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Mycobacterium tuberculosis: Bacterial fitness within the host macrophage

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Summary.

Mycobacterium tuberculosis (Mtb) has evolved to become the single greatest cause of death from an infectious agent. The pathogen spends most of its infection cycle in its human host within a phagocyte. The bacterium has evolved to block the normal maturation and acidification of its phagosome, and resides in a vacuole contiguous with the early endosomal network. Cytokine-mediated activation of the host cell can overcome this blockage and an array of anti-microbial responses can limit its survival.

The survival of Mtb in its host cell is fueled predominantly by fatty acids and cholesterol. Mtb's ability to degrade sterols is an unusual metabolic characteristic that was likely retained from a saprophytic ancestor. Recent results with fluorescent Mtb reporter strains demonstrate that bacterial survival differs with the host macrophage population. Tissue resident alveolar macrophages, which are biased towards an alternatively-activated, M2-like phenotype, are more permissive to bacterial growth than monocyte-derived, inflammatory, M1-like interstitial macrophages. The differential growth of the bacterium in these different phagocyte populations appears linked to host cell metabolism.

Introduction.

The foundations of our understanding of intracellular parasitism by a range of eukaryote and prokaryote pathogens has been laid using tissue culture infection models. These models, using defined cell lines or expanded primary cell cultures, have been invaluable in the generation of the knowledge base on which the field currently relies. However, the models artificially compress the heterogeneity that exists for all these pathogens in their natural *in vivo* infection cycle. It is the heterogeneity within the pathogen population that enhances a pathogen's capacity to adapt and survive under the differing immune pressures and tissue environments within its host (1–3).

The past few years has seen the development of a new generation of tools that will enable us to better understand the functional consequences of heterogeneity both in the pathogen population, and in the subsets of host cells present *in vivo* (4–6). *Mycobacterium tuberculosis* (Mtb) is a human pathogen and is the largest single cause of death by a single infectious agent. There are no effective vaccines against infection and no biomarkers for protective immunity (7–10). While there are drugs that are effective against Mtb, treatment requires a cocktail of 3–4 drugs taken continuously for 8–9 months. Such drug regimens are

a serious strain on the resources of the healthcare systems in many resource-challenged nations, and the emergence of drug resistant strains occurs with disturbing frequency in many countries. Understanding the consequences of bacterial heterogeneity *in vivo* with respect to both drug action and immune containment remains a serious challenge to the field.

The immune environment at site of infection.

While not an obligate intracellular pathogen, Mtb does spend the greatest part of its infection cycle within host phagocytes and the granuloma, the tissue response to Mtb infection, is an extremely macrophage-rich structure (11, 12). Recent data indicate that, following inhalation of infectious Mtb, the bacterium is phagocytosed by alveolar macrophages (AMs) patrolling the airway surface (13). Uptake of Mtb activates an inflammatory response through the stimulatory capacity of the multiple TLR ligands on the bacterial cell wall. The infected AM invades the subtending tissue of the lung and the pro-inflammatory response amplifies. This response leads to the generation of chemokines, such as CCL2, that are the primary drivers of the recruitment of interstitial macrophages (IMs) derived for peripheral blood monocytes in the circulation (14–17). This proinflammatory response persists until the development of an acquired immune response, which in the murine model system, is delayed until 3–4 weeks post-infection because it is dependent on dendritic cells (DCs) carrying Mtb antigen to draining lymph nodes to prime the initial T-cell response to infection (18, 19).

Upon acquisition of a specific immune response against Mtb the replication of the bacterium is restricted and the infection transitions into a containment state with relatively static bacterial burden, Figure 1A. In non-human primates (NHP) and, by inference in humans, the infection is paucibacillary, whereas in mice there is a much greater bacterial burden. This is one of the features of the murine infection that raises concerns regarding its usefulness as a model for human TB. During this phase of containment and cellular consolidation new macrophage phenotypes such as epithelioid macrophages, multi-nucleated giant cells and foamy macrophages appear within the granuloma (20). In NHPs and humans the granuloma is a highly stratified structure with distinct transcriptional signatures associated with the different regions, Figure 1B. The central, caseous region of the granuloma has a pro-inflammatory signature, while the region surrounding the caseum shows marked enrichment for transcripts associated with anti-inflammatory programs (21, 22). Intriguingly, each granuloma functions like an independent entity and, while the systemic immune response appears unchanged, some granulomas may progress to active disease whilst others continue to control the infection, or even progress to a sterile state (23). The factors that determine the localized progression to active disease have remained elusive (24).

This phenomenon reflects one of our greatest obstacles to combating this disease. There are no reliable biomarkers for protective immunity and therefore no surrogates to inform vaccine development programs (7–9). Increasingly sensitive indicators of early disease progression have been reported (25), but these indicators require initiation of the tissue damage that accompanies actual disease so they are not useful indicators of protective immune status. Mycobacterial Growth Inhibition Assays (MGIA) are the most utilized peripheral indicator of protective immunity (26). The data look compelling because they show a functional readout linked to bacterial survival. However, recent comprehensive evaluation of extensive

datasets on the application of MGIA on different human populations indicate that, while indicative of trained innate immunity, they do not correlate with the protected state of the individual (27).

Life or death in the Phagocyte.

M. tuberculosis is internalized by classic phagocytosis. Inert particles phagocytosed by macrophages are delivered to the acidic, hydrolytic environment of the phago-lysosome, but *M. tuberculosis* has evolved strategies to subvert the process of phagosome maturation (28). The compartment in which *M. tuberculosis* resides is slightly acidified (pH 6.4), remains interactive with the endosomal network, and shows limited acquisition of lysosomal hydrolases. Classic activation of the macrophage with interferon-gamma (IFN-γ) prior to infection enables the macrophage to overcome this process and deliver the bacterium to an acidic lysosome (29, 30). The killing of *M. tuberculosis* by activated macrophages is dependent on multiple factors, most significantly, the production of nitric oxide (NO), the low pH of the lysosome, and the delivery of antimicrobial peptides through the process of autophagy (31–33).

Several publications document the ability of *M. tuberculosis* to escape the phagosome and access the cytosol of its host cell (34–37). Escape from the phagosome appears to precipitate the necrotic death of the infected macrophage, and a marked growth spurt in the intracellular bacterial population (38, 39). This transient event that may have significance with respect to the pathology observed in late-stage disease, but may be of less significance to long-term survival of the pathogen in its host. Data indicate that, temporally and spatially, the intravacuolar population of *M. tuberculosis* likely represents the more significant target for therapeutics (40).

What are the underlying mechanisms of immune control and disease progression?

Our understanding of immune control of tuberculosis is shaped heavily by failed immunity in the form of knockout mouse studies, or catastrophic human genetic lesions (41, 42). IFN-γ is known to be important because mice deficient in IFN-γ fail to control Mtb infection and humans with genetic defects in their IFN-γ receptor are exquisitely susceptible to TB and to BCG'osis. IFN-γ Release Assays (IGRA) have also been used, unsuccessfully, as indicators of a protective immune response or treatment efficacy (43). However, our current knowledge indicates that while a Th1-biased immune response and the production of IFN-γ is required for an effective immune response to Mtb it is not sufficient to protect against either infection, or disease progression. Moreover, the assumption that disease progression is the consequence of failure in Th1-dependent immune control, while widely held, is actually unsubstantiated.

A recent study used fluorescent Mtb fitness reporter strains to identify those host phagocytes that best controlled Mtb growth, and those phagocytes that were permissive (44). The strains all expressed mCherry constitutively and expressed GFP either as a fusion protein with the single strand binding protein (SSB) as a readout for replication, or conditional expression of

GFP under regulation of the NO-responsive promoter for *hspX*(4–6), shown in Figure 2. Studies in vaccinated and naïve mice demonstrated that expression of *hspX'*::GFP correlated with the development of a Th1 immune response and the expression of iNOS in the host tissue, and that fluorescent SSB-GFP foci were less numerous in the face of a Th1 immune response (5). These data were generated on tissue sections from the murine granulomas. The phenotype of the bacterium at individual cell level was determined on cell suspensions from infected tissue (44). At 2 weeks post-infection the bacteria were present predominantly in neutrophils, AMs and IMs. Upon characterization of the reporter Mtb strains it was found that the levels of stress induction (*hspX'*:: GFP) were higher in Mtb in IMs and neutrophils, than it was in those bacilli in AMs. Conversely, the SSB-GFP puncti were more frequent in Mtb in AMs and neutrophils than they were in Mtb in IMs. These data suggested that Mtb in AMs experienced less stress and replicated more actively than those in IMs. This result was corroborated with Mtb expressing a clock plasmid, pBP10, which is lost from the bacteria at a fixed rate linked to replication (45, 46).

Clodronate liposome-mediated depletion of the macrophage subsets was conducted to demonstrate the functional significance of the IM and AM host cell populations. Delivery of clodronate liposomes to the lung airways depleted the AM population and intravenous inoculation of clodronate liposomes depleted the blood monocytes and therefore the IM population. In the mice with depleted AM the bacterial burden was reduced by approximately one log, while in the mice with depleted IMs the bacterial burden was increased by a log, Figure 3. These data demonstrate that by altering the relative proportion of IMs and AMs available to act as host phagocytes one can impact the bacterial load in the mice both positively or negatively, an observation consistent with previous macrophage depletion studies (14, 16, 17).

IMs and AMs adopt markedly differing metabolic states in response to Mtb infection.

Analysis of the transcriptional profiles of both Mtb-infected and uninfected IMs and AMs showed that all four phagocyte populations had their own discrete signature (44). Pathway analysis of the Mtb-infected AMs and IMs indicated that infected AMs were enriched for transcripts associated with fatty acid metabolism and cholesterol homeostasis. In contrast, infected IMs were up-regulated in transcripts linked to inflammatory responses, glycolysis, IFN-γsignaling and hypoxia. Treatment of infected mice with the non-hydrolyzable glucose analog 2-deoxyglucose (2-DG) led to a decrease in IM number without impacting the AM population. The reduction in IM number was accompanied by an increase in bacterial burden, providing independent demonstration that the reduction in the relative proportion of IM/AMs drives an expansion in the bacterial burden.

A functional link between host cell metabolism and bacterial growth was demonstrated through the manipulation of Mtb-infected bone marrow-derived macrophages (BMDMs) with the metabolic inhibitors 2-DG and the fatty acid oxidation inhibitor Etomoxir (ETO) *in vitro*. Inhibition of glycolysis in the infected BMDM enhanced bacterial growth, while

inhibition of fatty acid oxidation with ETO led to a reduction in bacterial growth. Neither compound had any impact on bacterial growth in rich Middlebrook 7H9 bacterial broth.

What is the basis of the difference between AM and IM?

Until very recently it was thought that all macrophages in the body were differentiated from peripheral blood monocytes derived from hematopoietic precursors in the bone marrow. This is now known not to be the case and most tissue resident macrophages, including alveolar macrophages, derive from fetal yolk sac and fetal liver stem cells during embryogenesis (47, 48). These tissue resident cells are self-maintaining and capable of replication, albeit at a slow rate during homeostasis. Interestingly, recent reports suggest that Mtb-infection arrests cell cycle in infected cells while increasing bystander macrophage replication within the infected tissue (44, 49). A similar state of monocytosis has been observed in human tuberculosis indicating that this response is not restricted to the murine infection model (50, 51). The induction of replication within the macrophage populations in the infected lung provides another route for the selective expansion of permissive AM population.

The larger question emerging from these studies is how the IM and AMs lineages, that experience the same immune milieu generated by Mtb infection, adopt such divergent metabolic states. IMs and AMs are ontologically-distinct macrophage lineages suggesting that ontogeny is the dominant determinant controlling their response to infection. This interpretation is supported by recent data from an acute lung injury model where IMs and AMs experiencing LPS in the lung responded divergently despite experiencing the same insult (52). The accepted tissue culture model for macrophage polarization invokes the adoption of an M1 (inflammatory and anti-microbial) state in response to IFN-γ, and the acquisition of an M2 (anti-inflammatory and tissue repair) state following exposure to IL-4 and IL-13 (53–55). While these definitions provide a useful sense of context, multiple labs report that *in vivo*, in both humans and mice, different macrophage populations co-express numerous proteins or transcripts that, *in vitro* are thought to associate exclusively with either M1 or M2 activation states (56, 57). Extensive analysis and modeling of macrophage subsets in NHP tuberculosis infection has detailed different populations of macrophages that express M1- (eNOS and iNOS) or M2- (Arg1 and Arg2) associated markers (58). In a subsequent model they suggest that the relative ratio of M1/M2 macrophage subsets is an accurate predictor of whether any individual granuloma is likely to progress to active disease (59). The model is consistent with data indicating that ontogenically-distinct macrophage populations, the AMs and IMs, are actually pre-programmed to respond divergently when experiencing the same immunological milieu during Mtb infection. Analysis of PBMC-derived macrophages and tissue resident macrophages under homeostatic conditions suggests that the bias towards M1-like and M2-like phenotypes in these different macrophage lineages exists prior to any insult or infection (60, 61).

Bacterial metabolism in the host environment.

The advances in our understanding of host cell metabolism and bacterial control now connects with our appreciation of bacterial metabolism within the host cell environment. Mtb's preference for lipids and fatty acids as carbon source has been discussed since the

1950's but the central significance of this metabolic dependence to the virulence and pathogenesis of Mtb has only been demonstrated experimentally recently. In 2000 McKinney and colleagues reported that mutants of Mtb deficient in expression of isocitrate lyase (*icl1*) could not sustain an infection in the face of immune pressure (62). Isocitrate lyase of Mtb is a bifunctional enzyme whose more significant activity is that of a methyl isocitrate lyase that is required for the Methylcitrate Cycle, which is the primary route for detoxification of the proponyl-CoA that accumulates upon degradation of cholesterol (63–66).

Mtb has specific transport systems dedicated to the acquisition of fatty acids and cholesterol. The Mce family of lipid transporters is conserved across the bacterial kingdom (67), and is present in Mtb as 4 distinct multi-genic transporter complexes (68). Mce1 and Mce4 are the preferred uptake transporters for fatty acids and for cholesterol respectively (69). The two transporters share some of their subunit proteins, which stabilize the transporter complexes, and most notably, all Mce transporters use a common motor, the ATPase MceG (69). The linking of fatty acid and cholesterol acquisition is not surprising given the requirement for the balanced production of downstream intermediates to feed the TCA cycle and provide building blocks for the synthesis of complex cell wall lipids (63, 66).

The significance of cholesterol to Mtb growth was also demonstrated by a large empirical screen to identify compounds active against intracellular Mtb (70). The screen identified several inhibitors that blocked specific steps in bacterial cholesterol degradation, or its regulation, Figure 5. In addition, transcriptional profiling from a panel of 15 clinical strains of Mtb that represented the global genetic diversity of the *Mycobacterium tuberculosis* complex confirmed that genes involved in the processing of cholesterol and fatty acids were up-regulated during intracellular growth as part of common core transcriptome shared across all isolates (71).

The coupled metabolism of host and pathogen.

While it is clear that the metabolism of Mtb is shifted towards heavy dependence on fatty acids and cholesterol, and that the predisposition of the AM population towards fatty acid oxidation appears to provide Mtb with a permissive host cell population, the modulation of host metabolism extends beyond the host cell to the surrounding tissue.

Figure 1B illustrates the caseous center of the human TB granuloma. TLC and mass spectrometry analysis of the lipid species in the human granuloma demonstrated that the major lipid species were triacylglycerols (TAG), cholesterol and cholesterol ester (CE) (21). The presence of abundant CE in in the caseum is strong evidence that these lipids came from lipid droplets present in the foamy macrophages that typically surround the caseous center of the granuloma (72, 73). When cells accumulate cholesterol, they usually esterify the sterol prior to transport from the ER and incorporation into the lipid droplet. This esterification is proposed to reduce the toxicity of the cholesterol. Mtb-infection in culture induces a foamy macrophage phenotype in the infected cell, and in uninfected bystander macrophages in the same culture. The mycobacterial cell wall lipid trehalose dimycolate (TDM) has been shown to induce this behavior (72). TDM is recognized by the scavenger receptor MARCO and

signals through both TLR2 and Mincle (74, 75). It is thought that the prolonged, chronic activation of the proinflammatory pathways in macrophages drives this transformation into foamy cells, Figure 6, similar to the cascade invoked in atherosclerotic plaques.

Concluding Remarks.

It is interesting to see how evolution appears to have driven Mtb to exploit the nutrient sources that it has the capacity to enhance at site of infection, thus maximizing its chance of success. But the odds are not entirely in favor of Mtb. Of those individuals that acquire an Mtb infection, during the course of their lifetime only 5–10 % of immunecompetent individuals will progress to develop active disease. These look pretty good odds for the human species. However, the problem comes with the extraordinary efficiency of transmission that enables a single individual with active TB to infect a large number of people. Recent estimates had indicated that approximately 25% of the world's population is sub-clinically infected with Mtb (76). In areas of high HIV endemnicity, such as Sub-Saharan Africa, this constitutes a major challenge to human health. Not only is Mtb that largest single cause of death by an infectious agent, it is also the single greatest cause of death in individuals living with HIV. The challenges remain, but our increased knowledge of the physiology and metabolism of intracellular Mtb, and its interplay with the different host macrophage populations, will likely provide new avenues to combat this pathogen.

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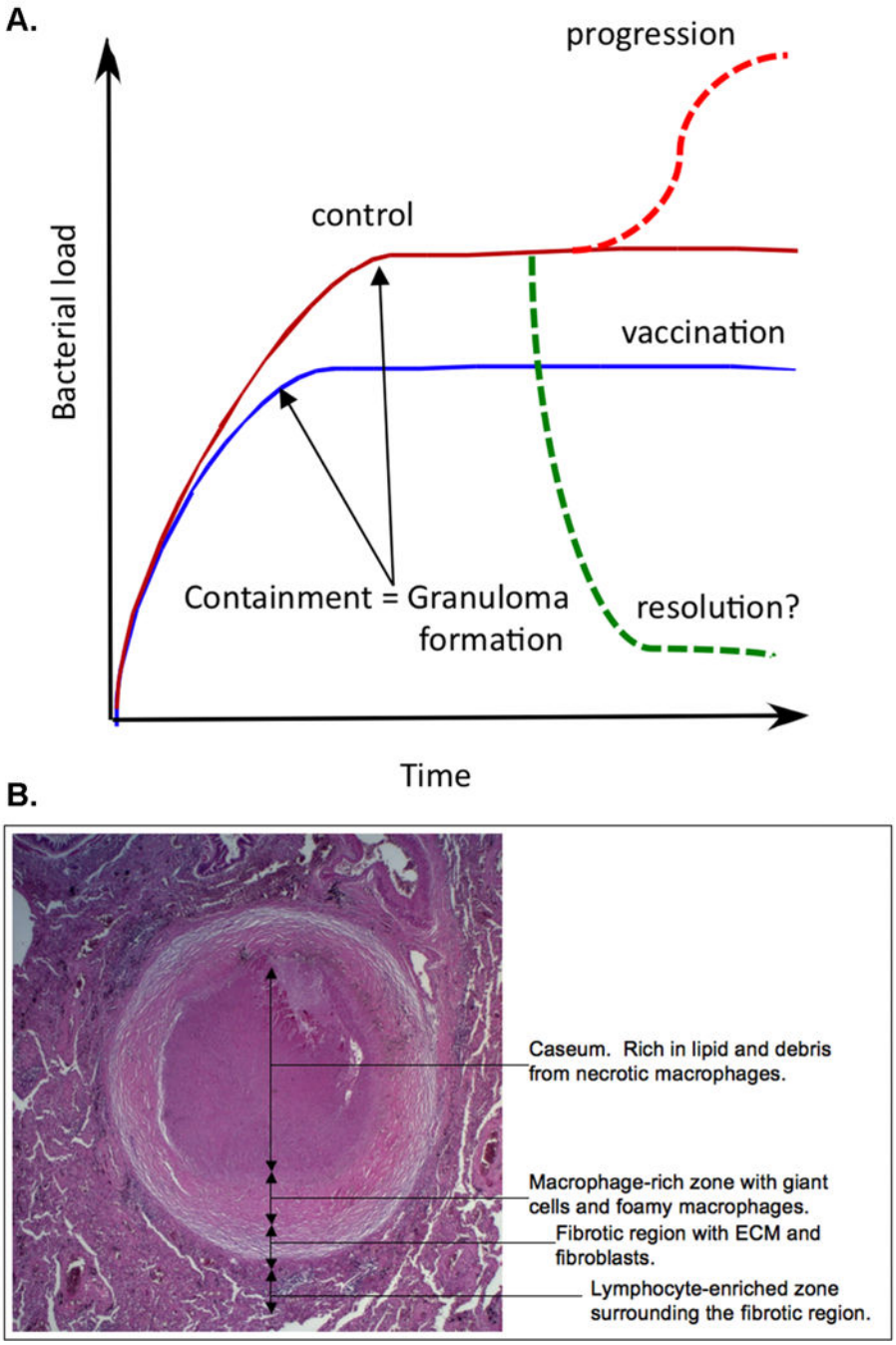

Figure 1.

A. A schematic illustration of the potential outcomes on infection with Mtb. In most hosts Mtb exhibits rapid expansion of the bacterial burden over the first 3–4 weeks of infection. At this point the acquired immune response has developed and controls the bacterial burden at a subclinical level but is unable to clear the infection. In vaccinated hosts this transition to control of the bacterial burden is achieved at around a log fewer bacilli. While resolution of infection is possible theoretically it is virtually impossible to demonstrate. Progression from latent disease to active disease appears to occur in the face of

a robust systemic immune response that is Th1 dominant. While there are candidate indicators of early disease progression the field lacks immunological markers to detect vaccine-induced protection. Published previously in (10). **B. The main features of the human TB granuloma.** A fully-formed human TB granuloma is an extremely stratified structure. The center of the granuloma is caseous in nature and rich in lipids, thought to be derived from the lipids present in foamy macrophages. The caseum is surrounded by a macrophage-rich layer that contains foamy macrophages, multi-nucleate giant cells and epithelioid macrophages. Mtb bacilli are observed in many of these cells. This structure is frequently encased in a fibrous capsule of collagen and other extracellular matrix proteins. Lymphocytes tend to be restricted to the periphery of the granuloma outside the fibrous outer layer. Published previously in (77).

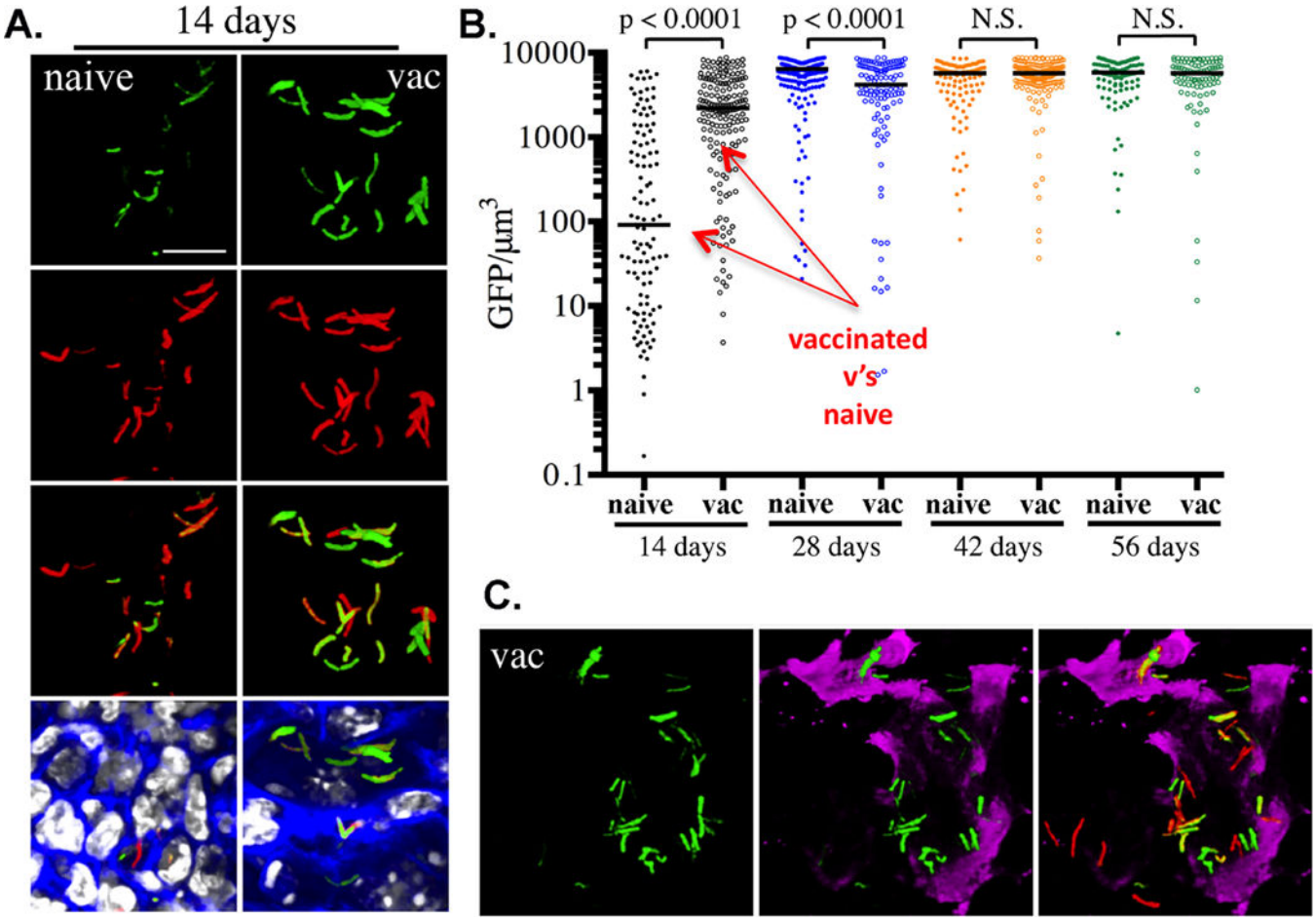

Figure 2. Demonstrating the usefulness of the *hspX'::GFP* reporter strain in assessing and reporting on the localized induction of iNOS at the site of infection.

PBS-immunized (naïve) and mice vaccinated with heat-killed *M. tuberculosis* (vac) were infected with *hspX'*::GFP, *smyc'*:: mCherry Erdman *M. tuberculosis* reporter strain. Fluorescence induction of the *hspX* promoter-dependent GFP is higher at 14 days in the vaccinated animals assessed by confocal microscopy of thick tissue sections (A), which were scored subsequently by Volocity (B). (C) The thick tissue sections were probed with antibodies against murine NOS2 (magenta) demonstrating the co-localization between GFP induction and NOS2 expression at the site(s) of infection. Data shown are detailed in Sukumar et al, (5).

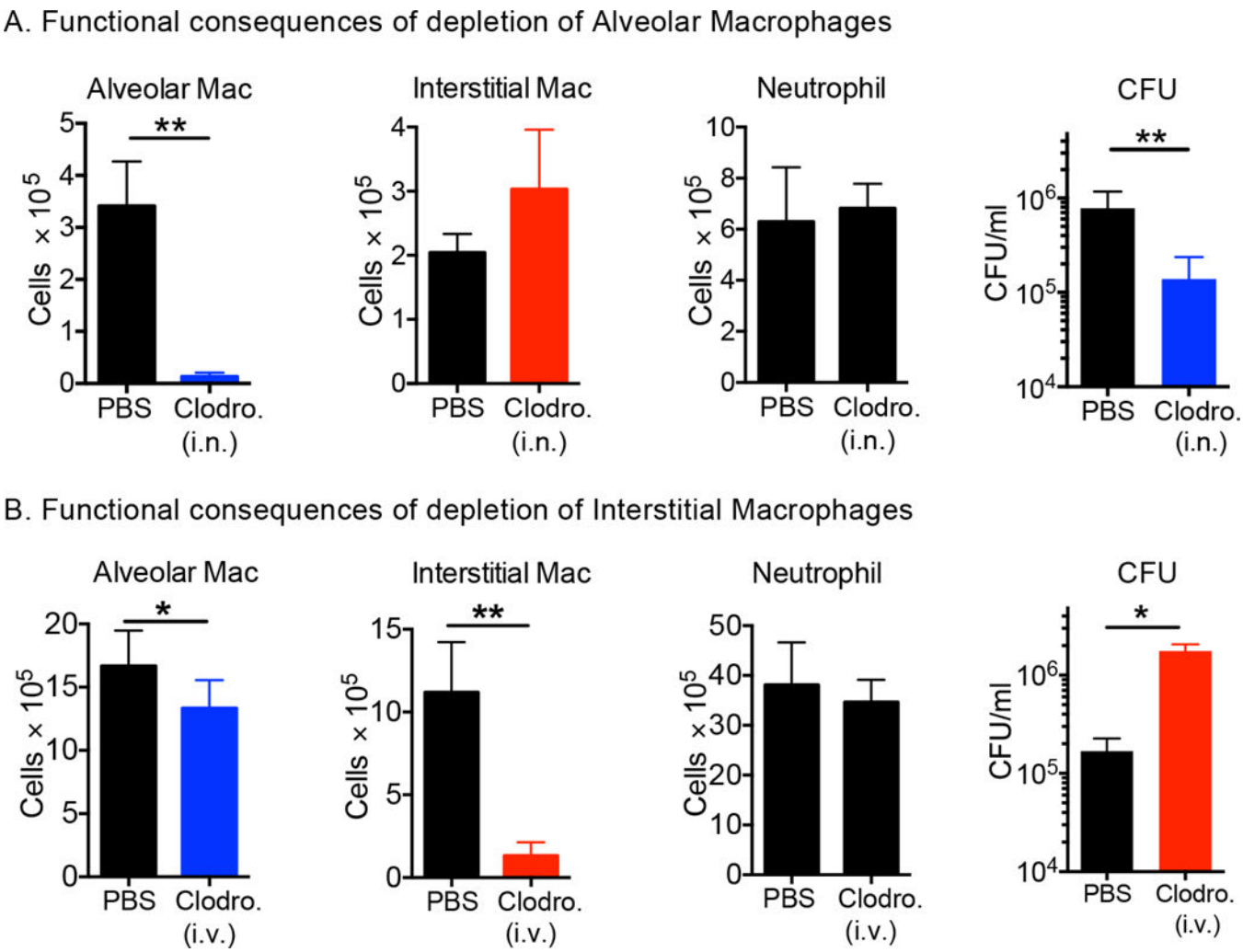

Figure 3. Selective depletion of AM and IM results in a decrease or an increase in bacterial burden respectively.

Treatment of mice by clodronate liposomes delivered either A. intranasally (i.n.) or B. intravenously (i.v.) to deplete the AM or the circulating monocytes, which depleted the recruited IM. Neither treatment impacted the neutrophil population within the infected lung tissue. Interestingly, depletion of AM lead to a reduction in bacterial burden, while depletion of IM lead to an increase in bacterial burden. The data demonstrate how modulation of the relative dimensions of the permissive (AM) and controller (IM) macrophage populations impacts directly on bacterial burden. Data taken from Huang et al. (44).

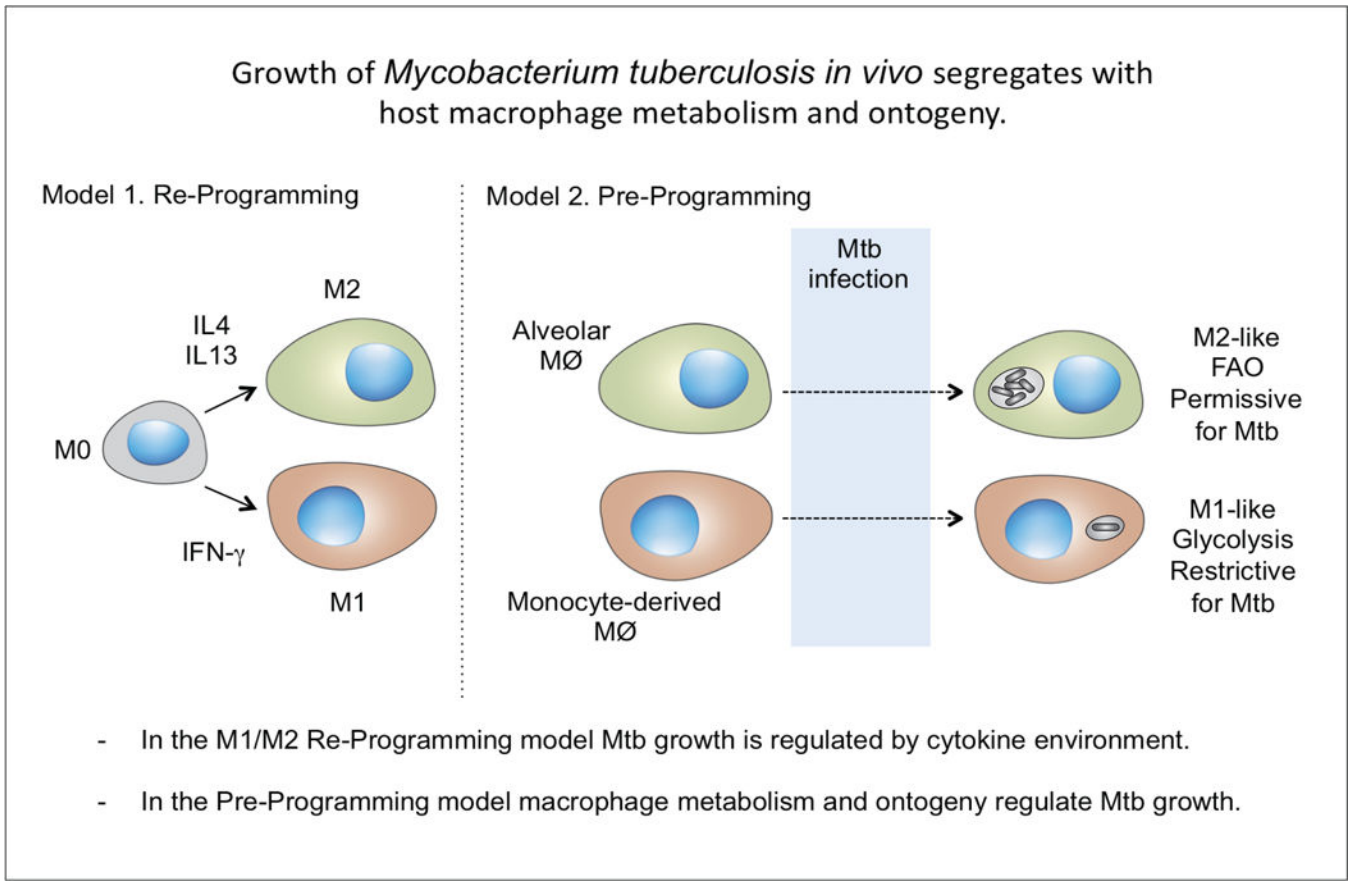

Figure 4. Models of Macrophage Reprogramming and Pre-programming.

Schematic representation illustrating how macrophage function in the Reprogramming model (A) is determined by immune signaling within the tissue niche. In the proposed Pre-Programming model (B), the function of co-existing macrophage lineages in the lung in Mtb infection is determined, in large part, by the origin of the macrophage. Published previously in Huang et al. (44).

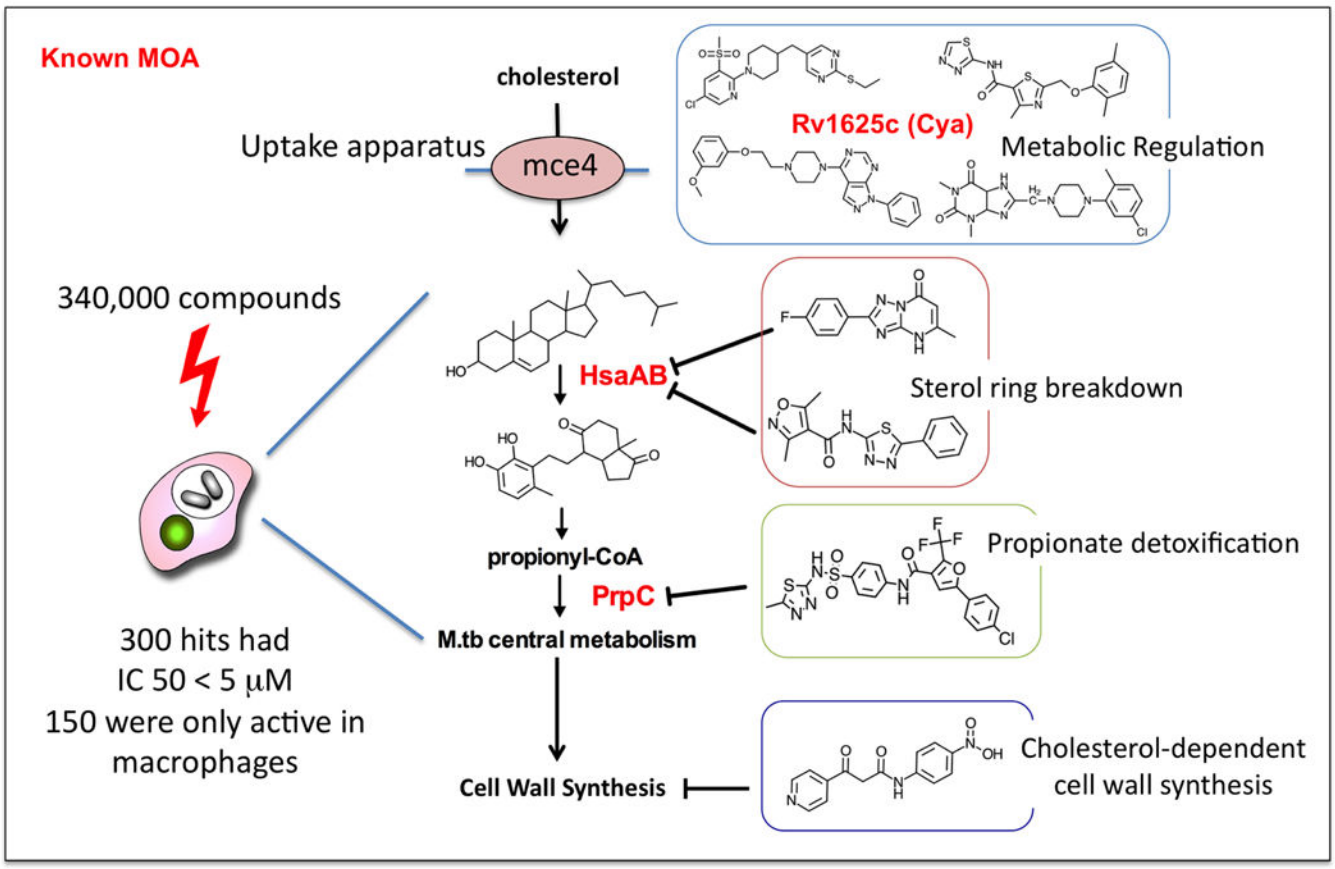

Figure 5. Summarizes the major classes of cholesterol-dependent anti-Mtb compounds identified in a screen against intracellular Mtb.

The primary screen of 340,000 compounds identified 300 hits with IC 50 of less than 5μM, 50 % of which only showed activity against intracellular bacteria and had no activity against Mtb in rich broth. However, the majority of these compounds recovered their activity when Mtb was grown in medium with cholesterol or fatty acids as the limiting carbon source. This figure summarizes the major targets or functions inhibited by the compounds. Activators of an adenylate cyclase (rv1625c, Cya) were shown to be involved in regulation of cholesterol utilization, as well as specific inhibitors of enzymes, HsaAB and PrpC, involved in cholesterol breakdown or propionyl-CoA detoxification. Data taken from VanderVen et al. (70).

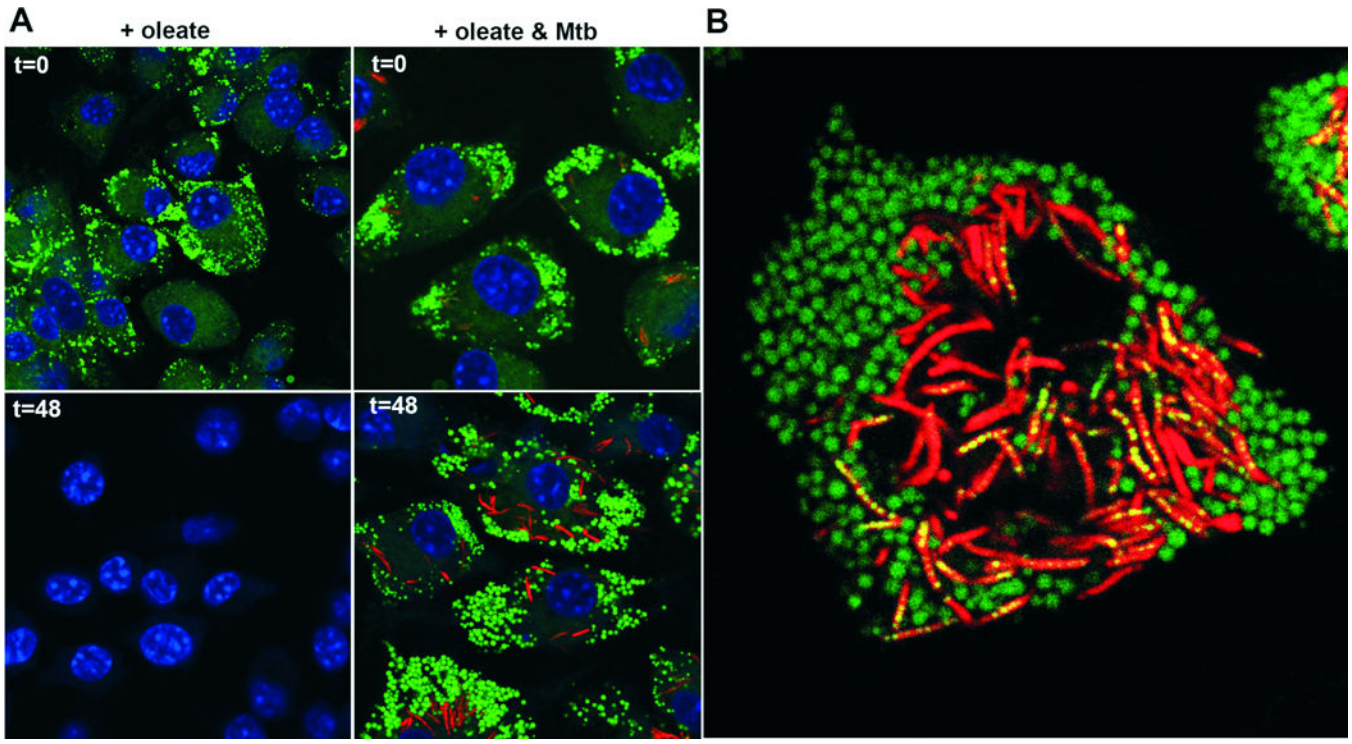

Figure 6. Mtb infection leads to retention of the foamy macrophage phenotype and facilitates bacterial access to host-derived lipids.

(A) Murine bone marrow-derived macrophages were induced to form foam cells through incubation with 400 μM 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 μM 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. Data published previously in Podinovskaia et al. (78).