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Am J Physiol Gastrointest Liver Physiol 293:G429-G437, 2007. First published 31 May 2007;
doi: 10.1152/ajpgi.00149.2007

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Bcl10 mediates LPS-induced activation of NF-κB and IL-8 in human intestinal epithelial cells

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Submitted 5 April 2007; accepted in final form 24 May 2007

Bhattacharyya S, Borthakur A, Pant N, Dudeja PK, Tobacman JK. Bcl10 mediates LPS-induced activation of NF-κB and IL-8 in human intestinal epithelial cells. *Am J Physiol Gastrointest Liver Physiol* 293: G429–G437, 2007. First published May 31, 2007; doi:10.1152/ajpgi.00149.2007.—Lipopolysaccharide (LPS) is recognized as an inducer of the inflammatory response associated with gram-negative sepsis and systemic inflammatory response syndrome. LPS induction proceeds through Toll-like receptor (TLR) in immune cells and intestinal epithelial cells (IEC). This report presents the first identification of Bcl10 (B-cell CLL/lymphoma 10) as a mediator of the LPS-induced activation of IL-8 in human IEC. Bcl10 is a caspase-recruitment domain-containing protein, associated with constitutive activation of NF-κB in MALT (mucosa-associated lymphoid tissue) lymphomas. The normal human IEC line NCM460, normal primary human colonocytes, and ex vivo human colonic tissue were exposed to 10 ng/ml of LPS for 2–6 h. Effects on Bcl10, phospho-IκBα, NF-κB, and IL-8 were determined by Western blot, ELISA, immunohistochemistry, and confocal microscopy. Effects of Bcl10 silencing by small-interfering RNA (siRNA), TLR4 blocking antibody, TLR4 silencing by siRNA, and an IL-1 receptor-associated kinase (IRAK)-1/4 inhibitor on LPS-induced activation were examined. Following Bcl10 silencing, LPS-induced increases in NF-κB, IκBα, and IL-8 were significantly reduced ($P < 0.001$). Increasing concentrations of LPS were associated with higher concentrations of Bcl10 protein when quantified by ELISA, and the association between LPS exposure and increased Bcl10 was also demonstrated by Western blot, immunohistochemistry, and confocal microscopy. Exposure to TLR4 antibody, TLR4 siRNA, or an IRAK-1/4 inhibitor eliminated the LPS-induced increases in Bcl10, NF-κB, and IL-8. Identification of Bcl10 as a mediator of LPS-induced activation of NF-κB and IL-8 in normal human IEC provides new insight into mechanisms of epithelial inflammation and new opportunities for therapeutic intervention.

lipopolysaccharide; inflammation; colonocytes; Toll-like receptor 4

BACTERIAL LIPOPOLYSACCHARIDE (LPS) is a structural component of the outer envelope of all gram-negative bacteria and is a highly proinflammatory molecule. When LPS is released into the circulation from the surface of replicating and dying gram-negative bacteria, LPS, either alone or in association with other endogenous factors, induces a strong response from the immune system that is responsible for much of the inflammatory response and vascular endothelial cell injury associated with gram-negative sepsis. LPS, or endotoxin, is composed of three parts, including the conserved, hydrophobic domain lipid A, the core oligosaccharide that is attached to lipid A, and the variable polysaccharide side chain, referred to as the O-antigen, that extends from the core oligosaccharide and confers antigenic specificity. LPS is detectable in the bloodstream of gram-negative septicemic patients, and levels of circulating LPS can be used to predict the development of multiorgan failure (2, 6).

Administration of LPS alone to experimental animals reconstitutes the endothelial cell injury seen after gram-negative bacterial challenge (7). LPS directly elicits several inflammatory responses in vitro including production of the proinflammatory cytokines IL-6, IL-8, and IL-1β and increased surface expression of the adhesion molecules E-selectin, ICAM-1, and VCAM-1 (2, 7, 9, 18, 25, 40). In addition to activation of these mediators in endothelial cells, distinct effects of LPS are identified in intestinal epithelial cells (IEC), including increased IL-1β, IL-6, and IL-8, and the production of these cytokines by the intestinal mucosa has been correlated with the endoscopic grade of inflammation in inflammatory bowel disease (17, 28, 31).

The present understanding of the mechanism of LPS-induced inflammation and intracellular signaling is derived predominantly from studies of immune cells; however, LPS-mediated inflammation has been characterized in alveolar epithelial cells and IECs, as well as endothelial cells and inflammatory cells, including lymphocytes, macrophages, and neutrophils (2, 7, 9, 11, 17, 18, 28, 31, 40). Toll-like receptors (TLRs) are receptors for lipid A in mammalian immune cells, human endothelial cells, and human IECs (1, 12, 27, 30). TLRs are innate immunity receptors that possess a large extracellular domain of leucine-rich repeats, a single transmembrane segment, and a smaller cytoplasmic signaling region that reacts with the adaptor protein myeloid differentiation factor 88 (MyD88) (30). The TLRs can recognize the pathogen-associated molecular pattern of LPS produced by bacteria, virus, or other pathogens, and TLR4 is recognized as an essential receptor for LPS signaling (4, 27). Mammalian TLRs share similar cytoplasmic domains with the IL-1 receptor family, and there is overlap in the signaling pathway upon ligand binding (21).

In immune cells, including lymphocytes and macrophages, several proteins mediate the downstream signaling cascades from TLR4, including MyD88, IL-1 receptor-associated kinases (IRAKs), and TNF receptor-activated factor 6 (TRAF6) (10). TRAF6 forms a complex with TAB1/TAB2/TAK1, and the kinase activity of TAK1 mediates additional downstream events (19, 32). Bcl10 (B-cell CLL/lymphoma 10), a caspase-recruitment domain containing protein composed of 233 amino acids, is recognized as a mediator of constitutive activation of

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NF-κB in mucosa-associated lymphomas (MALT) (36, 39). Bcl10 is increasingly recognized as an important mediator of signaling events (22, 38). In immune cells, Bcl10 is phosphorylated by TAK1 and combines with MALT1, leading to the activation and subsequent ubiquitination of NEMO (NF-κB essential modifier, also called IKK-γ) (8, 41). NEMO induces the phosphorylation and ubiquitination of IκBα, permitting nuclear localization of NF-κB. Nuclear translocation of NF-κB enables the activation of the IL-8 promoter and increased secretion of IL-8 (2, 4, 5, 8, 41). IL-8 induces leukocyte infiltration and additional tissue inflammatory responses.

Bacterial translocation from the intestinal lumen, defined as the passage of viable bacteria or their cell wall components from the gastrointestinal tract through the epithelial mucosa, can provoke a systemic inflammatory response, as well as the mucosal immune response. Mucosal epithelial cells can come into contact with LPS and be sites of initiation and propagation of inflammatory responses. We have recently reported a Bcl10-mediated pathway of activation of phospho-IκBα, NF-κB, and IL-8, initiated by exposure to the sulfated polygalactose carrageenan in normal human colonic epithelial cells, demonstrating for the first time that Bcl10 mediates an inflammatory cascade in human IECs, as well as in immune cells (5). Similarly, McAllister-Lucas et al. (26) determined that angiotensin II activates an inflammatory cascade in hepatocytes that is mediated by Bcl10 and involves CARMA3 and MALT1. Wang et al. (34) reported Bcl10 mediation of NF-κB activation induced by G-protein coupled receptors in human embryonic kidney 293T cells, and Klemm et al. (20) observed that Bcl10 and MALT1 control lysophosphatidic acid-induced NF-κB activation and IL-6 production in murine embryonic fibroblasts.

To determine whether the effects of LPS in IEC are also mediated by Bcl10, we investigated the pathway of LPS-induced activation of phospho-IκBα, NF-κB, and IL-8 in normal colonic epithelial cells and have identified Bcl10 as a critical mediator of an LPS-induced cascade leading to IL-8 activation.

MATERIALS AND METHODS

Cell culture of NCM460 cells and primary colonocytes. Nontransformed human colonic epithelial cell line NCM460 was grown in M3:10 media (INCELL, San Antonio, TX) and maintained at 37°C in a humidified, 5% CO_2 environment (29). Cells were harvested at 60–80% confluency following experimental treatments.

Primary cultures of normal human colonic epithelial cells were initiated from deidentified colon specimens obtained at the time of colectomy through an established protocol, approved by the Institutional Review Board of the University of Illinois at Chicago. Surgical specimens were accessed through the Tissue Bank of the University of Illinois at Chicago Hospitals and Clinics and the Department of Pathology. Patients consented to donate tissue to the Tissue Bank for research purposes. Primary cultures were established as previously described (5).

NCM460 and primary colon cells were treated with different concentrations (1, 10, and 20 ng/ml) of LPS (from *Escherichia coli* 026:B6, no. L2654, Sigma-Aldrich, St. Louis, MO) for durations of 2, 4, 6, or 24 h. The endotoxin level of the LPS from *E. coli* was reported as 500,000 endotoxin units (EU)/mg LPS. The secretion of IL-8 and the cellular content of Bcl10 and phospho-IκBα proteins in response to these treatments were assayed. In the majority of experiments, LPS 10 ng/ml for 6 h was selected as the optimal dose and duration. A second LPS (from *Salmonella enterica* serotype Minnesota; no. L4641, Sigma-Aldrich) was also used for some of the experiments. The endotoxin level of the batch of *S. enterica* was reported as 800,000 EU/mg LPS.

Immunohistochemistry of ex vivo colonic tissue. Immunohistochemistry for Bcl10 was performed on previously frozen ex vivo human colonic specimens, after 2 h of exposure to LPS (10 ng/ml). Control and treated tissue samples were processed simultaneously. Sections of 5-μm thickness were cut by microtome. Tissue sections on the slides were hydrated, and antigen retrieval was performed with DakoTarget Retrieval 10× citrate buffer solution (DakoCytomation) for 20 min in a steamer at 95°C. Slides were then equilibrated to room temperature in the same solution for 20 min, rinsed in dH_2O, placed in buffer solution for 15 min, placed in 3% H_2O_2 for 10 min, rinsed in buffer, and protein was blocked for 10 min. Slides were incubated with the Bcl10 primary antibody (Santa Cruz Biotechnology) at a 1:50 dilution overnight. Slides were rinsed in buffer, and an anti-mouse secondary antibody (DakoCytomation EnVision mouse monoclonal kit) was applied for 30 min. Slides were rinsed in buffer, then treated with 3,3′-diaminobenzidine for color detection, rinsed in H_2O, dehydrated, coverslipped with Permount, and photographed with Nikon imaging system. No enhancement or modification of the images was performed.

Confocal microscopy of human intestinal cells probed for Bcl10. NCM460 cells and primary human colonic epithelial cells were grown on collagen-coated Transwell inserts or four-chamber tissue culture slides for 24 h, and then treated preparations were exposed to LPS 10 ng/ml for 6 h. Cells were washed once in 1× PBS containing 1 mM calcium chloride (pH 7.4), fixed for 1.5 h with 2% paraformaldehyde, then permeabilized with 0.08% saponin. Preparations were washed with PBS, blocked in 5% normal goat serum, incubated overnight with Bcl10 monoclonal antibody (1:100, Santa Cruz) at 4°C, and then washed and stained with goat-anti-mouse IgG-FITC (1:100, Invitrogen). Cells were exposed for 1 h to phalloidin-Alexa Fluor 568 (Invitrogen) diluted 1:40 to stain actin and to Hoechst 33342 (1:20,000, Invitrogen) for nuclear staining. Preparations were washed thoroughly, mounted, and observed via a Zeiss LSM 510 laser scanning confocal microscope equipped with a ×63 water-immersion objective. Excitation was at 488 and 534 nm from an Ar-Kr laser and at 361 nm from a UV laser. Green and red fluorescence were detected through LP505 and 585 filters. The fluorochromes were scanned sequentially and images were recorded with Zeiss LSM Image Browser software. Single-channel and merged images are presented (14).

ELISA for IL-8. The secretion of IL-8 in the spent media of control and treated NCM460 cells and primary colon cells was measured by the DuoSet ELISA kit for human IL-8 (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. The IL-8 in the spent media was captured into the wells of a microtiter plate precoated with specific anti-IL-8 monoclonal antibody. Immobilized IL-8 was then detected by biotin-conjugated secondary IL-8 antibody and streptavidin-horseradish peroxidase (HRP). Hydrogen peroxide-tetramethylbenzidine chromogenic substrate was used to develop the color, and the intensity of color was measured at 450 nm with a reference filter of 570 nm in an ELISA plate reader (SLT, Spectra). The IL-8 concentrations were extrapolated from a standard curve plotted by using known concentrations of IL-8. The sample values were normalized with total protein content (BCA Protein assay kit; Pierce, Rockford, IL) and expressed as picograms or nanograms per milligram cellular protein.

ELISA for Bcl10. The expression of Bcl10 in NCM460 cells or primary colon cells was determined by a solid-phase sandwich ELISA designed to quantify cellular Bcl10. Control or treated cells were lysed in RIPA buffer (50 mM Tris·HCl containing 0.15 M NaCl, 1% Nonidet P40, 0.5% deoxycholic acid and 0.1% SDS, pH 7.4) and the cell extracts were stored at −80°C until assayed. Bcl10 molecules in the samples or standards were captured in the wells of a microtiter

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plate precoated with rabbit polyclonal antibody to Bcl10 (QED Bioscience, San Diego, CA). Immobilized Bcl10 molecules were detected by a mouse monoclonal antibody to Bcl10 (Novus Biologicals, Littleton, CO) and goat anti-mouse IgG-HRP complex (Santa Cruz Biotechnology, Santa Cruz, CA). The peroxidase enzyme activity bound to Bcl10 molecules was determined by chromogenic reaction with hydrogen peroxide-tetramethylbenzidine. Color development due to enzymatic activity was stopped by 2 N sulfuric acid, and intensity of the color was measured at 450 nm in ELISA plate reader (SLT, Spectra). Bcl10 concentrations of the samples were extrapolated from a standard curve derived by using known concentrations of recombinant Bcl10 (Calbiochem, EMD Bioscience, San Diego, CA). Sample values were normalized with the total cell protein concentrations determined by BCA protein assay kit (Pierce).

ELISA for phospho-IκBα. Cell extracts were prepared from treated and control NCM460 or primary colon cells by washing with PBS, then harvesting in ice-cold cell lysis buffer (Cell Signaling Technology, Danvers, MA) that contained 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF. Cells were sonicated twice for 20 s and spun at 13,500 *g* for 10 min at 4°C. The supernatant (cell lysate) was collected and stored at −80°C until phospho-IκBα assay was performed.

Phospho-IκBα was determined by commercially available PathScan Sandwich ELISA (Cell Signaling Technology), following the manufacturer's instructions. IκBα in the samples was captured in microtiter wells that were coated with monoclonal antibody to IκBα. Captured phospho-IκBα in the wells was detected by a specific rabbit phospho-IκBα antibody that detected Ser32 phosphorylation and was then recognized by an anti-rabbit IgG-HRP. The enzyme activity of bound HRP was determined by adding hydrogen peroxide-tetramethylbenzidine chromogenic substrate. The magnitude of the optical density for the developed color was measured in an ELISA reader at 450 nm, after stopping the reaction with 2 N sulfuric acid. The intensity of the developed color is proportionate to the quantity of phospho-IκBα in each sample. The sample values were normalized with the total cell protein and expressed as percent control.

Silencing of Bcl10 mRNA expression. Small-interfering RNA (siRNA) for Bcl10 (NCBI NM_003921) silencing and control siRNA labeled with rhodamine were procured (Qiagen, Valencia, CA), and the expression of Bcl10 was silenced in the NCM460 and the primary colon cells with the following protocol. Cells were grown to 60–70% confluency in a six-well tissue culture plate, and on the day of silencing the medium of the growing cells was replaced with 2.3 ml of fresh growth medium with serum. Then 0.6 μl/well of 20 μM siRNA (150 ng) was mixed with 100 μl/well of serum-free medium and 12 μl/well of HiPerfect Transfection Reagent (Qiagen). The mixture was incubated for 10 min at room temperature to allow the formation of transfection complexes and then was added dropwise onto the cells. The plate was swirled gently, and then the cells were incubated at 37°C in a humidified, 5% CO_2 environment. After 24 h the medium was changed to fresh growth medium. The entry of the transfection complexes into the cells was monitored by observing the fluorescence by microscopy of the control cells that were transfected with rhodamine-tagged control siRNA. The effectiveness of silencing of Bcl10 expression was determined by Western blot of the cell lysates with Bcl10 monoclonal antibody (Santa Cruz Biotechnology). The blot indicated that siRNA-4 was the most effective, followed by siRNA-2 (Fig. 2*C*). Subsequently, siRNA-4 was used in our experiments.

Neutralization of TLR-4 receptor by blocking antibody. NCM460 cells were grown in 12-well tissue culture plates. At 60–70% confluency, the cells were treated with fresh media containing either 10 or 20 μg/ml of TLR4 receptor antibody (BioLegend, San Diego, CA) or mouse IgG2a,κ (BioLegend no. 401502) for 1 h, prior to LPS (10 ng/ml) challenge for 6 h. After 6 h, the spent media were collected for IL-8 assay and the cells were harvested for total cell protein determination or Bcl10 assay.

Treatment of the NCM460 cells with IRAK-1/4 inhibitor. NCM460 cells, grown in 24-well plates, were incubated with 50 μM IRAK-1/4 inhibitor [*N*-(2-morpholinylethyl)-2-(3-nitrobenzoylamido)-benzimidazole; EMD Bioscience] for 2 h. After 2 h, the media were changed, and new media with or without LPS (10 ng/ml) added. Treatment was terminated at 6 h, and spent media and cells were collected for IL-8 and other assays.

Preparation of whole cell lysate and nuclear extract. To prepare the whole cell lysate, cells were washed with PBS and harvested. Complete lysis buffer (in mM: 10 Tris·HCl pH 7.5, 150 NaCl, 5 EDTA, 1 PMSF, 1% Triton X-100, 1× protease inhibitor cocktail; Roche Diagnostics, Indianapolis, IN) was added to the cell pellets, and cells were incubated on ice for 30 min with occasional shaking. Then cells were sonicated twice for 20 s and spun at 13,500 *g* for 20 min at 4°C. The supernatant (cell lysate) was collected and stored at −80°C.

Nuclear extracts were prepared as described previously (15). Cells were washed twice with PBS and transferred to an Eppendorf tube. Cells were lysed in lysis buffer [in mM: 10 HEPES (pH 7.9), 10 KCl, 1.5 $MgCl_2$, 0.1 EDTA, 1 DTT, and 1 PMSF], kept on ice for 10 min, and then dounced 10 times on ice in a Dounce homogenizer. The nuclear pellet obtained by centrifugation at 13,000 *g* for 10 min was washed with lysis buffer and resuspended in 50–80 μl of nuclear extraction buffer [in mM: 20 HEPES (pH 7.9), 400 NaCl, 1.5 $MgCl_2$, 1 EDTA, 1 DTT, 1 PMSF with 25% glycerol] and rotated at 4°C for 3 h. Supernatant (nuclear extract) obtained by centrifugation at 13,000 *g* for 20 min was stored at −80°C until further use.

Detection of nuclear NF-κB by ELISA. Nuclear extracts were prepared from treated and control NCM460 cells according to the procedure described above. Activated NF-κB in the samples was determined by oligonucleotide-based ELISA (Active Motif, Carlsbad, CA), following the manufacturer's instructions. Briefly, when the samples were added and incubated in the 96-well microtiter wells, the activated NF-κB in the nuclear extracts attached to a consensus nucleotide sequence (5′-GGGACTTTCC-3′) that was coated onto the microtiter wells. After washing off the unattached extract, the attached NF-κB molecules were captured by antibody to NF-κB (p65) and detected by an anti-rabbit-HRP-conjugated IgG. Colorimetric readout was performed with hydrogen peroxide-tetramethylbenzidine chromogenic substrate. After the reaction was stopped with 2 N sulfuric acid, the magnitude of optical density of the developed color was measured in an ELISA plate reader at 450 nm. The intensity of the developed color proportionately represents the quantity of NF-κB in each sample. The specificity of the binding of NF-κB with the coated nucleotide sequence was determined by comparison to the binding when either free consensus nucleotide or mutated nucleotide was added in the reaction buffer. The sample values were normalized with the total cell protein and expressed as percent control.

Western blot of Bcl10. Whole cell lysates or nuclear extracts were separated by SDS-PAGE on a 12% gel. Proteins were transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ) and probed with monoclonal antibody to Bcl10 (Santa Cruz Biotechnology), followed by IgG-HRP detection antibodies. Immunoreactive bands were visualized on X-ray film (Bio-Rad) using the ECL detection kit (Amersham Biosciences).

Comparison of effects of Bcl10 silencing on TNF-α, IL-1β, DSS, LPS, and λ-carrageenan activation of IL-8. Bcl10 knockdown was performed as described above in the NCM460 cells, and the effects of TNF-α (0.1 ng/ml), IL-1β (10 ng/ml), and DSS (1 μg/ml) exposure for 24 h on IL-8 secretion were determined by ELISA (R&D) and compared with the effects of LPS (10 ng/ml × 6 h) and λ-carrageenan (1 μg/ml × 24 h).

Statistical analysis. Data presented are means ± SD of three biological replicates with technical duplicates. Unless stated otherwise, statistical significance was determined by one-way ANOVA, followed by a post hoc Tukey-Kramer test for multiple comparisons,

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using GraphPad InStat Software (GraphPad Software, San Diego, CA). A P value of <0.05 is considered statistically significant. Data are expressed as means ± SD. Single asterisk in figures indicates $P <$ 0.001.

RESULTS

Increase in Bcl10 following exposure to LPS in IECs. When NCM460 cells were exposed to LPS (1–20 ng/ml for 6 h), significant increases ($P < 0.001$) in Bcl10 were detectable by Bcl10 ELISA (Fig. 1*A*). LPS exposures of 1, 10, and 20 ng/ml for 6 h produced successive increases in Bcl10 from baseline value of 1.32 ± 0.09 to 2.80 ± 0.29, 5.58 ± 0.56, and 8.08 ± 0.82 ng/ml. Western blot for Bcl10 (Fig. 1*B*), using a mouse monoclonal antibody (Santa Cruz Biotechnology) and exposing cells to LPS 10 ng/ml for 6 h, confirmed the LPS-induced increase in cellular Bcl10 protein.

Figure 2 presents immunohistochemical staining of Bcl10 in ex vivo sections of normal human colonic tissue obtained from surgery. Increase in intensity and extent of positive (brown) staining for Bcl10 in the tissue that was exposed to LPS (10 ng/ml for 2 h) vs. the unexposed control is apparent (Fig. 2, *A* vs. *B*).

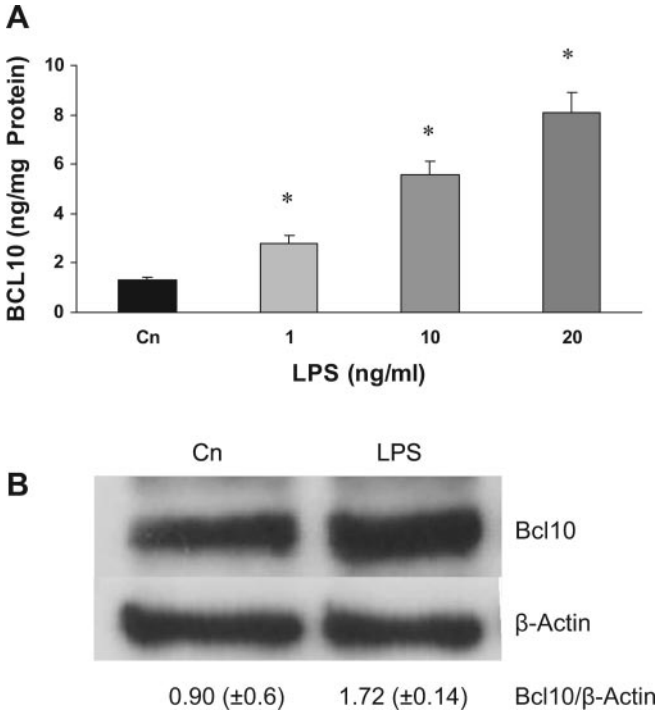

Fig. 1. Increase in Bcl10 following lipopolysaccharide (LPS) treatment. *A*: NCM460 cells grown in 12-well plates were treated with LPS from *Escherichia coli* 026:B6 (1, 10, and 20 ng/ml) for 6 h. Cells were assayed for Bcl10 by a solid-phase sandwich ELISA. LPS was found to stimulate Bcl10 protein expression in a dose-dependent manner. Concentrations of Bcl10 were 1.32 ± 0.09 (control), 2.80 ± 0.29, 5.58 ± 0.56, and 8.08 ± 0.82 ng/ml with successive doses of LPS. Statistical significance was determined by 1-way ANOVA followed by Tukey-Kramer posttest for multiple comparisons. Differences between the groups, control (Cn) vs. LPS 1, 10, and 20 ng/ml are statistically significant ($P < 0.001$). Data are means ± SD of 3 biological and 2 technical replicates. *Statistical significance ($P < 0.001$). *B*: increase in Bcl10 in response to LPS was also detected by Western blot. Whole cell extracts of control and LPS-treated cells were separated by SDS-PAGE on a 12% gel. Proteins were transferred to a nitrocellulose membrane and probed with a mouse monoclonal antibody for Bcl10. Densitometry was performed with Image J software and demonstrated Bcl10/β-actin ratio of 1.72 ± 0.14 following LPS (1 ng/ml for 6 h) vs. 0.90 ± 0.06 for control cells.

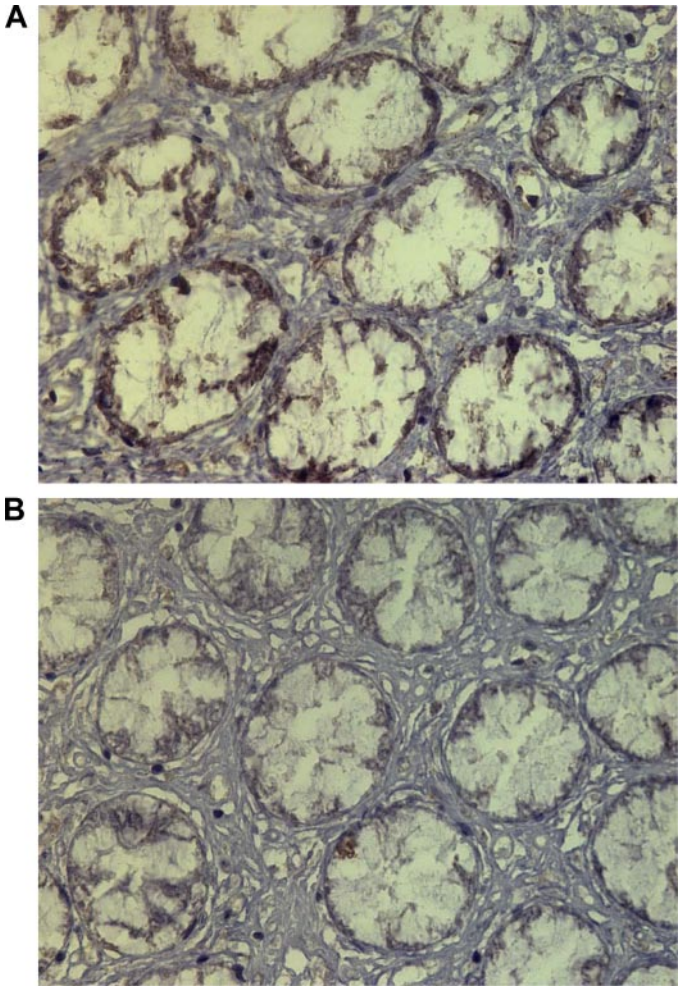

Fig. 2. Increased intensity and distribution of Bcl10 in ex vivo human colonic epithelial cells following exposure to LPS by immunohistochemistry. *A*: after treatment with LPS 10 ng/ml for 2 h, marked increase in intensity and extent of Bcl10 staining (brown) is evident in ex vivo human colonic cell preparations. *B*: control sections were processed simultaneously under similar conditions, but tissue was not exposed to LPS.

NCM460 cells and primary human colonic epithelial cells were exposed to LPS (10 ng/ml for 6 h), then fixed and prepared for confocal imaging. Images are presented in Fig. 3; Bcl10 is tagged with green fluorescence, cell nuclei with blue, and actin fibers with red. The LPS-exposed NCM460 cells (Fig. 3*B*) and primary colonic epithelial cells (Fig. 3*D*) have marked increase in staining of Bcl10, compared with the unexposed control cells that have minimal green fluorescence (Fig. 3, *A* and *C*).

Reduced IL-8 response to LPS following Bcl10 silencing by siRNA. Marked increases in IL-8 secretion were observed in response to LPS exposure of the NCM460 cell line (Fig. 4*A*) and the primary human colonic epithelial cells (Fig. 4*B*). LPS doses ranged from 1 to 20 ng/ml for 6 h. Following Bcl10 silencing by siRNA, there were significant declines in the LPS-induced responses ($P < 0.001$). Western blot of Bcl10 demonstrates marked decline in Bcl10 following silencing by siRNA-2 and -4 (Fig. 4*C*). The greatest silencing effect was by

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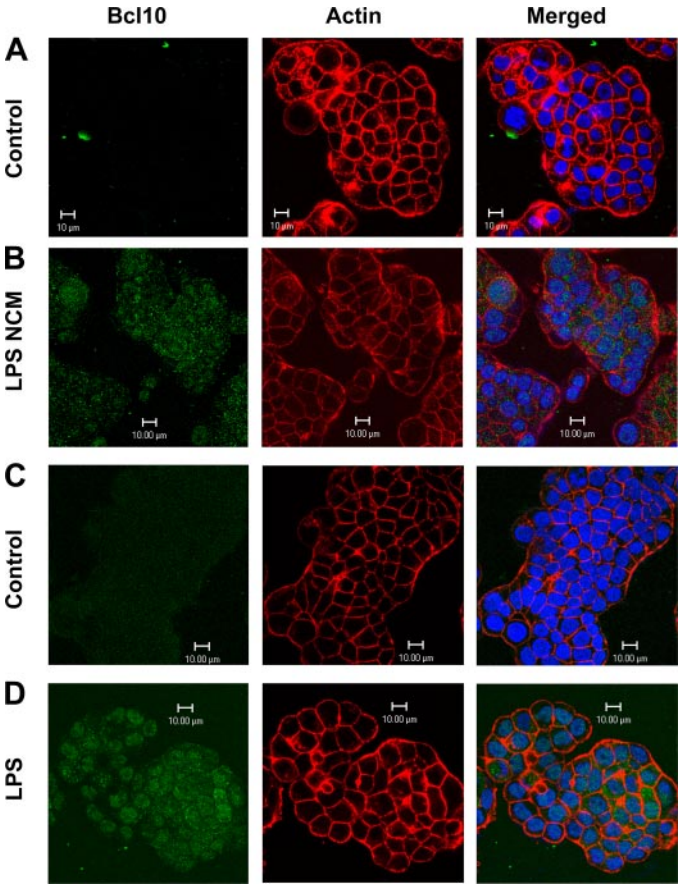

Fig. 3. Confocal images of Bcl10 in human colonic epithelial cells following LPS demonstrate marked increase in Bcl10. Confocal images of NCM460 cells (NCM) are presented in *A* and *B*, and primary human colonic epithelial cells are displayed in *C* and *D*. Green fluorescence identifies Bcl10, rhodamine-phalloidin labeled actin is red, and Hoechst 33342-labeled nuclei are blue. Marked increase in Bcl10 staining is evident in the PS-exposed (10 ng/ml for 6 h) cells (*B* and *D*), compared with the unexposed control cells (*A* and *C*) in which green fluorescence is virtually absent. Merged images present the combined Bcl10, actin, and nuclear staining.

siRNA-4, which was used in subsequent experiments. IL-8 declined by 50% following exposure to Bcl10 siRNA for 24 h in the NCM460 cells and by 56% in the primary colonic epithelial cells.

Reduced phospho-IκBα response to LPS following Bcl10 silencing. LPS exposure produced marked increase in phospho-IκBα, measured by a sandwich ELISA kit that detects phosphorylation of Ser32 (Fig. 5*A*). Following Bcl10 silencing, there was significant reduction ($P < 0.001$) in the LPS-induced increase of phospho-IκBα, with phospho-IκBα declining by 50% from a peak rise to 4.17 times baseline to 2.07 times baseline (Fig. 5*B*).

Decline in LPS-induced activation of NF-κB following Bcl10 siRNA. Nuclear extract of NCM460 cells was prepared and tested for NF-κB content, by use of a 96-well ELISA spotted with oligonucleotide specific for NF-κB (p65). Significant decline ($P < 0.001$) in LPS-stimulated nuclear NF-κB occurred, following Bcl10 silencing (Fig. 6). NF-κB declined from 3.97 times baseline to 2.04 times baseline.

TLR4 blocking antibody leads to reduction of LPS-induced increase in Bcl10. TLR4 is the cell receptor for LPS and mediates the subsequent LPS-induced inflammatory cascade. Blocking antibody to TLR4 produced a significant reduction ($P < 0.001$) in the LPS-stimulated secretion of IL-8 by the NCM460 cells (Fig. 7*A*). IL-8 declined 84% from peak value of 2.60 ± 0.05 to 0.54 ± 0.01 ng/ml, with baseline control value of 0.14 ± 0.01 ng/ml. When cells were stimulated by LPS following exposure to the TLR4 neutralizing antibody (20 ng/ml × 1 h), there was a statistically significant reduction ($P < 0.001$) in the baseline Bcl10 protein, measured by ELISA ($P < 0.001$) (Fig. 7*B*). Bcl10 declined to 2.41 ± 0.24 ng/ml, from an LPS-induced peak of 5.20 ± 0.08 ng/ml, with baseline control value of 1.31 ± 0.12 ng/ml, a reduction of 72% in the LPS-induced increase in Bcl10.

IRAK-1/4 inhibition and Bcl10. Inhibition of IRAK-1/4 was performed using the inhibitor *N*-(2-morpholinylethyl)-2-(3-nitrobenzoylamido)-benzimidazole. Statistically significant declines in IL-8 (Fig. 8*A*) and Bcl10 (Fig. 8*B*) occurred ($P <$

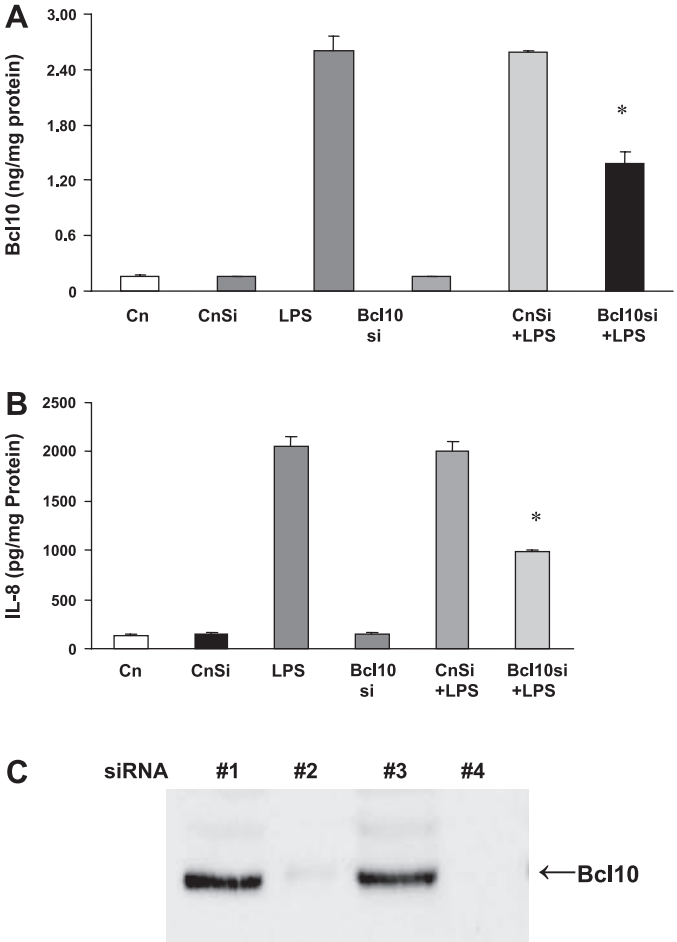

Fig. 4. Decline in IL-8 in human colonic epithelial cells following silencing of Bcl10 by small-interfering RNA (siRNA). *A*: IL-8 in the spent medium was assayed by ELISA. Silencing of Bcl10 by siRNA resulted in 50% reduction in the IL-8 response to LPS in the NCM460 cells, declining from a peak value of 2.60 ± 0.16 to 1.38 ± 0.13 ng/ml, with a baseline value of 0.16 ± 10 ng/ml. CnSi, control siRNA; Bcl10 si, Bcl10 siRNA. *B*: in primary human colonic epithelial cells, Bcl10 silencing reduced the effect of LPS on IL-8 by 56%, from a peak of 2.05 ± 0.10 to 0.98 ± 0.021 ng/ml. Differences are statistically significant ($P < 0.001$, 1-way ANOVA with Tukey-Kramer posttest). Data are means ± SD of 3 biological and 2 technical replicates. *C*: Western blot demonstrates effectiveness of silencing Bcl10 by siRNA. SiRNA-4 was used for all subsequent experiments. *$P < 0.001$, 1-way ANOVA with Tukey-Kramer posttest.

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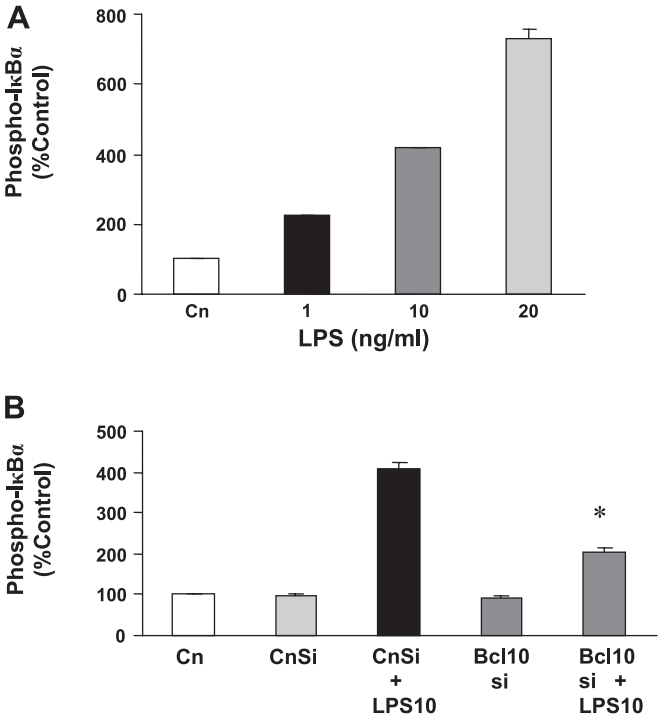

Fig. 5. LPS-induced increase in IκBα phosphorylation in NCM460 cells reduced by Bcl10 siRNA. *A*: semiquantitative determinations of phospho-IκBα, by ELISA that detected phosphorylation of Ser32, demonstrate 100% increases over baseline following exposure to LPS. LPS doses of 1, 10, and 20 ng/ml increased phospho-IκBα 2- to 8-fold. *B*: NCM460 cells were exposed to LPS 10 ng/ml for 6 h, following knockdown of Bcl10 for 24 h by siRNA. Phospho-IκBα was measured in the cell lysates by ELISA. A 50% reduction in the level of phospho-IκBα occurred. Data are means ± SD of biological and technical replicates. Differences between the groups are statistically significant (*$P < 0.001$, 1-way ANOVA with Tukey-Kramer posttest).

0.001), indicating that IRAK-1/4 mediates LPS-induced IL-8 activation and functions upstream of Bcl10. The LPS-induced increase in Bcl10 declined by 73% (from 5.18 ± 0.22 to 2.36 ± 0.08 ng/ml), and the IL-8 response declined by 60% (from 2.64 ± 0.31 to 1.14 ± 0.08 ng/ml).

Comparison of effects of LPS from E. coli and S. enterica. The effects of LPS from *E. coli* and from *S. enterica* on the responses of IL-8 and Bcl10 in the NCM460 cells were compared. Results demonstrate IL-8 increase from 2.51 ± 0.06 to 3.53 ± 0.11 ng/ml, a 1.4-fold difference with the less potent LPS from *E. coli* (Fig. 9*A*). Following exposure to LPS from *Salmonella*, Bcl10 increased 1.62-fold, consistent with the greater reported potency (800,000 vs. 500,000 EU/mg LPS) (Fig. 9*B*). *S. enterica* LPS increased Bcl10 from 1.25 ± 0.06 to 7.55 ± 0.51 ng/ml, compared with 5.16 ± 0.37 ng/ml from the *E. coli* LPS. The proportionate changes in LPS potency and Bcl10 level confirm the quantitative association between LPS exposure and Bcl10.

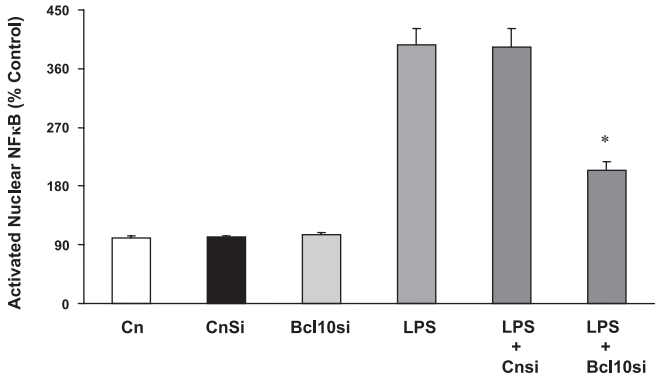

Fig. 6. Nuclear translocation of NF-κB declined following Bcl10 silencing. Semiquantitative determination of nuclear NF-κB (p65) by ELISA demonstrated decline from an LPS-induced increase of 2.97 ± 0.28 fold, to an increase of 1.04 ± 0.14 fold. Results are means ± SD of 3 biological and 2 technical replicates. Differences between the groups are statistically significant (*$P < 0.001$, 1-way ANOVA with Tukey-Kramer posttest).

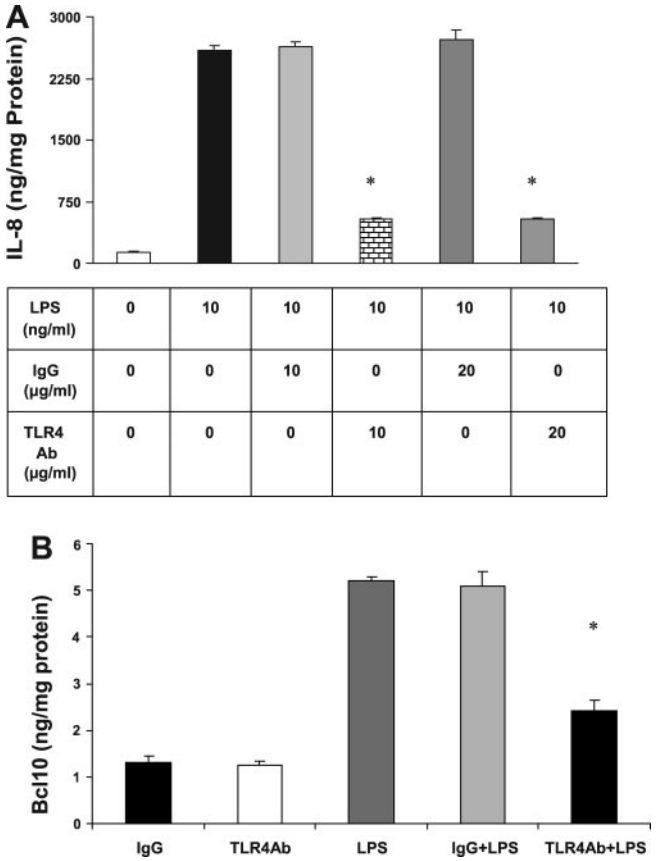

Fig. 7. Blocking TLR4 receptor by neutralizing antibody reduced the LPS-induced stimulation of IL-8 secretion and Bcl10 protein content of NCM460 cells. TLR4 receptors on NCM460 cells were blocked by neutralizing antibody (10 or 20 μg/ml) for 1 h prior to LPS challenge (10 ng/ml) for 6 h. Control experiments were done with IgG isotype control (10 and 20 μg/ml). Blocking of TLR4 receptors by antibody inhibited the LPS-induced secretion of IL-8 by 84% (*A*) and Bcl10 by 72% (*B*). Differences between the groups are statistically significant (*$P < 0.001$, 1-way ANOVA followed by Tukey-Kramer posttest).

Bcl10 does not mediate IL-8 activation by TNF-α, IL-1β, or DSS. Following Bcl10 silencing by siRNA for 24 h, NCM460 cells were exposed to TNF-α (0.1 ng/ml), IL-1β (10 ng/ml), DSS (1 μg/ml), carrageenan (1 μg/ml) for 24 h, or LPS (10 ng/ml) for 6 h (Fig. 10). In contrast, to the marked declines evident with LPS or carrageenan, Bcl10 silencing had no effect on the IL-8 responses to TNF-α, IL-1β, or DSS.

The LPS-induced increases in phospho-IκBα, NF-κB, and IL-8 were significantly reduced following silencing of Bcl10 by siRNA. Inhibition of IRAK-1/4 and neutralizing antibody for TLR4 produced marked reductions in the LPS-induced increases in Bcl10 and IL-8. However, the reductions in the

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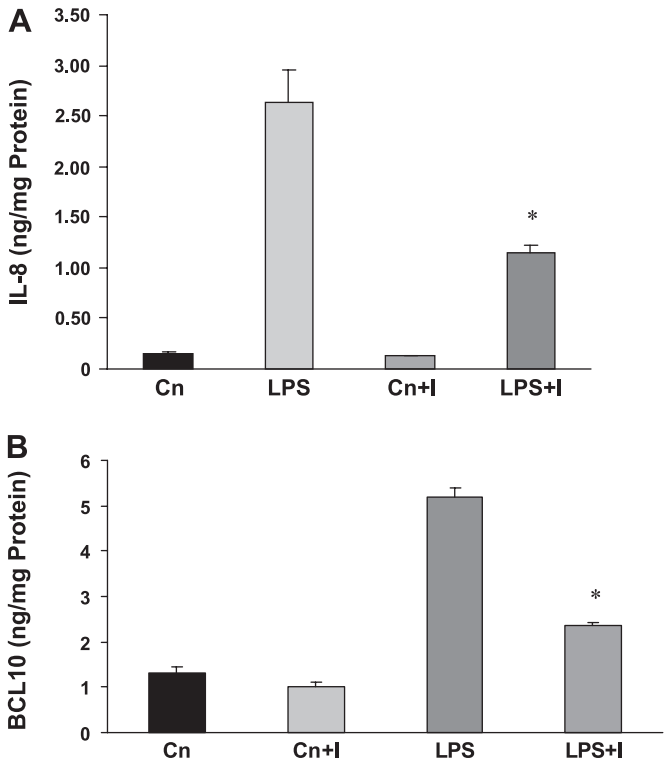

Fig. 8. Effect of IL-1 receptor-associated kinase (IRAK)-1/4 inhibitor on LPS-induced increases in IL-8 and Bcl10. NCM460 cells were pretreated with 50 μM of IRAK-1/4 inhibitor for 2 h prior to 6 h LPS exposure. Inhibition of IRAK-1/4 was performed by using the inhibitor *N*-(2-morpholinylethyl)-2-(3-nitrobenzoylamido)-benzimidazole. The LPS-induced increase in IL-8 response declined by 60% (*A*) and Bcl10 declined by 73% (*B*). Differences are statistically significant (*$P < 0.001$, 1-way ANOVA with Tukey-Kramer posttest) and suggest that IRAK-1/4 mediates LPS-induced IL-8 activation upstream of Bcl10.

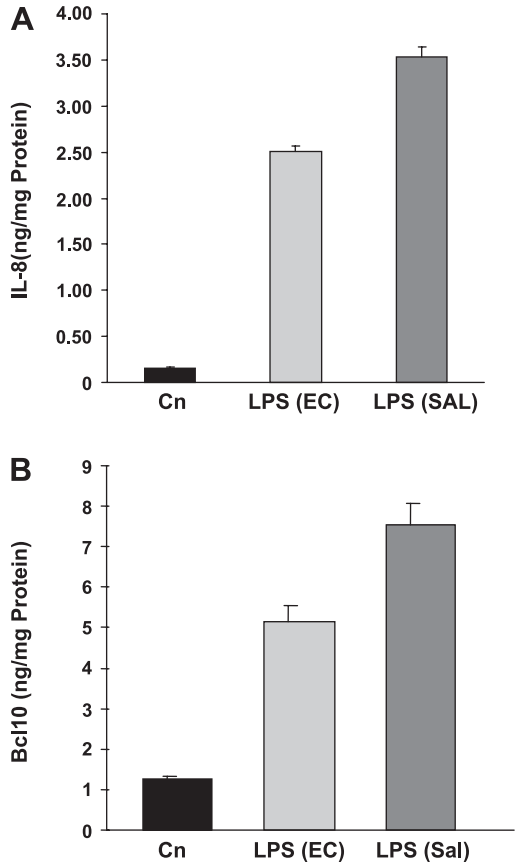

Fig. 9. Higher potency LPS yields proportionate increase in Bcl10. NCM460 cells were exposed to LPS (10 ng/ml × 6 h) from *E. coli* (EC; 500,000 EU/mg LPS) and from *Salmonella enterica* (SAL) of greater potency (800,000 EU/mg LPS). IL-8 response (*A*) increased 1.43-fold, and Bcl10 response (*B*) increased 1.62-fold.

LPS-induced effects are not complete, suggesting either incomplete inhibition by Bcl10 siRNA, TLR4Ab, or IRAK inhibitor or the presence of alternate and multiple cellular pathways of IL-8 activation stimulated by LPS. Since the IL-8 responses induced by TNF-α and IL-1β are not affected by silencing of Bcl10, interactions between LPS and pathways activated by these cytokines may provide alternative mechanisms of IL-8 activation following exposure to LPS that are not mediated by Bcl10.

DISCUSSION

Bcl10 is increasingly recognized as an important mediator of cellular inflammation. Here, we present data that indicate a role for Bcl10 in LPS-mediated inflammation in IECs. Previously, we reported that the commonly used food additive carrageenan, which is associated with development of intestinal ulcerations, inflammation, and neoplasms in animal models, activates IL-8 through a Bcl10-mediated pathway in human IECs (5). Reports of the Bcl10-mediated inflammatory pathway in lymphocytes and macrophages have provided evidence about the role of potential mediators in these pathways, including NEMO, IRAK-1/4, TRAF2/6, TAK1, TAB1/2, and MALT1 (10). These intermediates are candidates for functional roles in LPS-induced inflammation in the IEC, as well. The data that we have presented suggest that one of the important pathways of IL-8 activation induced by LPS in the normal human IEC proceeds through TLR4 → IRAK → Bcl10 → phospho-IκBα → NF-κB → IL-8 (Fig. 11). The evidence that we have compiled, including Bcl10 Western blots, immu-

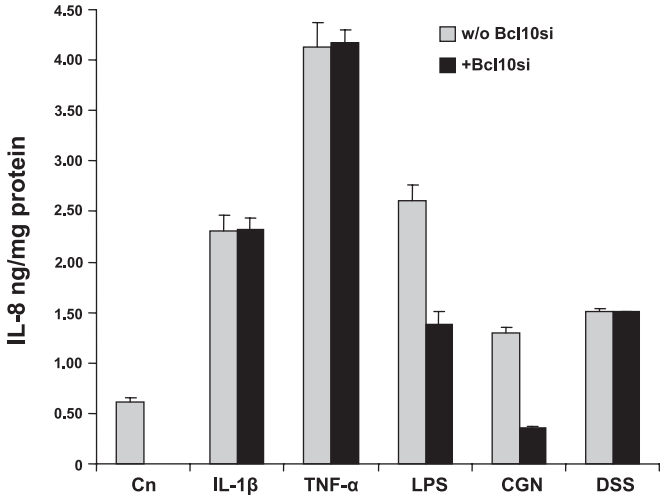

Fig. 10. Silencing of Bcl10 does not affect IL-8 response to TNF-α, IL-1β, or DSS in the NCM460 cells. In contrast to the declines in IL-8 secretion produced by Bcl10 silencing when IEC were exposed to LPS or λ-carrageenan (CGN), no reductions in IL-8 response occurred with Bcl10 silencing following exposure to TNF-α (0.1 ng/ml), IL-1β (10 ng/ml), or DSS (1 μg/ml) for 24 h. +, With, w/o, without.

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nohistochemistry and fluorescent imaging, quantitative determination of Bcl10 by ELISA, and effects of Bcl10 silencing by siRNA, demonstrates a direct relationship between LPS exposure and Bcl10 and downstream targets of LPS activation, including phospho-IκBα, NF-κB, and IL-8.

The increases in Bcl10 following LPS were significantly reduced following exposure of the IECs either to TLR4 neutralizing antibody or to IRAK inhibitor, thereby indicating the responsiveness of Bcl10 to recognized upstream mediators of an LPS-TLR4-induced cascade in immune cells (10). Until recently, the Bcl10 pathway of NF-κB activation had been described only in cells of immune origin. We reported Bcl10 activation of an inflammatory cascade in human IEC induced by the polysulfated polygalactan carrageenan (5). Recently, McAllister-Lucas et al. (26) described a Bcl10-mediated pathway involving CARMA3 and MALT1 in hepatocytes that was activated by angiotensin II. Klemm et al. (20) determined that lysophosphatidic acid-induced activation of NF-κB in murine embryonic fibroblasts was mediated by Bcl10 and MALT1. Wang et al. (34) identified Bcl10 as a critical mediator in human embryonic 293 kidney cells, in which G protein-coupled receptors activate NF-κB. They examined other potential mediators by Western blot and found lysophosphatidic acid and endothelin-1 pathways also involved Bcl10. In their study, TNF-α, LPS, or integrin-induced NF-κB activation were not affected in Bcl10 deficient cells (34). The results that we present are in contrast to their Western blot-based findings with regard to LPS. Our extensive analysis, which includes quantitative determinations of Bcl10 levels by solid-phase sandwich ELISA, immunohistochemistry, confocal imaging, and effective Bcl10 silencing by siRNA, indicates that in the human IECs, LPS activation of NF-κB and IL-8 is mediated largely by Bcl10. In contrast, we found that IL-8 activation by TNF-α, IL-1β, or DSS does not involve Bcl10. We tested LPS from *E. coli* and *S. enterica* of different potencies and found proportionate changes in Bcl10 and IL-8, providing additional quantitative support for the Bcl10 mediation of IL-8 activation by LPS.

Bcl10 has also been associated with other significant pathways of cell activation, including the JNK2 kinase pathway (3). Bcl10 may play a critical role in the cross talk among different cellular mechanisms that respond to exogenous immune stimuli. Bcl10 appears to have a prominent role in determination of cell fate, influencing activation of a pathway leading either to an inflammatory response, cell death, or cell proliferation (13, 23, 24, 33). Additional mechanisms of activation appear to be induced by LPS exposure, since Bcl10 silencing does not completely eliminate the increases in phospho-IκBα (Fig. 5*B*), NF-κB (Fig. 6), or IL-8 (Fig. 4*A*) that followed stimulation by LPS. These additional effects may involve cross talk with intermediates in the pathway mediated by Bcl10 but require further investigation. Additional future investigation is required to determine the interactions between Bcl10 and the downstream adaptor MALT1. The CARMA3-Bcl10-MALT1 interactions have been described as a signalosome, linking innate and adaptive immune responses to the NF-κB pathway (35). Cellular localization of complexes containing these molecules, as well as the cellular localization of Bcl10 and the TLR4 signaling complex, will facilitate the analysis of the complex interactions that occur. Since the TLR4 appears to have intracellular as well as extracellular localization, interaction with Bcl10 may occur not at the apical cell membrane, but in the Golgi apparatus (16).

Transmission of signals from the hydrophobic lipid A occurs via the leucine-rich TLR4 and via Bcl10. This suggests that the network by which these signals are integrated and transmitted may be sterically mediated by a series of hydrophobic interactions. Bcl10 and NOD2 are both CARD proteins, containing a caspase recruitment domain. The interactions among the different CARD-domain containing proteins, including Bcl10 and the CARMA proteins, which are membrane-associated guanylate kinases, appear to be crucial to the pathway of Bcl10-mediated intracellular inflammation in the immune cells studied previously (3, 13, 26). The CARD protein has leucine-rich repeats and interacts with other CARD proteins, regulating the activation of intracellular signaling mechanisms. Bcl10 has been considered as a potential transcriptional activator and found to act as a mediator of transcriptional activation induced by the transcription factor TFIIB (23). The nuclear translocation of Bcl10 was stimulated by Akt in association with TNF-α in MCF7 cells (37), suggesting the role of Bcl10 in transcription may not be limited to immune cells.

Further consideration of the role of Bcl10 and the interactions among leucine-rich-domain-containing proteins may help to explain the mechanism of signal transduction in the IECs, as well as in immune cells with TLRs. Since LPS-associated inflammation leads to clinically significant illness that is often refractory to the current pharmaceutical interventions, clarification of these signaling mechanisms may lead to therapeutic innovations, as well as improved understanding of the integration of cellular responses to inflammatory stimulation.

ACKNOWLEDGMENTS

The authors acknowledge Dr. Ravinder Gill, Dr. Mei Ling Chen, and Lu Leach for contributions to confocal imaging and immunohistochemistry.

GRANTS

This work was supported in part by National Institute of Diabetes and Digestive and Kidney Diseases grants to P. K. Dudeja (DK-54016 and DK-68324) and the Department of Veterans Affairs.

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