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Molecular mechanism of interaction of *Mycobacterium tuberculosis with* host macrophages under high glucose conditions

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ABSTRACT

Mycobacterium tuberculosis has the potential to escape various cellular defense mechanisms for its survival which include various oxidative stress responses, inhibition of phagosome-lysosomes fusion and alterations in cell death mechanisms of host macrophages that are crucial for its infectivity and dissemination. Diabetic patients are more susceptible to developing tuberculosis because of impairement of innate immunity and prevailing higher glucose levels. Our earlier observations have demonstrated alterations in the protein profile of *M. tuberculosis* exposed to concurrent high glucose and tuberculosis conditions suggesting a crosstalk between host and pathogen under high glucose conditions. Since high glucose environment plays crucial role in the interaction of mycobacterium with host macrophages which provide a niche for the survival of *M. tuberculosis*, it is important to understand various interactive mechanisms under such conditions. Initial phagocytosis and containment of *M. tuberculosis* by macrophages, mode of macrophage cell death, respiratory burst responses, *Mycobacterium* and lysosomal co-localization were studied in *M. tuberculosis* H37Rv infected cells in the presence of varied concentrations of glucose in order to mimic diabetes like conditions. It was observed that initial attachment, phagocytosis and later containment were less effective under high glucose conditions in comparison to normal glucose. *Mycobacterium* infected cells showed more necrosis than apoptosis as cell death mechanism during the course of infection under high glucose concentrations. Co-localization and respiratory burst assay also indicated evasion strategies adopted by *M. tuberculosis* under such conditions. This study by using THP1 macrophage model of tuberculosis and high glucose conditions showed immune evasion strategies adapted during co-pathogenesis of tuberculosis and diabetes.

1. Introduction

Epidemiological data for association between tuberculosis (TB) and diabetes (DM) is increasing continuously, however, the current literature and evidence regarding interplay between TB and DM is ambiguous and more importantly, the molecular mechanisms underlying this association are difficult to interpret. This association might be due to alterations in the immune response of patients with diabetes which leads to accelerated progression of active TB and make them more prone to develop tuberculosis [1,2]. Several studies have indicated that diabetic conditions specifically hyperglycemia weakens the innate immune response as depicted by reduced expression of mycobacterial recognition receptors [3,4], decreased phagocytosis, adaptive cytokine secretion [5,6] and macrophage activation [7].

Mycobacterium tuberculosis has the ability to survive intracellularly by escaping various host defense mechanisms. After phagocytosis by macrophages, *M. tuberculosis* grows well by inhibiting phagosome lysosomes fusion and its maturation [8] which further prevent downstream immunological functions thus delaying the initiation of adaptive immune responses [9]. Further *M. tuberculosis* has developed several immune evasion strategies which include alterations in the cell survival mechanisms crucial for *M. tuberculosis* infectivity [10]. Apoptosis is a sequential cell death pathway for effective clearance of the pathogen whereas necrosis allows escape and release of mycobacteria and its further dissemination. Several studies support the fact that virulent mycobacterium inhibits apoptosis and induces necrosis as survival strategy inside the host macrophages [11].

To elucidate the effect of high glucose conditions on priming the immune response by host macrophages during copathogenesis of tuberculosis and diabetes, we explored the effect of increasing

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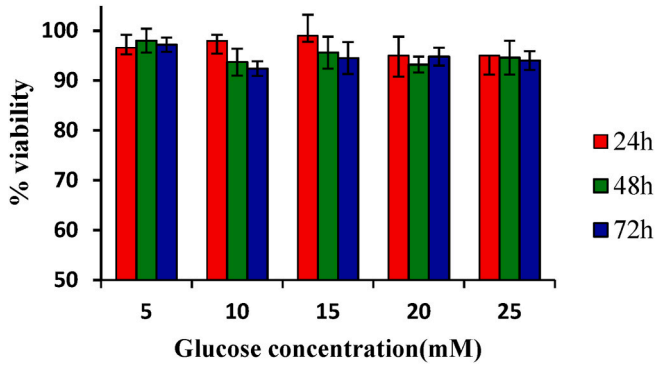


Fig. 1. Effect of different glucose concentrations on THP1 cell viability. The effect of different gluose concentrations (5, 10, 15, 20 and 25 mM) on cell viability was monitored by MTT assay at 24, 48 and 72 h. Values are represented as mean ± SE of 3 independent experiments.

concentrations of glucose on human macrophages *in vitro*. The initial attachment and phagocytic ability of THP1 derived macropahges for *M. tuberculosis* H37Rv followed by their capacity to restrain the mycobacterial growth was accessed. Further, respiratory burst ability and cell death mechanisms prevalent in *M tuberculosis* infected macrophages under high glucose conditions were evaluated.

2. Results

2.1. Effect of different glucose concentrations on ROS and NO production by THP1 cells co-infected with M. tuberculosis H37Rv

Cell viability as quantified by MTT assay, was not affected in the presence of different concentrations of glucose used in this study till 72h of treatment (Fig. 1). At all time points and glucose concentrations evaluated, cell morphology was healthy and the cells were adherent (Fig. 2). H_2DCFDA fluorescence intensity has been reliably used to evaluate total reactive oxygen species (ROS) generation including superoxide, hydrogen peroxide and peroxynitrite which are formed intracellularly. ROS production was significantly increased upon infection in the presence of high glucose (15 and 25 mM) in comparison to normal glucose (5.5 mM) and a more pronounced increase in the levels of ROS production was observed at 24 h and 48 h (Fig. 3). H_2DCFDA dye positive cells represented ROS producing cells. Nitric oxide (NO) levels as measured in terms of nitrite release were very low in non-infected cells as compared to the infected cells at each time point. A significant rise in NO production was observed at both 15 mM and 25 mM glucose. This increase was highly significant at 24 h and 48 h in comparison to 12 h post infection (Fig. 4).

2.2. Co-localization of late endosome markers with fluoresecent labeled M. tuberculosis in THP1 cells exposed to different glucose concentrations

Macrophages infected with *M. tuberculosis* H37Rv for 2 h were incubated for another 12, 24, and 48 h. After fixation, cells were stained for LAMP-1and LAMP-2 and binding was visualized by using FITC-labeled secondary antibody by confocal microscopy (Fig. 5).

It was observed that mean fluorescence intensity (counted for 100 cells) of co-localization decreased at both the glucose concentrations (15 mM and 25 mM) at 12, 24 and 48 h in comparison to the normal glucose concentration (Fig. 6).

2.3. Macrophage cell death studies under in-vitro tuberculosis and high glucose conditions

Effect of high glucose concentrations on the THP1 derived macrophages co-infected with *M. tuberculosis* H37Rv with respect to their effect on the cell death was studied using ethidium bromide and acridine orange dual staining. H_2O_2 treated cells were taken as apoptotic controls and boiling water treated cells were taken as necrosis control (Fig. 7). Under the experimental conditions it was observed that few cells underwent apoptosis at 12 h as represented by round green constricted nuclei (Fig. 8). At 24 h and 48 h, cell death occurred mainly by necrosis pathway at higher glucose concentration (15 mM and 25 mM) as compared to mostly by apoptosis at normal glucose concentration i.e.

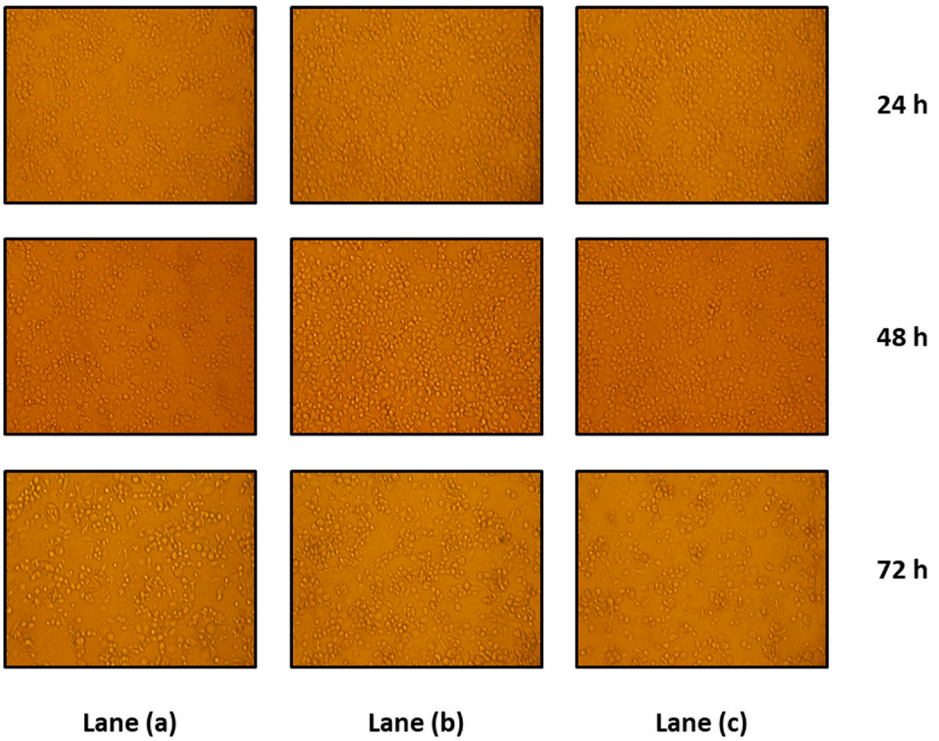


Fig. 2. Images (20 X) of PMA induced THP1 cells after treatment with different glucose concentrations at different time points. After Induction with PMA and incubation with different glucose concentrations; cells morphology and adherence was visualized by light microscopy. At all the time points cells retained healthy morphology and all the cells were adherent. Lane (a), Lane (b) and Lane (c) represents 5.5 mM, 15 mM and 25 mM glucose treated THP1 cells respectively after 24, 48 and 72 h of glucose treatment.

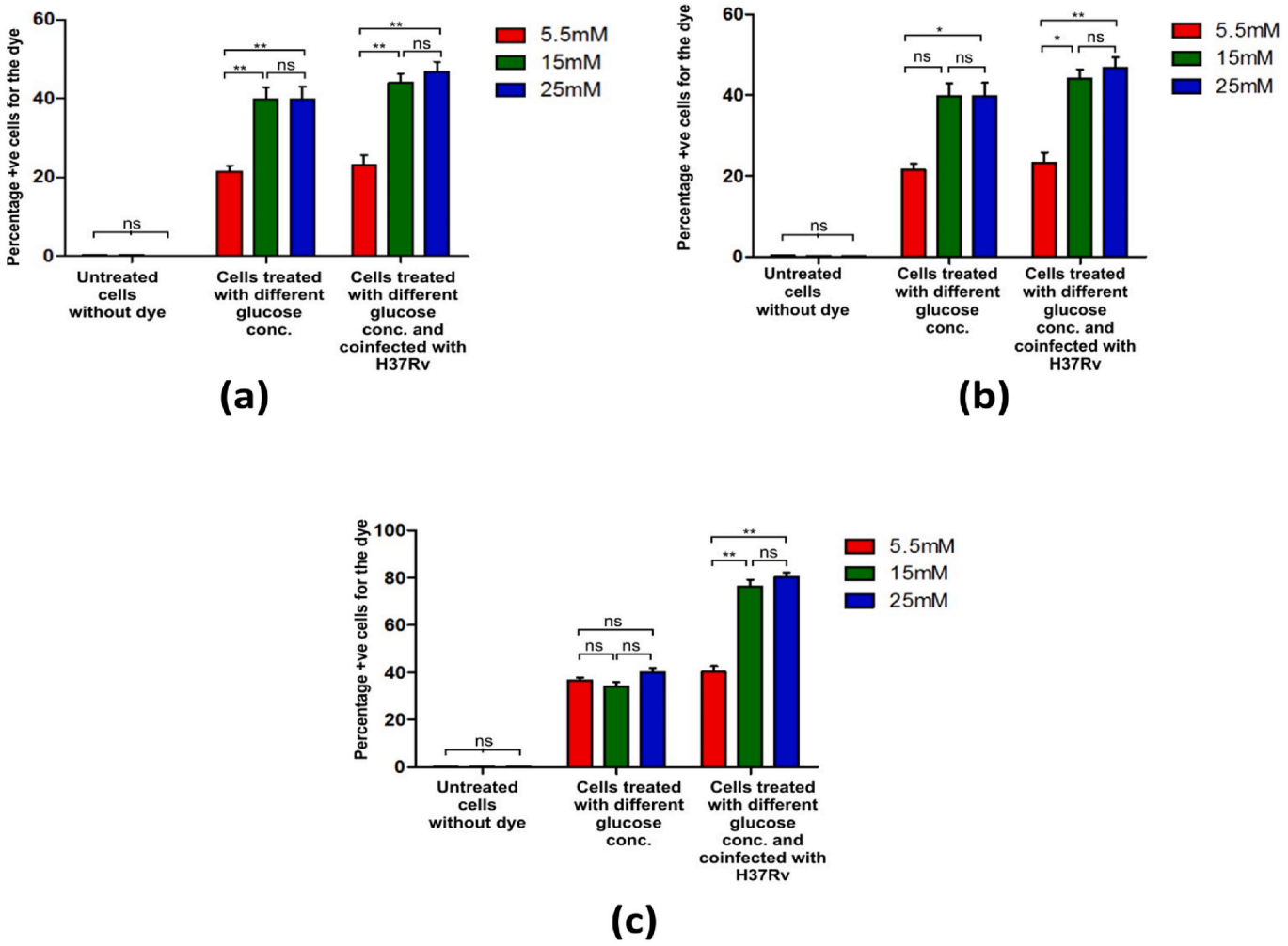


Fig. 3. Reactive oxygen species generation. ROS production was assayed by H2DCFDA staining of the cells and at 12 h **(a)**, 24 h **(b)** and 48 h **(c)** post infection in the THP1 macrophage model of concurrent tuberculosis and high glucose conditions. Minimum 10,000 events were acquired by flow cytometery from each flow tube. Stain (H2DCFDA) fluorescence positive cells were counted as ROS producing cells. Values are mean ± SD of 3 independent experiments, *$p < 0.01$, **$p < 0.001$, ns-non significant.

5.5 mM. Also minimal percentage of cell death was recorded under normal glucose in comparison to high glucose concentration. Further FITC-annexin binding assay was performed to see the effect of *in-vitro* infection and high glucose on the rate of apoptosis of THP1cells.

Increased apoptosis was observed at 15 mM and 25 mM glucose in infected cells in comparison to the infected cells under normal glucose conditions, though difference was not significant between 15 mM and 25 mM glucose conditions at 24 h and 48 h (Fig. 9). Total of 100 cells were counted from each well and scored as live (green staining) or necrotic (orange staining with disintegrated and swollen/disintegrated cell shape) or apoptotic (yellowish green condensed nuclei). Percentage Annexin PI positive cells depict percentage cell death at 12 h, 24 h and 48 h post infection under *in-vitro* TB-high glucose conditions compared to infection conditions in normal glucose conditions cultured THP1 macrophages.

2.4. *M. tuberculosis* phagocytosis and intracellular survival assay

CFUs enumerated at 12 h post infection reflect number of bacilli initially phagocytosed under different glucose concentrations. CFU count at 24 h and 48 h post infection represented intracellular replication of *Bacilli* (Fig. 10). Under normal glucose conditions, CFU count decreased at 24 and 48 h in comparison to number of initially phagocytosed Mycobacteria, whereas reverse was observed in the presence of 15 mM and 25 mM glucose.

At normal glucose conc. 5.56 ± 0.01 log CFUs were phagocytosed whereas under high glucose(15 mM and 25 mM), only 4.35 ± 0.01 log CFUs and 4.11 ± 0.10 log CFUs respectively were phagocytosed. At 24 h and 48 h post infection under normal glucose conditions, CFU count decreased to 4.88 ± 0.06 and 4.20 ± 0.05 log CFUs respectively, whereas log CFU count increased to 4.91 ± 0.01 log CFU at 24 h and 5.43 ± 0.01 log CFUs at 48 h post infection under 15 mM glucose conditions. Further at 25 mM glucose, CFUs increased from 4.10 ± 0.01 at 12 h to 4.61 ± 0.03 at 24 h and 5.42 ± 0.12 at 48 h post infection.

3. Discussion

Persistent hyperglycemia is the predominant cause of most of the complications observed in diabetes and is responsible for increased susceptibility to various infections including tuberculosis. Our earlier observations have demonstrated alterations in the proteomic profile of *M. tuberculosis* exposed to concurrent high glucose and tuberculosis conditions [12] suggesting a crosstalk between host and pathogen. In this context, we tried to analyse the interaction between intracellular mycobacteria and host macrophages under copathogenesis conditions of tuberculosis and high glucose concentration prevalent in diabetics. This analysis was performed using an *in-vitro* model of THP1 derived macrophage cell line grown under high glucose concentrations and

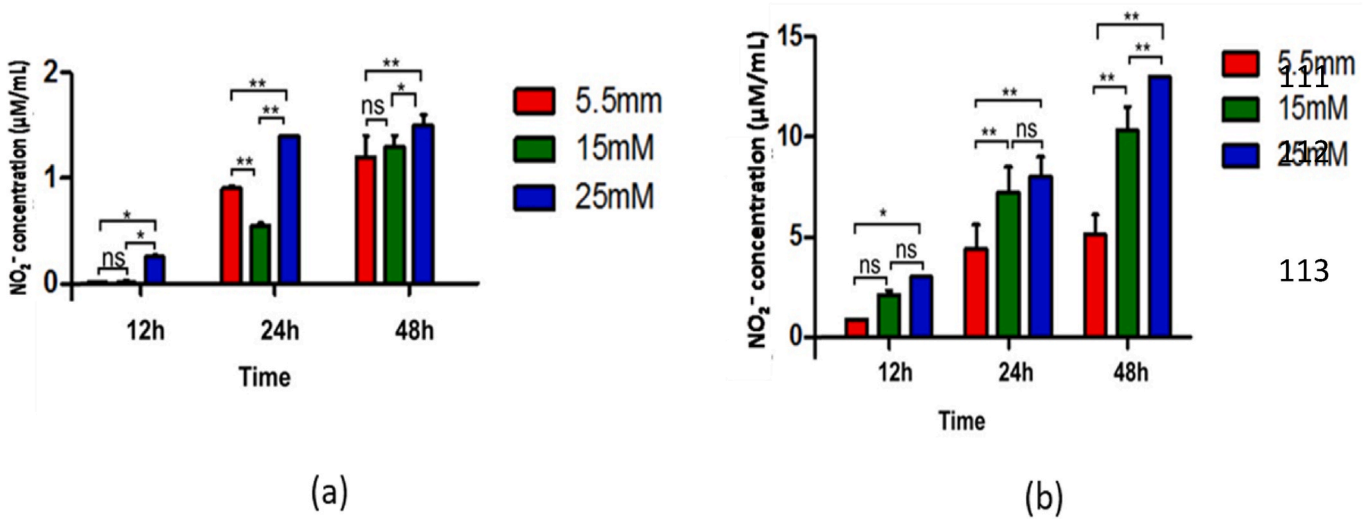


Fig. 4. NO production by the THP1 cells in the presence of different glucose concentrations. Griess method was used for NO quantification in the cell free supernatants from the cells cultured in the presence of under different glucose concentrations and coinfected with *M. tuberculosis* H37Rv **(a)** Nitrite levels in the THP1 macrophages under different glucose conditions without *M. tuberculosis* infection at 12, 24 and 48 h post glucose treatment, **(b)** Nitrite levels produced by the THP1 macrophages under different glucose conditions with simultaneous *M. tuberculosis* infection at 12, 24 and 48 h. Values are mean ± SD of 3 independent experiments, *p < 0.01, **p < 0.001, ns-non significant.

simultaneously infected with *M. tuberculosis* H37Rv to mimic the concurrent tuberculosis and high glucose conditions. This cell line was chosen because it mimics the human alveolar macrophages where *M. tuberculosis* mainly resides [13]. Similar THP1 cell line models have also been successfully used in previous studies [6,14].

Hyperglycemia have been shown to inhibit binding and phagocytosis of pathogen in diabetic host [15,16]. Adhesion and intake of *M. tuberculosis* by human monocytes has been shown to be decreased in diabetic patients [16] which support our observations. Further, after the initial phagocytosis, killing of *M. tuberculosis by* host macrophages under high glucose concentrations was decreased as shown by increased CFUs at 24 h and 48 h (Fig. 10). Similar findings have been observed in a mice model [17]. Studies have shown that in human disease, or in the model systems, NO production is not restrictive against mycobacteria and suggest that mycobacteria can utilize NO or other intermediates such as nitrite or nitrate for its survival [18–22].

Relatively lower co-localization of *M. tuberculosis* with the late phagolysosomal markers LAMP1 and LAMP2 was observed (Fig. 5) under high glucose conditions as compared to normal glucose. These results are in accordance with observed increase in the CFUs under high glucose conditions at different time points, because *M. tuberculosis* might have evolved a strategy to circumvent the immune response of the host cell inside the phagolysosome which results in its replication inside the host system leading to increased bacterial load. Our data is supported by the fact that *M. tuberculosis* has evolved a strategy to escape immune mechanisms and actually survives within macrophages. *M. tuberculosis* may be able to survive by preventing phagosomal maturation, acidification and fusion with the lysosomes, or may escape to cytosol [23–27]. ROS are produced by phagocytes such as macrophages and neutrophiles as a part of oxidative respiratory burst to eliminate antigens [28]. Mycobacteria have intrinsically developed the ability to resist and degrade these antimicrobial agents [29,30]. Mehrotra et al., 2014 documented that blood glucose levels also provide the pathogen a protective edge by affecting macrophage apoptosis [31]. It was observed that under high glucose conditions, virulent bacilli turns the cell death pattern towards increased necrosis thus escaping its clearance by the host (Fig. 8).

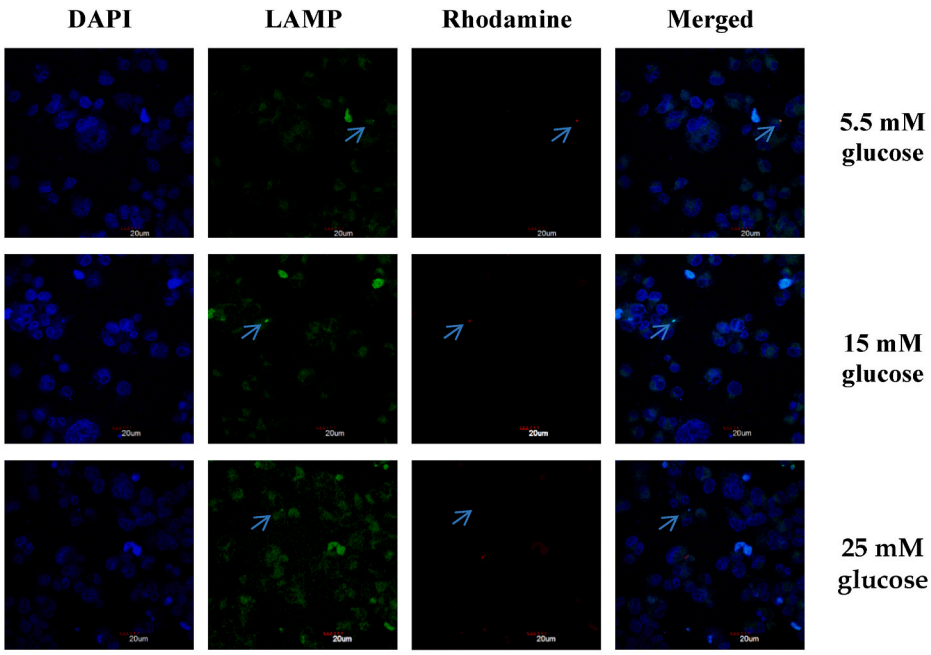
Increased cell death both by apoptosis and necrosis was seen under copathogenesis of tuberculosis and diabetes conditions (Figs. 8 and 9). Initially at 12 h, cell death percentage was low and most of the cells died by apoptosis whereas at 24 and 48 h, cell death mechanism switched from apoptosis to more necrosis under high glucose conditions. This might help mycobacteria to multiply as evident by the increased CFUs under high glucose conditions. However, under normal glucose conditions, most of the cells died of apoptosis thus correlating with the sharp decrease in the CFUs at 48 h in comparison to the initially phagocytosed bacteria. The respiratory burst response for reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) demonstrated an increase in both ROS and RNI of the *M. tuberculosis* infected THP1 macrophages with increasing glucose concentrations (Figs. 3 and 4). Significantly high production of ROS and RNI in the presence of high glucose is supported by earlier studies where it is shown that *M. tuberculosis* can resist ROS by developing certain mutations at *mel2* locus that provides protective cover to the pathogen against damage caused by ROS. Further studies have revealed a protective role of NO in animals but its role is not clear in humans [32]. Another study by Jung et al. have revealed that mycobacteria thrive well in the macrophages that produce NO as indicated by increased CFUs in a high glucose state at 24 h and 48 h post infection [33].

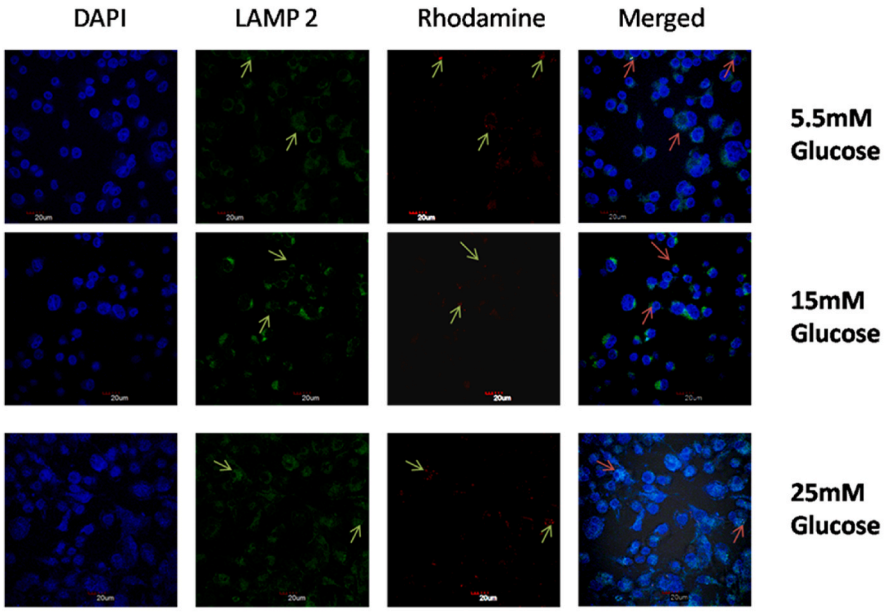
Our study supports the existing findings that *M. tuberculosis* develops several immune evasion strategies under diabetes conditions. *In-vitro* THP1 macrophage model of tuberculosis and high glucose copathogenesis will be helpful to study physiological interactions between the host macrophages and pathogen mimicing concurrent tuberculosis and hyperglycemia. The immune evasion strategies of *M. tuberculosis* such as switching from apoptosis to necrosis mode of cell death, adaptive protection of *M. tuberculosis* from macrophage oxidative respiratory burst plus phagolysosomal escape may explain the mechanism of exacerbated tuberculosis and delayed treatment response in diabetic like conditions.

4. Material and methods

4.1. Culture and maintenance of THP1 cell lines

Human leukemic macrophage like cell line THP-1 obtained from National Centre for Cell Sciences, Pune, India) was maintained as suspended cells in RPMI 1640 media, supplemented with 10% fetal bovine serum, at 37 °C in a CO_2 humidified incubator. Cells were grown to a density of 2–5 × 10^6 cells/ml and prior to infection with *M. tuberculosis,* THP-1 cells were passaged at least three times in supplemented





(b)

Fig. 5. Representative confocal microscopic images of co-localization of Rhodamine tagged *M. tuberculosis* and the late endosomal lysosomal marker LAMP-1 and LAMP-2 in THP1 cells. Infected cells were fixed and stained for LAMP-1 **(a)** and LAMP2 **(b)** together with a FITC-labeled secondary antibody at 12, 24, and 48 h and visualized using confocal microscopy.

antibiotic-free RPMI 1640 growth medium before expansion into six well flat bottom tissue culture plates. The cells grown to a density of 1–2 $\times 10^5$ cells per well were stimulated with 20 nM (12 ng/ml) phorbol 12-myristate13 acetate (PMA) for 24 h so as to allow the cells to adhere. Non-adherent cells were removed by washing twice in warm RPMI 1640 at 37 °C and the resulting monolayers were covered with 3 ml supplemented RPMI 1640 growth medium.

The cells were further cultured in RPMI containing either 5.5 mM (normal glucose), 15 mM, 25 mM (high glucose) D-glucose. 25 mM D-mannitol was used as osmotic control.

The effect of hyperglycemia on the cell viability was monitored in terms of changes in cellular viability using MTT assay. Briefly PMA differentiated THP1 macrophages were grown as monolayer in 24 well plate at a concentration of 2–5 $\times 10^5$ cells/ml in RPMI medium. The monolayer was cultured with different glucose concentrations for different time periods (24, 48 and 72 h). Wells containing medium alone without cells and MTT reagent were used as negative controls. The results were represented as percent viability taking control (cell under normal glucose conditions) as reference standard. Percent cell viability was calculated by using the formula: (Mean OD570 of test wells)/(Mean

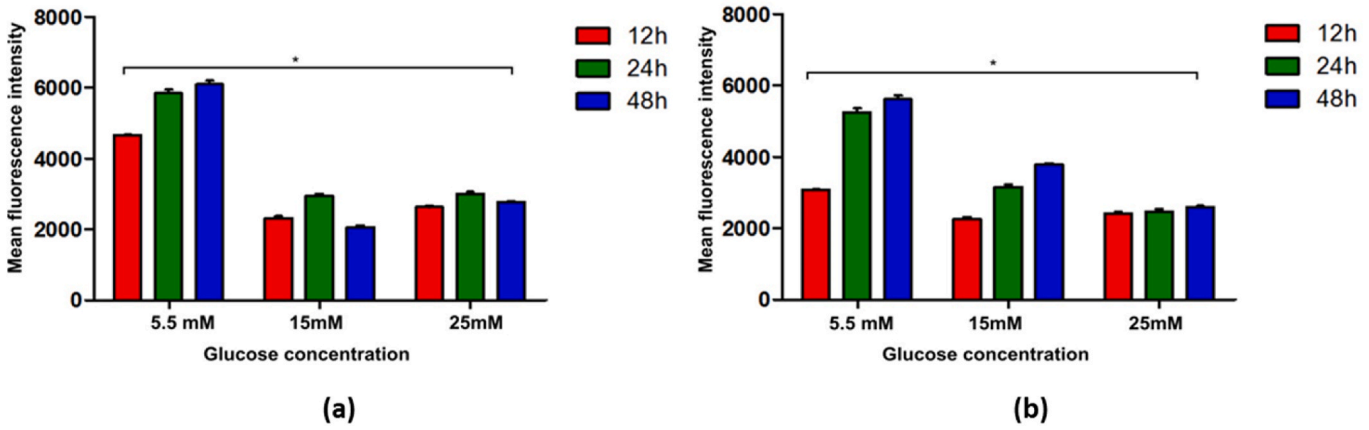


Fig. 6. Colocalization of labeled *M. tuberculosis* and the late endosomal-lysosomal protein LAMP-1 and LAMP-2. Infected cells were fixed and stained for LAMP-1 (a) and LAMP2 (b) together with a FITC-labeled secondary antibody at 12, 24, and 48 h. Mean fluorescence intensity of colocalization was calculated by quatifying the immunofluorescence with colocalized bacteria by ImageJ software. The data is represented as mean standard deviation of at least 100 cells for each glucose concentration and time point.

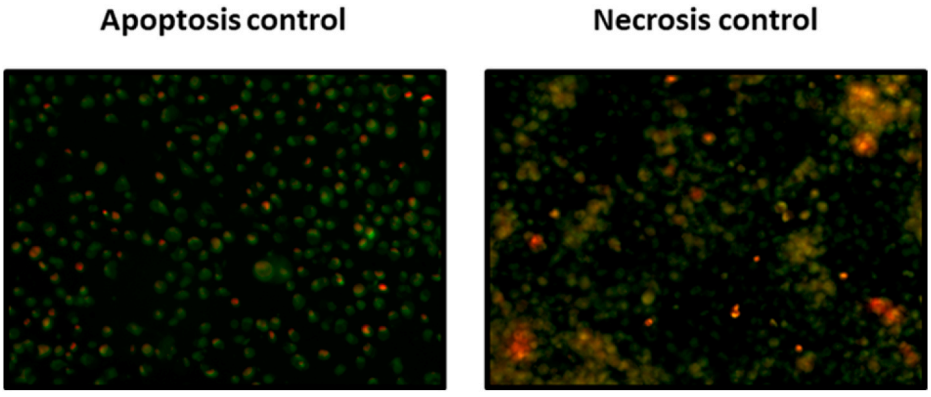


Fig. 7. Dual staining of *M. tuberculosis* H37Rv infected THP1 cells with Ethidium Bromide/ Acridine orange. H_2O_2 treated cells in the left image are showing apoptosis as the nuclei of the cells are condensed and orange/green and cells are round. Right image is showing necrosis as cells which are not intact and nuclei are disintegrated and thus have taken orange stain. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

OD570 of control wells) × 100.

4.2. *M. tuberculosis H37Rv culture and maintenance*

M. tuberculosis was originally obtained from National collection of type Culture (NCTC-7416), London, UK. The bacteria were cultured in Sautan's medium in the laboratory. The bacterial aliquots were prepared by counting them using McFarland's comparative standards and stored at −80 °C till further use.

4.3. *Infection of THP1 derived macrophages with M. tuberculosis H37Rv*

Mid-exponential phase mycobacterial cells (5×10^8 bacilli) were suspended in 1 ml RPMI 1640 growth medium and added to the adhered macrophage monolayer at an infection ratio of ten bacilli per macrophage and left to phagocytose for 12 h at 37 °C in a 5% humidified CO_2 incubator. After 12 h, extracellular mycobacteria were removed by decanting the supernatant and extensively washing the adhered cells twice in warm RPMI 1640. The infected macrophages were replenished with 3 ml complete RPMI 1640 medium containing amikacin (50 μg/ml) to prevent extracellular replication of *Mycobacteria*. Macrophage viability (never fell below 80%) was assessed by trypan blue exclusion. Incubations were carried out for further 5 days at 37 °C in a humidified CO_2 incubator. Different assays were performed to study various interaction parameters between macrophages and *M. tuberculosis* during *in vitro* TB and high glucose conditions.

4.4. *Cell viability and cytotoxicity assay*

The effect of high glucose conditions on the cell viability was monitored in terms of changes in cellular viability using MTT assay. The monolayer was cultured with different glucose concentrations in the presence of 5% CO_2 for different time periods (24, 48 and 72 h). After incubation, the culture supernatant from each well was removed and 150 μl of 1.2 mM MTT solution was added and incubated for 4 h at 37 °C. Wells containing medium alone without cells and MTT reagent were used as negative controls. After completion of incubation, 100 μl of supernatants were discarded and to the remaining 50 μl supernatant, 100 μl of DMSO was added. The solution was mixed thoroughly to dissolve formazan crystals. The absorbance was measured at 570 nm with reference at 630 nm on ELISA reader. The results were represented as percent viability taking control (cell under normal glucose conditions) as reference standard.

4.5. *M. tuberculosis uptake and phagocytosis assay*

After 12, 24 and 48 h,the media from wells was removed, monolayer was washed and lysed with 1 ml of 0.05% SDS-PBST for 10 min. Serial dilutions of the lysates in PBST were plated on 7H11 agar supplemented with 10% OADC for CFU enumeration. Colonies were counted after three weeks of incubation at 37 °C.

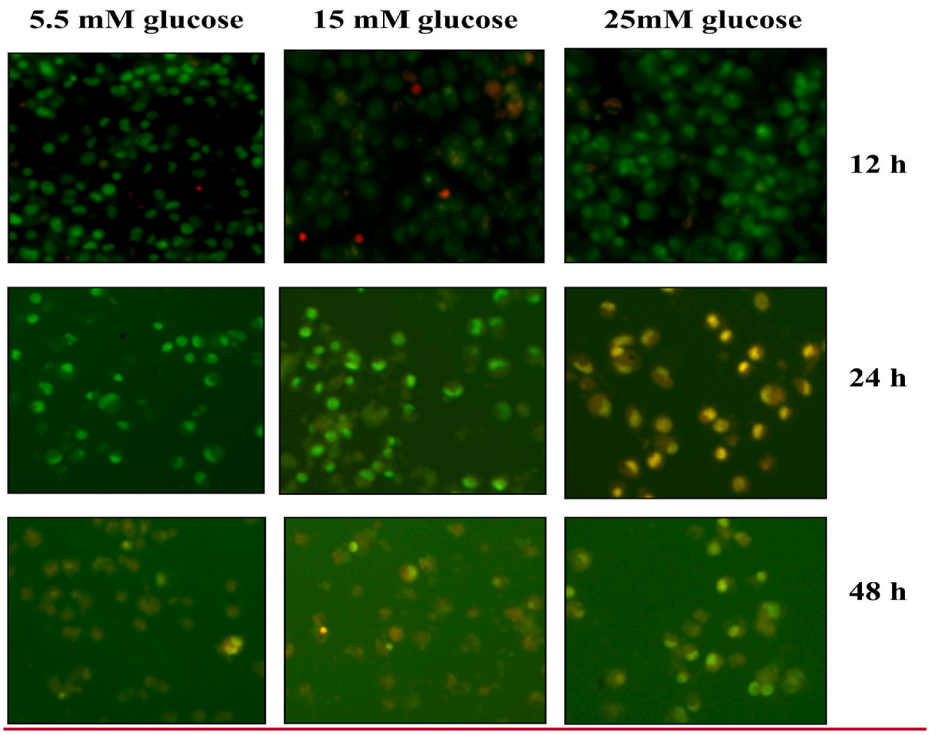


Fig. 8. Dual fluorescence staining with Etidium bromide/Acridine orange .A total of 100 cells were counted from each well and scored as live (green staining) or necrotic (orange staining with disintegrated and swollen/disintegrated cell shape), apoptotic (orange condensed nuclei). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

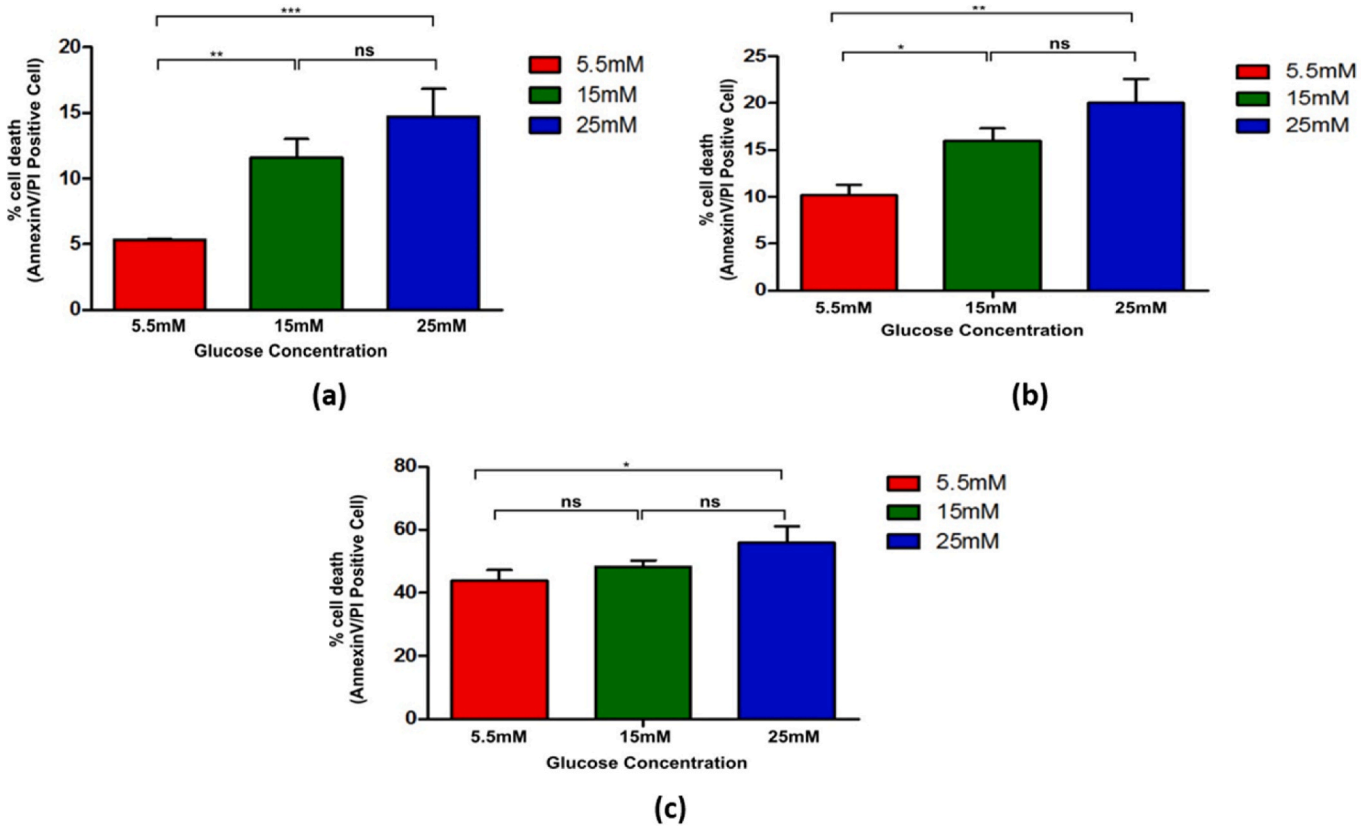


Fig. 9. Cell death assay by annexin PI staining for host macrophages under *in vitro* tuberculosis and high glucose conditions. Cells cultured under high and normal glucose conditions were harvested at (a) 12 h, (b) 24 h, and (c) 48 h and stained with FITC labeled annexin-V and propidium iodide. Fluorescence was acquired by flow cytometry in channels specified for the stains. For each tube at least 10,000 events were acquired for analysis. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ns-not significant.

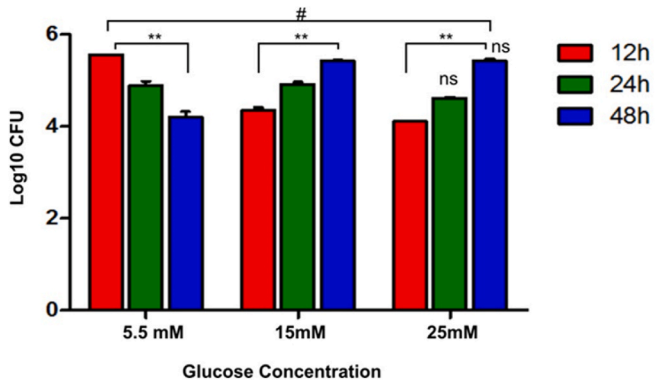


Fig. 10. *M. tuberculosis* uptake and phogocytosis assay: *M. tuberculosis* H37Rv attached and phaocytosed by the THP1 cells under different glucose conditions at 12, 24, and 48 h post infection were assayed by CFU enumeration from THP1 cells lysate plated on supplemented 7H11 agar and further incubated till three weeks. Results are mean ± SD of 3 independent experiments at each time point. #$p < 0.001$, **$p < 0.01$ and ns-not significant.

4.6. Macrophage respiratory burst assay

The generation of reactive oxygen species (ROS) after infection of THP1 macrophages with mycobacterium was measured by using 2′,7′-dichlorodihydrofluoresceindiacetate (H_2DCFDA). Briefly, THP1 cells (2×10^5 cells/well) cultured in the presence of PMA (20 nM) in 24 well plate were infected with *M. tuberculosis* as mentioned. After incubation, monolayers were washed with serum free RPMI and incubated with 5 μM H_2DCFDA for 10 min in dark at 37 °C. Cells were harvested, washed twice with ice cold PBS and placed on ice, and cell fluorescence was measured using excitation/emission wavelength of 490/525 nm by flow cytometry (FACS Caliber, BD Biosciences) [34].

Concentration of nitrite produced by the host cells, as a measure of the production of NO (nitric oxide) was quantified by the Griess assay [35]. Supernatants from THP1 cells incubated with different glucose concentrations and infected at MOI 10:1 were collected at different time points and assayed for nitrite. Cell free medium was used as blank for the assays. Briefly, 50 μl supernatant was removed from the culture well-sand incubated with 100 μl of Griess reagent (1% sulphanilimide, 0.1% napthylethylenediamindihydrochloride, 2.5% phosphoric acid) at room temperature for 10 min. Absorbance was read at 540 nm on ELISA reader. Units of NO were determined by comparison with a standard curve using sodium nitrite ($NaNO_2$) ranging from 3.1 to 100 μM.

4.7. Evaluation of cell death

Cells were harvested with 2 mM PBS-EDTA, washed in PBS-BSA 0.1% and centrifuged at 200×*g* for 5 min. The pellets were again suspended in 100 μL of staining-solution: 20 μl fluorescein isothiocyanate-labeled annexin-V (10 μg/ml) and 20 μl propidium iodide (PI) (50 μg/ml) in 1 ml phosphate buffered saline and incubated in dark for 15 min at room temperature, 400 μl phosphate buffered saline and processed for flow cytometry analysis using a FACS Calibur (Becton Dickinson System, San Jose, CA) with 488 nm excitation. Emitted light was split and collected at 530 nm band pass filter for fluorescein and 585 nm to PI. Data were analyzed using CELLQUEST software (Becton Dickinson).

To evaluate the cell death pattern induced by interaction of mycobacteria with THP1 cells, the cultured cells as stated earlier were infected with *M. tuberculosis* in 24 well tissue culture plate ($2–5 \times 10^5$ cells/well). After 24 h of incubation, cells were stained with 10 μL of aqueous Acridine orange(AO)/EthidiumBromide(EB) solution (100 μg/mL of AO in PBS; 100 μg/mL of EB in PBS) for 5 min. Cells were washed and changes in the nucleus of cells after AO/EB staining were observed under inverted phase fluorescent microscope. Viable cells stained only by AO were bright green with an intact structure; early apoptotic cells stained by AO have bright green area in the nucleus. Late apoptotic cells stained by AO and EB were red-orange with condensation of the chromatin visible as dense orange areas [36].

4.8. Colocalization of lysosome associated membrane protein (LAMP) with labeled M. tuberculosis using immunofluorescence

4.8.1. Labeling of M. tuberculosis with Rhodamine B

Log phase mycobacterial cell suspension was centrifuged at 5000×*g* for 15 min. Pellet was washed twice with PBST (PBS-0.05% tween 80). Pellet was passed five times through 27G needle to achieve single cell suspension. Pellet containing $4–8 \times 10^8$ cells was then suspended into 100 μL of Rhodamine B solution at a concentration of 0.1 mg/mL. Suspension was vortexed for 30sec and then incubated on ice for 60 min in the dark. Fluorescent bacteria were separated by centrifugation and washed with PBST to remove free fluorescent dye till the pellet comes clear. Pellet was suspended in RPMI media and covered with aluminium foil to protect from light.

4.8.2. Nuclear labeling of THP1 derived macrophages with DAPI

THP1 cells were fixed with 4% paraformaldehyde in PBS for 30 min and quenched by incubating with 50 mM NH_4Cl in PBS for 10 min. Cells were permeabilized with 0.1% Triton in PBS for 5 min. Fixed cells were washed and blocked with 1% BSA in PBS and incubated with 300 nM DAPI (4,6-diamidino-2-phenylindole, dihydrochloride) for nuclear staining, in 1% BSA/PBS for 10 min. Cells were mounted with glycerol and analyzed by confocal microscopy (Olympus, FLUOVIEW FV1000).

4.8.3. Immunofluorescence analysis of intracellular mycobacterial trafficking using late endosome markers LAMP1 and LAMP2

Macrophages were cultured on cover slips in 6-well plates and infected with labeled mycobacteria at MOI of 10:1(bacilli:macrophage) for 2 h at 37 °C. After 2 h, infection medium was replaced with fresh medium containing 0.2 mg/mL amikacin for 2 h to prevent growth of extracellular mycobacteria. Infected cells were washed with PBS, fixed in 4% paraformaldehyde for 30 min and incubated in 50 mM NH_4Cl for 10 min. Cells were permeabilized with 0.1% Triton in PBS for 5 min. Fixed cells were washed and blocked with 1% BSA in PBS and then sequentially incubated in 20 μL of PBS-1% BSA containing primary antibody (1:250 dilution) for 2 h and then washed with PBS thrice followed by secondary FITC conjugated antibody(1:1000 dilution) for an additional 1 h followed by washing with PBS and visualized using confocal imaging.

4.9. Statistical analysis

Colony forming units (CFUs) were log-transformed before analysis. Multiple comparisons between various groups were performed by one way and two way ANOVA (Analysis of Variance). The statistical analysis was done by using GraphPad Prism v.5.01 (GraphPad, San Diego, CA). The statistical evaluation of the confocal microscopy data was done by using Image J software.

Authors statement

The authors declare no financial and/or competing interests and roles described in the manuscript are final.

Declaration of competing interest

The authors declare that they have no conflicts of interests.

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