HHS Public Access

Author manuscript

Semin Immunopathol. Author manuscript; available in PMC 2017 March 01.

Published in final edited form as:

Semin Immunopathol. 2016 March ; 38(2): 213–220. doi:10.1007/s00281-015-0533-1.

A bug's life in the granuloma

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Abstract

The granuloma is the defining feature of the host response to infection with *Mycobacterium tuberculosis* (Mtb). Despite knowing of its existence for centuries, much remains unclear regarding the host and bacterial factors that contribute to granuloma formation, heterogeneity of presentation, and the forces at play within. Mtb is highly adapted to life within the granuloma and employs many unique strategies to both create a niche within the host as well as survive the stresses imposed upon it. Adding to the complexity of the granuloma is the vast range of pathology observed, often within the same individual. Here, we explore some of the many ways in which Mtb crafts the immune response to its liking and builds a variety of granuloma features that contribute to its survival. We also consider the multitude of ways that Mtb is adapted to life in the granuloma and how variability in the deployment of these strategies may result in different fates for both the bacterium and the host. It is through better understanding of these complex interactions that we may begin to strategize novel approaches for tuberculosis treatments.

Keywords

Tuberculosis; Granuloma; Phenotypic heterogeneity; Macrophage

Introduction

The clinical hallmark of tuberculosis is the granuloma. Despite decades of study, the host and bacterial determinants critical for granuloma formation, progression, and resolution remain unknown. The field is even at a loss as to whether granuloma formation ultimately benefits host or bacterium and which is the master of the situation. Here, we focus on the bacterial factors involved in shaping the granuloma and the bacterium's survival within it.

In discussing bacterial adaptation to the granuloma environment, it is important first to define the salient features of a human tuberculous granuloma. To satisfy the clinical definition of a granuloma, an organized foci of macrophages is the only requirement.

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However, this definition is alarmingly simple and does not convey the range of granuloma types that are associated with different pathophysiologic processes, especially those observed during tuberculosis infection.

The *Mycobacterium tuberculosis* (Mtb) bacillus is well adapted to life within at least some of the myriad of granuloma phenotypes found in patients. Indeed, the bacterium may participate in driving these different granuloma outcomes. The diversity of possible effects is highlighted by the striking heterogeneity of granuloma features, appearance, and bacterial burden within an infected individual. Recent work has begun to untangle how both the host and pathogen can contribute to disparate granuloma fates, and in this chapter, we will focus on Mtb's influence and highlight how novel experimental approaches may be the key to unraveling the complexities of the tuberculosis granuloma.

Granuloma formation and progression

While our focus in this review is bacterial determinants of granuloma formation and fate, it is important to define the host players and events in this process. In its simplest form, a granuloma is an organized collection of macrophages. As such, it is likely that granuloma formation begins when inhaled Mtb encounter alveolar macrophages, the sentinel cells of the lung. Other phagocytes such as neutrophils are called to the scene shortly thereafter. Monocytes are recruited from the blood and differentiate into macrophages. Dendritic cells arrive to take up antigen, both live bacteria, bacterial products, and dying infected cells and transit to the lymph nodes. Mtb's chance of survival in these various cell types differs as does the fate of the infected cell. Taken together, the innate action of these cells together is insufficient for control and the bacterial population expands until the onset of the adaptive immune response, which rapidly works to fully activate macrophage effector functions and control the bacterium.

Classically, Th1 polarized CD4+ T cells have been thought to be the prime mediators of adaptive immunity to tuberculosis. However, other T cell subsets are now known to contribute. Cytolytic activity and cytokine secretion from CD8+ T cells, Th17, and Tregs are also needed, but perhaps play different roles during different stages of the infection [1], and the precise mix of T cell subsets required for resolution versus progression to immunopathology is not known. Even less is understood about the roles of non-classically restricted and innate T cells and B cells, which can be abundant constituents of the granuloma [2–5].

Granuloma progression

Collectively, many host cell types acting both in concert and discordantly lead to the formation of the granuloma and influence both the fate of the bacterium within and the immunopathology observed. Autopsy studies of humans infected with tuberculosis classified mature granulomas into several histopathologic subtypes [6]. The classic tuberculous granuloma consists of a focus of organized macrophages encapsulated by fibroblasts, outside of which is a ring of lymphocytes. The center of the granuloma consists of caseum, a coagulative form of tissue necrosis so-called because of its crumbly, cheese-like consistency. If the host is able to control the mycobacteria, necrosis halts and the caseum may be

replaced by calcification and fibrosis over time. Progression of disease correlates with expansion of the caseum, cavitation, and conversion from coagulative to liquefactive type necrosis, with breakdown of the surrounding lung tissue and erosion into nearby airways, facilitating pathogen dissemination and transmission to new hosts.

These events are typically envisioned temporally such that in individuals with clinically latent TB one imagines granuloma that have progressed to fibrosis, while in individuals who are sick, necrosis and liquefaction have ensued. However, recent research utilizing non-human primates (NHP) indicates that granuloma evolution is more nuanced and less linear. Clinically latent NHP can have necrotic granulomas, and clinically active NHP can have quiescent and calcified granuloma [7]. Moreover, each individual granuloma behaves independently of others within a host [8]. The specific factors that drive an individual granuloma towards a particular fate are just beginning to be untangled, but it appears that the local balance of pro- and anti-inflammatory cytokines plays a critical role [9]. Adding to this complexity, we are also beginning to recognize that there is variability within a population of infecting bacteria as well, raising the possibility that diverse bacterial states both arise in response to and drive lesional heterogeneity.

Bacterial determinants of granuloma formation and heterogeneity

Before we further consider how granuloma heterogeneity might arise, we will first consider some potential forks in the road in lesion course and which bacterial factors have been implicated in these different paths.

Induction of altered macrophage phenotypes

Macrophages are a highly plastic cellular population. Bacterial infection will skew their transcriptional and functional phenotypes towards a “classical” activation phenotype. Classically activated macrophages assist in a Th1-dominant immune response, such as that required for proper Mtb control by secreting IL-12, TNF, and other pro-inflammatory cytokines and upregulating antimicrobial pathways. This is counterbalanced by “alternatively activated” macrophages, which are weakly antibacterial but stimulate anti-inflammatory pathways, wound healing, and damage resolution [10]. Alveolar macrophages typically display a mixed phenotype, in that they are charged with both surveying and destroying inhaled bacteria, but also must dampen inflammatory cascades that could otherwise damage the lung [11].

Despite possessing TLR agonists capable of skewing macrophages towards classical activation, the Mtb granuloma is made up of both classically and alternatively activated macrophages, where the ratio of classically to alternatively activated macrophages within a granuloma is predictive of bacterial control. The bacterial signals that contribute to the emergence of alternatively activated macrophages are unknown. However, microenvironments within each granuloma may perpetuate pockets of differentially activated macrophages [12]. Indeed, the emergence of alternatively activated macrophages may be largely host protective; while classically activated macrophages are highly protective early post infection, the switch towards alternative activation is required later to prevent rampant immunopathology [13].

Other specialized macrophage subsets also arise within the tuberculous granuloma and appear to be more directly driven by bacterial determinants. The best studied of these are foamy macrophages or foam cells. While foam cells are not unique to the tuberculosis granuloma, they are a hallmark of it. So-called due to the appearance of abundant intracellular lipid droplets, foam cells may serve as a nutrient-rich reservoir for Mtb. Mtb bacilli have been observed by electron microscopy in close juxtaposition and within these lipid droplets [14]. Transfer of lipids from host lipid droplets to the bacterium has been observed, suggesting that these fatty macrophages can act as a nutrient source for the bacterium.

Numerous Mtb cell wall-derived lipids have been implicated in foam cell development. Trehalose dimycolate, a major cell wall component, alone can induce foam cell appearance [15, 16]. Similarly, keto-mycolic acid, a major component of the Mtb cell wall, can induce foam cells and may also contribute to granuloma formation [17]. Cell wall components drive the emergence of other macrophage subsets as well. For example, lipoarbinomannan derived from the Mtb cell wall can drive multinucleated giant cell formation [18]. The implications of multinucleated giant cell formation, and the formation of even less well-understood macrophage subsets such as epitheliod macrophages for bacterial fate and lesion outcome are poorly understood.

Exosomes: a mechanism to extend bacterial influence?

Mtb can also exert its influence on immune cells from a distance, not just locally. It has been found that Mtb sheds cell wall components as exosomes, secreted vesicles which contain highly immunogenic cell wall lipids and proteins [19, 20]. These exosomes can be taken up by macrophages and surrounding immune cells [21]. Uptake of Mtb-derived exosomes by uninfected macrophages inhibits IFNγ-mediated activation and upregulation of mycobactericidal pathways [22]. These processes serve to extend Mtb's influence to bystander, non-infected cells and may serve to explain the long observed phenomenon of extensive host cellular involvement in granuloma, despite low bacterial numbers.

Exosomes may also aid Mtb in carefully fine-tuning the local immune and inflammatory environment. The regulation of exosome secretion, governed in part by the gene Rv0431, is tied to the bacterium's ability to strike the appropriate balance in inflammation that allows for transmission without incurring too much bacterial killing [23]. Furthermore, it underscores the bacterium's role in actively shaping the granulomatous environment. Variability in secretion of these immunomodulatory exosomes, either across bacterial populations within or between granuloma or over time could greatly influence both containment of the bacteria and disease progression.

Caseation: the great escape

Caseation, cavitation, and ultimately progression to airway erosion permit transmission and, as such, are critical events in the Mtb life cycle. These processes appear to be at least partially bacterially driven, as caseation may result from the Mtb-induced expression of matrix metalloproteinases (MMPs), which can degrade collagen and facilitate tissue destruction [24, 25]. One Mtb-induced MMP has also been implicated in the recruitment of

macrophages during early granuloma formation [26]. Thus, MMPs may play a role both in the formation and breakdown of the tuberculous granuloma.

Bacterial adaptations to life in the granuloma

Within an established granuloma, the bacterium encounters a number of stressors. Below, we consider some of most salient pressures and consider how mycobacteria have adapted to survive them.

Hypoxia

The Mtb genome encodes an extensive regulatory network dedicated to the response to hypoxia. The bacterium may encounter a hypoxic environment during latency, for example in calcified granuloma, and centers of caseous granuloma may also be hypoxic [27]. To counter hypoxic conditions, Mtb relies on a complex transcriptional network including the DosR regulon [28, 29]. Genes involved in the hypoxic response are important for stabilization of proteins, alternative electron transport chain proteins, and DNA damage repair, reflecting the physiologic stresses faced by Mtb in hypoxic conditions [30]. The bacterium responds to hypoxia by entering a state of nonreplicating persistence, characterized by slowed growth and significant metabolic shifts that decrease antibiotic susceptibility [31].

These classic observations have recently led to novel approaches to host directed therapy. In the zebrafish model, it was recently shown that mycobacterial infection induces angiogenesis at the site of the hypoxic granuloma. Angiogenesis was predicted to benefit the bacterium by increasing oxygen availability; blocking angiogenesis in this model with VEGFR inhibition increased the number of hypoxic granuloma and improved bacterial control [32]. The mechanism of anti-angiogenic treatment may be more nuanced in more complex lesions, however. In a rabbit model of tuberculosis, anti-VEGF treatment altered the quality of the neo-vascularization and actually promoted a more “normalized” vasculature that reduced lesional hypoxia and improved antibiotic efficacy [33].

Oxidative and nitrosative stress

Just as the bacterium employs an array of genes to survive the hypoxic environment of the granuloma, it also must contend with often lethal concentrations of reactive oxygen and nitrogen species. Immune cells, typically macrophages and neutrophils, release bursts of reactive species with the goal of damaging the cell wall, protein, and DNA. Surprisingly, the bacterium must contend with nitrites produced both by the macrophage but also as a byproduct of bacterial metabolism itself [34]. These compounds appear to exert bacteriostatic, not bactericidal pressure on the bacilli [35]. Nevertheless, inhibition of nitrosative stress allows for robust outgrowth of the bacteria in vivo [36].

To coordinate its transcriptional response to oxidative and nitrosative stress, the bacterium relies on the DosR system, which is also deployed during the hypoxic response [37]. It also requires several antioxidizing enzymes. Superoxide dismutase (SodA), tasked with disarming oxygen ions, plays an important and unique role. In Mtb, SodA is secreted, in contrast to other bacterial species where SodA is a cytosolic enzyme [38]. More recently, it

has been shown that SodA is the extracellular component of membrane associated oxidoreductase complex with DoxX and SseA. This macromolecular complex allows the cell to respond to oxidative stress not just by detoxifying radicals but by adjusting cytosolic redox homeostasis through the thiol pools [39].

Altered nutrient availability

Mtb must scavenge key nutrients from the host for successful infection. Host cells sequester nutrients such as carbon sources, metals, and amino acids as part of an antibacterial strategy referred to as “nutritional immunity” [40]. Many of these nutrient scavenging pathways have been described in previous reviews [41].

In addition, it has recently become clear that it is possible to have too much of a good thing —and that nutrient acquisition must be carefully regulated to avoid toxicity. This is clearest for metal acquisition, where the cell must actively avoid metal toxicity. Mtb encodes many metal exporters, which may be involved in surviving metal intoxication—specifically zinc and copper—as they are required for survival during macrophage infection [42, 43].

Carbon source utilization

Bacteria do not just need to eat to survive. Nutrient availability and utilization can have profound consequences on the host-pathogen interaction. This is most obvious in the emerging understanding of central carbon metabolism. Numerous publications have shown Mtb requires fatty acids, cholesterol, and gluconeogenesis to persist during infection [44–46]. These studies built on early work from the 1950s that demonstrated Mtb recovered from the lungs of infected mice preferentially metabolized fatty acids, in contrast to Mtb cultured in vitro, which metabolized carbohydrates. It was subsequently recognized that during infection of macrophages Mtb upregulates a large host of genes involved in fatty acid oxidation, consistent with the hypothesis that lipids, from either lipid bodies or intracellular membranes, are a key nutrient source during infection [47]. Indeed, deletion of both isocitrate lyases, *icl1* and *icl2*, enzymes in the glyoxylate cycle used for fatty acid catabolism, attenuated growth, and decreased virulence in mice, but only after the onset of adaptive immunity [48, 49]. This suggests a close link between bacterial metabolism and the host immune response. In line with this hypothesis, a recent study of bacterial mutants attenuated for growth in the setting of a CD4+ T cell response showed that CD4+ T cells activates the transcriptional upregulation of indoleamine-2,3-dioxygenase (IDO) in macrophages to convert tryptophan to immune signaling molecules. This robs Mtb of tryptophan, and tryptophan biosynthetic mutants are exquisitely sensitive to IFN-γ mediated killing [50].

Immunologic consequences of bacterial carbon metabolism

These studies have also demonstrated that carbon metabolism is an important factor in dictating the local cytokine milieu and granuloma pathology because of the relationship between central carbon metabolism and cell wall composition. For example, fatty acid metabolism generates proprionyl-CoA, which can in turn produce toxic byproducts [51]. To solve this problem, the Mtb cell shuttles proprionyl-CoA into the production of the virulence lipid, PDIM [52]. This switch may occur because of shifts in precursor pools but also may

be mediated through a thiol-disulfide redox “switch” in the transcription factor WhiB3, which senses the increase NADH pools associated with fatty acid metabolism. Macrophages infected with *whiB3* mutants are associated with altered cytokine profiles in macrophages, providing further evidence of the important link between bacterial metabolism, homeostasis, and virulence [53].

Antibiotic stress

Perhaps the ultimate stress experienced by a microbe is that imposed by antibiotic therapy. Mtb is notoriously resistant to chemotherapy, requiring multi-drug antibiotic courses lasting several months. It was long held that Mtb’s slow growth rate and its ability to enter a non-replicative “persister” state were the primary drivers of the requirement for prolonged antibiotic therapy. However, recent research indicates that the granuloma itself creates a complex diffusion barrier, permitting different drugs variable spatial and temporal access across the granuloma [54]. The resulting microenvironments within the granuloma may allow subpopulations of Mtb to experience subtherapeutic levels of drugs or even monotherapy, giving these bacteria the opportunity to develop resistance. Thus, by inhabiting a granuloma, mycobacteria may have inadvertently found the perfect niche to protect themselves from antibiotic treatment.

Diverse granulomatous environments, diverse antigenic repertoires?

As these data indicate, Mtb responds to specific environmental conditions with distinct responses. These different responses may account for temporal changes in Mtb gene expression that has been described in mice [55]. By extension, these differences may account for different antigen expression that has been described for proteins in the Antigen85 complex of cell wall mycolyl transferases [56]. The functional importance of altered antigen expression has been demonstrated by constitutively expressing Ag85B and showing that this improved CD4+ effector T cell recognition during the chronic stage of infection in mice, when the gene is naturally downregulated.

The lungs of infected mice represent a relatively homogeneous environment as compared to granuloma in NHP and, presumably, people. Nonetheless, these data establish a paradigm for understanding antigenic heterogeneity during infection. First, Mtb could express different antigens over time. Secondly, because different granulomas impose distinct environmental stresses on the bacterial population, the bacteria in these lesions may express different constellations of antigens and be differentially recognized by the adaptive T cell response. This could have profound implications if bacteria stop expressing antigens that the immune system was initially primed towards, rendering these bacteria effectively immunologically invisible.

Primary paths to phenotypic diversity in the bacterial population

In the previous section, we considered how different host environments might lead to differences in bacterial state that then might feedback onto the host immune response. However, these data do not address the problem of why different granulomas in the same host have different fates in the first place. This diversity in granuloma trajectories is likely

multifactorial, reflecting some combination of anatomic effects, variability in responding host cells, and variability in the bacterial population. Here, we will highlight recent advances in our understanding of bacterial phenotypic heterogeneity. These have been largely driven by efforts to understand cell to cell differences in bacterial survival in the face of antibiotic treatment. However, this phenotypic heterogeneity is likely to have important consequences in pathogenesis as well. This is especially true where each granuloma is founded by a single bacterium, providing ample opportunity for dramatic founder effects [8].

An emerging idea in the field is that transmissible but non-mutational processes generate important heterogeneity in the Mtb population. Upon encountering the stressful environment of the host, one strategy for the bacterial population to survive uncertainty is to diversify. One manifestation of this diversity is differential growth rates. Using a reporter of ribosome activity as a readout of metabolic state, McKinney and colleagues have demonstrated that upon entering a macrophage a population of bacteria become more phenotypically diverse than when grown in broth culture and bacteria from the lungs of infected mice are even more diverse still [57]. Furthermore, nutrient stress in vitro could also recapitulate the appearance of diversifying metabolic states. It is interesting to speculate that in different granuloma or under different host applied stresses the metabolic and growth rates of Mtb may change. The mechanisms that permit such phenotypic diversity within an isogenic population are just beginning to be uncovered.

DNA methylation

DNA methylation is perhaps the best understood epigenetic mechanism and an important means of regulating gene expression across both eukaryotes and prokaryotes [58, 59]. Methylated cytosine is the dominant DNA adduct in eukaryotes, while in prokaryotes, methylated adenine appears to be the most significant species. Methylation of DNA bases alters interactions between the nucleic acid sequence and DNA-binding proteins. While cytosine methylation induces repression in eukaryotes, the effects of adenine methylation in bacteria are more complex and the effects appear to be site specific, with the potential to either enhance or inhibit gene expression [58]. In Mtb, the predominant adenine methyltransferase has been identified and disruption of this gene attenuates survival in hypoxia [60]. This study did not, however, formally demonstrate that adenine methylation mediates epigenetic inheritance in Mtb and this question remains outstanding.

Bistability

DNA need not be physically altered in order to establish stable changes in gene expression patterns. Bistability refers to the existence of two (or more) stable states of gene expression: in the most simple case, a high expression state and a low expression state. Bistability is typically mediated by feedback loops that amplify stochastic noise in gene expression. The result is phenotypically distinct subpopulations within an isogenic population of cells growing under homogenous conditions. In Mtb, there is evidence to suggest bistability in the stringent response, a coordinated transcriptional response to nutritional stress important for Mtb to survive starvation as a potential “bet-hedging” survival strategy [61–63]. Another example of bistability in Mtb may help regulate the switch to persistence during periods of

stress [64]. As single-cell imaging and sequencing techniques become more accessible, examples of other bistable gene circuits may be discovered in Mtb.

Asymmetry

Bacterial offspring inherit not just a genome, but also cellular factors including proteins, cell wall components, and transcripts, thereby providing another opportunity for non-genetically encoded heritability. In some cases, these components are distributed equally between daughter cells during division, resulting in progeny with the same phenotype as the mother cell. In other cases, asymmetric distribution of cellular components maintains the phenotype of the mother cell in one daughter, but not the other. This strategy permits both phenotypic memory and plasticity. An example of this phenomenon was demonstrated in mycobacteria by Aldridge et al., who used microfluidic devices and microscopy to show that mycobacteria grow fastest from one pole [65]. Division therefore produces two daughter cells with different growth rates. Notably, these differences resulted in differential antibiotic susceptibility. Like bistability, this represents a built-in mechanism to generate pre-existing phenotypic heterogeneity that likely provides a survival advantage when the pathogen encounters stresses.

Conclusions

M. tuberculosis is one of humanity's first pathogens and remains a remarkably successful one. It originated in Africa as the human species emerged and left Africa with us in waves of exploration and expansion to spread as we did throughout the world [66]. From this perspective, it is not surprising that Mtb has evolved to survive and co-opt the various pressures imposed by the human immune response in its niche, the granuloma. Mtb stimulates the granulomatous response and ultimately requires the granuloma for efficient spread and thus continuation of the species, but can also be killed by the forces at play within it. Studies using knockout bacteria have helped uncover some of the pathways involved in mycobacterial survival and adaptation to the granuloma. However, these are complex interactions and single genetic perturbations only go so far. Work focusing on genetic and epigenetic differences in Mtb and relating these bacterial differences to the diverse pathologies observed in the host will more accurately resolve these complex webs of host-pathogen interactions.

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