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Importance of phagosomal functionality for growth restriction of *Mycobacterium tuberculosis* in primary human macrophages

Amanda Welin^{1*}, Johanna Raffetseder¹, Daniel Eklund¹, Olle Stendahl¹, Maria Lerm^{1,2}

¹Medical Microbiology, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, SE-58185 Linköping, Sweden.

²Center for Infectious Medicine, Department of Medicine, Karolinska Institute, SE-171 77 Stockholm, Sweden.

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Key words: Phagosomes, lysosomes, phagolysosomal fusion, phagosomal maturation, macrophages, mycobacteria, *Mycobacterium tuberculosis*

* Corresponding author

Mailing address: Medical Microbiology, Linköping University, SE-58185 Linköping, Sweden

Telephone: +46101032059

Fax: +46101034789

E-mail: amanda.welin@liu.se

Abstract

The best characterized survival mechanism of *Mycobacterium tuberculosis* inside the macrophage is the inhibition of phagosomal maturation. Phagosomal maturation involves several steps including fusion with lysosomes and acidification. However, it has not been elucidated which components of phagosomal maturation correlate with growth restriction of virulent mycobacteria in human macrophages, and we aimed to study this. We infected human monocyte-derived macrophages with *M. tuberculosis* and assessed bacterial replication, translocation of CD63 to the phagosome, and phagosomal acidification. We found that unstimulated human macrophages were able to control infection with *M. tuberculosis* upon inoculation at a low multiplicity of infection (MOI) of 1, but not at a high MOI of 10. This resulted in a macrophage-controlled balance between host cells and bacteria. Both H37Rv and H37Ra infection, at high and low MOI, led to equally ineffective translocation of CD63 to the phagosome. On the other hand, acidification of mycobacterial phagosomes was more efficient at MOI 1 than at MOI 10 with both mycobacterial strains, consistent with a direct or indirect role for phagosomal acidification in restricting *M. tuberculosis* growth. Furthermore, inhibition of the vacuolar H⁺-ATPase as well as of cathepsin D led to enhanced mycobacterial replication inside the macrophage. This again shows the importance of phagosomal acidification for control of mycobacterial growth, through the activation of lysosomal hydrolases. We conclude that acidification and related functional aspects of the mature phagosome are important factors for restriction of *M. tuberculosis* replication in human macrophages.

Keywords

Mycobacterium tuberculosis, macrophages, phagosomal maturation, phagolysosomal fusion

Introduction

The causative agent of tuberculosis, *Mycobacterium tuberculosis*, primarily infects alveolar macrophages, whose bactericidal mechanisms are modulated by the bacterium to create a niche suitable for survival and effective replication [1]. The best characterized survival mechanism of *M. tuberculosis* is that it inhibits maturation of the phagosome in which it resides in order to avoid lysosomal hydrolases and an acidic environment [2-5], and another is escape from the phagosome into the cytosol [6]. These evasion mechanisms allow for replication of the bacilli and subsequent spread to other cells. However, it has not been fully elucidated which factor or factors determine the outcome of the infection – whether the bacteria are able to replicate intracellularly or whether the macrophage can control the infection. This question is crucial in understanding why only a small portion of infected individuals actually develop tuberculosis during their life time.

The fact that phagosomal maturation is partially inhibited by *M. tuberculosis* suggests that evasion of this mechanism is important for survival or replication of the bacillus [7,8]. *M. tuberculosis* protects itself from phagosomal acidification by preventing incorporation of the vacuolar H⁺-ATPase into the phagosomal membrane [8-11], and from phagolysosomal fusion by preventing delivery of lysosomal components to the phagosome [12]. Mycobacterial mutants defective in inhibition of phagosomal maturation display reduced survival within mouse macrophages [13], whereas macrophages activated with IFN- γ or Toll-like receptor ligands have been shown capable of counteracting the bacterium's immunomodulating effects and restrict *M. tuberculosis* growth in some studies [8,11,14,15]. Nitric oxide production is crucial for growth restriction in mouse macrophages, while the mechanism in human macrophages is not as clear [14].

In this study, we investigated which components of phagosomal maturation correlate with inhibition of *M. tuberculosis* replication in unstimulated human macrophages, focusing on translocation of late endosomal/lysosomal proteins to the phagosome, phagosomal acidification, and other phagosomal

functions.

Materials and Methods

Bacterial strains and growth conditions

The virulent *M. tuberculosis* strain H37Rv and the attenuated H37Ra were obtained from ATCC. γ -irradiated *M. tuberculosis* was provided by Colorado State University, Fort Collins. For constitutive expression of green fluorescent protein (GFP), bacteria were transformed with the pFPV2 plasmid carrying the gene for GFP [16]. For selection of pFPV2-carrying mycobacteria, 20 μ g/ml kanamycin was used. For constitutive luciferase expression, bacteria were transformed with the pSMT1 plasmid carrying the *luxAB* gene, and selected using 100 μ g/ml hygromycin. Early passages of *M. tuberculosis* were prepared and stored at -70 °C. The bacteria were grown in Middlebrook 7H9 broth with 0.05 % Tween-80, 0.5 % glycerol and ADC enrichment (Becton Dickinson) at 37 °C for two to three weeks, and then passaged and incubated for 7 days before use in experiments.

Macrophage isolation

Human monocytes were isolated from heparinized peripheral human blood and differentiated into human monocyte-derived macrophages (hMDMs) as reported previously by us [17,18] and others [19,20]. Briefly, peripheral blood mononuclear cells were isolated and seeded in cell culture flasks, allowing selective enrichment of the adhesive monocytes and washout of lymphocytes. The monocytes were kept in Dulbecco's modified Eagle's Medium with glucose (DMEM, Gibco, supplemented with 25 mM Hepes, 100 U/ml penicillin, 100 μ g/ml streptomycin) and allowed to differentiate to human monocyte-derived macrophages (hMDMs) for 5-8 days in the presence of 10 % active human serum pooled from five donors and 80 μ M L-glutamine. Determination of macrophage differentiation was based on adherence and phagocytic capacity. The day before experiment, the hMDMs were trypsinized and re-seeded as required (2.5×10^5 per cover slip or 1×10^5 per well in 96-well plates), diluted in antibiotic-free medium with serum.

Experimental infection

The bacterial suspension was centrifuged twice at $3000 \times g$ for 10 minutes, in phosphate buffer saline (PBS) supplemented with 0.05 % Tween-80, the pellet was resuspended in plain DMEM and single bacilli were obtained by passaging the suspension 2×10 times through a sterile syringe equipped with a 27 gauge needle. The concentration of living bacilli was determined by using optical density (OD_{600}) as a function of colony forming units/ml, and microscopy confirmed efficient separation. Bacteria were added to the hMDMs at the indicated multiplicity of infection (MOI), diluted in plain DMEM to achieve non-opsonic conditions. The hMDMs were incubated with the bacteria at 37°C for 1 h after which the medium was replaced with antibiotic-free DMEM with serum (pulse-chase approach), before incubation for the indicated total times. Treatment of the hMDMs with bafilomycin A1 (Sigma-Aldrich, 100 nM) or diphenylene iodonium (DPI, Sigma-Aldrich, 5 μM) dissolved in DMSO (final concentration 0.1 %), or pepstatin A (Roche, 1 μM) dissolved in methanol (final concentration 0.03 %) was carried out 1 h pre-infection, and the inhibitor was then included throughout the experiment.

For phagocytosis of zymosan, hMDMs were incubated with complement-opsonized and FITC-labelled zymosan particles (10:1) diluted in plain DMEM for 1 h, after which the medium was changed as described above. Infection with a GFP-expressing strain of *Salmonella typhimurium* was performed in the same manner.

Measurement of intracellular bacterial growth

Bacterial growth inside hMDMs over three days was assessed using a luminometry-based method, as previously described [18]. Briefly, hMDMs seeded in triplicate in 96-well plates were infected as described above with luciferase-expressing H37Rv, and hypotonically lysed at the indicated time points before addition of substrate (1 % decanal) and measurement of luminescence using a Modulus Microplate Reader (Turner Biosystems). As previously validated, the amount of

luminescence corresponded to the number of viable bacilli in each sample. The median value of each triplicate was used, and the amount of luminescence in the cell lysate at each time point was normalized against the phagocytosis value of each treatment (4 h) to account for donor variability in uptake.

Determination of macrophage viability

For microscopic determination of hMDM viability, cells were incubated with Fixable Viability Dye eFluor 660 (eBioscience), diluted 1:1000 in PBS, at 4°C for 30 min prior to PFA fixation and subsequent immunofluorescence staining. The dye stains the nuclei of cells with damaged plasma membranes, allowing discrimination of live and compromised cells.

The relative number of viable hMDMs was assessed throughout the infection by a calcein acetoxymethyl (calcein-AM)-based method, as previously described [18]. Briefly, hMDMs seeded in black 96-well plates were infected, incubated for up to 7 days, washed with PBS and incubated with 4 μ M calcein-AM (Molecular Probes) at RT for 45 min. As metabolically active cells convert calcein-AM to highly fluorescent calcein, the resulting fluorescence corresponds to the number of viable cells. Fluorescence was measured in a Modulus Microplate Reader using a 490 nm filter, and the values were normalized against the value for uninfected hMDMs at each time point to obtain the relative proportion of live cells.

Immunofluorescence and F-actin staining

Infected hMDMs on cover slips were fixed in 4 % paraformaldehyde at RT for 30 min and washed three times. Staining was then performed in a humid chamber. The fixed cells were blocked and permeabilized in a solution containing PBS with 2 % BSA and 10 % normal goat serum (Sigma-Aldrich), supplemented with 0.1 % saponin (w/v) at RT for 30 min. After three washes in PBS, the cover slips were incubated with either mouse monoclonal anti-LAMP-1 antibody (Santa Cruz, 4

$\mu\text{g/ml}$) or mouse monoclonal anti-CD63 antibody (Sanquin, 4 $\mu\text{g/ml}$) diluted in blocking solution at RT for 1 h. After three 5-min washes, the cells were incubated with Alexa 594-conjugated goat anti-mouse IgG (Molecular Probes, 5 $\mu\text{g/ml}$), also diluted in blocking solution, at 37 °C for 30 min. Cover slips were washed and mounted using fluorescence mounting medium (Dako) and the preparations were re-coded to achieve unbiased analysis. For staining of F-actin, the hMDMs were permeabilized with 0.1 % saponin (w/v) post-fixation and incubated with 5 U/ml Alexa 594-coupled Phalloidin (Molecular Probes), diluted in PBS, for 30 min at RT, followed by washing and mounting in the same manner.

LysoTracker assay

hMDMs on cover slips were infected and acidified compartments were labelled using LysoTracker Red DND-99 (75 nM, Molecular Probes) [21], which was added during the final 2 h of incubation, diluted in antibiotic-free DMEM with serum. The hMDMs were washed, fixed, and mounted for confocal microscopy analysis, and images were obtained and evaluated in a blinded fashion. Phagosomes in the images were classified as positive or negative for LysoTracker staining.

Confocal microscopy

Images were obtained using a confocal microscope (BioRad Radiance 2000 MP with LaserSharp 2000 software), with an argon laser emitting dually at 488 nm for excitation of FITC or GFP and at 514 nm for Alexa 594, and a red diode laser for excitation of eFluor 660. The samples were blinded throughout image acquisition and all analyses, after which the collected data was decoded.

Statistical analysis

Paired Student's t-test was used for statistical analysis, and p values of ≤ 0.05 were considered significant.

Results

hMDMs are able to control infection with H37Rv at a low MOI

In order to study what conditions are necessary for growth restriction of mycobacteria, we developed an infection model where unstimulated hMDMs were able to control infection with virulent *M. tuberculosis*. It is conceivable that the hMDMs are better equipped to deal with a low bacterial burden than a high one, so we evaluated the use of a low-MOI infection model to obtain a balance between the bacteria and the infected cells. hMDMs were infected with luciferase-expressing H37Rv at MOI 10 and MOI 1, and light emission which was a correlate of the number of CFU in the hMDM lysates, was measured over a time period of one week. Inoculation of the macrophages at MOI 10 resulted in effective bacterial replication while inoculation at MOI 1 resulted in a balance between the macrophages and the bacilli, where very little replication occurred after the first few days of infection (Fig. 1A). A calcein AM-based cell viability assay showed that at MOI 1, the macrophages remained viable throughout the experiment (typically 80-100 % were viable after 1, 3 and 7 days, as compared to uninfected cells), while at MOI 10 the hMDMs were rapidly killed (typically less than 40 % were viable after 1 day and less than 20 % after 3 days, as compared to uninfected cells). After 4 h, however, there was no significant difference between the number of viable hMDMs in uninfected samples and after infection with H37Rv at MOI 10 or 1. The same assay showed that upon infection of hMDMs with H37Ra at MOI 10, the majority (typically 70-90 %) of the macrophages were still viable after 3 days.

It is known that H37Ra does not replicate inside macrophages [22], and we previously corroborated this finding in our macrophage model [18]. We confirmed this result by enumerating the number of GFP-expressing H37Rv and H37Ra inside fixed hMDMs over 2 days of infection, after inoculation at an MOI of 10 (Fig. 1B). Fluorescence microscopy images of hMDMs infected with H37Rv at MOI 10 for up to two days, and stained for F-actin, show increased numbers of intracellular bacteria growing in clusters (Fig. 1C). The luciferase assay showed that inoculation of DMEM (without

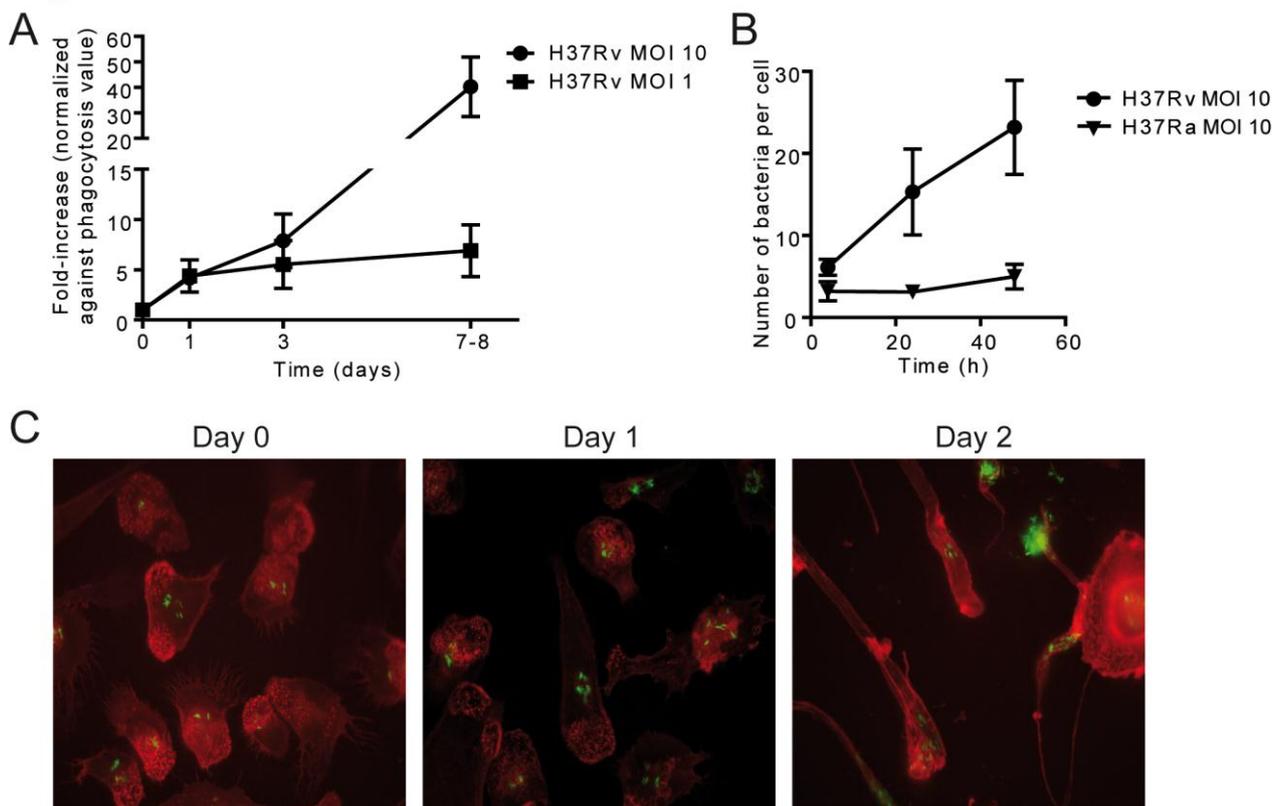


Figure 1. Intracellular replication of H37Rv and H37Ra. hMDMs were infected at different MOIs with H37Rv or H37Ra for the indicated times, and the number of intracellular bacteria was analyzed. **A)** Growth curve of luciferase-expressing H37Rv infecting hMDMs at MOI 10 or 1. The relative number of viable intracellular bacilli was measured at each time point. The values were normalized against the phagocytosis value of each MOI. The figure shows the mean and SEM from three independent experiments. **B)** Growth curve showing the mean number of bacilli per hMDM over 2 days of infection with GFP-expressing H37Rv or H37Ra at MOI 10. Enumeration of GFP-expressing bacteria was performed in 20 blinded images from each time point and donor. Means and SEM from three independent experiments are shown. **C)** Representative fluorescence microscopy images of F-actin-stained (red) hMDMs infected with GFP-expressing H37Rv (green) at MOI 10 over two days. The images are merges of the green and red channels.

antibiotics but with serum) with H37Rv or H37Ra in the absence of hMDMs, or in the presence of pre-lysed hMDMs, resulted in effective growth of both strains of bacteria (data not shown).

However, the same assay showed that the net increase in bacterial numbers inside hMDMs infected with H37Rv at MOI 10 was not due to extracellular growth and continuous phagocytosis because the addition of cytochalasin B, which inhibits actin polymerization and thus phagocytosis, did not affect H37Rv growth in the intracellular fraction (data not shown). Our data thus indicate that unstimulated macrophages are able to inhibit growth of H37Ra, even at high MOI, and of H37Rv when a low MOI is used. This observation was used in the following experiments to study what components of phagosomal maturation were important for the control of intracellular mycobacterial

replication.

Growth restriction at MOI 1 does not correlate with CD63 acquisition

The first component of phagosomal maturation that we studied was the translocation of lysosomal-associated membrane proteins (LAMPs) to mycobacterial phagosomes. LAMPs such as CD63 (LAMP-3) are often used as an indicator of phagolysosomal fusion, although they are not found exclusively on lysosomes, but also on intermediate/late endosomes, and their usefulness in the context of mycobacterial phagosomes has been questioned [8,23,24]. In order to investigate to what extent macrophages are able to translocate CD63 to the mycobacterium-containing phagosome, immunostaining for CD63 was performed in H37Rv- or H37Ra-infected hMDMs. For comparison, phagosomes carrying dead H37Rv or zymosan particles were studied. The images of the H37Rv-, H37Ra-, and zymosan-challenged hMDMs showed phagosomes that were either positive or negative for CD63 staining (Fig. 2A). To study the translocation of CD63 to the phagosome in macrophages infected with the different prey, the association of CD63 to phagosomes was quantified in the confocal images. In this assay, single bacilli and aggregates were both scored as one phagosome event. As mentioned, extensive cell death occurred in hMDM cultures infected with H37Rv at MOI 10, and these experiments thus included both viable and unviable macrophages. However, a control experiment where hMDMs were stained with a fixable fluorescent viability dye before CD63 staining showed that there was no difference in the proportion of CD63-positive phagosomes between live and dead H37Rv-infected hMDMs (data not shown). Maximal CD63 translocation of about 75-80 % was observed after 4 h to zymosan- and γ -irradiated H37Rv-containing phagosomes. In hMDMs infected with live H37Rv or H37Ra, however, CD63 translocation was partially inhibited, and only about 40-50 % of the phagosomes were positive for CD63 after 4 h of infection. This level was similar after two days (Fig. 2B). Thus, using this assay we confirm that live *M. tuberculosis* inhibits translocation of a CD63 to the phagosome, and show that H37Rv and H37Ra reduce CD63 translocation to a similar extent.

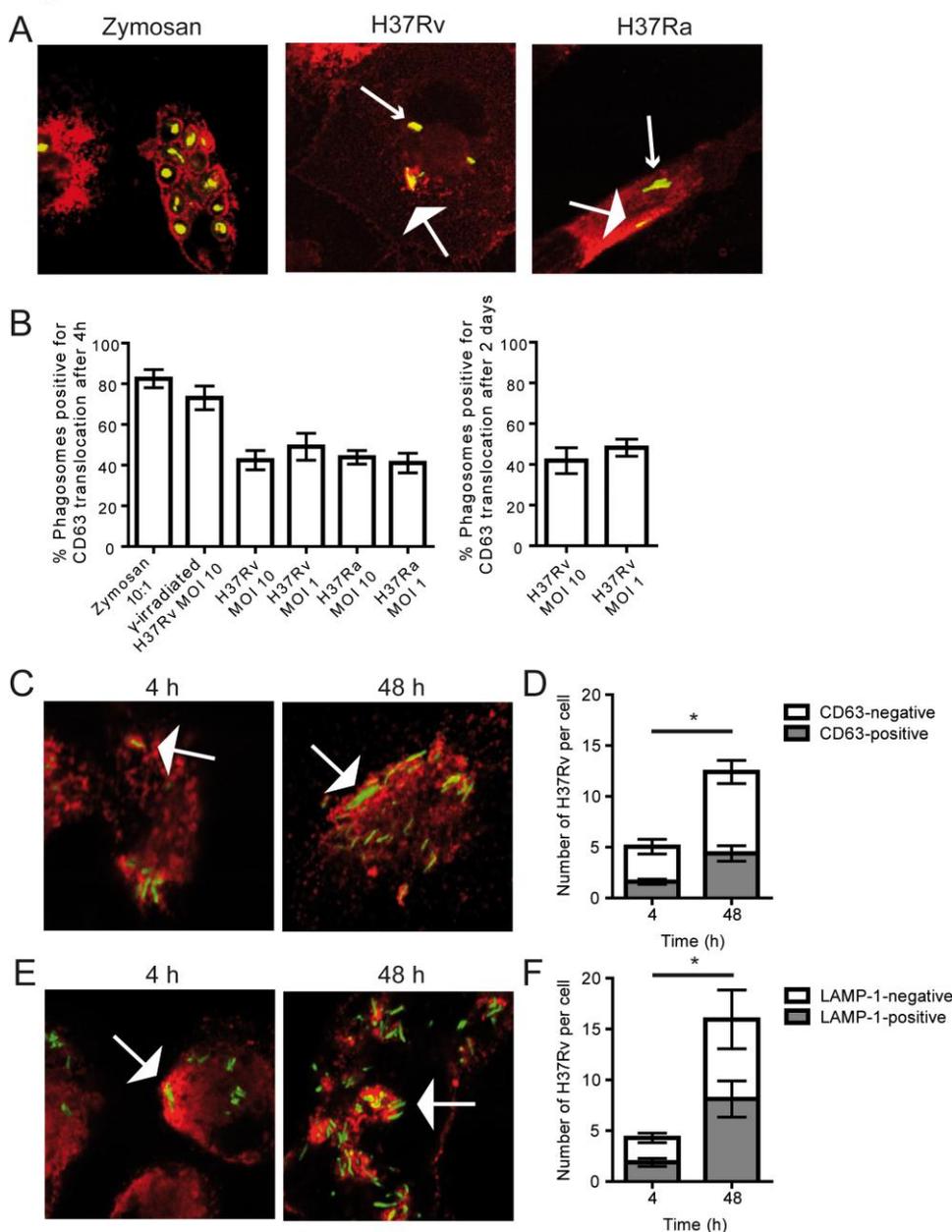


Figure 2. CD63 translocation to the phagosome after infection with different strains and MOI. hMDMs were challenged with GFP-expressing live or γ -irradiated H37Rv, live H37Ra or FITC-labelled zymosan for the indicated times, and the late endosomal/lysosomal markers CD63 or LAMP-1 were immunostained. **A)** Confocal images showing representative CD63-stained (red) zymosan-, H37Rv- and H37Ra (green)-containing hMDMs after 4 h of infection. The images are merges of the green and red channels. The large arrows indicate phagosomes positive for CD63 translocation, and the small arrows show phagosomes negative for CD63. **B)** hMDMs challenged with live or γ -irradiated H37Rv or H37Ra at MOI 10 or 1 or zymosan at 10:1 were fixed after 4 h or 2 days, and CD63 was stained. The histograms show the mean percentage of phagosomes that were positive for CD63 (blinded evaluation of > 40 phagosomes per preparation), and the SEM from at least four independent experiments. **C)** hMDMs infected with H37Rv (green) at MOI 10 for 4 h or 48 h were stained for CD63 (red). The confocal images show representative merges of GFP and CD63 after 4 h and 48 h. The arrows indicate CD63-positive phagosomes. **D)** The number of bacilli in CD63-positive and -negative compartments and the number of hMDMs were enumerated in CD63-stained hMDMs (blinded evaluation of ≥ 25 hMDMs per preparation). The mean number of bacilli per macrophage that were positive and negative for CD63 after 4 h and 48 h is shown ($n = 6$). Error bars show SEM and * indicates that $p < 0.05$. **E)** hMDMs infected with H37Rv (green) at MOI 10 for 4 h or 48 h were stained for LAMP-1 (red). The confocal images show representative merges of GFP and LAMP-1 after 4 h and 48 h. The arrows indicate LAMP-1-positive phagosomes. **F)** The number of bacilli in LAMP-1-positive and -negative compartments and the number of hMDMs were enumerated in CD63-stained hMDMs (blinded evaluation of ≥ 25 hMDMs per preparation). The mean number of bacilli per hMDM that were positive and negative for LAMP-1 after 4 h and 48 h is shown ($n = 4$). Error bars show SEM and * indicates that $p < 0.05$.

In order to investigate whether acquisition of CD63 correlated with the intracellular growth restriction observed with H37Rv at MOI 1, CD63 translocation was quantified in hMDMs infected with H37Rv or H37Ra at MOI 10 and 1. However, there was no difference between the percentage of CD63-positive phagosomes between MOI 10 and MOI 1 at either time point studied (Fig. 2B), indicating that the macrophages were able to control the infection despite the ineffective translocation of CD63 to the phagosome at the low MOI. It was not possible to compare CD63 translocation beyond 2 days of infection, as there were very few remaining intact hMDMs after H37Rv infection at MOI 10.

The dominating view is that *M. tuberculosis* divides inside phagosomes whose communication with lysosomes has been blocked [7]. However, having observed that the lack of CD63 acquisition did not correlate with effective growth of H37Rv at MOI 1 or H37Ra, we wanted to investigate whether replication of virulent *M. tuberculosis* could also occur inside phagosomes positive for LAMPs. We infected hMDMs with H37Rv at MOI 10 for 4 h or 48 h, fixed the cells and stained for the LAMPs CD63 (Fig. 2C) and LAMP-1 (Fig. 2E). The number of individual bacteria within CD63- or LAMP-1-positive and -negative compartments was enumerated, and each bacillus was scored as one event. There was an increase in the average number of CD63- (Fig. 2D), or LAMP-1- (Fig. 2F) positive individual bacteria per hMDM over 2 days (CD63 $p = 0.0101$, LAMP-1 $p = 0.0409$). This was probably not merely the result of fusion between phagosomes, as the total number of bacteria in the macrophages increased during this time (see Fig. 1). Furthermore, while the number of individual bacilli positive for the markers increased over two days, the proportion of CD63-positive phagosomes remained the same (see Fig. 2B). This may indicate that replication of bacilli can occur inside compartments positive for late endosomal/lysosomal markers as well as in LAMP-negative ones, or possibly that an increasing number of phagosomes form during replication. We conclude that the number of H37Rv bacteria residing in LAMP-positive compartments in hMDMs increases

as the bacteria replicate.

Acidification of the phagosome correlates with growth restriction at a low MOI

The absence of a correlation between inhibition of CD63 translocation and intracellular growth at different MOIs and with different strains prompted us to investigate whether another component of phagosomal maturation, acidification, instead correlated with mycobacterial growth restriction. We thus stained macrophages challenged for 4 h or 2 days with H37Rv or H37Ra at MOI 10 or MOI 1 with LysoTracker Red, fixed the macrophages and examined the phagosomes using a confocal microscope (Fig. 3A). In this assay, single bacilli and aggregates were both scored as one phagosome event, and *S. typhimurium*-containing hMDMs were used as a control because efficient acidification of *S. typhimurium*-containing phagosomes has previously been demonstrated [25]. When hMDMs were allowed to phagocytose *S. typhimurium* at MOI 10, an average of 67 % of phagosomes were positive for LysoTracker after 4 h. This value was reduced in both H37Rv- and H37Ra-infected hMDMs to about 20 % at MOI 10, again confirming that mycobacteria inhibit phagosomal maturation (Fig. 3B). The percentage of phagosomes positive for LysoTracker was significantly higher at MOI 1 than at MOI 10 after 4 h (H37Rv $p = 0.049$, H37Ra $p = 0.008$), suggesting more efficient acidification at the lower MOIs (Fig. 3B). The inefficient acidification at MOI 10 after 4 h was not due to cell death as hMDMs infected at both MOIs were viable at this time point, as evidenced by the cell viability assay mentioned above. The difference between MOI 10 and 1 observed at day 2, however, may be due to the extensive cell death at MOI 10 leading to a lack of LysoTracker staining. The loss of hMDM viability also made it difficult to examine whether there was an increase in the number of H37Rv bacilli in LysoTracker-positive and negative compartments, as studied for LAMPs in Fig. 2C-D.

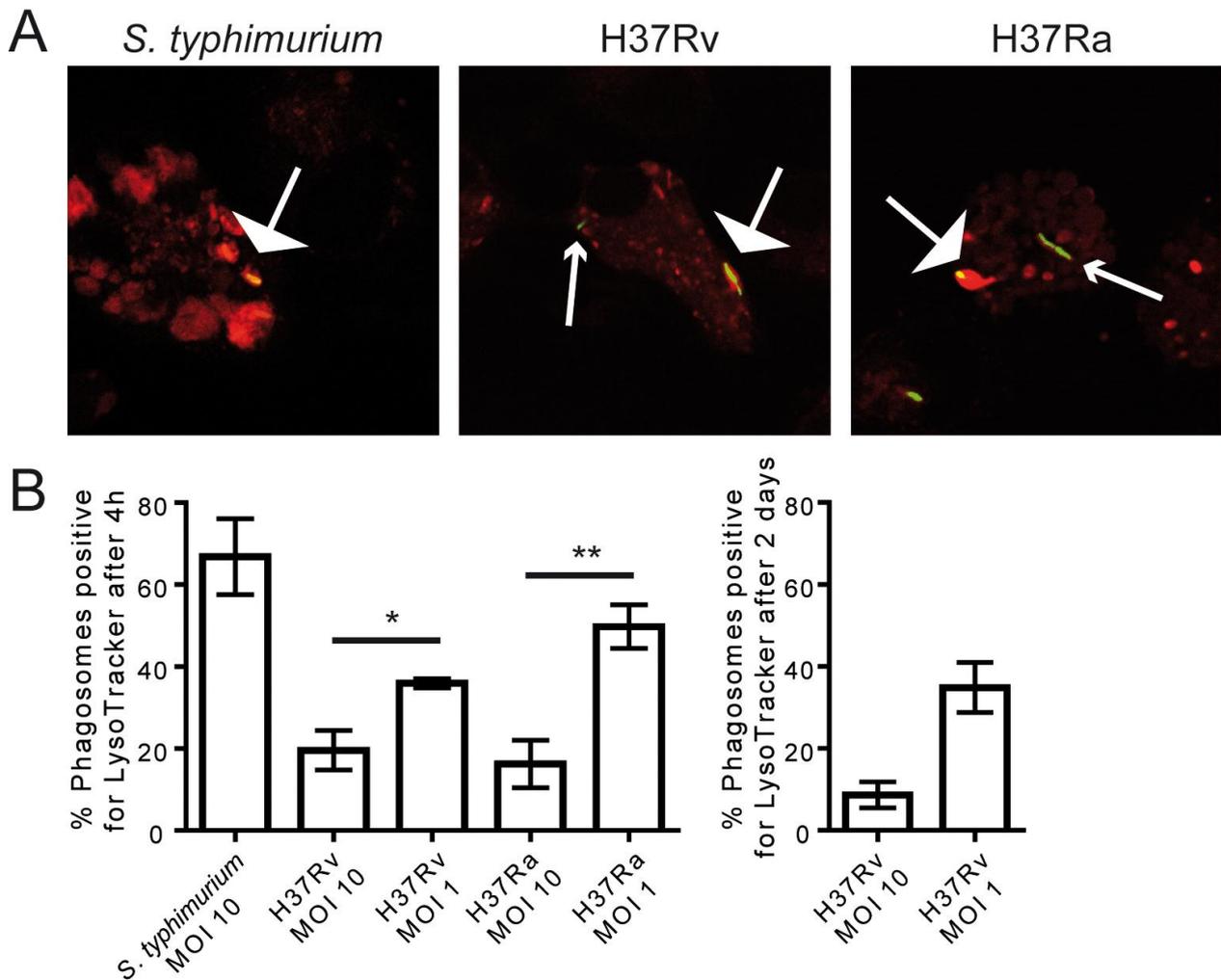


Figure 3. Acidification of the phagosome after infection with different strains and MOI.

hMDMs were infected with GFP-expressing *S. typhimurium*, H37Rv or H37Ra at MOI 10 or MOI 1, LysoTracker staining was performed, and the macrophages were fixed at the indicated time points. **A**) Confocal images showing *S. typhimurium*-, H37Rv- or H37Ra- (green) containing phagosomes stained with LysoTracker (red) after 4 h of infection. The images are merges of the green and red channels. The large arrows indicate phagosomes positive for LysoTracker, and the small arrows show negative phagosomes. **B**) Quantification of the number of acidified phagosomes in hMDMs challenged with *S. typhimurium*, H37Rv or H37Ra at MOI 10 or 1. The percentage of phagosomes that were positive for LysoTracker after 4 h and 2 days is shown. The figure depicts the mean and SEM from at least three independent experiments (blinded evaluation of >40 phagosomes per preparation). * indicates that $p < 0.05$, and ** indicates that $p < 0.01$.

The vacuolar H⁺-ATPase and cathepsin D are required for control of H37Rv growth

Having established that phagosomal acidification, but not CD63 translocation to the phagosome, correlated with growth inhibition of H37Rv at the low MOI, we wanted to further characterize the macrophage functions involved in the control of mycobacterial replication. To study the different aspects of phagosomal functionality necessary for the inhibition of mycobacterial growth, we

infected hMDMs with H37Rv at MOI 1 and inhibited the vacuolar vacuolar H⁺-ATPase using bafilomycin, cathepsin D using pepstatin, or the NADPH oxidase using DPI, prior to infection as well as throughout the experiment. The intracellular growth assay showed that addition of bafilomycin (Fig. 4A) or pepstatin (Fig. 4B) significantly increased the replication efficiency of the bacteria during the first 24 h of infection as compared to the control (bafilomycin p = 0.045, pepstatin p = 0.021). In a control experiment, bafilomycin was able to diminish the number of LysoTracker-positive latex bead phagosomes by 100 % (data not shown), showing that bafilomycin was inhibiting acidification. Addition of DPI, on the other hand, did not affect the growth rate of the bacteria upon infection at MOI 1 (Fig. 4C). The results show that the vacuolar H⁺-ATPase and cathepsin D are important for restricting the growth of H37Rv at the low MOI.

Discussion

M. tuberculosis can inhibit the fusion of the phagosome in which it resides with late endosomes and lysosomes, as well as prevent the compartment from acquiring vacuolar H⁺-ATPases. These events are thought to lead to survival and efficient replication of the bacilli. It is frequently assumed in the literature that since *M. tuberculosis* can inhibit the translocation of late endosomal/lysosomal markers to the phagosome, effective translocation representing phagolysosomal fusion must lead to killing of the bacilli [4,26,27]. However, the relationship between different components of phagosomal maturation and mycobacterial replication in human macrophages has not been extensively studied using virulent bacilli. Here, we investigated which components of phagosomal maturation correlate with inhibition of mycobacterial growth in unstimulated human macrophages, using a low MOI-infection with H37Rv.

By means of a luciferase-based method, we found that human macrophages could control H37Rv infection at MOI 1, resulting in a balance between the pathogen and the host cell, where there was little net change in bacterial numbers. Conversely, infection at MOI 10 led to effective replication

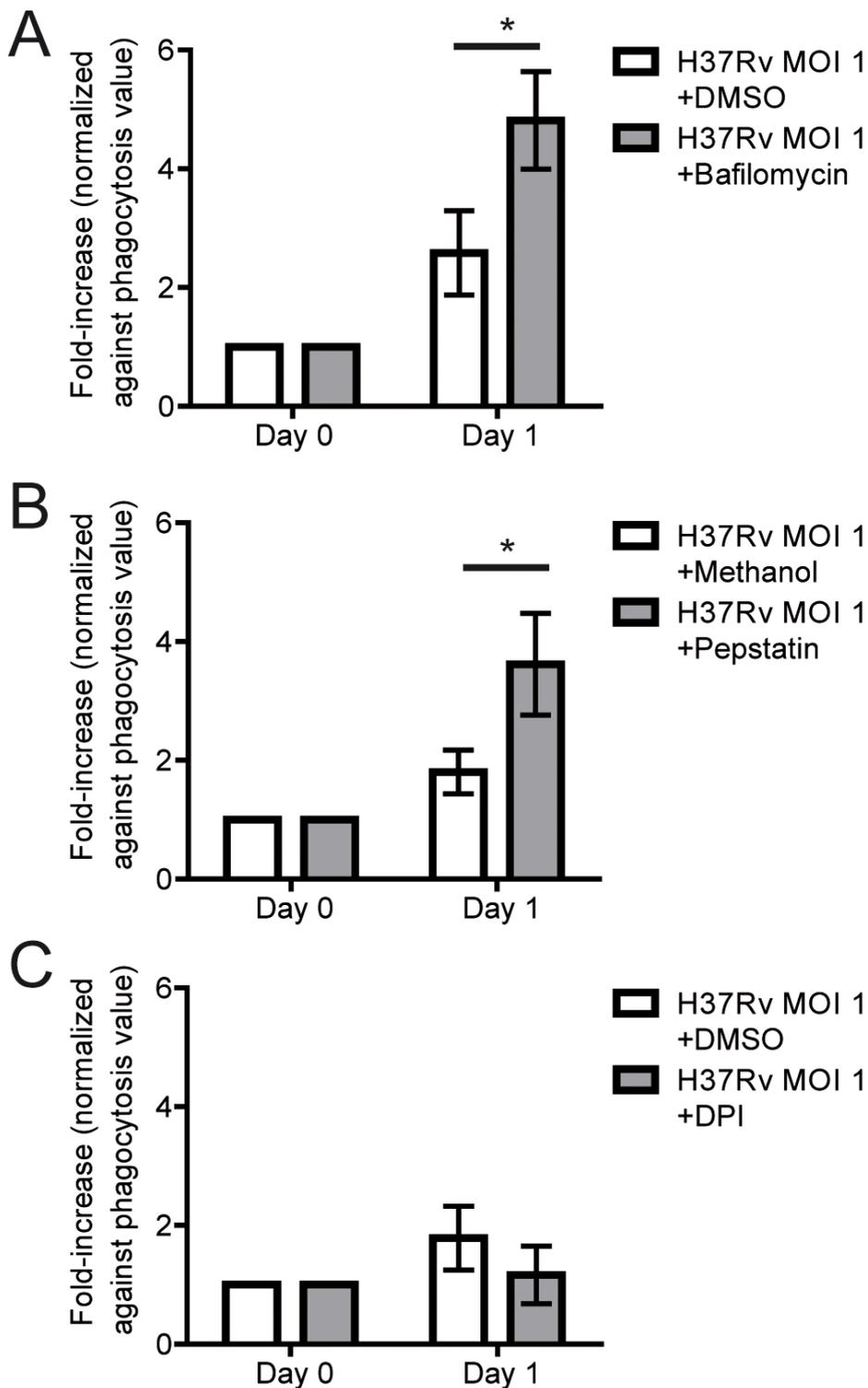


Figure 4. Replication of H37Rv upon inhibition of phagosomal functions. hMDMs were infected with luciferase-expressing H37Rv at MOI 1 in the presence or absence of different inhibitors and bacterial replication was assessed upon lysis of the hMDMs. The histograms show the fold-increase of H37Rv after 24 h, as compared to the 4 h value. **A)** The hMDMs were infected in the presence of bafilomycin (100 nM) or DMSO (0.1 %). **B)** The hMDMs were infected in the presence of pepstatin (1 μ M) or methanol (0.03 %). **C)** The hMDMs were infected in the presence of DPI (5 μ M) or DMSO (0.1 %). The mean and SEM of six (bafilomycin and pepstatin) or two (DPI) independent experiments where all values have been normalized against the phagocytosis value are shown. * indicates that $p < 0.05$.

and rapid host cell death. Comparison between different aspects of phagosomal maturation at the two MOIs proved a useful tool in studying which factors are necessary for control of intracellular replication of *M. tuberculosis*. Using a GFP-based method, we then confirmed that H37Rv at MOI 10 replicated efficiently inside hMDMs, forming large clusters, while H37Ra did not show any net change in bacterial numbers. H37Rv but not H37Ra infection at MOI 10 was coupled with decreased hMDM viability over time. Intra-macrophage replication of H37Rv but not H37Ra is in line with previous studies [18,22], and H37Ra and H37Rv have genomic and transcriptional regulation differences which make H37Rv better equipped for intramacrophage growth [22].

Through staining of CD63, which is a frequently used marker of fusion of phagosomes with late endosomes and lysosomes [3,28,29], we found that both the virulent H37Rv and the avirulent H37Ra strains were able to reduce translocation of late endosomal/lysosomal proteins to the phagosome in comparison to zymosan, consistent with many previous studies [3,4,27,30]. This result shows that our assay was capable of detecting differences in CD63 acquisition. The ability to reduce the fusion of the phagosome with late endosomes and lysosomes is thought to lead to protection against microbicidal hydrolases, facilitating replication in immature phagosomes [8]. The association between mycobacterial phagosomes and LAMPs is higher in our study than in another previous report using hMDMs [31], and lower than a third report [21], possibly reflecting differences in macrophage differentiation procedures.

A correlation between the ability to control low MOI-infections and translocation of LAMPs to the phagosome would suggest a major role for fusion of phagosomes with late endosomes/lysosomes in growth restriction at the low MOI. Thus, we studied CD63 translocation to phagosomes containing H37Rv at a high and low MOI. However, there were no differences in CD63 translocation between the high and low MOIs. This indicates that the ability to restrict acquisition of late endosomal/lysosomal proteins does not in itself mean that growth can occur. Furthermore, we found

that as H37Rv divided inside the macrophages, the number of bacteria in CD63- or LAMP-1-positive phagosomes increased, which could indicate that H37Rv was able to replicate within compartments that have acquired the markers. Replication in phagosomes positive for LAMPs has not previously been analyzed directly, but one study showed that fusion of the mycobacteria-containing vacuole with lysosomes does not lead to bacterial killing, but rather impairment in growth [32]. However, we cannot rule out the alternative explanation that the bacilli have replicated in an unfused phagosome and subsequently been taken up into an autophagosome that has matured [33], resulting in a higher number of LAMP-associated bacilli as well as a higher number of phagosomes over time. Altogether, acquisition of CD63 did not correlate with control of *M. tuberculosis* growth inside the phagosome in our experimental setup. The fact that *M. tuberculosis* avoids phagolysosomal fusion, however, indicates that inhibition of delivery of lysosomal components to the phagosome plays an important role in virulence. Our data rather support the notion that the presence of late endosomal/lysosomal markers, although frequently used to study mycobacterial phagosomes [12], does not completely reflect the environment within the phagosome [8,9]. CD63 and other LAMPs are present on intermediate and late endosomes as well as lysosomes, and thus do not reflect a complete maturation state [34].

Besides inhibiting the fusion between phagosomes and lysosomes, *M. tuberculosis* is also able to prevent the vacuolar H⁺-ATPase from being incorporated into the phagosomal membrane, thus protecting itself from acidification of the vacuole [8-10]. In order to further characterize the mycobacterial phagosomes in our infection model, LysoTracker staining was carried out, and we confirmed again that *M. tuberculosis* inhibits phagosomal maturation. The fact that H37Rv and H37Ra inhibited phagosomal maturation in terms of both CD63 acquisition and acidification to a similar extent despite the difference in intramacrophage growth ability suggests that inhibition of phagosomal maturation is not the mechanism explaining the difference in intracellular replication ability observed between H37Rv and H37Ra, since both strains achieved an equal inhibition of this

process. However, our study does not rule out a possible difference related to the sensitivity of the strains to lysosomal contents. The data furthermore revealed that acidification of the phagosome was more efficient at a low MOI, correlating with replication inhibition. The results obtained with hMDMs infected with *S. typhimurium* showed that the macrophages were able to handle acidification of many phagosomes in the same hMDM. The ability of *M. tuberculosis* to prevent acidification at higher bacterial loads is therefore not simply an exhaustion of the macrophage capacities, but must involve a specific bacterial mechanism, which is more efficient at high MOI and which results in more efficient reduction of acidification.

Inhibition of the vacuolar H⁺-ATPase and assessment of bacterial replication showed that lack of phagosomal acidification increased the replication efficiency of H37Rv, confirming data obtained with avirulent mycobacteria [35]. We suggest a major role for acidification in limiting growth of virulent *M. tuberculosis* inside human macrophages, which may be direct or indirect, consistent with previous studies [10,11]. One study showed that *M. tuberculosis* can survive acidic environments and suggested that inhibition of acidification is a strategy for avoiding other phagolysosomal functions [36], as acidification of the phagosome is important for the function of degradative lysosomal enzymes and the generation of hydrogen peroxide in the phagosome [37]. The fact that cathepsin D was also necessary for limitation of bacterial growth at MOI 1 suggests that acidification may indirectly contribute to the growth restriction. However, our data did not reveal a role for the NADPH oxidase and thus reactive oxygen intermediates in controlling mycobacterial growth. As reviewed by Huynh & Grinstein [37], the relationship between phagolysosomal fusion and phagosomal acidification is complex, since acidification is sometimes regarded as a determinant of phagosomal maturation, with a role in endosomal trafficking, and sometimes as a consequence, dependent on fusion with lysosomes. The mechanism by which the vacuolar H⁺-ATPase is recruited to the phagosome is also not completely clear, but previous studies suggest trafficking from the *trans* golgi network [38,39] or delivery from endosomes or lysosomes

[35,40]. Phagolysosomal fusion, on the other hand, is dependent on recruitment of the organelle tethering molecule early endosomal antigen 1 [12]. It was clear from our data that CD63 acquisition and phagosomal acidification occurred at different rates, especially at MOI 1, which makes us speculate that the vacuolar H⁺-ATPase and late endosomal/lysosomal proteins are acquired by different routes. A previous study showed that CD63 translocation occurred at similar levels to phagosomes containing both metabolically active and inactive *M. tuberculosis*, while LysoTracker only associated with inactive bacilli [21]. This is consistent with the notion that LAMP translocation does not truly reflect the phagosomal environment, but that acidification is a better marker for phagosome function.

In conclusion, we found that inhibition of CD63 translocation to the phagosome did not *per se* lead to effective mycobacterial growth inside the macrophage. On the other hand, phagosomal acidification and functional cathepsin D played important roles in growth restriction of virulent *M. tuberculosis* at a low MOI in unstimulated human macrophages. The importance of macrophage functionality *in vivo* should be the focus of future studies, to establish what factor or factors are critical for growth arrest of *M. tuberculosis* during natural infection.

Conflict of interest statement

The authors declare no conflict of interest.

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