

Foamy macrophages and the progression of the human tuberculosis granuloma

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The progression of tuberculosis from a latent, subclinical infection to active disease that culminates in the transmission of infectious bacilli is determined locally at the level of the granuloma. This progression takes place even in the face of a robust immune response that, although it contains infection, is unable to eliminate the bacterium. The factors or environmental conditions that influence this progression remain to be determined. Recent advances have indicated that pathogen-induced dysregulation of host lipid synthesis and sequestration serves a critical role in this transition. The foamy macrophage seems to be a key participant in both sustaining persistent bacteria and contributing to the tissue pathology that leads to cavitation and the release of infectious bacilli.

The pathogen *Mycobacterium tuberculosis* (Mtb) has evolved to cause infection in many, but active disease in few. The World Health Organization estimates that one third of the planet's population harbor this bacterium, yet only 2–23% will develop disease during their life span¹. Intriguingly, there are no biomarkers for disease progression because, as ascertained so far, the systemic immune response is similar in people who develop disease and those with effective containment. Progression to active disease is determined locally at the level of the infection site, the granuloma. Therefore, appreciating the interaction between the pathogen and the localized tissue response is critical for understanding the progression of infection to active disease and, ultimately, transmission.

Infection is initiated when inhaled bacilli are phagocytosed by alveolar macrophages, as demonstrated by the Mtb life cycle (Fig. 1). These cells are required to phagocytose toxic and inflammatory particles and are thought to be relatively quiescent to minimize potential damage to the lung tissue through vigorous proinflammatory responses. Once inside the phagocyte, Mtb modulates the activity of its phagosome by preventing its fusion with acidic, hydrolytically active lysosomes^{2,3}. In experimental hosts, this marks the start of a period of 'rapid' division in which the bacteria grow exponentially until the emergence of an acquired immune response. This is concomitant with the development of the granuloma, which signifies immune-mediated containment of the infection. The granuloma can proceed either to localized sterilization of the infection and min-

eralization of the lesion or to localized caseation and necrosis that culminates in the release of infectious bacteria into the airways. This review discusses some of the localized responses that seem to support bacterial persistence and lead, finally, to the tissue damage that facilitates the transmission of infection.

Localized consequences of the immune response

The tuberculosis granuloma is the outcome of the local interaction between the bacterium and the host cells at the site of infection. Since the first description of Ghon's complex one century ago⁴, pathologists have described the morphological parameters of these elaborate structures extensively, and the interaction is usually portrayed as a host-driven process to constrain the bacilli and prevent dissemination⁵. More recently, however, this viewpoint has been questioned, and there is a growing appreciation of the active role of the bacterium in this process⁶. The direct influence of the bacterium is clear even in 'pared-down' models, such as the zebrafish, that rely solely on the innate immune response at the early stage of development⁷.

In the mouse model, infected lungs initially do not have any lesions but do show an increase in cellularity between the epithelia and the lamina propria. At this time, although the tissue response is relatively unstructured⁸, the bacillary load increases more than a thousand times^{9,10}. The initial stages of granuloma formation are dependent on the production of tumor necrosis factor (TNF) by the infected macrophages and T cells. Sustained TNF signaling is needed to maintain chemokine concentrations for cellular recruitment and retention^{11–14}. It is interesting to note that although TNF-deficient mice do develop granulomatous responses, their onset is delayed¹⁵ and the structures remain amorphous and become necrotic.

Although TNF is needed to drive granuloma development, too much of it can lead to overt tissue damage¹⁶. Immune reconstitution inflammatory syndrome in patients with AIDS who are undergoing highly active antiretroviral therapy is caused by the population expansion of CD4⁺ cells in the face of an extensive antigen load or bacterial burden¹⁷. In such instances, the granulomatous response is too aggressive and

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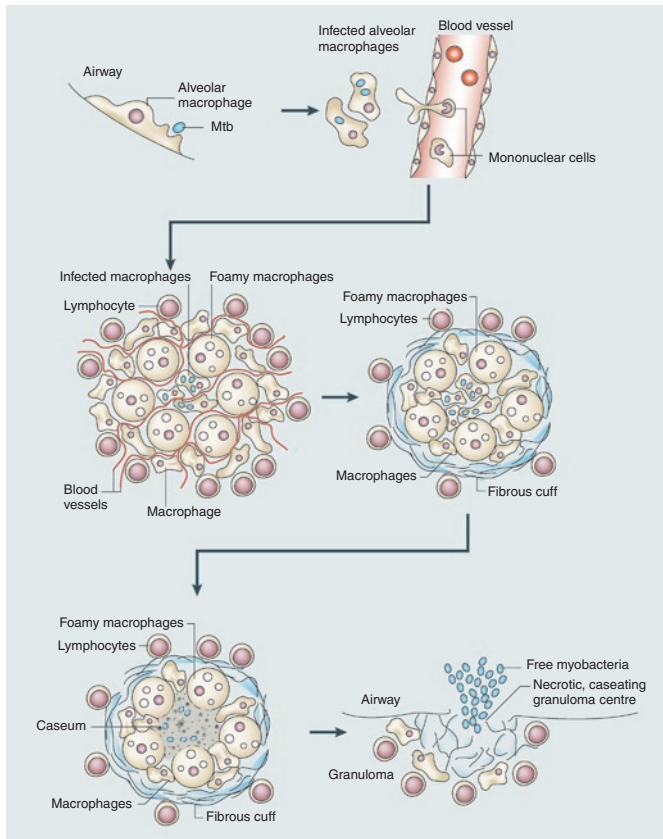


Figure 1 Progression of the human tuberculosis granuloma. Infection is initiated after inhalation of viable bacilli present in exhaled droplets or nuclei that are discharged into the atmosphere by a person with active infection. These droplets can persist in the atmosphere for several hours and, because the infectious dose is in the range of one to ten bacilli, this makes transmission an extremely efficient process. Once in the lung, the bacilli are phagocytosed by alveolar macrophages. Internalization of the bacilli triggers a proinflammatory response that induces the macrophage to invade the subtending epithelium. This response also leads to the recruitment of mononuclear cells from neighboring blood vessels. These monocytes form the cellular matrix of the early granuloma, which is the primary characteristic of this disease. In its early stage, the granuloma has a core of infected macrophages enclosed by foamy macrophages and other mononuclear phagocytes, surrounded by lymphocytes. This tissue response contains the infection and spells the end of the period of rapid replication of Mtb. As the granuloma matures, it develops an extensive fibrous capsule that encases the macrophage core and excludes the majority of lymphocytes from the center of the structure. Concomitant with this transition is a considerable decrease in the number of blood vessels penetrating the granuloma. At this stage there is a noticeable increase in the number of foamy macrophages in the fibrous capsule. We hypothesize that these cells are responsible for the accumulation of caseous debris in the center of the granuloma, which portends progression to active disease. In an immunocompetent person, this progression is localized to individual granulomas, and the same tissue site will contain other granulomas that seem to be under perfect immune containment. Nonetheless, in a progressive infection, the caseous, necrotic center of the granuloma liquefies and cavitates, spilling thousands of infectious Mtb into the airways. This damage to the lungs triggers the development of a productive cough, which facilitates generation of the infectious aerosol and completion of the bacterium's life cycle.

causes acute disease with extensive tissue destruction. From the perspective of the bacterium, the upper lobe of the lung, with its high oxygen pressure, seems to favor bacillary growth and represents a 'privileged site' because of the delayed immune response^{18–20}.

The development of the acquired immune response is promoted through the accumulation of infected dendritic cells in the regional lymph node^{21,22}. This immune response is based mainly on the induction of T helper type 1 cells and a small proportion of CD8⁺ T cells able to recognize infected macrophages and activate them by secreting interferon- γ ^{6,8,23}. Once the bacillary growth is stabilized, the presence of CD8⁺ T cells seems to gain importance, at least in the experimental mouse model, both for the production of interferon- γ and for the increase in cytotoxic activity^{24–26}. This is a period of 'stalemate' during which the bacillary load remains relatively constant and the infection is in a state of 'latency'.

The accepted view is that during this period of latency, the infection is sustained mainly by a population of nonreplicating bacilli rather than a population of growing bacilli whose numbers are regulated by immune-mediated killing. The evidence of this is, of necessity, circumstantial. In the mouse model, the absence of an accumulation of bacterial debris consisting of bacterial cell wall 'ghosts'²⁷ or bacterial genome equivalents²⁸ has been reported, which indicates that the bacterial number remains relatively static. It has been argued that the immune response is directed mainly toward antigens secreted by growing bacilli²⁹; therefore, nonreplicating bacilli will be less obvious to the protective cellular response. Finally, stressful conditions *in vitro* can induce a nonreplicative state in Mtb, and this physiological state correlates directly with an innate resistance to anti-Mtb drugs, most of which target processes active in replicating organisms.

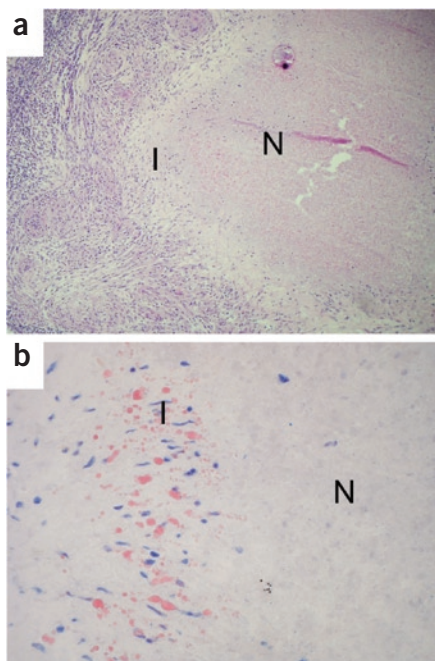
In the early stages of granuloma development, the structure becomes highly vascularized through a robust response mediated by vascular

endothelial growth factor^{30,31}. The blood vessels have extensive lymphocyte cuffing, which indicates that there is recruitment of cells, lymphocytes, macrophages and dendritic cells into the site of infection. As the site develops, the macrophages differentiate into several different morphotypes, including multinucleated giant cells, epithelioid cells, and foamy macrophages loaded with lipid droplets^{32–35}. At this time, the structure becomes much more stratified, a fibrous cuff starts to form outside the macrophage-rich layer, and the majority of lymphocytes are excluded from the granuloma center and aggregate outside the fibrous cuff.

The events described above take place in the face of an extremely strong antimycobacterial immune response, to which the bacterium minimizes its exposure by driving this tissue response that leads to the physical separation of the infected macrophages in the granuloma center from the peripheral lymphocytes. Several years ago, the spatio-temporal organization of cellular infiltration around classical granulomatous structures at secondary infectious sites in patients with tuberculosis was described³⁶. That study suggested that the center of the granuloma may not be the main site of the immune-mediated containment of the bugs but that peripheral cellular infiltrates containing both mycobacteria-handling antigen-presenting cells and T cell and B cell populations, and resembling secondary lymphoid organs, may be better shaped to orchestrate the host immune response, as suggested by the high proliferative activity found only in peripheral follicle-like structures. In addition, although patients with open cavitary pulmonary tuberculosis usually present with little vascularization of peripheral infiltrations, persistent nonprogressive tuberculous lesions, also called 'tuberculomas', are usually surrounded by highly vascularized tissues³⁷. These observations emphasize again how important the tissue remodeling may be for the fate of the physiopathology of tuberculosis.

The granuloma in this balanced, dynamic state is the 'stable unit' of infection, and in most people these granulomas do not develop into active

Figure 2 In lesions from patients with tuberculosis, foamy macrophages are located mainly in the interface region surrounding central necrosis. (a) Typical necrotic lesion from a thin section made through a lymph node biopsy from a patient with tuberculosis, stained with hematoxylin and eosin. As classically observed in human tuberculosis lesions, the lesion is well circumscribed and differentiated, with the central necrotic core (N) surrounded by a thin area called the 'interface' (I) that separates the necrotic area from the histiocyte area. At the periphery, the lymphocyte area delineates the boundary of the structure. (b) Oil red O staining of such tuberculosis lesions demonstrates the presence of large amounts of foamy macrophages in the interface region separating necrosis from the histiocytes, as shown in this enlarged view. Original magnification, $\times 15$ (a) or $\times 200$ (b). Adapted from ref. 35.



sites and can actually resolve. However, some granulomas show increasing accumulation of caseum in the granuloma center; this ultimately leads to necrosis and collapse of the granuloma center, which releases live, virulent bacilli into the airways³⁸. As the histology of people with active disease frequently reveals granulomas in all stages of development, disease progression seems to be determined locally at the level of the granuloma. So what determines granuloma progression, and how does the bacterium influence this tissue process?

Of mice and men

The shortcoming of the mouse as an experimental model for tuberculosis has been its inability to develop a well-defined and circumscribed granulomatous structure with the fibrotic capsule and caseous center that typifies an active tuberculosis granuloma in humans and primates. It has been reported that post-primary

Figure 3 Vesicles containing Mtb-derived cell wall lipids are released from infected macrophages. (a) Fluorescence microscopy of live macrophages infected for 24 h with *M. bovis* BCG labeled with Texas red hydrazide, showing substantial release of the Texas red label from the bacterial phagosome. (b) Fluorescence microscopy of the macrophages in a, incubated with dextran-fluorescein for 1 h, showing that the released lipids permeate the host macrophage and localize together with dextran-fluorescein; this demonstrates the penetrance of the endocytic system by the bacterial lipids. (c) Immunoelectron micrograph of an infected macrophage probed with antibodies to the Mtb cell wall glycolipids PIM (antibody to mouse IgM conjugated to 12-nm gold) and LAM (antibody to rabbit IgG conjugated to 18-nm gold). Bacterial lipids in multivesicular structures are labeled (arrows; B, bacteria). (d) Platinum replicas showing the surface of BCG-infected macrophages. Vesicles containing BCG cell wall lipids are exocytosed from macrophages infected with biotin hydrazide-labeled BCG. Bacterial components are detected with streptavidin-gold (15 nm; arrows). (e) High-magnification micrograph demonstrating the release of the vesicles in d at the cell surface. The cell membrane is free of label, which is restricted to the exocytosed vesicles. Original magnification, $\times 1,000$ (a,b), $\times 18,000$ (c) or $\times 50,000$ (d,e). Reproduced with permission from ref. 47 (a,b,d).

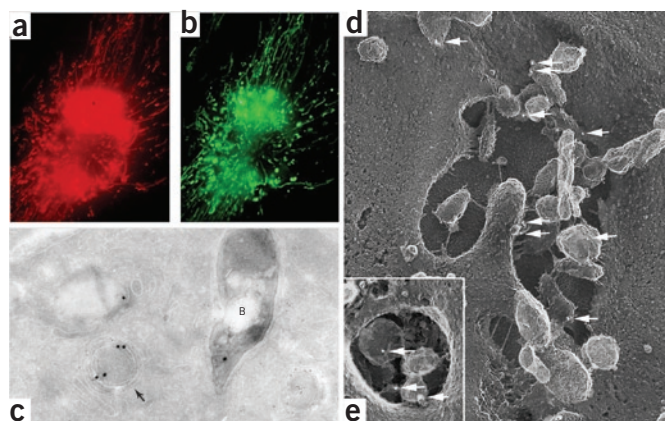
tuberculosis in mice recapitulates the pathology found in humans³⁹. In these studies, mice were experimentally infected with Mtb, treated with antibiotics and reactivated according to the 'Cornell' model. In brief, in this model, antibiotic treatment kills replicating bacilli 'preferentially', thus favoring the persistence of nonreplicating organisms that can reactivate and resume growth, either spontaneously or through treatment of the infected mice with immunosuppressive agents⁴⁰. Histopathological examination revealed focal lesions resembling lipid pneumonia typified by alveoli filled with foamy macrophages. Acid-fast staining demonstrated that these foamy macrophages were the predominant cell type that contained Mtb bacilli. In the central foci of necrosis, Mtb were associated with lipid droplets. The appearance of the foamy macrophage seemed to be a key component in this pathology.

In humans, there is a rich literature detailing the presence of foamy macrophages in tuberculosis granulomas^{35,39}. The most striking study has shown that in biopsy samples from patients with tuberculosis, foamy macrophages are located mainly at the interface region immediately flanking central necrosis (Fig. 2). Hence, these foamy macrophages are found only in necrotic lesions, thus suggesting that they serve an active function in necrosis formation and the accumulation of caseous debris at the heart of the granuloma³⁵.

Characterization of foamy macrophages

The induction of foamy macrophages packed with lipid bodies has been reported in many pathologies associated with chronic proinflammatory stimuli, ranging from atherosclerosis⁴¹ and septic arthritis to infection with a range of persistent pathogens such as Mtb, chlamydia⁴² and toxoplasma⁴³. In infection, the formation of lipid bodies is dependent on the activation of Toll-like receptors (TLRs) by a pathogen-derived agonist and the presence of proinflammatory signals such as TNF and monocyte chemoattractant protein 1 (CCL2)⁴⁴.

Macrophages convert into foam cells through a dysregulation in the balance between the influx and efflux of low-density lipoprotein (LDL) particles from the serum⁴¹. LDL is internalized by a receptor-mediated process through LDL receptors or, in the oxidized form, through scavenger receptors such as SRA and CD36. The LDL particles contain cholesterol, triacylglycerides and phospholipids, and although most of the phospholipids and triacylglycerides are metabolized, the cholesterol is retained by the macrophage mainly in an esterified form. After breakdown of the LDL, cholesterol is imported into the cell cytosol, where it is either esterified and sequestered into lipid droplets formed in the endoplasmic reticulum leaflet or pumped out of the cell by ATP-binding cassette (ABC) transporters. The ABC transporters



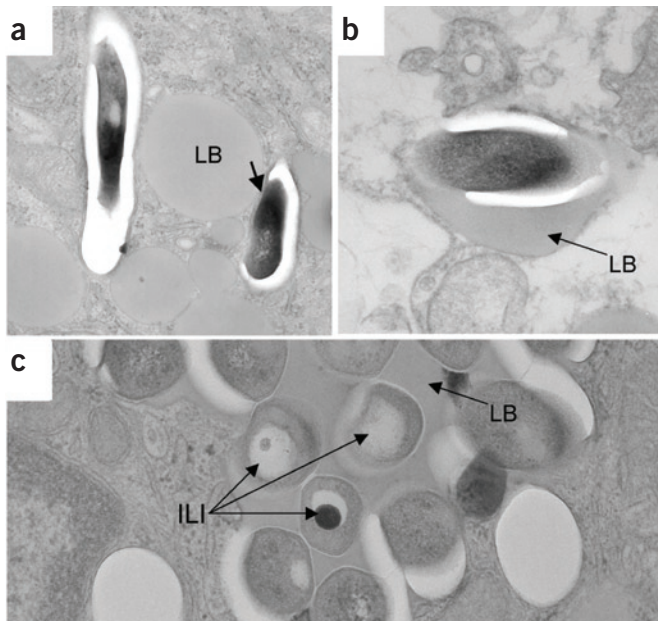


Figure 4 In foamy macrophages, tubercle bacilli-containing phagosomes have 'privileged' contact with cellular lipid bodies. (a) Tight apposition (arrow) between Mtb-containing phagosomes and host lipid bodies (LB). (b) Ultimately, at later stages, bacilli-containing phagosomes are found in lipid bodies, thus confirming that they have been engulfed. (c) Engulfed bacilli show intracytoplasmic lipid inclusions (ILI) typical of dormant bacteria, which suggests that they have accumulated host lipids³⁵. Reproduced with permission from ref. 35 (c).

ABCA1 and G1 are key mediators of cholesterol efflux, and genetic deficiency in these transporters exacerbates foam-cell formation.

Foamy macrophages not only are the product of an inflammatory response but also amplify that response through the production of prostaglandin E₂ and leukotrienes^{33,45}. ABCG1-deficient mice that have impaired cholesterol efflux have foamy macrophage formation and enhanced inflammation in the lung⁴⁶. This demonstrates that the foamy macrophage is itself a proinflammatory cell.

Mtb-dependent foam-cell formation

The tissue response to Mtb seems to exceed expectations, given the number of bacilli that seed the early granuloma. Studies have shown that Mtb lipids are 'overproduced' by intracellular bacilli; these lipids consolidate in the internal vesicles in the multivesicular body and are subsequently exocytosed into the extracellular milieu^{47,48} (Fig. 3). In a mouse granuloma model in which the bacterial lipids were adsorbed onto beads inoculated into mice, the tissue response mirrored several characteristics shared by the Mtb granuloma, including neovascularization, the formation of giant cells, epithelioid macrophages and foamy macrophages, and fibrosis⁴⁹. The most bioactive component of these released lipids was the trehalose dimycolates⁵⁰. In an *in vitro* model of human granulomas, Mtb-derived lipomannan was also shown to drive the differentiation of granuloma macrophages into multinucleated giant cells, in a TLR2-dependent manner⁵¹, thus emphasizing the involvement of mycobacterial lipids in the modulation of the host response.

More recently, it was demonstrated that foam cell formation is specifically induced by oxygenated forms of mycolic acid, such as oxygenated ketomycolic and hydroxyl-mycolic acids⁵⁵. These lipids are synthesized by pathogenic mycobacterial species such as *M. avium* and *Mtb* but not by saprophytic species such as *M. smegmatis*. However,

when *M. smegmatis* was transformed with the Mtb *hma* gene, which encodes a methyl transferase required for introducing the distal oxygen-containing modifications of mycolic acids, these bacteria induced foam-cell formation in macrophages. The phenotype was also inducible with isolated lipid.

M. bovis bacillus Calmette-Guérin (BCG) can induce foam-cell formation in a TLR2-dependent manner³³. The abundant cell wall lipid trehalose dimycolate is recognized by TLR2, in concert with the scavenger receptor MARCO, which seems to fulfill a tethering function⁵². As trehalose dimycolate is released and trafficked into and out of infected cells⁵³, this would be an ideal mediator for inducing a favorable metabolic shift in the macrophages of the lesion.

Other infections and proinflammatory stimuli result in the formation of foamy macrophages, and for some infectious agents, such as chlamydia, this has been linked to the nutritional needs of the pathogen⁵⁴.

Nutritional advantages to the bacterium

Foamy macrophages seem to sustain intracellular Mtb in a physiological state similar to the nonreplicating, vegetative state invoked to explain persistence³⁵. Electron microscopy has revealed bacilli in close apposition to the intracellular lipid droplets, which indicates that Mtb may access these structures as a nutrient source³⁵. Hence, at late stages, bacilli-containing phagosomes are also found in lipid bodies, and bacilli have intracellular lipid inclusions, which indicates that they are accessing and using host lipids (Fig. 4). The observation that Mtb can persist in human adipocytes⁵⁵, another lipid-rich cell type, is in agreement with the hypothesis that the bug is searching for the best places to survive in its human host and can even create such niches by manipulating the host cell metabolism.

In support of that hypothesis, earlier publications showed a requirement for isocitrate lyase activity in sustaining Mtb infection in the chronic phase of infection in the mouse model⁵⁶. Isocitrate lyase is the gating enzyme into the glyoxylate shunt and is mobilized when organisms are growing on fatty acids as their limiting carbon source. This enables the bacterium to retain carbon through gluconeogenesis,

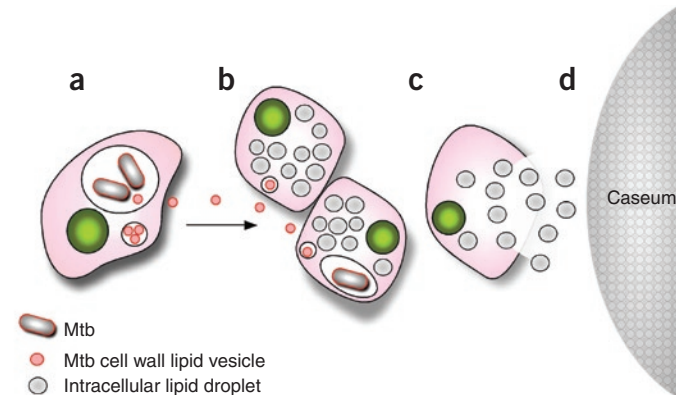


Figure 5 Model for caseum accumulation and granuloma progression. (a) Intracellular Mtb synthesize and release cell wall components inside their host cell. These lipids accumulate in the internal vesicles in multivesicular bodies, which are exocytosed from the cell in vesicular form. (b) Because of the release of such vesicles, both infected and uninfected macrophages are exposed to cell wall mycolates and are induced to form foam cells. (c) The foam cells die by an inflammatory, necrotic process and release their lipid droplets into the extracellular milieu in the granuloma. (d) As a result of the fibrotic capsule, the human granuloma is an enclosed, isolated structure. The enclosed nature of the human granuloma leads to the accumulation of necrotic debris as caseum. We propose that this process is an integral part of the pathology that leads to active disease and transmission.

rather than losing carbon to carbon dioxide. Mtb mutants defective in isocitrate lyase 1 (ICL1) have an impaired ability to maintain a chronic infection and to survive in interferon- γ -activated macrophages in culture⁵⁶. In addition, Mtb has been shown to be able to metabolize cholesterol⁵⁷. Bacteria deficient in the genes encoding Mce4, a cholesterol transporter, or HsaC, a key enzyme in cholesterol catabolism, are unable to grow in conditions in which cholesterol is the limiting carbon source. Intriguingly, when mice are infected with Mce4-deficient bacteria, the bacteria have a minimally altered phenotype early in infection but are impaired in sustaining bacterial numbers during the chronic, persistent state that occurs when an acquired immune response is established. This phenotype is similar to that of ICL1-deficient mutants. The metabolism of cholesterol increases the propionate pool in the bacterium⁵⁸, and ICL1 is required for propionate metabolism⁵⁹, so the genes encoding Mce4, HsaC and ICL1 may be functionally linked through cholesterol degradation. These data support the proposal that Mtb could exploit the lipid droplets in their host cells as a nutrient source³⁵.

Pathway to caseation and tissue destruction

The temporal and spatial correlation between foam-cell formation and the accumulation of caseum in the granuloma indicates a causal relationship (explored further in the model proposed in Fig. 5). If the caseum is derived from the lipids sequestered during foam-cell formation, then the lipids in the caseum should reflect their origin. Analysis of lipids from the caseum of human tuberculosis granulomas has shown that the main lipid species are cholesterol, cholesteryl ester and triacylglycerides, which is entirely consistent with the derivation of lipids from lipid droplets (M.-J.K. and D.G.R., unpublished results).

The induction of cell death in host macrophages has been the subject of many studies that have generated conflicting data as to whether the bacterium benefits or suffers from the death of its host cell. Apoptosis is generally regarded as a protective response, whereas necrosis is thought to favor inflammation and disease progression. Although studies have generated convincing data that Mtb blocks apoptosis through inhibition of crosslinking of exposed annexin I (ref. 60) and through the activity of its NADH dehydrogenase⁶¹, several groups have reported conditions in which Mtb is proapoptotic. Mtb activates the extrinsic apoptosis pathway in macrophages through a TNF autocrine loop⁶². This activity is thought to vary inversely with the virulence of the bacterium and would contribute to bacterial clearance through the phagocytosis of apoptotic bodies containing Mtb. However, if 'successful' or virulent Mtb strains benefit from blocking apoptosis early, the death of their host cell once a heavy intracellular infection has been reached would promote dissemination⁶³. Necrosis in mouse tuberculosis lesions has been correlated with bacterial burden, which indicates that with large bacterial numbers, damage and inflammation is the usual outcome¹⁵. Virulent Mtb has been shown to inhibit membrane repair, which drives the infected macrophage into necrosis⁶⁴. Such a response would lead to the death of foamy macrophages in the granuloma and accumulation of their lipid cargo in the extracellular milieu. The fibrotic nature of the enclosed human granuloma, which is now low in blood vessels, would lead to more aggregation of lipid debris forming the caseum at the center of the granuloma.

Closing remarks

In this review we have tried to present a new hypothesis for the tissue response that drives the progression of human tuberculosis to a state of active disease and transmission. Observations and experiments have indicated that Mtb promotes dysregulated lipid metabolism in its host macrophages. This promotes foam-cell formation, which supports bacterial persistence and ultimately leads to the accumulation of caseum in the granuloma. We feel that this is a pathogen-driven process that

subverts the host's immune response to induce the late-stage damage required for the completion of Mtb's life cycle.

If correct, such a model has implications for both vaccine and chemotherapy programs. The progression of tuberculosis seems to be determined locally, independently of the nature of the systemic immune response. In fact, the systemic immune response is actually a required component for late-stage damage. Therefore, to be maximally effective, vaccines need to limit bacterial growth before granuloma formation, because once formed the granuloma protects Mtb and prevents bacterial clearance. It is therefore difficult to imagine an anti-infection vaccine against tuberculosis, although decreasing the bacterial load would reduce the incidence of reactivation and should therefore reduce transmission.

The data also indicate that there might be avenues for manipulating the host tissue response to infection that could either interfere with the tissue state needed to support persistent organisms or render the bacilli more sensitive to drugs that selectively target replicating bacteria by preventing them from adopting a vegetative, nonreplicative state. Although this is mere speculation, it does encourage thinking more broadly about new strategies to combat this most enduring of pathogens!

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