

Differences in responses to the intracellular macrophage environment between *Mycobacterium bovis* BCG vaccine strains Moreau and Pasteur

Paloma Rezende Corrêa¹, Marcos Gustavo Araujo Schwarz¹, Renata Monteiro Maia^{1,2},
Fátima Maria Figueroa Vergara^{3,4}, Milton Ozório Moraes^{5,†}, Leila Mendonça-Lima^{1/+}

¹Fundação Oswaldo Cruz-Fiocruz, Instituto Oswaldo Cruz, Laboratório de Genômica Funcional e Bioinformática, Rio de Janeiro, RJ, Brasil

²Fundação Oswaldo Cruz-Fiocruz, Instituto Oswaldo Cruz, Laboratório de Biologia Molecular de Insetos, Rio de Janeiro, RJ, Brasil

³Fundação Oswaldo Cruz-Fiocruz, Instituto de Tecnologia em Fármacos, Laboratório de Farmacologia Aplicada, Farmanguinhos, Rio de Janeiro, RJ, Brasil

⁴Fundação Oswaldo Cruz-Fiocruz, Centro de Desenvolvimento Tecnológico em Saúde, Fiocruz, Rio de Janeiro, RJ, Brasil

⁵Fundação Oswaldo Cruz-Fiocruz, Instituto Oswaldo Cruz, Laboratório de Hanseníase, Rio de Janeiro, RJ, Brasil

BACKGROUND The Bacille Calmette-Guérin (BCG) vaccine comprises a family of strains with variable protective efficacy against pulmonary tuberculosis (TB) and leprosy, partly due to genetic differences between strains.

OBJECTIVES Previous data highlighting differences between the genomes and proteomic profiles of BCG strains Moreau and Pasteur led us to evaluate their behaviour in the macrophage microenvironment, capable of stimulating molecular responses that can impact the protective effect of the vaccine.

METHODS Strain infectivity, viability, co-localisation with acidified vesicles, macrophage secretion of IL-1 and MCP-1 and lipid droplet biogenesis were evaluated after infection.

FINDINGS We found that BCG Moreau is internalised more efficiently, with significantly better intracellular survival up to 96 h p.i., whereas more BCG Pasteur bacilli were found co-localised in acidified vesicles up to 6 h p.i. IL-1 β and MCP-1 secretion and lipid droplet biogenesis by infected macrophages were more prominent in response to BCG Pasteur.

MAIN CONCLUSION Overall, our results show that, compared to Pasteur, BCG Moreau has increased fitness and better endurance in the harsh intracellular environment, also regulating anti-microbial responses (lower IL-1b and MCP-1). These findings contribute to the understanding of the physiology of BCG Moreau and Pasteur in response to the intraphagosomal environment in a THP-1 macrophage model.

Key words: tuberculosis - BCG vaccine - *Mycobacterium bovis* BCG Moreau - intraphagosomal environment - THP-1 derived macrophages

Tuberculosis (TB) is a serious global health problem and occupied the first place in the world ranking of deaths by infectious diseases since 2015, being recently surpassed by coronavirus disease 19 (COVID-19).⁽¹⁾ Despite considerable efforts to control the disease, 10 million new cases were observed in 2020 with 1.5 million deaths. Most of the affected population is distributed in developing countries, due to low socioeconomic conditions.⁽¹⁾

The process of infection of the host by *Mycobacterium tuberculosis* (*Mtb*) occurs in a sequence of well-established events. Transmission occurs through inhalation of aerosols containing the bacillus, which is phagocytised by macrophages resident in the lung.⁽²⁾ The recognition and phagocytosis of *Mtb* by macrophages induces the expression of a variety of genes that con-

trol the immune function of these phagocytes, aiming to eliminate the pathogen. For this, several microbicidal mechanisms are employed, among them the production of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI), in addition to phagosomal acidification and the secretion of cytokines and chemokines.⁽³⁾

The phagosomal maturation process occurs through successive fusions and fissions with intracellular vesicles, proceeding from the initial phagosomes to late and then phagolysosomes. The phagolysosome is characterised by an acidic pH (4 - 4.5) and high hydrolase activity, causing the death of mycobacteria.^(4,5) Bacteria contained in phagosomes, such as *Mtb* and *M. bovis* Bacille Calmette-Guérin (BCG), not only interact with vesicles of various origins during maturation, but also with lipid storage compartments, such as lipid droplets.^(6,7)

Bacille Calmette-Guérin (BCG) is the only vaccine currently recommended and belonging to the World Health Organization (WHO) immunisation schedule in countries with high incidence of TB. It is an attenuated strain obtained in the 1920's by Calmette and Guérin after 231 passages of a *M. bovis* clinical isolate in glycerinated broth containing bovine bile.⁽⁸⁾ The BCG vaccine is effective in protecting children against severe forms of TB, including tuberculous meningoencephalitis and miliary TB, with greater than 80% efficacy.⁽⁸⁻¹³⁾ However, protection against pulmonary forms of TB in adolescents and

doi: 10.1590/0074-02760230070

Financial support: FIOCRUZ, CNPq (grant number CNPq-PAPES VI 421923/2017-2).

PRC was a scholarship recipient from CNPq.

† *In memoriam*

+ Corresponding author: lmlima@ioc.fiocruz.br

https://orcid.org/0000-0003-4388-5083

Received 14 April 2022

Accepted 05 September 2023



adults varies from 0 to 80%, depending on the study.^(14,15) The reason for this variable efficacy is still unknown, but may be associated with several factors, including differences between the BCG strains used in different countries.^(16,17,18) A BCG booster is used in Brazil for leprosy contacts, family members living near patients. A cohort study showed an increased protective effect of the second dose of the vaccine in leprosy outcomes.⁽¹⁹⁾

In Brazil, the BCG Moreau strain has been used for vaccine production since the 1920s, but it has only recently been characterised in more detail at the molecular level.^(20,21,22,23,24) BCG Pasteur, on the other hand, was the first BCG vaccine strain to have its genome completely sequenced.⁽²⁵⁾ It is considered a more recent strain and has been the focus of numerous studies.⁽²⁶⁾ The pathogen-host cell interaction is an important aspect impacting vaccine efficacy and given the differences observed between BCG strains. In this context we previously evaluated the impact of the loss of a transcriptional repressor (*rv3405c*) in BCG Moreau in a THP-1 cell infection model,⁽²⁷⁾ showing that loss of repression led to a possible functional gain. To expand this scenario, we compared the behaviour of BCG strains Moreau and Pasteur in this macrophage infection model. Our results show that BCG Moreau was better able to survive the hostile intracellular environment, reinforcing the differential physiological behaviour of the Brazilian strain when compared to BCG Pasteur.

MATERIALS AND METHODS

Culture of *M. bovis* BCG - *M. bovis* BCG Moreau (provided by Fundação Aulfo de Paiva - FAP) and BCG Pasteur (obtained from the Pasteur Institute, Paris, France) were grown in Middlebrook 7H9 liquid culture medium (Difco™) containing Tween 80 (Sigma) and glycerol (Sigma), supplemented with albumin, dextrose and catalase (ADC). The cultivation time was approximately three weeks, under constant agitation at 37°C, with weekly passages at 1:10 dilution. Upon reaching the optical density (DO_{600nm}) of approximately 1.0, the cultivation was interrupted, aliquoted in 15% glycerol (Sigma) and the seed lot was stored at -80°C.

To determine bacterial colony forming units (CFU), serial dilutions were made from the bacterial suspension and plated on Middlebrook 7H10 (Difco™) supplemented with ADC. Colonies were quantified on the 28th day.

THP-1 cell culture - THP-1 cells were cultured in RPMI-1640 medium containing L-glutamine (Sigma), 10% foetal bovine serum (FBS; Sigma) and 0.1% penicillin and streptomycin (PEES; Sigma). Cells were maintained in culture flasks at 37°C in atmosphere of 5% CO₂ by weekly replication.

Differentiation of THP-1 cells and BCG infection - For infection assays, THP-1 monocytes were stimulated with 200 nM phorbol 12-myristate 13-acetate (PMA; Sigma) in RPMI-1640 / 10% FBS medium for differentiation in dTHP-1 macrophages. After 48 h of stimulation, the cells were washed to remove PMA and pre-heated RPMI / 10% FBS medium was added for 48 h, with daily change. The THP-1 derived macrophages were infected with *M. bo-*

vis BCG in RPMI / 10% FBS medium at a multiplicity of infection (MOI) of 10: 1 by an infection pulse of 4 h at 37°C in an atmosphere of 5% CO₂. The choice of MOI (10 bacteria per cell) was based on previous studies.⁽²⁸⁾ In order to minimise clumping, typical of mycobacteria, bacterial suspensions were passaged 30 times through a 25-gauge needle attached to a 1 mL syringe prior to infection. At the end of the infection time, the culture medium was removed, and the cells were washed twice with RPMI / 10% FBS medium, to remove the bacteria that were not internalised, essentially as described.^(29,30)

Intracellular growth of BCG strains - For the intracellular growth assays, 2 X 10⁵ cells were used per well, in 24-well plates (Nunc), where the cells were differentiated and infected as previously described. Intracellular growth of BCG strains was evaluated through a time kinetics (4 h, 6 h, 24 h, 48 h, 72 h and 96 h) after infection. At the selected times, the medium was removed and the infected THP-1 derived macrophages lysed with 0.05% SDS. The bacterial pellet was resuspended in 1 mL of Middlebrook 7H9 liquid medium containing 10% ADC. Serial dilutions were made from this bacterial suspension and plated on Middlebrook 7H10 as described above. The colonies were quantified on the 28th day and the morphologies documented.

Co-localisation of bacteria with acidified vesicles - For the co-localisation assays, 2 X 10⁵ cells were used per well, in 24-well plates (Nunc), where the cells were differentiated on glass coverslips and infected as previously described, with the bacteria previously marked by the fluorophore SYTO9 (LIVE / DEAD® BacLight kit; Molecular Probes - Invitrogen).

After 4 h and 22 h of infection, the acidified vesicles of the infected THP-1 derived macrophages were stained with LysoTracker Red DND-99® fluorophore (Molecular Probes - Invitrogen), following the manufacturer's instructions. The cells were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature, protected from light. The nuclei of the eukaryotic cells were stained with 0.5 µg / mL DAPI (Sigma), for 10 min at room temperature and protected from light. Images of 10 random fields were captured using a fluorescence microscope (Nikon Eclipse Ci). At least 250 cells from each studied condition were analysed to observe the percentage of infected cells and 50 isolated bacteria to observe the percentage of bacteria co-localised with acidified phagosomes.⁽³¹⁾ These values were plotted and analysed using the GraphPrism software.

Quantification of lipid droplets - The labelling for lipid bodies by oil red O (ORO) was performed at 24 h of infection, essentially as described by Melo et al.⁽³²⁾ Briefly, cells were fixed with 3.7% formalin for 10 min, washed and incubated in 100% propylene glycol for 5 min followed by incubation with 0.5% ORO for 10 min. Cells were rinsed with 85% propylene glycol for 5 min, followed by water. The nuclei of eukaryotic cells were stained with DAPI (Sigma). Images from 20 random fields were captured totalling a minimum of 250 cells using a fluorescence microscope (Nikon Eclipse Ci).

Images were transformed into black and white pictures and analysed with a macro language, kindly provided by Dr Patricia Bozza (Fiocruz, Brazil), for the open-source image analysis platform Fiji to automatically extract and quantify information from microscopic images. Spots were determined by automatic spot detection and the total area of fluorescent lipid bodies was obtained for each field and divided by the number of cells in the respective field.⁽³³⁾

Cytokine assays - For cytokine dosage assays (IL-1 β and MCP-1) 3.2 X 10⁴ cells were used per well, in 96-well plates (Nunc). The cells were differentiated and infected, as described previously. Supernatants from 6 h, 24 h, 48 h, 72 h and 96 h were collected and stored at -80°C. Using the enzyme-linked immunosorbent assay (ELISA) technique, we determine the concentrations of IL-1 β (DY201 - R&D Systems) and MCP-1 (DY279 - R&D Systems), following the manufacturer's recommendations. The absorbance measurements were obtained on a Molecular Devices microplate reader with a 450 nm filter. The samples were analysed in four biological replicates and the concentration of each cytokine was calculated using the SoftMax program. The data were plotted and analysed using the GraphPrism program.

Statistical analysis - Data were expressed as mean \pm standard error of the mean (SEM) and statistically analysed using the analysis of variance test (ANOVA), followed by the Bonferroni post-test or the unpaired t-test. All experiments were carried out at least in biological triplicate. The number of replicates is indicated in the caption of the respective figures.

RESULTS

Mycobacterium bovis BCG Moreau has a higher internalisation rate in THP-1 derived macrophages - The internalisation rate for each BCG strain was observed after 4 h of bacteria-host cell interaction. Percentage of internalisation was related to the initial number of bacteria used for infection and common to both strains (2 x 10⁶ bacteria, MOI 10:1). BCG Moreau showed 20% of internalised bacteria, whereas the Pasteur strain showed only 8.5% internalisation (Fig. 1).

Mycobacterium bovis BCG Moreau has increased numbers of bacteria during infection - Growth kinetics was assessed from 4 to 96 h post-infection (Fig. 2A). The BCG Moreau intracellular growth profile shows a decrease at 6 h, with a strong and constant increase at subsequent time points. In comparison to 4 h, this decrease observed at 6 h p.i. for the Moreau strain is significant ($p < 0.05$; Fig. 2B). BCG Pasteur, on the other hand, shows a significant decrease in growth only at 24 h (Fig. 2C) but does not recover growth for the duration of the experiment. The comparison of the intracellular growth profile of these strains showed that BCG Moreau has a higher number of intracellular bacteria compared to BCG Pasteur at all times, except in the 6-hour period. Thus, BCG Moreau is internalised more efficiently than Pasteur, with significantly better intracellular survival up to 96h p.i.

Mycobacterium bovis BCG Moreau and Pasteur present changes in colony morphology after infection - The growth of bacteria in 7H10 plates (to determine CFUs) revealed alterations in the colony morphology of the two BCG strains. Fig. 3 shows representative images of BCG Moreau and Pasteur colonies from bacteria obtained from macrophages during time kinetics, compared to the morphology of bacteria obtained from the seed lots (no infection). Within 4 h p.i., both strains present a more spread-out and smooth colony morphology, quite different from the rough and raised colonies observed before infection. Although BCG Moreau colonies readily return to the pre-infection morphology, BCG Pasteur colonies maintain the altered morphology throughout the duration of the experiment. These colony morphology changes were reproducible and observed in all four biological replicates.

Mycobacterium bovis BCG Moreau phagosomes are more resistant to acidification - Another parameter evaluated was the co-localisation of the bacteria with acidified vesicles at 6- and 24-h p.i., using LysoTracker staining (Fig. 4A), a measure of phagosome maturation into phagolysosomes. Within 6 h, a greater percentage of cells were infected by BCG Moreau (29%) compared to the BCG Pasteur (17%), increasing to 38% (Moreau) and 26% (Pasteur) at 24 h (Fig. 4B). From the infected cells, we quantified the percentage of co-localisation of Syto-9 labelled bacteria with acidified vesicles (Fig. 4C). At least 50 bacteria distributed in five random fields were analysed by experiment.^(31,34) At 6 h, 45% of BCG Moreau and 56% of BCG Pasteur co-localised with acidified vesicles, decreasing to 36% (BCG Moreau) and 26% (BCG Pasteur) at 24 h p.i.

Infection of THP-1 derived macrophages with M. bovis BCG Pasteur induces higher accumulation of lipid droplets - The accumulation of lipid droplets induced by infection with SYTO9-labelled bacteria was evaluated by ORO staining of infected macrophages at

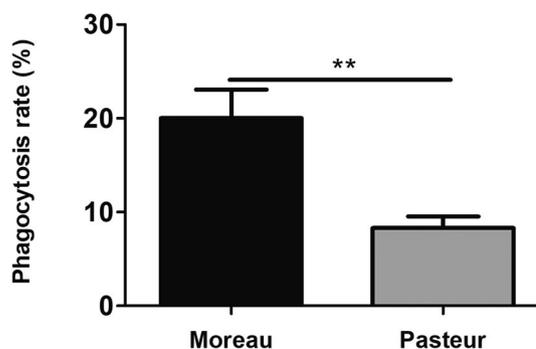


Fig. 1: comparison of the internalisation rate of Bacille Calmette-Guérin (BCG) Moreau and Pasteur strains by THP-1 derived macrophages after 4 h of bacteria-host cell interaction. THP-1 monocytes were differentiated into macrophages with 200 nM PMA for 48 h, with subsequent 48-hour rest. THP-1 derived macrophages were infected with the strains studied, using a multiplicity of infection (MOI) 10:1 for 4 h. Colony forming units (CFU) were determined 28 days after plating. Data are presented as mean \pm standard error of the mean (SEM). Statistical significance calculated by unpaired t-test ** $p < 0.01$. N = 4.

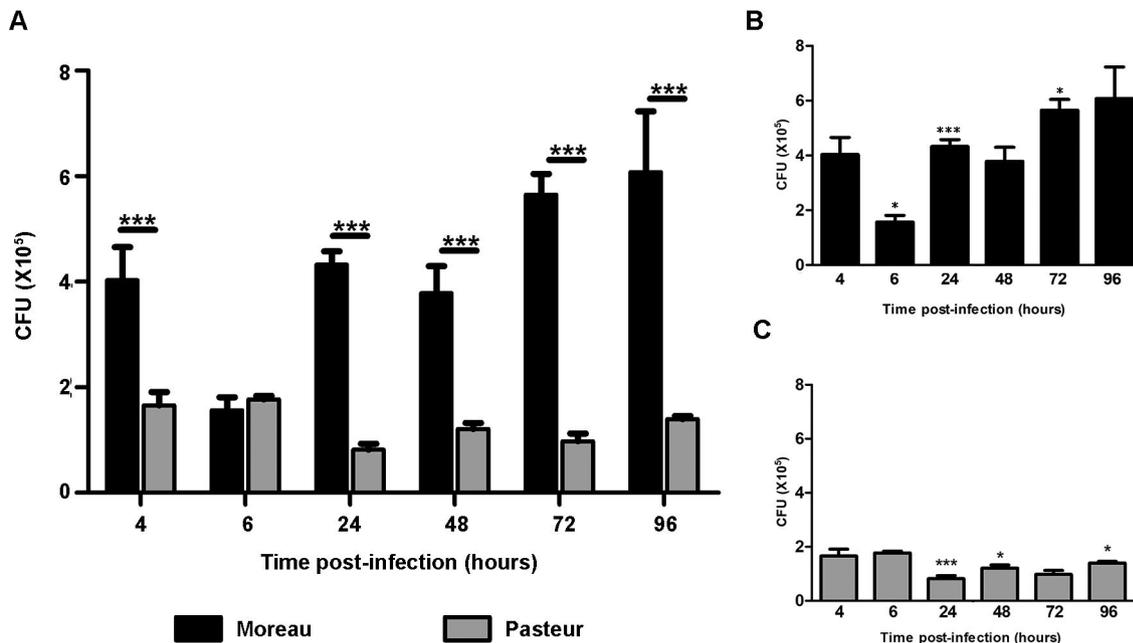


Fig. 2: (A) Comparison of the growth profile of Bacille Calmette-Guérin (BCG) strains Moreau and Pasteur in THP-1 derived macrophages. THP-1 monocytes were differentiated into macrophages with 200 nM PMA for 48 h, with subsequent 48-hour rest. THP-1 derived macrophages were infected with the strains studied, using a multiplicity of infection (MOI) 10:1 for 4 h. Colony forming units (CFU) were determined 28 days after plating. Data are presented as mean \pm standard error of the mean (SEM). Statistical significance calculated by unpaired t-test *** $p < 0.001$. $N = 4$. Growth kinetics of BCG strains Moreau (B) and Pasteur (C) after infection of THP-1 cells. Unpaired t test referring to the previous time * $p < 0.05$ and *** $p < 0.001$. $N = 4$.

24 h p.i. (Fig. 5B-C). Quantification shows that the accumulation of lipid droplets in macrophages infected with BCG Pasteur was approximately two times higher when compared to uninfected cells, with only a slight increase (around 1.3 times) when cells were infected with BCG Moreau (Fig. 5D).

Macrophages infected by BCG Pasteur secrete higher levels of IL-1 β and MCP-1 - THP-1 derived macrophages infected with BCG Pasteur showed significant higher secretion of IL-1 β cytokine at 6 h and 24 h p.i when compared to uninfected cells or to those infected with BCG Moreau (Fig. 6), peaking at 24 h p.i. After 48 h, the secretion of this cytokine falls to levels detected in uninfected macrophages.

MCP-1 secretion was also significantly higher in BCG Pasteur infected THP-1 derived macrophages at 48 h p.i, falling to levels detected in uninfected macrophages after 72 h p.i (Fig. 7).

DISCUSSION

The main purpose of this work was to evaluate the behaviour profile of the Brazilian vaccine strain, BCG Moreau, compared to BCG Pasteur using a THP-1 macrophage model of infection.

Genetic differences between BCG strains have already been characterised and associated with different immunogenic profiles. For example, regarding the two strains chosen for this work, BCG Moreau is deficient in phthiocerol dimycocerosate (PDIM) biosynthesis,⁽³⁵⁾ while Pasteur is not, and loss of PDIM has been associ-

ated with reduced reactogenicity.⁽³⁶⁾ Older strains such as Moreau produce methoxymycolates, strong immunostimulatory molecules, a capacity lost in more recent strains (such as BCG Pasteur).⁽³⁷⁾ Differences related to the wall composition, such as the presence of PDIM, can interfere in the binding to receptors, phagocytosis and in the subsequent processes underlying infection.^(38,39,40,41)

Both strains were readily taken up by THP-1 derived macrophages after 4 h of bacterial-host cell contact, with markedly different internalisation rates, reflecting the distinct lipid composition of the bacterial wall in each strain. Giovannini et al. demonstrated that a low PDIM composition in H37Rv strains would be sufficient to favour the non-aggregation of bacteria and consequently result in a higher phagocytosis rate.⁽⁴²⁾ Therefore, the absence of PDIM in the Moreau strain could be contributing to the increased phagocytosis profile compared to the Pasteur strain.

A comparison of intracellular survival between BCG strains Moreau and Pasteur reveals significantly different profiles. Except for a reduction in viability at 6 h p.i., BCG Moreau showed a constant increase in bacterial numbers up to the final 96 h time point. BCG Pasteur failed to do so. Furthermore, the number of intracellular viable bacteria was always significantly higher for BCG Moreau, except at 6 h p.i. This initial decline may reflect an adaptation period to intracellular stress, but may also result from loss of bacteria due to the possibility that host cells may undergo apoptosis in response to infection;⁽⁴³⁾ the removal of medium prior to CFU determination would deplete extracellular bacteria liberated from lysed cells.

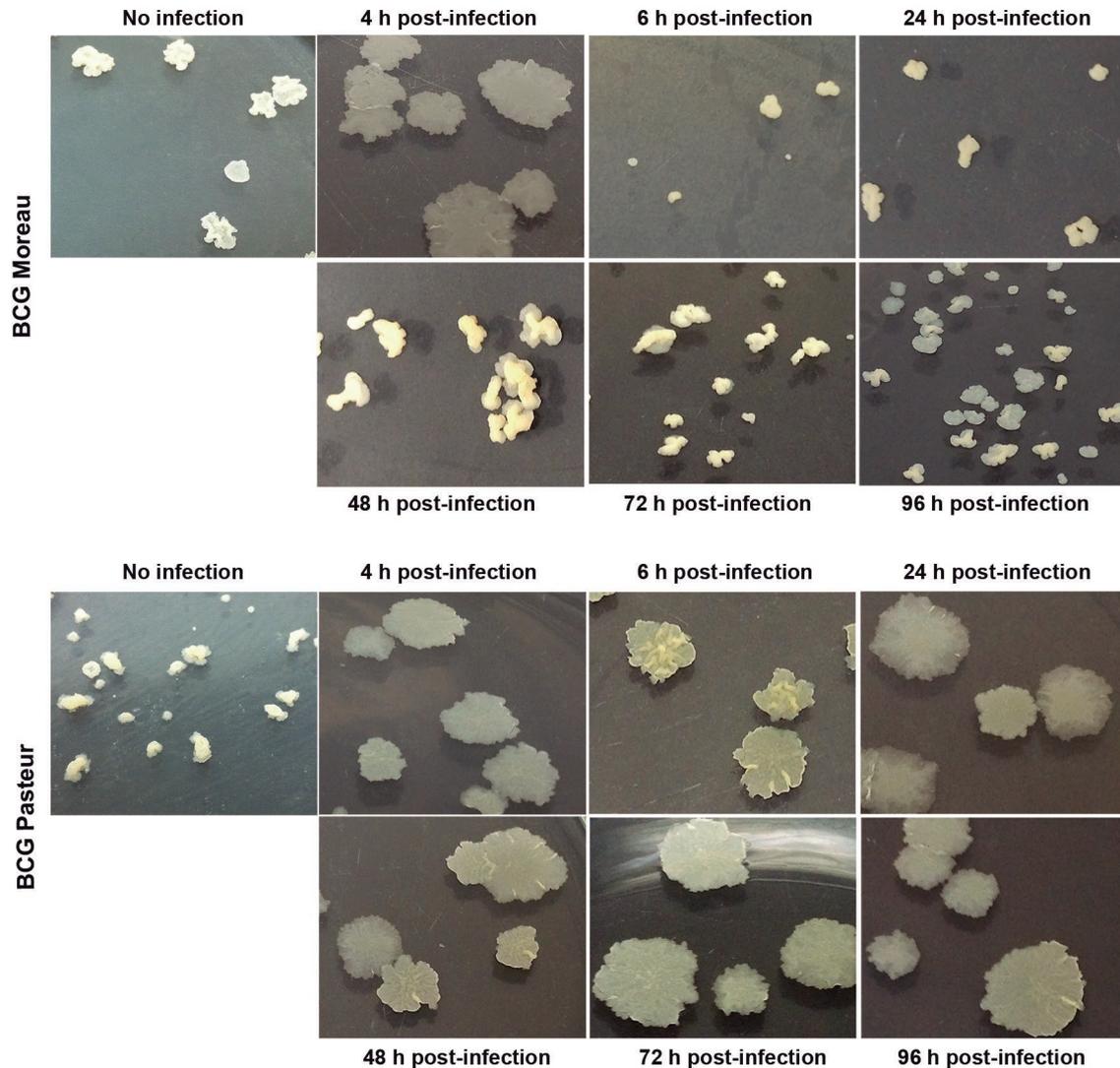


Fig. 3: representative profile of Bacille Calmette-Guérin (BCG) Moreau (top panel) and Pasteur (lower panel) colonies after infection of THP-1 derived macrophages. THP-1 monocytes were differentiated into macrophages with 200 nM PMA for 48 h, with subsequent 48-hour rest. THP-1 derived macrophages were infected with the strains studied, using a multiplicity of infection (MOI) 10:1 for 4 h and samples for colony forming units (CFU) counting were removed up to 96 h p.i., as indicated. Images show representative bacterial colonies obtained from lysed macrophages, serially diluted and plated on Middlebrook 7H10, after 28 days. Colonies obtained directly from the serially diluted seed lots prior to infection were used for comparison (no infection). N = 4.

The intracellular growth profiles of *M. bovis* and BCG Pasteur in J774 macrophages showed that viability for both strains is reduced in up to 1 day (*M. bovis*) or three days (BCG Pasteur). However, *M. bovis* can revert to a growth phase, while BCG Pasteur maintains constant levels of viable bacteria until the seventh day of infection.⁽⁴⁴⁾ This profile partially reflects what we observed in the THP-1 cell model, where BCG Moreau shows a less attenuated phenotype compared to BCG Pasteur. Indeed, BCG Moreau showed a decrease in viability prior to the Pasteur strain but was capable of regaining growth, while BCG Pasteur maintained the number of intracellular bacilli practically stable until the end of the studied kinetics. This phenomenon may reflect the BCG vaccine genealogy, since the BCG

Moreau strain is genetically closer to the original BCG, and consequently to *M. bovis*.⁽⁴⁵⁾

Changes in the colony morphology of bacteria recovered from macrophages were observed while plating to determine CFU counts. Both strains showed morphological changes as early as the first evaluated time (4 h p.i.), from the original small, elevated, rough colonies to a much more spread-out, smooth phenotype. However, while the original morphology was already restored in BCG Moreau at the next evaluated time (6 h p.i.), with few smooth colonies still present, BCG Pasteur colonies remained altered throughout the study period (up to 96 h p.i.). This change in colony morphology probably occurred due to alterations in the lipid composition of the mycobacterial surface, as a form of adaptive plastic-

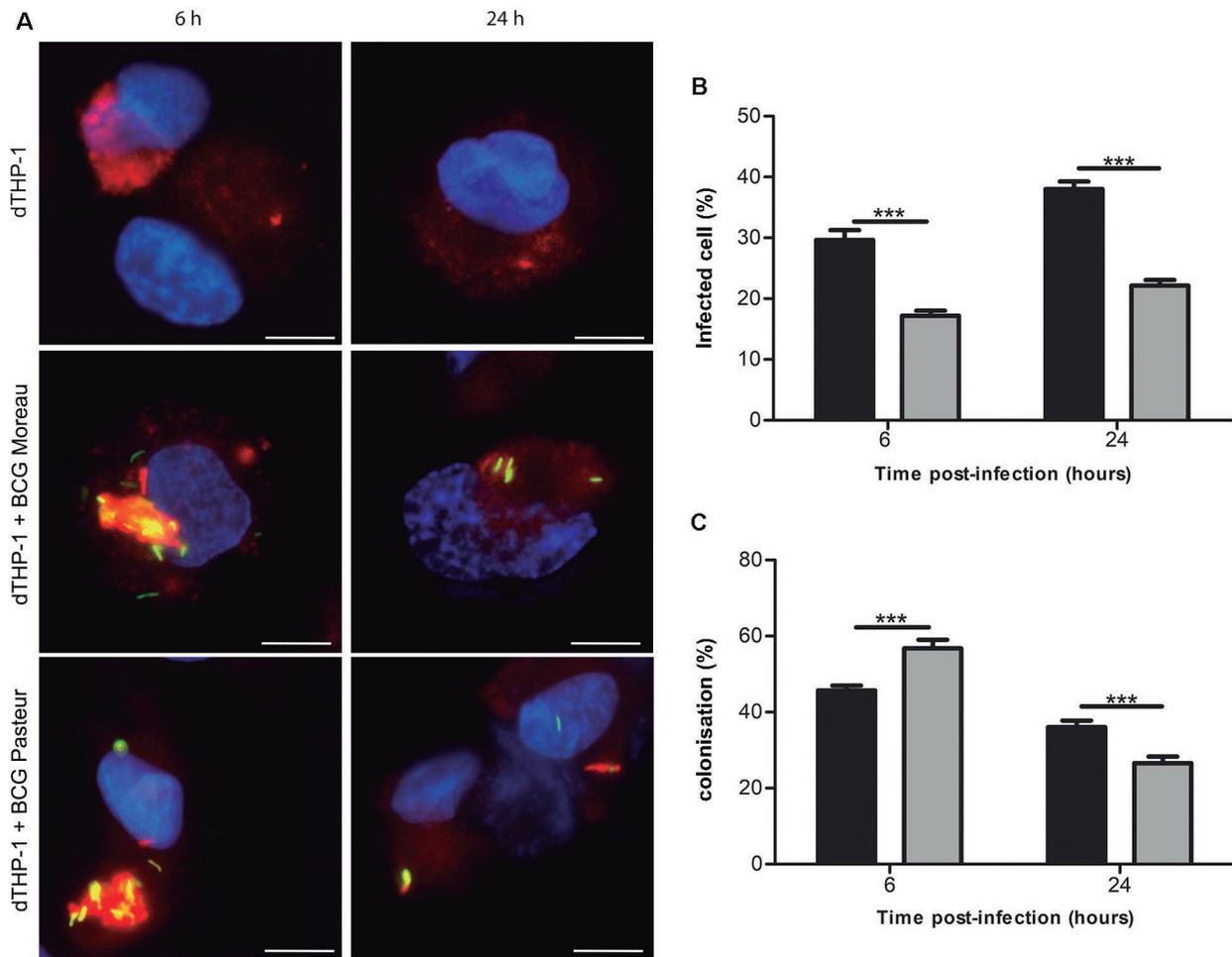


Fig. 4: representative co-localisation profile of Bacille Calmette-Guérin (BCG) strains with acidified vesicles at 6 and 24 h p.i. (A) THP-1 macrophages were differentiated into macrophages with 200 nM phorbol 12-myristate 13-acetate (PMA) for 48 h, with subsequent 48-hour rest. Macrophages were infected with the strains studied, using a multiplicity of infection (MOI) 10:1 for 4 h. THP-1 derived macrophages not infected and infected with BCG Moreau or Pasteur were visualised by fluorescence. Nuclei were labelled with DAPI (blue); BCG Moreau and Pasteur labelled with SYTO9 (green); acidified vesicles labelled with LysoTracker (red). Virtual overlay of the images (merge). Scale bar = 10 μ m. N = 3. (B) Percentage of THP-1 cells infected with BCG Moreau (black bar) or Pasteur (gray bar) at 6 and 24 h p.i. At least 250 cells were analysed in each condition. (C) Co-localisation of BCG Moreau (black bar) and Pasteur (gray bar) with acidified vesicles at 6 and 24 h. At least 50 isolated bacteria were analysed in each condition. Data are presented as mean \pm standard error of the mean (SEM). Statistical significance calculated by unpaired t-test ***p < 0.001. N = 3.

ity in response to the intracellular environment.⁽⁴²⁾ The phenotypic changes in colonies of mycobacteria are observed in *Mtb*, *M. avium*, and *M. abscessus*. The colony's morphology is highly correlated with the severity of the disease, with the rough morphotype being associated with more severe and persistent infections compared to the smooth morphotype.^(42,46,47,48) In *Mtb* H37Rv strain, the smooth colony phenotype was detected only after passage in monocyte-derived macrophages (MDM), and not in bacteria from axenic culture.⁽⁴²⁾ The smooth colony pattern in solid media is associated to attenuated microorganisms, unable to form surface biofilms when grown in liquid media,⁽⁴⁹⁾ and would result from nutrient deprivation and oxidative stress, characterising survival within activated macrophages.⁽⁵⁰⁾ Alteration of the lipid composition of the cell wall and reduction of bacterial multiplication, so that it remains undetectable to

the host's immune system, are adaptive strategies of *Mtb* to survive the adverse intra-phagosomal environment. Bacteria with this smooth colony phenotype would be less virulent, consisting of a population of heterogeneous intracellular bacilli, better able to coexist with the human host. In this way, the host's immune system would be weakly activated, until favourable conditions were resumed.⁽⁴²⁾

In order to gain an initial insight regarding possible differences in the host cell immune response to these two BCG strains, we assayed supernatants for the production of IL-1 β and MCP-1. These were chosen due to their known role in control of bacterial load (IL-1 β) and ROS response (MCP-1)⁽⁵¹⁾ in the context of BCG infected THP-1 derived macrophages. The secretion of the cytokine interleukin-1 β (IL-1 β) that controls bacterial load through the recruitment of microbial effectors⁽⁵²⁾ was as-

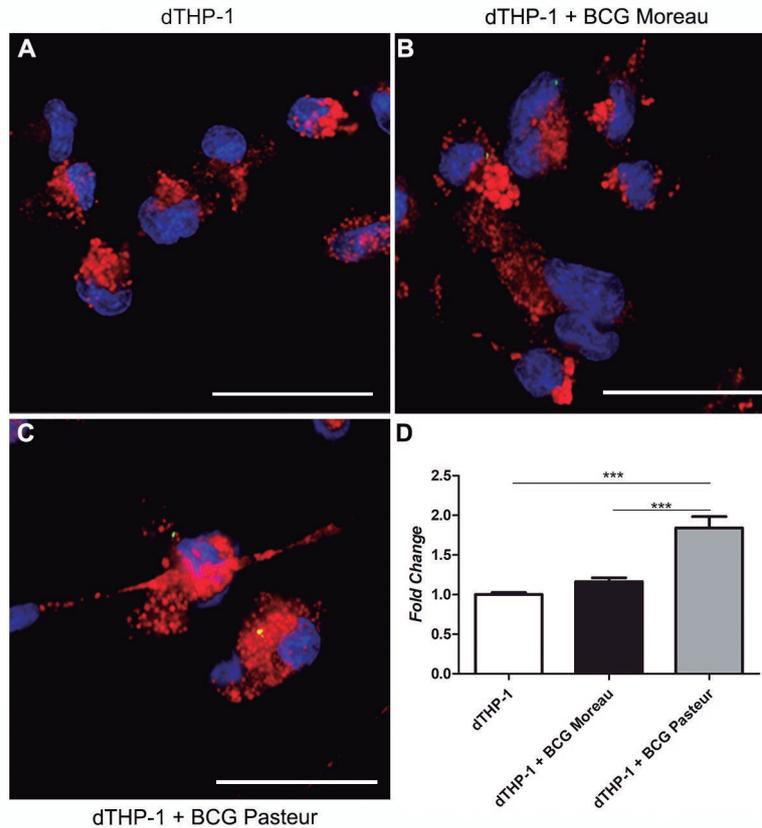


Fig. 5: representative profile of the presence of lipid droplet in cells infected with Bacille Calmette-Guérin (BCG) Moreau and Pasteur at 24 h p.i. THP-1 monocytes were differentiated into macrophages with 200 nM phorbol 12-myristate 13-acetate (PMA) for 48 h, with subsequent 48-hour rest. THP-1 derived macrophages were infected with the strains studied, using a multiplicity of infection (MOI) 10:1 for 4 h. THP-1 derived macrophages not infected (A) and infected with BCG Moreau (B) or Pasteur (C). Immunofluorescence detecting the nucleus, labelled with DAPI (blue); BCG Moreau and Pasteur labelled with SYTO9 (green); lipid droplets labelled with oil red O (ORO) (red). Virtual overlay of the images (merge). Scale bar = 50 μ m. N = 3. (D) Relative quantification of lipid droplets through ORO staining. At least 250 cells were analysed in each condition. Data are presented as mean \pm standard error of the mean (SEM). Statistical significance calculated by analysis of variance (ANOVA) test with Bonferroni post-test *** $p < 0.01$. N = 3.

essed by ELISA in the supernatant of infected THP-1 derived macrophages. An increase in its concentration was observed at 6 h p.i., reaching a peak at 24 h, with a tendency to return to levels like those of uninfected macrophages at other times. This 24-hour peak of expression can lead to the induction of antimicrobial effectors, such as tumour necrosis factor (TNF), to contain the infection. TNF would then trigger the apoptosis mechanism, which would lead to decreased viability in macrophages and bacilli.^(43,53) In addition, the intracellular bacillary load remained low and constant, suggesting that the microbicidal mechanism triggered by IL-1 β would be affecting the viability of the BCG Pasteur strain. However, for cells infected with BCG Moreau, the increase in the secretion of the cytokine IL-1 β was less pronounced, perhaps insufficient to generate a response in the same proportion as that observed for BCG Pasteur.

The increased secretion of the chemokine MCP-1 (CCL2) observed in macrophages infected with BCG Pasteur at 48 h p.i. but not with BCG Moreau, may be reflecting an increased immune response against infection with this strain. The induced secretion of chemokines CCL2,

CCL3, CCL4 and CCL5 has already been observed in THP-1 and PBMC-derived macrophages when infected with BCG.^(51,54) *M. bovis* BCG infection induces the intracellular formation of ROS, generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The accumulation of ROS plays an important role as a signalling molecule in the regulation of CCL2 chemokine secretion, contributing to the molecular response in the signalling of the inflammatory process.⁽⁵¹⁾ Thus, in a similar way, we could infer that the BCG Pasteur strain would be inducing a greater formation of reactive oxygen species, which would be neutralised due to the higher production of antioxidants in this strain when compared to the more primitive strains such as BCG Moreau.⁽⁴⁵⁾ These preliminary results should be further explored, expanding the analysis of host molecules differentially expressed in response to the infection with different BCG strains.

Fluorescence analyses of infected THP-1 derived macrophages with the labelled BCG strains indicated a higher percentage of cells infected with the BCG Moreau strain in 6 and 24 h, suggesting that in 6 hours there are less bacilli per cell in macrophages infected

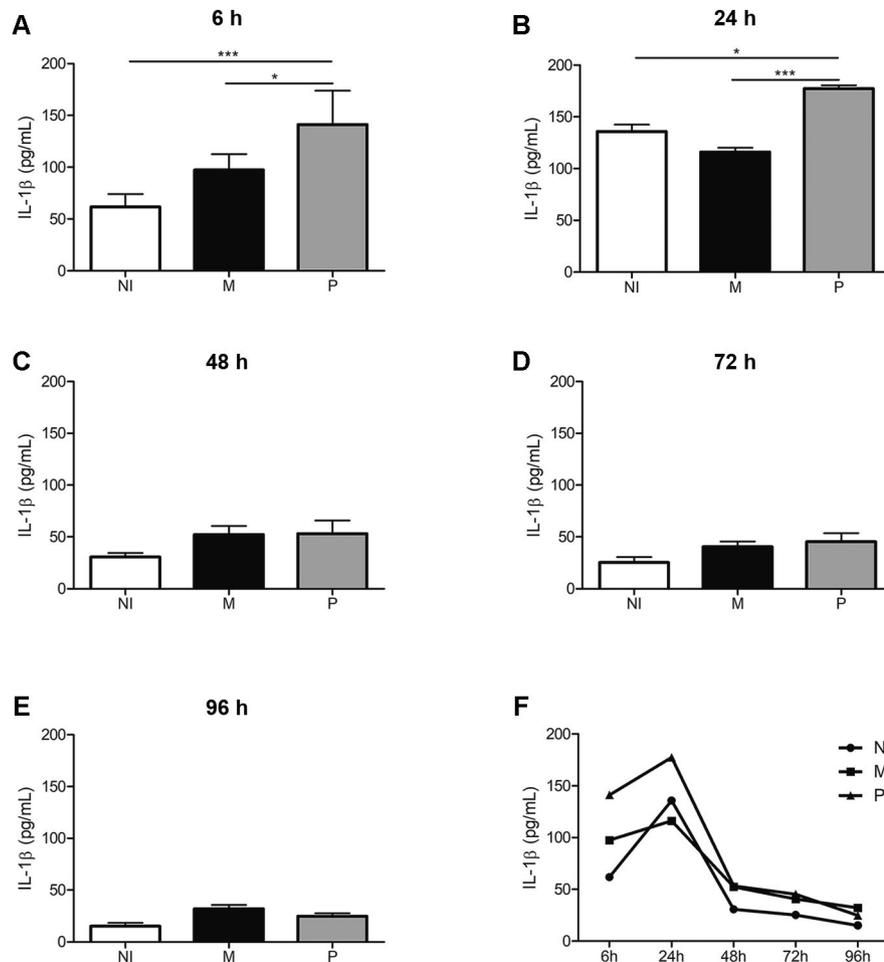


Fig. 6: IL-1 β secretion profile by THP-1 derived macrophages in response to Bacille Calmette-Guérin (BCG) Moreau (M) and Pasteur (P) infection at 6, 24, 48, 72 and 96 h p.i. (A-E). THP-1 monocytes were differentiated into macrophages with 200 nM phorbol 12-myristate 13-acetate (PMA) for 48 h, with subsequent 48-hour rest. THP-1 derived macrophages were infected with the strains studied, using a multiplicity of infection (MOI) 10: 1 for 4 h. The culture supernatant was collected every 24 h, starting at 6 h. Non-infected THP-1 derived macrophages (NI) used as controls. Kinetics of secretion profile throughout infection is shown in F. Data are presented as mean \pm standard error of the mean (SEM). Statistical significance calculated by analysis of variance (ANOVA) test with Bonferroni post-test *** $p < 0.001$, * $p < 0.05$. $N = 4$.

by BCG Moreau, since the number of viable bacilli was similar when compared to the BCG Pasteur strain. The possible reinfection of cells by extracellular bacteria liberated earlier in infection⁽⁴³⁾ could contribute to the increase in the percentage of infected cells at subsequent time points. The higher rate of co-localisation of cells infected with BCG Pasteur in 6 h suggests that these bacilli are failing to successfully evade microbicidal mechanisms and are likely to be eliminated. In comparison, co-localisation (%) for BCG Moreau was less variable between these two time points.

The survival of pathogenic mycobacteria within a phagosome is based on the bacterium's ability to control phagosomal maturation differently from non-pathogenic. J774 cells infected with BCG Pasteur for 3 h show about 40% of the bacilli co-localised with acidified vesicles and a decrease in the intracellular viability at 24 h p.i.,⁽⁵⁵⁾ corroborating with our data.

Macrophage infection with bacteria also triggers the modulation of lipids, which favour the escape of the

pathogen.⁽⁵⁶⁾ The accumulation of lipid droplets (LDs) in macrophages infected by *M. bovis* BCG was associated with Toll-Like Receptor-2 (TLR-2), with the modulation of cytokines IL-10 and TNF- α , as well as the presence of prostaglandin E2 (PGE2).⁽⁷⁾ The use of fatty acids as a carbon source by *Mtb* during infection is widely recognised.⁽⁵⁷⁾ Mycobacteria can store fatty acids in the form of triacylglycerol, detected during dormancy^(58,59) and also in the sputum of TB patients.^(60,61) The use of this triacylglycerol occurs when *Mtb* is deprived of nutrients⁽⁶²⁾ and when BCG is reactivated after a non-replicative state during hypoxia,⁽⁶³⁾ showing an important role of intracellular lipid storage during the non-replicative phase and in reactivation.⁽⁶⁴⁾ Thus, the greater accumulation of LDs observed in macrophages infected by BCG Pasteur could favour its persistence within the intra-phagosomal environment, since the formation of LD is correlated with the inhibition of autophagy and phagosomal acidification,⁽⁶⁵⁾ possibly suggesting that BCG Pasteur is in a viable but non-replicative state.

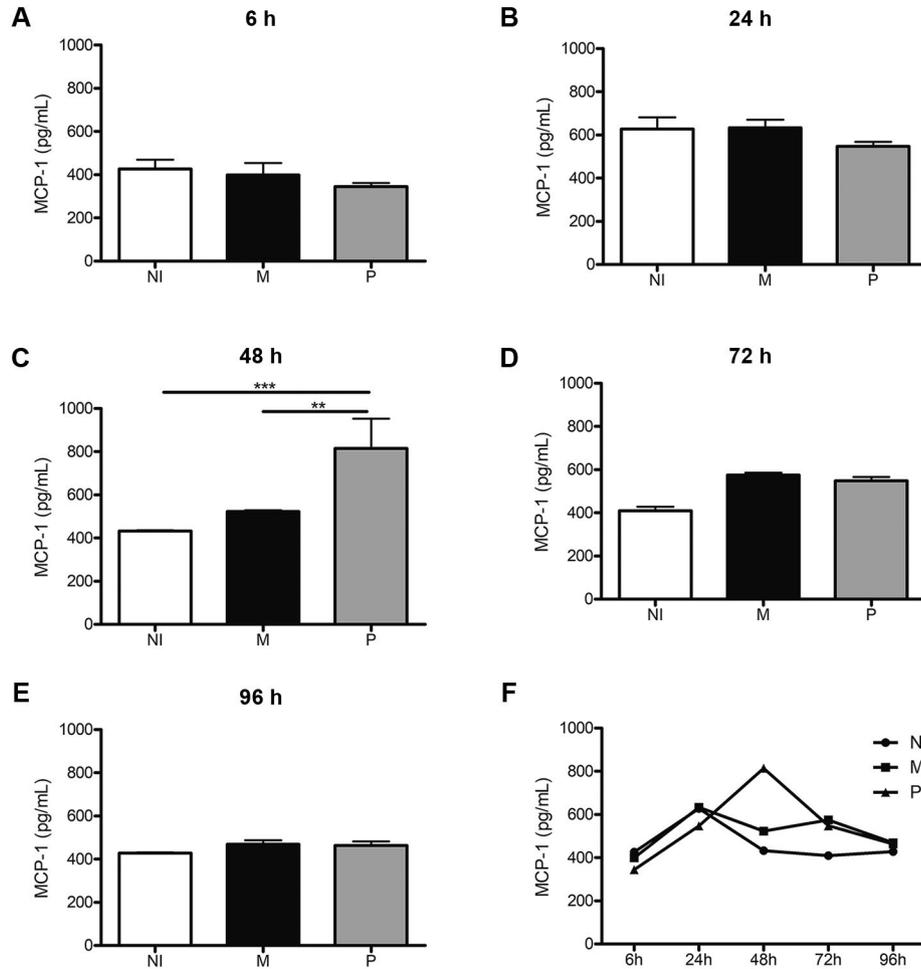


Fig. 7: MCP-1 secretion profile by THP-1 derived macrophages in response to Bacille Calmette-Guérin (BCG) Moreau (M) and Pasteur (P) infection at 6, 24, 48, 72 and 96 h p.i. (A-E). THP-1 monocytes were differentiated into macrophages with 200 nM phorbol 12-myristate 13-acetate (PMA) for 48 h, with subsequent 48-hour rest. THP-1 derived macrophages were infected with the strains studied, using a multiplicity of infection (MOI) 10: 1 for 4 h. The culture supernatant was collected every 24 h, starting at 6 hours. Non-infected THP-1 derived macrophages (NI) used as controls. Kinetics of secretion profile throughout infection is shown in F. Data are presented as mean \pm standard error of the mean (SEM). Statistical significance calculated by analysis of variance (ANOVA) test with Bonferroni post-test *** $p < 0.001$, ** $p < 0.01$. N = 4.

Overall, our results show that, compared to Pasteur, BCG Moreau can infect more efficiently and grow steadily in THP-1-derived macrophages, important characteristics in the development of an efficient immune memory by a live vaccine.^(66,67) The observed lower production of IL β -1b and MCP-1 by BCG Moreau infected macrophages point in the direction of a reduced capacity, by this strain, to signal a bactericidal response. These data should be further expanded in order to allow correlations to the vaccine's protective efficacy, but already indicate important differences between the two strains.

AUTHORS' CONTRIBUTION

PRC, MOM and LM-L - Conceptualisation; PRC, MGAS, FMFV and RMM - methodology; PRC and FMFV - formal analysis and investigation; MOM and LM-L - resources, funding acquisition and supervision; PRC - writing original draft preparation; PRC, MGAS, RMM, FMFV, MOM and LM-L - writing review and editing; PRC, MOM and LM-L - project

administration. All authors have read and agreed to the published version of the manuscript. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

1. WHO - World Health Organization. Global tuberculosis report 2021. 2021. Available from: <https://www.who.int/publications/item/9789240037021>.
2. Russell DG. *Mycobacterium tuberculosis*: here today, and here tomorrow. *Nat Rev Mol Cell Biol.* 2001; 2: 569-78. doi:10.1038/35085034.
3. Awuh JA, Flo TH. Molecular basis of mycobacterial survival in macrophages. *Cell Mol Life Sci.* 2017; 74: 1625-48. doi:10.1007/s00018-016-2422-8.
4. Vandal OH, Pierini LM, Schnappinger D, Nathan CF, Ehrt S. A membrane protein preserves intrabacterial pH in intraphagosomal *Mycobacterium tuberculosis*. *Nat Med.* 2008; 14: 849-54. doi:10.1038/nm.1795.

5. Baker JJ, Johnson BK, Abramovitch RB. Slow growth of *Mycobacterium tuberculosis* at acidic pH is regulated by *phoPR* and host-associated carbon sources. *Mol Microbiol*. 2014; 94: 56-69. doi:10.1111/mmi.12688.
6. Melo RCN, Dvorak AM. Lipid body-phagosome interaction in macrophages during infectious diseases: host defense or pathogen survival strategy? *PLoS Pathog*. 2012; 8: e1002729. doi:10.1371/journal.ppat.1002729.
7. D'Avila H, Melo RCN, Parreira GG, Werneck-Barroso E, Castro-Faria-Neto HC, Bozza PT. *Mycobacterium bovis* Bacillus Calmette-Guérin induces TLR2-mediated formation of lipid bodies: intracellular domains for eicosanoid synthesis *in vivo*. *J Immunol*. 2006; 176: 3087-97. doi:10.4049/jimmunol.176.5.3087.
8. Liu J, Tran V, Leung AS, Alexander DC, Zhu B. BCG vaccines: their mechanisms of attenuation and impact on safety and protective efficacy. *Hum Vaccin*. 2009; 5: 70-8. doi:10.4161/hv.5.2.7210.
9. Colditz GA, Berkey CS, Mosteller F, Brewer TF, Wilson ME, Burdick E, et al. The efficacy of Bacillus Calmette-Guérin vaccination of newborns and infants in the prevention of tuberculosis: meta-analyses of the published literature. *Pediatrics*. 1995; 96: 29-35. doi:10.1542/peds.96.1.29.
10. Trunz BB, Fine P, Dye C. Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. *Lancet*. 2006; 367: 1173-80. doi:10.1016/S0140-6736(06)68507-3.
11. Dye C. Global epidemiology of tuberculosis. *Lancet*. 2006; 367: 938-40. doi:10.1016/S0140-6736(06)68384-0.
12. Mangtani P, Abubakar I, Ariti C, Beynon R, Pimpin L, Fine PEM, et al. Protection by BCG vaccine against tuberculosis: a systematic review of randomized controlled trials. *Clin Infect Dis*. 2014; 58: 470-80. doi:10.1093/cid/cit790.
13. Lahey T, von Reyn CF. *Mycobacterium bovis* BCG and new vaccines for the prevention of tuberculosis. *Microbiol Spectr*. 2016; 4. doi:10.1128/microbiolspec.TNMI7-0003-2016.
14. Colditz GA. Efficacy of BCG vaccine in the prevention of tuberculosis. *JAMA*. 1994; 271: 698. doi:10.1001/jama.1994.03510330076038.
15. Brewer TF. Preventing tuberculosis with Bacillus Calmette-Guérin vaccine: a meta-analysis of the literature. *Clin Infect Dis*. 2000; 31: S64-7. doi:10.1086/314072.
16. Behr MA. BCG — different strains, different vaccines? *Lancet Infect Dis*. 2002; 2: 86-92. doi:10.1016/S1473-3099(02)00182-2.
17. Young SL, Slobbe L, Wilson R, Buddle BM, de Lisle GW, Buchan GS. Environmental strains of *Mycobacterium avium* interfere with immune responses associated with *Mycobacterium bovis* BCG vaccination. *Infect Immun*. 2007; 75: 2833-40. doi:10.1128/IAI.01826-06.
18. Abdallah AM, Hill-Cawthorne GA, Otto TD, Coll F, Guerra-Assunção JA, Gao G, et al. Genomic expression catalogue of a global collection of BCG vaccine strains show evidence for highly diverged metabolic and cell-wall adaptations. *Sci Rep*. 2015; 5: 15443. doi:10.1038/srep15443.
19. Hacker MA, Sales AM, Duppre NC, Sarno EN, Moraes MO. Leprosy incidence and risk estimates in a 33-year contact cohort of leprosy patients. *Sci Rep*. 2021; 11: 1947. doi:10.1038/s41598-021-81643-4.
20. Gomes LHF, Otto TD, Vasconcellos EA, Ferrao PM, Maia RM, Moreira AS, et al. Genome sequence of *Mycobacterium bovis* BCG Moreau, the Brazilian vaccine strain against tuberculosis. *J Bacteriol*. 2011; 193: 5600-01. doi:10.1128/JB.05827-11.
21. Berrêdo-Pinho M, Kalume DE, Correa PR, Gomes LH, Pereira MP, da Silva RF, et al. Proteomic profile of culture filtrate from the Brazilian vaccine strain *Mycobacterium bovis* BCG Moreau compared to *M. bovis* BCG Pasteur. *BMC Microbiol*. 2011; 11: 80. doi:10.1186/1471-2180-11-80.
22. Galvão TC, Lima CR, Gomes LHF, Pagani TD, Ferreira MA, Gonçalves AS, et al. The BCG Moreau RD16 deletion inactivates a repressor reshaping transcription of an adjacent gene. *Tuberculosis*. 2014; 94: 26-33. doi:10.1016/j.tube.2013.11.004.
23. Pagani TD, Guimarães ACR, Waghbi MC, Corrêa PR, Kalume DE, Berrêdo-Pinho M, et al. Exploring the potential role of moonlighting function of the surface-associated proteins from *Mycobacterium bovis* BCG Moreau and Pasteur by comparative proteomic. *Front Immunol*. 2019; 10. doi:10.3389/fimmu.2019.00716.
24. Schwarz MGA, Corrêa PR, Malaga W, Guilhot C, Mendonça-Lima L. *Mycobacterium bovis* BCG Moreau is naturally deficient in homologous recombination. *Tuberculosis*. 2020; 123: 101956. doi:10.1016/j.tube.2020.101956.
25. Brosch R, Gordon SV, Garnier T, Eiglmeier K, Frigui W, Valenti P, et al. Genome plasticity of BCG and impact on vaccine efficacy. *PNAS*. 2007; 104: 5596-601. doi:10.1073/pnas.0700869104.
26. Ponte C, Hacker M, Moraes M, Castello-Branco L, Silva F, Antas P. The patterns of *in vitro* cell-death and inflammatory cytokines induced by distinct BCG vaccine strains are differentially induced in human mononuclear cells. *Hum Vaccin Immunother*. 2018; 14: 28-35. doi:10.1080/21645515.2017.1382788.
27. Monteiro-Maia R, Correa PR, Sousa-Vasconcelos PS, de Pinho RT, Mendonça-Lima L. Gain of function in *Mycobacterium bovis* BCG Moreau due to loss of a transcriptional repressor. *Mem Inst Oswaldo Cruz*. 2018; 113(11): e180267. doi:10.1590/0074-02760180267.
28. Oliveira MM, Charlab R, Pessolani MCV. *Mycobacterium bovis* BCG but not *Mycobacterium leprae* induces TNF- α Secretion in Human Monocytic THP-1 Cells. *Mem Inst Oswaldo Cruz*. 2001; 96(7): 973-8. doi:10.1590/S0074-02762001000700015.
29. Santucci P, Greenwood DJ, Fearn A, Chen K, Jiang H, Gutierrez MG. Intracellular localisation of *Mycobacterium tuberculosis* affects efficacy of the antibiotic pyrazinamide. *Nat Commun*. 2021; 12: 3816. doi:10.1038/s41467-021-24127-3.
30. Nkwouano V, Witkowski S, Rehberg N, Kalscheuer R, Nausch N, Mayatepek E, et al. A novel mycobacterial *in vitro* infection assay identifies differences of induced macrophage apoptosis between CD4⁺ and CD8⁺ T cells. *PLoS One*. 2017; 12: e0171817. doi:10.1371/journal.pone.0171817.
31. Alves L, Lima LM, da Silva ME, et al. *Mycobacterium leprae* infection of human Schwann cells depends on selective host kinases and pathogen-modulated endocytic pathways. *FEMS Microbiol Lett*. 2004; 238(2): 429-37. doi:10.1016/j.femsle.2004.08.007.
32. Melo RCN, D'Ávila H, Bozza PT, Weller PF. Imaging lipid bodies within leukocytes with different light microscopy techniques. *Methods Mol Biol*. 2011; 689: 149-61. doi:10.1007/978-1-60761-950-5_9.
33. Dias SSG, Soares VC, Ferreira AC, Sacramento CQ, Fintelman-Rodrigues N, Temerozo JR, et al. Lipid droplets fuel SARS-CoV-2 replication and production of inflammatory mediators. *PLoS Pathog*. 2020; 16: e1009127. doi:10.1371/journal.ppat.1009127.
34. Barker LP, George KM, Falkow S, Small PL. Differential trafficking of live and dead *Mycobacterium marinum* organisms in macrophages. *Infect Immun*. 1997; 65: 1497-504. doi:10.1128/iai.65.4.1497-1504.1997.
35. Chen JM, Islam ST, Ren H, Liu J. Differential productions of lipid virulence factors among BCG vaccine strains and implications on BCG safety. *Vaccine*. 2007; 25: 8114-22. doi:10.1016/j.vaccine.2007.09.041.
36. Tran V, Ahn SK, Ng M, Li M, Liu J. Loss of lipid virulence factors reduces the efficacy of the BCG vaccine. *Sci Rep*. 2016; 6: 29076. doi:10.1038/srep29076.
37. Hayashi D, Takii T, Fujiwara N, Fujita Y, Yano I, Yamamoto S, et al. Comparable studies of immunostimulating activities *in vitro* among *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) substrains. *FEMS Immunol Med Microbiol*. 2009; 56: 116-28. doi:10.1111/j.1574-695X.2009.00559.x.

38. Rens C, Chao JD, Sexton DL, Tocheva EI, Av-Gay Y. Roles for phthiocerol dimycocerosate lipids in *Mycobacterium tuberculosis* pathogenesis. *Microbiology (Reading)*. 2021; 167(3). doi:10.1099/mic.0.001042.
39. Astarie-Dequeker C, Le Guyader L, Malaga W, Seaphanh F-K, Chalut C, Lopez A, et al. Phthiocerol dimycocerosates of *M. tuberculosis* participate in macrophage invasion by inducing changes in the organization of plasma membrane lipids. *PLoS Pathog*. 2009; 5: e1000289. doi:10.1371/journal.ppat.1000289.
40. Quigley J, Hughitt VK, Velikovsky CA, Mariuzza RA, El-Sayed NM, Briken V. The cell wall lipid PDIM contributes to phagosomal escape and host cell exit of *Mycobacterium tuberculosis*. *mBio*. 2017; 8. doi:10.1128/mBio.00148-17.
41. Singh A, Crossman DK, Mai D, Guidry L, Voskuil MI, Renfrow MB, et al. *Mycobacterium tuberculosis* whiB3 maintains redox homeostasis by regulating virulence lipid anabolism to modulate macrophage response. *PLoS Pathog*. 2009; 5: e1000545. doi:10.1371/journal.ppat.1000545.
42. Giovannini D, Cappelli G, Jiang L, Castilletti C, Colone A, Serafino A, et al. A new *Mycobacterium tuberculosis* smooth colony reduces growth inside human macrophages and represses PDIM operon gene expression. Does an heterogeneous population exist in intracellular mycobacteria? *Microb Pathog*. 2012; 53: 135-46. doi:10.1016/j.micpath.2012.06.002.
43. Keane J, Remold HG, Kornfeld H. Virulent *Mycobacterium tuberculosis* strains evade apoptosis of infected alveolar macrophages. *J Immunol*. 2000; 164: 2016-20. doi:10.4049/jimmunol.164.4.2016.
44. Jordao L, Bleck CKE, Mayorga L, Griffiths G, Anes E. On the killing of mycobacteria by macrophages. *Cell Microbiol*. 2008; 10(2): 529-48. doi:10.1111/j.1462-5822.2007.01067.x.
45. Kernodle DS. Decrease in the effectiveness of Bacille Calmette-Guérin vaccine against pulmonary tuberculosis: a consequence of increased immune suppression by microbial antioxidants, not over-attenuation. *Clin Infect Dis*. 2010; 51: 177-84. doi:10.1086/653533.
46. Torrelles JB, Ellis D, Osborne T, Hofer A, Orme IM, Chatterjee D, et al. Characterization of virulence, colony morphology and the glycopeptidolipid of *Mycobacterium avium* strain 104. *Tuberculosis*. 2002; 82: 293-300. doi:10.1054/tube.2002.0373.
47. Supply P, Marceau M, Mangenot S, Roche D, Rouanet C, Khanna V, et al. Genomic analysis of smooth tubercle bacilli provides insights into ancestry and pathoadaptation of *Mycobacterium tuberculosis*. *Nat Genet*. 2013; 45: 172-9. doi:10.1038/ng.2517.
48. Pawlik A, Garnier G, Orgeur M, Tong P, Lohan A, Le Chevalier F, et al. Identification and characterization of the genetic changes responsible for the characteristic smooth-to-rough morphology alterations of clinically persistent *Mycobacterium abscessus*. *Mol Microbiol*. 2013; 90: 612-29. doi:10.1111/mmi.12387.
49. Hunter RL, Venkataprasad N, Olsen MR. The role of trehalose dimycolate (cord factor) on morphology of virulent *M. tuberculosis in vitro*. *Tuberculosis*. 2006; 86: 349-56. doi:10.1016/j.tube.2005.08.017.
50. Jacob EB, Becker I, Shapira Y, Levine H. Bacterial linguistic communication and social intelligence. *Trends Microbiol*. 2004; 12(8): 366-72. doi:10.1016/j.tim.2004.06.006.
51. Méndez-Samperio P, Pérez A, Alba L. Reactive oxygen species-activated p38/ERK 1/2 MAPK signaling pathway in the *Mycobacterium bovis* Bacillus Calmette Guérin (BCG)-induced CCL2 secretion in human monocytic cell line THP-1. *Arch Med Res*. 2010; 41: 579-85. doi:10.1016/j.arcmed.2010.10.009.
52. Jayaraman P, Sada-Ovalle I, Nishimura T, Anderson AC, Kuchroo VK, Remold HG, et al. IL-1 β promotes antimicrobial immunity in macrophages by regulating TNFR signaling and caspase-3 activation. *J Immunol*. 2013; 190: 4196-4204. doi:10.4049/jimmunol.1202688.
53. Keane J, Balcewicz-Sablinska MK, Remold HG, Chupp GL, Meek BB, Fenton MJ, et al. Infection by *Mycobacterium tuberculosis* promotes human alveolar macrophage apoptosis. *Infect Immun*. 1997; 65: 298-304. doi:10.1128/iai.65.1.298-304.1997.
54. Luo Y, Chen X, O'Donnell MA. *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) induces human CC- and CXC-chemokines *in vitro* and *in vivo*. *Clin Exp Immunol*. 2007; 147: 370-8. doi:10.1111/j.1365-2249.2006.03288.x.
55. Butler RE, Cihlarova V, Stewart GR. Effective generation of reactive oxygen species in the mycobacterial phagosome requires K⁺ efflux from the bacterium. *Cell Microbiol*. 2010; 12: 1186-93. doi:10.1111/j.1462-5822.2010.01463.x.
56. Nicolaou G, Goodall AH, Erridge C. Diverse bacteria promote macrophage foam cell formation via toll-like receptor-dependent lipid body biosynthesis. *J Atheroscler Thromb*. 2012; 19: 137-48. doi:10.5551/jat.10249.
57. Russell DG. Phagosomes, fatty acids and tuberculosis. *Nat Cell Biol*. 2003; 5: 776-8. doi:10.1038/ncb0903-776.
58. Daniel J, Deb C, Dubey VS, Sirakova TD, Abomoelak B, Morbidoni HR, et al. Induction of a novel class of diacylglycerol acyltransferases and triacylglycerol accumulation in *Mycobacterium tuberculosis* as it goes into a dormancy-like state in culture. *J Bacteriol*. 2004; 186: 5017-30. doi:10.1128/JB.186.15.5017-5030.2004.
59. Deb C, Lee C-M, Dubey VS, Daniel J, Abomoelak B, Sirakova TD, et al. A Novel *In vitro* multiple-stress dormancy model for *Mycobacterium tuberculosis* generates a lipid-loaded, drug-tolerant, dormant pathogen. *PLoS One*. 2009; 4: e6077. doi:10.1371/journal.pone.0006077.
60. Garton NJ, Christensen H, Minnikin DE, Adegbola RA, Barer MR. Intracellular lipophilic inclusions of mycobacteria *in vitro* and in sputum. *Microbiology (Reading)*. 2002; 148(Pt 10): 2951-8. doi:10.1099/00221287-148-10-2951.
61. Garton NJ, Waddell SJ, Sherratt AL, Lee S-M, Smith RJ, Senner C, et al. Cytological and transcript analyses reveal fat and lazy persistor-like bacilli in tuberculous sputum. *PLoS Med*. 2008; 5: e75. doi:10.1371/journal.pmed.0050075.
62. Deb C, Daniel J, Sirakova TD, Abomoelak B, Dubey VS, Kolattukudy PE. A Novel lipase belonging to the hormone-sensitive lipase family induced under starvation to utilize stored triacylglycerol in *Mycobacterium tuberculosis*. *J Biol Chem*. 2006; 281: 3866-75. doi:10.1074/jbc.M505556200.
63. Low KL, Rao PSS, Shui G, Bendt AK, Pethe K, Dick T, et al. Triacylglycerol utilization is required for regrowth of *in vitro* hypoxic nonreplicating *Mycobacterium bovis* Bacillus Calmette-Guérin. *J Bacteriol*. 2009; 191: 5037-43. doi:10.1128/JB.00530-09.
64. Low KL, Shui G, Natter K, Yeo WK, Kohlwein SD, Dick T, et al. Lipid droplet-associated proteins are involved in the biosynthesis and hydrolysis of triacylglycerol in *Mycobacterium bovis* Bacillus Calmette-Guérin. *J Biol Chem*. 2010; 285: 21662-70. doi:10.1074/jbc.M110.135731.
65. Singh V, Jamwal S, Jain R, Verma P, Gokhale R, Rao KVS. *Mycobacterium tuberculosis*-driven targeted recalibration of macrophage lipid homeostasis promotes the foamy phenotype. *Cell Host Microbe*. 2012; 12: 669-81. doi:10.1016/j.chom.2012.09.012.
66. Fine PEM. The BCG Story: Lessons from the Past and Implications for the Future. *Clin Infect Dis*. 1989; 11: S353-9. doi:10.1093/clinids/11.Supplement_2.S353.
67. Kaveh DA, Garcia-Pelayo MC, Hogarth PJ. Persistent BCG bacilli perpetuate CD4 T effector memory and optimal protection against tuberculosis. *Vaccine*. 2014; 32: 6911-18. doi:10.1016/j.vaccine.2014.10.041.