Integrity of the Actin Cytoskeleton of Host Macrophages is Necessary for Mycobacterial Entry

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Abstract
Macrophages are the primary hosts for Mycobacterium tuberculosis (M. tb), an intracellular pathogen, and the causative organism of tuberculosis (TB) in humans. While M. tb has the ability to enter and survive in host macrophages, the precise mechanism of its internalization, and factors that control this essential process are poorly defined. We have previously demonstrated that perturbations in levels of cholesterol and sphingolipids in macrophages lead to significant reduction in the entry of Mycobacterium smegmatis (M. smegmatis), a surrogate model for mycobacterial internalization, signifying a role for these plasma membrane lipids in interactions at the host–pathogen interface. In this work, we investigated the role of the host actin cytoskeleton, a critical protein framework underlying the plasma membrane, in the entry of M. smegmatis into human macrophages. Our results show that cytochalasin D mediated destabilization of the actin cytoskeleton of host macrophages results in a dose-dependent reduction in the entry of mycobacteria. Notably, the internalization of Escherichia coli remained invariant upon actin destabilization of host cells, implying a specific involvement of the actin cytoskeleton in mycobacterial infection. By monitoring the F-actin content of macrophages utilizing a quantitative confocal microscopy-based technique, we observed a close correlation between the entry of mycobacteria into host macrophages with cellular F-actin content. Our results constitute the first quantitative analysis of the role of the actin cytoskeleton of human macrophages in the entry of mycobacteria, and highlight actin-mediated mycobacterial entry as a potential target for future anti-TB therapeutics.

Graphical Abstract

Keywords Actin cytoskeleton · Cytochalasin D · F-actin quantitation · Macrophages · Mycobacterium

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Extended author information available on the last page of the article
Introduction

Intracellular pathogens employ diverse strategies to invade and establish infection in host cells, the success of which depends mainly on their utilization of host cellular components and averting host defense responses (Asrat et al. 2014; Bagga and Bouchard 2014; Liu et al. 2017; Thakur et al. 2019). Mycobacterium tuberculosis, the causative organism of tuberculosis (TB) in humans, has been identified to utilize multiple mechanisms for establishing infection (Stanley and Cox 2013; Awuh and Flo 2017; Chaurasiya 2018; Fraga et al. 2018). A comprehensive understanding of these events is crucial in deciphering the processes underlying infection by this deadly pathogen, which remains a major cause of mortality and morbidity worldwide. An estimated 10 million new cases and 1.5 million deaths were reported in 2020 alone, which is further worsened by its opportunistic infection among HIV positive patients and the shortfall in case notifications due to the ongoing COVID-19 pandemic (World Health Organization 2021). Importantly, immediate attention toward designing novel therapeutic strategies to treat this disease has become necessary due to the emergence of multi- and extensively drug-resistant TB (Gandhi et al. 2006; Dye 2009; Keshavjee and Farmer 2012; Lange et al. 2018; Singh et al. 2020; Allué-Guardia et al. 2021).

As a prerequisite to gain access into host cells, mycobacteria interact with and traverse the host cell plasma membrane. It is therefore critical to understand the events and players involved at this first step of mycobacterial entry into its host, which later determines its growth, dissemination and pathogenesis. Targeting this step has the potential advantage of preventing infection early enough, most likely before the manifestation of clinically relevant pathology (El-Etr and Cirillo 2001). Over the years, several key cell surface components of the host and the pathogen have been identified to facilitate efficient host–pathogen interaction. These include a number of candidate receptors on the cell surface of host macrophages, important for the recognition and entry of mycobacteria such as complement receptors, mannose receptor, CD-14, scavenger receptors, DC-SIGN, and Dectin-1 (Ernst 1998; Killick et al. 2013; Awuh and Flo 2017). In this context, we have previously demonstrated a role for host membrane cholesterol and sphingolipids in the entry of mycobacteria into macrophages, by modulating the availability of these lipids using a combination of complementary approaches (Viswanathan et al. 2015, 2018).

The actin cytoskeleton, a dynamic protein network that is involved in cellular processes such as trafficking, motility and signaling, lies beneath the lipid bilayer and has emerged as an integral component in the contemporary model of the plasma membrane (Janmey 1998; Honigmann and Pralle 2016; Chakrabarti et al. 2021). Interestingly, membrane cholesterol and the actin cytoskeleton are believed to exhibit dynamic interplay in terms of their content and organization (Kwik et al. 2003; Sun et al. 2007; Ganguly and Chattopadhyay 2010; Sarkar P, Kumar GA, Shrivastava S, Chattopadhyay A (unpublished observations)). Since the host plasma membrane serves as the first portal of entry for pathogens (Kumar et al. 2016), investigating the role of host actin cytoskeleton in the entry of mycobacteria could prove to be extremely insightful in understanding the mechanics of this essential process. In the present work, we explored the role of the host actin cytoskeleton in the entry of Mycobacterium smegmatis into human THP-1 macrophages. Since the composition and architecture of their cell surface components are conserved, M. smegmatis serves as an effective surrogate model for M. tuberculosis in addressing the mechanisms of mycobacterial entry (Sani et al. 2010; Zhang 2013).

In order to explore the role of actin cytoskeleton in the entry of M. smegmatis, we employed cytochalasin D (CD), a potent inhibitor of actin polymerization. Actin is an abundant cytosolic protein found in eukaryotes both as monomeric G-actin and polymeric F-actin, with F-actin being maintained in a dynamic equilibrium with soluble G-actin (Dominguez and Holmes 2011). In vitro studies have shown that CD stops actin polymerization by binding to the barbed end (the fast growing end) of the actin filament, thereby shifting the equilibrium toward depolymerization (Schliwa 1982; Sampath and Pollard 1991). Our results show that destabilization of the actin cytoskeleton of host macrophages by CD results in a dose-dependent reduction in the entry of M. smegmatis into macrophages. We monitored F-actin content of macrophages utilizing a quantitative confocal microscopy-based technique, previously developed by us (Ganguly et al. 2011). Notably, we observed a close correlation between the entry of mycobacteria into host macrophages with cellular F-actin content, a pointer to the molecular basis for the involvement of actin cytoskeleton in mycobacterial entry. To the best of our knowledge, these results constitute the first quantitative analysis on the role of the actin cytoskeleton of human macrophages in the entry of mycobacteria.

Materials and Methods

Materials

MgCl₂, CaCl₂, penicillin, streptomycin, gentamycin sulfate, CD, DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), NaCl, Phorbol 12-myristate 13-acetate (PMA), Triton X-100 and Tween 80 were obtained from...
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Sigma Chemical Co. (St. Louis, MO). Middlebrook 7H9 broth and 7H10 agar were obtained from Becton, Dickinson DiFco (Sparks, MD). RPMI-1640 medium and fetal bovine serum were obtained from Gibco/Life Technologies (Grand Island, NY). Alexa Fluor 546 phalloidin was from Molecular Probes/Invitrogen (Eugene, OR). Vectashield® antifade mounting medium containing DAPI was obtained from Vector Laboratories (Burlingame, CA). All chemicals used were of the highest available purity. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

Cell Culture

The human monocyte cell line THP-1 (American Type Culture Collection) was maintained and differentiated as described previously (Tiwari et al. 2012, 2014). Briefly, cells were grown in RPMI-1640 medium supplemented with 2 g/l of sodium bicarbonate, 60 μg/ml penicillin, 50 μg/ml streptomycin, 30 μg/ml gentamycin sulfate and 10% fetal bovine serum in a humidified atmosphere with 5% CO₂ at 37 °C. THP-1 monocytes were differentiated into macrophages by treatment with 5 ng/ml PMA for 24 h and were subsequently grown in PMA-free medium for 48 h, prior to further treatments.

Bacterial Culture

*Mycobacterium smegmatis* mc²6 and *E. coli* DH5α were cultured as described previously (Viswanathan et al. 2015, 2018). *M. smegmatis* was grown in Middlebrook 7H9 broth and 7H10 agar supplemented with albumin dextrose complex (5 g/l BSA, 2 g/l glucose and 0.85 g/l NaCl), 0.5% (v/v) glycerol and 0.05% (v/v) Tween 80 under shaking conditions at 37 °C. *E. coli* DH5α was grown in Luria Bertani broth under similar conditions.

Cytochalasin D Treatment of THP-1 Macrophages

Destabilization of the actin cytoskeleton of THP-1 macrophages was carried out using CD as described previously (Ganguly et al. 2008, 2011; Roy et al. 2014) with some modifications. Briefly, a stock solution of CD (2 mM) was prepared in DMSO and further concentrations required for treating macrophages were prepared by diluting it in serum-free RPMI-1640 medium. Prior to treatment, macrophages were incubated in serum-free medium for 3 h at 37 °C, and were then treated with increasing concentrations of CD for 30 min at 37 °C in the same medium. After the treatment, medium was removed to take away excess CD and macrophages were washed twice with PBS prior to the next steps. During the treatments, the amount of DMSO was always maintained at <0.5% (v/v). Treatment of control cells with similar amount of DMSO did not affect cellular morphology.

MTT Viability Assay

The viability of THP-1 macrophages upon treatment with CD was assessed using an MTT assay as described earlier (Viswanathan et al. 2015) with some modifications. In brief, macrophages were plated at a density of ~2 × 10⁶ in 24 well plates and were treated with CD as described above. Cells were then washed with serum-free RPMI-1640 medium and were incubated with MTT dissolved in the same medium at a final concentration of 0.4 mg/ml at 37 °C for 2.5 h. In live cells, mitochondrial enzymes would reduce the MTT salt to formazan (Vistica et al. 1991), the formazan crystals formed were dissolved in DMSO and the color obtained was measured by absorbance at 540 nm in a PowerWave XS2 microplate spectrophotometer (BioTek Winooski, VT).

F-Actin Labeling

Labeling of F-actin in THP-1 macrophages was carried out as described earlier (Ganguly et al. 2011; Roy et al. 2014) with some modifications. Macrophages were plated at a density of ~2 × 10⁶ on glass coverslips and were treated with CD. The macrophages were subsequently washed with PBS containing 1 mM CaCl₂ and 0.5 mM MgCl₂ (buffer A) and were then fixed with 4% (w/v) formaldehyde for 10 min. Cells were permeabilized with 0.5% Triton X-100 (v/v) in buffer A for 6 min. Cells were further washed and stained with Alexa Fluor 546 phalloidin for 1 h and mounted for subsequent confocal imaging.

Confocal Microscopy and Quantitation of F-Actin

The effect of CD treatment on actin cytoskeleton in macrophages was assessed by quantitation of F-actin from images acquired using confocal microscopy. Confocal microscopic images of THP-1 macrophages were acquired on an inverted Zeiss LSM 880 confocal microscope (Jena, Germany). F-actin labeled with Alexa Fluor 546 phalloidin (as described above) was imaged by exciting at 543 nm and collecting emission from 550 nm to 650 nm. F-actin was quantified using a technique previously developed by us (Ganguly et al. 2011) with some modifications. Briefly, images of z-sections were acquired with a 63x/1.4 NA oil immersion objective under 1 airy condition, with a fixed step size of 0.4 μm. Maximum intensity projections were generated from 11 sections (~4 μm from the base into the cell), and area bound by the projected images was determined manually using the Zen software provided with Zeiss LSM 880 confocal microscope. Images of z-sections corresponding to each projected image were processed by fluorescence...
intensity thresholding, followed by the application of a Gaussian filter using Imaris 8.0.0 software (Bitplane AG, Zurich, Switzerland). Contours generated upon joining voxels of equal fluorescence intensity, called iso-surfaces, were generated from z-sections. The volumes enclosed by iso-surfaces were normalized to the projected area of cells for each field and used as a quantitative estimate of cellular F-actin levels.

Quantification of Entry of Bacteria Into Macrophages

Entry of *M. smegmatis* and *E. coli* into THP-1 macrophages was quantified by scoring colony forming units (CFU), as described earlier (Tiwari et al. 2012, 2014). Macrophages plated on 6-well plates at a density of $\sim 2 \times 10^6$ were infected with exponentially growing *M. smegmatis* or *E. coli* at a multiplicity of infection (MOI) of 100:1 (bacteria to macrophage). Prior to infection, *M. smegmatis* culture was passed 5–6 times through 26½ gauge needles to obtain single cell suspensions. Macrophages treated with (or without) CD were incubated with bacteria for 2 h and were washed with PBS. Extracellular bacteria were killed by treatment with gentamycin (50 µg/ml) in serum-free RPMI-1640 for 30 min at 37 °C. After washing at least 3 times with antibiotic-free media, macrophages were lysed with 0.1% Triton X-100 (v/v). For *M. smegmatis*, macrophage cell lysates were plated on Middlebrook 7H10 agar upon preparing appropriate dilutions in Middlebrook 7H9 media. In case of *E. coli*, lysates were plated onuria Bertani agar after dilutions were made inuria Bertani broth. To enumerate colony forming unit (CFU) counts, bacterial culture plates were incubated at 37 °C till visible colonies appeared.

Fluorescence Confocal Microscopy of Macrophages Infected with *M. smegmatis*

The effect of CD treatment on the entry of *M. smegmatis* into THP-1 macrophages was visualized by confocal microscopic imaging as described previously (Tiwari et al. 2012, 2014), with some modifications. Briefly, macrophages were plated on glass coverslips at a density of $\sim 2 \times 10^6$ and were treated with varying concentrations of CD. Macrophages were then infected with *M. smegmatis* stably expressing the fluorescent protein dsRed2 (strain transformed with *pMSP12::dsRed2*) (Cosma et al. 2004). Cells were washed, fixed with 4% formaldehyde (w/v) and mounted using Vectashield® antifade mounting medium containing DAPI. Images of z-sections were acquired on an inverted Zeiss LSM 880 confocal microscope (Jena, Germany) with a 63x/1.4 NA oil immersion objective under 1 airy condition, with a fixed step size of 1 µm. Confocal z-sections were then merged to generate maximum intensity projections.

Statistical Analysis

Significance levels were calculated using Student’s two-tailed paired t-test using Graphpad Prism software (version 4.0; San Diego, CA) with 95% confidence interval. The correlation between *M. smegmatis* CFU counts and F-actin content of host macrophages with increasing concentrations of CD was analyzed using the same software. Plots were generated using OriginPro software, version 8.0 (OriginLab, Northampton, MA).

Results

Cytochalasin D Induced Alteration of Actin Organization in THP-1 Macrophages

To explore the role of actin cytoskeleton of human macrophages in the entry of mycobacteria, we treated THP-1 macrophages with CD, a potent inhibitor of actin polymerization. The effect of CD on the macrophage actin

![Fig. 1 Actin organization in THP-1 macrophages upon treatment with cytochalasin D. THP-1 macrophages were treated with increasing concentrations of CD and actin organization was evaluated using confocal microscopy. Alexa Fluor 546 phalloidin was used to stain F-actin in macrophages. Maximum intensity projections obtained by merging 11 sections from the base of the coverslip (~4 µm from the base into the cell) are shown. Representative projected images for control macrophages in panel (A) and the corresponding images for macrophages treated with 2.5 µM, 5 µM and 10 µM CD are shown in panels (B–D), respectively. Upon treatment with increasing concentrations of CD, reduction in F-actin filaments and formation of F-actin aggregates were observed. The scale bar represents 10 µm. See Methods for more details](image-url)
cytoskeleton was monitored by labeling the F-actin filaments with Alexa Fluor 546 phalloidin. Figure 1 shows maximum intensity projections of confocal images of the actin cytoskeleton of THP-1 macrophages, with progressive loss of F-actin filaments and formation of F-actin aggregates due to actin depolymerization with increasing concentrations of CD. We did not observe any significant change in cellular morphology of THP-1 macrophages upon treatment with CD under our experimental conditions (see below). Although visual inspection of the confocal images showed a reduction in F-actin filaments upon treatment with CD, we employed a high-resolution microscopy-based quantitative approach to get a better estimate of the loss of F-actin in macrophages (see later).

Viability of THP-1 Macrophages is Not Compromised Upon Treatment with CD

In order to quantitatively assess the effect of CD on the viability of THP-1 macrophages, cells were treated with CD and were tested for viability using the MTT assay, a cell proliferation assay that provides an estimate of cell growth rate and viability (Vistica et al. 1991). Figure 2 shows no significant change in the viability of macrophages upon treatment with increasing concentrations of CD relative to the control.

Quantitative Estimate of F-Actin Content Upon Treatment with CD

Intensity-based analysis is a suboptimal method to evaluate the effect of CD on the F-actin content of macrophages. This is because F-actin aggregates that are formed due to fragmentation of actin filaments appear brighter in fluorescence microscopy relative to intact filaments. We therefore employed a high-resolution microscopy-based quantitative approach previously developed by us (Ganguly et al. 2011), to estimate changes in F-actin content in macrophages more robustly. This method works by generating iso-surface renditions from confocal z-sections of THP-1 macrophages labeled with Alexa Fluor 546 phalloidin. The volume enclosed by the iso-surfaces is normalized to the area enclosed by maximum intensity projections from confocal z-sections of cells, thereby providing a reliable quantitative estimate of the cellular F-actin content (Ganguly et al. 2011). Figure 3A–D show iso-surface images corresponding to the projected images of macrophages treated with increasing concentrations of CD (see Fig. 1). Figure 3E shows a quantitative estimate of F-actin content in macrophages generated using this method and a concentration-dependent reduction in cellular F-actin levels upon treatment with CD.

Actin Destabilization Inhibits Mycobacterial Entry Into Host Macrophages

In order to probe the role of the actin cytoskeleton of host macrophages in the entry of mycobacteria, THP-1 macrophages treated with increasing concentrations of CD were infected with M. smegmatis. Following infection, colony forming units (CFU) of M. smegmatis were scored upon plating the lysate of infected macrophages and are shown in Fig. 4. The figure shows a dose-dependent reduction in the entry of M. smegmatis into host macrophages upon treatment with increasing concentrations of CD. We observed a reduction of ~30% in the entry of mycobacteria upon mild actin destabilization with 2.5 μM CD. Upon treatment with higher concentrations (5 μM and 10 μM) of CD, we observed a progressive reduction of ~40% and ~50% in the entry of M. smegmatis into THP-1 macrophages, respectively, relative to the control (see Fig. 4). These results show that the actin cytoskeleton of host macrophages is essential for the entry of mycobacteria and any disruption of actin organization of macrophages significantly affects mycobacterial entry.

Fluorescence Imaging Validates the Requirement of the Actin Cytoskeleton for the Entry of M. smegmatis

We further validated the bacterial entry phenotype observed upon destabilization of the actin cytoskeleton by performing confocal microscopy of THP-1 macrophages infected with M. smegmatis (stably expressing dsRed2 (Cosma et al. 2004)). Figure 5 shows representative confocal microscopy images of macrophages infected with M. smegmatis with increasing concentrations of CD. The
The figure shows a concentration-dependent reduction in the entry of *M. smegmatis* into macrophages upon treatment with increasing concentrations of CD, in agreement with the observations depicted in Fig. 4. These results reinforce the requirement of the actin cytoskeleton for the entry of *M. smegmatis*.

**Effect of Actin Destabilization is Specific for Mycobacterial Entry**

In order to evaluate the specificity of the observed effect of actin destabilization in the entry of mycobacteria into host macrophages, we monitored the effect of CD treatment on the entry of *Escherichia coli* into THP-1 macrophages (see Fig. 6). Interestingly, the data show that the entry of *E. coli* is invariant under actin-destabilized conditions in sharp contrast to the significant reduction observed in the entry of *M. smegmatis* under similar conditions (Fig. 4). These results indicate that although the entry of *M. smegmatis* is dependent on mechanisms requiring host actin cytoskeleton, the entry of *E. coli* is independent of destabilization of the actin cytoskeleton. The requirement of the intact actin cytoskeleton therefore appears to be specific for mycobacterial entry.

**Entry of *M. smegmatis* Into Host Macrophages Exhibits a Strong Correlation with Cellular F-Actin Content**

To examine a possible correlation between the reduced F-actin content of host macrophages (upon treatment with increasing concentrations of CD) and the corresponding decrease in the entry of *M. smegmatis*, we plotted (shown...
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In Fig. 7, levels of cellular F-actin (from Fig. 3E) vs. CFU counts of M. smegmatis (from Fig. 4) are correlated. Correlation of M. smegmatis CFU counts and F-actin content of host macrophages upon treatment with increasing concentrations of CD. Data for the figure are from Figs. 3 and 4. Linear regression analysis yielded a correlation coefficient (r) of ~0.93. All data points were bound by the 95% confidence band (plotted as red dashed lines), implying a significant dependence of mycobacterial entry on the host actin cytoskeleton. See Methods and text for further details (Color figure online)
correlation of ~0.93. The figure shows that all the data points are bound by the 95% confidence band, which implies a significant dependence of mycobacterial entry on host actin cytoskeleton.

**Discussion**

The actin cytoskeleton constitutes a dynamic protein framework within cells that has been shown to be involved in essential functions including cellular mobility, trafficking and signaling (Janmey 1998). An important emerging feature associated with the organization of actin involves its intimate association and interplay with the cellular plasma membrane. Such an interplay has been implicated in functional outcomes such as regulation of membrane stiffness, morphological polarity and migration of cells, and dynamics of G protein-coupled receptors (GPCRs) (Pierini et al. 2003; Byfield et al. 2004; Tsai et al. 2006; Sun et al. 2007; Ganguly and Chattopadhyay 2010). In view of the fact that the host plasma membrane serves as the first portal of entry for intracellular pathogens to gain access into the host cell interior (Kumar et al. 2016), these observations on actin-membrane interplay make the exploration of the role of the actin cytoskeleton in the entry of intracellular pathogens extremely relevant. From this perspective, our results demonstrating the role of the integrity of host actin cytoskeleton for mycobacterial entry assume significance. To the best of our knowledge, these results constitute one of the first reports employing a quantitative microscopy-based approach to explore the effect of actin reorganization in mycobacterial entry. The close correlation exhibited by these two processes further enhances the significance underlying such observed effects.

In an earlier study, we demonstrated the requirement of host actin cytoskeleton in the entry of the protozoan parasite *Leishmania donovani* (Roy et al. 2014). Although there are reports on *Mycobacterium*-induced changes in the organization of intracellular actin, especially in the context of the maturation of pathogen-containing phagosomes and their fusion with lysosomes (Guérin and de Chastellier 2000; Hestvik et al. 2005), direct evidence on the role of the host actin cytoskeleton in the entry of mycobacteria into their hosts is lacking, and the precise mechanism by which Mycobacteria enter host cells remains poorly understood. While in vitro studies have identified roles for specific receptors in internalization (Ernst 1998; Killick et al. 2013; Awuh and Flo 2017), it is plausible that Mycobacteria could engage a combination of receptors for their entry, depending on cellular identity. Mycobacterial uptake into epithelial cells, a non-phagocytic cell type, has been shown to require actin cytoskeleton rearrangement, which is regulated by ADP-ribosylation factor 1 and phospholipase D1, and is dependent on the M3 muscarinic receptor (Song et al. 2018). It is conceivable that the entry defect we observe on inhibition of actin polymerization reflects a compromise in the endocytic pathway involved in internalization. Exploring if this defect lies in a specific endocytic pathway (such as clathrin- or caveolin-mediated phagocytosis) as has been described by us for the entry of *Leishmania donovani* into murine macrophages (Kumar et al. 2019) could potentially open avenues for the development of therapeutic approaches targeting the specific route of entry.

With an overall objective to explore the role of the host plasma membrane in the entry of mycobacteria, we have recently shown that *M. smegmatis* requires an optimum level of host membrane cholesterol for efficient entry into host cells (Viswanathan et al. 2015). Cholesterol depletion from membranes has been shown to induce changes in the underlying actin cytoskeleton by loss or redistribution of phosphatidylinositol-4,5-bisphosphate (Kwik et al. 2003). In addition, cholesterol depletion could affect membrane rigidity and neutrophil migration in a manner dependent on the polymeric state of actin (Pierini et al. 2003; Byfield et al. 2004). Although the mechanistic basis underlying the interplay between the actin cytoskeleton and membrane cholesterol remains a subject of active investigation (Sarkar P, Kumar GA, Shrivastava S, Chattopadhyay A, unpublished observations), the consequences of such an interplay in active membrane-associated events such as pathogen entry cannot be ruled out. In addition, we demonstrated that metabolic inhibition of host sphingolipid synthesis leads to reduction in the entry of *M. smegmatis* into host cells (Viswanathan et al. 2018). We believe that a comprehensive understanding of key membrane components encompassing membrane lipids such as cholesterol and sphingolipids, along with the underlying actin cytoskeleton, would provide a comprehensive molecular insight into the process of mycobacterial entry.

The entry of mycobacteria involves host membrane proteins such as complement receptors, mannose receptor, CD-14, scavenger receptors, DC-SIGN, and dectin-1 that act as receptors for pathogen recognition (Ernst 1998; Killick et al. 2013; Awuh and Flo 2017). In addition, a recent study has reported that by manipulating GPCRs in host cells, mycobacteria could suppress host cell signaling pathways (Alaridah et al. 2017). In this overall context, previous work from our group has highlighted the role of the actin cytoskeleton in the function and dynamics of GPCRs (Ganguly et al. 2008; Shrivastava et al. 2020). The ability of the actin cytoskeleton to modulate function of membrane proteins involved in pathogen recognition by host cells could provide a molecular basis underlying the role of the actin cytoskeleton in mycobacterial entry. Taken together along with our previous work on *Leishmania donovani* (Roy et al. 2014), our present results on *M. smegmatis* highlight...
a potential common theme involving the role of the host actin cytoskeleton in the entry of intracellular pathogens. We envision that our results could assume significance in developing novel therapeutic strategies targeting actin-mediated mycobacterial entry in host cells.

The entry of intracellular pathogens into host cells is dependent on multiple factors, most important of these being the interaction between pathogenic and host molecules at the plasma membrane. In the context of the emerging concept of the interplay between membrane cholesterol and the actin cytoskeleton, and in view of our previous observations on the role of cholesterol in mycobacterial entry, our results (using a robust quantitative confocal microscopy-based technique) on the role of actin cytoskeleton in the entry of mycobacteria into human macrophages assume significance. Our results comprehensively demonstrate a link between actin organization and efficiency of mycobacterial entry, with reduced levels of F-actin correlating with a significant reduction in pathogen internalization. We believe that, in addition to cholesterol and sphingolipids, these results now add a third factor intimately associated with the plasma membrane as a major player in mycobacterial entry into macrophages. This opens up an additional point of possible therapeutic intervention targeting host entry as an anti-TB strategy, which has important connotations in the context of the alarming rise in *M. tb* infections resistant to the currently available antibiotics that target the pathogen.

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Author Contributions MJ, TRR and AC conceptualized the project; AD, MJ, RPM and GAK performed experiments and analyzed data; GAK, MJ, TRR and AC designed experiments; AD, MJ, GAK and AC wrote the manuscript; TRR and AC edited the manuscript, organized access to research facilities and funding, and provided overall supervision and mentoring.

Declarations

Conflict of Interest The authors declare that they have no conflicts of interest.

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