Original Article

Effect of mycobacterial secretory proteins on the cellular integrity and cytokine profile of type II alveolar epithelial cells

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ABSTRACT

Background: Pulmonary tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (*M. tb*). In lungs, alveolar macrophages and type II alveolar epithelial cells serve as a replicative niche for this pathogen. Secretory proteins released by actively replicating tubercle bacilli are known to interact with host cells at the initial stages of infection. To understand the role of these cells in TB pathogenesis, it is important to identify the mycobacterial components involved in interaction with alveolar epithelial cells. **Materials and Methods:** We fractionated the whole secretory proteome of *M. tb* H_{37}Rv into 10 narrow molecular mass fractions (A1-A10; <20 kDa to >90 kDa) that were studied for their binding potential with A549; type II alveolar epithelial cell line. We also studied the consequences of this interaction in terms of change in epithelial cell viability by MTT assay and cytokine release by ELISA. **Results:** Our results show that several mycobacterial proteins bind and confer cytolysis in epithelial cells. Amongst all the fractions, proteins ranging from 35-45 kDa (A5) exhibited highest binding to A549 cells with a consequence of cytolysis of these cells. This fraction (A5) also led to release of various cytokines important in anti-mycobacterial immunity. **Conclusion:** Fraction A5 (35-45 kDa) of mycobacterial secretory proteome play an important role in mediating *M. tb* interaction with type II alveolar epithelial cells with the consequences detrimental for the TB pathogenesis. Further studies are being carried out to identify the candidate proteins from this region.

KEY WORDS: Cytokines, cytotoxicity, *Mycobacterium tuberculosis (M. tb)*, type II alveolar epithelial cells

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INTRODUCTION

Mycobacterium tuberculosis (M. tb), the causative agent of tuberculosis (TB), claims nearly 2 million deaths annually. *M. tb* primarily infects lungs after inhalation of bacilli-laden droplet nuclei via respiratory route. In the lung microenvironment, alveolar macrophages constitute the principal host cells involved in *M. tb* invasion and replication.[1] They function to engulf and mount the foremost line of defense to tubercle bacilli; however, *M.tb* resists the phagocytic cell's bactericidal mechanism and manages to replicate within.[2] Although numerous facets of phagocytic cell- *M. tb* interactions have been studied, there is mounting evidence that bystander alveolar epithelial cells (AEC) are also cellular targets for *M. tb* infection. Various studies have documented mycobacterial invasion and replication potential within type II AEC.[3-5] It is considered that mycobacterial invasion of AEC possibly creates a niche for the bacterium to replicate and establish the infection, avoiding potentially hostile environment of macrophages. Additionally, epithelial cell cytolysis owing to mycobacterial infection results in alveolar barrier disruption and subsequently contributes to the dissemination of both free and macrophage-ingested mycobacteria from the lungs.[6-8]

Formerly, AEC were looked upon as bystander cells performing only their physiological roles, while it has

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also been documented that type II AEC are ideally suited to play a role in local regulation of inflammatory response within alveolar space.[9] These cells produce inflammatory mediators such as interleukin (IL)-8,[10] prostaglandin,[11] nitric oxide,[12] and complement proteins[13] capable of modulating local immune response.

Secreted proteins by *M. tb* are believed to mediate important biological functions by interacting with host cells and are potentially important for virulence and pathogenesis of the bacterium.[14,15] Though the complete biology of mycobacterial antigens and their role in directing the fate of an infection is not deciphered, yet it is a well-established fact that extracellular proteins secreted by actively growing bacilli rather than mycobacterial subcellular components are the key molecules to come in contact of host cells at the initial stages of infection both in human[16] and in various experimental animal models.[17] These proteins have been studied for immuno-dominant antigens as these were considered to be rapidly recognized by host lymphocytes.[18] However, till now, no systematic study has been carried out to dissect the components of mycobacterial secretory proteome in order to identify the candidate proteins involved in the interaction with AEC. Therefore, present study has been designed to elucidate the interaction of mycobacterial short term culture filtrate (STCF) components with A549 cells, with the consequences imperative for the dissemination or control of disease. As it is not possible to elute and evaluate all the secretory proteins present in the culture filtrate, here fractionation of the mycobacterial secretory proteome into narrow molecular mass fractions was performed, and the interaction of these fractions with type II AEC was investigated.

MATERIALS AND METHODS

Cell Line Culture and Maintenance

A549 (ATCC CCL185); a human type II AEC line, was obtained from ATCC (American Type Culture Collection, Rockville, MD) and maintained in RPMI-1640 supplemented with 25 mM HEPES, 1 mM L-glutamine, and 1 mM sodium pyruvate and 10% heat inactivated fetal bovine serum (FBS, Sigma chemical, St Louis, MO) at 37°C and 5% CO_2 in T50 and T250 tissue culture flasks (Grenier Bio-one Bioscience, Germany). Before use in experiments, the cells were released from the culture flasks with trypsin-EDTA solution, washed with incomplete media (RPMI without FBS), seeded onto tissue culture plates (Nunc, Waltham, MA) as needed, and were allowed to grow to 80-90% confluency.

Fractionation of *M. tb* STCF into Narrow Molecular Mass Fractions

Mycobacterial crude STCF was obtained from the *M. tb* H_{37}Rv log phase cells grown as stationary culture for 3-4 weeks as described earlier.[19] 5 mg of STCF was separated on 16% preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) overnight on protein II xi cell (BioRad). Constant current of 10 mA was applied for stacking of protein samples, followed by resolution at 30 mA. After the electrophoresis run, the gel was pre-equilibrated in tris borate buffer (pH 8.7; 50 mM tris and 250 mM boric acid) and were transferred to whole gel elutor and electroeluted at 250 mA for 30 min, and the current was reversed for 10 sec at the end of the elution period to loosen molecules sticking to the cellophane membrane at the bottom of the wells. Fractions were harvested from the unit with the help of vacuum pump and designated as A1 to A10 in the order of increasing molecular mass and were kept frozen at -20°C until use. All fractions were analyzed by separation on SDS-PAGE (16%) followed by silver staining. Protein concentration of various fractions, obtained upon fractionation of STCF, was estimated using bicinchoninic acid (BCA) kit (Sigma chemicals, Ltd.) according to manufacturer's instructions.

Binding of STCF Fractions with Host Cells

A549 cells cultured for 4 days were harvested and were washed 3 times with RPMI-1640 after centrifuging at 400 × g for 10 min, suspended in PBS and were counted by trypan blue dye exclusion method. 5×10^5 cells/tube/ L were taken for each test and were treated with different culture filtrate's fractions at optimized concentration of 20 μg/mL and kept for incubation for 30 min at 37°C. After 3 washings with PBS (containing 0.1% bovine serum albumin), the cells were incubated with anti-STCF antisera (available in lab) at the dilution of 1:100 for 45 min at 4°C. Subsequently 6 washings with RPMI-1640 were given, and the cells were again incubated with anti-mouse FITC (1:250) for 20 min at 4°C. The pelleted cells after 3 washings with RPMI-1640 were suspended in 300 μL of fixative (1% paraformaldehyde in phosphate buffered saline). Flow cytometric analysis was performed in FACS scan flow cytometer (Becton Dickinson, Mountview, CA). Data analysis was done using WinMDI 2.8 software. Debris in the cellular suspension was excluded from the analysis by suitable gating. The cell population was identified on the basis of forward vs. side scatter and gated appropriately to determine the percentage of positively stained cells. Appropriate antibody control, cell control were included, and the data obtained was represented as percent binding. The experiments were conducted thrice in duplicate.

Cytotoxicity Assay

For MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, 4×10^5 A549 cells/mL were cultivated, counted with trypan blue dye exclusion method, and 100 μL of cell suspension was dispensed in each well of 96-well plates. Cells were maintained in a 5% CO_2 incubator at 37°C. After 72 hr, supernatant was removed leaving adhered cells, and 20 μg/mL of each of the fractions (culture filtrate protein) was added to the wells, followed by 72 hr incubation in a 5% CO_2 incubator at 37°C. Supernatant was removed, and 110 μL MTT reagent was added to each well. After 4 hr incubation at 37°C, 85 μL of MTT reagent was removed and to remaining 25 μL, 50 μL of dimethyl sulfoxide was added for cell lysis and

solubilization. After 10 min incubation, the absorbance was read at 545 nm, and the results were represented as percent cytotoxicity, taking control (cells without proteins, with MTT) as reference standard. The experiment has been conducted twice in triplicate wells.

Cytokines Release Assay

4×10^5 A549 cells/mL were cultured, in 96-well plates in 5% CO_2 at 37°C. Concentration of various cytokines; interferon (IFN)-γ, tumor necrosis factor (TNF)-α, IL-6, IL-12(p40) was measured in the culture supernatants removed from the cell monolayers stimulated with 20 μg/mL of each of the fractions for 72 hr by ELISA kits (BD Pharmingen) according to manufacturer's instructions.

RESULTS

Fractionation of STCF in Narrow Molecular Mass Fractions

Short-term culture filtrate was obtained from the stationary culture of *M. tb* H_{37}Rv, which was allowed to grow for 4-5 weeks. The protein concentration of STCF was found to be 4.44 mg/mL. The protein profile of STCF resolved on 16% SDS-PAGE [Figure 1] showed the presence of numerous bands with ~30 prominent protein bands ranging in molecular weight from 3 kDa to more than 100 kDa.

Fractionation of STCF by whole gel elutor resulted into 10 narrow molecular mass fractions (A1 to A10) ranging from <20 kDa to >90 kDa of molecular mass [Table 1] and each fraction consisting of about 2-5 visible protein bands [Figure 1]. The fractionation was carried out using the combination approach of preparative high resolution SDS-PAGE, followed by whole gel electro-elution. The elutor acts as an electro-dialyzer and removes SDS from protein, leaving culture filtrate protein fractions in a non-toxic physiological buffer, which was then used directly in various cellular assays.

Binding of STCF Fractions with Host Cells

To identify proteins from the mycobacterial secretory proteome that could have some role in pathogenesis of *M. tb* disease, the binding of STCF fractions with AEC was checked by flow cytometric analysis, and binding capacity was recorded as the percentage of positively stained cells by anti-mouse FITC [Figure 2]. The background staining observed in control i.e. without protein treatment, was ~5.1 % [Figure 2]. The minimal cut-off for the binding capacity was taken to be 15% (3 times the background fluorescence) and has been subtracted from the fluorescence obtained after treatment with each fraction. The fractions A1, A2, A4, A5, A6, A7, and A8 exhibited good binding with A549 cells, with fraction A5 (35-45 kDa) showing maximum binding, followed by fraction A6 (45-55 kDa) [Figure 2]. These observations clearly demonstrate that the 35-55 kDa region of mycobacterial STCF predominantly contains the proteins involved in binding and interaction with host AEC.

Cytotoxicity Assay

The effect of *M. tb* secretory proteins interaction with AEC was measured in terms of cell viability by MTT assay. Wells containing medium alone (without cells) and MTT

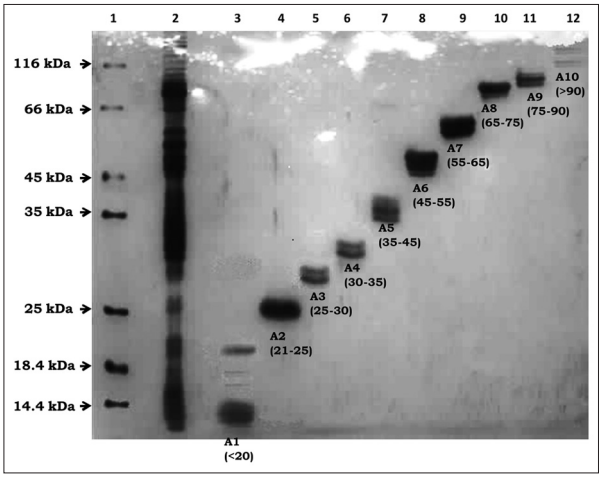

Figure 1: Protein profile of STCF of narrow molecular mass fractions obtained from the fractionation of *M. tb* H_{37}Rv on 16% SDS-PAGE

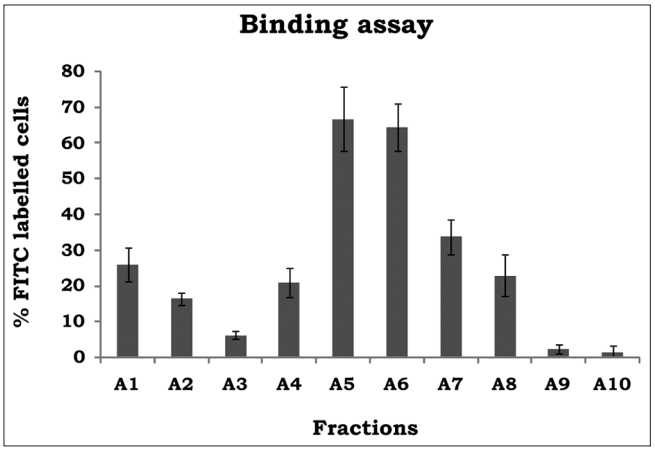

Figure 2: Percent binding of mycobacterial secretory proteins with A549 cell line. Bars represent mean values ± SD, $n = 3$

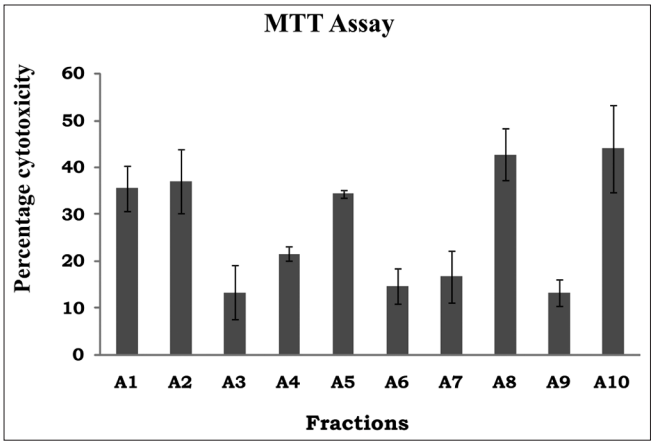

Figure 3: Percent cytotoxicity to A549 cells by mycobacterial secretory proteins. Bars represent mean values ± SD; $n = 6$

reagent were used as negative control. The results are represented as percent cytotoxicity taking control (cells without protein) as reference standard. As it is evident from the Figure 3, all those fractions showing binding above the cut-off value also exerted cytotoxic effects on the host cells although there was no direct correlation between the extent of binding and cytotoxicity. Fraction A7 (55-65 kDa) with ~34% binding to A549 cell line and A6 fraction (45-55 kDa) with ~67% binding displayed a similar cytotoxic potential despite the difference in their binding potential. Similarly, A5 and A6 fractions having almost similar binding exhibited different cytotoxicity. Amongst all the fractions, A10 (>90 kDa) was the only fraction that did not bind to A549 cells, yet it showed maximum cytotoxicity to host cells. Thus, the proteins present in molecular range 35-55 kDa showed high binding with A549 cell line as well as cytotoxicity to AEC.

Cytokine Release

To determine the immuno-modulatory effect of *M. tb* secretory proteins on AEC, cytokine profile of A549 monolayer treated, with narrow molecular mass fractions of mycobacterial STCF, was determined by sandwich ELISA. Amongst various narrow molecular mass fractions of the mycobacterial secretory proteome, A1 (<20 kDa), A5 (35-45 kDa), A6 (45-55 kDa), and A7 (55-65 kDa) resulted into higher IFN-γ secretion [Figure 4a]. Only proteins corresponding to 35-45 kDa (A5) were able to induce prominent release of TNF-α [Figure 4b]. Similar to IFN-γ and TNF-α, high levels of IL-6 [Figure. 4c] were observed in response to A5 fraction corresponding to region of 35-45kDa. However, two more regions containing the proteins of 20-30 kDa were also identified as potent stimulator of IL-6 secretion from A549 cells. Maximum level of secretion of IL-12(p40), a Th1 cytokine considered to be important for protective immunity against mycobacterial infection presented in Figure 4d (> 180 pg/mL), was observed in response to A5 fraction.

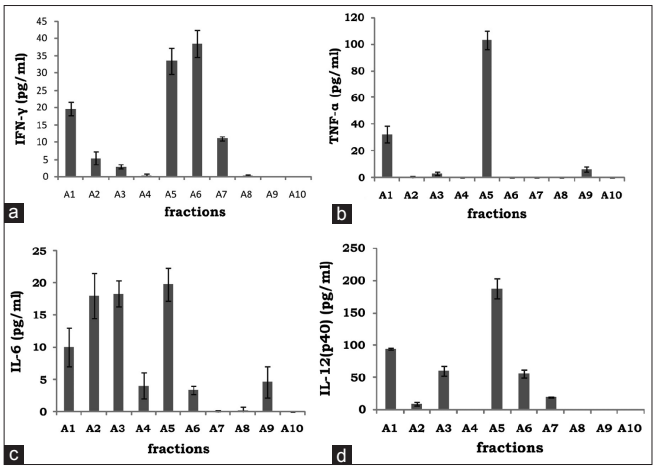

Figure 4: Cytokine release from the A549 cells treated with narrow molecular mass fractions of mycobacterial STCF (a) IFN-γ, (b) TNF-α, (c) IL-6, and (d) IL-12. Bars represent mean values ± SD; *n* = 3

The comparative analysis [Table 1] clearly indicated the significance of proteins present in 35-45 kDaregion to be important in the interaction with A549 cells with a consequence of effect on cellular viability as well as cytokine release. Therefore, there is a need to characterize various proteins present in this molecular range (35-45kDa) to have an insight in the significance of these proteins in host AEC interaction and pathogenesis of tuberculosis.

DISCUSSION

The secretory proteins released by actively replicating mycobacteria have been suggested to play an important role in anti-mycobacterial immunity as infection with killed mycobacteria failed to evoke cellular immunity.[20] Both in human and murine models, *M. tb* secretory proteins have been studied for immuno-dominant antigens based on their potential to generate protective immune responses during *M. tb* infection.[19,21-23] While the roles for various *M.tb* secretory proteins are ascribed based on their interaction with lymphocytes/macrophages, no study has clearly investigated the interaction of these proteins with lung epithelia. Although alveolar macrophages are believed to be principal host cells for the intracellular multiplication of tubercle bacilli, various studies have also reported type II AEC as one of the major portals of mycobacterial entry as well as replicative niche in the lung parenchyma.[4,5,24]

In the present study, we fractionated STCF into 10 fractions to explore their role in interaction with type II AEC. We studied various aspects of host-pathogen interactions such as binding, cytotoxic, and immuno-modulatory potential of mycobacterial proteins on A549 cells. The elution pattern

Table 1: Combined overview of the interaction of STCF fractions with A549 cells

Fractions	Molecular weight	Percent binding	Percent cytotoxicity	IFN-γ (pg/mL)	TNF-α (pg/mL)	IL-6 (pg/mL)	IL-12 (pg/mL)
A1	<20 kDa	28.025	35.64273	20.7	29.4	8.47	93.1
A2	21-25 kDa	16.445	37.164	6.3	-	16.2	9.8
A3	25-30 kDa	6.86	13.46	3.15	3.676	19.22	63.726
A4	30-35 kDa	18.54	21.73333	-	-	5.08	-
A5	35-45 kDa	71.26	34.4975	31.53	106.6	18.46	196
A6	45-55 kDa	67.025	14.775	39.63	-	3.76	58.8
A7	55-65 kDa	34.145	16.9	11.26	-	-	19.6
A8	65-75 kDa	19.665	42.88571	-	-	-	-
A9	75-90 kDa	3.11	13.4	-	7.352	5.46	-
A10	>90 kDa	0.295	44.125	-	-	-	-

DISCUSSION

of culture filtrate fractions on SDS-PAGE employed in the current study was found to be nearly similar as described previously by Andersen and Heron[19] and reported from our lab.[25] Bacterial pathogens typically gain entry to host tissues using some adherence molecules that bind host cells.[26] *In vitro* studies using cultured epithelial cells have also shown that mycobacteria are able to bind type II AEC.[27] However, till date, only a few mycobacterial proteins have been explored for their role in interaction with non-phagocytic cells. Studies of interaction of *M. tb* with human respiratory epithelial cells (Hep-2) have shown binding of a 28 kDa protein, which was found to be heparin-binding hemagglutinin (HBHA). HBHA has also been demonstrated to mediate bacterial attachment to epithelial cells[28] and is further suggested to involve in the extra pulmonary dissemination of these bacteria.[29] Mycobacterial cell entry protein (Mce-1A, 48 kDA),[30] mycobacterial DNA-binding protein1 (MDP1, 21 kDa)[31] are some other proteins identified as a mycobacterial adhesins employed in the mycobacterium-epithelial cell interaction. Another mycobacterial protein, culture filtrate protein-10 (CFP-10) has been shown to bind murine macrophage cell line,[32] but it failed to bind to A549 monolayers.[33] The present study shows that various mycobacterial secretory proteins are capable of binding with A549 cells and predominantly proteins in the regions of <20-25 kDa, 35-55 kDa, and 55-65 kDa are involved in binding and interaction with type II AEC. Thus, mycobacterial secretory proteome contains numerous proteins, including some of the well-characterized proteins as well as some other yet uncharacterized proteins that might aid mycobacteria to bind AEC. Epithelial cells have been suggested to play a significant role in dissemination of mycobacterial infection. Infection of A549 cells with virulent *M. tb* strains Erdman and CDC1551 demonstrated significant cell monolayer clearing,[34] suggesting that mycobacteria cause epithelial cell cytotoxicity, which may result in breaching of the single-layered alveolar barrier. Barrier disruption allows the passage of both, free and macrophage-ingested mycobacteria, and thus facilitates dissemination.[33,35] Till date, only two well-characterized proteins ESAT-6 and HBHA have been implicated in disrupting alveolar epithelial barrier. Our previous work with ESAT-6 has identified this protein to be a bacterial toxin as it causes contact lysis after binding to AEC lines.[33] The results of the present study demonstrate that mycobacteria secrete certain proteins that following binding to epithelial cells result in cytotoxicity of these cells. A5 fraction comprising 35-45 kDa proteins was found to exhibit both maximum binding as well as cytotoxicity to A549 cells. Surprisingly, A10 fraction lead to cytolysis of A549 cells, even without exhibiting any binding to these cells. This could be due to the presence of one or more high molecular weight protein(s) having the inhibitory effect on the binding of other proteins in A10 fraction with A549 cells. However, further studies are required to isolate and purify all the proteins of this fraction and study their interaction with A549 cells individually.

Earlier during experimental TB in mouse model, two regions (4-11 kDa and 26-35 kDa) of mycobacterial secretory proteins have been reported to be good IFN-γ inducers in T-lymphocytes.[23] Phagocytic cells, especially macrophages, are programmed to mediate host defense by releasing pro-inflammatory cytokines such as TNF-α[36] and IL-6[37] that are important for control of mycobacterial growth. Type II AEC has also been shown to contribute to lung immunity by presenting mycobacterial antigens to immune $CD4^+$ T cells by using the class II MHC pathway.[38] A549 cells have been reported to express iNOS, release nitric oxide under the influence of tubercle bacilli conditioned media[39] or cytokine stimulation,[40] and IFN-γ[41] contributing to the control of microbial infection. Our results show that various mycobacterial proteins in narrow molecular mass fractions (A1, A5, A6, and A7) resulted into higher IFN-γ secretion. While, only proteins corresponding to 35-45 kDa (A5) were able to induce prominent release of TNF-α, IL-6, and IL-12. Thus, our study demonstrates that proteins from 35-45 kDa region modulate epithelial cells to release pro-inflammatory and immuno-regulatory cytokines, which could further aid in mycobacterial infection control.

In summary, the present study illustrates that amongst various narrow molecular mass fractions, A5 fraction comprising 35-45 kDa proteins is predominantly involved in the interaction with AEC, thereby leading to modulation of their cellular integrity as well as immune status. Further studies to identify the proteins from this region that are either involved in the disruption of AEC barrier or in the immuno-modulation of these cells are needed to have the better understanding of the pathogenesis of TB. At the same time, host epithelial cell surface molecules utilized for the interactions with mycobacteria also need to be investigated, and such information could be important to develop the novel anti-mycobacterial therapeutic strategies to block this interaction.

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REFERENCES

1. Kaufmann SH. How can immunology contribute to the control of tuberculosis? Nat Rev Immunol 2001;1:20-30.
2. Russell DG. Mycobacterium tuberculosis: Here today, and here tomorrow. Nat Rev Mol Cell Biol 2001;2:569-77.
3. McDonough KA, Kress Y. Cytotoxicity for lung epithelial cells is a virulence-associated phenotype of *Mycobacterium tuberculosis*. Infect Immun 1995;63:4802-11.
4. Bermudez LE, Goodman J. *Mycobacterium tuberculosis* invades and replicates within type II alveolar cells. Infect Immun 1996;64:1400-6.
5. Mehta PK, King CH, White EH, Murtagh JJ Jr, Quinn FD. Comparison of *in vitro* models for the study of *Mycobacterium tuberculosis* invasion and intracellular replication. Infect Immun 1996;64:2673-9.
6. Birkness KA, Deslauriers M, Bartlett JH, White EH, King CH, Quinn FD. An *in vitro* tissue culture bilayer model to examine early events in *Mycobacterium tuberculosis* infection. Infect Immun 1999;67:653-8.
7. Bermudez LE, Sangari FJ, Kolonoski P, Petrofsky M, Goodman J. The efficiency of the translocation of *Mycobacterium tuberculosis* across

a bilayer of epithelial and endothelial cells as a model of the alveolar wall is a consequence of transport within mononuclear phagocytes and invasion of alveolar epithelial cells. Infect Immun 2002;70:140-6.
8. Danelishvili L, McGarvey J, Li YJ, Bermudez LE. *Mycobacterium tuberculosis* infection causes different levels of apoptosis and necrosis in human macrophages and alveolar epithelial cells. Cell Microbiol 2003;5:649-60.
9. Standiford TJ, Kunkel SL, Basha MA, Chensue SW, Lynch JP 3rd, Toews GB, *et al*. Interleukin-8 gene expression by a pulmonary epithelial cell line. A model for cytokine networks in the lung. J Clin Invest 1990;86:1945-53.
10. Wickremasinghe MI, Thomas LH, Friedland JS. Pulmonary epithelial cells are a source of IL-8 in the response to *Mycobacterium tuberculosis*: Essential role of IL-1 from infected monocytes in a NF-kappa B-dependent network. J Immunol 1999;163:3936-47.
11. Schneeberger EE. Alveolar type II cells. In: Crystal RJ, West JB, editors. The Lung: Scientific Foundations. New York: Raven Press; 1991. p. 736-58.
12. Asano K, Chee CB, Gaston B, Lilly CM, Gerard C, Drazen JM, *et al*. Constitutive and inducible nitric oxide synthase gene expression, regulation, and activity in human lung epithelial cells. Proc Natl Acad Sci U S A 1994;91:10089-93.
13. Ferguson JS, Schlesinger LS. Pulmonary surfactant in innate immunity and the pathogenesis of tuberculosis. Tuber Lung Dis 2000;80:173-84.
14. Beatty WL, Russell DG. Identification of mycobacterial surface proteins released into subcellular compartments of infected macrophages. Infect Immun 2000;68:6997-7002.
15. Pieters J, Gatfield J. Hijacking the host: Survival of pathogenic mycobacteria inside macrophages. Trends Microbiol 2002;10:142-6.
16. Demissie A, Ravn P, Olobo J, Doherty TM, Eguale T, Geletu M, *et al*. T-cell recognition of *Mycobacterium tuberculosis* culture filtrate fractions in tuberculosis patients and their household contacts. Infect Immun 1999;67:5967-71.
17. Pollock JM, Andersen P. Predominant recognition of the ESAT-6 protein in the first phase of interferon with Mycobacterium bovis in cattle. Infect Immun 1997;65:2587-92.
18. Abou-Zeid C, Smith I, Grange JM, Ratliff TL, Steele J, Rook GA. The secreted antigens of *Mycobacterium tuberculosis* and their relationship to those recognized by the available antibodies. J Gen Microbiol 1988;134:531-8.
19. Andersen P, Heron I. Simultaneous electroelution of whole SDS-polyacrylamide gels for the direct cellular analysis of complex protein mixtures. J Immunol Methods 1993;161:29-39.
20. Orme IM. Induction of nonspecific acquired resistance and delayed-type hypersensitivity, but not specific acquired resistance in mice inoculated with killed mycobacterial vaccines. Infect Immun 1988;56:3310-2.
21. Sonnenberg MG, Belisle JT. Definition of *Mycobacterium tuberculosis* culture filtrate proteins by two-dimensional polyacrylamide gel electrophoresis, N-terminal amino acid sequencing, and electrospray mass spectrometry. Infect Immun 1997;65:4515-24.
22. Laal S, Samanich KM, Sonnenberg MG, Zolla-Pazner S, Phadtare JM, Belisle JT. Human humoral responses to antigens of *Mycobacterium tuberculosis*: Immunodominance of high-molecular-mass antigens. Clin Diagn Lab Immunol 1997;4:49-56.
23. Andersen P, Askgaard D, Gottschau A, Bennedsen J, Nagai S, Heron I. Identification of immunodominant antigens during infection with *Mycobacterium tuberculosis*. Scand J Immunol 1992;36:823-31.
24. Tomioka H, Sato K, Sano C, Sano K, Shimizu T. Intramacrophage passage of *Mycobacterium tuberculosis* and M. avium complex alters the drug susceptibilities of the organisms as determined by intracellular susceptibility testing using macrophages and type II alveolar epithelial cells. Antimicrob Agents Chemother 2002;46:519-21.
25. Sable SB, Kaur S, Verma I, Khuller GK. Immunodominance of low molecular weight secretory polypeptides of *Mycobacterium tuberculosis* to induce cytotoxic T-lymphocyte response. Vaccine 2005;23:4947-54.
26. Fearon DT, Locksley RM. The instructive role of innate immunity in the acquired immune response. Science 1996;272:50-3.
27. Reddy VM, Hayworth DA. Interaction of *Mycobacterium tuberculosis* with human respiratory epithelial cells (HEp-2). Tuberculosis (Edinb) 2002;82:31-6.
28. Menozzi FD, Rouse JH, Alavi M, Laude-Sharp M, Muller J, Bischoff R, *et al*. Identification of a heparin-binding hemagglutinin present in mycobacteria. J Exp Med 1996;184:993-1001.
29. Pethe K, Alonso S, Biet F, Delogu G, Brennan MJ, Locht C, *et al*. The heparin-binding haemagglutinin of *M. tuberculosis* is required for extrapulmonary dissemination. Nature 2001;412:190-4.
30. Arruda S, Bomfim G, Knights R, Huima-Byron T, Riley LW. Cloning of an *M. tuberculosis* DNA fragment associated with entry and survival inside cells. Science 1993;261:1454-7.
31. Aoki K, Matsumoto S, Hirayama Y, Wada T, Ozeki Y, Niki M, *et al*. Extracellular mycobacterial DNA-binding protein 1 participates in mycobacterium-lung epithelial cell interaction through hyaluronic acid. J Biol Chem 2004;279:39798-806.
32. Trajkovic V, Singh G, Singh B, Singh S, Sharma P. Effect of *Mycobacterium tuberculosis*-specific 10-kilodalton antigen on macrophage release of tumor necrosis factor alpha and nitric oxide. Infect Immun 2002;70:6558-66.
33. Kinhikar AG, Verma I, Chandra D, Singh KK, Weldingh K, Andersen P, *et al*. Potential role for ESAT6 in dissemination of *M. tuberculosis* via human lung epithelial cells. Mol Microbiol 2010;75:92-106.
34. Dobos KM, Spotts EA, Quinn FD, King CH. Necrosis of lung epithelial cells during infection with *Mycobacterium tuberculosis* is preceded by cell permeation. Infect Immun 2000;68:6300-10.
35. Menozzi FD, Reddy VM, Cayet D, Raze D, Debrie AS, Dehouck MP, *et al*. *Mycobacterium tuberculosis* heparin-binding haemagglutinin adhesin (HBHA) triggers receptor-mediated transcytosis without altering the integrity of tight junctions. Microbes Infect 2006;8:1-9.
36. Mohan VP, Scanga CA, Yu K, Scott HM, Tanaka KE, Tsang E, *et al*. Effects of tumor necrosis factor alpha on host immune response in chronic persistent tuberculosis: Possible role for limiting pathology. Infect Immun 2001;69:1847-55.
37. Vanham G, Toossi Z, Hirsch CS, Wallis RS, Schwander SK, Rich EA, *et al*. Examining a paradox in the pathogenesis of human pulmonary tuberculosis: Immune activation and suppression/anergy. Tuber Lung Dis 1997;78:145-58.
38. Debbabi H, Ghosh S, Kamath AB, Alt J, Demello DE, Dunsmore S, *et al*. Primary type II alveolar epithelial cells present microbial antigens to antigen-specific CD4+ T cells. Am J Physiol Lung Cell Mol Physiol 2005;289:L274-9.
39. Kwon OJ, Kim JH, Kim HC, Suh GY, Park JW, Chung MP, *et al*. Nitric oxide expression in airway epithelial cells in response to tubercle bacilli stimulation. Respirology 1998;3:119-24.
40. Roy S, Sharma S, Sharma M, Aggarwal R, Bose M. Induction of nitric oxide release from the human alveolar epithelial cell line A549: An *in vitro* correlate of innate immune response to *Mycobacterium tuberculosis*. Immunology 2004;112:471-80.
41. Sharma M, Sharma S, Roy S, Varma S, Bose M. Pulmonary epithelial cells are a source of interferon-gamma in response to *Mycobacterium tuberculosis* infection. Immunol Cell Biol 2007;85:229-37.

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