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Mycobacterium bovis hosted by free-living-amoebae permits their long-term persistence survival outside of host mammalian cells and remain capable of transmitting disease to mice

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Summary

Bovine tuberculosis (TB) is a zoonotic disease caused by *Mycobacterium bovis*. Despite intensive TB control campaigns, there are sporadic outbreaks of bovine TB in regions declared TB free. It is unclear how *M. bovis* is able to survive in the environment for long periods of time. We hypothesized that Free-living amoebae (FLA), as ubiquitous inhabitants of soil and water, may act as long-term reservoirs of *M. bovis* in the environment. In our model, *M. bovis* would be taken up by amoebal trophozoites, which are the actively feeding, replicating and mobile form of FLA. Upon exposure to hostile environmental conditions, infected FLA will encyst and provide an intracellular niche allowing their *M. bovis* cargo to persist for extended periods of time. Here, we show that five FLA species (*Acanthamoeba polyphaga*, *Acanthamoeba castellanii*, *Acanthamoeba lenticulata*, *Vermamoeba vermiformis* and *Dictyostellium discoideum*) are permissive to *M. bovis* infection and that the *M. bovis* bacilli may survive within the cysts of four of these species for over 60 days. We further show that exposure of *M. bovis*-infected trophozoites and cysts to Balb/c mice leads to pulmonary TB. This work describes for the first time that FLA carrying *M. bovis* can transmit TB.

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Introduction

Bovine tuberculosis (TB) is a zoonotic disease caused by *Mycobacterium bovis* (*M. bovis*). The disease has a vast impact on the health of domestic and wildlife species as well as humans (Fitzgerald and Kaneene, 2013). Although cattle are the main hosts of this pathogen, other domesticated and wildlife animals can suffer from *M. bovis*-mediated TB. The disease is found throughout the world and is more prevalent in most of Africa, parts of Asia and the Americas. Expensive eradication programs consisting of postmortem meat inspection, surveillance, culling of infected and contact animals as well as movement restrictions of livestock have been successful at reducing and eliminating the disease. These control measures have led to the declaration of bovine-TB-free areas. However, sporadic reemergence of bovine TB has occurred preventing eradication (Gallagher and Muirhead, 1999; Karolemeas *et al.*, 2011; Fitzgerald and Kaneene, 2013; Gallagher *et al.*, 2013; Gormley *et al.*, 2016). In countries such as Ireland, the UK, Spain, New Zealand and Northeast Michigan in the United States, reemergence of the TB disease is strongly associated with *M. bovis* infected wildlife reservoirs such as badgers (*Meles meles*) (Byrne *et al.*, 2014), possums (*Thricosurus vulpecula*) (Porphyre *et al.*, 2011), white-tailed deers (*Odocoileus virginianus*) (Schmitt *et al.*, 1997; Kaneene *et al.*, 2002) and feral pigs (Aranaz *et al.*, 2004; Fitzgerald and Kaneene, 2013). Grazing of *M. bovis* infected livestock and wildlife also contaminates pasture, feed, water and soil by bacterial shedding via feces and urine (Gallagher *et al.*, 1998; Fine *et al.*, 2011). Thus, contaminated environmental sources and reactivation of latent infection are believed to be causes of 'residual' infection in cases of reemergence of bovine TB (Karolemeas *et al.*, 2011; Gallagher *et al.*, 2013). Some *M. tuberculosis* complex mycobacteria including *M. bovis* (Drancourt, 2014) are also known to retain capability to survive in soil and environment despite suboptimal environmental conditions (Williams and Hoy, 1930), but understanding of how *M. bovis* survives conditions imposed upon it by the environment remains poorly understood.

Lower eukaryotic organisms have been studied as a potential feral intracellular niche for *M. bovis* survival in the environment (Taylor *et al*., 2003). Growing evidence suggests that free-living amoebae (FLA) may provide such niches for several *Mycobacteria* spp. including *M. bovis* (Taylor *et al*., 2003; Greub *et al*., 2004; Mba Medie *et al*., 2011). There are striking similarities in the biology of FLA and macrophages, the established eukaryotic intracellular niche within host organisms of mycobacteria (Rue-Albrecht *et al*., 2014). Trophozoites are the actively replicating and feeding form of FLA with similar phagocytic capacity to macrophages (they can uptake particles and microorganisms larger than 0.5 μm) and are capable of forming enveloping phagocytic vacuoles followed by formation of phagolysosomes which, in some cases, kill and digest both pathogenic and nonpathogenic microbes (Guimaraes *et al*., 2016). For these reasons, FLA are sometimes termed 'feral macrophages'. A unique feature of the FLA lacking in macrophages is their capacity to switch from a free and motile trophozoite form to an immobile cyst depending on the physical and nutritional status of their microenvironment. Cysts are the dormant form of FLA that render them capable of survival for decades under extreme physical and chemical stresses (e.g., starvation, temperature, pH, desiccation and antibiotics) (Steinert *et al*., 1998). Recently, a study found that FLA trophozoites of *Acanthamoeba castellanii* support *M. bovis* survival for at least 5 months (Mardare *et al*., 2013). Moreover, it remains to be determined how long *M. bovis* persists in other amoeba species and whether infected trophozoites or cysts may transmit TB disease to a mammalian host.

The hypothesis driving these studies was that survival of *M. bovis* within ubiquitous environmental FLA (e.g., in soil and water) is possible and that, while inside the FLA, *M. bovis* can retain sufficient virulence to transmit infection and disease to a mammalian host. A second hypothesis was that *M. bovis* could survive hostile microenvironmental conditions (e.g., starvation, temperature, pH and desiccation) within encysted FLA. These two scenarios would help explain how and why a rather fastidious facultative intracellular organism such as *M. bovis* can survive a seemingly 'hostile' environment and remain fully capable of transmitting disease. Indeed, some *Mycobacterium* spp. such as *Mycobacterium avium* and *Mycobacterium leprae* have been reported to use protozoan cysts as a means to survive long-term outside a mammalian host (Ben Salah and Drancourt, 2010; Wheat *et al*., 2014). It is also possible that, by virtue of surviving within FLA, mycobacteria can be transmitted therein and potentially by-pass host innate immune barriers. This strategy would allow the bacilli to survive utilizing the FLA as a biological 'Trojan horse'. Coculturing *M. bovis* strain # 95-1315 isolated from a white-tail deer (Waters *et al*., 2014) with *Acanthamoeba lenticulata*, *A. castellanii*, *Acanthamoeba polyphaga*, *Vermamoeba vermiformis* (*Hartmannella vermiformis*) and *Dictyostelium discoideum*, we here demonstrated that *M. bovis* is capable of surviving for extended periods of time within both trophozoites and cysts of FLA of *Acanthamoeba* and *Vermamoeba spp*. but not *Dictyostelium*. Furthermore, we demonstrated that both *M. bovis*-infected *Acanthamoeba* trophozoites and cysts can transmit pulmonary mycobacterial infection to mice with subsequent development of TB-associated pathology. Evidence was provided for the first time that *M. bovis* can find long-term refuge ($>$ 60 days) in common FLA cysts and retains sufficient capacity for transmission of infection and disease to a mammalian host.

Results

Infection of various amoeba species by M. bovis

The main goal of these studies was to quantify the uptake and persistence of *M. bovis* in a variety of amoeba species. To facilitate analysis and visualization of the *M. bovis* bacilli within amoebae, *M. bovis* was modified to express the green fluorescence protein (GFP) by transformation with the pMSP12:GFP plasmid as detailed in Material and Methods. To demonstrate successful insertion and expression of GFP in *M. bovis*, bacilli fluorescence was examined by flow cytometry and confocal microscopy (Fig. 1A and B). Thereafter, trophozoites of *Acanthamoeba* spp. and *V. vermiformis* were infected with *M. bovis* GFP at an MOI of 1:10 (amoeba:bacilli) and incubated for 12 h at 28°C. The intracellular compartmentalization of *M. bovis*-GFP within *A. polyphaga* trophozoites was demonstrated using confocal Z-stack imaging (Fig. 1C). Some uninfected encysted amoebae (Fig. 1D) were observed but typically represented 0.5–1% of the total amoeba population. Similar observations were made following the infection of *Acanthamoeba* spp. and *V. vermiformis* trophozoites with *M. bovis*-GFP (Fig. 1D and G). We concluded that all five species of amoebae were able to take up *M. bovis*-GFP resulting in the intracellular localization of the bacilli.

Persistence of M. bovis-GFP inside trophozoites

Cultures of amoebae (*Acanthamoeba* spp. and *V. vermiformis*) infected with *M. bovis*-GFP for 12 h at 28°C were incubated in their corresponding medium for 5 days and aliquots of the cocultures collected 12, 48, 72 and 120 h postinfection were prepared for further evaluation by confocal microscopy. Z-stacks of confocal images were used to localize the bacteria within the FLA. After collection of multiple Z-stack per image, two independent readers without knowledge of the study determined the total number of cells (trophozoites and cysts) in the fields analysed, number of infected trophozoites and number of infected and uninfected cysts (Fig. 2). The number of encysted FLA in

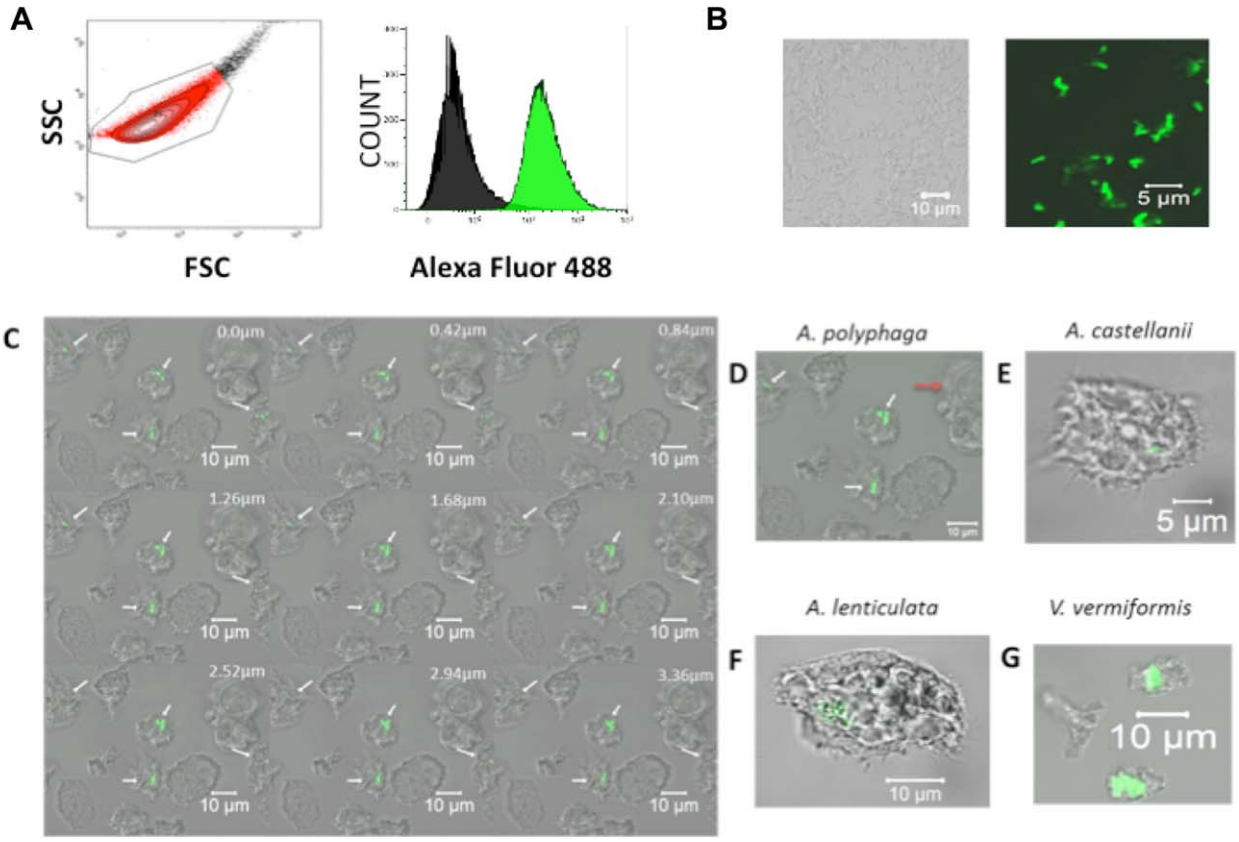

Fig. 1. Generation of *M. bovis* bacilli expressing green fluorescence protein; evaluation and infection of *Acanthamoeba* spp. and *Vermamoeba vermiformis*.
M. bovis-GFP was generated by transformation of *M. bovis* with plasmid pMSP12:GFP. Positive constructs were evaluated and compared against control (*M. bovis*) using (A) flow cytometry. Plot for FSC versus SSC shows gating of bacilli population and histogram shows fluorescence overlay of *M. bovis* (black) and *M. bovis-GFP* (green).
B. Confocal imaging of *M. bovis* (left) and *M. bovis*-GFP (right).
C–G. Confocal images of trophozoites infected with *M. bovis*-GFP at an MOI of 1:10 for 12 h at 28°C. (C) *A. polyphaga* trophozoites in a confocal Z-stack image demonstrating the presence of *M. bovis* inside the FLA. Similar images were obtained with other FLA (data not shown). (D) Merged image of (C) with white arrows showing a green fluorescent bacillus within an amoeba and a red arrow pointing at a cyst of *A. polyphaga*,. (E) Trophozoite of *A. castellanii* containing an intracellular bacillus. (F) Trophozoite of *A. polyphaga* with multiple intracellular bacilli. (G) Trophozoites of *Vermamoeba vermiformis* infected with *M. bovis*-GFP. Scale bars are 5 and 10 µm.

the uninfected and *M. bovis*-GFP-infected cultures was similar after 5 days of coculture (for all four FLA species) (Fig. 2), suggesting that FLA infection with *M. bovis* did not induce any significant encystment of the trophozoites. Note that *V. vermiformis*, whether infected or uninfected, tended to encyst more readily than *Acanthamoeba* spp. (Fig. 2D). Samples were further analysed by confocal microscopy for changes in the number of intracellular bacilli over time. Trophozoites contained between 1 and 3 intracellular bacilli after 12 h of infection, and this number did not change noticeably over time (data not shown).

The presence of viable intracellular bacilli in each of the cocultures was also determined by culturing lysates of these samples on agar plates. The results confirmed a similar number of CFU on days 1 and 5 postinfection (Fig. 2E).

Additionally, the kinetics of infection of *M. bovis*-GFP in *D. discoideum* was also studied up to 5 days postinfection. However, only the samples recovered 12 h postinfection established positive CFU (data not shown) suggesting that *M. bovis*-GFP was not able to survive in *D. discoideum* for more than 12 h. We thus conclude that the outcome of *M. bovis* association with amoebae is species dependent with *Acanthamoebae* and *V. vermiformis* showing greater permissiveness to *M. bovis* infection than *D. discoideum*. Whereas *M. bovis* persisted for at least 5 days in *Acanthamoeba* and *V. vermiformis*, there was no apparent proliferation of the bacilli in these FLA over this time period.

Persistence of M. bovis-GFP inside cysts

Amoebae (*Acanthamoeba* spp., *V. vermiformis* and *D. discoideum*) infected *with M. bovis* as detailed above were induced to encyst by culturing in encystment medium and

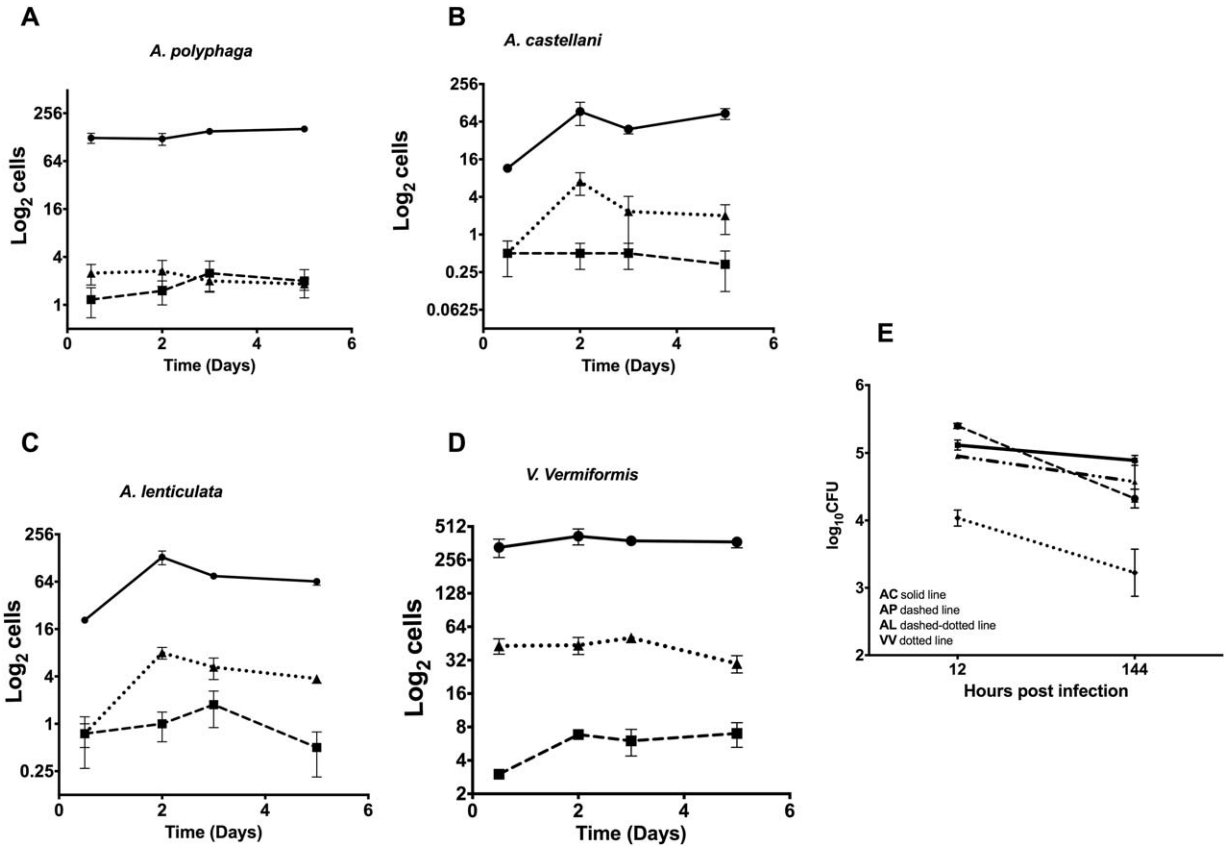

Fig. 2. Growth and persistence of *M bovis*-GFP inside amoebae.
A. *A. polyphaga*, (B) *A. castellanii*, (C) *A. lenticulata* and (D) *V. vermiformis*. FLA cultures were infected as described in Fig. 1 and cells were fixed in 4% PFA, washed and analysed by confocal microscopy after 12 , 48, 72 and 120 h (5 days) of infection. Z-stacks of confocal images were used to localize the bacteria within the FLA. Two independent readers without knowledge of the study determined the total number of cells representing both trophozoites and cysts (solid lines), infected trophozoites (dotted lines) and total cyst (infected and uninfected cysts) (dashed lines).
E. Viable bacilli obtained at 12 and 144 h (5 days) of FLA cultures infected with *M. bovis* as described in Fig. 1 and represented as $\log_{10}$CFU. AP = *A. polyphaga*; AC = *A. castellanii*; AL = *A. lenticulata* and VV = *V. Vermiformis*.

remaining therein for 30–60 days. Using light microscopy, we observed that, after 30 days, at least 95% of the amoebae had encysted. To determine viable bacterial load within cysts as shown in Fig. 3C, infected amoebae recovered after 30 and 60 days of culturing in encystment medium were transformed back to trophozoites by changing the culture medium. Lysates of excysted-trophozoites were then cultured on agar plate to determine viable *M. bovis* CFU (Fig. 3A). The results show that *M. bovis* can survive within *Acanthamoeba* and *V. vermiformis* cysts for at least 60 days with *A. castellanii* cysts presenting the highest bacillary burden among the amoebae spp. tested.

The possibility that extracellular *M. bovis* could survive in encystment medium (i.e., outside the cysts) during the 60 days of culture and potentially proliferate within this medium was discarded by culturing the axenic bacilli on encystment medium during 15 days followed by enumeration of CFU after culturing on agar plates. As shown in Fig. 3B, no CFU were recovered on agar plates cultured during 3 weeks with samples from axenic *M. bovis* cultured on encystment medium. Thus, *M. bovis* is not able to survive in encystment medium alone; it thus follows that the CFU recovered from infected FLA cyst cultures were associated with the cysts.

Infection of mice with M. bovis-infected A. polyphaga *trophozoites*

Balb/c mice were infected with a cell suspension containing trophozoites of *A. polyphaga* infected with *M. bovis*. Briefly, Balb/c mice were randomly separated in groups (Table 1) and challenged with 50 μl of a cell suspension containing *A. polyphaga* infected with *M. bovis*. Our previous *in vitro* results (Fig. 2) demonstrated that cocultures of *A. polyphaga* and *M. bovis* resulted in an average extent of 20% infection. Accordingly, the high dose (HD) and low dose (LD) inocula administered to mice was prepared as 5×10^4 and 5×10^2 infected *A. polyphaga* trophozoites,

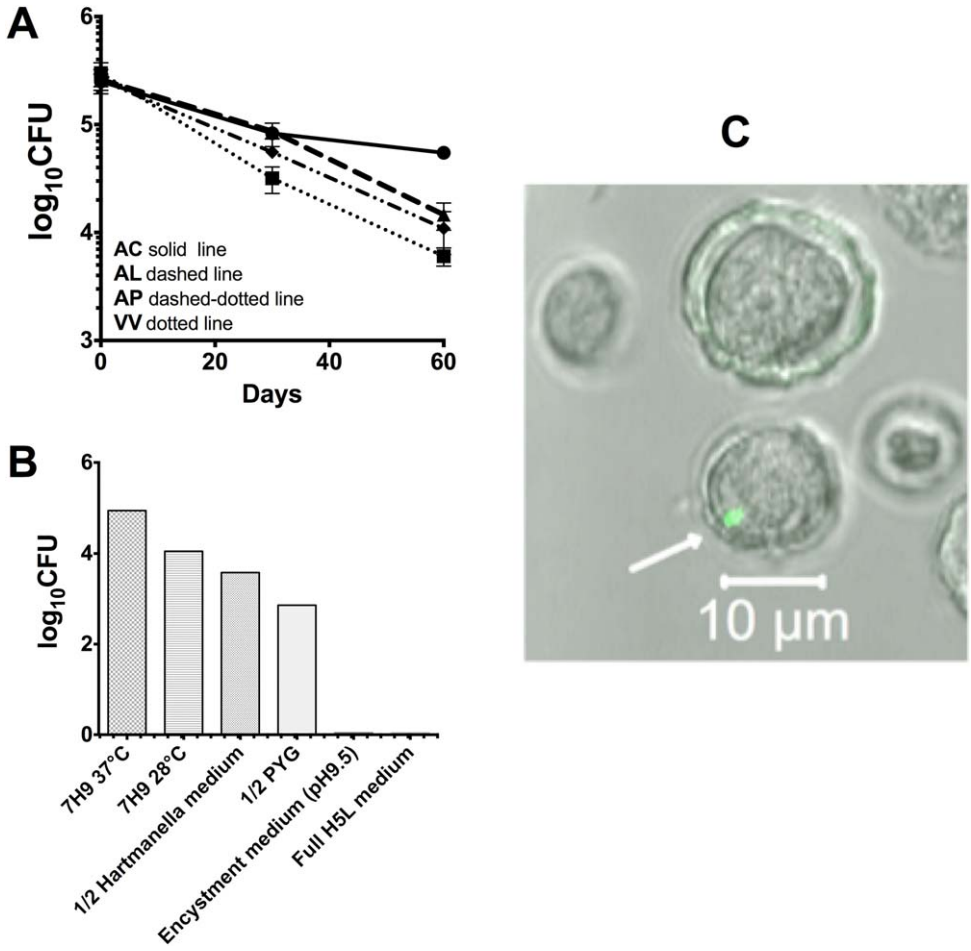

Fig. 3. Growth and persistence of *M bovis*-GFP inside the cysts of four amoebae spp.
A. Trophozoites were infected with *M. bovis* as described above, followed by culture in encystment medium for 30 and 60 days. The cultures were monitored for percentage of encystment until 100% of FLA appeared as cysts. Long-term encysted cocultures were forced to excyst by culturing the samples in their corresponding amoeba growth medium and temperature optimum. *M. bovis* was then extracted from the excysted trophozoites by lysing the samples with 0.5% SDS. CFU enumeration was performed after serial dilutions of each sample were plated onto 7H11 agar plates followed by incubation for 3 weeks at 37°C. AP = *A. polyphaga*; AC = *A. castellanii*; AL = *A. lenticulata*; VV = *V. vermiformis*.
B. The viability of axenic *M. bovis*-GFP after 15 days of culture either in encystment medium, amoeba culture media or 7H9 medium at 37 or 28°C was determined by plating serial dilutions of each culture onto 7H11 agar and incubating the plates at 37°C during 3–4 weeks for CFU enumeration.
C. Confocal image of a cyst infected with *M. bovis*-GFP (white arrow).

respectively, carrying an estimated 1×10^4 and 1×10^2 CFU bacilli. One control group of mice received 1×10^4 and 1×10^2 *M. bovis* CFU (as free bacilli) while another control group received 5×10^4 and 5×10^2 uninfected *A. polyphaga* trophozoites. On day 1 (D1) postinfection, lung homogenates from FLA/*M. bovis*-infected mice and the *M. bovis*-infected control animals were plated onto agar plates yielding the following CFU counts: 2.04 $\log_{10}$ CFU for FLA/*M. bovis* HD, 2.45 $\log_{10}$ CFU for *M. bovis* HD and 0.3 $\log_{10}$ CFU for FLA/*M. bovis* LD and *M. bovis* LD. These results demonstrated the successful deposition of bacteria into the lungs. Similarly, the bacterial burden on days 14, 30 and 60 postinfection from FLA/*M. bovis* and *M. bovis* groups had increased by 2–4 $\log_{10}$ over D1 postinfection confirming the establishment of the infection and the progressive proliferation of the bacilli within the lungs. As expected during pulmonary TB, the infection entered a chronic state and the bacterial burden in all groups remained relatively constant after 30 days of infection (Fig. 4A). Importantly, it was possible to enumerate CFU after culturing spleen homogenates harvested at 30 and 60 days postinfection (Fig. 4A) demonstrating that dissemination of bacilli is also possible.

Table 1. Groups of mice and dose.

Group	Targeted dose (in 50 μl)
FLA	5×10^4 trophozoites of *A. polyphaga*
M. bovis low dose (control)	*M. bovis* (1×10^2 CFU)
M. bovis High dose (control)	*M. bovis* (1×10^4 CFU)
FLA infected with *M. bovis* high dose (FLA–*M. bovis* HD)	5×10^{4a} trophozoites of *A. polyphaga* infected with *M. bovis*
FLA infected with *M. bovis* low dose (FLA–*M. bovis* LD)	5×10^{2a} trophozoites of *A. polyphaga* infected with *M. bovis*

a. As shown in Fig. 2, between 5% and 20% of the FLA carry intracellular *M. bovis* bacilli. Thus, we estimated that each LD and HD inoculum carry an approximated load of 1×10^2 or 1×10^4 CFU *M. bovis*, respectively.

Infection of mice with M. bovis-infected A. polyphaga *cysts*

Mice were further infected via intrapulmonary delivery with a cell suspension containing 1-month-old cysts of *A. polyphaga* infected with *M. bovis*. Mice were sacrificed on days 1 and 60 postinfection and lung and spleen homogenates prepared for CFU determination as described above. On day 1 postinfection, 2 $\log_{10}$ CFU were recovered from the lungs of mice, whereas an average of 6 $\log_{10}$CFU was recovered from the same organ 60 days postinfection (Fig. 4B). Importantly, the spleens also harboured an average of 5 $\log_{10}$CFU 60 days postinfection. *M. bovis* housed in amoebic cysts was thus able to establish a proliferative and disseminating mycobacteria infection in the lungs and spleen of mice.

Histopathological assessment

Gross lesions (Fig. 5A) and histopathology (Fig. 5B) were evaluated on days 1, 14, 30 and 60 postinfection. As expected, there were no gross lesions in any of the groups on the first day of the infection. Similarly, lung histology of day 1 samples showed minimal signs of inflammation suggesting that the FLA by itself had no effect on lung pathology. This state of quiescence remained for 14 days postinfection (data not shown). On day 30, small lesions resembling early stages of granulomatous formations were present in tissue sections obtained from the lungs of *M. bovis* and FLA/*M. bovis*-infected groups of mice. On day 60, larger granulomas and a progressive stage of inflammation were present in the same animal groups. Cell aggregations resembling tuberculous granulomas and consisting of an aggregation of macrophages and lymphocytes were present. In contrast, no signs of inflammation were observed in the group of mice infected only with FLA trophozoites, suggesting that non-infected FLA did not cause apparent histopathology or disease when administered directly into the lungs of mice. We attempted to localize amoebae within the lung tissues of all groups of mice on days 1, 30 and 60 postinfection by using the Gimenez's staining method but no amoebae were detected at any of the time points (data not shown). The morphometric's analysis shown in Fig. 5C indicated there were no statistical differences in the number, size and type of cellular infiltration of granulomas between the groups of mice that had received axenic *M. bovis* or FLA/*M. bovis* either at high or low dose. Thus, there appeared to be no significant differences between the pathogenicity of the bacilli derived from axenic cultures or those housed within FLA.

Discussion

TB is an insidious disease yet to be controlled in most parts of the world and far from being eradicated because of reemergence in areas formally declared TB-free. The exact manner in which TB disease is transmitted is not fully understood. *M. bovis*, the causative agent of TB in livestock and wildlife, is a facultative intracellular pathogen capable of surviving within the host in a latent-dormant stage for long periods of time (Durr *et al.*, 2000; Andrade *et al.*, 2012; Muller *et al.*, 2013). Macrophages provide a safe niche for long-term survival within mammals (Andrade *et al.*, 2012), however, within the lower eukaryotes organisms, FLA are also hosts to several *Mycobacterium* species, including *M. bovis* (Mardare *et al.*, 2013). The current growing interest in amoebae as important mediators of emerging epidemics of human and animal diseases (Rue-Albrecht *et al.*, 2014; Scheid, 2014) stimulated these studies on the persistence of *M. bovis* within amoebae as a potential environmental source of TB infection.

We demonstrated that at least two genera of ubiquitously found amoebae (*Acanthamoeba* and *Vermamoeba*) may support the intracellular survival of *M. bovis*. Whereas *M. bovis* was not able to survive for more than 12 h within *D. discoideum*, all three spp. of *Acanthamoeba* and *Vermamoeba* supported the viability of the bacilli for at least 5 days. Other *Mycobacterium* spp., including *M. avium*, *M. leprae* and *M. avium* subsp. *paratuberculosis* were reported to infect and survive in *Acanthamoeba* (Mura *et al.* 2006; Drancourt, 2014; Wheat *et al.*, 2014). Survival within *D. discoideum*, in contrast, seems to be more variable depending on the *Mycobacterium* spp. as *M. marinum*, for instance, was reported to survive at least five days longer in this amoebal host (Solomon *et al.*, 2003).

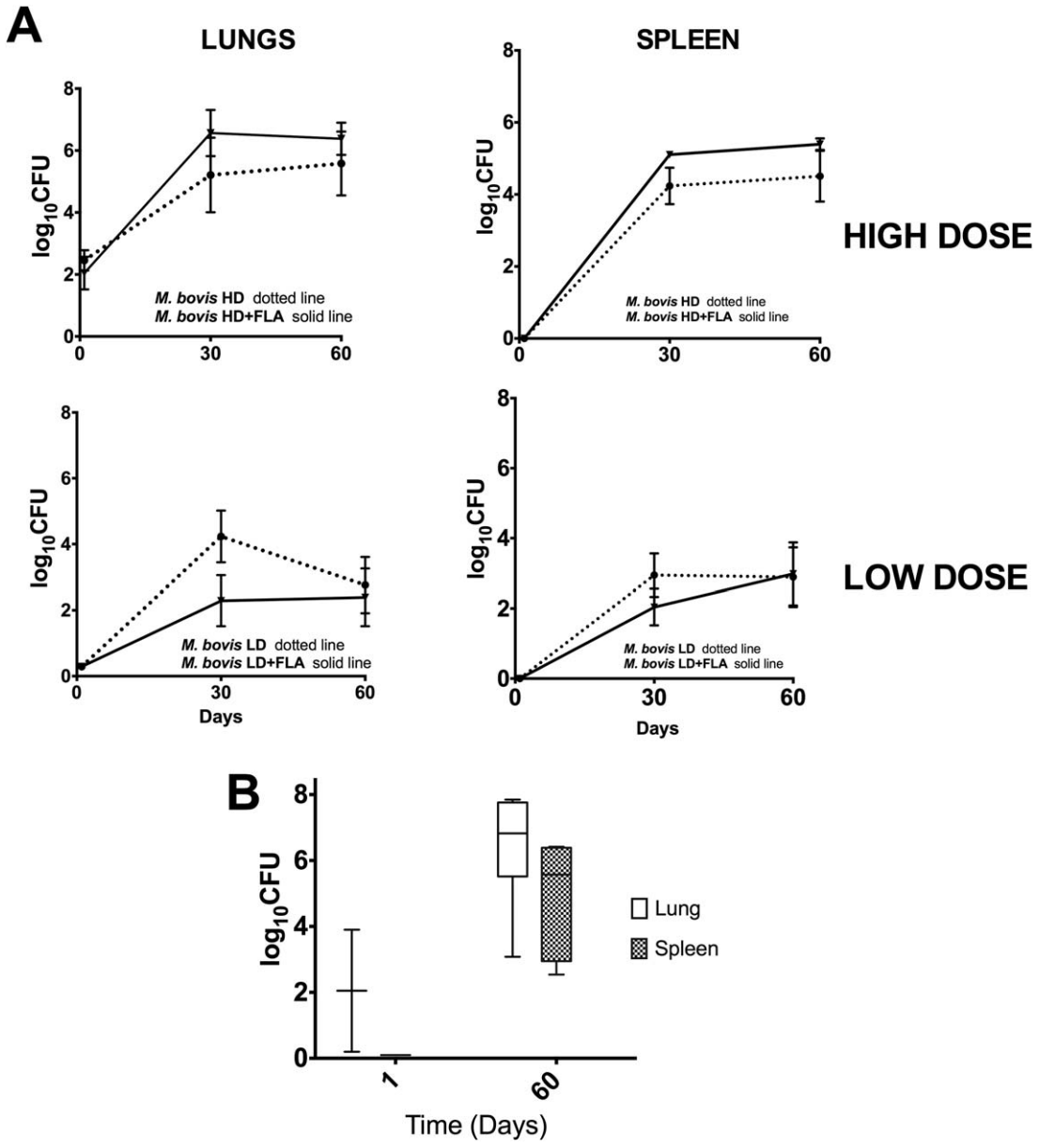

Fig. 4. Mouse infection with *M. bovis*-infected trophozoites.
A. Balb/c mice were infected with *A. polyphaga* trophozoites harbouring *M. bovis* via the intrapulmonary route and viable CFU in the lung and spleen were enumerated on days 1, 30 and 60 days postinfection. Infection with *M. bovis*-infected FLA is represented as solid lines and infection with axenically grown *M. bovis* is represented as dotted lines; high dose (HD) = 1×10^4 CFU; low dose (LD) = 1×10^2 CFU). Mice were sacrificed on 1 ($n = 3$), 14 ($n = 5$) and 60 ($n = 5$) days postinfection for the HD groups; and on days 1 ($n = 4$), 30 ($n = 5$) and 60 ($n = 5$) postinfection for the LD groups. The left lung lobule of each mouse was homogenized and serial dilutions were plated onto 7H11 agar for CFU determination.
B. Course of infection in mice infected with amoeba cysts carrying *M. bovis*. Sixty-day cyst-*M. bovis* cocultures were prepared as in Fig. 3 and used to infect mice ($n = 7$) via intrapulmonary delivery. The targeted dose of *M. bovis* infection in the lungs was 10^2 CFU. Animals were monitored and sacrificed, and lung (solid bars) and spleen (empty bars) were harvested and processed as in Fig. 4A.

Recent studies have highlighted the conservation of the basic mechanisms of interaction of several pathogens with lower and higher eukaryotes (Bozzaro and Eichinger, 2011). Successful intracellular bacteria like *Legionella pneumophila* and *Salmonella typhimurium* display virulence traits that are essential for productive infection of susceptible host cells whether these be aquatic amoebae or phagocytic cells of the human lung (Bozzaro and Eichinger, 2011). Such observations suggest that pathogenic bacteria may acquire their nefarious traits by virtue

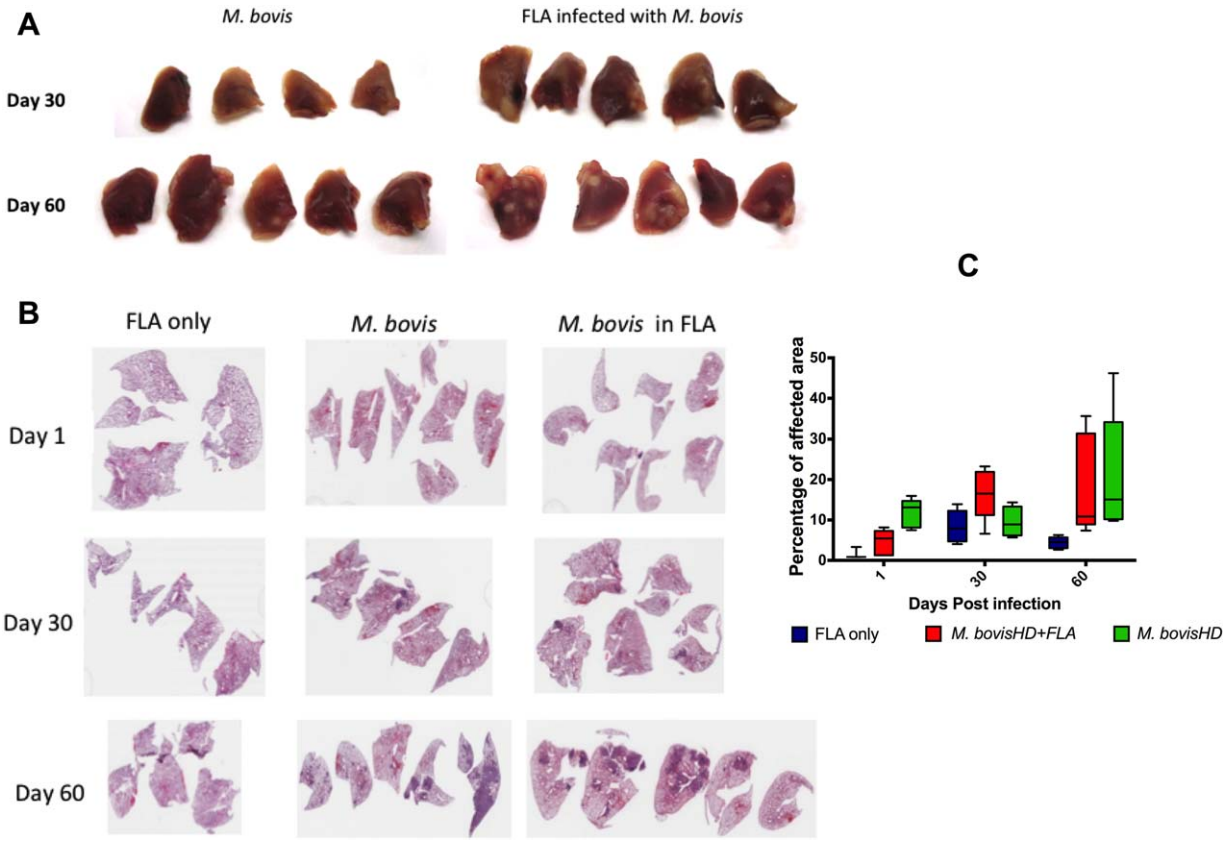

Fig. 5. Gross lesions and histopathology in lungs of mice infected with *M. bovis*-infected trophozoites.
A. Photographs of lungs harvested from mice infected with *M. bovis* (left) and *A. polyphaga* infected with *M. bovis* (right) showing gross lesions.
B. H&E of lung tissue sections from mice infected with FLA only, *M. bovis* high dose and FLA infected with *M. bovis* high dose on days 1, 30 and 60 postinfection.
C. Morphometric analysis of the histological sections shown in B. Shown is the average percentage area occupied by granulomatous lesions in the tissue section.

of adaptation against predation by amoebae. In a similar manner to macrophages, access to the intracellular niche of amoebae is via a broad repertoire of pattern recognition receptors (mannose receptors, Lectin, Gal/GalNac) that recognize and bind microorganisms (Bozzaro and Eichinger, 2011; Clarke *et al*., 2013). Interestingly, our data indicate that each of the amoeba tested in this study differs in its capacity to internalize *M. bovis* suggestive of different interaction mechanisms. Likewise, it is important to mention that the virulence of the bacterial strain used to infect amoebae is also a critical determinant of these interactions as others have reported that Bacillus Calmette–Guerin (BCG), an attenuated version of *M. bovis* (and currently the only vaccine available against bovine and human TB), was not able to survive within *A. castellanii* (Taylor *et al*., 2003). Recent studies have begun to shed light on the *M. bovis* genes required for the infection of bovine macrophages and amoebae (Stewart, 2016).

Although *Acanthamoeba* housed *M. bovis* for at least 5 days (trophozoites) or 60 days (cysts), the bacilli did not appear to undergo intra-amoebic proliferation. One possible interpretation of these data is that there is as much intracellular killing as there is intracellular replication. Alternatively, it is also possible that the bacilli remained in a latent-dormant form that may resemble that described for infection within macrophages in the mammalian host. Whether the same genes governing *M. bovis* dormant stage in the mammalian host (Boon and Dick, 2002) are also activated during dormant stage in the lower eukaryotic host needs to be determined. Furthermore, a previous study has shown that *M. marinum* infect and replicate within *A. castellanii* and that the bacilli escape via lysis of the amoeba host cell and actin tail formation (Kennedy *et al*., 2012). In this study, the possibility of *M. bovis* escaping amoebae and potentially infecting other amoebae via a bystander mechanism is unlikely because we did not observe extracellular bacteria or increased rate of infected

amoebae over time. Moreover, other bacteria–amoeba interactions such as those involving virulent strains of *Francisella tularensis* and *Acanthamoeb*a have been shown to induce the rapid formation of cysts (El-Etr *et al.*, 2009). Our infections of *Acanthamoeba* trophozoites with *M. bovis* did not reveal a higher encystment rate compared to uninfected *Acanthamoeba* cultures. Thus, interactions of *M. bovis* with trophozoites have no adverse effects on the amoebae leading to encystment. Encystment of *M. bovis*-infected *Acanthamoeba* trophozoites, however, was possible when environmental conditions became unfavourable (in our study, upon shifting the coculture medium to a low-nutrient encystment buffer). Thus, it is possible that hostile environmental conditions (such as winterized weather conditions, changes in pH and desiccation) cause *M. bovis*-infected amoebae to encyst. The length of the survival of uninfected cysts under natural conditions is variable, however it has been shown that cysts of *Acanthamoeba* spp. are capable of surviving and retaining their invasive properties after 24 years of encystment at 4°C; however, their capacity to cause infection in mice is greatly reduced (Mazur, *et al*. 1995; Khan, 2003).

Finally, our studies demonstrated that *M. bovis* housed inside trophozoites or cysts of *A. polyphaga* retain full capacity to establish infection when delivered to a mammalian host (mice in this instance). The bacilli isolated from long-term amoeba cocultures were fully capable of establishing a pulmonary infection and proliferating within the host tissue. Moreover, the bacilli were able to disseminate to other organs (spleen). When measured in terms of bacterial burden in the lung and spleen, infection of mice with bacilli derived from amoebae did not differ substantially from infections caused by axenic bacilli. Furthermore, no differences were noted at the level of pulmonary pathology between these two groups of mice. Thus, passaging *M. bovis* bacilli through amoebae did not appear to affect pathogenesis but rather provide a persistence realm for the pathogen.

It is important to note that a very small number of *M. bovis* bacilli are sufficient to cause TB in a susceptible host. Our overarching hypothesis is that, in the environment, amoebae become infected with *M. bovis* released by infected animals. A recent report (Mardare *et al.*, 2013) was able to isolate *Acanthamoeba* with intracellular acid-fast bacilli from soil sampled at latrines of *M. bovis*-infected badgers. Unfortunately, attempts at culturing those samples and testing them by IS6110 PCR for the presence of *M. bovis* rendered negative results. Thus, the hypothesis that environmental amoebae play a role in the survival of *M. bovis* in the environment still needs to be proven, but the studies presented here provide strong support for the hypothesis that *M. bovis* bacilli housed within trophozoites or encysted amoebae may establish a TB infection in a mammalian host. Thus, the possibility rationally exists that a few environmentally infected trophozoites/cysts would be sufficient to infect a mammalian host (badger, possum, or cattle, goats, deer) and thus initiate a new sequence of events leading to re-emergence of bovine TB disease.

Experimental procedures

Mice

Balb/c female mice were purchased from Jackson Laboratories and maintained at Colorado State University Biosafety Level 3 (BSL-3) facility. Mice were kept in a pathogen free environment, with free access to standard mouse chow and water. Protocols for use of mice in these studies were approved by the Animal Care and Use Review Office (ACURO) of USAMRMC Protocol No. 68103-LS-DRP.01 under title 'Feral Macrophages: Dynamics of Free-Living Amoeba Interactions with Pathogens' and the Institutional Animal Care and Use Committee Animal (IACUC) at Colorado State University under the protocol number 15–6154A. Infected mice were monitored daily by trained animal laboratory technicians at Laboratory Animal Resources and researchers with experience in animal handling certified by the University.

Amoeba strains

Stocks of axenic *A. lenticulata* ATCC 30841, *A. castellanii* ATCC 30232, *A. polyphaga* CCAP 1501/18, *V. vermiformis* (*H. vermiformis*) ATCC 50237 and *D. discoideum* NC4A1:DBS0236602 were obtained from the American Type Culture Collection (Manassas, VA, USA) and the Dicty.org organization. Amoebae were cultured as previously reported (Wheat *et al.*, 2014). Briefly, *Acanthamoeba* trophozoites were cultured in 1× peptone yeast glucose medium [PYG medium prepared by adding 900 ml of Page's amoebae saline (PAS) (60 mg NaCl, 2 mg $MgSO_4{\cdot}7H_2O$, 68 mg KH_2PO_4, 71 mg $NaHPO_4$ and 2 mg $CaCl_2$ in 500 ml dH_2O, pH = 6.9)] to 100 ml of 10× PYG solution [50 g proteose peptone (Difco); 5 g yeast extract (Difco); 2.45 g $MgSO_4{\cdot}7H_2O$; 2.5 g sodium citrate$\cdot 2H_2O$; 0.05 g ammonium iron sulfate (NH_4) $2Fe(SO_4)2{\cdot}6H_2O$; 0.85 g KH_2PO_4; 0.89 g $Na_2HPO_4{\cdot}7H_2O$; 22.5 g α-D-glucose; 0.295 g $CaCl_2$ in 250 ml dH_2O (pH 6.6)]. *Vermamoeba* trophozoites were cultured in modified PYNFH medium (pH 6.9) (ATCC medium 1034). *D. discoideum* was maintained in HL5 medium (pH 6.4–6.9) (Fey *et al.*, 2007).

Free living-amoeba (FLA) trophozoites (in monolayers) of *Acanthamoeba* were cultured at 28°C and passaged in PYG medium twice a week. *V. vermiformis* was cultured at 28°C and passaged in PYNFH medium three times a week. *D. discoideum* was cultured at room temperature (21–23°C) and passaged in HL5 medium twice a week. All amoeba strains were cultured with penicillin–streptomycin and passaged at most six times before freezing to avoid down regulation of genes essential for encystment (Johnston *et al.*, 2009).

Generation of M. bovis bacilli expressing green fluorescence protein

M. bovis strain # 95–1315 was originally isolated from an infected deer in the United States (Waters *et al.*, 2014). *M.*

bovis expressing green fluorescence protein (*M. bovis*-GFP) was generated by electroporation of the plasmid pMSP12::gfp. To confirm the positive expression of GFP in *M. bovis* bacilli, colonies were harvested, expanded by culturing in 7H9 medium (7H9 agar base 4.3 g, OADC 50 ml, Tween 80 0.05% and 4.1 g l^{-1} sodium pyruvate, 50 μg ml^{-1} of kanamycin and 1000 ml of water) at 37°C for 2 weeks and analysed by confocal microscopy and flow cytometry. Transformants with highest GFP expression (as determined by mean fluorescence intensity) were subsequently subcultured in the same medium and stored at – 80°C until further use.

M. bovis-GFP infection of free-living amoebae

Amoeba trophozoites (in monolayers) were infected with viable *M. bovis*-GFP at a multiplicity of infection (MOI) 1:10 (amoeba:bacilli) and incubated for 12 h in medium diluted 1:2 with PAS at the same temperature as the amoeba culture. Prior to mixing FLA with bacilli, the *M. bovis* cell suspension was passed through a $26\frac{1}{2}''$ needle 15 times to break any clusters of bacteria. Cocultures of FLA–*M. bovis* were washed three times with PAS medium. Thereafter to remove extracellular bacteria the same cultures were also incubated with 100 μg ml^{-1} of amikacin during 2 h and then washed as previously reported (Waters *et al.*, 2014). To evaluate if viable extracellular bacteria were remaining in these cultures, 500 μl of supernatant harvested from last wash were cultured onto 7H11 agar plates (as described before). From these same samples and to enumerate if viable bacilli were present within FLA trophozoites, the pellets were re-suspended in their corresponding amoeba medium and half of the sample was lysed by incubating with sterile sodium dodecyl sulfate (SDS) at a final concentration of 0.5% for 5 min followed by serial dilutions (1:5 with PAS media) of the resulting lysate and culturing onto 7H11 agar plates as above. The number of colony forming unit (CFU) in each sample was enumerated after incubating agar plates at 37°C for 3 weeks. The other half of the sample was processed for confocal microscopy as explained below.

Confocal microscopy and flow cytometry analysis

Samples of *M. bovis*-GFP infected and control FLA were fixed with 4% Paraformaldehyde (PFA) for an hour, washed with sterile phosphate buffer solution (PBS), and resuspended in 50–100 μl of PBS. Samples were analysed using a Zeiss LSM 510 confocal microscope equipped with the Zen 2009 software (Zeiss). To determine the spatial localization of *M. bovis* within amoebae, serial optical sections of infected FLA were taken at 0.42 nm intervals using a 488 nm excitation laser. FACSCanto II (BD) flow cytometer was used to determine the percentage and intensity of GFP fluorescence in cocultures of *M. bovis*-GFP and FLA.

Kinetics of M. bovis within trophozoites of free-living amoebae

The five species of FLA trophozoites (uninfected and *M. bovis* infected) were incubated in triplicates in their corresponding culture conditions (as explained above) for 5 days. There were no antibiotics added during this time except for 50 μg ml^{-1} of kanamycin to maintain the GFP-plasmid in the *M. bovis*-GFP bacilli. At 48, 72 and 120 (5 days) h of culture, samples of 100 μl were taken from each well and processed for confocal microscopy analysis. Alternatively, CFU were enumerated from coculture lysates by plating on 7H11 agar plates. *D. discoideum* infections were exclusively monitored by culturing lysates of these cells onto 7H11 agar plates and enumerating the CFU because we were unable to visualize positive fluorescent bacilli in these cocultures by confocal microscopy.

Persistence of M. bovis within encysted free-living amoebae

Trophozoites of *A. polyphaga*, *A. castellanii*, *A. lenticulata*, *V. vermiformis* and *D. discoideum* were infected as described above. After the last wash, trophozoites were forced to encyst by changing the culture medium to encystment buffer (0.1 M KCl, 0.02 M Tris-HCl, pH 8.0, 8 mM $MgSO_4$, 0.4 mM $CaCl_2$ and 1 mM $NaHCO_3$) followed by incubation at 28°C for all amoeba strains except for *D. discoideum* that was incubated at RT (~ 21°C) for 2 months. Samples from each culture were collected after 1 and 2 months of infection. The encystment rate of each culture was monitored on weekly basis by light microscopy. Thereafter, long-term encysted cocultures were forced to excyst by culturing the samples in their corresponding complete amoeba growth medium and temperature optimum. *M. bovis* was then extracted from the excysted trophozoites by lysing the samples with 0.5% SDS and plating onto 7H11 agar plates as described above.

Culture of axenic M. bovis in encystment medium

Axenic *M. bovis*-GFP was cultured in encystment medium and, for comparison of viability/growth, in 7H9 medium at 28 and 37°C for 15 days. After 15 days, the viability of *M. bovis*-GFP was determined by plating serial dilutions of each culture onto 7H11 agar plates and incubating the plates at 37°C during 3–4 weeks.

Infection of mice with M. bovis-infected trophozoites of A. polyphaga

Mice ($n = 5$ per group) were randomly assigned to groups as shown in Table 1. Inoculum for infection was prepared as explained above. Briefly, *A. polyphaga*-trophozoites were infected with *M. bovis*. Twelve hours postinfection, amoeba cultures were washed three times followed by treatment for 2 h with amikacin (100 μg ml^{-1}). The resulting cell suspension was counted and adjusted to 10^6 FLA ml^{-1}. Thereafter, mice were anesthetized using a mix of isofluorane and oxygen and 50 μl of cell suspensions were administered via intrapulmonary delivery to each mouse. As controls and to evaluate the potential pathogenicity of FLA and bacilli individually, groups of mice ($n = 5$) were infected with 50 μl per animal of a cell suspension containing 5×10^4 trophozoites of *A. polyphaga* or a HD or LD dose of axenically-grown *M. bovis* containing 1×10^4 and 1×10^2 CFU, respectively. Mice were sacrificed on days 1, 14, 30 and 60 postinfection.

Enumeration of bacterial burden in the lungs and spleen

Determination of bacterial load at different times post infection (days 1, 14, 30 and 60) was as follows: after euthanasia, mouse tissues (lung and spleen) were homogenized using the Next Advance Bullet Blender® (Averill Park, NY, USA). Briefly, the left lobe of the lung or spleen were placed in a 1.5 ml sterile, safe lock Eppendorf tubes containing 0.5 ml of sterile saline and 3 mm × 3.2 mm, sterile stainless steel beads. The tubes were then placed in the Bullet Blender and homogenized for 4 min at 8000 rpm. Serial dilutions of homogenized organs in PBS were plated on 7H11 agar plates and cultured for 3 weeks at 37°C to determine CFU counts.

Histopathological analysis

The postcaval lobe of the lung of each mouse was fixed in 4% PFA. Samples were inactivated in 4% PFA solution for 48hrs and transferred to histology cassettes for processing using standard histological protocols for sectioning and staining with Haematoxylin-Eosin (H&E). H&E staining was visualized using the Aperio Digital Scanner (Leica Biosystems) and analysed with the Image Scope software. Morphometric analysis was done by determining the percentage of the surface of lung lobule in each section occupied by granulomatous lesions in each animal group. Gimenez stain. Amoebae (trophozoites or cysts) were visualized using the Gimenez stain as previously described (El-Sayed and Hikal, 2015). Briefly, tissue sections were incubated with filtered carbol fuschin solution for 2 min. Slides were rinsed in tap water and incubated in malachite green (counterstain) for 3 min or until a red colour emerged. Slides were rinsed in tap water and air-dried.

Infection of mice with *M. bovis*-infected cysts of *A. polyphaga*

Trophozoites of *A. polyphaga* were infected with *M. bovis* and forced to encyst as explained above. Encystment was monitored at least twice a week using light microscopy. After a month, all cells appeared as cysts by microscopy. In preparation for infection of mice with cysts infected with *M. bovis*, cysts were washed with sterile and low endotoxin 0.9% saline solution (Teknova, Cat. Number S5818) two times, and thereafter were re-suspended in low endotoxin 0.9% saline solution. Mice were infected with cysts of *A. polyphaga* infected with *M. bovis* at a dose of 5×10^3 cysts in 50 μl, following the same procedures as described above. On days 1 ($n = 2$) and 60 ($n = 7$) postinfection, mice were sacrificed to evaluate bacterial burdens as described above.

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