**Mycobacterium bovis** Bacillus Calmette-Guérin Induces CCL5 Secretion via the Toll-Like Receptor 2–NF-κB and –Jun N-Terminal Kinase Signaling Pathways

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In response to *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), CC chemokines are secreted from host cells to attract components of the innate and adaptive immune systems to the site of infection. Toll-like receptor 2 (TLR2) has been shown to recognize *M. bovis* BCG and to initiate signaling pathways that result in enhanced secretion of CC chemokines. Despite the essential requirement of TLR2 in *M. bovis* BCG infection, the mechanisms by which it induces secretion of CC chemokines are not well defined. In this study, we report that stimulation of HEK293 cells expressing human TLR2 with *M. bovis* BCG resulted in increased CCL2 and CCL5 secretion, as determined by an enzyme-linked immunosorbent assay. *M. bovis* BCG infection resulted in the activation of c-Jun N-terminal kinase (JNK), and the inhibition of JNK activity had a significant effect on *M. bovis* BCG-dependent CCL5 secretion in TLR2-expressing cells but no effect on *M. bovis* BCG-dependent CCL2 secretion from infected HEK293 cells expressing human TLR2. The *M. bovis* BCG-induced CCL5 release was attenuated by sulfasalazine (a well-described inhibitor of NF-κB activity), BAY 11-7082 (an IκB phosphorylation inhibitor), and ALLN (a well-described inhibitor of NF-κB activation that prevents degradation of IκB and eventually results in a lack of translocated NF-κB in the nucleus). In addition, stimulation of TLR2-expressing cells with *M. bovis* BCG resulted in translocation of NF-κB subunits from the cytoplasmic to the nuclear fraction, and stimulation of cells with *M. bovis* BCG activated IκB kinase αβ. These findings indicate that *M. bovis* BCG induces CCL5 production through mechanisms that include a TLR2-dependent component that requires JNK and NF-κB activities.

Protective immunity against human tuberculosis requires productive infection of the host by *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), the world’s most widely used tuberculosis vaccine (39). The early innate immune response to mycobacteria includes Toll-like receptor (TLR) stimulation of cells (18). The TLR family includes more than 12 members, with different ligand specificities and differential expression among cell types (27, 41, 42). Signal transduction pathways activated by TLRs have continued to be a major focus of research for investigators interested in the initiation of innate immune responses and the induction of chemokines during mycobacterial infection. In general, two major pathways are activated by TLRs (23). The first of these kinases in the activation of nuclear factor κB (NF-κB), which acts as a master switch for inflammation, regulating the transcription of many genes that encode proteins involved in immunity and inflammation (8, 36). The second leads to the activation of several protein kinases, such as mitogen-activated protein kinases (24), phosphatidylinositol 3’-kinase, Jun amino-terminal kinase (JNK), and IκB kinases (IKKs) (17). Activation of the kinases leads to the nuclear translocation of corresponding nuclear transcription factors and thus regulates host cell responses to mycobacteria, including those associated with chemokine gene expression (7, 10).

Chemokines are a large family of low-molecular-mass (8- to 10-kDa) polypeptides involved in directing the recruitment and activation of leukocytes to sites of infection or inflammation (40). The chemokines have been broadly divided into CC, CXC, and CX3C subgroups, based on the positioning of amino acids relative to the first two conserved cysteine residues (44). TLR signaling leads to the secretion of CC chemokines, which include monocyte chemotactic protein 1 (MCP-1, also known as CCL2), macrophage inflammatory protein 1α (MIP-1α, also known as CCL3), MIP-1β (CCL4), and regulated upon-activation, normal T-cell-expressed and -secreted (RANTES, also known as CCL5) (2). Elevated levels of CCL2 and CCL5 in response to *M. bovis* BCG stimulation have been reported for pulmonary tuberculosis patients compared with controls (20). Recently, it has been demonstrated that a functional promoter polymorphism in CCL2 is associated with increased susceptibility to pulmonary tuberculosis (11). It has been reported that CCL5 is released by human alveolar macrophages upon infection with *Mycobacterium tuberculosis* and that tuberculous granulomas contain cell types potentially recruited by CCL5 (32). Furthermore, it has been demonstrated that anti-CCL5 antibodies decrease the sizes of pulmonary granuloma lesions in *M. bovis* BCG-infected mice, suggesting a functional role for CCL5 in murine mycobacterial granulomas (28). Our results have provided evidence that in vitro, *M. bovis* BCG stimulates human monocytes to produce CC chemokines and that mobilization of intracellular Ca2+ and the CD40 molecule are critical for the induction of CCL5 (25). However, the effects of JNK and NF-κB on TLR2-mediated CCL2 or CCL5 pro-
duction in epithelial cells are mostly unknown. In the present study, we explored the intracellular signaling pathway involved in *M. bovis* BCG-induced CCL2 or CCL5 production in TLR2 cells. The results show that TLR2 signals mediate responses to *M. bovis* BCG via activation of JNK and NF-κB, suggesting an important role of TLR2 in CCL5 secretion in response to *M. bovis* BCG.

**MATERIALS AND METHODS**

**Bacteria.** *M. bovis* BCG (ATCC 35733) was obtained from the American Type Culture Collection (Manassas, VA). *M. bovis* was grown at 37°C in Sauton medium for 2 weeks. Cultures were centrifuged at 800 rpm for 10 min and then washed three times in medium. Aliquots of the stock were kept at −70°C.

**Cell culture.** Human embryonic kidney cells of the HEK293 line (HEK cells) were obtained from the American Type Culture Collection. HEK cells were routinely cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1× penicillin-streptomycin (Gibco). HEK293 cells stably transfected with human TLR2 (HEK-hTLR2, referred to below as TLR2 cells) were purchased from InvivoGen (San Diego, CA). In preliminary experiments, the presence or absence of TLR2 in HEK cells was examined by immunoblotting (data not shown). TLR2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and with basic insulin (10 μg/mL) at 37°C under 7.5% CO₂. FBS contained <5 pg/100 ml lipopolysaccharide as certified by the manufacturer. The cells (10⁵/well) were then infected with mycobacteria at a multiplicity of infection (MOI) of 3:1. Control cultures with no mycobacteria were always included. Following the removal of culture supernatants for chemokine analysis, cell viability was assessed by resuspending the medium and adding 500 μg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma). After incubation for 3 h at 37°C, water-insoluble dark blue formazan crystals that formed from the cleavage of MTT in actively metabolizing cells were dissolved in isoy busfer containing 20% sodium dodecyl sulfate (SDS) and 50% dimethylformamide, and the absorbance at 570 nm was determined.

**Specific reagents.** The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): anti-JNK, anti-phospho-IκBα, anti-IκBα, and horseradish peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulin G. Anti-phospho-JNK was purchased from Promega (Madison, WI). Antibodies against the β6 and β3 subunits of NF-κB were purchased from BioMol Research Laboratories. The JNK inhibitor SP600125 and the IkBα phosphorylation inhibitor Bay 11-7082 were purchased from Calbiochem (La Jolla, CA). N-Acetyl-Leu-Leu-norleucinal (ALLN) was purchased from Boehringer Mannheim (Indianapolis, IN). Curcumin, sulfasalazine, and FK-506 were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Dimethyl sulfoxide (Sigma) was used at 1% (vol/vol) as a solvent control. An MTT assay indicated that the viability of cells was not altered by culture in the presence of SP600125 (10 μM), curcumin (5 μM), FK-506 (100 ng/ml), sulfasalazine (2 mM), ALLN (50 μM), or Bay 11-7082 (10 μM) for the duration of the experiment.

**ELISA.** Cells were treated in triplicate with *M. bovis* BCG at an MOI of 3:1. Twenty-four-hour culture supernatants were collected, centrifuged for CCL2 or CCL5 production by commercial enzyme-linked immunosorbent assay (ELISA) kits (R & D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

**Western blotting.** Cells were lysed on ice with 1 ml of cold lysis buffer (50 mM Tris [pH 7.3], 1% Triton X-100, 1 mM EDTA, 1 mM NaVO₄, 1 mM NaF), and centrifuged at 8°C for 15,000 × g for 1 min. Supernatants containing cytosolic proteins were collected. A pellet containing nuclei was resuspended in hypotonic buffer (20 mM HEPES [pH 7.5], 25% glycerol, 1.5 mM MgCl₂, 4 mM EDTA, 0.05 mM dithiothreitol, 10 mM aprotinin, 10 mM leupeptin, and 20 mM phenylmethylsulfonyl fluoride) for 15 min on ice and vortexed for 10 s. Nuclei were pelleted by centrifugation at 15,000 × g for 1 min. Supernatants containing nuclear proteins were collected. A pellet containing nuclei was resuspended in hypotonic buffer (20 mM HEPES [pH 7.5], 25% glycerol, 1.5 mM MgCl₂, 4 mM EDTA, 0.05 mM dithiothreitol, 10 mM aprotinin, 10 mM leupeptin, and 20 mM phenylmethylsulfonyl fluoride) for 30 min on ice. Supernatants containing nuclear proteins were collected by centrifugation at 15,000 × g for 2 min and then stored at −70°C. The protein levels of p50 and p65 in the cytosolic and nuclear fractions were determined by Western blot analysis, performed as described above.

**Statistical analysis.** All values are given as means ± standard errors of the means (SEM). When a significant difference was detected, statistical analysis was further performed by Student’s t test. A P value of <0.01 was taken to indicate a significant difference.

**RESULTS**

*M. bovis* BCG induces increased CCL2 or CCL5 secretion from epithelial cells that express TLR2. *M. bovis* BCG has been shown to play an important role in the regulation of *M. bovis* BCG-induced CXC chemokine production by human cells in vitro (13). In this study, to evaluate the contribution of TLR2 expression to CCL2 and CCL5 secretion, we took advantage of HEK293 cell lines that stably express TLR2 and HEK293 cells that do not express this receptor. Initially, we measured the CCL2 and CCL5 protein levels of culture supernatants from 24-h *M. bovis* BCG-infected HEK or TLR2 cells by ELISA. Figure 1A and B show that the levels of CCL2 and CCL5 produced by infected TLR2 cells were significantly higher than those secreted by parental HEK cells (P ≤ 0.01). These data indicate that secretion of CCL2 and CCL5 in response to *M. bovis* BCG infection is enhanced by the presence of TLR2.

The JNK activation pathway regulates *M. bovis* BCG-induced CCL5 protein upregulation. Given that JNK is a critical factor involved in CC chemokine gene activation (15), we measured the activation of JNK in cell lysates from infected HEK and TLR2 cells by Western blot analysis with phosphospecific antibodies. In HEK cells, *M. bovis* BCG infection induced the activation of JNK (Fig. 2). Moreover, the results in Fig. 2 reveal that infected TLR2 cells also showed an *M. bovis* BCG-dependent activation of JNK, indicating that there are no intrinsic differences between the HEK and TLR2 cell lines in their abilities to activate JNK. We next examined the importance of JNK activity for *M. bovis* BCG-induced CCL2 or CCL5 production by using a potent, cell-permeant, and selective inhibitor of the JNK signaling pathway, SP600125. Cells were treated with SP600125, and CCL2 or CCL5 production from *M. bovis* BCG-infected cells was measured. As shown in Fig. 3A, CCL2 secretion remained constant, whereas this inhibitor effectively blocked protein secretion of CCL5 after *M. bovis* BCG infection (Fig. 3B). It has been reported that a small dose of curcumin (5 to 10 μM) inhibits JNK activation (21). As evident in Fig. 3A and B, pretreatment with a low dose of curcumin (5 μM) did not influence *M. bovis* BCG-induced CCL2 protein upregulation but significantly inhibited CCL5 protein secretion after *M. bovis* BCG infection. This was consistent with the SP600125 result, indicating that the induction mechanisms of these two CC chemokines are different.

Role of NF-κB in TLR2-mediated CCL5 secretion by epithelial cells. Since the NF-κB binding site in the CCL5 promoter is required for optimal transcription in response to infection (26), we hypothesized that the JNK-dependent increase
in CCL5 secretion exhibited by TLR2-expressing cells might result from increased phosphorylation of NF-κB. In this study, HEK and TLR2 cells were pretreated with sulfasalazine, a well-described inhibitor of NF-κB activity. As shown in Fig. 4A, the effect of sulfasalazine was dose dependent: it was first evident at 0.2 mM and maximal at 2 mM (93.8% inhibition). In order to confirm these results, in this work we used another specific inhibitor of NF-κB activity (ALLN, a well-described inhibitor of NF-κB activation which prevents degradation of IκB and eventually results in a lack of translocated NF-κB in the nucleus) (30). Our results indicate that ALLN significantly diminished the effect of *M. bovis* BCG as well (Fig. 4B). In addition, HEK and TLR2 cells were pretreated with different concentrations of Bay 11-7082, an IκBα phosphorylation inhibitor (29). Figure 4C shows that 10 μM Bay 11-7082 decreased *M. bovis* BCG-induced CCL5 secretion by 91% ± 8%.
Taken together, our results indicate that *M. bovis* BCG-induced CCL5 secretion is NF-κB dependent.

To assess whether TLR2 requires NF-κB transactivation in order to increase CCL5 chemokine production, NF-κB activation was evaluated by analyzing the translocation of NF-κB from the cytosol to the nucleus. As evident in Fig. 5A, treatment of cells with *M. bovis* BCG resulted in a marked translocation of p65 and p50 from the cytosol to the nucleus in a time-dependent manner. In an experiment to further determine the upstream molecules involved in *M. bovis* BCG-induced NF-κB activation, stimulation of cells with *M. bovis* BCG induced an increase in IKKαβ phosphorylation in a time-dependent manner, reaching a maximum after 30 to 60 min of treatment (Fig. 5B).

We next sought to determine whether TLR2-mediated CCL5 secretion by *M. bovis* BCG-stimulated cells involves calcineurin. For this purpose, we employed FK-506 to probe the possible role of calcineurin in the observed CCL5 response to TLR2 stimulation. As shown in Fig. 6, FK-506 greatly reduced the magnitude of CCL5 protein levels induced by stimulation of TLR2 cells with *M. bovis* BCG, suggesting a role for calcineurin signaling in the process of TLR2-mediated CCL5 secretion induced by *M. bovis* BCG.

**DISCUSSION**

Currently, *M. bovis* BCG is the only available tuberculosis vaccine for routine use in humans. Even though BCG may not protect adults against pulmonary tuberculosis, it still confers good protection against disseminated tuberculosis and tuberculous meningitis in children. Furthermore, BCG provides efficient cross-protection against leprosy, and this vaccine is used for treatment of certain chronic diseases, including cancer. In fact, Reale et al. reported elevated serum CCL2 and CCL5 levels in bladder cancer patients after intravesical BCG instillation, indicating that human CC chemokines are responsive to BCG stimulation in vivo (31).

TLR activation is crucial for protecting the host against invading pathogens (3, 5). Because the absence of TLR2 signaling affected mostly the long-term control of chronic *M.
infection (1, 9), it is important that TLR signaling be tightly regulated in order to ensure favorable protective activities. Here we show for the first time that TLR2 initiated signaling events leading to JNK activation and enhanced CCL5 secretion. In this regard, we found that TLR2-mediated induction of CCL5 was markedly attenuated by the JNK inhibitors SP600125 and curcumin. These results are in perfect accordance with previous findings showing that the JNK activation pathway is involved in lipopolysaccharide-mediated CCL5 mRNA expression in epithelial cells (15). These data are also consistent with an independent study showing that the JNK signaling pathway regulates CCL5 production in influenza virus-infected human bronchial epithelial cells (19). It should be noted that although treatment with the JNK inhibitors resulted

FIG. 4. Chemical inhibition of NF-κB signaling decreases TLR2-mediated CCL5 secretion in epithelial cells. HEK and TLR2 cells were pretreated with the indicated concentrations of sulfasalazine (A), ALLN (B), or Bay 11-7082 (C) for 30 min. After incubation, cells were either left uninfected or stimulated with *M. bovis* BCG for a further 24 h. Culture supernatants were analyzed for CCL5 secretion by ELISA. The data shown are from one experiment representative of five independent experiments showing similar results. *, *P < 0.01 for comparison with *M. bovis* BCG cultures that did not receive an inhibitor. DMSO, dimethyl sulfoxide.

tuberculosis infection (1, 9), it is important that TLR signaling be tightly regulated in order to ensure favorable protective activities. Here we show for the first time that TLR2 initiated signaling events leading to JNK activation and enhanced CCL5 secretion. In this regard, we found that TLR2-mediated induction of CCL5 was markedly attenuated by the JNK inhibitors SP600125 and curcumin. These results are in perfect accordance with previous findings showing that the JNK activation pathway is involved in lipopolysaccharide-mediated CCL5 mRNA expression in epithelial cells (15). These data are also consistent with an independent study showing that the JNK signaling pathway regulates CCL5 production in influenza virus-infected human bronchial epithelial cells (19). It should be noted that although treatment with the JNK inhibitors resulted
in a dramatic inhibition of *M. bovis* BCG-induced CCL5 secretion, it did not reduce CCL5 secretion to the levels observed in uninfected cells, indicating that there are JNK-independent pathways that remain active in the presence of these inhibitors. In our present study, inhibition of JNK had no effect on *M. bovis* BCG-induced CCL2 production. However, previous investigations using mouse tubular epithelial cells and leptospiral membrane proteins have suggested that these proteins stimulate JNK and enhance the secretion of CCL2 (16). A possible explanation for these divergent results may lie in the mouse tubular epithelial cells used in that study, which differ in many respects from the TLR2 cells used in this study.

We propose that activation of JNK in TLR2 cells may contribute to the enhanced CCL5 secretion observed upon *M. bovis* BCG infection. Exactly how this occurs is not currently known. It is possible that increased JNK activation in TLR2-expressing cells might result in elevated CCL5 production through increased phosphorylation of NF-κB. The results of this study demonstrate *M. bovis* BCG-induced increases in IKKαβ phosphorylation, as well as translocation of p65 and p50 from the cytosol to the nucleus. Importantly, we could show that sulfasalazine, ALLN, and Bay 11-7082, all inhibitors of the NF-κB-dependent signaling pathway, significantly reduced *M. bovis* BCG-induced CCL5 production, indicating that CCL5 production in response to *M. bovis* BCG is dependent on NF-κB activation. Maximal NF-κB transcriptional activity requires interaction with other components of the transcriptional machinery, such as p300/CREB-binding protein (12, 43). We are currently investigating this possibility.

In activated T cells, calcineurin inhibition by FK-506 has been found to have differential effects on chemokine production (33, 35). Our data demonstrate that calcineurin plays an important role in the mediation of TLR2-stimulated CCL5 production by *M. bovis* BCG-infected cells, since we found that TLR2-mediated induction of CCL5 was greatly reduced by the calcineurin inhibitor FK-506. This finding is in excellent agreement with previous studies showing that another calcineurin inhibitor, cyclosporine, achieved significant inhibition of TLR2-mediated induction of CXCL8 secretion (38). It is interesting to consider the potential effects of calcineurin on several different transcription factors that could be involved in CCL5 production. In this regard, an effect of calcineurin on NF-κB itself is possible (37), particularly since mitochondrial stress can lead to NF-κB activation in skeletal muscle C2C12 cells through a calcineurin-mediated mechanism (4, 6). It is also possible that calcineurin modulates several other proinflammatory signaling pathways that could be involved in TLR-mediated chemokine secretion, such as NFAT and C/EBP (14, 22, 34). In fact, C/EBP has been strongly implicated in CC chemokine secretion (34). Further studies will be needed to determine whether calcineurin activates NF-κB directly or via the activation of other proinflammatory signaling pathways.

In conclusion, the signaling pathway involved in *M. bovis* BCG-induced CCL5 secretion in TLR2 cells has been explored. *M. bovis* BCG increases CCL5 secretion by binding to

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**FIG. 5.** *M. bovis* BCG activates NF-κB in epithelial cells. (A) Cells were infected with *M. bovis* BCG for the indicated times, and the levels of cytosolic and nuclear p65 and p50 were determined by immunoblotting with p65- and p50-specific antibodies, respectively. (B) Cells were incubated without (Con) or with *M. bovis* BCG for the indicated times. Cell lysates were prepared and immunoblotted with antibodies for phospho-IKKαβ (upper panel) or IKKαβ (lower panel), respectively. Typical traces are representative of three experiments with similar results.

**FIG. 6.** TLR2-mediated CCL5 production by epithelial cells involves calcineurin. HEK and TLR2 cells were pretreated with FK-506 (100 ng/ml) for 1 h. After incubation, cells were either left uninfected or stimulated with *M. bovis* BCG for a further 24 h. Culture supernatants were analyzed for CCL5 secretion by ELISA. Results are means ± SEM from five independent experiments performed in triplicate. *, *P* < 0.01 for comparison with *M. bovis* BCG cultures that did not receive an inhibitor. DMSO, dimethyl sulfoxide.
TLR2 and by activation of JNK and NF-κB. The existence of a TLR2 pathway for recognition of M. bovis BCG will provide a rational basis for optimization of vaccine potency by the recruitment and activation of inflammatory cells during immunization.

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