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Formation of *Mycobacterium abscessus* colonies in cellular culture in an *in vitro* infection model

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ABSTRACT

Mycobacterium abscessus is one of the most important nontuberculous mycobacteria that cause lung diseases. *In vitro* infection models developed to analyze the immune response are frequently based on the addition of mycobacteria to mononuclear cells or neutrophils from peripheral blood. An important requirement of these assays is that most cells phagocytose mycobacteria, only accomplished by using large multiplicities of infection (1 or more bacteria per cell) which may not adequately reflect the inhalation of a few mycobacteria by the host. We propose modifications that try to mimic some of the conditions in which immune cells deal with mycobacteria. For the preparation of the inoculum mycobacteria are grown in solid media followed by preparation to a single cell suspension. Multiplicities of infection (number of bacteria per cell) are below 0.01. Serum-free cellular media is used to allow the growth of *M. abscessus*. After several days of incubation Bacterial Colonies in Cellular Culture (BCCC) develop, which are enumerated directly under an inverted microscope. These colonies may represent biofilm formation during chronic infections.

- Low multiplicity of infection (below 0.01 bacteria per cell) reflects more realistically conditions encountered by immune cells in the lungs.
- The surface of mycobacteria prepared for infection assays that are grown in solid media are less affected than that of mycobacteria grown in liquid media with detergents.
- Colony formation in the infected cells may reflect the aggregation and biofilm formation in the lungs during chronic infection.

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Method details

Introduction

Mycobacterium abscessus is a pathogen of increasing clinical and epidemiological importance, included in the group of rapidly growing mycobacteria (RGM), very difficult to treat [1]. Mycobacteria are hard to kill because their thick wall protects them from antibiotics and the host's immune system [2]. *M. abscessus* has traditionally been considered as an opportunistic pathogen but may be evolving into a true pathogen involved in chronic infections that may last for several years [3]. It is particularly problematic for individuals with susceptibility to pulmonary infections, including patients with bronchiectasis, chronic obstructive pulmonary disease or cystic fibrosis [3]. The immune response to the infection is complex and a challenge to reproduce *in vitro*. Frequently used cellular models are monocytes, peripheral blood mononuclear cells (PBMC) and polymorphonuclear neutrophils (PMN) [4], although cells such as dendritic cells, epithelial cells, endothelial cells and others, have also been used. In these assays the main outcome analyzed is usually the inhibition of mycobacterial growth, although killing activity has been reported [5]. Current protocols generally add a large number of bacteria, with a multiplicity of infection of 1 or more bacteria per cell [6], although a lower number, such as 0.1, are sometimes used [7]. Cells and bacteria are then incubated for less than eight hours and non-phagocytosed bacteria are washed away with warm cellular medium [8]. To avoid extracellular multiplication of bacteria, antibiotics are frequently added to the medium. Initial protocols added streptomycin, until it was found that it also affected intracellular mycobacteria [9]. Other antibiotics, such as amikacin, are now used for this purpose [10]. Infected cells are maintained in cellular media supplemented with fetal bovine or autologous serum [11], although occasionally serum-free media has been used [12]. After a predefined length of time (1 h to 2–3 days) cells are lysed with detergents to release intracellular bacteria. Several detergents have been used, including sodium dodecyl sulfate, which requires neutralization with bovine serum albumin [13] or Triton X-100 [10,14]. To enumerate colony forming units (CFU), lysates are incubated at 37°C on solid media, such as 7H10 or 7H11, and RGM form colonies in less than a week. The term CFU is used because not always all viable bacteria form colonies.

We present several modifications that try to represent with higher fidelity the events that take place after the inhalation of mycobacteria. *M. abscessus* is the most common etiological agent of pulmonary disease caused by RGM and has two presentations, the fibrocavitary form and the nodular bronchiectatic form [15]. It is generally considered that an infection becomes chronic when symptoms persist for more than 4 - 6 weeks. The formation of biofilm is an inherent feature of chronic infection, and mycobacterial infections have been shown to be capable of biofilm formation [16]. Our model presents the following features that are different from current methodology:

1. Use of serum-free media, to standardize the protocol conditions, avoiding the interference of biological molecules variably present in the serum, such as eicosanoids [17]. This media has the additional advantage that allows the growth of mycobacteria, because we have observed that human serum may inhibit bacterial colony formation. Because bacterial colonies are formed in serum-free media, any growth inhibition in the presence of cells may be attributed to a cellular activity.
2. Use of an ultra-low MOI, 0.01 bacteria per cell or less. The number of non-phagocytosed bacteria is so low that no cytotoxic effect is expected and, consequently, no washes are required, avoiding the removal of loosely attached cells.
3. The outcome of the experiments will be expressed in two ways. First, the enumeration of mycobacterial colonies directly formed in the cell culture and visualized under an inverted microscope. These colonies will be referred to as Bacterial Colonies in Cellular Culture (BCCC) and may represent the development of biofilm. And second, the number of released bacteria after cellular lysis by ultrasonication, in the form of CFU. Mechanical means of lysis do not require the addition of detergents and help to disrupt mycobacterial aggregates.

The study of BCCC provides information different from mycobacterial survival expressed as CFU. The usual detection of mycobacteria in sputum as isolated bacilli may indicate that they do not always develop biofilms. Colonies may be considered as biofilm in the form of aggregates [18], which are increasingly viewed as important in chronic infections [19]. We will detail the protocol in four steps. First, production of individualized mycobacteria for infection; second, purification of blood immune cells; third, the *in vitro* infection assay and fourth, analysis of the assay outcomes.

Bacterial individualization

Growth of *M. abscessus* in the usual 7H9 liquid media supplemented with 10% ADC enrichment (albumin, dextrose and catalase) and 0.05% Tween-80 is avoided because the detergent will affect the normal lipid composition of the mycobacterial cell wall [20]. Solid media 7H11 supplemented with 10% OADC (oleic acid, albumin, dextrose and catalase, Becton Dickinson) is preferred.

Protocol

1. Scrape bacteria from 7H11 solid media to prepare a suspension in PBS, seed a new plate and recover bacteria grown as a lawn for 4 – 7 days, depending on the strain, with a microbiological loop. Suspend it in an Eppendorf with 1 ml of CTS AIM-V serum-free media (Gibco). Smooth strains will readily disperse in the medium, but aggregates from rough strains are more difficult to break.
2. Sonicate the suspension 5 – 10 s with a microtip sonifier (Branson Ultrasonics), at an amplitude of 10 % (2 W). Alternatively, an ultrasonic bath may be used, but treatments of 30 s or longer will be needed and lower disruption of clumps will be achieved.
3. Centrifuge at 100×g for 90 s at room temperature. Individualized bacteria or small clumps will remain in the supernatant and large clumps will pellet at the bottom of the tube. Recover supernatant, taking care not to touch the pellet.
4. Aseptically filter the supernatant through a syringe filter with a pore size of 5 μm [21]. Aggregated bacteria will be retained in the filter and individualized bacteria will pass through.
5. Confirm the quality of single bacterial suspension and viability with the fluorescence LIVE/DEAD Bac Light Bacterial Viability kit (Thermo Fisher). Viable bacteria are green stained and dead bacteria are red stained. If more than 5 % of bacteria are red, the stock suspension is discarded.
6. Add glycerol to 20 %, aliquot in Eppendorfs tubes (usually 20 μl) and freeze at −80 ˚C. In our experience two or three cycles of freeze-thawing will not affect mycobacterial viability.
7. Quantify stock by tenfold serial dilutions in 7H9 liquid medium and inoculate dilutions in 96 well plates. Concentration of bacteria are usually from 10^7 to 10^8/ml, which requires high levels of dilution before cellular infection. *M. abscessus* colonies are visible under an inverted microscope after 36 – 48 h [22]. Do not allow overgrowth because colonies will mix and become difficult to count. Colony counting may be performed days later if the plate is stored in a fridge, because growth will stop at 4 ˚C.

The use of tissue culture serum-free media appropriate for primary leukocytes, such as CTS AIM-V, is one of the main features of the protocol. We have observed that most mycobacterial species of clinical importance will grow in these media, although to a lower degree than in 7H9 medium. This characteristic is very convenient to compare the growth of mycobacteria in the absence or presence of leukocytes, as will be commented below in the "*In vitro* infection" section. To ensure that this medium is appropriate for leukocyte culture me measured the viability of PBMC and PMN with the MTT assay (3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide, Thermo Scientific) [23] at different time points in both CTS-AIM-V medium and RPMI/10 % autologous serum, frequently used for the maintenance of cells of hematopoietic origin. Autologous serum is obtained from clotted blood. We also included the medium RPMI/2 % autologous serum to determine the importance of serum. The basal level of MTT reduction in PBMC the day of isolation (day 0) was significantly higher in RPMI/10 % than in the other media (Fig 1.A), but it was not significant in PMN (Fig 1.B). The viability remained high for PBMC for two days and it decreased after three days. The viability of PMN sharply decreased in one day. No statistical differences were found for the three media in PBMC at any day, and only at days 3 and 4 in PMN, when medium CTS AIM-V and RPMI/10 % serum, but not RPMI/2 % serum, were compared. From these results we conclude that viability of cells was not different for the three media in PBMC and only in days 3 and 4 in PMN, when the viability of PMN is already very low. It seems that the metabolic activity of PBMC is higher in RPMI/10 % serum than in RPMI/2 % serum or CTS AIM-V, although differences were not statistically significant after day 0. Therefore, all of the three media are suitable for PBMC and PMN maintenance, although PMN viability rapidly decreases after isolation.

Cellular purification

Cellular fractions are purified by density gradient sedimentation. PBMC may be isolated using solutions with density = 1077 g/ml, such as Ficoll-Paque Plus (GE Healthcare), following manufacturer's instructions. PMN may be isolated directly using Polymorphprep (Serumwerk), with a 1113 g/ml density, but a double gradient is preferred because the separation between the mononuclear cells band and the polymorphonuclear cells band is wider, minimizing cross-contamination.

PMN isolation protocol

1. Carefully and sequentially lay one volume of Polymorphprep, enough Ficoll-Paque Plus to form a 1 cm layer and approximately two volumes of undiluted blood. For example, 3.5 ml of Polymorphprep, 1 ml of Ficoll-Paque Plus and 7 – 8 ml of undiluted blood in a 16×100 mm tube.
2. Centrifuge at 460×g for 30 min without brake, at 20 – 25 ˚C.

The PBMC layer will form above the 1077 g/ml solution, the PMN layer in the 1113 g/ml solution, and erythrocytes will pellet to the bottom of the tube. The layer of PMN is frequently wide and close to the erythrocytes fraction (Fig 2.A). A significant contamination of PMN with erythrocytes will be usually visible.

4. Transfer the PBMC and PMN layers to different tubes and wash twice with 10 vol of PBS by centrifugation of cells at 500×g 5 min, at room temperature.

Leukocytes and erythrocytes are readily distinguished using a phase contrast microscope and are quantified in a Neubauer chamber. PMN enrichment is high (>50 %) with this protocol. Hypotonic lysis of remaining erythrocytes is usually performed, but this is a procedure that may affect some PMN functions [24] and we have found that erythrocytes do not usually interfere with *in vitro* infection assays (see below *Experimental example*). Alternatively, a second Polymorphprep sedimentation gradient of PMN diluted in

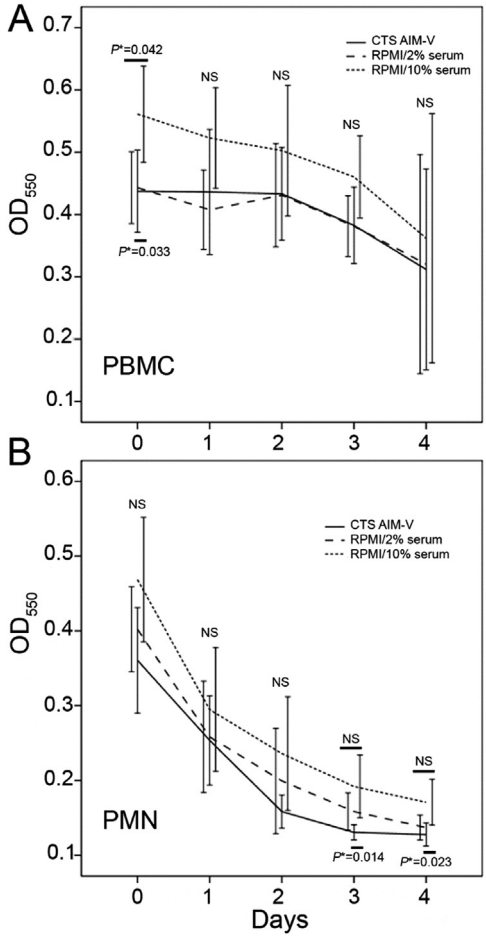

Fig. 1. Viability of PBMC and PMN in different media. Cells were purified as described in the "Cellular purification" section. Fifteen wells were seeded with 2×10^5 cells of each cellular type (PBMC or PMN) and incubated for 4 days at 37ºC and 5 % CO_2 in 3 different media (5 wells each): CTS AIM-V (Gibco), RPMI/10 % autologous serum and RPMI/2 % autologous serum. The first day, and each of the following four days, 10 µl of MTT (5 mg/ml) dissolved in phosphate buffered saline (PBS) was added to one well of each cellular type maintained in each of the three different media, and incubated at 37ºC for four hours to allow the reduction of MTT and formation of formazan, a violet-blue water-insoluble molecule [23]. To solubilize formazan, 100 µl of a solubilization solution (40 % dimethylformamide/16 % sodium dodecyl sulfate in 2 % glacial acetic acid) was added and mixed. Absorbance was recorded at 550 nm. Data represent the mean and standard deviation of absorbance (n = 5). Comparison of groups was performed by ANOVA and the Tukey's test for pairwise comparisons. P* <0.05 was considered significant. NS: Not significant.

Table 1
Erythrocyte contamination in cellular fractions purified from whole blood using Ficoll-Paque and Polymorphprep in a single step.

Cellular fraction	Erythrocyte (%)
Peripheral Blood Mononuclear Cells	1.14 SD 0.84
Polymorphonuclear neutrophils (1st purification)	38.76 SD 20.30
Polymorphonuclear neutrophils (2nd purification)	3.28 SD 2.91**

** P = 0.007 (n = 6). 1st and 2nd purification mean comparison by Student's t-testSD (Standard Deviation).

autologous serum, this time without Ficoll-Paque Plus, will decrease erythrocyte contamination usually below 5 % (Table 1). The second gradient is performed by suspending PMN in 800 µl of autologous serum, layering the cells on 800 µl of Polymorphprep in a 11×70 mm tube, or similar, and centrifuge it at 460×g 30 min at 20 – 25ºC (Fig. 2.B). This procedure will remove more than 90 % of the erythrocyte contamination, but will increase the manipulation of cells.

Monocytes are an alternative cellular model, and may be isolated from PBMC using anti-CD14 antibodies coupled to magnetic nanoparticles. Purified monocytes may be allowed to differentiate to monocyte derived macrophages, or differentiated with cytokines such as M-CSF (macrophage colony stimulating factor).

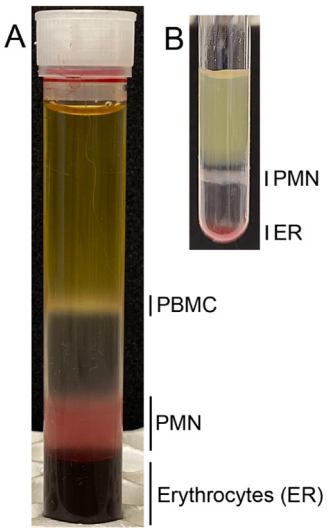

Fig. 2. Purification of PBMC and PMN. (A) Undiluted whole blood is applied to a layer formed with Polymorphprep/Ficoll-Paque Plus and centrifuged. PBMC will form a band between plasma and gradient solutions. PMN will usually form a wide band in contact with the erythrocyte fraction. Contaminating erythrocytes in the PMN band may be removed by hypotonic lysis or by an additional Polymorphprep gradient (B). In the latter, a band of PMN will form in the Polymorphprep solution, and the erythrocytes will pellet at the bottom of the tube.

In vitro *infection*

We have observed that *M. abscessus* multiply and form colonies in serum-free media for cellular culture [25]. Mycobacterial growth is not affected by 1 % autologous serum. In media with 10 % fetal bovine serum or 2 - 4 % autologous serum colonies may form, but they are smaller. No colonies will develop with ≥ 5 % autologous serum in the first four days, although mycobacteria remain viable. Infection in serum-free media allows a direct comparison of the mycobacterial growth in either the presence or the absence of cells. The lower recovery of bacteria after cell lysis as compared with growth in serum-free media is an indication of the cellular inhibitory activity. *M. abscessus* colonies may be detected in serum-free media in two-three days.

Anti-mycobacterial activity may be observed at very low multiplicity of infection (MOI, number of bacteria per cell). Infection of 1 to 3×10^5 phagocytes (neutrophils or monocytes) in 96-well plates with 10^1 to 10^3 mycobacteria (MOI = 0.00003 to 0.01 bacteria per cell), will likely increase the probability of bacteria to be phagocytosed. Larger MOI (1 or more) are usually applied for less than eight hours and the culture is washed to get rid of non-phagocytosed mycobacteria. Washes will select cells by removing loosely attached neutrophils or monocytes. Remaining extracellular mycobacteria are killed by antibiotics such as amikacin [8]. The purpose of this approach is to increase the number of cells that have phagocytosed mycobacteria and analyze anti-mycobacterial cellular activity. The variations that we propose may more closely reflect *in vitro* the natural process, because a lower MOI is used and manipulation of cells in minimized (no use of antibiotics and no washes). Incubation of the infected cells for several days, allowing the formation of BCCC, may simulate some aspects of mycobacteria multiplication in lung immune cells, when the infection becomes chronic and biofilm is formed.

Lysis of infected cells to release intracellular mycobacteria is preferentially performed with a sonifier, using a microtip suitable for 96-well plates. An amplitude low enough to avoid foaming is recommended. Cells will lyse easily without affecting bacterial viability. Most protocols lyse cells by adding detergents such as sodium dodecyl sulfate (SDS), which will afterwards be neutralized with bovine serum albumin (BSA). The use of physical means such as ultrasounds produced by a sonifier avoids the addition of reagents to the well and disperse small mycobacterial clumps, resulting in the development of a larger number of CFU.

Protocol

1. Purify immune cells and suspend the appropriate dilution of *M. abscessus* in serum-free media suitable for maintenance of blood immune cells. Mix them and dispense 1 to 3×10^5 cells in 100 µl/well in 96-well plates. If BCCC will be analyzed, 1000 or less viable bacteria should be added, to allow the accurate enumeration of colonies under an inverted microscope.
2. Incubate the *in vitro* infection at 37 ˚C and 5 % CO_2 for the planned length of time, generally one or more days. It is important to note that PMN has a low viability after one day (Fig. 1).
3. Lyse cells with a microtip sonifier, at the highest amplitude that do not produce foam (10 % in a Branson 450 digital sonifier) for 3 – 5 s.
4. Prepare tenfold dilutions of the lysates in supplemented 7H9 medium, and inoculate 100 µl/well in 96-well plates [22].
5. Incubate plates in a cell culture incubator at 37 ˚C for 36 – 48 h.
6. Enumerate CFU under an inverted microscope.

The whole protocol (bacterial individualization, cellular purification and *in vitro* infection) is summarized in Fig. 3.

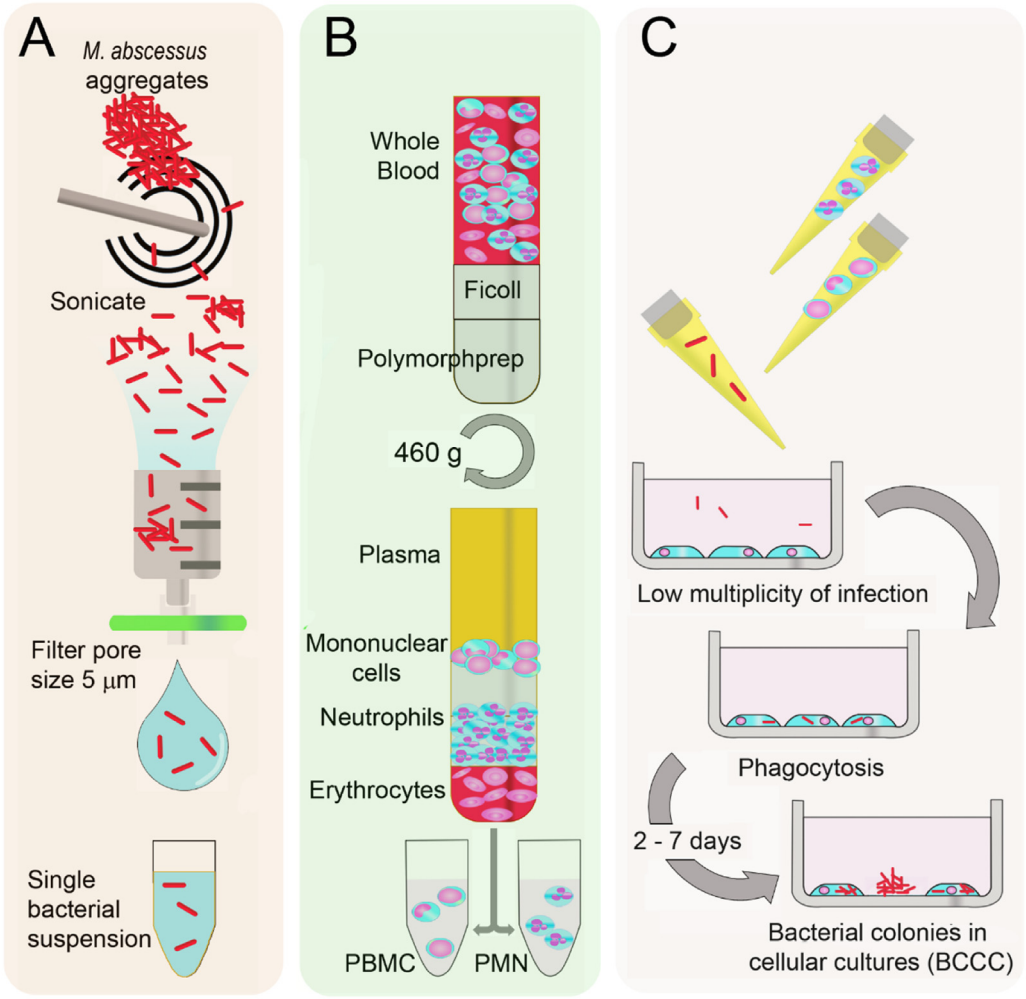

Fig. 3. *M. abscessus* infection procedure. (A) Mycobacteria individualization. *M. abscessus* is obtained as aggregates from a 7H11 culture. Large aggregates are dispersed by sonication, which generates some individualized mycobacteria. To separate these bacteria from the remaining aggregates, the suspension is filtered through a syringe filter with a pore size of 5 μm, which allows single bacteria to pass through. (B) Cellular purification. PBMC and PMN may be simultaneously obtained from a Polymorphprep/Ficoll-Paque Plus gradient. PBMC will form a band bellow plasma, separated from PMN by the gradient solutions. The PMN band is wide and usually contaminated with erythrocytes. For their removal, an additional Polymorphprep gradient may be performed. (C) Infection. Wells seeded with purified cells (PBMC and/or PMN), are infected with mycobacteria and incubated for 2 – 7 days to allow the formation of BCCC.

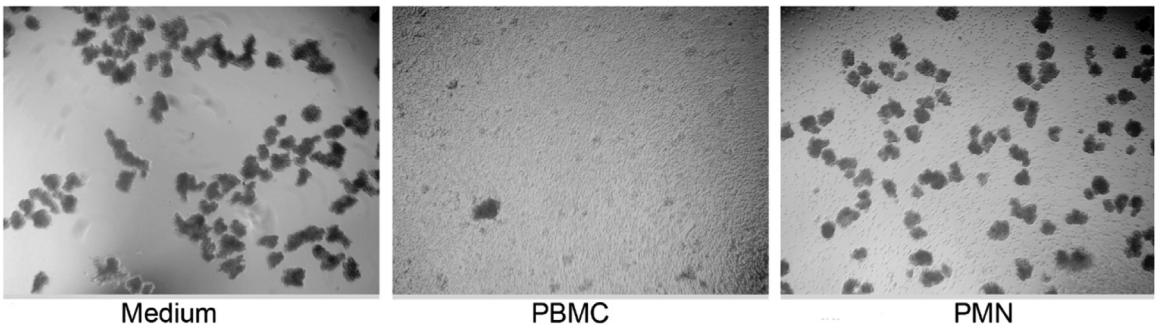

Fig. 4. Colony formation in medium or cellular culture (BCCC). Medium, PBMC or PMN (10^5 cells) were inoculated with *M. abscessus* 239aba (10^2 CFU) and incubated for three days at 37 ˚C and 5 % CO_2 in CTS AIM-V medium. Microphotographs were taken at 40× magnification.

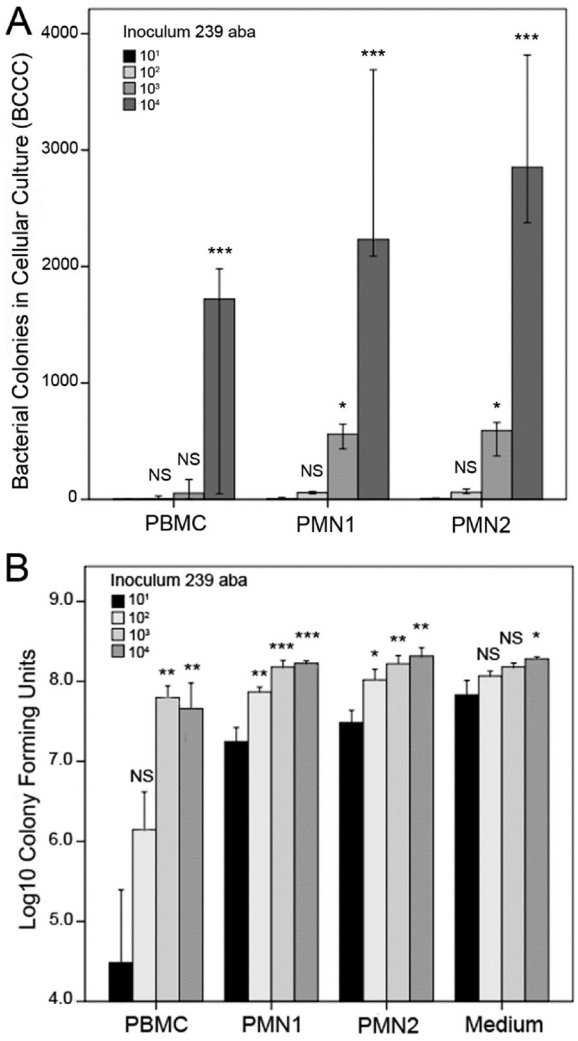

Fig. 5. Mycobacterial multiplication and colony formation in PBMC and PMN. PBMC were isolated from a Ficoll-Paque Plus gradient and PMN from two rounds of a Polymorphprep gradients. The first isolation round included a layer of Ficoll Paque-Plus gradient (PMN1), but the second was performed with only Polymorphprep (PMN2). The indicated number of individualized mycobacteria (*M. abscessus* 239aba) was added to wells with 10^5 cells and incubated for three days at 37 ˚C in CTS AIM-V medium. (A) Number of BCCC visible under the inverted microscope. Data represent the median and 95 % confidence interval ($n = 6$). The Kruskall Wallis test was performed for group comparisons, and pairwise comparison of the 10^1 group with the other groups by the Dunn's test. (B) Cells were lysed by ultrasonication and 100 µl of tenfold dilutions of lysates in 7H9 medium were inoculated in 96-well plates. After 36–48 h, CFU were enumerated. Data represent the mean and standard deviation of $\log_{10}$ CFU ($n = 6$). Comparison of groups was performed by ANOVA and the Tukey's test for pairwise comparisons between the 10^1 group and the other groups. $P < 0.05$ was considered significant. $P^{*}<0.05$; $P^{**}<0.01$ and $P^{***}<0.001$. NS: Not significant.

Outcomes

It is very difficult for immune cells to exert mycobactericidal activity against *M. tuberculosis* [4] but also against non-tuberculous mycobacteria. Cellular activity is usually displayed as inhibition of mycobacterial growth, quantified by cellular lysis and CFU enumeration. Nevertheless, an alternative measure of mycobacterial inhibition by cells is the enumeration of BCCC [25]. Mycobacterial microcolonies may be considered as biofilm in the form of aggregates [19], and could represent one of the mycobacterial presentations in the lungs in chronic infections.

Experimental example

To obtain practical examples of the described outcomes, the proposed protocol was applied to PBMC and PMN, which were infected with the *M. abscessus* smooth strain 239aba. Informed consent was obtained from healthy donors of peripheral blood that was collected in BD Vacutainer Blood Collection Tubes with heparin (Becton Dickinson). PBMC and PMN (PMN1) were isolated in a Ficoll-Paque Plus/Polymorphprep gradient. Cells were washed with PBS, and half of the neutrophils were suspended in autologous

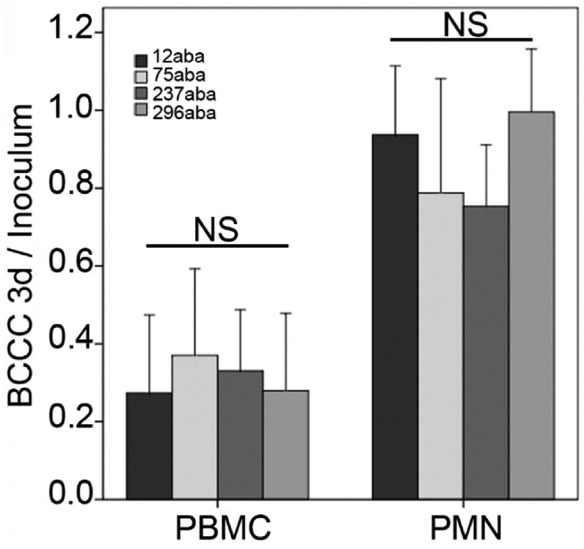

Fig. 6. Influence of *M. abscessus* colony morphology in the formation of BCCC. PBMC and PMN were isolated from a Polymorphprep/Ficoll-Paque Plus gradient. Erythrocytes from the PMN fraction were removed by a second round of Polymorphprep gradient. Viable individualized mycobacteria (100 of each *M. abscessus* strain) were added to wells with 10^5 cells and incubated for three days at 37 °C in CTS AIM-V medium. Data represents the mean and standard deviation of the number of BCCC ($n = 4$). The ANOVA test was performed for group comparisons. $P < 0.05$ was considered significant. NS: Not significant.

serum and applied to a new tube with Polymorphprep. PMN from this additional gradient (PMN2) were washed with PBS and all the fractions (PBMC, PMN1 and PMN2) were suspended in serum-free medium. An increasing number of individualized mycobacterium was inoculated in wells wit 10^5 leukocytes and incubated for three days at 37 °C and 5 % CO_2. Colonies in the infected cells, visible under an inverted microscope (Fig 4), were enumerated.

Although mycobacteria multiplied several times after infection, the number of BCCC was always lower than the number of inoculated viable bacteria, indicating that many of them did not form visible colonies. The pattern of visible colonies was very similar for PMN1 and PMN2 (Fig 5.A). Pairwise comparison of BCCC between PMN1 and PMN2 at each inoculum showed no significant differences by the Mann Whitney test. PMN1 *vs*. PMN2 number of BCCC median (*P* value) at 10^1 inoculum were 5.5 vs. 8.0 ($P = 0.310$); at 10^2 inoculum were 59.5 vs. 61.5 ($P = 0.310$); at 10^3 inoculum were 560.0 vs. 590.0 ($P = 0.589$) and at 10^4 inoculum were 2232.0 vs. 2853.0 ($P = 0.132$).

Cells were then lysed with a sonifier and 100 µl of tenfold dilutions in 7H9 medium were inoculated in 96-well plates. After 36 – 48 h, CFU were enumerated. Even in wells with no visible BCCC, mycobacteria multiplied and more than 10^4 CFU were recovered from the lysed cells (Fig 5.B). The observed mycobacterial growth inhibition degree was higher when the inoculum is low for the different cellular fractions.

To further explore the performance of this assay, the formation of BCCC from different clinical isolates of *M. abscessus* were studied. Four strains of different colony morphology, 12aba, 296aba (smooth), 75aba and 237aba (rough) were used to infect PBMC and PMN (Fig. 6). To normalize the results, the number of BCCC after 3 days was divided by the number of bacteria inoculated. Normalized BCCC were similar for the four strains in both PBMC and PMN. The results from this experiment do not support the hypothesis that the number of BCCC depends on the colony morphology of the strains.

Lysis of cells for CFU analysis was performed with a microtip sonifier as indicated above. An advantage of ultrasonication is the disruption of the mycobacterial aggregates. For comparison purposes, PBMC were infected with MOI=0.1 and were lysed after 3 days by either 4 % Triton X-100 in CTS AIM-V for 5 min or ultrasonication. Viability of mycobacteria after lysis was assessed with the Bac Light kit and it was observed that bacilli were not affected (Fig 7). Nevertheless, some bacterial clumps remained after Triton X-100 lysis. In the subsequent enumeration of CFU, each clump would generate a single CFU in mycobacterial media. Triton X-100 does not lyse the cellular nuclear membrane, and nuclei appear red stained.

Shortcommings and advantages of the protocol

The characterization of chronic infections by this protocol is only partial. It focuses on the interaction of cells and mycobacteria that may translate into the regulation of biofilm development in chronic infections. But the duration of the clinical infection, measured in months or years, cannot be reproduced with these kind of assays. Another common shortcoming of these assays is that only a few of the multiple components present in lung microenvironments are included, such as other cell types, mucus or cytokines and other molecules. Nevertheless, any of the aforementioned host biological components may be added to the *in vitro* infection to study their influence. On the other hand, the low MOI limits the analysis of cellular responses because most cells do not phagocytose mycobacteria.

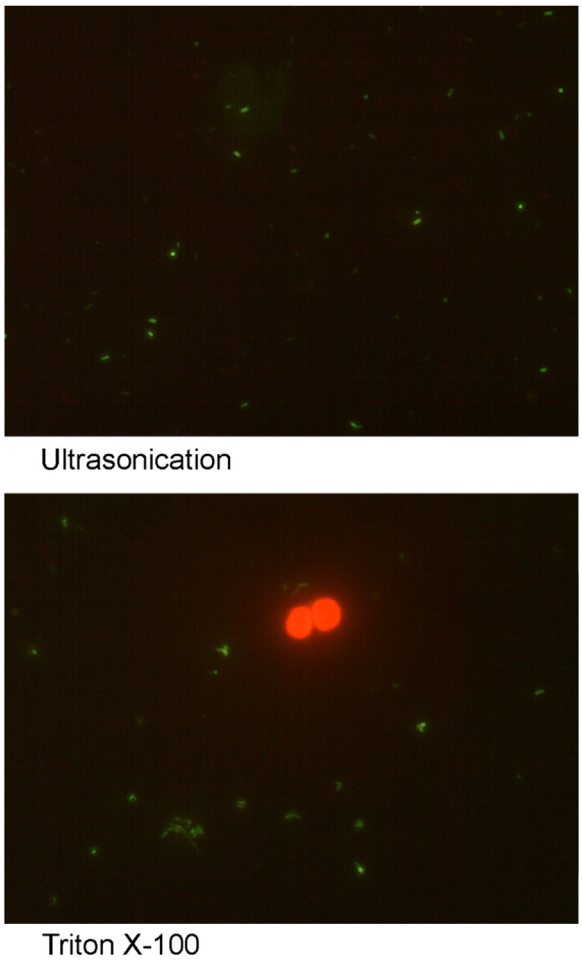

Fig. 7. Viability and aggregation after cellular lysis. PBMC (10^5) were infected with *M. abscessus* 239aba (10^4) and incubated for three days in CTS AIM-V medium. Cellular lysis to release intracellular mycobacteria was accomplished by either sonication or incubation with 4 % Triton X-100 in CTS AIM-V for 5 min. Lysates were stained using the LIVE/DEAD Bac Light Bacterial Viability kit, that stain live bacteria green and dead bacteria red.

In this regard, even though the number of cells per bacillus (100 or more) is expected to facilitate internalization by any one of the phagocytic cells, we are uncertain about the actual proportion of phagocytosed mycobacteria. At this point, we do not know whether BCCC develop from either free or intracellular mycobacteria or both, because intracellular mycobacteria may multiply to a point of cellular destruction making it difficult to determine whether it was initially phagocytosed or remained out of cells. Nevertheless, the number of colonies change in various conditions, and the mechanisms involved in the regulation of their formation need to be investigated. There are also technical problems that must be taken into consideration. It has recently been reported that sonication of mycobacteria may introduce experimental artifacts, including alteration of host inflammatory signaling and intracellular bacterial survival [26]. Nevertheless, it would be expected that any individualization technique will influence the final status of mycobacteria. In the *in vitro* infection procedure, the absence of opsonins from serum in the serum-free medium may interfere with phagocytosis, although it has long been known that the mycobacterial surface provides ligands for many cellular receptors [27].

Several advantages may be accounted for this protocol. It allows the study of bacterial aggregation, that may be considered a form of biofilm, an outcome that is not considered when analyzing CFU. In a previous work we showed that growing mycobacteria do not always form BCCC [25], indicating that the kind of interaction between immune cells and mycobacteria determine whether colonies will develop. The proposed protocol will be useful to analyze *in vitro* the factors, either cellular or mycobacterial or both, that may regulate biofilm formation. For this purpose, the addition of serum to infected cells incubated in serum-free media is optional, allowing the discrimination of the roles played by cells and serum. Furthermore, the use of a low MOI minimizes manipulations, because washes and the addition of antibiotics are avoided, decreasing the variability of the assays. From the practical point of view, the isolation of both PBMC and PMN in a single separation step is convenient. Polymorphprep manufacturer specifies that the reagent allows the separation of both cellular types, but with blood samples from patients we frequently fail to obtain that result because bands are too close and erythrocyte contaminated.

Ethics statements

This study was carried out at the Complejo Asistencial Universitario de León (CAULE) in accordance with the principles of the Declaration of Helsinki (2013), following approval of the protocol by the Hospital of León Clinical Research Ethics Board (reference number 1941) and collection of informed consent from cellular donors.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ramiro López-Medrano: Conceptualization, Methodology, Investigation, Writing – review & editing. **Miriam Retuerto-Guerrero:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Sara Blanco-Conde:** Methodology, Validation, Investigation, Writing – review & editing. **María Belén Morán-Fernández:** Investigation. **Octavio Miguel Rivero-Lezcano:** Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing – original draft.

Data availability

Data will be made available on request.

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