-2 in colonic epithelial cell lines (58) and down-regulatory signals from adjacent lamina propria immune cells. Previous results have shown that bacterial mediated activation of IEC is modulated by the presence of immunocompetent cells using transwell co-cultures (42, 43). Interestingly, the presence of human peripheral blood lymphocytes inhibited *B. vulgatus*-induced NF-κB activity by blocking IκBα degradation. However, *B. vulgatus*-induced IRAK-1 degradation and RelA phosphorylation is not inhibited in IEC/lymphocyte co-cultures, whereas IκBα degradation is strongly suppressed. This suggests that

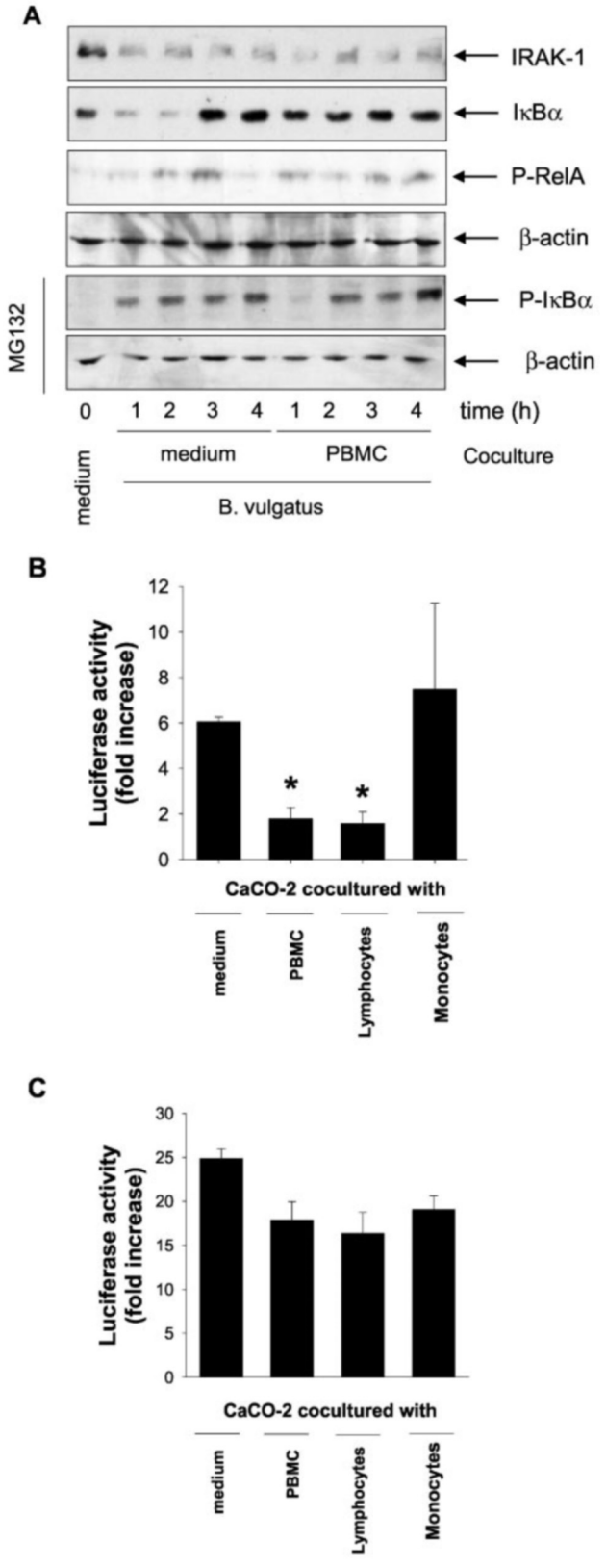

FIG. 9. ***A–C*, immune-mediated inhibition of *B. vulgatus*-induced NF-κB activation in CaCO-2 transwell cultures.** *A*, CaCO-2 cells were stimulated with *B. vulgatus* (5×10^7 cfu/ml) for 0–4 h. Where indicated CaCO-2 cells were preincubated with the proteasome inhibitor MG132 (20 μM) for 30 min. Total protein was extracted from CaCO-2 cells, and 20 μg of protein was subjected to SDS-PAGE followed by IRAK-1, IκBα, phospho-IκBα, phospho-RelA, and β-actin immunoblotting using the ECL technique. *B* and *C*, CaCO-2 cells were infected with Ad5κB-LUC (5 m.o.i.) for 12 h. PBMC, PBL, and monocytes were purified from healthy donors ($n = 3$) as described under "Materials and Methods." PBMC, PBL, or monocytes (2×10^6/ml) were added to the basolateral side of the transwell cultures (filter membrane pore size 0.4 μm), and CaCO-2 cells were apically stimulated with *B. vulgatus* (5×10^7 cfu/ml), IL-1 (5 ng/ml), or medium alone for 12 h. Luciferase activity was determined as described under "Materials and Methods." These results are representative for 3 different experiments including 3 different donors. Statistical analysis was performed by using Student's *t* test, and significance is indicated as follows: *, $p < 0.05$.

the upstream signaling cascade leading to IκBα and RelA phosphorylation is not blocked by the presence of lymphocytes but rather points out an inhibition at the level of proteosome and/or ubiquitination. Interestingly, non-virulent *Salmonella* strains inhibit NF-κB activity by preventing IκB ubiquitination, possibly through inhibition of the IκB ubiquitin ligase (67). Further studies would be required to dissect the precise mechanism of immune cell-mediated blockade of IκBα degradation. In addition, our recent studies[2] show that the immunoregulatory cytokine TGF-β1 inhibits *B. vulgatus*-mediated NF-κB transcriptional activity in IEC through the induction of the Smad signaling pathway. This suggests that the responsiveness of IEC to luminal enteric bacteria in a complex environment such as the mucosal immune system may depend on a network of communication between immune and epithelial cells and their secreted mediators.

Although various rodent models of inflammatory bowel diseases demonstrated the key role of enteric bacteria and bacterial products in initiating and perpetuating chronic immune-mediated intestinal inflammation in genetically susceptible hosts, the molecular mechanisms of bacterial signaling in the intestinal epithelium remain to be elucidated. Recent studies showed that NF-κB blockade in IEC by tissue-specific expression of the IκBαAA super-repressor exacerbates dextran sodium sulfate-induced experimental colitis (68), suggesting that in contrast to previous observations of protective effects of NF-κB blockade in trinitrobenzene sulfonic acid (TNBS)-induced chronic colitis (69), NF-κB-derived IEC gene expression may have a protective role during acute intestinal inflammation. This concept was further supported by the finding that p50 and p65 gene-deleted mice compared with wild type mice have more severe inflammation in a model of *Helicobacter hepaticus*-induced typhlocolitis (70). In addition, blocking NF-κB activity with pharmacological inhibitors during the resolution phase of carrageenan-induced acute inflammation is deleterious to the host (71). This is in line with recent findings showing that activation of TLR9 signaling by unmethylated CpG bacterial DNA decreased dextran sodium sulfate- and hapten-induced colitis (72). Therefore, NF-κB activation may have a beneficial role in the early onset of inflammation, but at the same time may be detrimental if activation persists. We hypothesize that low levels of NF-κB activity induced by non-pathogenic commensal bacteria play a role in the maintenance of intestinal homeostasis in a normal host, whereas it may be deleterious in a genetically susceptible host. Alternatively, epithelial cell NF-κB activation may be protective, whereas NF-κB activation in lamina propria cells may induce activation. In summary, our data demonstrate that components of the Gram-negative non-pathogenic *B. vulgatus* have the potential to signal through the TLR4 system to activate NF-κB and gene expression in IEC, independent of viable bacterial adhesion and invasion. Therefore, a more complete understanding of bacterial signaling in IEC will provide additional insights into the mechanisms of mucosal homeostasis and injury and may yield new therapeutic targets for intestinal inflammation.

[2] D. Haller, L. Holt, R. B. Sartor, and C. Jobin, manuscript in preparation.

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