

Mycobacterium and the coat of many lipids

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Pathogenic *Mycobacterium* reside inside vacuoles in their host macrophages. These vacuoles fail to fuse with lysosomes yet interact with early endosomes. Glycoconjugates released by the intracellular bacilli traffic through the host cell and are released through exocytosis. These molecules represent both antigens for immune recognition and modulators of immune function. The molecules play key roles in the induction and maintenance of the granuloma, a tissue response that limits bacterial spread yet ensures persistence of the infection.

Introduction

Pathogenic *Mycobacterium* species are responsible for a wide variety of human and animal ailments. In humans, the most notable are tuberculosis (*Mycobacterium tuberculosis*) and leprosy (*Mycobacterium leprae*), whereas *Mycobacterium bovis*, *Mycobacterium avium*, and *Mycobacterium marinum* are common pathogens of cattle, birds, and fish, respectively. Despite the variety of hosts and diseases, all pathogenic *Mycobacterium* spp. survive within their host phagocytes (Fig. 1) and induce a localized, inflammatory response that is granulomatous in nature.

Intracellular survival: remodeling of the endosome

The foundation of our appreciation of the cell biology of the interplay between bacteria and phagocyte was laid by the elegant studies of Philip D'Arcy Hart in the 1970's who demonstrated that *M. tuberculosis*-containing phagosomes fail to fuse with lysosomes after internalization by macrophages (Hart et al., 1972). Moreover, the absence of fusion correlated with the viability of the infecting bacilli to the extent that dead bacteria are delivered to the host cell's lysosomes. Work on this phenomenon exploded in the 1990's, and our understanding of the biology of this cellular compartment is extensive, although the mechanism(s) behind its generation and maintenance remain elusive.

Vacuoles containing pathogenic *Mycobacterium* spp. in

resting macrophages exhibit limited acidification (pH 6.2–3) (Sturgill-Koszycki et al., 1994); however, contrary to early suggestions the vacuoles are dynamic and retain the capacity to fuse with other intracellular vesicles (Russell et al., 1996; Sturgill-Koszycki et al., 1996). In essence, these bacilli manipulate the endosomal system to arrest the maturation of their compartment and retain access to the rapid recycling endosome system of their host cell (Clemens and Horwitz, 1995; Clemens and Horwitz, 1996; Sturgill-Koszycki et al., 1996). The restricted progression of *Mycobacterium*-containing vacuoles limits the hydrolytic capacity of the compartment, facilitates access to nutrients internalized by the host cell, and restricts the interface between the pathogen and the antigen processing and presentation machinery of the macrophage (Russell, 2001).

There has been a plethora of studies describing the aberrant association or retention of early endosomal “markers” with vacuoles containing virulent *Mycobacterium*. In a notable early study, Via et al. (1997) demonstrated that, in contrast to vacuoles formed around inert particles, phagosomes containing *M. bovis* BCG (bacille Calmette-Guérin) remained associated with the small GTPase rab 5 that is known to modulate the fusion behavior of early endosomes. More recently, the same lab reported that vacuoles containing *Mycobacterium* fail to acquire EEA 1, the rab 5- and PI₃P-binding protein, and show degradation of the early endosomal v-SNARE cellubrevin (Fratti et al., 2001, 2002). The former observation indicates that vacuole maturation might be arrested through modulation of PI₃-kinase activity. Intriguingly, inhibitors of these enzymes, including antibodies against VPS34, a class III PI₃-kinase, produce a profile of phagosome-associated proteins comparable to that observed for *Mycobacterium* (Fratti et al., 2001). The degradation of vacuole-associated cellubrevin is also an interesting observation, and the authors suggested that this could lead to diminished delivery from both the TGN and the rapid recycling pathway, which in turn could limit the maturation of the phagosome. Data from previous studies show that *Mycobacterium*-containing vacuoles can access transferrin from the recycling endosomal pathway (Clemens and Horwitz, 1996; Sturgill-Koszycki et al., 1996; Ullrich et al., 1999) and that the kinetics of transferrin flux through the *Mycobacterium*-containing vacuoles is comparable to that of uninfected cells. In addition, cholera toxin B subunit bound to GM1 ganglioside on the surface of infected cells appears rapidly and

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Key words: mycobacterium; macrophage; phagosome; tuberculosis; lipidoglycan

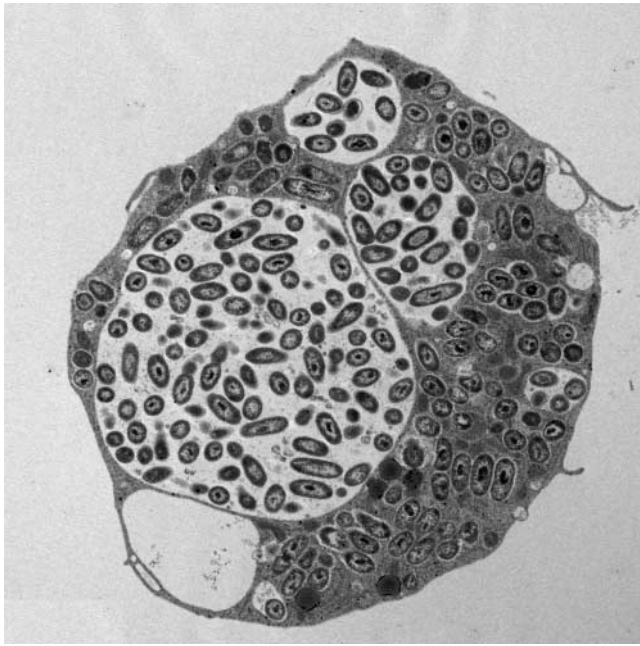


Figure 1. Electron micrograph of an alveolar macrophage isolated by bronchial lavage from a tuberculosis patient in Malawi. The cell is heavily loaded with bacteria that reside within vacuoles. The vacuoles vary both in size and in the number of bacteria. This degree of heterogeneity in vacuole morphology is not observed in infections in culture.

uniformly in vacuoles containing *M. tuberculosis* (Fig. 2) (Russell et al., 1996), supporting the contention that these vacuoles retain access to early endosomes. Vesicular trafficking from the TGN is more difficult to evaluate, although the presence of cathepsin D in its proenzyme form has been detected in *M. avium*-containing vacuoles and is presumably derived from the synthetic pathway and not fusion with lysosomes (Ullrich et al., 1999). Nonetheless, the degradation of cellubrevin could lead to modulation in vesicular trafficking undetected by these assays.

Ferrari and Pieters described the acquisition and retention of the actin-binding coat protein coronin I (TACO) by *Mycobacterium*-containing phagosomes (Ferrari et al., 1999). Coronin I had been shown previously to play a role in phagosome formation in *Dictyostelium* (Maniak et al., 1995) and through the heroic study of Morrissette et al. (1999) is also known to be associated with latex bead phagosomes after internalization. Ferrari and Pieters went on to demonstrate that the mouse coronin I gene, on transfection into fibroblasts, facilitated infection of these cells and suggested that the protein sequestered the bacteria-containing vacuoles and blocked fusion with other intracellular vesicles. However, examination of the original data suggest that, although uptake was clearly different, survival rates were affected minimally by the presence of coronin I (Ferrari et al., 1999). The role of coronin I in regulating maturation of *Mycobacterium bovis* BCG-containing phagosomes was also examined by Schuller et al. (2001) who reported that, although

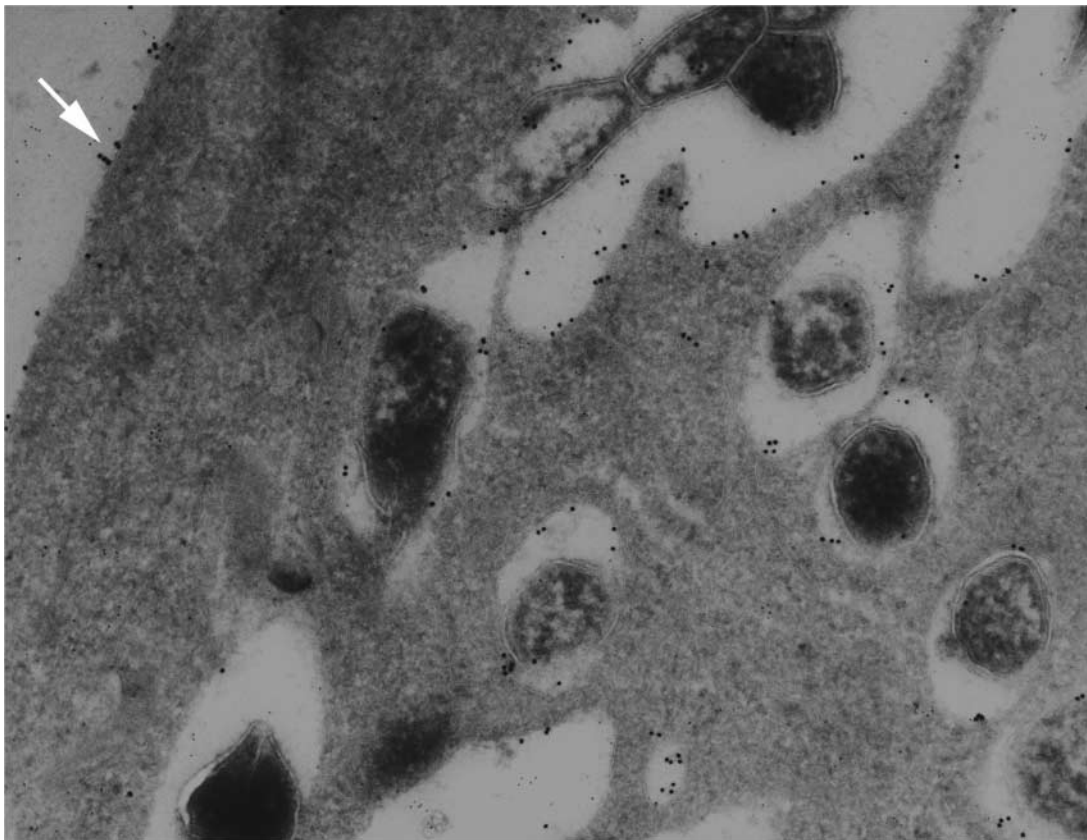


Figure 2. Bone marrow-derived macrophages infected 72 h previously with *M. tuberculosis*, incubated in 10 µg/ml biotinylated cholera toxin B for 15 min, washed, and placed in prewarmed medium for a 45 min chase period. The section was probed with streptavidin/antistreptavidin (anti-rabbit IgG 18 nm gold). Cholera toxin B can be detected in all of the bacterial vacuoles visible in this field, demonstrating the continued interaction between the host cell plasmalemma and the vacuoles harboring the bacilli. Reproduced from Russell et al. (1996).

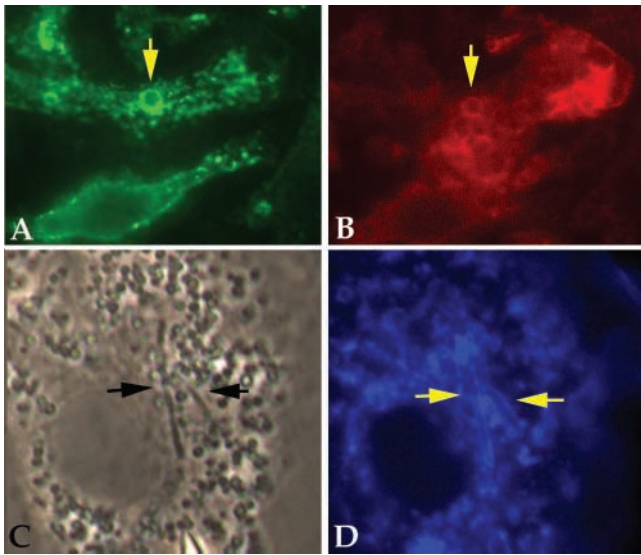


Figure 3. Immunofluorescent analysis of macrophage phagosomes formed around IgG beads (A/B) and *M. tuberculosis* (C/D). Fluorescent analysis of macrophage after uptake of IgG-coated beads (A and B) and infection with *M. tuberculosis* (C and D). Macrophages fixed 15 min after internalization of IgG-coated beads (arrows) probed with antibodies against LAMP1 (A) and coronin I (B). The labeling pattern shows strong colocalization of both LAMP1 and coronin I around the early phagosome. C and D shows phase-contrast and fluorescence micrographs, respectively, of macrophages fixed and probed with filipin after overnight infection with *M. tuberculosis*. Filipin associates directly with cholesterol and shows the distribution of the lipid in the cell. There is a clear halo around the intracellular bacteria (arrows).

coronin I was involved in bacterial uptake, it did not remain associated with vacuoles containing viable bacilli. This is corroborated by the immunofluorescence image of IgG bead phagosomes that shows colocalization of coronin I and LAMP1 (Fig. 3), demonstrating that acquisition of LAMP1, i.e., phagosome maturation, is unaffected by the presence of coronin I.

More recently, Gatfield and Pieters (2000) reported that *Mycobacterium*-containing vacuoles had a high cholesterol content and that depletion of cholesterol from the macrophages before incubation with bacteria rendered the cells refractory to infection. The authors suggested that cholesterol in the host plasmalemma bound directly to the bacterium facilitating uptake. However, the suggestion that the bacteria bind directly to cholesterol is in conflict with the recent work of Peyron et al. (2000) who demonstrated that the resistance of cholesterol-depleted cells to infection was due to an effect on receptor signaling rather than binding. Gatfield and Pieters (2000) reported previously that *M. tuberculosis*-infected macrophages label intensely with filipin, a cholesterol marker (also shown in Fig. 3). This may well reflect the fact that *Mycobacterium* resides in a vacuole accessed readily by glycosphingolipid “rafts” that are known to be cholesterol rich (Fig. 2) (Russell et al., 1996).

Kusner and colleagues noted that uptake of virulent *M. tuberculosis* via the complement receptor CR3 failed to induce the increase in cytosolic Ca concentration observed on internalization of inert particles via the same receptor (Malik et al., 2000). Artificial induction of a calcium flux with the ionophore A23187 lead to increased fusion of the bacteria-con-

taining vacuoles with lysosomes, culminating in a reduction in the number of viable bacilli. This transition correlated with an increase in the amount of phosphorylated calcium/calmodulin-dependent protein kinase II associated with the cytosolic face of the phagosome (Malik et al., 2001).

All of the above studies show tantalizing snapshots of the biology of the bacterium-containing compartment, yet the mechanism(s) employed by the bacterium to achieve this degree of control over their vacuoles remains to be defined. de Chastellier and Thilo (1998) have implicated the hydrophobic nature of the bacterial surface in the process, whereas the classic studies by D’Arcy Hart suggested that ammonia production was responsible for the absence of acidification (Gordon et al., 1980). More recently, Fratti et al. (2000) demonstrated that beads coated with the mycobacterial cell wall lipidoglycan, lipoarabinomannan (LAM),* were internalized into phagosomes that showed diminished fusion with lysosomes. Interestingly, this result was not observed for particles coated in phosphatidylinositol mannoside, which is a form of LAM without the elongated carbohydrate chain. Whether this is a role of LAM in intact bacteria and how the lipidoglycan mediates the reduced acquisition of proteins such as EEA1 remains to be determined. Recent breakthroughs in the application of emerging genetic techniques to *Mycobacterium* may be useful in answering these questions. Traditional screens that rely on bacterial death are unlikely to work because the bacilli takes so long to die inside macrophages that death could result from a broad range of defects not connected to modulation of the vacuole. Nonetheless, genetic screens based on enrichment protocols for bacteria unable to block progression down the endosomal continuum are extremely attractive and offer an unbiased way of identifying genes implicated in modulation of the host cell phagosome.

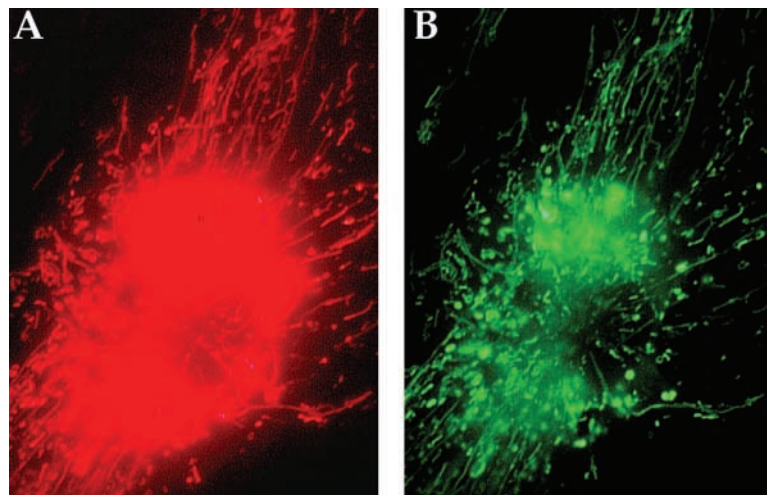
Shedding the fat

Despite the fact that vacuoles containing pathogenic *Mycobacterium* spp. fail to fuse with lysosomes, they fuse readily with plasmalemma-derived, early endosomes, and therefore, material released by the bacterium accesses both host cell lysosomes and the cell surface. The release of pathogen-derived material in the infected macrophage can be extreme, and both cell wall lipids and bacterial proteins can be detected in the host cell (Harth et al., 1994, 1996; Beatty et al., 2000; Beatty and Russell, 2000; Fischer et al., 2001; Neyrolles et al., 2001). The consequences of this behavior are essentially twofold. First, the macrophage is an antigen-presenting cell; therefore, material released by the bacterium are potential antigens. Second, the released material might modulate the function of both the host cell and the surrounding tissue. The observation by Fratti et al. (2000) on the properties of LAM suggests a role in vacuole modulation, whereas other reports implicate cell wall components in numerous aspects of immune modulation from immune suppression to induction of proinflammatory responses, apoptosis, and granuloma formation.

We have observed that *Mycobacterium* labeled with Texas red hydrazide after mild periodate treatment of live bacteria released copious amounts of label inside the host macrophage (Fig. 4). Solvent extraction of labeled bacteria followed by thin layer chromatography indicated that the majority of fluo-

*Abbreviation used in this paper: LAM, lipoarabinomannan.

Figure 4. Release of labeled mycobacterial cell wall lipids into the macrophage endocytic network. (A) A live bone marrow–derived macrophage infected for 24 h with Texas red hydrazide–labeled BCG were analyzed by fluorescence microscopy, with striking release of Texas red label from the bacterial phagosome. (B) Infected macrophages were incubated with dextran-fluorescein for 1 h followed by a chase period of 3 h. Fluorescent label (Texas red) released from the bacteria permeated the host macrophage and colocalized with dextran-fluorescein, revealing the accessibility of mycobacterial constituents to endocytic compartments. Reproduced from Beatty et al. (2000).



rescent glycoconjugates released were lipid in nature (Beatty et al., 2000). Although the arrangement of the lipids in the cell wall is still a matter of considerable debate, there is consensus concerning lipid identity (Besra and Chatterjee, 1994). Two-dimensional thin layer chromatography analysis of released lipids indicated that a heterogeneous mix of phospholipids, glycolipids, and waxes is released. These lipids comprise phosphatidylinositol mono- and dimannosides (PIMs), phosphatidylglycerol and cardiolipin, phosphatidylethanolamine, trehalose mono- and dimycolates, and a *M. bovis*–specific phenolic glycolipid mycoside B (unpublished data). Cell fractionation of infected macrophages by density gradient electrophoresis demonstrated that the bacterial lipids tend to traffic in bulk fashion through the cell and accumulate in a late endosome/lysosome-like compartments (Beatty et al., 2000). Ultrastructural studies revealed multilamellar vesicles accumulating in late infections by *M. tuberculosis*, suggesting an impressive overproduction of cell wall constituents. These compartments also show an abundance of MHC class II molecules, suggesting a possible connection with the antigen presentation machinery (Beatty and Russell, 2000; Beatty et al., 2001).

In terms of energy, continual lipid production is a very expensive behavior leading one to postulate that such a wasteful indulgence would need to be rewarded to be conserved through evolution. There are many studies imbuing mycobacterial lipids with a range of mystical properties. This is perhaps not surprising when one remembers that dead mycobacteria were the key ingredient in Freund's Complete Adjuvant. The obvious proinflammatory activity of these lipids seems in contradiction to the covert phenotype of many pathogens. Therefore, the properties of the released material is best discussed in two discrete sections: one dealing with their effects on acquired immunity and one dealing with their proinflammatory properties.

Effects on adaptive immunity

The majority of molecules released by intracellular mycobacterium represent antigens recognized by the immune system. Some of these antigens are proteins and some are lipids, yet both can induce a cellular immune response capable of stimulating T cells that demonstrate protective activity. The pathogen appears to cope with this conundrum through two

strategies: reducing the antigenicity of the infected macrophage to prevent its recognition and active modulation of the local immune response to limit its efficacy at the site of infection. With respect to reducing the host cell's antigenicity, antigen presentation by the infected macrophage is minimized by several mechanisms. First, the vacuoles in which the bacteria reside do not lie within the antigen-sampling continuum within the cell (Pancholi et al., 1993; Ullrich et al., 2000). Although shortly after infection the vacuoles do possess MHC class II molecules, these molecules are surface derived and already peptide loaded and therefore limited in their ability to sample new antigens. This strategy does not work on macrophages activated with IFN γ before infection. In these cells, the vacuoles acquire the MHC class II molecule chaperone H2-M (mouse) or HLA-DM (human) (Schaible et al., 1998; Ullrich et al., 2000). These chaperones are responsible for removal of the invariant chain peptide that blocks antigen loading, and the presence of H2-M/HLA-DM signifies that the vacuoles are no longer sequestered outside the antigen-sampling machinery. The functional significance of this transition was demonstrated elegantly by Ramachandra et al. (2001) who showed that the MHC molecules in *Mycobacterium*-containing vacuoles in activated macrophages are loaded with bacterial antigen and are competent to induce T cell stimulation.

Infected cells show diminished expression of both classical (MHC class I and class II) and nonpolymorphic (CD1) antigen presentation and costimulatory molecules. The suppression is partial, most groups reporting a 50–70% reduction in the surface expression of these molecules; however, this reduction does appear to generate a measurable depression in T cell stimulation in *in vitro* assays (Gercken et al., 1994; Wadee et al., 1995; Wojciechowski et al., 1999; Giuliani et al., 2001). Lipidated moieties of mycobacteria contribute to this diminution of antigen presentation and T cell responsiveness. Noss et al. (2001) demonstrated that the 19-kD lipoprotein down-regulated surface expression of MHC class II molecules and reduced the antigen-presentation capacity of the infected macrophage. Phenolic glycolipids bearing long chained acyl tails suppress lymphoproliferation *in vitro* (Fournie et al., 1989), and LAM interferes with IFN γ -mediated activation of macrophages (Sibley et al., 1988). All of these effects would aid a persistent pathogen in maintaining its infection.

However, there is some measure of conflict here because in addition to confounding antigen presentation and the adaptive immune response mycobacterial lipids may themselves be presented to lymphocytes in the context of CD1 molecules, nonpolymorphic MHC I-like antigen presentation molecules (Jullien et al., 1997). Most studies demonstrating CD1-dependent recognition of mycobacterial lipids have used human cells that express a more diverse set of CD1 molecules than mouse cells. The acyl chains of LAM, PIMs, and glycosylated mycolates can be loaded into the deep hydrophobic binding pocket of CD1b in the acidic environment of late endosomes (Ernst et al., 1998). Once presented on the cell surface, lymphocytes recognize the lipids in the context of CD1, responding with cytolytic activity and lymphokine production. It has been speculated that an infected macrophage, compromised in MHC class II-mediated antigen presentation, might still be capable of presenting mycobacterial lipids in the context of CD1, thereby continuing to elicit some degree of protective lymphocyte responses. Antibody-mediated blocking of CD1d results in exacerbated bacterial growth and impaired expression of macrophage-activating cytokines in mice (Szalay et al., 1999). CD1d-deficient mice contain mycobacterial infections successfully; however, this demonstrates that CD1-mediated events are not required for the development of a protective immune response. These findings illustrate that mycobacterial lipids fulfill multiple, apparently contradictory roles with respect to the acquired immunity.

Active modulation in host responsiveness: whose granuloma is this anyway?

Granuloma formation is the defining characteristic of most all mycobacterial infections. The tuberculosis granuloma in humans is a highly organized structure that represents a balance between host and pathogen. For the host, the granuloma provides a focal point for the immune response, walls off the infection, and thereby limits dissemination of bacteria. For the pathogen, the proinflammatory environment of the granuloma recruits potential host cells and maintains lymphocytes at the periphery of the structure where macrophage activation is likely to be less effective. The “poor” distribution of lymphocytes is likely the result of the cytokine microenvironments in the granuloma. The bacterial lipids released by the intracellular bacilli induce cytokines that, like IL-6 (VanHeyningen et al., 1997), can be suppressive to T cell proliferation, and the exocytosis of these lipids from infected cells would extend the sphere of influence beyond the confines of the host cell. Such a strategy would favor bacterial persistence even in the face of a robust immune response.

The contribution of mycobacterial lipids to the formation of the granuloma has been examined in several studies. For example, cord factor (trehalose dimycolate) or LAM, when added to particles and instilled into the lung, induce formation of epithelioid granulomas reminiscent of tubercles (Juffermans et al., 2000; Lima et al., 2001). The proinflammatory cytokines released by macrophages upon stimulation with mycobacterial cell wall lipids are in very close agreement with the expression profiles obtained more recently by microarray analysis of monocytes exposed to either dead or live *M. tuberculosis* (Ehrt et al., 2001) and would lead to the

recruitment of leukocytes to the site of infection (Rhoades et al., 1995; Peters et al., 2001; Mendez-Samperio et al., 2002).

The lipidated cell wall constituents are recognized by Toll-like receptors, a group of structurally related transmembrane proteins that are “pattern” receptors, recognizing structural motifs in microbial products (Brightbill et al., 1999; Underhill et al., 1999). Gram negative bacterial LPS is recognized by TLR-4, whereas the majority of *Mycobacterium*-derived moieties stimulate cells via TLR-2. These include the structurally related lipidoglycans LAM, PIM, and the 19-kD lipoprotein (Underhill et al., 1999; Noss et al., 2001). Both PIM’s and the 19-kd lipoprotein are released actively by bacteria inside host macrophages, and PIMs are known to trigger cytokine and chemokine induction in macrophages and dendritic cells (unpublished data). The potent biological responses induced by these molecules provide a clear justification of why such apparently wasteful behavior would be sustained.

Concluding remarks

Persistence of infection is the hallmark of tuberculosis where the infecting bacteria survive even in the face of a strong immune response. The success of the bacterium is dependent on its ability to manipulate both the host cell and the surrounding tissues. The mechanisms behind these processes are just beginning to emerge and appear to rely on the host’s capacity to recognize and respond to nonproteinaceous antigens and molecules released by the bacteria. Research in this area promises to be extremely informative across a range of disciplines beyond the perverse eccentricities of the bug the Western world forgot.

This research was supported by National Institutes of Health grants AI33348 and HL55936. H. Mwandumba is supported by the Wellcome Trust.

Submitted: 9 May 2002

Revised: 18 June 2002

Accepted: 18 June 2002

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