Published in final edited form as:

Cell Microbiol. 2013 June ; 15(6): 843–859. doi:10.1111/cmi.12092.

Infection of macrophages with *Mycobacterium tuberculosis* induces global modifications to phagosomal function

Maria Podinovskaia, Wonsik Lee, Shannon Caldwell, and David G. Russell*

Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA.

Abstract

The phagosome is a central mediator of both the homeostatic and microbicidal functions of a macrophage. Following phagocytosis, *Mycobacterium tuberculosis* (Mtb) is able to establish infection through arresting phagosome maturation and avoiding the consequences of delivery to the lysosome. The infection of a macrophage by Mtb leads to marked changes in the behavior of both the macrophage and the surrounding tissue as the bacterium modulates its environment to promote its survival. In this study, we use functional physiological assays to probe the biology of the phagosomal network in Mtb-infected macrophages. The resulting data demonstrate that Mtb modifies phagosomal function in a TLR2/TLR4-dependent manner, and that most of these modifications are consistent with an increase in the activation status of the cell. Specifically, superoxide burst is enhanced and lipolytic activity is decreased upon infection. There are some species- or cell type-specific differences between human and murine macrophages in the rates of acidification and the degree of proteolysis. However, the most significant modification is the marked reduction in intra-phagosomal lipolysis because this correlates with the marked increase in the retention of host lipids in the infected macrophage, which provides a potential source of nutrients that can be accessed by Mtb.

INTRODUCTION

Mycobacterium tuberculosis (Mtb) is an intracellular pathogen and the causative agent of the disease tuberculosis, which affects millions of people worldwide every year. At initiation of infection, Mtb is ingested by macrophages, the first line of defense against bacterial pathogens. Mtb, however, is able to arrest normal phagosome maturation, avoid fusion with lysosomes, and render the intraphagosomal environment more compatible with bacterial survival and replication (Russell, 2011; de Chastellier *et al.*, 2009; Deretic *et al.*, 2006; Russell, 2001). Furthermore, Mtb releases cell wall lipids and other effectors that modulate both the function of the host cell and the surrounding tissue (Russell, 2011; Geisel *et al.*, 2005; van den Elzen *et al.*, 2005; Rhoades *et al.*, 2003; Beatty *et al.*, 2000). The impact of infection, and released bacteria effectors such as the bioactive lipids mentioned above, or the substrates of Mtb's ESX secretory systems (Romagnoli *et al.*, 2012; Uplekar *et al.*, 2011; Smith *et al.*, 2008; McLaughlin *et al.*, 2007), on macrophage function have not been fully established. This is particularly true of the phagosomal network in the host cell, which has not been characterized beyond the observation that model particles go to phagolysosomes in infected cells (de Chastellier *et al.*, 2009). Modifications to phagocytic function might impact Mtb survival through influencing nutrient availability for the bacterium, antigen presentation to lymphocytes, and the microbicidal capacity of the infected macrophage. In

*Corresponding author: Tel: +1 607 253 4272; Fax: +1 607 253 4058; dgr8@cornell.edu.

this paper we explore functional changes within the phagosomal network of the macrophage associated with Mtb infection.

The macrophage phagosome exhibits functional plasticity, with outcomes determined by the physiological state of the macrophage. The main role of a resting macrophage is to perform a range of homeostatic tasks, such as tissue repair and removal of debris and apoptotic cells in a non-inflammatory manner. For this, the phagosome is programmed to degrade internalized material with high efficiency, through rapid acidification and fusion with lysosomes rich in proteolytic and lipolytic enzymes (Yates *et al.*, 2007). Upon exposure to TNF- α or IFN- γ , or TLR agonists, the macrophage becomes activated and, as an immune effector cell, shifts focus to microbicidal activity and antigen presentation (Russell *et al.*, 2009a). Increased microbicidal capacity of the phagosome is achieved through production of reactive oxygen and nitrogen species (MacMicking *et al.*, 1997), whereas hydrolytic enzyme activities have to be carefully balanced between efficient generation of T-cell epitopes and effective elimination of the pathogen (Rybicka *et al.*, 2010; Russell *et al.*, 2009a; Yates *et al.*, 2007). The ability of the macrophage to modulate phagosome function clearly offers opportunities to Mtb to adjust the global behavior of its host to impact its own survival.

We studied both murine bone marrow-derived macrophages, and human monocyte-derived macrophages to explore impact of Mtb infection on phagosomal function in both species and cell type. To investigate phagosome function in Mtb-infected macrophages, we examined the physiological and hydrolytic properties of phagosomes containing IgG-coated beads internalized by infected versus uninfected macrophages. The beads were complexed with different fluorogenic reporters that allow the quantitative monitoring of phagosome function in real-time at the level of the individual phagosome for a range of different activities including superoxide burst, acidification and proteolytic and lipolytic activities. Infected cells were examined using (1) fluorescence plate reader, which allows monitoring of multiple conditions with many replicates; (2) confocal microscopy, which provides excellent spatial and temporal detail at the level of the individual phagosome but has limited statistical power; and (3) flow cytometry, which provides detail at individual cell level across the entire cell population. The three approaches complement one another to generate a dataset of considerable sensitivity, and spatial and temporal resolution.

Our findings reveal that Mtb infection produced changes in phagosome function that were broadly comparable to those changes observed following activation with IFN- γ or LPS, and which were in part TLR2/TLR4 dependent. We observe an increase in the intensity of superoxide burst and a reduction in lipolytic activity, in both human and murine macrophages infected with Mtb. Infection of human macrophages led to a delay in acidification of bead-bearing phagosomes and an associated decrease in proteolytic activity, but no significant alteration in rates of acidification or proteolytic activity was observed upon infection of murine macrophages. This suggests that there are, not surprisingly, some species, or cell-type specific differences in the modulation of macrophage function by Mtb. Confocal microscopy-based characterization of superoxide burst and the kinetics of acidification revealed considerable heterogeneity in both the amplitude of superoxide burst and the initiation of acidification amongst individual phagosomes. Of greatest potential significance is the decrease in phagosomal lipolysis, which is accompanied by an increased retention of lipids by Mtb-infected versus uninfected macrophages. The lipid droplets in these foamy macrophages were also observed within Mtb-containing phagosomes, and we could traffic fluorescently-tagged lipids to both the lipid droplets and the lipid inclusions in Mtb itself. Acquiring more detailed knowledge of phagosome biology and functional modifications of the phagosomal network upon Mtb infection is important in improving our understanding of host-pathogen interactions and their impact on the outcome of infection.

RESULTS

Individual phagosomes display heterogeneity in generation of superoxide burst

Generation of oxidative burst within phagosomes is an essential part of microbicidal response of macrophages to infection (Fang, 2004). Upon phagocytosis, NADPH oxidase complex is assembled in the phagosomal membrane which leads to the reduction of oxygen to produce superoxide (O_2^-) within the phagosomal lumen (Groemping and Rittinger, 2005). Interaction of superoxide with halides, amines and other phagosomal radicals results in the formation of highly cytotoxic secondary radicals, such as peroxynitrite, which contribute to microbial killing (Nathan and Shiloh, 2000). NADPH oxidase activity is regulated by the recruitment of the guanosine triphosphate Rac2, which fine tunes the onset, extent and duration of superoxide production (Quinn and Gauss, 2004). Detailed analysis of superoxide burst activity in phagosomes would help define changes in oxidizing potential of macrophages upon Mtb infection.

Detection of superoxide burst activity in phagosomes in real time using beads coated with oxidation-sensitive fluorochromes has been described by VanderVen and colleagues (VanderVen *et al.*, 2009). In these assays, a spectrofluorometer was used to measure overall generation of superoxide burst of approximately 3.5×10^4 macrophages, and was shown to be sufficiently sensitive to distinguish oxidation profiles between resting and activated macrophages. The aim of the current experiment was to investigate oxidative capacity at individual phagosome level in real-time by confocal microscopy. Oxidation-sensitive H2DCFDA-OxyBURST coated beads were added to murine bone marrow-derived macrophages (BMDM) and monitored by confocal microscopy over 50 min, with frames acquired every 1 min. The extent of substrate oxidation, as measured by fluorescence ratio of the substrate over calibration fluorophore, varied between phagosomes, even within the same macrophage, although the timing of superoxide burst generation showed minimal variation and ceased 10–25 min post-induction for each phagosome (Fig. 1). A proportion of phagosomes remained negative for substrate oxidation. This is in line with previous studies showing phagosome-to-phagosome heterogeneity (Henry *et al.*, 2004). To confirm heterogeneity was truly phagosomal, and not related to variation between individual beads, the beads were tested for uniformity by treatment with horseradish peroxidase and 0.003% hydrogen peroxide, with prior de-esterification of the substrate with 1.5 M hydroxylamine (pH8). Confocal microscopy confirmed all of the beads were oxidized by the hydrogen peroxide treatment and fluoresced with minimal variation between the beads (Fig. 1B).

M. tuberculosis infection of macrophages leads to an enhanced generation of superoxide burst in bead-containing phagosomes

Next we tested the effect of mycobacterial infection of macrophages on superoxide burst generation in bead-containing phagosomes. Macrophages were infected with Mtb for 5 days, at multiplicity of infection (MOI) of 5:1, 2:1, or 10 heat-inactivated bacteria per macrophage. Subsequently, H2DCFDA-OxyBURST beads were added to the infected macrophages for fluorescence analysis by plate reader, flow cytometry and confocal microscopy. It is important to note that this assay measured oxidative activity in bead-containing phagosomes, which are believed not to fuse with Mtb vacuoles within the same macrophage, confirmed in current experiments by electron microscopy (Fig. 2). As observed in BMDM in Fig. 3A, there was an increase in phagosomal substrate oxidation which correlated with MOI, detectable with both plate reader and confocal microscopy analyses. Mock infection of macrophages with heat-inactivated bacteria produced a small increase in oxidative activity, comparable to that observed with low multiplicity of infection with live bacteria. The enhanced generation of superoxide burst in phagosomes of infected macrophages is similar to that seen in activated macrophages, previously reported by

VanderVen and colleagues (VanderVen *et al.*, 2009). A detailed analysis of phagosome data acquired by confocal microscopy revealed that, following infection, bead-containing phagosomes displayed higher efficiency of superoxide burst, both in terms of phagosome numbers, timing, and amplitude of oxidation at individual phagosome level (Fig. 3B). Superoxide burst lasted approximately 30 min in resting macrophages and 20 min in infected or activated macrophages. Interestingly, combined infection and activation of macrophages led to a more rapid induction of superoxide burst in bead-containing phagosomes, that ceased 6 min post-induction (data not shown). As with murine BMDM, human monocyte-derived macrophages (HMDM) displayed enhanced superoxide burst activity upon infection and activation, as measured by plate reader and by confocal microscopy (Fig. 3D,E).

To investigate whether the enhanced superoxide burst was a phenotype specific to *Mtb* infection or was merely a reflection of cell activation, as documented previously (VanderVen *et al.*, 2009), we repeated these assays by flow cytometry (Fig. 3F–H) and by confocal microscopy (data not shown) using infected BMDM from TLR2/TLR4 double-knockout mice. Flow cytometric dotplot in Fig. 3F reveals two macrophage populations, with and without phagocytosed beads, detected as positive and negative for calibration fluor Alexa 633 fluorescence, respectively. Only the Alexa 633 positive population was used for analysis of superoxide burst activity and is the population seen to increase in H2DCFDA-OxyBURST fluorescence over time. At 24 h post infection, only wild-type macrophages, but not TLR2/TLR4 deficient macrophages, showed enhanced oxidative activity compared to resting macrophages, suggesting that TLR2/TLR4-mediated activation was solely responsible for this early enhancement in superoxide burst activity (Fig. 3G). However, at 5 days post infection, even TLR2/TLR4 deficient macrophages displayed a partial increase in oxidative activity upon infection, although not to the same extent as wild-type macrophages (Fig. 3H). This indicates that factors other than TLR2/TLR4-mediated activation play a role in enhancing oxidative burst in established infection.

Phagosome acidification is a rapid process, taking 2–4 min to complete after the onset of acidification

Phagosome acidification is prerequisite to optimal hydrolytic enzyme function and phagosomal content degradation. Furthermore, acidic pH establishes a hostile environment for an internalized pathogen. Phagosome acidification takes place early in phagosome maturation and is predominantly a direct result of recruitment of vacuolar proton-ATPase (V-ATPase) to the phagosomal membrane (Russell *et al.*, 2009a; Lukacs *et al.*, 1990).

In preliminary experiments, pH-sensitive carboxyfluorescein-coated beads were used to characterize phagosome acidification in uninfected macrophages by confocal microscopy at individual phagosome level. Beads were added to macrophages and 40 beads were tracked by confocal microscopy over 40 min, with frames recorded every 1–2 min. Relative pH of individual phagosomes was calculated based on the ratio of bead fluorescence emitted at 520 nm following alternate excitation at pH-sensitive and pH-insensitive wavelengths of 490 nm and 450 nm respectively. Using polynomial regression of a standard curve (data not shown), as described (Yates and Russell, 2005), we calculate initial phagosomal pH to be 7.5, and final phagosomal pH to be 5.5. Detailed analysis revealed that there is minimal variation in pH of acidified phagosomes or in the rate of acidification once the process initiated. However, there is substantial variation in the onset of acidification between individual phagosomes, ranging between 5 and 40 min (mean = 19 min, standard deviation = 16 min) for murine BMDM (Fig. 4A). For each phagosome, the acidification process took 2–4 min to complete upon initiation. Human macrophage phagosomes displayed similar acidification kinetics upon initiation (Fig. 4B), however, although some phagosomes acidified within 2 min after the onset of acidification, others took up to 14 min to acidify completely. In a

separate study sampling a greater number of individual phagosomes (n=200), the proportion of acidified phagosomes at 80 min was 95% for murine BMDM and 80% for HMDM (data not shown). It is unclear if this delay in acidification represents delayed internalization of surface-bound particles or delayed initiation of acidification of internalized particles.

***M. tuberculosis* infection of macrophages delays acidification of bead-containing phagosomes of human macrophages but not of mouse bone marrow derived macrophages**

Following detailed characterization of phagosome acidification, we went on to determine whether or not *Mtb* infection had any effect on acidification of bead-containing phagosomes of resting or activated macrophages. While *Mycobacterium* is known to prevent acidification of its own phagosome (Mwandumba *et al.*, 2004; Sturgill-Koszycki *et al.*, 1994; Crowle *et al.*, 1991), the results of our plate reader and confocal microscopy studies reveal that bead-containing phagosomes of infected murine BMDM acidify normally, irrespective of multiplicity of infection, viability of mycobacteria, or activation status of the macrophages (Fig. 5A and data not shown). A detailed analysis of fluorescence intensity of individual beads showed identical acidification patterns for resting and infected macrophages (Fig. 5B). In contrast, infected or activated HMDM showed a distinct delay in phagosome acidification compared to resting macrophages (Fig. 5C). At 40 min 46% of phagosomes in uninfected macrophages were acidified compared to 20% in infected macrophages (Fig. 5D).

***M. tuberculosis* infection of macrophages leads to differential modulation of proteolysis in murine versus human macrophages**

To test whether or not *Mtb* infection of macrophages affects proteolytic activity of bead-containing phagosomes, IgG beads coated with the fluorogenic substrate DQ green BSA were added to infected or activated macrophages. A small increase in proteolytic activity was evident in infected and activated murine BMDM compared to uninfected macrophages, becoming more marked later in infection (Fig. 6A–D). The slight increase in proteolytic activity was independent of TLR2/4-mediated activation (Fig. 6D). Phagosomal proteolytic activity is the product of many factors, including the extent of phagosomal superoxide burst (Rybicka *et al.*, 2010), the degree of acidification, the extent of phagosome-lysosome fusion, and proteolytic capacity of lysosomes (Yates *et al.*, 2007). Oxidative burst has previously been reported to inactivate phagosomal cysteine cathepsins and to inhibit phagosomal bulk proteolysis (Rybicka *et al.*, 2010). Indeed, infection experiments using IgG beads coated with a cysteine protease-specific substrate revealed a decrease in cysteine protease activity that correlated with MOI (Fig. 6E). The observed increase in bulk proteolytic activity upon infection could be generated through an increase in phagosome-lysosome fusion or in proteolytic capacity of lysosomes, possibly through increased expression of other hydrolases such as cathepsin D. The expression levels of transcripts for cathepsin D, and other cathepsins, are markedly up-regulated in macrophages in human TB granulomas (Kim *et al.*, 2010). In contrast to murine BMDM, HMDM showed a delay in bulk proteolytic activity upon infection or activation, with synergistic effect upon combined activation and infection (Fig. 6F–G). This is consistent with the observed delay in acidification (Fig. 5C), the process, which is integral to efficient proteolysis (Fig. 3D).

***M. tuberculosis* infection of macrophages leads to a decrease in lipolytic activity of bead-containing phagosomes**

The macrophage has a key role in lipid homeostasis through the recycling of low-density lipoprotein (LDL) from serum as well as processing lipid from phagocytosed cellular debris (Schmitz and Grandl, 2008). The internalized lipids are hydrolyzed by a variety of phagosomal and lysosomal lipases (VanderVen *et al.*, 2010; Hornick *et al.*, 1992). Persistent

inflammation, such as seen in *Mtb* infection, can lead to the induction of foamy macrophages which are characterized by lipid sequestration and lipid body formation within the macrophage (Kim *et al.*, 2010; Peyron *et al.*, 2008; Bobryshev, 2006).

In order to determine whether or not *Mtb* infection affects lipolytic activity in bead-containing phagosomes, beads coated with a fluorogenic pyrene-based esterase substrate were added to infected macrophages and monitored by fluorescence plate reader. Lipolytic activity was measured by the increase in pyrene fluorescence upon esterolysis. As shown in Fig. 7A, there was decrease in lipolytic activity in infected murine BMDM that correlated with MOI, and that was further accentuated by macrophage activation (Fig. 7B). However, this decrease in lipolytic activity in phagosomes in infected or activated BMDM was not observed in parallel experiments conducted on TLR2/TLR4 double-knockout macrophages (Fig. 7C). These data indicate TLR2/TLR4-mediated activation is responsible for the observed decrease in lipolytic activity in infected macrophages. Consistent with murine BMDM, lipolytic activity in HMDM was also decreased upon infection or activation (Fig. 7D). Analysis of phagosomal lipolytic activity in macrophages by confocal microscopy confirmed the steady degradation of the triglyceride substrate. However, in contrast to the other hydrolytic assays, the fluorescent product of hydrolysis was relatively rapidly exported out of the phagosome as observed by the increase in false-color red fluorescence throughout macrophages in Fig. 7F. Confocal microscopy (Fig. 7E) may therefore underestimate the extent of lipolytic activity, particularly at later time points and is therefore a less favored method of analysis for intraphagosomal lipolysis compared to the plate reader-based assay.

***M. tuberculosis* infection leads to retention of lipids and the maintenance of foamy macrophage phenotype**

The foamy macrophage phenotype can be induced by *Mtb*-infection, shown in Fig. 2, or by feeding macrophages exogenous lipids such as oleate. We decided to probe lipid turnover/retention in *Mtb*-infected and uninfected BMDM by incubating the cells in 400 μ M oleate, complexed with BSA for 24 hrs, then infecting the macrophages and following retention of the sequestered lipid over several days. Lipid droplets were detected by treatment of macrophages with the hydrophobic dye Bodipy-493/503 prior to imaging by confocal microscope (Fig. 8A). Comparable levels of fluorescent label were observed in both cells following loading with oleate, however, the uninfected macrophages rapidly lost the lipid upon further culture. The retention of lipid by infected macrophages is likely due to many factors beyond the reduction in phagosomal lipolysis, however these data are consistent with the decreased degradation and/or turnover of lipids in infected macrophages.

Lipid droplets are formed from the ER membrane and thought to exist as distinct cytosolic organelles. However, as reported previously by Peyron and colleagues (Peyron *et al.*, 2008), we observe lipid droplets within *Mtb*-containing vacuoles (Fig. 2). Finally, to determine if *Mtb* could access lipid taken up by their host cell we infected BMDM and allowed the infection to establish for 4 days. The infected cells were then incubated in oleate overnight and pulsed with Bodipy FL-C16. The fluorescent lipid was incorporated into both the lipid droplets of the host cell and the lipid inclusions within *Mtb* itself (Fig. 8B), demonstrating that *Mtb* has access to lipids sequestered within the host cell.

DISCUSSION

The detailed live-cell analysis approaches employed in this study to explore phagosome function in macrophages revealed some marked trends at individual phagosome level, which both complement our current understanding of phagosome maturation (Russell *et al.*, 2009a) and raise some new questions with respect to the phenotype of individual phagosomes. For example, we find that, with respect to the superoxide burst, the amplitude of response is

highly variable at the level of the individual particle, and a proportion of phagosomes remain negative for superoxide burst activity even in activated macrophages. In contrast, phagosome acidification appears to rely on a trigger that, once tripped, uniformly and rapidly reduces pH from neutral to below 5.5, but the timing of that trigger event varies widely between phagosomes. These early events also impact hydrolytic function, which may in turn explain the variation seen in proteolytic activity between individual phagosomes. Variation in the maturation of individual phagosomes has been reported previously by Henry and colleagues using markers such as Rab5 and LAMP-1 (Henry *et al.*, 2004) and this heterogeneity is an important consideration when designing studies to probe small differences in phagosome maturation under varying conditions.

Infection of macrophages with Mtb led to functional changes comparable to those observed previously upon activation of macrophages with LPS (VanderVen *et al.*, 2009; Yates *et al.*, 2007), these included more efficient superoxide burst, reduced lipolysis and reduced cysteine proteinase activity. These previously characterized traits were global changes in macrophage behavior (VanderVen *et al.*, 2009; Yates *et al.*, 2007; Yates and Russell, 2005) and not the localized phagosomal responses reported by Medzhitov and colleagues (Blander and Medzhitov, 2004), which remain the subject of debate (Russell and Yates, 2007b; Russell and Yates, 2007a; Blander and Medzhitov, 2006). In the case of murine BMDM, these phenotypic shifts were shown to be mediated via TLR2/TLR4 signaling. Activation is an important switch for a macrophage to shift from its role in homeostasis, in tasks such as tissue repair and removal of debris and apoptotic cells, to an immune-centered role of bacterial killing, degradation and antigen presentation (Russell *et al.*, 2009a; Yates *et al.*, 2007).

Interestingly, the effect of activation on phagosome function differed between murine BMDM and human macrophages. This could be attributed to differing baseline phagocytic properties of the two cell types. For example, murine BMDM phagocytosed beads more efficiently, showed a greater extent of superoxide burst and could generally be described as more “active” under resting conditions than HMDM. This in turn could be due to culturing conditions as well as the source of macrophages. The resting state properties of phagosomes might be responsible for the differences observed under activated conditions. Variation in strain background of mice did not affect observed changes in phagosome function upon macrophage activation, as previously reported for C57BL/6 and C3H/HeJ mice (Yates and Russell, 2005) and consistent trends were obtained with human macrophages from several donors.

The differences in superoxide burst activities of resting murine and human macrophages may contribute to the patterns of functional changes observed with acidification and proteolytic activity upon infection of the two cell types. Extremely low superoxide burst activity in resting HMDM combined with efficient oxidation in activated states is predicted to cause alkalization of the phagosomal lumen (Savina *et al.*, 2006) which in turn would limit proteolytic capacity of the phagosome of an activated or infected macrophage. Limited proteolysis is predicted to delay pathogen degradation and has previously been shown to favor antigen presentation (Delamarre *et al.*, 2005). It is possible that the less pronounced change in superoxide burst activity following infection of murine BMDM, though effective at inhibiting cysteine cathepsin activity through oxidative inactivation (Rybicka *et al.*, 2010), was insufficient to lead to a detectable reduction in acidification or bulk proteolytic activity. The enhanced bulk proteolytic activity observed in murine BMDM following infection could be due to increased expression and/or delivery of hydrolytic enzymes to phagosomes, which would be consistent with the increased transcription of lysosomal protease genes observed in human TB granulomas (Kim *et al.*, 2010).

Alterations in phagosome function following infection were observed throughout the cell culture, even in cells not harboring intracellular Mtb (unpublished observations), with all cells showing activation-like changes. It has previously been shown that intracellular mycobacteria shed lipids, such as trehalose dimycolate (TDM) and phosphatidylinositol dimannosides (PIM2), which spread via endocytic/exocytic networks throughout the macrophage as well as to uninfected macrophages (van den Elzen *et al.*, 2005; Rhoades *et al.*, 2003; Russell *et al.*, 2002; Beatty *et al.*, 2000). It is possible that the spread of such bacterial components triggers culture-wide TLR2/TLR4-dependent microbicidal response (Bowdish *et al.*, 2009; Geisel *et al.*, 2005). The delayed activation observed in infected TLR2/TLR4-deficient macrophages indicates that other TLR-independent pathways of activation are triggered. Other potential mediators of this response may involve CD14, Mincle and NOD receptors (Bowdish *et al.*, 2009; Ishikawa *et al.*, 2009; Matsunaga and Moody, 2009; Divangahi *et al.*, 2008; Yang *et al.*, 2007; Ferwerda *et al.*, 2005).

One of the more potentially significant alterations in phagosomal properties is the reduction in lipolysis following Mtb infection. It is known that Mtb induces lipid-rich foam cell phenotype in its host macrophage (Figure 2) (Kim *et al.*, 2010; Russell *et al.*, 2009b; Peyron *et al.*, 2008), possibly via TLR2 and TLR6 activation (Mattos *et al.*, 2009). Consistent with the observed reduction in phagosomal triacyl glycerol (TAG) hydrolysis following Mtb infection, TAG has been shown to accumulate in the lipid droplets of Mtb-infected macrophages (Daniel *et al.*, 2011). Mtb has access to host TAG and utilizes TAG-derived fatty acids to synthesize mycobacterial lipids (Daniel *et al.*, 2011). These findings were confirmed and extended in the current study where we readily observed lipid droplets within Mtb-containing vacuoles and could traffic fluorescently-tagged lipids to intracellular Mtb. These observations are entirely consistent with the extensive re-programming of host lipid metabolism induced by Mtb infection (Mahajan *et al.*, 2012; Singh *et al.*, 2012). The appearance of lipid droplets within the endosomal network, and Mtb-containing vacuoles, appears unique to this infection and was also observed in the study of Peyron and colleagues (Peyron *et al.*, 2008). Whether this indicates that Mtb infection has induced the trafficking of lipid droplets from the cytosol into the endosome or lysosome, or that this is a normal cellular function and it is the degradation of the droplets that is prevented through the reduction in lipolytic activity, remains to be determined. Nonetheless, such reprogramming of lipid metabolism by the host likely aids bacterial nutrition and contributes to the accumulation of caseum within granuloma that is thought to correlate with disease progression at the level of the tissue (Russell, 2011; Kim *et al.*, 2010; Russell *et al.*, 2009b; Hunter *et al.*, 2007). Lipid availability within the caseum is important for dormant Mtb to maintain their cell wall integrity and as an energy source for replication following disease reactivation. The realignment of lipids metabolism in host macrophages and the shifting utilization of different carbon sources by Mtb during the course of infection (Daniel *et al.*, 2011; Russell *et al.*, 2010; Pandey and Sasseti, 2008; McKinney *et al.*, 2000) remains one of the more important and less appreciated areas of this host-pathogen interplay.

EXPERIMENTAL PROCEDURES

Cells and materials

Macrophages were harvested from the bone marrow of C57BL/6 mice (Jackson Laboratories, USA) and TLR2/4 double knock-out mice and maintained in DMEM supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, 20% L-cell-conditioned media and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) at 37°C and 7.0% CO₂. TLR2/4 double knock-out mice were created by Shizuo Akira (Osaka University), generously supplied by Lynn Hajar (University of Washington), and bred at the Cornell University Transgenic Mouse Core Facility (Bowdish *et al.*, 2009). For lipid trafficking studies, macrophages were derived from the bone marrow

of Balb/c mice (Jackson laboratories). Purified human peripheral blood mononuclear cells (PBMCs) were obtained from Elutriation Core Facility, University of Nebraska Medical Center and maintained in DMEM supplemented with 10% human serum, 2 mM L-glutamine, 1 mM sodium pyruvate and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). For plate reader assays, fully differentiated macrophages were transferred to 96-well plates. For flow cytometric studies, cells were transferred to 6-well plates. For microscopy studies, cells were transferred to Ibidi µ-dishes or to 8-well glass-bottom chambers for 18–24 h to establish a confluent monolayer. For lipid staining, cells were transferred to sterile cover slips in 24-well plates. Macrophages were activated with ultrapure LPS isolated from *Salmonella minnesota* (List Biological Laboratories, Campbell, CA, USA; 10 ng/mL) and recombinant IFN-γ (Preprotech, Rocky Hill, NJ, USA; 100 U/mL) for a minimum of 18–24 h. Antibiotics were omitted from cell culture medium 24 h prior to and during infection.

Infection of Macrophages

Mycobacterium tuberculosis strain CDC1551 expressing fluorescent reporter protein mCherry from P_{smyc} was used. This strain was shown to bear no fitness defects *in vitro* or in macrophages (Carroll *et al.*, 2010). It was chosen for easy visualization using excitation and emission wavelengths of 587 nm and 610 nm respectively. Bacteria were grown in standing, vented tissue culture flasks in 7H9 Middlebrook medium, supplemented with OADC, 0.05% Tween-80, and 50 µg/mL hygromycin. For infection, bacteria were added to macrophages at required multiplicity of infection (MOI). After 2.5 h of infection, extracellular mycobacteria were removed by washing. Infected macrophages were maintained in cell culture medium at 37°C and 7% CO₂ for 5 days for the infection to establish, unless otherwise specified. For experiments with heat-inactivated bacteria, *M. tuberculosis* culture was incubated at 65°C for 20 min, prior to infection at high MOI.

Preparation of H2DCFDA-OxyBURST beads

Carboxylated, 3 µm silica beads (Kisker Biotech, Germany) were washed three times in 1 mL PBS by vortexing and centrifugation in a tabletop microfuge at 2000 g for 1 min. Beads were resuspended in PBS with 40 mg/mL carbodiimide and agitated for 15 min. Excess carbodiimide cross-linker was removed by washing the beads twice in 1 mL PBS and once in 1 mL coupling buffer (0.1 M sodium borate, pH 8.0). Beads were resuspended in 1 mL coupling buffer with 1.0 mg defatted BSA and 0.1 mg human IgG (Sigma-Aldrich, USA) for 2 h. Beads were washed twice in coupling buffer, resuspended in 1 mL coupling buffer with 20 µg dichlorodihydrofluorescein diacetate (Molecular Probes, USA) and agitated for 1 h under argon. Beads were washed once in coupling buffer and H2DCFDA labeling repeated. The coated beads were washed twice with coupling buffer, resuspended in 1 mL coupling buffer with 1 µg Alexa Fluor 633-SE (Molecular Probes) and agitated for 1 h under argon. The beads were washed three times in 1 mL PBS, and stored in PBS under argon. Oxidized substrate emits a fluorescent signal at 520 nm when excited at 490 nm, and Alexa Fluor 633 emits at 647 nm when excited at 633 nm. The degree of substrate oxidation was determined by the ratio of the two fluorescence intensities.

Preparation of pH and DQ-BSA beads

Carboxylated, 3 µm silica beads were coated with defatted BSA and IgG as described for OxyBURST beads, and subsequently labeled with pH-sensitive reporter carboxyfluorescein-SE, as detailed by Yates and Russell (Yates and Russell, 2008). Relative pH was reflected in the ratio between fluorescence intensities at 520 nm upon the pH-sensitive excitation at 490 nm and the pH-insensitive excitation at 450 nm. Excitation ratios could be approximated to actual pH values through polynomial regression of a standard curve as described by Yates and colleagues (Yates and Russell, 2005). Beads for general proteolytic activity were

prepared similarly using DQ green BSA as the coupled protein that was subsequently derivatized with the calibration fluor Alexa Fluor 633-SE. Hydrolyzed substrate emits at 520 nm when excited at 490 nm. The degree of substrate hydrolysis was determined by the ratio of the substrate and calibration fluor fluorescence intensities.

Preparation of Cathepsin B/L beads

Cysteine proteinase substrate (Biotin-LC-Phe-Arg)₂-Rhodamine 110 was used for labeling beads coated with IgG and streptavidin as previously described (Yates and Russell, 2005). Briefly, incubation of beads with 0.2 mg/mL substrate in PBS for 1 h was followed by washing and labeling with Alexa Fluor 633-SE. Hydrolyzed substrate emits a fluorescent signal at 515 nm when excited at 490 nm.

Preparation of fluorescent triglyceride substrate beads

Lipid monolayer-bearing particles were prepared as described previously (Yates and Russell, 2005) with a substitution of the described calibration fluor with Bodipy FL C₁₆ fluorescent reagent (Molecular probes; 22 µg/mL). Briefly, 2.0 mg of 3 µM silica Nucleosil C₁₈ reverse phase HPLC beads (Macherey-Nagel, Easton, PA) were coated with 25 µg lipase substrate trinitrophenyl-amino-dodecanoyl-2-pyrenedecanoyl-3-O-hexadecyl-glycerol (kindly provided by Albin Hermetter, Graz University of Technology, Austria), together with 5 µg calibration fluor Bodipy FL C₁₆, as well as 300 µg 1,2-dipalmitoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (Avanti Polar Lipids, Alabaster, AL), 5 µg 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(Cap Biotinyl) (Avanti Polar Lipids) and 50 µg cholesterol, with subsequent opsonisation with IgG. Hydrolyzed triglyceride substrate emits a fluorescent signal at 400 nm when excited at 342 nm and the Bodipy FL fluorescent signal emits at 520 nm when excited at 490 nm.

Phagosomal bead assays

Beads were washed three times in PBS and resuspended in assay buffer [PBS (pH 7.2), 1 mM CaCl₂, 2.7 mM KCl, 0.5 mM MgCl₂, 5 mM dextrose and 10% FCS]. Confluent macrophage monolayers were washed and medium replaced with pre-warmed assay buffer. Beads were added to macrophages at five beads per macrophage. A washing step to synchronize phagocytosis was omitted as all beads settled onto cell monolayers and were bound by macrophages within 2–3 min, as observed by microscopy of the synchronized movement of the macrophage pseudopodia and the adjacent beads, as well as by flow cytometry analysis of consistent bead numbers per macrophage at early and late time points. Addition of beads to the macrophages was considered as the start of assay, and real-time fluorescence acquisition by microscopy or plate reader or sample processing for flow cytometry was initiated within 2–3 min of bead addition, as described below. Following fluorescence measurements by plate reader or just prior to cell fixation for flow cytometry, specimens were examined under a standard light microscope to ensure macrophage viability and internalization of the beads. At least two independent experiments were conducted for each represented technique with a combined total of at least 5 experiments for each studied function for each macrophage type.

Fluorescence acquisition and analysis by confocal microscopy

Images were acquired with a Leica SP5 confocal laser-scanning system with an inverted microscope (Leica Microsystems GmbH, Germany). For analysis, the chamber was maintained at 37°C with a stage heating system, and imaging was performed with an HCX PL APO CS 63.0_1.20 oil objective. Fluorescence signals sequentially acquired using applicable excitation laser lines, and emission was detected using applicable excitation wavelengths with a 30 nm range (+/- 15 nm of a given emission wavelength), respectively.

Images were acquired with a 1.0 Airy unit pinhole and scanning speed of 400 Hz, and fifteen 512×512 optical slices at $1.4 \mu\text{m}$ z-axis intervals were collected at each time-point. Post acquisition, a single three-dimensional maximum projection was performed for each time-point. Leica Application Suite Advanced Fluorescence (LAS-AF 1.8.2) was used for acquisition and analysis of images. Volocity image analysis software (PerkinElmer Life Sciences, USA) was used for tracking beads over time and for quantification of fluorescence. Beads that never made contact with the macrophage and remained motionless were excluded from analysis. GraphPad Prism software was used for dotplots and statistical analyses. Bars in dotplots represent median values and P values were determined using Mann-Whitney test. Dotplots for pH beads represented binomial population prompting their classification into acidified and non-acidified subpopulations with an arbitrary cut-off value and subsequent application of Fisher's Exact test to establish differences in acidification profiles between different samples.

Fluorescence acquisition and analysis by fluorescence plate reader

For fluorescence monitoring of multiple samples by plate reader, beads were added to confluent layers of macrophages in 96-well plates at 5 beads per macrophage at room temperature before transfer to 37°C in a thermostat- regulated fluorescence microplate reader and initiation of fluorescence data collection. Wallac 2100 EnVision multilabel plate reader (PerkinElmer Life Sciences, USA) with top read was used for fluorescence measurements of infectious samples. Gemini EM plate reader and SoftMax Pro acquisition software (Molecular Devices, USA) was used for bottom read fluorescence measurements for all other experiments. Fluorescent emission at applicable wavelengths was recorded during excitation at applicable wavelengths of approximately 1×10^5 cells. At least 3 replicate wells were monitored for each experimental condition.

Fluorescence acquisition and analysis by flow cytometry

For flow cytometric measurements, macrophages containing beads were washed at required time points with cold PBS, scraped into cold PBS and fixed with 4% paraformaldehyde. Samples were run using BD LSR II flow cytometer and FACSDiva acquisition software, and fluorescence intensity analyzed using FlowJo analysis software (BD Biosciences, USA).

Electron microscopy

Carboxylated $3 \mu\text{m}$ latex beads (Polysciences, Warrington, PA, USA) were coated with defatted BSA and IgG as described for OxyBURST beads. In parallel with phagosome bead assays, latex beads were added to infected macrophages for 80 min prior to fixation with buffered glutaraldehyde solution (2.5% glutaraldehyde in 0.1 M sodium cacodylate, 5 mM CaCl_2 , 5 mM MgCl_2 , 0.1 M sucrose, pH 7.2). The samples were stained as described previously (Rohde *et al.*, 2012). Briefly, cells were post-fixed with 1% osmium tetroxide, then treated with 1% aqueous uranyl acetate, with subsequent dehydration in a graded ethanol and propylene oxide series, and followed by gradual infiltration of Spurr's resin. Polymerized blocks were cut into 70nm sections and contrasted with lead citrate and uranyl acetate.

Foamy macrophage induction and analysis

Lipid droplet induction was performed in murine BMDM as described (Listenberger and Brown, 2007). Briefly, 400 μM oleate, conjugated to de-fatted BSA (8:1), was added to macrophages for 24 hours. The induced cells were infected with Mtb at MOI of 5 bacteria per macrophage or left uninfected. To assess the decay of lipid droplets in uninfected versus Mtb-infected macrophages, cells were fixed in 4% paraformaldehyde at specified time points and the lipid droplets were stained with BODIPY 493/503.

To study bacterial access to the labeled lipids, murine BMDM were infected with Mtb for 5 days. The medium was then supplemented with 400 μ M oleate complexed with de-fatted BSA as detailed above, 24 hours prior to imaging. Macrophages were pulsed with 5 μ M Bodipy FL-C16 (Molecular Probes) complexed with de-fatted BSA at 1:1.5 molar ratio for 60 min, rinsed and imaged at excitation wavelength of 488 nm and emission range of 505–535 nm.

Acknowledgments

Authors thank Albin Hermetter for providing the fluorogenic reagent for measuring phagosomal lipolysis. We are grateful to Tanya Parish for sharing the pmCherry3 plasmid. We thank Brian C. VanderVen for his help and advice with experimental procedures. These studies were supported by USPHS NIH awards AI607027 and AI095519 to DGR. Electron microscopy facilities were supported in part by NSF award DMR-1120296.

REFERENCES

- Beatty WL, Rhoades ER, Ullrich HJ, Chatterjee D, Heuser JE, Russell DG. Trafficking and release of mycobacterial lipids from infected macrophages. *Traffic*. 2000; 1:235–247. [PubMed: 11208107]
- Blander JM, Medzhitov R. Regulation of phagosome maturation by signals from toll-like receptors. *Science*. 2004; 304:1014–1018. [PubMed: 15143282]
- Blander JM, Medzhitov R. On regulation of phagosome maturation and antigen presentation. *Nat Immunol*. 2006; 7:1029–1035. [PubMed: 16985500]
- Bobryshev YV. Monocyte recruitment and foam cell formation in atherosclerosis. *Micron*. 2006; 37:208–222. [PubMed: 16360317]
- Bowdish DM, Sakamoto K, Kim MJ, Kroos M, Mukhopadhyay S, Leifer CA, et al. MARCO, TLR2, and CD14 are required for macrophage cytokine responses to mycobacterial trehalose dimycolate and *Mycobacterium tuberculosis*. *PLoS Pathog*. 2009; 5:e1000474. [PubMed: 19521507]
- Carroll P, Schreuder LJ, Muwanguzi-Karugaba J, Wiles S, Robertson BD, Ripoll J, et al. Sensitive detection of gene expression in mycobacteria under replicating and non-replicating conditions using optimized far-red reporters. *PLoS One*. 2010; 5:e9823. [PubMed: 20352111]
- Crowle AJ, Dahl R, Ross E, May MH. Evidence that vesicles containing living, virulent *Mycobacterium tuberculosis* or *Mycobacterium avium* in cultured human macrophages are not acidic. *Infect Immun*. 1991; 59:1823–1831. [PubMed: 1902198]
- Daniel J, Maamar H, Deb C, Sirakova TD, Kolattukudy PE. *Mycobacterium tuberculosis* uses host triacylglycerol to accumulate lipid droplets and acquires a dormancy-like phenotype in lipid-loaded macrophages. *PLoS Pathog*. 2011; 7:e1002093. [PubMed: 21731490]
- de Chastellier C, Forquet F, Gordon A, Thilo L. *Mycobacterium* requires an all-around closely apposing phagosome membrane to maintain the maturation block and this apposition is reestablished when it rescues itself from phagolysosomes. *Cell Microbiol*. 2009; 11:1190–1207. [PubMed: 19388907]
- Delamarre L, Pack M, Chang H, Mellman I, Trombetta ES. Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. *Science*. 2005; 307:1630–1634. [PubMed: 15761154]
- Deretic V, Singh S, Master S, Harris J, Roberts E, Kyei G, et al. *Mycobacterium tuberculosis* inhibition of phagolysosome biogenesis and autophagy as a host defence mechanism. *Cell Microbiol*. 2006; 8:719–727. [PubMed: 16611222]
- Divangahi M, Mostowy S, Coulombe F, Kozak R, Guillot L, Veyrier F, et al. NOD2-deficient mice have impaired resistance to *Mycobacterium tuberculosis* infection through defective innate and adaptive immunity. *J Immunol*. 2008; 181:7157–7165. [PubMed: 18981137]
- Fang FC. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat Rev Microbiol*. 2004; 2:820–832. [PubMed: 15378046]
- Ferwerda G, Girardin SE, Kullberg BJ, Le Bourhis L, de Jong DJ, Langenberg DM, et al. NOD2 and toll-like receptors are nonredundant recognition systems of *Mycobacterium tuberculosis*. *PLoS Pathog*. 2005; 1:279–285. [PubMed: 16322770]

- Geisel RE, Sakamoto K, Russell DG, Rhoades ER. In vivo activity of released cell wall lipids of *Mycobacterium bovis* bacillus Calmette-Guerin is due principally to trehalose mycolates. *J Immunol.* 2005; 174:5007–5015. [PubMed: 15814731]
- Groemping Y, Rittinger K. Activation and assembly of the NADPH oxidase: a structural perspective. *Biochem J.* 2005; 386:401–416. [PubMed: 15588255]
- Henry RM, Hoppe AD, Joshi N, Swanson JA. The uniformity of phagosome maturation in macrophages. *J Cell Biol.* 2004; 164:185–194. [PubMed: 14718518]
- Hornick CA, Thouron C, DeLamatre JG, Huang J. Triacylglycerol hydrolysis in isolated hepatic endosomes. *J Biol Chem.* 1992; 267:3396–3401. [PubMed: 1737793]
- Hunter RL, Jagannath C, Actor JK. Pathology of postprimary tuberculosis in humans and mice: contradiction of long-held beliefs. *Tuberculosis (Edinb).* 2007; 87:267–278. [PubMed: 17369095]
- Ishikawa E, Ishikawa T, Morita YS, Toyonaga K, Yamada H, Takeuchi O, et al. Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle. *J Exp Med.* 2009; 206:2879–2888. [PubMed: 20008526]
- Kim MJ, Wainwright HC, Locketz M, Bekker LG, Walther GB, Dittrich C, et al. Caseation of human tuberculosis granulomas correlates with elevated host lipid metabolism. *EMBO Mol Med.* 2010; 2:258–274. [PubMed: 20597103]
- Listenberger LL, Brown DA. Fluorescent detection of lipid droplets and associated proteins. Chapter 24. *Curr Protoc Cell Biol.* 2007 Unit 24 22.
- Lukacs GL, Rotstein OD, Grinstein S. Phagosomal acidification is mediated by a vacuolar-type H(+)-ATPase in murine macrophages. *J Biol Chem.* 1990; 265:21099–21107. [PubMed: 2147429]
- MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. *Annu Rev Immunol.* 1997; 15:323–350. [PubMed: 9143691]
- Mahajan S, Dkhar HK, Chandra V, Dave S, Nanduri R, Janmeja AK, et al. *Mycobacterium tuberculosis* modulates macrophage lipid-sensing nuclear receptors PPARgamma and TR4 for survival. *J Immunol.* 2012; 188:5593–5603. [PubMed: 22544925]
- Matsunaga I, Moody DB. Mincle is a long sought receptor for mycobacterial cord factor. *J Exp Med.* 2009; 206:2865–2868. [PubMed: 20008525]
- Mattos KA, D'Avila H, Rodrigues LS, Oliveira VG, Sarno EN, Atella GC, et al. Lipid droplet formation in leprosy: Toll-like receptor-regulated organelles involved in eicosanoid formation and *Mycobacterium leprae* pathogenesis. *J Leukoc Biol.* 2009; 87:371–384. [PubMed: 19952355]
- McKinney JD, Honer zu Bentrup K, Munoz-Elias EJ, Miczak A, Chen B, Chan WT, et al. Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature.* 2000; 406:735–738. [PubMed: 10963599]
- McLaughlin B, Chon JS, MacGurn JA, Carlsson F, Cheng TL, Cox JS, Brown EJ. A mycobacterium ESX-1-secreted virulence factor with unique requirements for export. *PLoS Pathog.* 2007; 3:e105. [PubMed: 17676952]
- Mwandumba HC, Russell DG, Nyirenda MH, Anderson J, White SA, Molyneux ME, Squire SB. *Mycobacterium tuberculosis* resides in nonacidified vacuoles in endocytically competent alveolar macrophages from patients with tuberculosis and HIV infection. *J Immunol.* 2004; 172:4592–4598. [PubMed: 15034077]
- Nathan C, Shiloh MU. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci U S A.* 2000; 97:8841–8848. [PubMed: 10922044]
- Pandey AK, Sasseti CM. Mycobacterial persistence requires the utilization of host cholesterol. *Proc Natl Acad Sci U S A.* 2008; 105:4376–4380. [PubMed: 18334639]
- Peyron P, Vaubourgeix J, Poquet Y, Levillain F, Botanch C, Bardou F, et al. Foamy macrophages from tuberculous patients' granulomas constitute a nutrient-rich reservoir for *Mtuberculosis* persistence. *PLoS Pathog.* 2008; 4:e1000204. [PubMed: 19002241]
- Quinn MT, Gauss KA. Structure and regulation of the neutrophil respiratory burst oxidase: comparison with nonphagocyte oxidases. *J Leukoc Biol.* 2004; 76:760–781. [PubMed: 15240752]
- Rhoades E, Hsu F, Torrelles JB, Turk J, Chatterjee D, Russell DG. Identification and macrophage-activating activity of glycolipids released from intracellular *Mycobacterium bovis* BCG. *Mol Microbiol.* 2003; 48:875–888. [PubMed: 12753183]

- Rohde KH, Veiga DF, Caldwell S, Balazsi G, Russell DG. Linking the Transcriptional Profiles and the Physiological States of *Mycobacterium tuberculosis* during an Extended Intracellular Infection. *PLoS Pathog.* 2012; 8:e1002769. [PubMed: 22737072]
- Romagnoli A, Etna MP, Giacomini E, Pardini M, Remoli ME, Corazzari M, et al. ESX-1 dependent impairment of autophagic flux by *Mycobacterium tuberculosis* in human dendritic cells. *Autophagy.* 2012; 8:1357–1370. [PubMed: 22885411]
- Russell DG. *Mycobacterium tuberculosis*: here today, and here tomorrow. *Nat Rev Mol Cell Biol.* 2001; 2:569–577. [PubMed: 11483990]
- Russell DG. *Mycobacterium tuberculosis* and the intimate discourse of a chronic infection. *Immunol Rev.* 2011; 240:252–268. [PubMed: 21349098]
- Russell DG, Yates RM. Toll-like receptors and phagosome maturation. *Nat Immunol.* 2007a; 8:217. author reply 217–218. [PubMed: 17304226]
- Russell DG, Yates RM. TLR signalling and phagosome maturation: an alternative viewpoint. *Cell Microbiol.* 2007b; 9:849–850. [PubMed: 17346307]
- Russell DG, Mwandumba HC, Rhoades EE. *Mycobacterium* and the coat of many lipids. *J Cell Biol.* 2002; 158:421–426. [PubMed: 12147678]
- Russell DG, Vanderven BC, Glennie S, Mwandumba H, Heyderman RS. The macrophage marches on its phagosome: dynamic assays of phagosome function. *Nat Rev Immunol.* 2009a; 9:594–600. [PubMed: 19590530]
- Russell DG, Cardona PJ, Kim MJ, Allain S, Altare F. Foamy macrophages and the progression of the human tuberculosis granuloma. *Nat Immunol.* 2009b; 10:943–948. [PubMed: 19692995]
- Russell DG, VanderVen BC, Lee W, Abramovitch RB, Kim MJ, Homolka S, et al. *Mycobacterium tuberculosis* wears what it eats. *Cell Host Microbe.* 2010; 8:68–76. [PubMed: 20638643]
- Rybicka JM, Balce DR, Khan MF, Krohn RM, Yates RM. NADPH oxidase activity controls phagosomal proteolysis in macrophages through modulation of the luminal redox environment of phagosomes. *Proc Natl Acad Sci U S A.* 2010; 107:10496–10501. [PubMed: 20498052]
- Savina A, Jancic C, Hugues S, Guernonprez P, Vargas P, Moura IC, et al. NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. *Cell.* 2006; 126:205–218. [PubMed: 16839887]
- Schmitz G, Grandl M. Lipid homeostasis in macrophages - implications for atherosclerosis. *Rev Physiol Biochem Pharmacol.* 2008; 160:93–125. [PubMed: 18425439]
- Singh V, Jamwal S, Jain R, Verma P, Gokhale R, Rao KV. *Mycobacterium tuberculosis*-Driven Targeted Recalibration of Macrophage Lipid Homeostasis Promotes the Foamy Phenotype. *Cell Host Microbe.* 2012; 12:669–681. [PubMed: 23159056]
- Smith J, Manoranjan J, Pan M, Bohsali A, Xu J, Liu J, et al. Evidence for pore formation in host cell membranes by ESX-1-secreted ESAT-6 and its role in *Mycobacterium marinum* escape from the vacuole. *Infect Immun.* 2008; 76:5478–5487. [PubMed: 18852239]
- Sturgill-Koszycki S, Schlesinger PH, Chakraborty P, Haddix PL, Collins HL, Fok AK, et al. Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase. *Science.* 1994; 263:678–681. [PubMed: 8303277]
- Uplekar S, Heym B, Friocourt V, Rougemont J, Cole ST. Comparative genomics of Esx genes from clinical isolates of *Mycobacterium tuberculosis* provides evidence for gene conversion and epitope variation. *Infect Immun.* 2011; 79:4042–4049. [PubMed: 21807910]
- van den Elzen P, Garg S, Leon L, Brigl M, Leadbetter EA, Gumperz JE, et al. Apolipoprotein-mediated pathways of lipid antigen presentation. *Nature.* 2005; 437:906–910. [PubMed: 16208376]
- VanderVen BC, Yates RM, Russell DG. Intraphagosomal measurement of the magnitude and duration of the oxidative burst. *Traffic.* 2009; 10:372–378. [PubMed: 19183302]
- VanderVen BC, Hermetter A, Huang A, Maxfield FR, Russell DG, Yates RM. Development of a novel, cell-based chemical screen to identify inhibitors of intraphagosomal lipolysis in macrophages. *Cytometry A.* 2010; 77:751–760. [PubMed: 20653015]
- Yang Y, Yin C, Pandey A, Abbott D, Sasseti C, Kelliher MA. NOD2 pathway activation by MDP or *Mycobacterium tuberculosis* infection involves the stable polyubiquitination of Rip2. *J Biol Chem.* 2007; 282:36223–36229. [PubMed: 17947236]

- Yates RM, Russell DG. Phagosome maturation proceeds independently of stimulation of toll-like receptors 2 and 4. *Immunity*. 2005; 23:409–417. [PubMed: 16226506]
- Yates RM, Russell DG. Real-time spectrofluorometric assays for the luminal environment of the maturing phagosome. *Methods Mol Biol*. 2008; 445:311–325. [PubMed: 18425459]
- Yates RM, Hermetter A, Taylor GA, Russell DG. Macrophage activation downregulates the degradative capacity of the phagosome. *Traffic*. 2007; 8:241–250. [PubMed: 17319801]

Fig. 1. Intensity of superoxide burst varies between individual phagosomes

Beads were coated with H2DCFDA-OxyBURST and evaluated for oxidation in macrophages. (A–C) The kinetics of oxidation of individual beads were plotted as calibrated fluorescence using the ratio of H2DCFDA-OxyBURST fluorescence to Alexa-633 fluorescence. Measurements were taken every 1 min. Each line represents a single bead tracked over time. (A) Beads were added to macrophages at 0 min. (B) To test for bead homogeneity, horseradish peroxidase and hydrogen peroxide were added to beads in assay buffer at 0 min. (C) Beads in assay buffer. (D) Serial confocal images of phagosomal H2DCFDA-OxyBURST beads in resting macrophages. H2DCFDA-OxyBURST signal is displayed in green, Alexa-633 signal is displayed in purple, overlay appears in white. Scale bar, 50 μ m.

Fig. 2. Mtb-containing vacuoles do not fuse with bead-containing phagosomes

Human monocyte-derived macrophages were infected with Mtb for 5 days, and incubated with 3 μm beads for 80 min. (A & B) Survey electron micrographs illustrating the relative distribution of beads (b), lysosomes (Ly), lipid droplets (L), nucleus (N) and Mtb bacilli (some indicated with arrowheads) in the macrophages. The majority of bacilli are present in vacuoles (Va) distinct from lysosomes, however some bacilli can be seen in lysosomal compartments (defined by their denser content). The majority of beads are in separate phagosomes and exhibit little fusion with lysosomes at this time. (C) Lipid droplets (L) can also be observed within Mtb-containing vacuoles (arrow), and some bacilli contain lipid inclusions.

Fig. 3. Mtb infection increases intensity of superoxide burst

Beads were coated with H2DCFDA-OxyBURST and evaluated for oxidation. The kinetics of superoxide burst activity of individual beads were plotted as calibrated fluorescence using the ratio of H2DCFDA-OxyBURST fluorescence to Alexa-633 fluorescence. Beads were added to macrophages at 0 min. Macrophages were infected with Mtb 5 days prior and/or activated with LPS. Beads only = beads in assay buffer without macrophages. (A–C) Murine bone marrow derived macrophages. (D, E) Human monocyte-derived macrophages. (A, D) Average calibrated fluorescence of 4 wells tracked over time using a plate reader; (B, E) dotplots of bead calibrated fluorescence, recorded by confocal microscopy, each dot represents one bead; bars represent median values; P values were determined using Mann-

Whitney test. (C) Confocal microscopy images of macrophages at 20min post bead addition, with bacteria displayed in red, beads displayed in green Scale bar, 50 μ m. (F–H) Flow cytometry of murine bone marrow derived macrophages. (F) Overlaid dotplots representing fluorescence intensities in the FITC and APC channels of resting and infected macrophages at 10 min and 60 min after bead addition, representing superoxide burst activity and the presence of phagocytosed beads in each macrophage, respectively; (G, H) each time point represents ratio of FITC/APC median fluorescence intensities of 10,000 events; (G) macrophages were infected with Mtb 1 day prior to the bead assay. Continuous line (◆) = wild-type macrophages, broken line () = TLR2/4 double knock-out macrophages.

Fig. 4. Although the rate of acidification is comparable between phagosomes, the induction of the acidification process exhibits considerable heterogeneity

Beads were coated with carboxyfluorescein and evaluated for pH by confocal microscopy. The kinetics of acidification of individual phagocytosed beads were plotted as calibrated fluorescence using the ratio of fluorescence emitted at 520 nm, when excited at pH-sensitive and pH-insensitive 488 nm and 450 nm wavelengths, respectively. Beads were added to macrophages at 0 min. Each line represents calibrated fluorescence of individual beads. (A) Murine bone marrow derived macrophages; (B) human monocyte derived macrophages.

Fig. 5. Mtb infection delays phagosome acidification in human macrophages

Beads were coated with carboxyfluorescein and evaluated for pH. The kinetics of acidification of phagocytosed beads were plotted as calibrated fluorescence using the ratio of fluorescence emitted at 520 nm, when excited at pH-sensitive and pH-insensitive 488 nm and 450 nm wavelengths respectively. Beads were added to macrophages at 0 min. Macrophages were infected with Mtb 5 days prior and/or activated with LPS. Beads only = beads in assay buffer without macrophages. (A, B) Murine bone marrow derived macrophages. (C, D) Human monocyte derived macrophages. (A, C) Average calibrated fluorescence of 4 wells tracked over time using a plate reader. (B, D) Dotplots of bead calibrated fluorescence, recorded by confocal microscopy, each dot represents one bead. P values were obtained using Fisher's Exact test applied to acidified and non-acidified subpopulations (separated by broken line in the dotplots).

Fig. 6. Mtb differentially modulates proteolysis in mouse and human macrophages

Beads were coated with DQ-BSA green or Cathepsin B/L substrate and evaluated for proteolytic activity. The kinetics of proteolysis of phagocytosed beads were plotted as calibrated fluorescence using the ratio of DQ-BSA green fluorescence to Alexa-633 fluorescence, except (E) in which the ratio of Rhodamine 110 fluorescence to Alexa-633 fluorescence was used for the evaluation of Cathepsin B/L substrate hydrolysis activity. Beads were added to macrophages at 0 min. Macrophages were infected with Mtb 5 days prior and/or activated with LPS. Beads only = beads in assay buffer without macrophages. (A–E) Murine bone marrow derived macrophages. (F, G) Human monocyte derived macrophages. (A, E, F) Average calibrated fluorescence of 4 wells tracked over time using a

plate reader; (B, G) dotplots of bead calibrated fluorescence, recorded by confocal microscopy, each dot represents one bead; bars represent median values; P values were determined using Mann-Whitney test. (C, D) Flow cytometry; (C) overlaid dotplots representing fluorescence intensities in the FITC and APC channels of resting and infected macrophages at 10 min and 120 min after bead addition, representing proteolytic activity and the presence of phagocytosed beads in each macrophage, respectively; (D) each time point represents ratio of FITC/APC median fluorescence intensities of 10,000 events. Continuous line (◆) = wild-type macrophages, broken line () = TLR2/4 double knock-out macrophages.

Fig. 7. Mtb infection markedly down-regulates intraphagosomal lipolysis

Beads were coated with triglyceride substrate and evaluated for lipolytic activity. The kinetics of lipolysis of phagocytosed beads were plotted as calibrated fluorescence using the ratio of pyrene fluorescence to Bodipy FL fluorescence. Beads were added to macrophages at 0 min. Macrophages were infected with Mtb 5 days prior and/or activated with LPS, except (B, C) where macrophages were infected 1 day prior to infection. Beads only = beads in assay buffer without macrophages. (A–C) Murine bone marrow derived macrophages; (B) Wild-type macrophages; (C) TLR2/4 double knock-out macrophages. (D–F) Human monocyte derived macrophages. (A–D) Average calibrated fluorescence of 4 wells tracked over time using a plate reader. (E) Dotplots of bead calibrated fluorescence, recorded by confocal microscopy, each dot represents one bead, phagocytosed by uninfected resting macrophages; bars represent median values; (F) corresponding serial confocal images.

Triglyceride substrate is displayed in red, Bodipy FL is displayed in green, overlay appears yellow. Scale bar, 50 μm .

Fig. 8. Mtb infection leads to retention of the foamy macrophage phenotype and facilitates bacterial access to host-derived lipids

(A) Murine bone marrow-derived macrophages were induced to form foam cells through incubation with 400 μ M oleate for 24 h. The cells were subsequently infected with Mtb or left uninfected. At 0 h and 48 h after infection (t=0 and t=48) cells were fixed and stained with BODIPY 493/503. Mtb are displayed in red, BODIPY 493/503 is displayed in green, and DAPI-stained nuclei are shown in blue. Absence of green stain in uninfected cells at 48 h indicates loss of oleate-induced lipid droplets. (B) Visualization of trafficking of host-acquired lipids into intracellular Mtb. Murine bone marrow-derived macrophages were infected with Mtb for 5 days and treated with 400 μ M oleate for 24 h. The cells were incubated with the fluorescent fatty acid Bodipy FL-C16 for 60 min prior to analysis by confocal microscopy. Mtb are displayed in red, Bodipy FL-C16 is displayed in green, and co-localization of Mtb with the fluorescent lipid appears in yellow.