Foamy macrophages and the progression of the human TB granuloma

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Abstract

The progression of tuberculosis from a latent, sub-clinical infection to active disease that culminates in 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, while 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 plays a critical role in this transition. The foamy macrophage appears to be a key player in both sustaining persistent bacteria and contributing to the tissue pathology that leads to cavitation and release of infectious bacilli.

Introduction

The pathogen Mycobacterium tuberculosis (Mtb) has evolved to cause infection in many, but active disease in few. The WHO estimates that 1/3 of the planet's population harbor this bacterium, yet only 2-23% will develop disease during their lifespan¹. Intriguingly, there are no biomarkers for disease progression because, as far as has been ascertained to date, the systemic immune response is comparable in those individuals developing disease versus those with effective containment. Progression to active disease is determined locally, at the level of the infection site, the granuloma. Therefore, appreciating the interplay between the pathogen and the localized tissue response is critical to understanding the progression of infection to active disease, and ultimately, transmission.

The infection is initiated when inhaled bacilli are phagocyted by alveolar macrophages, as illustrated in the life cycle diagram shown in Box 1. These cells are required to phagocyte toxic and inflammatory particles and are thought to be relatively quiescent to minimize potential damage to the lung tissue via vigorous proinflammatory responses. Once inside the...
phagocyte, Mtb modulates the behavior 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, where 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 mineralization 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 appear to support bacterial persistence and lead, finally, to the tissue damage that facilitates transmission of the infection.

**Localized consequences of the immune response**

The tuberculosis (TB) granuloma is the outcome of the local interplay between the bacterium and the host cells within the site of infection. Since the first description of the “Ghon’s complex” one century ago\(^4\), pathologists have described the morphological parameters of those complex structures extensively, and it is usually portrayed as a host-driven process to constrain the bacilli and prevent dissemination\(^5\). More recently, however, this viewpoint is being questioned and there is a growing appreciation of the active role played by the bacterium in this process\(^6\). 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 development\(^7\).

In the murine model, infected lungs initially do not show any lesion but an increase in the cellularity between the epithelia and the lamina propria. At this time, while the tissue response is relatively unstructured\(^8\), the bacillary load increases more than a thousand times\(^9, 10\). Initial stages of granuloma formation are dependent on tumor necrosis factor (TNF) production by the infected macrophages and T cells. Sustained TNF signaling is required to maintain chemokine concentrations for cellular recruitment and retention\(^11-14\). It is interesting to note that while TNF-deficient mice do develop granulomatous responses, their onset is delayed\(^15\) and the structures remain amorphous and become necrotic.

Although TNF is required to drive granuloma development, too much of it can lead to overt tissue damage\(^16\). Immune reconstitution inflammatory syndrome (IRIS) in AIDS patients under the treatment with highly active antiretroviral therapy (HAART) treatment is caused by expansion of CD4 cells in the face of an extensive antigen load or bacterial burden\(^17\). In such instances the granulomatous response is too aggressive and causes acute disease with extensive tissue destruction. From the perspective of the bacterium, the upper lobe of the lung, with its high oxygen pressure, appears to favor the bacillary growth and presents a privileged site due to a delayed immune response\(^18-20\).

The development of the acquired immune response is promoted through the accumulation of infected dendritic cells (DCs) in the regional lymph node\(^21, 22\). This immune response is based mainly on the induction of type 1 T helper (T\(_{H1}\)) cells and a low proportion of CD8+ T cells, able to recognize infected macrophages and activate them by secreting interferon \(\gamma\) (IFN-\(\gamma\))\(^6, 8, 23\). Once the bacillary growth is stabilized, the presence of CD8+ specific T cells appears to gain importance, at least in the experimental murine model, both for the production of IFN-\(\gamma\) and an increase in the cytotoxic activity\(^24-26\). This is a period of stalemate where 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 predominantly by a population of non-replicating 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 murine model an absence of bacterial debris accumulation consisting of bacterial cell wall ghosts\(^27\), or bacterial genome equivalents\(^28\) has been reported, implying that the bacterial number remains relatively static. It is argued that the immune response is mainly
directed towards antigens secreted by growing bacilli, therefore non-replicating bacilli will be less obvious to the protective cellular response. Finally, stressful conditions *in vitro* can induce a non-replicative 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 vascular endothelial growth factor (VEGF)-mediated response. The blood vessels exhibit extensive lymphocyte cuffing indicating that there is recruitment of cells, lymphocytes, macrophages and DCs, into the site of infection. As the site develops the macrophages differentiate into several different morphotypes, including multi-nucleated giant cells, epithelioid cells, and foamy macrophages loaded with lipid droplets. 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.

This takes place in the face of an extremely strong anti-mycobacterial immune response, to which the bacterium minimizes its exposure through 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 TB patients was described. This study suggested that the centre 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, T 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 only observed in peripheral follicle-like structures. In addition, whereas patients with open cavitary pulmonary TB usually present with low vascularization of peripheral infiltrations, persistent non-progressive tuberculous lesions, also called tuberculomas, were usually surrounded by highly vascularized tissues. These observations underline again how important the tissue remodeling may be for the fate of the physiopathology of TB.

The granuloma in this balanced, dynamic state is the “stable unit” of infection, and in most individuals these granulomas do not develop into active sites and can actually resolve. However, some granulomas show increasing accumulation of caseum in the granuloma center, which leads, ultimately, to necrosis and collapse of the granuloma center which releases live, virulent bacilli into the airways. As histology of individuals with active disease frequently reveals granulomas in all stages of development, disease progression appears 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 TB has been its inability to develop a well-defined and circumscribed granulomatous structure with the fibrotic capsule and caseous center that typifies an active TB granuloma in humans and primates. Recently it was reported that post-primary TB in mice recapitulated the pathology observed in humans. Mice were experimentally infected with Mtb, treated with antibiotics and reactivated according to the “Cornell” model. In brief, antibiotic treatment kills replicating bacilli preferentially, thus favoring persistence of non-replicating organisms that can reactivate and resume growth, either spontaneously or through treatment of the infected mice with immunosuppressive agents. Histopathological examination revealed focal lesions resembling lipid pneumonia typified by alveoli filled with foamy macrophages. Acid-fast stain demonstrated that these foamy
macrophages were the predominant cell type that harbored Mtb bacilli. In the central foci of necrosis Mtb were observed associated with lipid droplets. The appearance of the foamy macrophage appeared to be a key component in this pathology.

In humans, there is a rich database detailing the presence of foamy macrophages in TB granulomas\(^{35, 39}\). The most striking study showed that within TB patients biopsy samples, foamy macrophages were mainly located at the interface region immediately flanking central necrosis (Fig. 1). Hence, these foamy macrophages were only observed within necrotic lesions, thus strongly suggesting that they play and active role in necrosis formation and the accumulation of caseous debris at the heart of the granuloma\(^ {35}\).

**Characterization of foamy macrophages**

The induction of foamy macrophages packed with lipid bodies have been reported in many pathologies associated with chronic proinflammatory stimuli, ranging from atherosclerosis\(^ {41}\) and septic arthritis to infection with a range of persistent pathogens such as Mtb, *Chlamydia*\(^ {42}\), and *Toxoplasma*\(^ {43}\). 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 chemotactic protein-1 (also known as 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 serum\(^ {41}\). LDL is internalized via a receptor-mediated process through LDL receptors, or, in their oxidized form, through the scavenger receptors such as scavenger receptor A (SRA) and CD36. The LDL particles contain cholesterol, triacylglycerides (TAG) and phospholipids, and, while most of the phospholipids and TAGs are metabolized, the cholesterol is retained by the macrophage in a predominantly esterified form. Following breakdown of the LDL, cholesterol is imported into the cell cytosol where it is either esterified and sequestered into lipid droplets formed within the endoplasmic reticulum (ER) leaflet, or pumped out of the cell by ATP-binding cassette (ABC) transporters. The ABC transporters ABCA1 and G1 are key mediators of cholesterol efflux and genetic deficiency in these transporters exacerbates foam cell formation.

Foamy macrophages are not only the product of an inflammatory response but amplify that response through the production of prostaglandin E2 and leukotrienes\(^ {33, 45}\). ABCG1-deficient mice that have impaired cholesterol efflux show foamy macrophage formation and enhanced inflammation in the lung\(^ {46}\). This demonstrates that the foamy macrophage is itself a pro-inflammatory cell.

**Mtb-dependent foam cell formation**

The tissue response to Mtb seems to exceed expectation given the number of bacilli that seed the early granuloma. Previous studies demonstrated that Mtb lipids are “over-produced” by intracellular bacilli, these lipids consolidate in the internal vesicles in the multi-vesicular body and are subsequently exocytosed into the extracellular milieu\(^ {47, 48}\) (Fig. 2). In a murine granuloma model in which the bacterial lipids were adsorbed onto beads that were inoculated into mice, the tissue response induced mirrored several characteristics shared by the Mtb granuloma, including neovascularization, formation of giant cells, epithelioid macrophages and foamy macrophages cells, and fibrosis\(^ {49}\). The most bioactive component of these released lipids was the trehalose dimycolates (TDM)\(^ {50}\). In an *in vitro* model of human granulomas, Mtb-derived lipomannan has also been shown to drive the differentiation of granuloma macrophages into multinucleated giant cells, in a TLR2-dependent manner\(^ {51}\), thus emphasizing the role of mycobacterial lipids in the modulation of the host response.
More recently, Peyron and colleagues demonstrated that foam cell formation was specifically induced by oxygenated forms of mycolic acid, such as oxygenated ketomycolic and hydroxyl-mycolic acids. These lipids are synthesized by pathogenic *Mycobacterium* 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.

*Mycobacterium bovis* bacillus Calmette-Guérin (BCG) can induce foam cell formation in a TLR2-dependent manner. The abundant cell wall lipid TDM is recognized by TLR2, in concert with the scavenger receptor MARCO, which appears to fulfill a tethering function. As TDM is released and trafficked in and out of infected cells 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 foamy macrophage formation, and in some infectious agents, such as *Chlamydia*, this has been linked to the nutritional needs of the pathogen.

**Nutritional advantages to the bacterium**

Foamy macrophages seem to sustain intracellular Mtb in a physiological state similar to the non-replicating, vegetative state invoked to explain persistence. Electron microscopical analysis revealed bacilli in close apposition to the intracellular lipid droplets implying that Mtb may access these structures as a nutrient source. Hence, at late stages, bacilli-containing phagosomes are also observed within lipid bodies (LB) and bacilli exhibit intracellular lipid inclusions implying that they are accessing and utilizing host lipids (Fig. 3). The recent observation that Mtb can persist within human adipocytes, another lipid-rich cell-type, is in agreement with this hypothesis that the bug is searching for the best places to survive within its human host, and can even create these niches through manipulation of host cell metabolism.

In support of this hypothesis, earlier publications showed a requirement for isocitrate lyase activity to sustain Mtb infection in the chronic phase of infection in the murine model. 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, rather than lose carbon to carbon dioxide. Mtb mutants defective in ICL1 show impaired ability to maintain a chronic infection and to survive in IFN-γ-activated macrophages in culture. In addition, Mtb has recently been shown to be capable of metabolizing cholesterol. Bacteria deficient in the genes encoding either *mce4*, a cholesterol transporter, or *hsaC*, a key enzyme in cholesterol catabolism, are unable to grow under conditions where cholesterol is the limiting carbon source. Intriguingly, when mice are infected with *mce4*-deficient bacteria the bacteria exhibit minimal phenotype early in infection but are impaired for sustaining bacterial numbers during the chronic, persistent state observed when an acquired immune response is established. This is a similar phenotype to the *icl1*-deficient mutants. Metabolism of cholesterol increases the propionate pool in the bacterium, and ICL1 is required for propionate metabolism so the genes *mce4*, *hsaC* and *icl1* may be functionally linked through cholesterol degradation. These data support a proposal that Mtb could exploit the lipid droplets in their host cells as a nutrient source.

**Pathway to caseation and tissue destruction**

The temporal and spatial correlation between the foam cell formation and the accumulation of caseum in the granuloma indicates a causal relationship, which is explored further in the model proposed in Fig. 4. If the caseum is derived from the lipids sequestered during foam cell formation...
formation then the lipids in the caseum should reflect their origin. Analysis of lipids from the caseum of human TB granulomas revealed the major lipid species to be cholesterol, cholesteryl ester and triacylglycerides (TAG), which is entirely consistent with the lipids deriving from lipid droplets (MJK and DGR, unpublished results).

The induction of cell death in host macrophages has been the subject of multiple 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. While studies have generated convincing data that Mtb blocks apoptosis through inhibition of cross-linking of exposed annexin I\textsuperscript{60}, and through the activity of its NADH dehydrogenase\textsuperscript{61}, several groups report conditions under which Mtb is proapoptotic. Mtb activates the extrinsic apoptosis pathway in macrophages through a TNF autocrine loop\textsuperscript{62}. This behavior 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 dissemination\textsuperscript{63}. Necrosis in murine TB lesions has been correlated with bacterial burden, indicating that at high bacterial numbers damage and inflammation is the usual outcome\textsuperscript{15}. Virulent Mtb have recently been shown to inhibit membrane repair, which drive the infected macrophage into necrosis\textsuperscript{64}. Such a response would lead to death of foamy macrophages within 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 increased aggregation of lipid debris forming the caseum at the center of the granuloma.

**Closing Remarks**

In this article we have tried to present a new hypothesis for the tissue response that drives the progression of human TB to a state of active disease and transmission. Recent 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 leads, ultimately to the accumulation of caseum within 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 completion of Mtb’s life cycle.

If correct, such a model has implications for both vaccine and chemotherapy programs. The progression of TB appears 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 prior to granuloma formation, because once formed the granuloma protects Mtb and prevents bacterial clearance. It is therefore difficult to imagine an anti-infection vaccine against TB, although reduction of bacterial load will reduce the incidence of reactivation and should therefore reduce transmission.

The data also imply that there might be avenues for manipulation of the host tissue response to infection that could either interfere with the tissue state required to support persistent organisms, or render the bacilli more sensitive to drugs that selectively target replicating bacteria by preventing them from adopting a vegetative, non-replicative state. While this is mere speculation, it does encourage us to think more broadly about novel strategies to combat this most enduring of pathogens!
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Literature cited


Box 1. Histological Progression of the Human Tuberculosis Granuloma

The early events following infection with *Mycobacterium tuberculosis* appear relatively consistent. Infection is initiated following inhalation of viable bacilli present in exhaled droplets or nuclei that are discharged into the atmosphere by an individual with an active infection. These droplets can persist in the atmosphere for several hours and, because the infectious dose is in the range of 1-10 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 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 for 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 marked reduction in the number of blood vessels penetrating the granuloma. At this stage there is a noticeable increase in the number of foamy macrophages within 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 harbor other granulomas that appear 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 development of a productive cough facilitating generation of the infectious aerosol and completion of the bacterium's life cycle.
Figure 1.
Within TB patients’ lesions, foamy macrophages are mainly located within the interface region surrounding central necrosis. Adapted from Peyron et al. (a). Typical necrotic lesion from a thin section made through a lymph node biopsy from a TB patient, stained with Haematoxylin and Eosin. As classically observed in human TB lesions, the lesion is well-circumscribed and differentiated with the central necrotic core (N) surrounded by a thin area called Interface (I) separating the necrosis from the histiocytes area. At the periphery, the lymphocyte area delineates the boundary of the structure. (b). Oil red-O staining of such TB lesions demonstrated the presence of large amounts of foamy macrophages within the interface region (I) separating necrosis (N) from the histiocytes, as shown in this enlarged views.
Figure 2.
Vesicles containing *Mycobacterium*-derived cell wall lipids are released from infected macrophages. (a) Live macrophages infected for 24 h with Texas Red hydrazide-labeled *Mycobacterium bovis* BCG were analyzed by fluorescence microscopy, with striking release of Texas Red label from the bacterial phagosome. (b) These macrophages were incubated with dextran-fluorescein for 1 h demonstrating that the released lipids permeated the host macrophage and colocalized with dextran-fluorescein, revealing the penetrance of the endocytic system by the bacterial lipids. (c) Immunoelectron micrograph of an infected macrophage probed with Ab's against Mtb cell wall glycolipids PIM (anti-mouse IgM-12 nm gold) and LAM (anti rabbit IgG–18 nm gold). Labeling of bacterial lipids can be seen in multivesicular structures (arrowed, b=bacteria). (d) Platinum replicas revealing the surface of BCG-infected macrophages. BCG cell wall lipid-containing vesicles are exocytosed from macrophages infected with biotin hydrazide-labeled BCG. The presence of bacterial components were detected with Streptavidin-gold (15 nm, arrowed). (e) Shows a high magnification micrograph demonstrating the release of these vesicles at the cell surface. Notice that the cell membrane is free from label, which is restricted to the exocytosed vesicles. Panels (a), (b), and (d) are reproduced, with permission, from reference 47.
Figure 3.
Within foamy macrophages, tubercle bacilli-containing phagosomes display privileged contact with cellular lipid bodies. (a). Tight apposition between Mtb-containing phagosomes and host lipid bodies (LB) can be observed (arrow). (b) Ultimately, at later stages, bacilli-containing phagosomes are observed within lipid bodies (LB), thus confirming that they have been engulfed. (c). Engulfed bacilli display intra-cytoplasmic lipid inclusions typical of dormant bacteria (ILI), which suggests strongly that they have accumulated host lipids\textsuperscript{35}. 

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Figure 4.
A 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 multi-vesicular bodies, which are exocytosed from the cell in vesicular form. (b). Because of the release of these vesicles, both infected and uninfected macrophages are exposed to cell wall mycolates and induced to form foam cells. (c) These cells die via an inflammatory, necrotic process and release their lipid droplets into the extracellular milieu within 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 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.