

# On the killing of mycobacteria by macrophages

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## Summary

Both pathogenic and non-pathogenic mycobacteria are internalized into macrophage phagosomes. Whereas the non-pathogenic types are invariably killed by all macrophages, the pathogens generally survive and grow. Here, we addressed the survival, production of nitrogen intermediates (RNI) and intracellular trafficking of the non-pathogenic *Mycobacterium smegmatis*, the pathogen-like, BCG and the pathogenic *M. bovis* in different mouse, human and bovine macrophages. The bacteriocidal effects of RNI were restricted for all bacterial species to the early stages of infection. EM analysis showed clearly that all the mycobacteria remained within phagosomes even at late times of infection. The fraction of BCG and *M. bovis* found in mature phagolysosomes rarely exceeded 10% of total, irrespective of whether bacteria were growing, latent or being killed, with little correlation between the extent of phagosome maturation and the degree of killing. Theoretical modelling of our data identified two different potential sets of explanations that are consistent with our results. The model we favour is one in which a small but significant fraction of BCG is killed in an early phagosome, then maturation of a small fraction of phagosomes with both live and killed bacteria, followed by extremely rapid killing and digestion of the bacteria in phago-lysosomes.

## Introduction

Infection with mycobacteria remains a major health problem. Although in the recent years infections by

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*M. avium* are gaining importance among immunosuppressed individuals, tuberculosis is still the major problem. Mycobacteria from *Mycobacterium tuberculosis* complex, *M. africanum*, *M. bovis* and *M. tuberculosis*, are the aetiological agents of this disease (Wedlock *et al.*, 2002; Baker *et al.*, 2006). In many countries, *M. bovis* is responsible for important economic losses due to bovine tuberculosis (Pollock and Neill, 2002; Biet *et al.*, 2005). This *Mycobacterium* can also infect humans, causing tuberculosis, with a pathobiology indistinguishable from that one caused by *M. tuberculosis* (Hedvalle, 1949; Wedlock *et al.*, 2002). The success of these pathogens is, to a large part, dependent on their ability to inhibit host defence mechanisms and persist in a potentially hostile environment, the macrophage phagosome, and is also facilitated by the emergence of multidrug-resistant strains and extensively drug-resistant strains (Gandhi *et al.*, 2006).

The crucial difference between non-pathogenic and pathogenic mycobacteria is that the latter arrest maturation of phagosomes at an early stage (Clemens, 1996; Via *et al.*, 1998; Vergne *et al.*, 2004; Stewart *et al.*, 2005). Although the mechanism responsible for this process is poorly understood, the arrested phagosome provides a friendly environment for pathogen survival and even growth (Hart *et al.*, 1972; Russell, 2001; Pethe *et al.*, 2004). The mycobacterial phagosome has particular characteristics such as low content of vacuolar ATPase (V-ATPase) (Sturgill-Koszycki *et al.*, 1994), and thereby incomplete luminal acidification, as well as a low content of mature lysosomal enzymes. Collectively these pathogen-altered conditions are assumed to facilitate survival of the mycobacteria (Russell, 2001; Vergne *et al.*, 2004).

The question of how and where mycobacteria are killed by macrophages is still open, for both the non-pathogenic and pathogenic forms (Kusner, 2005). From the literature two extreme models can be envisaged. In the first, the killing only occurs *after* phagolysosome fusion; in this model, the macrophage-killing potential depends on its ability to fuse the phagosome with late endocytic organelles, here, often simplified as 'lysosomes'. Thereby, it is presumed that the combination of low pH and a battery of lysosomal hydrolases induces both killing and digestion/clearance of the bacteria. In agreement with this hypothesis are extensive data correlating a high incidence of phagolysosomal fusion with a high rate of killing, and a low extent of this fusion with high survival (Silva *et al.*, 1987; Russell, 2001; Kusner, 2005). This model is widely

favoured and is also supported by an early study by Cohn (1963a,b), who first showed that lysosomal extracts are potentially bacteriocidal; that study showed that extracts of macrophage or neutrophil lysosomes can induce killing of *E. coli* within 1 min. In the case of *M. smegmatis* in J774 cells, a rapid 1–4 h killing period coincides with extensive phagolysosome fusion, and the internalization of a cocktail of lysosomal enzyme inhibitors by endocytosis into infected macrophages strongly reduced the potency of this early killing period Anes *et al.* (2006).

[2] An alternative hypothesis emerged from the pioneering study by Armstrong and D'Arcy Hart (1971). Based on a thorough EM analysis in which endocytosed ferritin was used as a lysosomal marker, these authors came to the conclusion that the live *M. tuberculosis* actively avoided phagolysosome fusion, a point that is widely appreciated. Less appreciated is their conclusion that only *after* being killed in a non-matured phagosome could the phagosome fuse with lysosomes. In contrast, when *M. tuberculosis* opsonized with a specific anti-*M. tuberculosis* antibody before internalization, the majority of the bacterial phagosomes now fused with lysosomes. However, *M. tuberculosis* nevertheless multiplied at the same rate as the non-opsonized bacteria, that were predominantly in non-mature phagosomes (Hart *et al.*, 1972). A similar conclusion was made later by Gomes *et al.* (1999). In these experiments, the accumulation of pathogenic mycobacteria in a mature phagolysosome (that also contained *Coxiella burnetii*) did not induce killing of *M. tuberculosis* or *M. avium*. More recently, elegant assays were used to select for *M. tuberculosis* mutants that can survive in mature phagosomes (Pethe *et al.*, 2004; MacGurn and Cox, 2007; Majlessi *et al.*, 2007). All these observations are clearly at odds with the model in which mycobacteria are killed in mature phagosomes.

[3] Nitric oxide (NO) produced from iNOS is a more well-defined component of host defence against intracellular pathogens, including *M. tuberculosis* (Shiloh and Nathan, 2000). Phagocytes produce NO and a plethora of other potent mycobacteriocidal reactive nitrogen intermediates following stimulation with mycobacteria, and this process is accentuated by inflammatory cytokines like  $\gamma$ -IFN and/or bacterial lipopolysaccharides (LPS) (Chan *et al.*, 2001; Webb *et al.*, 2001; Morris *et al.*, 2003). The tolerance of mycobacteria *in vitro* to RNI is strain-, dose- and time-dependent, with the pathogens being inherently more resistant than the non-pathogens (O'Brien *et al.*, 1994; Rhoades and Orme, 1997; Long *et al.*, 1999; Long *et al.*, 2005). In our recent study of the non-pathogenic *M. smegmatis*, the macrophage needs at least 4 h, and up to 24–48 h after infection to completely kill the bacteria. Nevertheless, the expression of iNOS and the production of NO, which facilitated bacterial killing, was restricted to the first 2 h (Anes *et al.*, 2006).

Most published studies have compared up to two different cell types and two different mycobacteria, often with different virulence, such as *M. tuberculosis* H37Ra and Rv. We decided to make a broader analysis of mycobacterial–macrophage interactions. The main goal was to try to find a pattern of killing or survival that could be clearly correlated with macrophage-killing mechanisms. Towards this goal, after preliminary studies with *M. smegmatis*, we tested different mycobacteria (*M. bovis* and *M. bovis* BCG) in up to six different cell types: mouse [bone marrow macrophages (BMM), J774 and Raw], human [monocyte-derived macrophages from human peripheral blood (HMDM) and THP1] and bovine macrophages (monocyte-derived macrophages from bovine peripheral blood: BMDM). For *M. bovis* spp. we first monitored intracellular survival following colony-forming units (cfu) and then investigated the role of NO. Subsequently, different assays for phagolysosome fusion and acidification were applied.

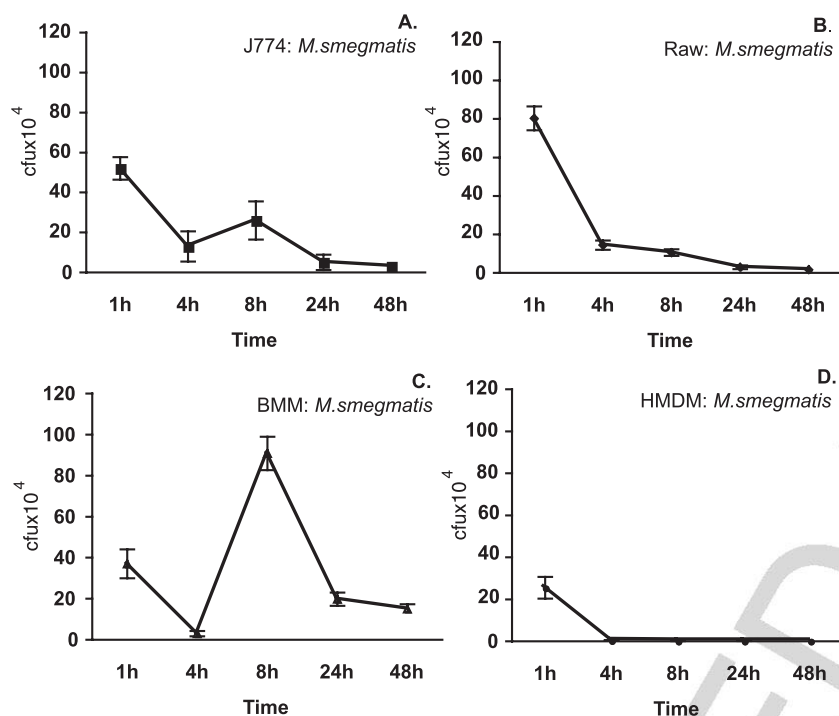
Our results suggest that a specific pathogen-induced macrophage 'program' is induced upon infection that correlates with the bacterial doubling time, in which phases of high macrophage-killing potential often alternate with phases conducive to bacterial growth. RNI and low pH play key roles in killing during the first day(s) of infection of pathogenic mycobacteria. Subsequently, RNI- and pH-independent mechanisms kill the bacteria within phagosomes. Our results, in conjunction with theoretical modelling for BCG infection, argue that a significant killing of these mycobacteria must occur in non-matured, early phagosomes, in agreement with Armstrong and D'Arcy Hart (1971). However, the majority of BCG is more likely to be killed and digested after phagolysosome formation.

## Results

### *Kinetics of M. smegmatis survival in different host macrophages*

From previous studies, *M. smegmatis* infecting J774 are killed completely within 48 h under all infection conditions (Kuehnel *et al.*, 2001; Anes *et al.*, 2003; Anes *et al.*, 2006). *M. smegmatis* survival kinetics inside J774 is complex and was characterized in detail previously (Anes *et al.*, 2006). A striking observation was a dynamic interplay between bacteria and J774 cells (Fig. 1A). A first phase of killing (4 h) was followed by growth (4–8/9 h) and subsequent killing phases between 9 and 48 h.

Here we used GFP-expressing *M. smegmatis* and followed the cfu in BMM derived from C57BL/6 mice and in HMDM. In BMM, the dynamics of *M. smegmatis* showed a super-imposable pattern to that seen in J774 macrophages (Fig. 1A and C). In HMDM (Fig. 1D) and Raw



**Fig. 1.** Intracellular fate of *M. smegmatis* in different macrophages. Colony-forming unit (cfu) estimates for *M. smegmatis* (GFP) are shown in J774 macrophages (A), Raw macrophages (B), BMM (C) and HMDM (D).

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(Fig. 1B), the initial killing phase was seen but, thereafter, different kinetics were observed, with the remaining *M. smegmatis* being killed slowly in a continuous fashion. For HMDM, the same pattern of killing by 4 h was also seen at higher levels of infection (results not shown). It is striking, and surely no coincidence, that in all four cell types, the first killing period is found between 1 and 4 h and, when growth is permitted, it peaks in both cells around 8 h. These data suggest that the timing of these cycles is likely to be induced by the bacteria that divide every 3 h.

#### Kinetics of *M. bovis* spp. survival in different host macrophages

The above data suggested that the cycles of killing and growth might be induced by factors related to the cell cycle of the pathogen. This idea made it interesting to investigate pathogenic mycobacteria, which have a far longer division time (around 24 h) than *M. smegmatis*. We therefore tested BCG and *M. bovis* in six different cell lines: J774, Raw, BMM, the human monocytic like cell line THP1, HMDM and BMDM (Fig. 2A–F). We first established infection conditions for BCG-GFP in J774. This GFP strain grows at identical rates, *in vitro* and in macrophages, to the parental non-GFP strain (results not shown). Bacteria were added at OD<sub>600</sub> 0.1 for 3 h; this gave on average 2–9 bacteria per cell. For the other cells, the same conditions were used, except for HMDM and BMDM, in which case OD<sub>600</sub> 0.1 appeared to be toxic for

the cells. We therefore used an OD<sub>600</sub> of 0.01, which allowed the cells to survive normally for at least 7 days. In all our reported experiments, the total number of live macrophages remained almost constant over the period of infection, as assessed by cell counts.

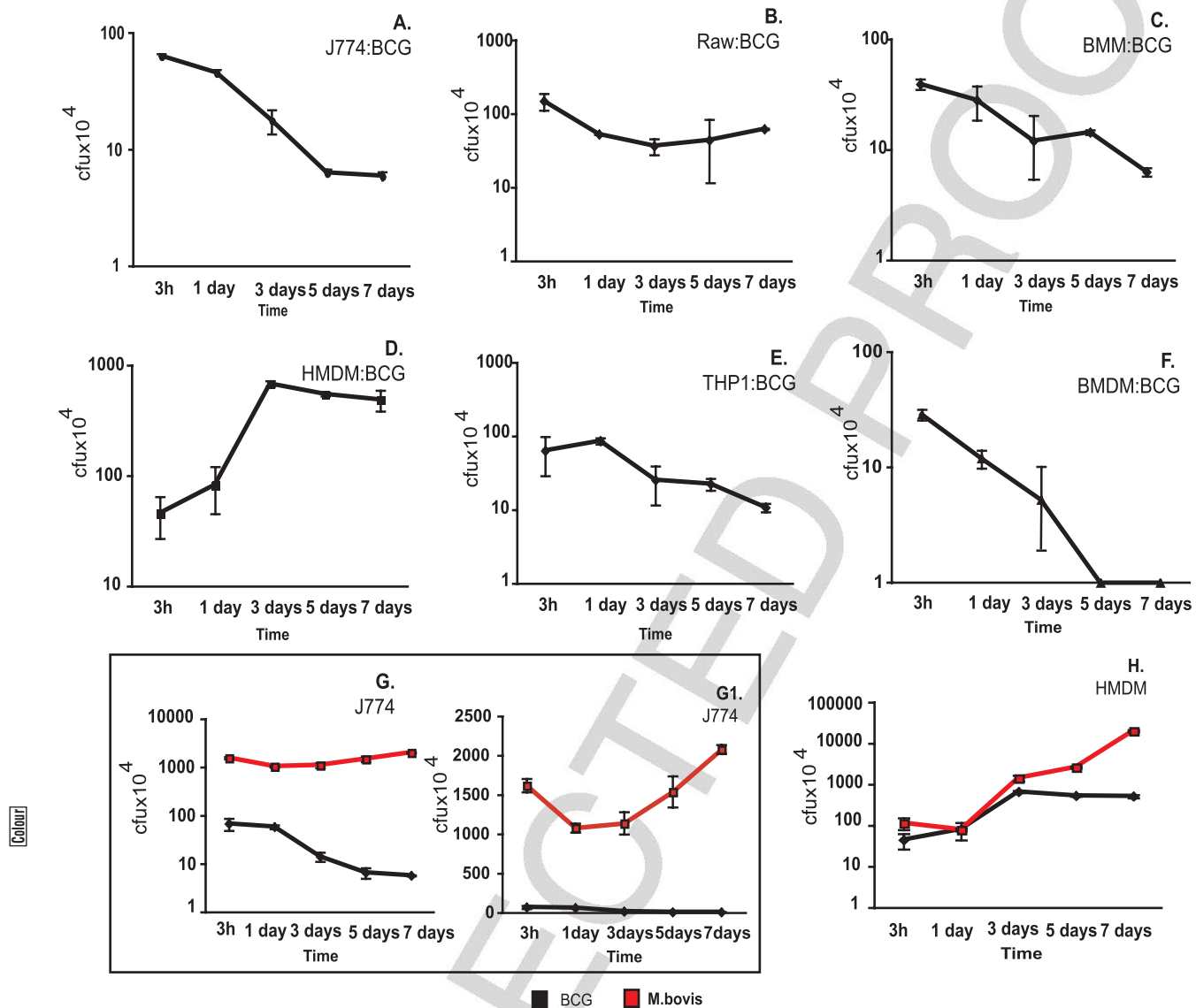
Using the trypan blue exclusion method, less than 1% macrophage death was detected under all conditions. Thus, in this study macrophage cell death could be excluded as having any significant role in the killing of mycobacteria.

As shown in Fig. 2A–F, we saw three different patterns of bacterial survival. In BMM, THP1 and J774, an overall pattern of killing was observed over the 7 day period. With Raw, neither significant growth nor killing was observed. In contrast, in HMDM, BCG grew up to 3 days, and thereafter, neither growth nor killing was seen. We initially used time scales of hours for these experiments, but it soon emerged that the switches from killing to growth, when they occurred, happened on a much slower time scale with the pathogenic mycobacteria, in agreement with the hypothesis that timing of the macrophage cycles of killing and growth is related to the bacterial growth/division time.

We next compared BCG with the virulent strain of *M. bovis* in J774 and HMDM. In J774, whereas BCG could be steadily killed over the course of 7 days infection (Fig. 2G), *M. bovis* could be killed only during the first 24 h that was following a growth phase; this is more easily observed using a linear scale (Fig. 2G1) rather than the semilog scale used in Fig. 2G. With HMDM, both BCG and *M. bovis* could steadily grow at a level easily seen on

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**Fig. 2.** Intracellular fate of BCG (GFP) or *M. bovis* in different host macrophages. Colony-forming unit (cfu) estimates for BCG (GFP) in J774 macrophages (A), Raw macrophages (B), BMM (C), HMDM (D), THP1 cells (E) and BMDM (F). A comparison between the cfu estimates for BCG (GFP) and *M. bovis* in J774 macrophages (G) and HMDM (H) is also shown. G and G1 show the same data plotted differently (see y-axis).

a semilog scale (Fig. 2H). Thus, both bacteria behave quite differently in the two macrophage cell types and are better adapted to survive and grow exponentially in the human primary macrophages.

It is difficult to visualize any meaningful trends in these data, although some cells (THP1 and BMM) tended towards a similar gradual killing of BCG. There was no obvious ranking of macrophage 'killer cells' when we compared their abilities to kill *M. smegmatis*, BCG or *M. bovis*. For example, J774 kills BCG better than Raw, but Raw is much more effective than J774 cells in clearing *M. smegmatis*.

#### Role of inducible NO synthase and NO release

We next related the pattern of intracellular growth/killing to the pattern of macrophage activation that is known to contribute to killing of mycobacteria. We first investigated NO production using the Griess reagent. In our recent study on *M. smegmatis*, in J774 cells we showed that NO is only produced up to 2 h after infection, where it contributed to the first killing phase (Anes *et al.*, 2006). Here we focused on BCG and *M. bovis*.

First, we tested the susceptibilities of different mycobacteria to NO *in vitro*. For these, we used two NO

sources NOC-18 (Fig. 3A and B) and acidic solutions of sodium nitrite (not shown). BCG could be killed by 0.1  $\mu\text{M}$  NOC-18 after 1 day, but after 14 days in culture, a 100-fold higher concentration of NOC-18 was needed for efficient killing (Fig. 3A). In contrast, *M. bovis* was highly resistant; after 1 day in culture, only partial killing was seen with 1  $\mu\text{M}$  NOC-18 (a 10-fold higher concentration than that needed to kill BCG) and, to achieve complete killing, a 1000-fold higher concentration of NOC-18 was required (Fig. 3B).

We next investigated cell production of NO in response to BCG and *M. bovis*. NO was not detected in supernatants of HMDM under any conditions, in agreement with other studies on human macrophages (Clemens and Horwitz, 1995).  $\gamma$ -IFN is known to induce strong iNOS activity and often also an increase in mycobacterial killing (Nozaki *et al.*, 1997; Bonecini-Almeida *et al.*, 1998; Carpenter *et al.*, 1998; Schaible *et al.*, 1999). As seen in Table 1, overnight treatment of J774 cells with  $\gamma$ -IFN before infection led to a highly significant increase in NO levels after 1 and 2 day infection with BCG or *M. bovis*.

We next tested the effects of NO on *M. bovis* spp. survival in J774. For this, we either increased NO artificially, using NOC-18 or  $\gamma$ -IFN, or decreased NO levels by blocking the enzyme (iNOS) that synthesizes it, with a specific inhibitor: L-NAME. At 1 day post infection, 0.1 or 1  $\mu\text{M}$  NOC-18 was able to significantly increase the killing of BCG and *M. bovis* (around 40% increase in killing) respectively (Fig. 3C and D). The extent of killing by NOC-18 and  $\gamma$ -IFN was similar for *M. bovis*, whereas  $\gamma$ -IFN had a small but insignificant effect on BCG survival at day 1 (Fig. 3C and D). With L-NAME, a reproducible and significant (30 and 40%) increase in survival was observed after 1 day infection of BCG- and *M. bovis*-infected macrophages (Fig. 3C and D). After 1 day, neither NOC-18 nor L-NAME affected mycobacterial survival (results not shown). Taking in account the limit of quantification of NO using the Griess method, we observed low but detectable amounts of NO production during the early infection periods with both strains (Table 1: 3 h and 1 day post infection).

Figure 3E and F shows the effects of an extended treatment of infected cells with  $\gamma$ -IFN, until 7 day infection. These results show clearly that, for BCG,  $\gamma$ -IFN treatment modestly but significantly increases bacterial killing until day 3, with little effect thereafter (Fig. 3E). With *M. bovis* (Fig. 3F), the effect of  $\gamma$ -IFN was predominantly seen between 3 h and 1 day. It is notable (and will become more evident in Fig. 7) that the main effect of  $\gamma$ -IFN in enhancing killing coincides with an innate early phase of bacterial killing. In another series of experiments, we found the same pattern with *M. tuberculosis* in response to  $\gamma$ -IFN (Fig. S1). We presume that the main effect of  $\gamma$ -IFN in the killing process operates via the RNI system.

Because the mouse macrophages made NO in response to the mycobacteria, we next correlated the expression of iNOS, the enzyme that makes it after infection of J774 cells with BCG and *M. bovis*. Using immunofluorescence microscopy, we could not detect any signal for iNOS in uninfected cells (Fig. 4A) whereas detected a significant increase in cytoplasmic labelling for iNOS already after 1 h infection with *M. bovis* (Fig. 4C) or BCG (Fig. 4B). No specific association of iNOS with phagosomes of either BCG or *M. bovis* was seen, in agreement with the data of Anes *et al.* (2006) and other studies (Miller *et al.*, 2004). After treatment of uninfected (Fig. 4D) or infected macrophages (Fig. 4E) with  $\gamma$ -IFN, a general increase in iNOS labelling was clearly observed. Immunofluorescence microscopy was a more sensitive estimator of iNOS than Western blotting. Although by Western blot, iNOS could be detected after treatment with  $\gamma$ -IFN, no band was detectable in untreated infected cells (Fig. 4F).

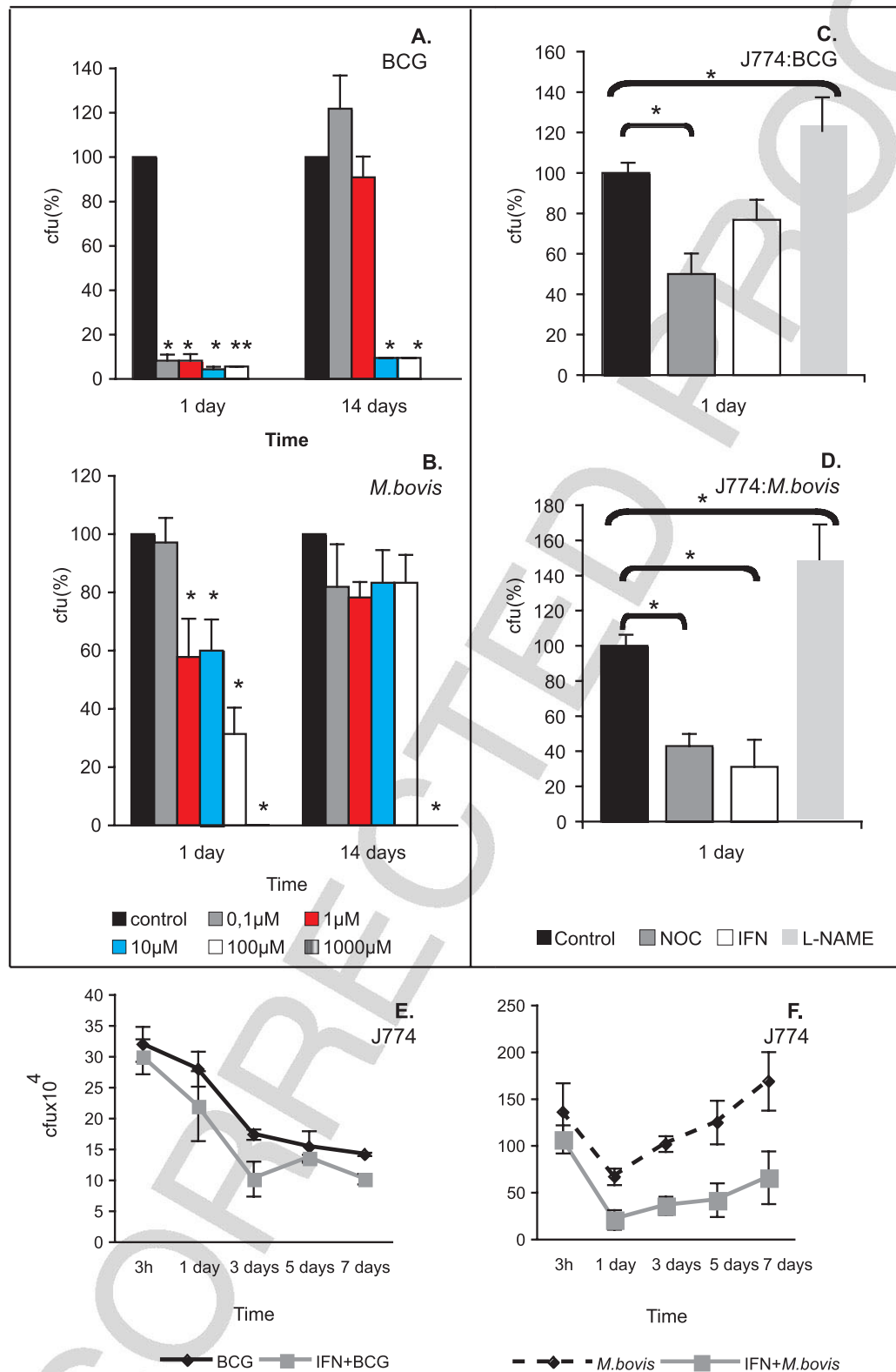
Thus, conditions where NO levels are high correlate with an increase in iNOS protein levels, as expected. Collectively, these data lead us to conclude that increased iNOS activity early, but not late in infection plays a role in killing mycobacteria in mouse cells.  $\gamma$ -IFN treatment enhances the early killing phase, predominantly by stimulating iNOS activity. However, the fact that human macrophages have a similar capacity to kill non-pathogenic and pathogenic mycobacteria but have no iNOS activity, shows clearly that RNI is not only or the main killing mechanism in macrophages.

#### Phagosome-lysosome fusion and pH

We next addressed the role of phagosome maturation in mycobacteria killing, focusing on phagosome-late-endosome/lysosome acidification and fusion. This is often assumed to be the major factor in the ability of the bacteria to survive (no fusion) and for the macrophage to kill them (fusion). If this is indeed the key factor, we would predict that, by following phagosome maturation using a range of markers in different cell types infected with BCG and *M. bovis*, we would see significant correlations between the extent of fusion and the degree of killing.

Late endocytic organelles as well as late phagosomes are predominantly acidic, and the acquisition of a low pH is an accepted marker for mature phagosomes (Malik *et al.*, 2000; Kusner and Barton, 2001; Vieira *et al.*, 2002; Kusner, 2005; Russell, 2005; Deretic *et al.*, 2006). Using the fluorescent dye lysotracker DND99, which accumulates in acidic compartments (pH < 6), the acidification of BCG and *M. bovis* was monitored by confocal microscopy, focusing on J774 and HMDM cells (Via *et al.*, 1998; Anes *et al.*, 2006). In J774 cells, only around 10% of both types of mycobacterial phagosomes were acidic 3 h after infection, and this increased to 20% at day 1. However, by

Colours



**Fig. 3.** Role of NO in *M. bovis* spp. survival. *In vitro* susceptibility of *M. bovis* BCG (GFP) (A) and *M. bovis* (B) to different concentrations of the NO donor NOC-18. Effects of L-NAME, NOC-18 and  $\gamma$ -IFN on intracellular survival of BCG (GFP) (C) or *M. bovis* (D) evaluated after 1 day infection. E and F. Colony-forming unit (cfu) estimates after 7 days of *M. bovis* BCG (GFP) (E) or *M. bovis* (F) in J774 macrophages treated with or without  $\gamma$ -IFN. \* $P < 0.05$ ; \*\* $P < 0.01$ .

**Table 1.** Nitric oxide release ( $\mu\text{M}$ ) by infected J774 macrophages.

Time post infection	BCG				<i>M. bovis</i>			
	Control		$\gamma$ -IFN		Control		$\gamma$ -IFN	
	Average	SD	Average	SD	Average	SD	Average	SD
3 h	< 1.0 <sup>a</sup>	0.40	< 1.0 <sup>a</sup>	0.40	< 1.0 <sup>a</sup>	0.40	< 1.0 <sup>a</sup>	0.40
1 day	1.0	0.5	18.7	0.12	1.0	0.05	25.4	1.23
2 days	2.8	0.40	20.5	0.09	1.3	0.08	25.3	0.91
3 days	3.2	0.20	4.4	0.03	1.8	0.05	5.6	0.06

NO estimated by the Griess reagent at different times after infection of J774 cells with BCG or *M. bovis* with and without  $\gamma$ -IFN. At values below 1  $\mu\text{M}$  the results are less precise.

a. Extrapolated from the reference standard curve.

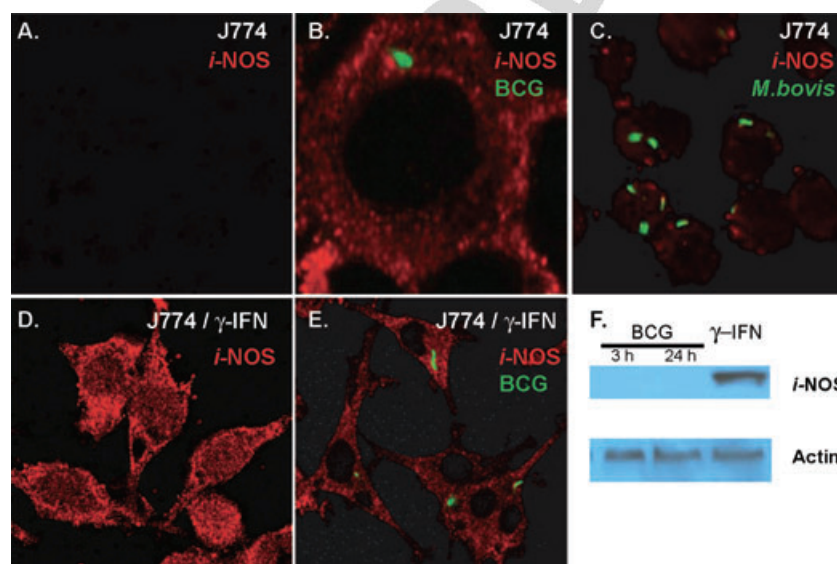
day 3 these values decreased again to around 10% (Fig. 5A). In contrast, in HMDM more BCG-containing phagosomes acidified at 3 h, but thereafter, the fraction of acidified phagosomes remained below 10%. The fraction of acidic *M. bovis*-containing phagosomes never exceeded 10% and, after 3 days, only 5% were acidic enough to accumulate lysotracker (Fig. 5B).

We next followed phago-‘lysosome’ (late endosome and lysosome) fusion using an assay we recently introduced, in which nanogold particles labelled with rhodamine are internalized by late endocytic organelles (Fig. 5C and D; Anes *et al.*, 2006). Under all conditions, in J774 and HMDM cells less than 10% of the phagosomes containing BCG or *M. bovis* fused with rhodamine-labelled ‘lysosomes’. In HMDM cells, at later time points less than 3% *M. bovis* phagosomes had fused with this marker.

When J774 were fed with heat-killed BCG, the fraction of acidified phagosomes increased from 25% at 3 h to around 80% after 1 day (Fig. 5E). These argue that phagosome containing dead bacteria matured efficiently, as

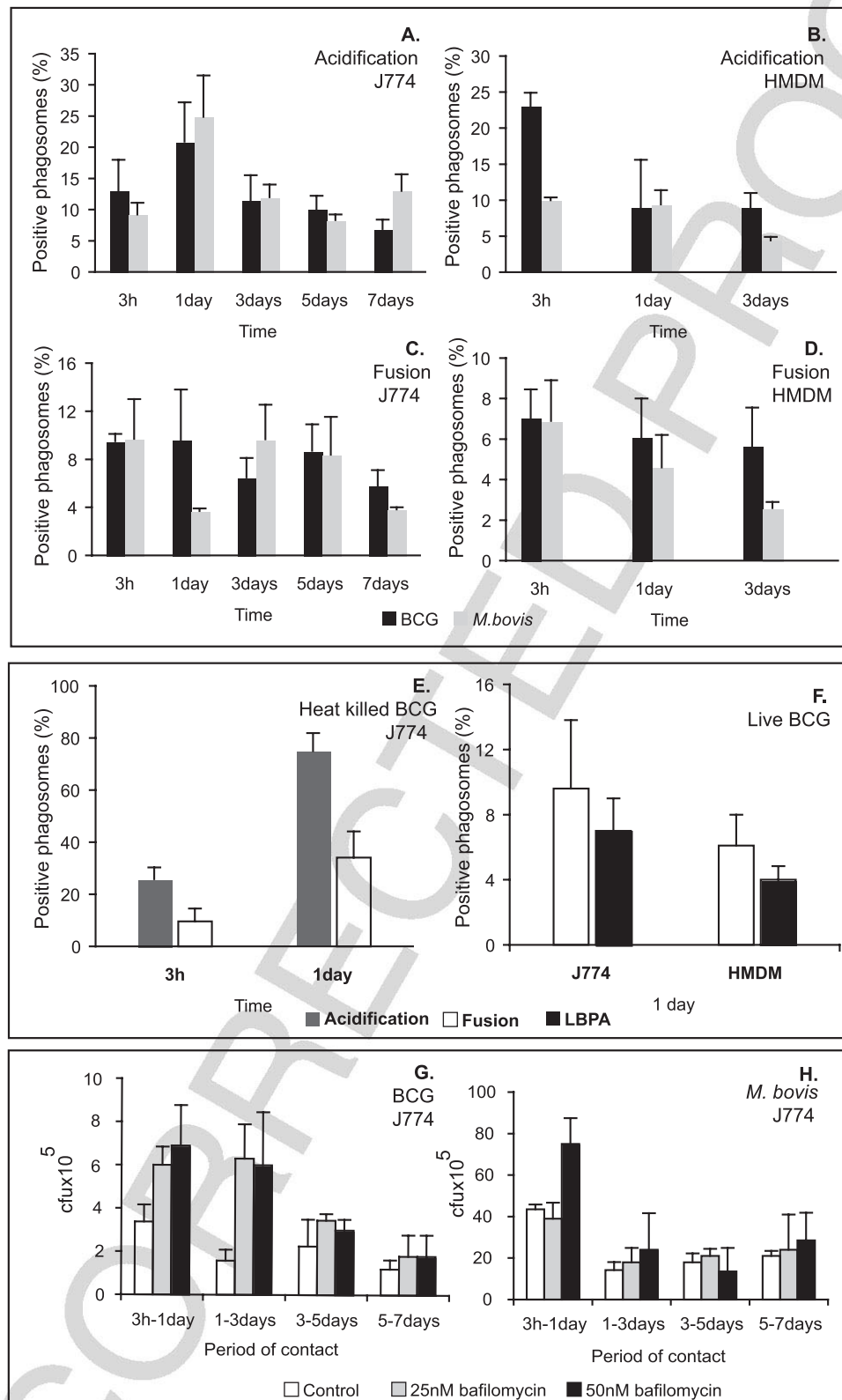
expected (Clemens and Horwitz, 1995; Clemens, 1996; Vergne *et al.*, 2005). The fraction of phagosomes that fused with lysosomes also increased between 3 h and 1 day; however, whereas only 40% of the heat-killed BCG phagosomes had fused with lysosomes, the fraction that acidified was 80%. This difference in the levels of acidification and fusion can be rationalized by our recent observation (Anes *et al.*, 2006) showing that, in J774 cells, the bulk of V-ATPase is delivered to phagosomes from a late endocytic vesicle that is distinct from the ‘classical’ late endosomes that label with LAMP-1 (Griffiths *et al.*, 1988; Xu *et al.*, 1994; Clemens and Horwitz, 1995).

Using a different late endosome marker lysobisphosphatidic acid (LBPA) (Schmid and Cullis, 1998) with live BCG-infected J774 and HMDM cells, a similar low fraction of phagosomes was found to colocalize with this marker (Fig. 5F). These data collectively show that overall, less than 10% of BCG or *M. bovis* fused with late endosomes and lysosomes, or acidified in J774 or HMDM cells. When one compares the overall pattern of phagosome maturation over the infection period with the growth of BCG or



**Fig. 4.** Localization of inducible NO synthase (iNOS) in J774 macrophages. Immunofluorescence labelling for iNOS in untreated and uninfected J774 cells (A), in macrophages infected with BCG (GFP) (B) or with *M. bovis* (C) for 3 h. D and E. The effects of  $\gamma$ -IFN treatment on uninfected cells (D) or cells infected with BCG (GFP) (E). In all images, iNOS was labelled with Cy3 (red channel) and *Mycobacterium* spp. were labelled with GFP (BCG) or Oregon green (*M. bovis*) (green channel). Western blots for iNOS during J774 macrophage infection with BCG (GFP) during 3 h and 1 day. Macrophages treated with  $\gamma$ -IFN were used as positive control. Actin was used as loading control (F).





**Fig. 5.** Phagosome acidification and fusion with late endosomes and lysosomes. Acquisition of lysotracker and rhodamine gold by live BCG or *M. bovis* phagosomes in J774 (A and C) or HMDM (B and D) and heat-killed BCG phagosomes in J774 (E). Shown in panel F is the acquisition of LBPA and gold by live BCG phagosomes either in J774 or in HMDM. The effects of bafilomycin A1 in macrophages infected with BCG are seen in panel G and with *M. bovis* spp. (H).



**Table 2.** Effects of pH on BCG and *M. bovis* *in vitro*.

pH	Mycobacteria killed compared with the control (%)			
	<i>M. bovis</i>		BCG	
	1 day	7 days	1 day	7 days
6.5	–	–	20	10
5.5	–	–	20	10
4.5	40	40	84	84

Colony-forming unit (cfu) estimate of BCG and *M. bovis* incubated at different pH values. The data are presented as the percentage of bacteria killed relative to the control (bacteria grown in standard medium) after 1 and 7 days.

–, no difference relative to the control.

*M. bovis* (Fig. 2G and H), it is difficult to observe any clear correlations.

In our earlier study on *M. smegmatis* in J774 cells, we showed that a low pH in the phagosome contributes to the killing of these bacteria because the V-ATPase inhibitor bafilomycin A1 reduced the extent of killing (Anes *et al.*, 2006). Given that even the early phagosome stage has a pH around 6.2 (Russell, 2001; Yates *et al.*, 2005) that will not be detected by lysotracker, we asked whether lowering the pH had any effect on the survival of BCG or *M. bovis* *in vitro*. A prior study (Chapman and Bernard, 1962) had shown that *M. tuberculosis* has an optimal pH for growth *in vitro* at pH 6.2 but there was significant less growth as the pH was reduced. In contrast, *M. smegmatis* is highly robust and can grow well in a wide pH range (from 4.5 to above 8), as might be expected for an organism found naturally in soil. As observed in Table 2, while *M. bovis* was not affected in our experiments by pH down to 5.5, BCG was more susceptible, with 20% even being killed at pH 6.5. At pH 4.5, however, 40% of *M. bovis* and over 80% of BCG were killed.

If we extrapolate these pH values to those found in the different phagosomes in macrophages – early phagosomes around pH 6.2 and late phagosomes down to pH 4.5 (Yates *et al.*, 2005), these data predict that, in ‘non-matured’ early phagosomes, BCG could be exposed to a partial bactericidal effect of pH whereas *M. bovis* would not be affected by the mild acidity. In contrast, should either bacteria find themselves in fully matured phagosomes (pH 4.5–5), both would be susceptible to low pH killing. In order to test this hypothesis, we used the V-ATPase inhibitor bafilomycin A1 to neutralize the pH of all endocytic compartments. We showed earlier that this treatment significantly reduced the killing of *M. smegmatis* in J774 cells (Anes *et al.*, 2006).

J774 cells infected with BCG or *M. bovis* were treated with bafilomycin A1 at the different time windows indicated in Fig. 5G and H. The treatment with 25 or 50 nM of the drug led to a significant increase in growth of BCG between

the 3 and 24 h time points and at the 1–3 day period, but it had no effect at subsequent times up to day 7 (Fig. 5G). For *M. bovis*, a similar result was seen with bafilomycin (at 50 but not 25 nM) between 3 and 24 h, whereas no effect was seen at later times (Fig. 5H). These data show that: (i) a low pH does contribute significantly to killing of both BCG and *M. bovis*, but this effect is restricted to the early phases of the infections; and (ii) the period (up to day 1) in which BCG is resistant to pH is significantly longer (up to 3 days) than for *M. bovis* (up to 1 day). As shown below, these periods coincide precisely with initial strong bacteriocidal stages in macrophages that are different for the two mycobacteria.

#### *All mycobacteria are exclusively in phagosomes*

Although it is generally accepted that intracellular mycobacteria in macrophages are always enclosed by membranes of phagosomes or phagolysosomes, two earlier EM studies suggested that *M. tuberculosis* could under some conditions escape out of the phagosome (Myrvik *et al.*, 1984; McDonough *et al.*, 1993). This idea, received with scepticism in the field, was recently re-awakened by a more convincing EM study by Peters and collaborators (van der Wel *et al.*, 2007). Using the Tokuyasu cryo section EM method following chemical fixation, these authors provided a striking scenario in which *M. tuberculosis* H37 Rv-enclosing phagosomes in human blood macrophages and dendritic cells fused significantly with lysosomes after 2 day infection (as monitored via acquisition of LAMP-1, CD63 and cathepsin D). After this period, the phagosomes were found to lyse and *M. tuberculosis* escaped into the cytoplasm, where bacterial growth was observed. In contrast, BCG remained within phagosomes at all times after infection. This result fits well with the behaviour of the closely related pathogen *M. marinum* (Stamm *et al.*, 2003; 2005), which was even able to move actively through the cytoplasm by actin comets, similar to *Listeria* and other pathogens (Cossart and Sansonetti, 2004).

The possibility that *M. bovis* in particular could escape into the cytoplasm in our experiments offered an attractive alternative hypothesis to explain the lack of obvious correlation between its intracellular growth/killing and the fraction of fully matured phagosomes. We found no conditions in this study (even after treatment with  $\gamma$ -IFN; results not shown) in which BCG-, *M. bovis*- or *M. tuberculosis*-containing phagosomes showed an index of phagolysosome fusion much above the 10% value, at least up to 7 days. Similar conclusions were drawn by Armstrong and Hart (1971) after monitoring the infection with *M. tuberculosis* up to 15 days in primary macrophages. Nevertheless, perhaps this abrupt maturation of pathogen-containing phagosomes seen by Van der Wel *et al.* (2007) might have occurred too rapidly in our cell systems. If the bacteria were in the cytoplasm, they

could not be expected to contain any lysosomal marker. Clearly, this question could only be addressed using electron microscopy. An obvious worry for any electron microscopic (EM) study using conventional fixation is the question of fixation artefacts, a problem that can be particularly difficult in preserving membranes (Griffiths, 1993). Only by use of cryo-immobilization methods such as freeze substitution or cryo-electron microscopy of vitrified sections (CEMOVIS) (Al Amoudi *et al.*, 2004) can one be sure to avoid all chemically induced artefacts. However, if pathogens are to be investigated by these more modern technologies, it requires the combination of a high-pressure freezer machine in a biosafety level 3 facility, a combination that we currently do not have.

There is, however, one proviso for using conventional chemical embedding that makes this approach suitable for analysing pathogen phagosomes. While there is always the possibility that phagosomal membranes can lyse, or even perhaps disappear in response to fixation, we consider it highly unlikely that a bacterium free in the cytoplasm can acquire a membrane artifactually as a result of this procedure. This discussion is highly relevant to interpretation of EM images of chemically fixed specimens, and to the data we now present.

We chemically fixed J774 or HMDM infected with BCG, *M. bovis* or *M. tuberculosis* with glutaraldehyde, post-fixed in osmium and uranyl acetate, and embedded the samples in epoxy resin. Alternatively, after only glutaraldehyde fixation the cells were infused with sucrose and prepared for cryo-sectioning (Tokuyasu, 1973); these were contrasted with either uranyl acetate or ammonium molybdate in combination with methyl cellulose (Griffiths, 1993). Using both approaches, thin-section EM analysis showed unequivocally that all of the bacteria we observed within the macrophages were invariably surrounded by a phagosome membrane at all times up to 6 day infection. Examples of these images are shown in Fig. 6. This rules out the possibility that any cytoplasmic mycobacteria exist under our conditions and supports the observations of Armstrong and D'Arcy Hart (1971), who found *M. tuberculosis* to be enclosed by a phagosomal membrane at all times over 2 weeks of infection.

#### *Killing of mycobacteria occurs in mature and non-mature phagosomes*

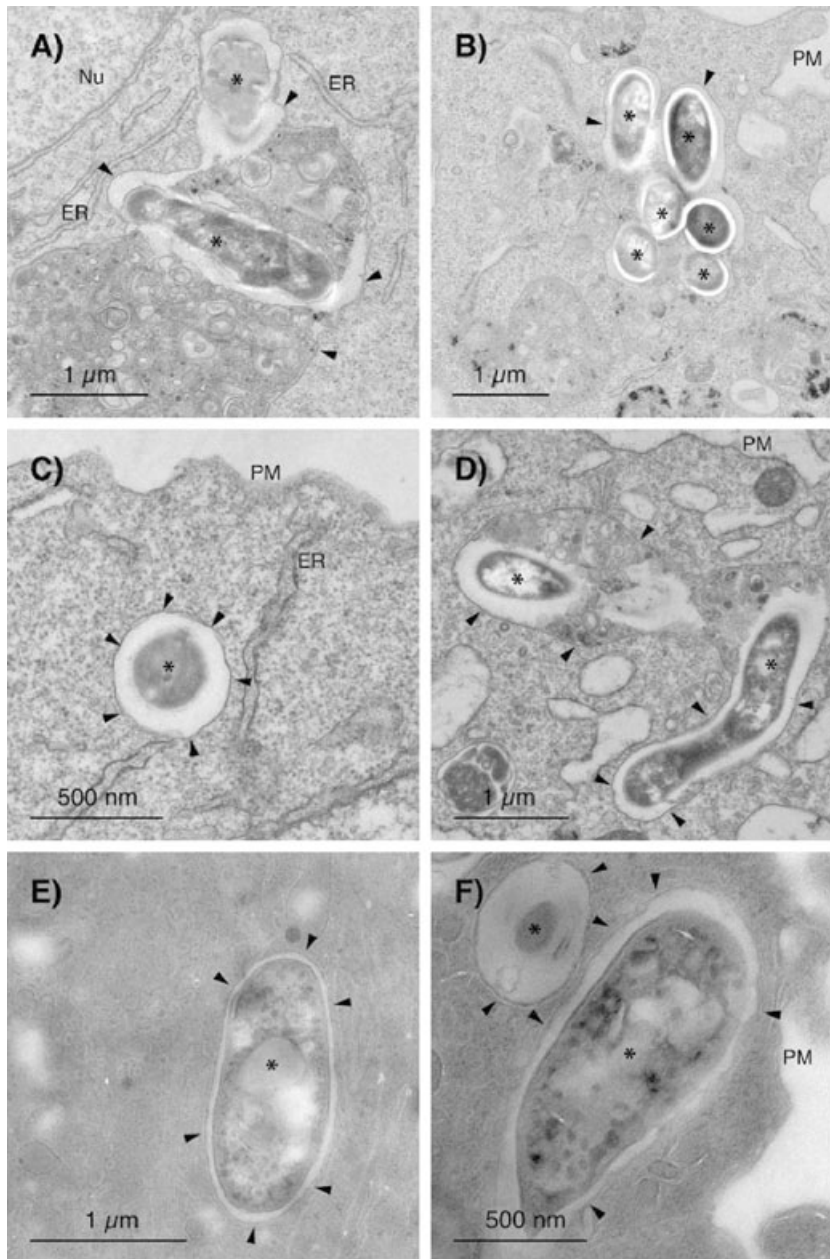
From the above results, both BCG and *M. bovis* reside in macrophages in phagosomes that we can operationally separate into 'early' or non-matured phagosomes (not fused with 'lysosomes' and lysotracker negative) and 'late' or mature phagosomes (fused with lysosomes and lysotracker positive). The vast majority (90%) of both BCG

and *M. bovis* phagosomes were in early (non-mature) phagosomes. We wanted to distinguish between the two extreme models presented in *Introduction*: first, killing occurs only *after* a live bacterium finds itself in a matured phagosome; and second, killing occurs in early phagosomes and is perhaps a prerequisite for some of these phagosomes to mature into a hydrolytic compartment that degrades the pathogens.

To address this issue, we set up an experiment in which we could distinguish between live and dead bacteria per cell, using an approach similar to that described by Anes *et al.* (2006) and Barker *et al.* (1997). For this, we infected J774 cells with *M. bovis* that had been surface-stained with Oregon green or with GFP-BCG. Subsequently, the cells were lysed and the recovered bacteria were stained with propidium iodide, a red dye that is unable to cross intact bacterial membranes but stains nucleic acid in dead bacteria. This allowed us to distinguish between dead (red or yellow) and live bacteria (green) and, by separately counting the number of macrophages, we could relate the numbers of bacteria to the numbers of cells. Importantly, the viability and the total number of macrophages during the time of the experiment remained constant. Less than 1% of macrophages could be stained with trypan blue in infected cultures for 7 days.

The quantitative analysis of this experiment was quite revealing (Fig. 7A). With both BCG and *M. bovis*, the number of green (live) bacteria dropped at a similar initial rate, giving a total killing of around 40% during the first day of infection (Table 3). From the data in Figs 3C and D and 5G, it seems likely that RNI and a low pH are the major factors responsible for this early killing of both bacteria. In the case of *M. bovis*, day 1 was the start of a continuous growth period, whereas for BCG, killing proceeded until day 3, when a static condition was reached. The initial decrease in the number of green bacteria, up to day 3, was, however, not correlated with an increase in the number of red/yellow bacteria, as we expected. Rather, this number was kept at a consistently low value of around 0.5–1 bacteria per cell, for both *M. bovis* and BCG. Our interpretation at this point is that we have dead bacteria in different stages of digestion and only the best-preserved ones would be recovered after macrophages lysis and propidium iodide staining. Clearly, these killed bacteria must be cleared relatively fast, and at a similar rate for both bacteria. Taking this into account, we consider that the rate of disappearance of the green bacteria provides the best independent estimate of the killing rate. This assumption was supported by the excellent correlation between cfu (Fig. 7B) and the different kinetics of disappearance of BCG and *M. bovis* green bacteria (Fig. 7A).

During the first 24 h, the rate of killing for BCG and *M. bovis* was similar (Table 3). Thus, both BCG and



**Fig. 6.** *Mycobacterium* spp. reside in a phagosome compartment at all infection times. A–D. Epoxy resin plastic section of J774 macrophages infected after 3 day infection with *M. bovis* (A and B) or *M. tuberculosis H37Rv* (C and D). E and F. Thawed cryo-sections of HMDM infected for 3 days (E) and 5 days (F) with *M. bovis*; sections stained with ammonium molybdate and methyl cellulose. In all panels, mycobacteria are marked with an asterisk (\*), and arrowheads indicate the membrane of phagosomes that enclose the mycobacteria. Plasma membrane (PM), nucleus (Nu) and endoplasmic reticulum (ER) are also indicated.

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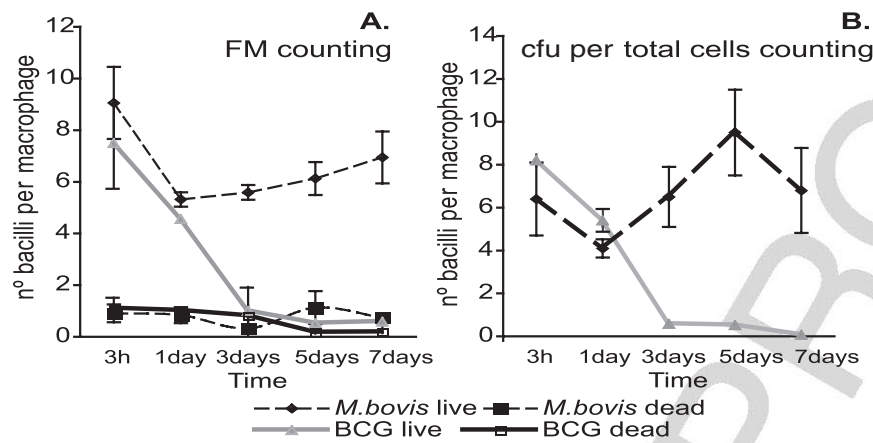
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1 the pathogenic *M. bovis* are subjected to a similar  
2 macrophage-killing potential early in infection. After 1 day,  
3 *M. bovis* strikingly switched to a state of growth, with the  
4 number of green (live) bacteria increasing to give an  
5 average of 8 bacteria per cell after 7 days, as seen by direct  
6 microscopy counts and by cfu estimations (Fig. 7A and B).  
7 So the major difference between BCG and *M. bovis* is that  
8 BCG continuously suffers a net loss until a steady state is  
9 reached after 3 days, whereas *M. bovis* is able to switch to  
10 a growth phase after 1 day – we presume that this involves  
11 a signalling switch from a pro-inflammatory signalling state  
12 of macrophages to an anti-inflammatory one (L. Jordao  
13 *et al.*, submitted). Nevertheless, because a constant

number of dead bacteria are seen at all times for *M. bovis*  
(Fig. 7A and B), it argues that the net growth seen for  
*M. bovis* reflects a balance between growth and a constant  
rate of killing; a similar conclusion was made for *M. mari-*  
*num* in phagosomes (Barker *et al.*, 1997). Although no net  
growth is seen for BCG at any time, the constant ratio of live  
to dead bacteria argues that this bacterium is also at a  
steady state with continuous growth and killing. Because  
roughly half of the BCG population in these late infection  
period is dead, and only 5–10% are in phagosomes that  
have fused with lysosomes, we interpreted these data to  
mean that some bacteria must be killed in non-matured  
phagosome.

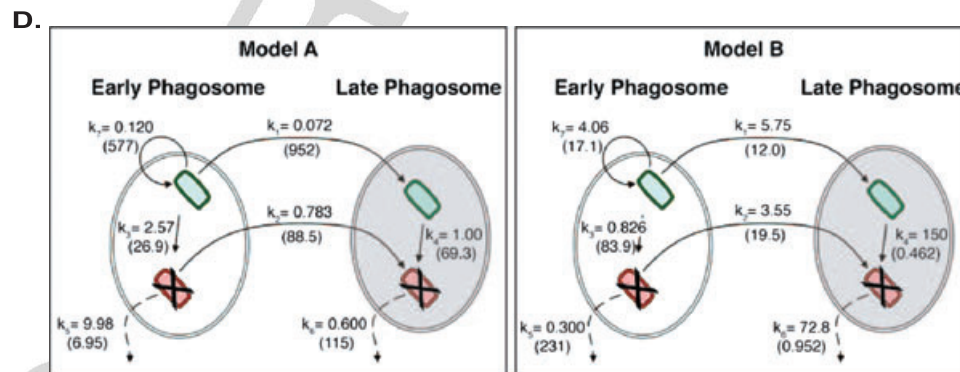
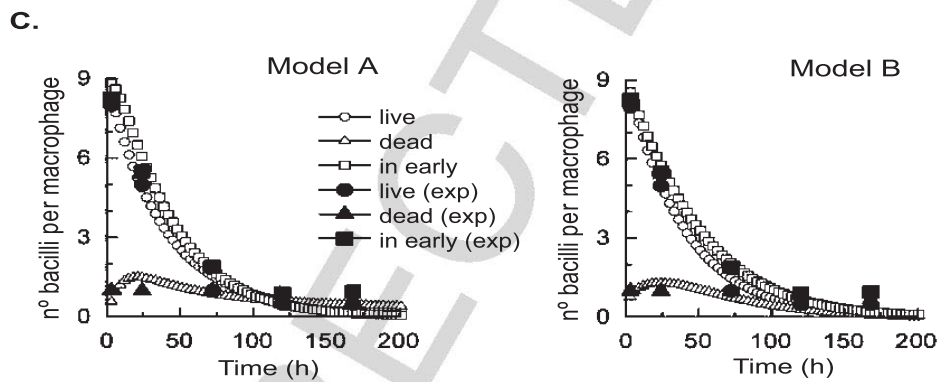
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**Table 3. Live versus dead intracellular bacteria**

Time post-infection	Numbers of bacilli per macrophage		
	Live BCG	Live <i>M. bovis</i>	Dead BCG or <i>M. bovis</i>
3 h	7.50	9.10	2.00
1 day	4.56	5.32	0.65
3 days	1.00	5.59	0.17
5 days	0.52	6.13	0.00
7 days	0.58	6.94	0.00



Colour



**Fig. 7.** Evaluation of mycobacteria growth/survival by different methods and the COPASI models for BCG transport and killing in J774 macrophages. Evaluation of live and dead *M. bovis* spp. in J774 macrophages over 7 days by microscopy (A) or by cfu (B). Table 3 shows a numerical summary of parameters calculated from panels A and B. C. The results of two modelling scenarios (model A and model B). Filled symbols represent the experimental data from panel A, whereas open symbols indicate the values obtained from the models. D. The seven reactions modelled – for more details see *Supplementary material* (COPASI file). In the models, two phagosomal compartments are considered (early or non-matured, and late or matured). The bacteria can divide, and be killed, digested or transported from early to late compartments. Kinetics constants are: k1, rate of transport (by maturation) from early to late compartments for live BCG; k2, rate of transport from early to late compartments for killed BCG; k3, rate of killing of BCG in early compartments; k4, rate of killing of BCG in late compartments; k5, rate of disappearance of BCG from early compartments; k6, rate of disappearance of BCG from late compartments; k7, rate of duplication of BCG in early compartments. The first numerical value for each arrow is the kinetic constant  $k$  expressed as  $1 \text{ h}^{-1} \times 10^2$ . The numbers in parentheses give the half-life for reaction 1–6 (time in hours to transport, kill or digest 50% of the bacteria) or the doubling time for reaction 7 (time in h to double the bacterial number).

We also analysed the rate of disappearance of heat-killed GFP-BCG and *M. bovis* taken up by phagocytosis by J774 cells. The rate of clearance was identical for both bacteria (Table 3), with 67% of these killed bacteria having disappeared from cells between 3 h and 1 day. Between 1 and 3 days, almost all remaining bacteria were eliminated (n.b. for this experiment, we used a low number of bacteria for infection in order to more clearly visualize the bacteria; see below).

A comparison of Fig. 7A and B with Fig. 5G and H reveals an interesting correlation. For both BCG (with net killing until day 3) and *M. bovis* (killed until day 1), these periods of killing coincided precisely with the times that blocking phagosomal acidification prevents killing. This provides more compelling evidence that low pH is directly involved in the initial net killing period, but not thereafter.

Under some conditions, particles within phagosomes can exit cells by exocytosis of the phagosomes (Di *et al.*, 2002). We therefore hypothesized that the potential live (green) bacteria that were 'lost' from cells might be exocytosed after (or even before) killing. However, extensive microscopy and cfu counts of live and killed bacteria on the surface of cells or released into medium showed no significant release of bacteria to the extracellular space (results not shown). This indicates that the digestion and clearance of bacteria must occur in the lumen of the early or late phagosomes.

#### Modelling intracellular transport and killing of BCG

We then assessed whether the data in Fig. 7A and B for BCG could be fitted to a simple model considering two compartments (early and late) and the rates of bacterial growth, killing, disappearance and transport between compartments (i.e. phagosome maturation). We described the system by defining the seven different reactions shown in Fig. 7D, each having a rate constant, k1–7. A set of differential equations were generated in the COPASI modelling program (see *Experimental procedures* and Fig. 7C). This elegant program allows one to change each constant independently, and then the

program is run to see what pattern of curves emerge for the number of bacteria over time. COPASI can be instructed to find the set of constants that best reproduce the observed values. For this exercise, we used all available experimental evidence we had. The data for dead and live bacteria at different times after internalization were taken from Fig. 7A (BCG). The values for the fraction of bacteria in early compartments were taken from Fig. 5C (BCG). The dynamics of transport to late compartments (along with phagosome maturation) and the disappearance from macrophages of dead BCG were those shown in Fig. 5E and Table 3.

The general features for BCG intracellular transport and survival in J774 macrophages i.e. most bacteria present in early compartments at all times after infection, a stable amount of dead microorganisms, and an exponential decrease of live bacteria could be reproduced quite well by two totally different scenarios (Fig. 7C and D). In the first (model A), the majority of the bacteria is killed in, and disappears from, early compartments, whereas in the second (model B), the majority of the bacteria are first transported to late compartments, where they are quickly killed and degraded (Fig. 7D). It is interesting to notice that both models require killing in the early compartments, as postulated by Armstrong and D'Arcy Hart (1971), in order to fit the experimental data. However, whereas this process is the main player in model A, it plays a quantitatively less important role in model B. The time-limiting step for BCG disappearance in the first model is the killing and degradation in the early compartments, whereas for the second, it is the transport to late compartments. It is worth mentioning that we could not find any other set of parameters that fit the experimental data, although calculations were started from eight different initial conditions.

At first glance, model A was attractive because it seems to be consistent with the low fraction of bacteria in phagolysosomes and the low rate of killed bacteria. If the majority of the bacteria are cleared from an early compartment (a phenomenon that has never been observed), it might explain why we detected such apparent low values for

phagolysosome fusion as well as the relatively low numbers of dead bacteria. However, a deeper analysis makes us favour much more the second model B, in which an important small fraction of bacteria are killed in early phagosomes. Both live and killed bacteria phagosomes have a relatively low chance of undergoing full maturation, but when they do, they face a compartment where the live bacteria are rapidly killed (30 min), and both this pool, and the bacteria already killed in early phagosomes, are rapidly digested and disappear (60 min). In model B the almost perfect fitting of theoretical values to our experimental data is observed with a duplication time for BCG ( $k7$  in Fig. 7D) of 17 h. This value, which was important for the curve fitting, is within the range estimated for the cell division time of 23 h for BCG growing *in vitro* (Beste *et al.*, 2005). In contrast, in model A the fitting of theory to experimental data necessitated a  $k7$  value of over 500 h, a value that is totally unrealistic.

## Discussion

Here, we addressed those factors that were believed to contribute to killing mycobacteria in macrophages and investigated the interactions between three mycobacteria, *M. smegmatis*, BCG and *M. bovis*, in up to six different cell types. We started our analysis by further investigating *M. smegmatis*, which we have recently characterized in great detail in J774 cells (Anes *et al.*, 2006). In these cells, *M. smegmatis* is exposed to a first killing phase (1–4 h), a subsequent growth phase (4–8/9 h) and two further killing stages with different kinetics (9–24 h). It was striking that the other three cell types we tested (Raw, mouse BMM and human HMDM) all showed the same kinetics of efficient killing between 1 and 4 h; this argues that the bacteria bind receptors which induce the same dynamics of early signalling in all the cells. At subsequent times, the pattern of *M. smegmatis* growth in BMM cells was almost identical to J774 cells, whereas different patterns were seen with Raw and HMDM, although all the cells effectively cleared *M. smegmatis* by 48 h.

Differences between cells became much more apparent when we compared six different macrophage types following infection with *M. bovis* BCG (GFP). No easily discernable pattern emerges from this analysis, arguing that each combination of cell–mycobacterium tends to behave differently. Further, the ability of a cell type to kill BCG or *M. bovis* could not be easily correlated with their ability to kill *M. smegmatis*.

Extensive literature argues that NO produced by iNOS in macrophages is a crucial killing factor for mycobacteria in macrophages (Macmicking *et al.*, 1997; Rich *et al.*, 1997; Jagannath *et al.*, 1998; Long *et al.*, 1999). Our data lend support to this hypothesis, and to the notion that the

pathogenic *M. bovis* is more resistant to the killing effects of NO *in vitro* compared with BCG. There is also significantly less NO produced from *M. bovis*- relative to BCG-infected mouse macrophages, although these levels could be increased dramatically in both infections with  $\gamma$ -IFN treatment; this resulted in a slight increase in killing of BCG and a significant increase in killing *M. bovis* after 1 day, but not at later days of infection. Effects of  $\gamma$ -IFN in killing mycobacteria in macrophages have been described earlier (Denis, 1991; Via *et al.*, 1998; Schaible *et al.*, 1999). In mouse cells, there was a good correlation between the levels of NO and the killing of mycobacteria, with both higher levels of NO and iNOS-dependent killing being restricted to the first 1–2 day infection. A similar pattern was seen earlier with *M. smegmatis* in J774 cells, in which the NO production and iNOS-dependent killing was restricted to the first 2 h of infection. That RNI is not the only killing mechanism is also seen by the fact that human macrophages are unable to show iNOS activity but are still capable of killing mycobacteria. Here, HMDM produced undetectable levels of NO but were nevertheless capable of killing *M. smegmatis* in the first 4 h infection at the same rate as the three types of mouse cells (Fig. 1A–D).

An additional factor that was important for the early killing potential of macrophages was a low pH. When BCG- or *M. bovis*-infected J774 macrophages were treated with the V-ATPase inhibitor bafilomycin A1, there was a significant increase in bacterial growth at the 3–24 h period for *M. bovis*, and between 3–24 h and 1–3 days for BCG. However, this drug had no effect at later times of infection for either bacteria up to 7 days (Fig. 5G). The early infection period where a low pH facilitates killing coincides with the period of iNOS activity, and it is known that a low pH synergizes with the RNI system to induce more potent killing activity (Rhoades and Orme, 1997). It is striking that the period where a low pH is needed for net killing of both bacteria coincides precisely with the early infection period in which the bacterial counts were decreasing strongly (the pro-inflammatory phase), as assessed by both direct microscopy estimates and cfu (Fig. 7A and B). We propose that the combination of RNI and a lowered compartment pH synergize to provide the first killing process. The early mycobacterial phagosomes have a pH of around 6.2, while the late ones are at 5 or slightly below (Sturgill-Koszycki *et al.*, 1994; Clemens and Horwitz, 1995; Yates *et al.*, 2005). Because NO and its relatives are freely diffusible, it is conceivable that its combination with a lowered pH in early or late endocytic organelles could induce killing in either compartment; our data do not allow us to distinguish between these options. The observation that blocking phagosome acidification at days 3–7 had no effect on the survival of BCG or *M. bovis* was especially surprising. Because both these bacteria

1 must be continually killed, even after the RNI wave, it  
2 argues that whatever mechanism that kills them is not  
3 dependent on a low pH.

4 The interpretation of our data was not straightforward,  
5 given the many different potential reactions that operate in  
6 the highly dynamic system – in which a variable fraction of  
7 the pathogens can divide and a different, variable fraction  
8 can be killed at any time by the macrophage. We therefore  
9 carried out a more detailed analysis of the BCG and  
10 *M. bovis* infections by estimating the total numbers of live  
11 and killed bacteria in the cells at different times of  
12 infection. Both bacteria were initially killed rapidly until day  
13 1 (*M. bovis*) or day 3 (BCG). Thereafter, *M. bovis* switched  
14 to a robust net growth phase while BCG retained a constant  
15 low level of live bacteria per cell until day 7. Surprisingly,  
16 the fraction of bacteria that were rod-shaped but  
17 accessible to propidium iodide, and therefore presumed  
18 to be dead, remained at a constant value of around one  
19 dead bacterium per cell at all infection times for both BCG  
20 and *M. bovis*. Clearly, the killed bacteria must be digested,  
21 and the number of dead ones identified at any stage  
22 represents the balance between the rate of killing and the  
23 rate of digestion.

24 The use of the COPASI program to model our data  
25 turned out to be a very powerful tool. When one deals  
26 with complex multistep processes such as phagosome  
27 maturation, pathogen growth and killing processes, it is  
28 difficult to make an intuitive interpretation of the data. In  
29 fact, without such modelling we would have concluded  
30 that the quantitatively most significant killing of the bac-  
31 teria occurs in an early compartment, in agreement with  
32 the classical study by Armstrong and D'Arcy (1971).  
33 However, the theoretical modelling led us to a different  
34 conclusion. In this exercise the overall process of infec-  
35 tion is separated into seven distinct reactions with their  
36 own rate or activity constants. The COPASI program then  
37 allows one to find estimates for these seven parameters  
38 that gives the best fit of the dynamics of the bacterial  
39 growth/killing with the observed experimental data  
40 (Fig. 7A and B). We found two quite different potential  
41 'solutions' that accurately mimicked the experimental  
42 data (Fig. 7D). The main difference between the two  
43 models is that in model A, the majority of bacteria are  
44 killed and cleared from early phagosomes whereas in  
45 model B, these processes predominantly occur in late  
46 compartments. As already pointed out, the latter model  
47 makes more sense, for example because it assumes a  
48 reasonable doubling time for BCG in the macrophage of  
49 17 h whereas model A predicts a totally unrealistic value  
50 for this parameter of over 500 h. Moreover, the input  
51 value of 17 h in model A was essential for the good fit  
52 between theory and experiment; with slightly higher or  
53 lower values, there was poor agreement between theory  
54 and experimental data.

55 Of course, a model that accurately fits the data is far  
56 from guaranteeing that its assumptions are correct; after  
57 all, model A, which we believe is unrealistic, also gives an  
58 excellent concordance between theory and experiment.  
59 Nevertheless, a model can lead to predictions that can be  
60 tested in further experiments. The preferred model B pre-  
61 dicting that: (i) a small but significant degree of BCG killing  
62 (and presumably *M. bovis*) must occur in early phago-  
63 somes; (ii) a rate-limiting step occurs for the maturation of  
64 phagosomes containing both live and killed bacteria; (iii)  
65 once phagosomes have matured, the live bacteria are  
66 killed very rapidly, on average in 30 min; and (iv) bacteria  
67 that were killed in early phagosomes and those that are  
68 killed in late phagosomes are rapidly digested, and dis-  
69 appear from the system within 1 h. A recent study by  
70 Majlessi *et al.* (2007) provides support for the hypothesis  
71 that both the early and late mycobacterial phagosome can  
72 show hydrolytic activity because the same level of myco-  
73 bacterial antigen presentation was seen, irrespective of  
74 whether the bacteria resided in early or late phagosomes.  
75 We are currently setting up experiments to test some of  
76 these predictions, most notably the idea that the bacteria  
77 are killed and removed from the late compartments in a  
78 relatively short period, using live cell-imaging methods.

79 Our data collectively argue that, for BCG and  
80 *M. bovis*, the killing by macrophages occurs in two  
81 distinct phases: the first, an early low pH- and RNI-  
82 dependent process, and the second, independent of  
83 both. The hypothesis emerges that multiple bacteriocidal  
84 factors must be delivered into the pathogenic myco-  
85 bacterial phagosome. The first, early phase may be  
86 explained by the combination of a pH below neutrality  
87 and RNI. The second, later phase that occurs for sure  
88 after (and perhaps also before) the RNI wave evidently  
89 occurs independently of a low pH. We presume that two  
90 sets of hydrolytic enzymes or factors must contribute to  
91 the small degree of killing postulated to occur in early  
92 phagosomes, and to the more extensive killing that likely  
93 occurs in late phagosomes. Factors such as cathelicidin,  
94 which has recently been implicated in killing *M. tubercu-*  
95 *losis* (Liu *et al.*, 2006), can be expected to contribute to  
96 the overall killing process. Although hydrolases are gen-  
97 erally more active at a lowered pH, the late killing factors  
98 that we observed in this study were not affected by  
99 blocking the proton pump with bafilomycin. However,  
100 some lysosomal enzymes have an optimal activity at a  
101 neutral pH (Butor *et al.*, 1995), and even some enzymes  
102 with a low pH optimum may still retain significant activity  
103 at neutral pH values. The identification of all the factors  
104 within both early and late phagosomes that have the  
105 potential to kill pathogenic mycobacteria, especially  
106 *M. tuberculosis*, is now crucial to our understanding of  
107 the macrophage-killing potential, and to evolve therapies  
108 that could boost this process.



## Experimental procedures

### Bacterial culture conditions

*Mycobacterium tuberculosis* H37 Rv, *M. bovis* BCG Pasteur (ATCC35734), *M. bovis* BCG harbouring a pMN437 plasmid, and a strain of *M. bovis* genetically characterized isolated from a bovine were grown on Middlebrook's 7H9 broth medium (Difco) supplemented with 10% OADC (v/v) and 0.05% Tween 80 (v/v) until exponential phase at 37°C/5% CO<sub>2</sub>. Media were supplemented with 50 µg ml<sup>-1</sup> hygromycin (Roche) for selection of recombinant mycobacteria.

### Cell line culture conditions

The mouse macrophage cell lines J774.A1 and Raw 264.7 were cultured as described previously. Two days before infection, macrophages were seeded onto 24-well culture dishes and left for 2 days in a 5% CO<sub>2</sub> incubator at 37°C (10<sup>5</sup> macrophages ml<sup>-1</sup>).

THP1 cells were grown in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% HEPES (Gibco), penicillin and streptomycin (Gibco). The cells were seeded onto 24-well culture dishes at a density of 5 × 10<sup>5</sup> cells ml<sup>-1</sup> and treated overnight with 5 nM phorbol myristate acetate (Sigma). Then cells were washed three times with PBS and incubated for one more day.

### Human monocyte-derived macrophage (HMDM) isolation and culture

Human monocyte-derived macrophages were prepared from venous blood from healthy volunteer donors using density gradient centrifugation as described before (Mendez-Samperio *et al.*, 2004). Cells were plated at a density of 2 × 10<sup>6</sup> cells ml<sup>-1</sup> and incubated for 3 days with RPMI 1640 supplemented with 30% FCS and 10% human-type AB serum (Sigma). Then cells were washed three times with warm 1% FCS in PBS and cultured for 7 days before infection.

### Bovine monocyte-derived macrophage (BMDM) isolation and culture

Bovine monocyte-derived macrophages were prepared from venous blood from healthy, 8- to 9-month-old male Holstein Friesian, bovines using density gradient centrifugation. The protocol used was similar to that described above for HMDM with the following differences: density gradient (Nycoprep 1.077A from AXIS-SHIELD) and the supplementation of the RPMI 1640 medium. Instead of 10% human-type AB serum, 10% homologous bovine serum was used.

### Bone marrow-derived macrophage (BMM) isolation and culture

Experiments were performed with 3-week-old C57BL/6 male mice from Jackson Laboratory. Mice were killed by cervical dislocation, and the femur and tibia bones were removed. The bones were trimmed at both ends, and the marrow was flushed out with

5–10 ml of RPMI containing 10% FCS using a 23-gauge needle. The cell suspension was centrifuge for 4 min at 900 g, and the pellet was gently resuspended in RPMI supplemented with 10% FCS, 1% penicillin/streptomycin, 1% nonessential amino acids (Gibco) and 15% L929 supernatant, and plated into bacterial Petri dishes. After 3 days, monolayers were washed with warm 1% FCS in PBS and incubated for another 3 days. At this point, more than 95% of the cells were CD14 (BD Pharmingen) positive by flow cytometry. Cells were scraped and plated at a density of 2.5 × 10<sup>5</sup> cells ml<sup>-1</sup> and incubated overnight before infection.

### Macrophage infection

Bacterial cultures on exponential grown phase were pelleted, washed twice in PBS pH 7.4, and resuspended in PBS to a final concentration of 5–10 × 10<sup>9</sup> cells ml<sup>-1</sup>. Single-cell suspensions were generated by 2 min pulse in a water-bath sonicator (room temperature), followed by passage through a 23-gauge needle to disrupt remaining bacterial clumps. Before infection, residual bacterial aggregates were removed by low-speed centrifugation (120 g) for 2 min. Single-cell suspension was verified by light microscopy.

Before infection, non-adherent cells were removed by wash with PBS, and the medium was replaced by antibiotic-free medium supplemented with 10% FBS. To achieve 2–8 bacilli per macrophage after 3 h uptake, an OD<sub>600</sub> ~0.1 was used for all the macrophages used except HMDM. For these macrophages, an OD<sub>600</sub> ~0.01 was used instead. The cells were washed twice with PBS to remove free mycobacteria. At infection time (3 h) and after several days (3, 5 and 7 days), infected macrophages were washed with PBS and lysed with 1% Igepal (Sigma) solution in water. Serial dilutions of the lysate were prepared in water and plated at Middlebrook 7H10 medium supplemented with OADC. After about 3 weeks of incubation at 37°C, colonies were counted.

For *M. smegmatis*, a similar protocol was used with the following differences: (i) an OD<sub>600</sub> ~0.1 was used for all the macrophages; and (ii) cells were allowed to uptake bacteria only for 1 h.

### Treatment with γ-IFN, inhibitors and NOC-18

When required, cells were treated with murine γ-IFN (50 IU) overnight before infection. γ-IFN was a kind gift from the Centralised Facility for AIDS Reagents.

When treated with iNOS inhibitor N-(G)-nitro-L-arginine methyl ester hydrochloride (500 µg ml<sup>-1</sup> L-NAME; Sigma), this compound was added to the medium 1 h before infection and left until the end of the experience. When treated with NOC-18 (0.1 or 1 µM; Calbiochem), this compound was added to the medium at time zero and left until the end of the experience. Chemicals were renewed during the course of the experiments according to their half-lives. The V-ATPase inhibitor, bafilomycin A1, 25 or 50 nM (Sigma), was added to the medium in the following time windows: 3 h–1 day, 1–3 days, 3–5 days and 5–7 days.

### Nitric oxide

Culture supernatants were collected at several time points for determination of nitrite contents. Nitrite concentration indicating the NO production was performed using the standard Griess reaction adapted to microplate.



*Mycobacterium bovis* spp. *in vitro* sensitivity to NO was determined using serial dilutions of an 1000  $\mu$ M NOC-18 solution in DMEM supplemented with 10% FCS. After 1, 7 and 14 days, serial dilutions of control and samples were plated on 7H10 supplemented with OADC supplement. After about 3 weeks of incubation at 37°C, colonies were counted.

#### *Epifluorescence and confocal fluorescence microscopy*

Macrophages grown on glass coverslips were allowed to uptake *M. bovis* BCG harbouring a pMN437 plasmid (live/heat killed) or *M. bovis* stained with Oregon green (Molecular Probes). Cells were fixed with 3% paraformaldehyde at room temperature for 15 min.

Lysotracker Red DND-99 (Molecular Probes) staining of acidic organelles was carried out by adding a 1:10 000 dilution in DMEM that was added for the last 30 min of the experiments. Cell permeabilization, when required, was achieved with 20 min treatment with 0.1% Triton X-100 (Sigma) in PBS. After 30 min blocking with 5% goat serum (Sigma) in 0.1% BSA/PBS, cells were incubated for 1 h with primary antibody, followed for further 30 min by secondary antibody. For LBPA, blocking was made with 0.1% BSA/PBS, followed by simultaneous permeabilization/incubation with primary antibodies diluted in a 0.05% saponin (Sigma) solution in PBS, followed for further 30 min incubation with secondary antibodies in PBS.

Confocal microscopy images were collected using the LSM510 from Zeiss, and the percentage of colocalisation was calculated using either the Image J software system or manually. Fluorescence labelling and viability of mycobacteria was performed as described (Anes *et al.*, 2003).

The following antibodies were used for immunofluorescence microscopy. The mouse iNOS antibody was purchased from BD Pharmigen, and the LBPA antibody was a kind gift from Jean Gruenberg. The secondary antibodies used were linked to Cy3 (Molecular Probes).

#### *Fusion assay*

Analysis of phagosome–lysosome fusion was carried out using 7 nm gold particles prepared as describe before (Anes *et al.*, 2006). Macrophages were pulsed for 1 h with these gold particles, and washed three times with PBS. Then one of two treatments was carried out: (i) chase for 1 h in complete culture medium, infection with mycobacteria 3 h in complete medium without antibiotics; or (ii) chase for 4 h in medium without antibiotics. The first treatment was applied for 3 h time point, and the second for all the others. Macrophages were fixed with 3% PFA in PBS and processed for microscopy.

#### *Estimation of live versus dead bacteria*

Three hours, 1, 3, 5 and 7 days post infection, macrophages were scraped, stained with trypan blue (Sigma) and counted in a Neubauer chamber. Infected macrophages were then lysed with 1% Igepal in water. Dead mycobacteria were stained with PI as described by Anes *et al.* (2006). Mycobacteria pellets were resuspended in PBS, and dead (red or yellow) and live (green) bacteria were counted in a Neubauer chamber by fluorescence microscopy.

#### *Preparation of whole-cell extracts and immunoblot analysis*

Whole-cell extracts were prepared as previously described (Anes *et al.*, 2006). Equal amounts of protein were loaded on a 6% SDS-PAGE, transferred to nitrocellulose membrane and probed with a mouse anti-iNOS (BD Pharmigen) and mouse anti-actin (BD Pharmigen) antibodies. Enhanced chemiluminescence (Pierce biotechnology) was used to visualize antibody binding. Actin was used to assess the amount of total protein in the different membrane isolates.

#### *Electron microscopy*

Macrophages (J774.A1, HMDM) infected with *M. bovis* spp. as described previously in this section were fixed with 1% glutaraldehyde (EM grade, Sigma) in cell culture medium without serum for 10 min at 37°C. Cell cultures were washed several times with 200 mM Hepes pH 7.4 and incubated overnight at room temperature with 1% glutaraldehyde in 200 mM Hepes pH 7.4. Samples were processed for cryo-sectioning as described by Tokuyasu (1973). Negative staining of the cryo sections using ammonium molybdate-methyl celluloase was carried out as described by Griffiths (1993).

After fixation, some samples were post-fixed in osmium and uranyl acetate and embedded in epoxy resin as described previously (Bozzola *et al.*, 1973). Thin sections were stained with lead citrate and uranyl acetate and analysed by transmission electron microscopy.

#### *Modelling the intracellular transport and killing of M. bovis BCG*

A set of seven differential equations were generated in the COPASI 4.0.20 modelling program (<http://www.copasi.org>) (Hoops *et al.*, 2006), describing the duplication, killing in early and late compartments, transport of live and dead bacteria from early to late compartments (= phagosome maturation), and disappearance (digestion) of bacteria in early and late compartments. Simple mass action reactions were used for the kinetics of all steps. The same program was used to find a set of parameters that minimize the weighted sum of square differences between values predicted for the model and the experimental data. The COPASI file is provided as *Supplementary material*.

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### Supplementary material

The following supplementary material is available for this article  
 online:

**Fig. S1.** Comparison of cfu of *M. tuberculosis* in J774 macroph-  
 ages up to 7 day infection with and without  $\gamma$ -IFN treatment.  
 COPASI FILE-Model B (COPASI).cps at [http://www.ff.ul.pt/  
 paginas/eanes:COPASI](http://www.ff.ul.pt/paginas/eanes:COPASI)

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q3	Au: <b>Gomes <i>et al.</i> 1999</b> has not been included in the list	
q4	Au: <b>Armstrong &amp; D'Arcy Hart 1971</b> has not been included in the list	
q5	Au: <b>Armstrong &amp; Hart 1971</b> has not been included in the list	
q6	Au: <b>Van der Wel 2007</b> has been changed to <b>Van der Wel et al. 2007</b> so that this citation matches the list.	
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# MARKED PROOF

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Please use the proof correction marks shown below for all alterations and corrections. If you wish to return your proof by fax you should ensure that all amendments are written clearly in dark ink and are made well within the page margins.

<i>Instruction to printer</i>	<i>Textual mark</i>	<i>Marginal mark</i>
Leave unchanged	... under matter to remain	Ⓟ
Insert in text the matter indicated in the margin	∧	New matter followed by ∧ or ∧ <sup>Ⓢ</sup>
Delete	/ through single character, rule or underline or ┌───┐ through all characters to be deleted	Ⓞ or Ⓞ <sup>Ⓢ</sup>
Substitute character or substitute part of one or more word(s)	/ through letter or ┌───┐ through characters	new character / or new characters /
Change to italics	— under matter to be changed	↙
Change to capitals	≡ under matter to be changed	≡
Change to small capitals	≡ under matter to be changed	≡
Change to bold type	~ under matter to be changed	~
Change to bold italic	≈ under matter to be changed	≈
Change to lower case	Encircle matter to be changed	≡
Change italic to upright type	(As above)	⊕
Change bold to non-bold type	(As above)	⊖
Insert 'superior' character	/ through character or ∧ where required	Υ or Υ under character e.g. Υ or Υ
Insert 'inferior' character	(As above)	∧ over character e.g. ∧
Insert full stop	(As above)	⊙
Insert comma	(As above)	,
Insert single quotation marks	(As above)	ʹ or ʸ and/or ʹ or ʸ
Insert double quotation marks	(As above)	“ or ” and/or ” or ”
Insert hyphen	(As above)	⊞
Start new paragraph	┌	┌
No new paragraph	┐	┐
Transpose	┌┐	┌┐
Close up	linking ○ characters	○
Insert or substitute space between characters or words	/ through character or ∧ where required	Υ
Reduce space between characters or words		↑