**Mycobacterium bovis** BCG induces CXC chemokine ligand 8 secretion via the MEK-dependent signal pathway in human epithelial cells

Patricia Méndez-Samperio *, Artemisa Trejo, Elena Miranda

Departamento de Inmunología, Escuela Nacional de Ciencias Biológicas, IPN, Carpio y Plan de Ayala, Mexico, D.F. 11340, Mexico

Received 23 February 2005; accepted 15 April 2005

**Abstract**

Current knowledge of the cellular signaling by *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) in epithelial cells is still limited. In this study, we provide evidence that the signaling events induced by *M. bovis* BCG in these cells included phosphorylation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK). Our data also demonstrate that *M. bovis* BCG-induced CXC chemokine ligand (CXCL)8 release in epithelial cells was reduced by a mitogen-activated protein/ERK kinase (MEK) inhibitor (PD98059), but not by a p38 MAPK (SB203580) inhibitor. In addition, we found that a second and more potent MEK inhibitor (U0126) significantly blocked CXCL8 release in epithelial cells by *M. bovis* BCG. Evaluation of CXCL8 RNA messages by reverse transcription-polymerase chain reaction (RT-PCR) revealed that the inhibitory effect of PD98059 and U0126 was associated with a reduction in this parameter. Moreover, the induction of CXCL8 secretion in epithelial cells by *M. bovis* BCG occurs at the transcription level. Collectively, the findings reported in the present study suggest that MEK signaling is essential for the induction of CXCL8 in epithelial cells in response to *M. bovis* BCG.

Keywords: CXCL8; Epithelial cells; *Mycobacterium bovis*; MEK; p38 MAP kinase

**1. Introduction**

Worldwide, one of the major causes of death by infectious disease is *Mycobacterium tuberculosis* infection [1]. Bacillus Calmette-Guérin (BCG) is a live, attenuated strain of *Mycobacterium bovis* widely used as a vaccine against *M. tuberculosis* infection [2]. During infection, mycobacteria induces increased expression of chemokines, including the CC chemokine subfamily members and the CXC chemokine subfamily members, such as interleukin (IL)-8 (CXCL8) [3,4]. CXCL8 is an important chemokine which stimulates chemotactic action in T cells, neutrophils, and basophils in tuberculosis [5,6].

At a cellular level, phagocytosis of *M. tuberculosis* by monocytic cells or epithelial cells is an important stimulus to CXCL8 production [7,8]. Recently, we reported that human monocytes infected with *M. bovis* induce the secretion of CXCL8 [9]. Since it has been reported that the respiratory epithelium is a primary target for microbial infection [10,11], and that epithelial cells are considered as an important cellular source of chemokines [8], we also have investigated the induction of CXCL8 by *M. bovis* BCG in human epithelial cells infected with *M. bovis* [12]. However, the molecular mechanisms underlying the *M. bovis*-induced CXCL8 secretion in the human epithelial cells are not completely understood.

Cellular signaling by mycobacteria is mediated through activation of mitogen-activated protein (MAP) kinases [13,14]. These kinases include three major members: the extracellular signal-regulated kinases (ERKs),
the c-Jun N-terminal kinase (JNK)/stress-activated protein kinases, and the p38 kinase [15,16]. The role of MAPK pathways in generation of cytokines and chemokines during mycobacterial infection has been investigated in vitro. For example, infection of human monocytes with *Mycobacterium avium* induces phosphorylation of all three MAP kinases, and subsequent ERK1/2 activation is required for TNF-α secretion [17,18]. In addition, we have demonstrated that *M. bovis* BCG induction of TNF-α is dependent on the ERK pathway [19]. Recently, it has been demonstrated that mannolysated phosphatidylinositol, isolated from *M. tuberculosis*, stimulated ERK1/2 phosphorylation in human alveolar macrophages and activation of this MAP kinase was required for production of CC-chemokines [20]. Little is known, however, about the cellular signaling pathways by which *M. bovis* mediate CXC-chemokine induction. In this study, we have investigated the involvement of MAP kinases in *M. bovis* BCG-induced CXCL8 expression in human epithelial cells.

## 2. Materials and methods

### 2.1. Reagents

The specific inhibitor of p38 MAP kinase pathway, SB203580, and the MEK1/2 inhibitors, PD98059, and U0126 (Calbiochem-Novabiochem, San Diego, CA) were used and dissolved in DMSO (Sigma–Aldrich). Actinomycin D (ActD) was purchased from Sigma–Aldrich (St. Louis, MO).

### 2.2. Mycobacteria

*Mycobacterium bovis* (ATCC 35733) was obtained from the American Type Culture Collection (Rockville, MD, USA). *M. bovis* was grown at 37°C in Sauton medium for 2 weeks. Cultures were harvested by centrifugation and quantified by a colony forming unit (CFU) assay. Aliquots of the stock were kept at −70°C.

### 2.3. Cell culture

The human epithelial HEp-2 cell line was originally acquired from the American Type Culture Collection (Rockville, MD, USA). Cells were grown at 37°C in a humidified 5% CO₂ incubator using minimum essential medium Eagle with 2mM of l-glutamine, 1mM of sodium pyruvate, 0.1mM of non-essential amino acids, and Earle’s BSS adjusted to contain 1.5g/L of sodium bicarbonate and 10% heat-inactivated fetal bovine serum (Gibco-BRL, Rockville, MD, USA). The human alveolar epithelial A549 cell line was maintained in a humidified 5% CO₂ atmosphere in Dulbecco’s modified Eagle’s medium. Cells (10⁵/well) were infected with mycobacteria using an oncopsonized bacteria-to-cell ratio of 3:1. Control cultures with no mycobacteria were always included. SB203580, PD98059, U0126 or ActD were added to some of the cultures. Each experiment was repeated a minimum of three times with different donors.

### 2.4. Western blot analysis

HEp-2 cells were infected with *M. bovis* BCG at a bacteria/cell ratio of 3:1 in RPMI-1640 media and incubated at 37°C in 5% CO₂. After 30 min cells were washed three times with cold PBS and cell lysates were prepared using ice-cold lysis buffer (150mM NaCl, 1mM phenylmethylsulfonyl fluoride, 1μg/ml aprotinin, 1μg/ml leupeptin, 1μg/ml pepstein, 1 mM pervanadate, 1 mM EDTA, 1% igepal, 0.25% deoxycholic acid, 1 mM NaF, and 50 mM Tris–HCl (pH 7.4)). Following lysis the cellular suspension was centrifuged, and the clarified lysates were stored at −20°C. Equal amounts of protein were separated on SDS–polyacrylamide gel electrophoresis and electroblotted to nitrocellulose membranes (Bio-Rad Laboratories, Florida, USA). After blocking with 5% nonfat milk in TBS-T (Tris-buffered solution, pH 7.6, 0.05% Tween 20), the membranes were incubated overnight with primary antibodies phospho-p38, total p38, phospho ERK1/2, or total ERK1/2 from New England Biolabs (Schwalbach, Germany) as indicated by the manufacturer’s instructions. The primary antibody reaction was followed by 1h incubation with the appropriate horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G at room temperature. The peroxidase-positive bands were detected by immersing the blots in a developing solution (73mM sodium acetate, pH 6.2) containing 0.3% diaminobenzidine tetrahydrochloride and 0.04% H₂O₂ at room temperature for 5 min. The enzyme reaction was terminated by washing the blots in 0.1 M H₂SO₄.

### 2.5. CXCL8 ELISA

CXCL8 levels were determined in culture supernatants from control and *M. bovis*-infected HEp-2 cells by using the kit human CXCL8 enzyme-linked immunosorbent assay (ELISA) system (R&D Systems, Minneapolis, MN, USA). The ELISA assays were carried out according to the manufacturer’s instructions.

### 2.6. Assessment of CXCL8 gene expression by RT-PCR

HEp-2 cells were treated with PD98059 (50μM) or U0126 (30μM) for 30 min, and were then infected with *M. bovis* BCG at a MOI=3. Total RNA from cells was isolated by the method of Chomczynski and Sacchi [21]. Briefly, total RNA was extracted with TRIzol (Life Technologies, Rockville, MD) for 5 min at room temperature, transferred to a microfuge tube, and then 200μl of 145 chloroform was added, vortex mixed, and incubated for 146
10 min. After centrifugation for 5 min, the aqueous layer was transferred to a fresh tube, and RNA was precipitated by adding an equal volume of isopropanol. After centrifugation, the RNA pellet was dried and dissolved in 50 μl of sterile water. The RNA concentration was determined by optical density measurements. Cellular RNA (2 μg) was reversibly transcribed using random hexamer primers (Gibco-BRL, Rockville, MD) and Superscript II reverse transcriptase (Gibco-BRL). The resulting cDNA was amplified for a total of 20 cycles in the standard reaction mixture using Taq DNA polymerase and the appropriate CXCL8 primers (Clontech Laboratories, Palo Alto, CA, USA). PCR products were electrophoresized on a 2% agarose gel, visualized by ethidium bromide staining and photographed. Expression of GAPDH was used to check RNA integrity.

2.7. Statistical analysis

Results were analyzed by Student’s t test. p ≤ 0.05 was considered significant.

3. Results

3.1. Mycobacterium bovis BCG activates MAPKs in epithelial cells HEp-2

In a previous study, secretion of CXCL8 in epithelial cells infected with M. bovis was determined [12]. In this study, we examined whether M. bovis stimulates MAPKs in HEp-2 cells by using Western blot analysis with anti-phospho-specific Abs against p38 MAPK or ERK1/2 kinases. The data indicate that M. bovis BCG infection induced strong phosphorylation of p38 and ERK1/2 MAP kinases in HEp-2 cells (Figs. 1A and B). To confirm that M. bovis BCG activates MAPKs in HEp-2 cells, phosphorylation of p38 MAP kinase by M. bovis BCG in HEp-2 cells was inhibited by the P38 MAP kinase inhibitor, SB203580 (Fig. 1A). A similar inhibitory effect of an inhibitor of MEK PD98059, was observed on activation of ERK(1/2) MAP kinase by M. bovis BCG in HEp-2 cells (Fig. 1B).

To gain an insight into molecular mechanisms leading to M. bovis BCG-induced CXCL8 production, we studied the effect of specific inhibitors on signal transduction through the p38 MAPK and MEK/ERK pathways in HEp-2 cells. We preincubated HEp-2 cells with different concentrations of SB203580, to specifically inhibit p38 MAP kinase activity [22] or with PD98059, an inhibitor of MEK via upstream activator-dependent phosphorylation [23] before M. bovis infection. Results were that M. bovis BCG-induced CXCL8 production was significantly reduced by PD98059 in a dose-dependent manner, beginning at 5 μM PD98059 (p < 0.01) (Fig. 2B), whereas SB203580 had no significant effect (Fig. 2A). To confirm the regulatory role of ERK pathway, we also evaluated the effect of a second and more potent MEK inhibitor (U0126, a direct inhibitor of MEK-1 and -2) [24]. Fig. 3 shows that this MEK inhibitor was also capable of significantly blocking (92% inhibition at 30 μM) M. bovis BCG-induced CXCL8 secretion in a dose-dependent manner. A similar effect was observed with other epithelial A549 cell line (Fig. 3). The next step in the investigation led us to examine whether the CXCL8 inhibition reported in Fig. 2B and 3 occurred at the pre- or post-transcriptional level. Therefore, the impact of PD98059 and U0126 on CXCL8 gene expression was monitored. As shown in Fig. 4, inhibition of MEK1/2 reduced M. bovis BCG-mediated CXCL8 mRNA expression, a reduction that correlated with the high level of downregulation observed for the CXCL8 protein secretion (Figs. 2B, 2C and 3). These results indicate that abrogation of CXCL8 production by blocking MEK1/2-dependent signals is a result of pretranscriptional downregulation of CXCL8. In order to verify or exclude whether transcriptional upregulation of the CXCL8 gene is the predominant mechanism through which M. bovis BCG induces CXCL8 secretion, HEp-2 cells were pretreated with or without various concentrations of the transcriptional inhibitor actinomycin D for 30 min at 37 °C prior to the addition of BCG (MOI = 3) for 48 h at 37 °C. Then the culture supernatants were assayed for CXCL8 content by ELISA. Results from all five experiments showed that, addition of actinomycin D significantly reduced in a concentration-dependent manner the ability of M. bovis BCG to induce CXCL8 secretion (Fig. 5).

Fig. 1. Mycobacterium bovis BCG-induced phosphorylation of p38 and ERK1/2 in HEp-2 cells. HEp-2 cells were exposed to M. bovis BCG (3:1 bacteria/cell), and after a period of 30 min cell lysates were prepared and assessed for p38 (A) and ERK(1/2) (B) MAP kinases activation by Western blot. HEp-2 cells were pretreated with SB203580 (30 μM) (A) or PD98059 (50 μM) (B) for 30 min and then stimulated with M. bovis. Cell extracts were prepared and subjected to immunoblot analysis. These results are representative of one of three experiments performed independently.
4. Discussion

The pivotal roles of MAPKs in CXCL8 production have been documented [25,26]. However, depending on the stimuli and cell types, three MAPKs have been reported to differentially mediate CXCL8 expression [27–29]. In this study, we show that M. bovis BCG indeed can rapidly stimulate p38 MAPK and ERK1/2 phosphorylation in HEp-2 cells. Despite this, we provide evidence leading to the observation that M. bovis BCG-induced CXCL8 production depends on MEK, but not on p38 MAPK. First, M. bovis BCG-stimulated CXCL8 protein secretion was reduced by a MEK inhibitor (PD98059), but not by a p38 MAPK (SB203580) inhibitor. Second, these results were confirmed by using a more specific MEK inhibitor (U0126) with different mechanism of action [24]. Third, both inhibitors significantly reduced CXCL8 mRNA expression.

Although previous reports have demonstrated mycobacterial-induced CXCL8 production [7,8], the upstream signaling mechanism involved in this M. bovis BCG-inducible epithelial cell function has not been fully elucidated. Therefore, in this study, we for the first time have demonstrated the involvement of the MEK1/2 MAPK cascade in the regulation of CXCL8 in M. bovis BCG-infected epithelial cells.
these data. First, p38α and p38β isoforms might exert distinct action in regulating CXCL8 synthesis. Second, that the upstream signal transduction for CXCL8 protein expression varies among different activating inducers. Third, SB203580 might have p38 MAPK-independent nonspecific action, which possibly can influence CXCL8 protein translation and/or stability [36]. On the other hand, Toll-like receptor 2 (TLR2) has been implicated in the recognition of M. bovis BCG particulate or soluble antigens leading to macrophage proinflammatory cytokine production [37]. However, a recent report has demonstrated that the proinflammatory response to live M. bovis BCG is independent of TLR2 and TLR4 [38]. Since abundant expression of TLR2 mRNA in human lung epithelial cells has been documented [39], and TLR2 mediated responses involve p38 MAPK activation, it may be a valuable undertaking to carry out an analysis of the dependency on TLR2 and TLR4 for M. bovis-induced CXCL8 production by human epithelial cells.

Collectively, the findings reported in the present study suggest that MEK1/2-dependent signaling events play pivotal roles in M. bovis BCG-induced CXCL8 expression in human epithelial cells. Further experimental work is needed to know whether the effect of MEK1/2 on M. bovis BCG-induced CXCL8 secretion may represent a significant regulatory mechanism in vivo. However, these data may represent an important regulatory mechanism during the immune response to BCG, since CXCL8 induction during mycobacterial infection is a major neutrophil-activating factor and chemotactic.

Acknowledgments

This research was supported by a grant from the Coordinación General de Posgrado e Investigación (CGPI) No. 20050019. P.M.S. is a COFAA, EDI, and SNI fellow.

References


induced IL-8 expression via an effect on the IL-8 promoter in intestinal epithelial cells, Immunology 108 (2003) 502–512.


