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Development of infection model for studying intracellular gene expression of *mycobacterium tuberculosis*

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Summary

Mycobacterium tuberculosis complex owe their ability to cause infection because of their intracellular survival ability in professional phagocytic cells of human and the ability to enter into stage of dormancy. The aim of this study was to develop an infection model that could be used to study *M. tuberculosis* and macrophage interactions at molecular level. Four infection models were examined namely opsonised *M. bovis* BCG / J774.2 macrophage cell line, non-opsonised *M. bovis* BCG / J774.2 macrophage cell line, opsonised *M. tuberculosis* / J774.2 macrophage cell line, and non-opsonised *M. tuberculosis* / J774.2 macrophage cell line infection models. A J774.2 macrophage cell line was synchronously infected with *M. bovis* (BCG strain) and *M. tuberculosis* (H37Rv), respectively at different multiplicity of infections (M.O.I). For opsonisation, the organisms were pre-incubated with human serum prior to infection. The infected cell lines were examined by light microscopy and electron microscopy with viable bacterial counts. Macrophage viability was assessed by trypan blue exclusion staining. The results showed higher significant level of infection of J774.2 macrophage cell line by opsonised *M. bovis* BCG (30 – 40%) compared to non-opsonised *M. bovis* BCG (<0.1%) at an M.O.I of 50 ($p < 0.05$) with high macrophage viability. In contrast, there was no significant statistical difference ($p > 0.05$) in high infectivity (30 - 42%) with high macrophage viability achieved with using non-opsonised *M. tuberculosis* and opsonised *M. tuberculosis*, respectively, at an M.O.I of 10. In conclusion, opsonisation is not required for *M. tuberculosis* / J774.2 infection model in contrast to *M. bovis* BCG / J774.2 infection model where opsonisation is necessary to achieve high level of infection.

Keywords: *Infection, intracellular, mycobacterium tuberculosis, macrophage*

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Résumé

Le but de cette étude était de développer un modèle d'infection qui pourrait être utilisée pour étudier le M tuberculose et les interactions des macrophages au niveau moléculaire. Quatre modèles infections étaient examinés : cellules opsonisees et non-opsonisees M.Bovis BCG/J774.2 ; Cellules macrophages opsonisees et non-opsonisees J774.2 synchronisées par différent infections de multiplication. Pour l'opsonisation les organismes étaient pré-incubes dans les sérum humain avant l'infection. Les cellules infectées étaient examinées a l'aide de microscope a lumière et a électron. L'habilite des macrophages était évalué par la teinture de Trypan bleue. Les résultants démontraient un niveau élevé d infection des macrophages du J774.2 par l ;'opsonisation de M.bovis BCG (30-40%) comparée aux moins opsonisees M bovis BCG(<0.1%) a M.O.I de 50 ($p<0.05$) avec des macrophages plus habiles. Au contraire, il n'y avait pas de différence significative($P>0.05$) a l'injectivité plus élevée (30-42%) avec des macrophages plus habiles sans opsonisation des M. tuberculose. a M.O.I de 10. En conclusion, l'opsonisation n'est pas nécessaire pour le *M. tuberculose*/J774.2 modèle d'infection comparée a *M.Bovis* BCG/J774.2 modèle d'infection ou l'opsonisation est nécessaire pour achever un niveau d'infection plus élevée.

Introduction

Tuberculosis (TB) is still one of the most important infectious diseases even in recent time. Estimate for 2000 indicates that incident cases have risen to nearly 8.5 million, with over 1.8 million deaths [1,2]. There were an estimated 9.2 million new cases of TB in 2006 (139 per 100,000) [3]. This is an increase of 0.7 million cases from 2000 incident cases. This fact is attributed partly to human immunodeficiency virus (HIV) pandemic and the inability to control this infection effectively despite potent anti-mycobacterial agents cum population growth. India, China, Indonesia, South Africa, and Nigeria rank first to fifth respectively in terms of absolute number of cases

according to the World Health Organization report [3]. Statistics has shown that 38% of African TB patients were HIV positive, and 31% of TB cases and 39% of TB deaths were considered directly attributable to HIV [1].

Members of the *Mycobacterium tuberculosis* complex are the aetiological agents of TB in humans and animals. They are intracellular pathogens that are phagocytosed by mononuclear phagocytes. *M. tuberculosis* enters macrophages by receptor mediated phagocytosis [4], in which several major host cell receptors play a role. These receptors include complement receptors (CRs), the mannose receptor, and type A scavenger receptors [5,6]. A study conducted by Schlessinger *et al.* has shown that Fcã receptors do not play a role in the phagocytosis of *M. tuberculosis* in the absence of specific antibody [4]. There is evidence for the direct interaction between *M. tuberculosis* surface components and CR3 during phagocytosis based on *in-vitro* studies using human and murine macrophages as well as CR3-transfected Chinese hamster ovary (CHO) cell lines [7-10]. Monoclonal antibodies against CRs have been shown to significantly inhibit the phagocytosis of bacteria by human macrophages in the absence of serum [7]. The binding site for *M. tuberculosis* on CR3 of murine macrophages in the absence of serum was found to be distinct from the C3bi-binding site as determined by monoclonal antibody-blocking experiments [8]. Both elicited and activated murine macrophages bound mycobacteria poorly despite expressing CR3.

The upsurge in the incidence of TB and multi-drug resistance TB in recent times in both developing and developed countries despite the availability of anti-TB drugs calls for development and discovery of anti-TB drugs in order to curb the mortality and morbidity rates due to aetiological agents of TB. This can only be achieved through understanding the basic biology of *M. tuberculosis* complex. The release of complete genome sequence of *M. tuberculosis* [11] has provided the necessary information that can be exploited in designing vast array of experiments to tackle this problem. Studying the intracellular gene expression of *M. tuberculosis* during intracellular infection requires developing an infection model that would be able to provide sufficient materials in reproducible manner in order to study differential genes expression at transcriptional or translational level. This study was therefore aimed at developing infection model for studying mycobacterial gene expression during intracellular infection of macrophage using murine J774.2 cell line and *M. tuberculosis*.

Materials and methods

Study design

The design of the study was experimental. The study was carried out in the Department of Infection, School of Medicine, University of Birmingham, Birmingham, United Kingdom.

Maintenance and culture of mycobacteria

Mycobacterial species used in this study were *M. bovis* BCG (Statens Seruminstitut vaccine strain ST1077, obtained from Evans Medical Ltd., Leatherhead, UK) and *M. tuberculosis* H37Rv (NCTC 7416/ATCC 9360) obtained from the National Culture Type Collection, Colindale, UK. Mycobacteria were grown to mid log or late log phase in Middlebrook 7H9 broth (Difco Laboratories Ltd., West Molesey, UK) supplemented with 10% albumin-dextrose-catalase enrichment (ADC; Difco) and 0.02% Tween 80 (Sigma, Poole, UK) at 37°C (in the presence of 5% CO_2 for *M. tuberculosis)* before harvesting at 0.5-1.0 OD_{600}. *M. tuberculosis* H37Rv cultures were also grown for 3 weeks at 37°C on thick 7H10 agar plates supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment (OADC) (Difco, UK) and 0.2% glycerol and then stored at 4°C. For long term storage, *M. bovis* BCG broth cultures were stored at -80°C. All works involving live *M. tuberculosis* was performed in a Class I biohazard safety cabinet under Category 3 containment conditions in the Department of Infection, University of Birmingham, Birmingham, UK.

Maintenance and culture of macrophage cell line

The murine macrophage cell line J774.2.2 was used in all infection experiments. This cell line was derived from a reticulum cell sarcoma, which arose in a female BALB/c/NIH mouse in 1968 during a plasmacytoma induction programme. J774.2.2 cells exhibit the cytological, adherent and phagocytic properties of macrophages. J774.2.2 macrophages (obtained from the European Collection of Cell Cultures, Centre for Applied Microbiology Research, Salisbury, UK) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% heat-inactivated foetal bovine serum (Life Technologies, Paisley, UK) with no antibiotic supplements. Macrophage monolayer was maintained at 37°C in humidified air containing 5%

CO_2 before and after infection. For storage purposes, cell pellets were suspended in 9% dimethyl sulphoxide (DMSO) in foetal bovine serum (FBS) at a concentration of approximately 4×10^6 cells/ml and aliquoted into 1 ml cryo-vials (Nunc, UK). The vials were placed in a polysytrene box and then placed at -20°C overnight before transferring to liquid nitrogen.

Macrophage infection

Studies were performed to determine the infectivity ratio of *M. bovis* BCG to J774.2.2 macrophages using the synchronised infection method of Schlesinger *et al* [4], with slight modification covering the range from 1:1 to 100:1 (bacilli to macrophages, respectively) multiplicity of infection (M.O.I). The bacilli were de-clumped by sonication by placing the culture in a sonicator waterbath (Nusonic, BDH, Leicester, UK) for 3 min. The bacilli were then incubated in the presence or absence of 1 ml of a positive human serum for 30 min at 37°C. J774.2 macrophage monolayers (in 75cm² flasks) were detached from the bottom of the flask using a disposable tissue scraper (Greiner, UK) and the macrophage suspension was transferred to polystyrene universal tubes for centrifugation at 2,500×g for 5 min. The cell pellets were re-suspended in 20 ml of cold DMEM (4°C) containing 10% FBS and either opsonised or non-opsonised bacilli were added at different M.O.I (i.e. 1:1, 5:1, 10:1, 20:1, 50:1 and 100:1). Subsequently, the bacilli and macrophages were centrifuged together at room temperature for 5 min at 2,500×g. The supernatant was discarded and the cells were re-suspended in 1 ml of pre-warmed (37°C) DMEM containing 10% FBS and placed in a 37°C, 5% CO_2 incubator for 5 min. The cells were then transferred to a 75cm² tissue culture flask containing 25 ml of pre-warmed DMEM and 10% FBS and incubated for a further 4 hr. After the 4 hr incubation period, both the infected and non-infected macrophages had typically adhered to the plastic making it possible to wash off any extracellular, non-phagocytised bacilli. The extracellular bacilli were removed by washing the monolayer three times with Hanks' Balanced Salt Solution (HBSS). The infected cell line was covered with fresh 10% FBS in DMEM and the cells were incubated overnight (typically ~15 hr) as above.

The infectivity of the macrophages was determined by Ziehl Neelsen staining and in some cases, transmission electron microscopy was undertaken to determine the intracellular localisation of the bacilli as described below. The viability of the macrophages following infection was determined using the trypan blue exclusion method [12]. Briefly, 20 l of trypan blue (Sigma, UK) were added to 80 µl of the macrophage suspension and placed in an improved Neubauer haemocytometer. The number of unstained and stained macrophages was determined by counting one of the nine large squares under an inverted light microscope. The number of cells per ml = average total cell count per square × 1.25 (dilution factor) × 10^4. The cell viability was then calculated using the following formula: cell viability (%) = [total viable cells (unstained) ÷ total cells (stained and unstained cells)] × 100.

Quantification of intracellular bacilli

Macrophages infected with opsonised *M. bovis* BCG at an M.O.I of 50:1 were harvested after 15 housr of infection (i.e. 15 hours after the washing step to remove extracellular bacteria). The harvested macrophages were resuspended in 5 ml sterile distilled water (at room temperature) to lyse the macrophages. The mycobacteria within the lysate were dispersed with water bath sonication as before. Ten-fold serial dilutions were performed and 0.5 ml of each dilution was seeded onto thick Middlebrook 7H10/OADC agar plates, in duplicate, and incubated at 37°C in a CO_2 incubator for three weeks. The number of colony forming units per ml (c.f.u/ml) was determined using the following formula: c.f.u/ml = average number of colonies counted x 2 x dilution (10^n). The intracellular *M. tuberculosis* was quantified using the above procedure using non-opsonised *M. tuberculosis* at an M.O.I of 10.

Confirmation of intracellular bacilli

The infectivity of the cell lines was confirmed by Ziehl Neelsen staining using the cold staining method. To confirm intracellular infection, transmission electron microscopy (TEM) was performed. The infected macrophage pellet was re-suspended in 1 ml of freshly prepared 2.5% (v/v) glutaraldehyde in 0.1M sodium phosphate buffer and was processed for TEM at the Department of Physiology, University of Birmingham, UK. Briefly, sections were stained with 30% (w/v) uranyl acetate in 70% (v/v) methanol and counterstained with lead citrate. These were then examined on a Jeol JEM-100CXII transmission electron microscope at an acceleration voltage of 80 kv. Photomicrographs were taken of representative samples.

Statistical analysis

The results of each infection model were subjected to statistical analysis using the statistical programmes within Microsoft XL. Significance was determined by student t-test method, where the difference was considered to be statistically significant when the p value obtained was less than 0.05 ($p < 0.05$).

Results

Infection of macrophage cell line by M. bovis BCG strain

Infection experiments performed using non-opsonised *M. bovis* BCG bacilli and a non-synchronised method of infecting the macrophages (whereby the bacilli were simply added to macrophage monolayer) resulted in a very low level of infection, even at M.O.I of 50:1. Similarly, low level of infection was achieved using synchronised method of infection using non-opsonised *M. bovis* (BCG strain) (Table 1a).

Table 1a: Infection of J774.2 macrophages with non-opsonised *M. bovis* BCG

M.O.I	Macrophage viability (%)	Level of infection (%)
1:1	95 ±0.3	Nil
5:1	92±1.5	<0.1
10:1	87±2.2	<0.1
20:1	84±0.6	<0.1
50:1	80±1.5	<0.1
100:1	70±2.0	0.1 – 0.5

M.O.I - multiplicity of infection, bacilli to macrophages. The level of infection was determined by ZN staining and macrophage viability was determined by trypan blue exclusion staining after 15 hr incubation. Values are the means of triplicate samples.

Table 1b: Infection of J774.2 macrophages with opsonised *M. bovis* BCG

M.O.I	Macrophage viability (%)	Level of infection (%)
1:1	90±0.1	Nil
5:1	95±0.2	<0.1
10:1	85±0.1	0.1 - 0.5
20:1	86±0.4	0.8 - 1.0
50:1	84±1.1	30 - 40
100:1	70±0.5	30 - 40

M.O.I - multiplicity of infection, bacilli to macrophages. The level of infection was determined by ZN staining and macrophage viability was determined by trypan blue exclusion staining after 15 hr incubation. Values are the means of triplicate samples.

Ziehl Neelsen staining of the infected macrophages showed that there was high number of extracellular bacilli present following the washing step of the infected monolayers, indicating that the macrophage infection process was inefficient. At the 50:1 M.O.I, the number of viable macrophages dropped to level as low as 70% of the starting cell number indicating that the high number of bacilli (despite being extracellular) were detrimental to macrophage viability. However, a high level of infection with a concomitantly high level of macrophage viability was achieved using the synchronised method of infection after 15 hr of infection when opsonised as opposed to non-opsonised *M. bovis* BCG bacilli were used at an M.O.I of 50:1 (Tables 1a and b). Statistically, comparing the level of infection in opsonised *M. bovis* BCG (30 to 40%) to non-opsonised BCG (<0.1%) was found to be statistically significant (t-test, $p < 0.01$), suggesting an enhancement of macrophage infection either by antibodies or/and complement. This was confirmed by electron microscopy (Figure 1). There was significant statistical difference at the macrophage viability (t- test, $p < 0.05$) when the non-opsonised *M. bovis* BCG (80±1.5%) was compared to opsonised *M. bovis* BCG (84±1.5%) (Tables 1a and b).

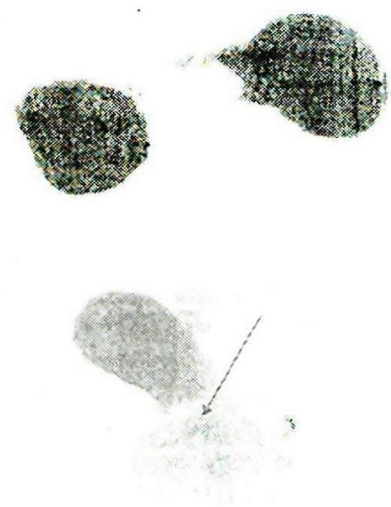

Fig. 1: Photograph of Ziehl Neelsen stained J774.2 macrophages infected with *M. bovis* BCG indicating that the bacilli (seen as acid fast, carbol fuschin stained rods) are located intracellularly. The macrophages were infected overnight (-15hr) with opsonised bacilli at M.O.I of 50:1 (magnification x 2,000).

Infection of macrophage cell line by M. tuberculosis H37Rv strain

At low M.O.I for opsonised and non-opsonised *M. tuberculosis*, the levels of infection were very low

Table 2a: Infection of J774.2 macrophages with non-opsonised *M. tuberculosis*

M.O.I	Macrophage viability (%)	Level of infection (%)
1:1	90±1.5	Nil
5:1	95±2.1	<0.1
10:1	85±2.0	33 - 42
20:1	86±1.5	35 - 41
50:1	71±0.3	30 - 40
100:1	74±0.2	30 - 40

M.O.I - multiplicity of infection, bacilli to macrophages
The level of infection was determined by ZN staining and macrophage viability was determined by trypan blue exclusion staining after 15 hr incubation. Values are the means of triplicate samples.

Table 2b: Infection of J774.2 macrophages with opsonised *M. tuberculosis*

M.O.I	Macrophage viability (%)	Level of infection (%)
1:1	94	Nil
5:1	93	<0.1
10:1	84±1.5	30 - 40
20:1	82±2.2	32 - 40
50:1	69±1.5	30 - 40
100:1	70±4.5	30 - 40

M.O.I - multiplicity of infection, bacilli to macrophages
The level of infection was determined by ZN staining and macrophage viability was determined by trypan blue exclusion staining after 15 hr incubation. Values are the means of triplicate samples.

(Tables 2a and 2b). Interestingly, there was no significant statistical difference (t-test, $p > 0.05$) between opsonised and non-opsonised *M. tuberculosis* H37Rv when comparing the level of infections in macrophages at an M.O.I. of 10 (Tables 2a and b). Furthermore, there was no statistical difference in macrophage viability in opsonised *M. tuberculosis*/J774.2 macrophage and non-opsonised *M. tuberculosis*/J774.2 infection models (t-test, $p > 0.05$). The results of the macrophage infection experiments using non-opsonised *M. tuberculosis* H37Rv at an M.O.I 10:1 showed a higher level of infection than the opsonised *M. bovis* BCG as observed by both Ziehl Neelsen staining (Tables 1b and 2a) and by transmission electron microscopy (Figures 2 and 3) reflecting the more virulence nature of *M. tuberculosis* over *M. bovis* BCG. As opsonisation of *M. tuberculosis* bacilli was not required to generate a high level of infection of the macrophages, the infection strategy used in the *M. tuberculosis* studies could be performed using non-opsonised bacilli. The number of *M. bovis* BCG associated with macrophages 15 hours post infection (assessed by counting the number of viable bacilli) was about 70.7% of the infective dose indicating successful phagocytosis or/and adherence of the bacilli using an M.O.I 50:1. A similar result (74%) was obtained using non-opsonised *M. tuberculosis* at an M.O.I 10:1. This also confirmed that pre-opsonisation of *M. tuberculosis* was not required for phagocytosis to take place.

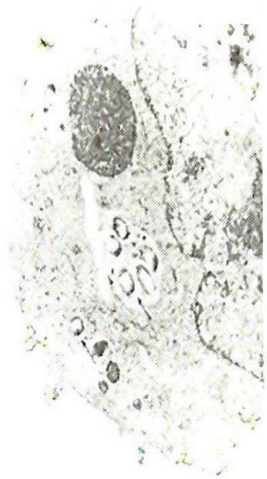

Fig. 2: Electron photomicrograph of a J774.2 macrophage confirming the intracellular localisation of *M.bovis* BCG. The macrophages were infected overnight (-15hr) with opsonised bacilli at M.O.I. of 50:1 (magnification x 10,000)

Fig. 3: Electron photomicrograph of a J774.2 macrophage confirming the intracellular localisation of *M. tuberculosis* (H37Rv.). The macrophages were infected overnight (-15hr) with non opsonised bacilli at M.O.I. of 10:1 (magnification x 5,400)

Discussion

Macrophage cell lines have proven to be an important tool for studying bacteria-host cell interactions, particularly those involving facultative intracellular microorganisms [13-16]. The importance of using a macrophage cell line in the infection model stems from the role the macrophage plays in limiting the spread of most bacterial infections. The role of the macrophage in the recognition and subsequent uptake of *M. tuberculosis* is undisputed and many studies have been performed using J774.2 macrophages and mycobacteria with high degree of success [14, 16]. The murine J774.2 macrophage-like cell is useful as a model cell to study bacteria-host interactions because it is considered to be fully differentiated and has active phagocytic properties. It is easier to use than macrophages prepared from human venous blood, the murine peritoneal cavity or the human macrophage cell line THP-1. The J774.2 cells are easy to grow, providing large numbers of macrophages to infect with good reproducibility between infection experiments. This is an important factor when studying intracellular gene expression of *M. tuberculosis* where a lot of intracellular bacilli are required.

The synchronised method of infecting the macrophages was chosen to enable the bacilli to be at the same stage of infection when the experiment was terminated. This would result in the same genes being expressed at the same time enhancing the 'fingerprinting' of the majority of the genes being expressed at a particular time point. In addition, because the RNA yield obtainable from mycobacteria is low, despite current improvements in extraction strategies, the synchronised method is anticipated to maximise the recovery of *M. tuberculosis* mRNA thus reflecting the genes being expressed at the same stage of intracellular infection.

It is interesting that variation was observed between the infection levels of opsonised *M. bovis* BCG and non-opsonised M. bovis BCG, and no significant variation between the infection levels of macrophage infected with non-opsonised *M. tuberculosis* and opsonised *M. tuberculosis* bacilli, suggesting that the degree of mycobacterial virulence can determine whether or not opsonisation is required to achieve high level of macrophage infection. The inability of non-opsonised *M. bovis* BCG to achieve high level of infection, despite using the synchronised method of infecting the macrophages, was in marked contrast to the results obtained with non-opsonised *M. tuberculosis* where a high level of infection was attained. This finding is in line with the results of a study on the interaction between *M. tuberculosis* and Chinese hamster ovary cells [10]. The authors found under their conditions, that the binding of *M. tuberculosis* H37Rv to CR3 was serum and complement independent. However, they used *M. smegmatis* as a control rather than *M. bovis* BCG, where serum or complement was very important for the efficient binding of *M. smegmatis* to Chinese hamster ovary cells. It is difficult, however, to rule out the role of complement in the infection model used in this study, since macrophages have been found to synthesise the complement component C3 [17-19]. Moreover, the fact that *M. bovis* BCG behaved differently under these conditions indicates that there are other bacterial factors influencing these host-pathogen interactions. It is likely that there are pathogenic traits in *M. tuberculosis*, which are lacking in *M. bovis* BCG. *M. bovis* BCG is an attenuated vaccine strain of *M. bovis*, which differs from *M. bovis* because of the deletion of genetic elements within the chromosome of *M. bovis* BCG [20]. *M. bovis* and *M. tuberculosis* exhibit the same clinical spectrum of disease in patients with TB despite the minor differences in their genetic make up. Therefore, it is not particularly surprising that a variation in infection results was obtained using *M. bovis* BCG and *M. tuberculosis*. Moreover, it has been shown that a component of non-opsonic binding may be mediated by the mannose receptor [7], although this is limited to *M. tuberculosis* strains with mannose-capped lipoarabinomannan moieties [21], and may be restricted to resting macrophages since activated macrophages downregulate expression of this receptor [22,23]. On the other hand, serum complement has been found to enhance the adherence of *M. tuberculosis* to mononuclear phagocytes by approximately three fold [4,5,7].

The suggested *M. tuberculosis* infection model is therefore based on using synchronised method of infecting the J774.2 cell line with non-opsonised *M. tuberculosis*. This is most likely to mimic the first encounter of *M. tuberculosis* with the host cells as it is unlikely that antibodies are present when the bacilli encounter the alveolar macrophages as complement components may be limited in the alveolar space in the quiescent lung [24]. Moreover, because the bacilli are not opsonised they are likely to enter the macrophage through different receptors and this may be important as it has been suggested that the route of entry determines the survival strategy employed by the mycobacteria [4,21]. However, this has since been disputed by the findings of Zimmerli *et al.* who showed that selective

receptor blockade during phagocytosis does not alter the survival and growth of *M. tuberculosis* in human macrophages [6].

One of the shortcomings of the infection model used in this study is the inability to fully simulate the host environment since professional phagocytes, such as macrophages and neutrophils are not the only host cells that contribute to the immune response during the initial stages of infection in the host. Lung, intestinal epithelial cells and vascular endothelial cells can also be infected and, in some cases, have been shown to be involved in the production of tissue-damaging cytokines and chemokines [25,26].. Despite this, the infection of J774.2 macrophages with *M. tuberculosis* represents a valid model for studying the genes involved in the key stage of intracellular survival. This infection model has been used in obtaining RNA for studying gene expression [27] and determining the level of expression of *mig* homologue in *M. tuberculosis* [28].

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