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Evaluation of the role of *Mycobacterium tuberculosis* pili (MTP) as an adhesin, invasin, and cytokine inducer of epithelial cells

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ABSTRACT

This study was undertaken in order to assess the involvement of *Mycobacterium tuberculosis* pili (MTP) as an adhesin, invasin, and cytokine inducer in the *M. tuberculosis*-epithelial cell interaction. A MTP-deficient strain of *M. tuberculosis* demonstrated a significant reduction of 69.39% ($p = 0.047$) and 56.20% ($p = 0.033$) in its ability to adhere to and invade A549 pulmonary epithelial cells, respectively, in comparison with the wild-type strain. Complementation of the MTP-deficient mutant restored its adhesion and invasion capacity back to the wild-type levels. Overall, it was found that similar concentrations of IL-1β, IL-4, IL-6, IL-8, G-CSF, IFN-γ, MCP-1, and TNF-α were induced in A549 cells infected with the MTP-proficient and MTP-deficient strains. However, at 48 h post-infection, the MTP-deficient mutant induced significantly lower levels of TNF-α than the wild-type strain ($p = 0.033$). Furthermore, at 72 h post-infection, the mutant induced significantly higher levels of IL-8 than the wild-type ($p = 0.005$). We conclude that MTP is an adhesin/invasin of epithelial cells and, while playing a role in *M. tuberculosis* entry, they do not appear to largely influence the epithelial cell cytokine response.

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Introduction

Mycobacterium tuberculosis, the notorious causative agent of tuberculosis (TB), remains one of the main causes of human mortality by an infectious disease. The lack of effective treatment, diagnostic, and preventative strategies continue to impede TB control, which is further compounded by the increasing prevalence of multi-, extensively, and totally drug-resistant strains of the pathogen and the HIV/AIDS co-epidemic. A better understanding of the virulence factors that are associated with the pathogenesis of this organism is crucial to improve strategies to control and reduce global TB burdens.

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Adhesins are molecules or structures that are present extracellularly on the bacterial cell surface. They play an important role in the initial host-pathogen interaction by mediating adherence, a precursor to host cell entry. A major *M. tuberculosis* adhesin of epithelial cells is the heparin-binding hemagglutinin adhesin (HBHA), which has also been implicated in extrapulmonary dissemination of the pathogen.[1] Bacterial species are known to produce multiple adhesins to effectively infect host cells. Therefore, other adhesins may contribute to *M. tuberculosis* adhesion to epithelial cells.[2]

Pili are a well-characterized family of bacterial adhesins. Two pili types have been described for *M. tuberculosis*, namely curli and type IV pili.[3] The curli or coiled pilus morphotype, first identified in *Escherichia coli* and *Salmonella* spp., are known to be mediators in biofilm formation, adherence to and colonization of the host, and induction of host inflammation.[4] The curli-like pili of *M. tuberculosis*, MTP, identified by Alteri et al.,[5] resemble the pili fibers of *E. coli* and *Salmonella enterica* and are encoded by the *mtp* (Rv3312A) gene. These researchers also reported on the ability of MTP to bind to the extracellular matrix protein laminin *in vitro*, which suggested that MTP may function as an adherence factor.[5]

A central focus of our research group currently is elucidating the function of MTP. We have previously identified the role of MTP in *in vitro* biofilm formation[6] and in the adherence to and invasion of macrophages.[7] We have also shown that the *mtp* gene sequence is unique to *M. tuberculosis* complex strains and highly conserved amongst clinical isolates and, thus, MTP may be a suitable biomarker for a TB diagnostic test.[8]

In this study, we compared adhesion and invasion of a wild-type, Δ*mtp* mutant, and *mtp*-complemented *M. tuberculosis* strains to the A549 pulmonary epithelial cell line. We also compared cytokine production by A549 cells infected with these strains.

Materials and methods

Bacterial strains and growth conditions

M. tuberculosis clinical isolate V9124[9] and the previously constructed MTP-deficient Δ*mtp* mutant and MTP-overexpressing *mtp*-complemented strains[6] were used in this study. Cultures were grown at 37 °C in Middlebrook 7H9 broth (Difco), supplemented with 10% (v/v) oleic acid-albumin-dextrose-catalase (OADC; Becton Dickinson), 0.5% (v/v) glycerol (Sigma), and 0.05% (v/v) Tween 80 (Sigma). Hygromycin (75 μg mL^{-1}; Roche Applied Sciences) or kanamycin (30 μg mL^{-1}; Sigma) were included for the mutant and complemented strain cultures, respectively.

Epithelial cell preparation

The A549 human type II alveolar epithelial cell line (ATCC CCL-185) was maintained in Eagle's Minimum Essential Medium with Earle's Balanced Salt Solution and 2 mM L-glutamine (Lonza), supplemented with 10% (v/v) fetal bovine serum (Biowest). Following trypsinization, 2×10^5 cells were seeded into 24-well plates (NEST Biotechnology) and incubated for 24 h at 37 °C in 5% CO_2.

Infection of epithelial cells

Logarithmic phase mycobacterial cultures were pelleted by centrifugation (2000 × *g* for 10 min; Heraeus Multifuge 3S-R Centrifuge, Thermo Scientific) and resuspended in fresh cell culture media. The epithelial cells were infected with the bacterial strains at a multiplicity of infection (MOI) of 1:1 for the adhesion and invasion assays. A MOI of 10:1 was used for the cytokine assay to ensure sufficient cytokine production.[10] Plates were incubated at 37 °C and 5% CO_2 at the desired time intervals. The inocula were also serially diluted and plated in triplicate onto Middlebrook 7H11 (Difco) agar plates, containing 10% (v/v) OADC and 0.5% (v/v) glycerol, to enumerate viable bacilli used for infection. Trypan blue exclusion was used to determine the number of viable epithelial cells at the time of infection, to confirm the MOI.

Adhesion assay

The adhesion assay was performed as previously described.[7] At the end of the 1 h incubation period, media was removed and the epithelial cells washed thrice with 1 mL phosphate-buffered saline (PBS; Oxoid). Adherent epithelial cells were lysed with 1 mL of 0.1% (v/v) Triton X-100 (Sigma) for 20 min at 37 °C and 5% CO_2. The number of adherent bacteria was determined by plating ten-fold serial dilutions of the lysate, in triplicate, onto 7H11 agar plates. Colonies were counted after 3 weeks of incubation at 37 °C.

Invasion assay

The invasion assay was performed as previously described.[7] After 2 h incubation, monolayers were treated with amikacin (300 μg mL^{-1}; Sigma) for 1 h at 37 °C in a 5% CO_2 atmosphere. The media was removed and plated onto 7H11 plates to confirm the death of non-invaded microbes. The amikacin was removed by washing thrice with PBS. Adherent epithelial cells were lysed and the number of viable invaded organisms was determined as in the adhesion assay.

Cytokine analysis

Following 4 h of infection, spent media was removed, the wells washed with PBS, and fresh tissue culture media added. At 24, 48, and 72 h post-infection, cell culture supernatants were removed and filtered through a 0.2 μm filter (PALL Life Sciences). Bovine serum albumin (BSA; Sigma) was added at a final concentration of 0.5% (w/v) and the samples were stored at −80 °C. The cytokine levels in supernatants were measured using a Bio-Plex Pro Human Cytokine Multi-Plex Panel (Bio-Rad) in a Bio-Plex 200 System (Bio-Rad), according to the manufacturer's instructions.

Statistical analysis

Adhesion/invasion assays were performed at least three independent times in duplicate. Cytokine experiments were performed at two independent times and assayed in duplicate. The significance of differences between the strains was determined by one-way analysis of variance (ANOVA). All data

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