CONTENTS

REVIEW: Understanding the pathophysiology of the human TB lung granuloma using in vitro granuloma techniques
*Future Microbiol.* Vol. 11 Issue 8

COMMENTARY: Mechanisms of mycobacterial transmission: how does *Mycobacterium tuberculosis* enter and escape from the human host
*Future Microbiol.* Vol. 11 Issue 12

EDITORIAL: New tests for detection of *Mycobacterium tuberculosis* infection: sufficient to meet the WHO 2035 targets?
*Future Microbiol.* Vol. 11 Issue 9
Understanding the pathophysiology of the human TB lung granuloma using *in vitro* granuloma models

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Tuberculosis (TB) remains a major human health threat, with one in three individuals in the world infected and approximately 1.5 million deaths worldwide each year [1]. The *Mycobacterium tuberculosis* complex comprises several human and animal associated species and subspecies, with human TB primarily caused by *M. tuberculosis* (*Mtb*) and *M. africanum* [2,3]. The shift between latent and active infection requires an understanding of host and bacterial dynamics within the milieu of a granuloma [4], which is influenced by bacterial and host factors. Thus, it is no surprise that the disease is more prominent in populations of immunosuppressed individuals (e.g., AIDS patients) [5]. The need for immunotherapeutic vaccines and new antimycobacterial agents [6–8], highlights the importance of studying *Mtb* in the context of a rational infection model. *Mtb* uses various methods in its attempts to evade the host immune system, including the prevention of phagosome-lysosome fusion/maturation, avoidance of lysosomal acidic proteases, avoidance of exposure to bactericidal mechanisms within lysosomes, as well as prevention of both mycobacterial degradation and presentation of mycobacterial antigens to the immune system.

The question arises, why study in vitro models of *Mtb*: These models may provide an improved understanding of host–pathogen interactions and immune responses during mycobacterial infection. These models might improve our understanding of correlation of immunity with infection, as well as the activity of new antimycobacterial agents against *Mtb* within a granuloma-like structure.
This review describes advances made in in vitro granuloma models, which have improved our understanding of how TB granulomas develop in the lungs of humans, as well as caveats to these models and their applicability to human disease. Current theories surrounding infection, granuloma development and the progression of lesions are examined, along with a rationale for the need of in vitro granuloma models. Additionally, this review highlights the importance of in vitro granuloma models in understanding the host–mycobacteria interactions at stages of granuloma formation that may not be reasonably achieved with animal models.

Initiation of infection: the basics

The steps during the initiation of infection are well described elsewhere [9] and will not be covered in this review in detail. Briefly, Mtb is spread in aerosolized droplets from the sputum of an infected person. Alveolar macrophages (AMs) are thought to be the primary target for Mtb once it enters the lung. Figure 1A, illustrates how inhaled droplets enter the lung alveoli, where the bacteria are phagocytosed by AMs [9–11]. The interaction between Mtb and AMs decides the subsequent progression of infection (Figure 1B). If the host fails to eradicate the bacteria, the infection is preserved in a latent condition and the infection does not transform into active disease, but is instead contained in the form of granuloma (Figure 1C). Alternatively, if the host fails to eradicate the bacteria, the infection is transformed into active TB disease (Figure 1D). A granuloma is an active lesion that has the capacity to influence T cell migration both locally and distally. As the lung is the primary site of infection, this review focuses on how in vitro models can be used to understand the development of pulmonary granulomas.

The infectious process described is specific for the lung. After inhalation of Mtb, the bacterium is deposited in the alveoli, but can infect human lung epithelial cells and activate mucosal associated invariant T cells [12]. Although lung epithelium can become infected, macrophages in the alveoli are the primary target for Mtb once it enters the lung. The interaction between Mtb and the alveolar macrophages determine the progression of infection. Lung hydrolases present in human surfactant alter the envelope of the invading Mtb, thereby reducing the ability of the Mtb interaction to grow within alveolar macrophages [12]. Mtb pathogenicity in the lung is linked to the capacity of the bacterium to express lipids on its cell surface. Specifically, the Mtb molecule, phthiocerol dimycocerosate (PDIM), limits innate recognition of Mtb, thereby reducing recruitment of Toll-like receptor (TLR) activated macrophages [13]. In addition, other lipids on the Mtb surface, such as the phenolic glycolipids, determine the recruitment of macrophages via chemokine CCL2 and the chemokine receptor CCR2 pathway [13].

The classic understanding of pulmonary granuloma formation

After initial infection, dendritic cells and monocyte-derived macrophages phagocytose the bacillus [14–16], Mtb can survive inside macrophages by inhibiting the fusion of phagosomes with the lysosomes thereby preventing the formation of a phagolysosome, the mature form of a phagosome [17]. During the initiation of the granuloma formation, there is an early recruitment and clustering event involving inflammatory macrophages. New macrophages and other immune cells are then recruited to the site of infection, and develop into granulomas, the characteristic lesions of tuberculosis [9–11,18]. After the initiation of the acquired immune responses, T cells migrate from the circulation into the parenchyma of the lung and then into the infected site, composed largely of macrophages and dendritic cells [19–21]. Mature granulomas form as multicellular structures composed of infected and uninfected macrophages, epithelioid cells, giant cells (multinucleated cells derived from fused macrophages), T cells and B cells [22–24] that can contain the bacilli and prevents spread of the infection. Inward migrating dendritic cells (DCs) express IL-12p40 [25], and the receptor IL-12Rβ1 [26]. Major histocompatibility complex (MHC) class II expressing DCs are required for T-cell activation but outward migrating DCs also end up transporting bacteria to local draining lymph nodes [27]. Inhibition of growth or death of Mtb is in part due to enhanced macrophage activation and the creation of an oxygen and nutrient deprived environment [28,29].

As shown in Figure 2, a spectrum of granulomas is observed in humans [30]. Figure 2A describes solid granulomas, where mycobacteria are most likely dormant [31]. Figure 2B describes necrotic granulomas, which are present in early stages of active TB. Figure 2C describes caseous granulomas found in end-stage or severe TB. The solid noncaseating granuloma consists of CD4+
Figure 1. Pathogenesis of tuberculosis. *Mtb* enters the host when inhaled droplets are transmitted to the lungs (A). The bacteria is phagocytized by alveolar macrophages and eliminated by different mechanisms, which include apoptosis and autophagy (B). When *Mtb* growth is contained inside granulomas, it is preserved in a latent condition seen in 90–95% of infected individuals and infection does not transform into disease (C). *Mtb* is replicated in the macrophages and transformed into active tuberculosis with symptoms of tuberculosis and can also be disseminated to other tissues and organs as seen in 5–10% of cases (D).


Figure 2B. A layer of fibroblasts surrounds this specific granuloma, with fibrosis occurring as the granuloma controls the infection and the inflammatory process is limited. In a latently infected individual, one or more granulomas controlling the infection can sometimes be observed; these granulomas can also be calcified [32,33]. As shown in Figure 2B, necrosis first occurs in the center of the structure and may further develop into a caseous necrosis of human lung tissue [34]. Figure 2C illustrates caseous granulomas can progress to form cavities within the lung, leading to erosion of the granuloma into a bronchus, and the subsequent release of bacteria into the airways [29]. They have a central necrotic area that contains extracellular bacteria surrounded by macrophages and phagocytes, and contains lymphoid-like structures that are rich in T and B cells, as well as macrophages that contain tubercle bacilli.

Multiple components are involved in controlling the development of a granuloma including immune cells, cytokines and chemokines (Figure 3). CD4+ Th1 cells [33,35–36], γ/δ T cells, CD4+ CD25+ FoxP3+ regulatory T cells [36,37], CD8+ T cells [35,38–39], as well as NKT cells [40] are involved in the control of TB infections.

and CD8+ T cells, B cells and macrophages harboring few tubercle bacilli (Figure 2A). A layer of fibroblasts surrounds this specific granuloma, with fibrosis occurring as the granuloma controls the infection and the inflammatory process is limited. In a latently infected individual, one or more granulomas controlling the infection can sometimes be observed; these granulomas can also be calcified [32,33]. As shown in Figure 2B, necrosis first occurs in the center of the structure and may further develop into a caseous necrosis of human lung tissue [34]. Figure 2C illustrates caseous granulomas can progress to form cavities within the lung, leading to erosion of the granuloma into a bronchus, and the subsequent release of bacteria into the airways [29]. They have a central necrotic area that contains extracellular bacteria surrounded by macrophages and phagocytes, and contains lymphoid-like structures that are rich in T and B cells, as well as macrophages that contain tubercle bacilli.

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The tuberculous granuloma is a compact, organized aggregate of epithelioid macrophages that tightly interlink cell membranes of epithelioid cells and adjacent cells. Depending on the immune response at the time of infection, the granulomas can either be solid, caseating or necrotic. In a solid granuloma, the infected macrophages are in the middle surrounded by other immune cells, such as CD4+ and CD8+ T cells, as well as macrophages that fuse to form multinucleated giant cells or have differentiated into foamy cells. A solid noncaseating granuloma (A) is seen during latent infection where the bacteria can be dormant and may have survived for decades. In a necrotic granuloma (B), the bacteria have multiplied and promoted the death of macrophages. In a caseous granuloma (C), the center of the granuloma is liquefied, which ultimately results in the dissemination of the bacteria and their spread to other parts of the body. The bacteria are also transmitted to other individuals due to their release via droplets.

$Mtb$: Mycobacterium tuberculosis.

Figure 2. Changing stability of the tuberculosis granuloma. The tuberculous granuloma is a compact, organized aggregate of epithelioid macrophages that tightly interlink cell membranes of epithelioid cells and adjacent cells. Depending on the immune response at the time of infection, the granulomas can either be solid, caseating or necrotic. In a solid granuloma, the infected macrophages are in the middle surrounded by other immune cells, such as CD4+ and CD8+ T cells, as well as macrophages that fuse to form multinucleated giant cells or have differentiated into foamy cells. A solid noncaseating granuloma (A) is seen during latent infection where the bacteria can be dormant and may have survived for decades. In a necrotic granuloma (B), the bacteria have multiplied and promoted the death of macrophages. In a caseous granuloma (C), the center of the granuloma is liquefied, which ultimately results in the dissemination of the bacteria and their spread to other parts of the body. The bacteria are also transmitted to other individuals due to their release via droplets.

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The $\gamma/\delta$ T cells that secrete IL-17 and NK T cells that express both TCR and NK cell markers act as intermediaries between the innate and adaptive immune responses [40,41].

Proinflammatory cytokines, such as TNF, IL-1β [42–45] and IL-17 [46], and anti-inflammatory cytokines, such as IL-4, IL-10 and TGF-β [45,47–48] maintain the granuloma. An appropriate equilibrium between pro- and anti-inflammatory cytokines has been shown to be important in sterilizing the granulomas [45] (Figure 3). Individuals that have mutations in IL-12p40 or IL-12Rβ1 are deficient in both IL-12 and IL-23 signaling, and have reduced IFN-γ T-cell responses [49–51]. In addition, signaling impairment, subsequent to IFN-γ deficiency and clinical phenotype was reported to vary between individuals [52]. These individuals are difficult to treat with currently available anti-TB drugs and fail to develop granulomas in tissues infected with $Mtb$, demonstrating the requirement for IFN-γ signaling in granuloma formation [49].

Chemokines are produced by monocytes, macrophages and dendritic cells, and influence cellular migration, recruitment and activation of monocytes, macrophages and leukocytes. Alveolar epithelial cells and human bronchial epithelial cells produce chemokines CCL-2 and CXCL-8 in response to $Mtb$ infection [53,54].
Many questions remain as to how host immune cells are recruited into sites of Mtb infection in the lung. Although it is well known that T-cell migration to the parenchyma is important for the control of Mtb growth, it is still not fully understood how T cells migrate from the vasculature into the parenchyma and whether they remain within the parenchyma. Expression of homeostatic chemokines CXCL13 and its receptor CXCR5 on T cells can act to recruit T cells to the infected macrophage areas [55–57].

The role of neutrophils within the Mtb granuloma is also currently not fully understood. In individuals with active TB infection, neutrophils can be seen in airway samples [58] and in peripheral blood analysis [59]. Although neutrophils might have a protective role immediately after exposure, it is evident that neutrophils are associated with the progression to active disease. One way in which neutrophils could interfere

Figure 3. Immune responses to tuberculosis infection. Following infection with Mycobacterium tuberculosis, the bacteria move to the lower respiratory tract where they are recognized by alveolar macrophages and trigger innate immune signaling pathways that lead to the production of various chemokines and cytokines, which promotes recruitment of other immune cells to the site of infection. Control of Mycobacterium tuberculosis is mainly dependent on the balance of pro- and anti-inflammatory cytokines. The CD4⁺ T-helper (Th) cells polarize into different subsets. Th1 cells produce IL-2 for T-cell activation, IFN-γ, or TNF for macrophage activation. Th17 cells, contribute to the early formation of protective immunity in the lung. Th2 cells and T regulatory cells (Treg) suppress Th1-mediated protection via IL4 and TGFβ or IL10, respectively. CD8⁺ T cells produce IFN-γ and TNF, which activate macrophages. The effector T cells (T_{eff}) and memory T cells (T_{mem}) cells produce cytokines IL-12, IFN-γ and TNF.
with the protective response to Mtb is to limit the interaction between infected phagocytes and antigen-specific T cells [58,59].

Lesion fate trajectories are controlled by host & bacterial factors
The fate of an individual granuloma is most likely controlled by a variety of bacterial and host factors. Apart from the mediators described above and in Figure 3, these driving forces may also be impacted by the factors described in the following sections.

- Is the RD1 region of Mtb required for the creation of a granuloma?
Recent literature suggests that the genomic and proteomic nature of an Mtb strain could have a direct impact on its ability to create a granuloma or other pathophysiological events. A variety of genes have been proposed to play a role in the persistence of Mtb in hosts [68]. Complete genome sequencing has now been carried out on a variety of Mtb complex strains including H37Rv, CDC1551, H37Ra and M. bovis BCG. It is clear that there are differences in both the presence and the expression of specific gene products amongst members of the Mtb complex [69,70]. DNA sequence analysis identified the region of difference-1 (RD1) as an approximately 9.5-kb region of Mtb [70] responsible for virulence that is absent in M. bovis BCG [71]. The RD1 region contains genes that encode two secretory proteins, EsxA (coding for ESAT-6) and EsxB (coding for CFP-10), as well as the rv3877 gene, a putative translocation pore in the cytoplasmic membrane; all are located within the ESX-1 locus that encodes a secretory system [71,72]. RD1 is associated with pathogenesis of TB and contributes to secretion of specific proinflammatory cytokines (e.g., IL-1) [24,72]. RD1 may also be driving nonspecific cell damage (e.g., mitochondrial damage), as RAW264 cell infection models showed that strains of H37Rv lacking RD1 have less measurable mitochondrial damage and did not have depleted ATP levels when compared with cells infected with wild-type H37Rv [24]. The soluble RD1 component ESAT-6 may also play a role in tissue remodeling and cell recruitment to support granuloma formation. ESAT-6 has been shown to induce MMP-9 production in epithelial cells and the recruitment of macrophages to the site of infection [73]. MMP-9 might drive tissue remodeling that would allow for intact solid granuloma production [74–76]. However, sequence analysis alone is not enough to determine the differences between strains as seen with Mtb. H37Ra, which contains the RD1 region but has noticeable downregulation

The fate of granulomas are independent of each other in the same host: different developmental trajectories of lesions within an individual host tissue
There is growing evidence that within the lung, each granuloma may be on its own course of fate, and that the lung should not be considered an environment where all granulomas are synchronized to a common state. The use of [18F]-fluorodeoxyglucose (FDG) positron emission tomography (PET)-CT scanning in human lungs during linezolid treatment studies indicated diversity in the local inflammatory response leading to variability in the size of lesions and FDG avidity [60]. This has been cited as evidence that lesions within the lung are controlled not only by a systemic host immune response, but also by local factors that would determine the fate of individual lesions [61]. Furthermore, in nonhuman primate models, molecular tracking has been used to indicate the variability in the fate of multiple lesions within a host, possibly due to differences in host-mediated killing at the individual lesion level [62]. Finally, a recent mathematical model proposes that there may actually be no steady state for granulomas and that instead, there is a continuous progression of disease with each granuloma progressing at a different rate over time.

One hypothesis is that independent lesion fates would require locally acting agent(s) or factor(s) to modulate the pathophysiology of the granuloma environment in response to the pathogen. It is still not known what these local agents might be. It is possible that locally acting agents are the product of lipid metabolism at the site of an infection or other pathophysiologic process as described elsewhere in other organ systems [63]. These could include pro- and anti-inflammatory eicosanoids (lipid mediators derived from arachidonic acid, prostaglandins, lipoxins and resolvins), those that modulate lesion resolution [63–65], or drive the lesion towards disease exacerbation and the development of necrotic foci [66]. It might be possible that these agents provide fine tuning to more systemic changes driven by cytokines and could possibly change the direction of lesion development within the host lung [61,67].
Host genetic polymorphisms may impact the balance of granuloma stability-instability & the progression to active disease

Previous work has suggested that some host genetic polymorphisms, such as the polymorphism in the promoter (−403G/A and −28C/G) and intron (In1.1T/C) regions of the CcID gene, may play a role in host resistance to Mtb infection [78,79]. However, once infection has been established, it is also possible that there are several human polymorphisms that may play a role in increasing patient risk for a destabilized granuloma and active disease. A functional promoter polymorphism in the −2518A>G of the MCP-1 gene Ccl2 (chromosome region 17q11.2) has been associated with increased susceptibility to Mtb infection in some non-BCG vaccinated populations [80]. The proposed model is that the polymorphism is associated with increased levels of MCP-1, which in turn is associated with decreased levels of IL-12p40 and greater likelihood of progression to active disease [80]. Another functional polymorphism is found in the promoter region of Mmp1 (chromosome region 11q22.2), an insertion of a guanine at position −1607 (−1607_1608insG), creates an Ets-1 transcription factor binding site [81] and enhances gene expression [82]. It is thought that polymorphisms in CcID2 and Mmp1 may jointly act to increase the chance of active disease in the hosts [82].

The rationale for supporting studies using in vitro granuloma models

There are varieties of approaches for understanding the pathophysiology of Mtb, as well as bacterial responses to antimycobacterial agents. The rationale for using in vitro models to study Mtb infections are presented below.

In vitro studies outside of the granuloma milieu only give a partial picture of bacterial-host interactions

So why not avoid an in vitro granuloma model and just study the impact of specific environmental or host components on Mtb? There is already a significant history of research using different aspects of the granuloma environment (e.g., starvation, changes ion oxygen concentration) to study Mtb alone in culture without host cells [83–86]. These perturbed environmental conditions can then be used to understand mycobacterial growth and the efficacy of antimycobacterial agents/growth inhibitors [85,87–88]. Although host cells are not present in these models, this work should not be discounted. For example, starvation studies of Mtb in the absence of host cells have identified possible roles for toxin-antitoxin systems in the response to nutrient starvation conditions [89]. Other models of phosphate depletion and nutrient starvation have been used to understand environmental impacts on mycobacterial two-component cell signaling systems [90]. These models may be ideal for the initial analysis of varying culture conditions on Mtb growth and survival responses but they are limited because that they do not represent the dynamic environment where cells are dying, changing or being recruited. The perturbation of mycobacteria in a liquid or pellicle environment does not represent the true physiological environment that exists within the lung granuloma.

The granuloma provides a distinctly different environment for drug-induced bacterial killing than within normal lung or blood

Growing evidence suggests that bacteria within the granuloma are not subject to the same host and environmental pressures as bacteria within the normal lung or within blood. It has been proposed that the concentration of antimycobacterial drugs in the blood represents the drug concentration in the lesions of the lung. However, initial work in rabbits indicates that drug penetration into lesions varies among individuals, and among drugs, and differs from the penetration into the lung in general as estimated by penetration coefficients. Work by Kjellson et al. has indicated that there may be no numerical differences for drug penetration across lesion type for rifampin (RIF) and isoniazid (INH), while for pyrazinamide (PZA) and moxifloxacin (MXF) there were modest numerical differences in the penetration for the suppurative and coalescing lesions compared with caseous and solid types of lesions [91]. Within lesions, other work has reported a lower concentration of MXF in the caseous versus the cellular fraction of the granuloma [92,93]. The lesions themselves are completely different environments than the normal lung. Recent modeling suggests that; antibiotics are frequently below effective concentrations
inside granulomas leading to bacterial growth between doses and prolonged treatment times, and antibiotic concentration gradients form within granulomas, with lower concentrations toward their centers [92].

- **In vivo experimental models to study the TB granuloma**
  There are several nonhuman models that can be used to study the pathophysiology of the TB granuloma, (1) the humanized murine model, (2) the guinea pig model, (3) the rabbit model; and (4) the primate model. The mouse model has been very useful in obtaining the information on the cytokine and immune cell responses for granuloma formation. Benefits of the mouse model include the inexpensive nature of the assay system, the ease in handling mice, the different variant strains available and reagents available for the perturbation of the system [94,95]. However, significant differences exist between the granulomatous response to *Mtb* infection in the lung between mice and humans [96]. Necrosis is lacking or difficult to achieve in murine models (Figure 4B) and may not represent the necrosis seen in human granulomas (Figure 4A) [97–101]. Finally, although latency can be studied, there is no standardized model of latency in the murine model [102]. In guinea pig and rabbit models, the benefits include ease of handling and the ability to produce necrosis within the granuloma [103,104]. There are now latent models of infection that have been created in the guinea pig host but these have not been widely tested [105]. The rabbit model is the only animal model that can represent the progressive disease as seen in human infection [106,107]. However, drawbacks to both the guinea pig and rabbit models include the limited variety of reagents to study pathophysiology when compared with human or murine models. Finally, primate models of infection allow for granuloma production that is similar to humans and latency can be established [45,108], with necrosis also produced in granulomas [109]. Drawbacks to using primates for this type of work include the expense, difficulty in handling and ethical concerns in primate research.

- **Mathematical & computational models of TB infection are not sufficient**
  Mathematical and computational models have been used to predict the granulomatous response in TB infection based on experimental observations and the available information about the disease [110–117]. Mathematical models are inexpensive and allow investigators to test a variety of new hypotheses and incorporate a number of complex parameters without the cost and time issues encountered in wet laboratory experiments. These models have been useful to address questions in TB that are difficult to approach experimentally. For example, differential equation (DE) based models describe a relationship between numbers of cells and concentrations of molecules and their rates of change in the granuloma and time [113–114,117]. In contrast to DE based models, individual based models, or agent-based models (ABMs), are rule-based models that capture the events occurring in the immune systems (e.g., immune cells, bacteria, environmental factors) using a 2D grid representing a section of lung tissue [110,115–116]. Although these models have been useful in answering complex questions, they are highly dependent on the parameters chosen, and require previous observations in different systems to extrapolate the results. As a result, they can miss unknown factors.

*In vitro granuloma models*

- **The experimental human lung tissue model**
  Recently, an early granuloma model was established using experimental human lung tissue [118], which used a previously described 3D tissue model of the human lung mucosa [119]. Macrophages were infected with *Mtb* strains prior to infection of the model and then cointroduced with PKH26 red fluorescent dye-labeled monocytes into the lung model. Using confocal microscopy, only virulent strain infections (e.g., H37Rv) and not avirulent strains (H37Ra, BCG, RD1 and ΔESAT-6) were associated with monocyte/macrophage clustering at the infection sites [118]. As an indicator of necrosis, another group stained for HMGB-1 protein, a marker released from cells undergoing necrosis [120]. They observed a significantly higher level of HMGB-1 staining in tissues infected with H37Ra than in uninfected areas or in tissues infected with avirulent or ΔRD1 or ΔESAT-6 strains [118]. However, it should be noted that this is a very preliminary model which uses indirect indicators (e.g., macrophage, monocytes clustering and HMGB-1 protein production) and only involves macrophages, monocytes, fibroblasts and lung-specific epithelial cells [118].
Figure 4. Differences in granuloma structure between mouse and human granulomas. (A) In human granulomas, the infected macrophages are in the middle surrounded by other immune cells such as CD4⁺ and CD8⁺ T cells, as well as macrophages that fuse to form multinucleated giant cells or differentiated into foamy cells. This type of granuloma is seen during latent infection where the bacteria can be dormant and survive for decades. (B) Granuloma in mice are comprised of loose nonnecrotic aggregates, surrounded by lymphocytes and macrophages that have fused and differentiated into foamy cells.

Mtb: Mycobacterium tuberculosis.

The key message in this study is that mycobacterial factors may be important in initiating early phases of granuloma production, as well as the development of necrosis.

**Peripheral blood mononuclear cell models**

Peripheral blood mononuclear cell (PBMC) granuloma models allow for the systematic determination of bacterial and host factors that drive granuloma formation and pathophysiology [121]. Over the last 10 years, a variety of approaches have been used.

In early models, proof of principle studies used *Mtb* antigens attached to agarose beads instead of viable mycobacteria [122]. These antigen-bead complexes, when incubated with PBMCs, induced the production of granuloma-like structure. The composition of immune cells within these structures was similar to that found in natural *Mtb* granulomas. Later studies used cyanogen bromide (CNBr)-activated Sepharose beads coated with purified protein derivative (PPD) [123]. In a study by Puissegur *et al.*, blood samples were collected from healthy BCG-vaccinated, PPD-reactive nontuberculous control individuals [124]. Monocyte-like cells were recruited to the bead surface on day one. By day four, lymphocyte-like cells were recruited and were seen to bind to the attached monocyte-like cells. By day 5, bead surfaces were completely
covered by recruited cells and multilayers of monocytes and lymphocytes were formed with pseudopodia (indicating cell differentiation) identified [124]. Staining at day 9 identified CD68-positive staining cells that might represent multinucleated giant cells (MGC). These MGC-like cells were also surrounded by CD3-stained lymphocytes. Potential macrophages stained strongly positive for CD168, and possible epithelioid cells were weakly stained for CD168.

Other PBMC models have avoided antigen-coated beads and utilized viable BCG to create granuloma-like structures. This approach also showed that PBMCs from healthy donors were able to form granuloma like structures around BCG within 9 days [125]. These granulomas included activated lymphocytes in tight contact with macrophages, as well as multinucleated giant cells. BCG-containing phagosomes were also present in what appeared to be epithelioid cells.

However, it is not clear how BCG generates a granuloma in the absence of RD1 and how these models would vary from other \textit{Mtb} models. BCG granulomas are possible as BCG pulmonary granulomas have been described in highly immunocompromised patients [125]. The growing number of models using human donor PBMCs suggest that the immune status, previous PPD or BCG exposure, previous \textit{Mtb} infection status and health (including acute infections) of the PBMC donor must be accounted for when \textit{in vitro} granuloma models are studied [124].

Concerns remain about these \textit{in vitro} PBMC granuloma models. Are these models in fact just cell aggregates with similar cellular characteristics to human granulomas? Perhaps they have some key characteristics that may improve our knowledge about \textit{Mtb} infections. They allow us to study \textit{Mtb} infections of cells in a mixed multicellular environment. They may also account for PBMC donor factors that might have a downstream impact on how \textit{Mtb} infections occur, and how granulomas are formed. These models can be quite dynamic and can be used to study the proliferation of specific cell types during a mock infection.

\section*{Dormancy models of TB infection}

As described earlier, host cell free \textit{Mtb} culture experiments have often been used to mimic the conditions encountered by the bacteria within host granulomas [126–128]. These have then been applied to simple infection models with some success. For example, using a hypoxic induced environment model, it was shown that \textit{Mtb} accumulates triacylglycerides and goes into a dormant state but can regrow after reexposure to oxygen [127]. When this information was applied to a lipid loading THP-1 infection model, the \textit{Mtb} was found to be dormant [127,128]. We believe that this work has been useful in improving our understanding of the metabolic adaptation of \textit{Mtb} during processes resembling dormancy. However, these models have been unable to demonstrate resuscitation under conditions that mimic immune suppression, which is a key element in \textit{Mtb} pathophysiology leading to active TB. To achieve this goal, 3D \textit{in vitro} models of granulomas have been studied.

\subsection*{3D \textit{in vitro} models}

Seitzer and Gerdes were the first to report the 3D granuloma model using PBMCs infected with \textit{Mtb} [129]. In their studies, PBMCs were infected with \textit{Mtb} strain H37Rv at different MOI (number of bacteria/cell) and seeded into agarose-coated wells. After 4 days of incubation, the lowest MOI in their study (1:150) produced host cell aggregates. However, infection at a higher MOI did not result in a large aggregate formation but rather numerous very small aggregates. An increase in the number of dead cells was also observed. By histological analysis the aggregates were confirmed to have similar phenotypical characteristics (e.g., cell population type) to natural \textit{Mtb} granulomas. Another study combined human PBMCs, autologous macrophages and \textit{Mtb} in ultralow attachment tissue culture plates and the nonadherent PBMCs were added on day 2 and 5 after infection to mimic the natural infection process in which additional lymphocytes are recruited to the infection site [130]. The formation of granuloma was observed and acid-fast bacilli were observed within the host cells composing the granuloma. This study also showed that addition of IL2, IFN-\(\gamma\) and/or TNF-\(\alpha\), enhanced the formation of the granuloma and host cell recruitment. Although these models have provided researchers with important information about granuloma cell differentiation, these models were unable to establish dormancy and resuscitation inside the 3D-generated granulomas.

Kapoor \textit{et al.} developed a 3D \textit{in vitro} granuloma model in which \textit{Mtb} progresses to dormancy and subsequently resuscitates under conditions that mimic weakening of the immune
In vitro models are potentially amenable to microdissection approaches

New developments in microdissection have raised the possibility that individual granulomas within an infection model could be interrogated. The previous PBMC granuloma models described above have all been approached as a single system with no attempt to dissect individual granulomas. Given the previous discussion that each granuloma may be on its own developmental trajectory, it is possible that dissection approaches could provide more information on how individual granulomas develop within a larger system. Microdissection studies have previously been used to study the expression of host cell genes in lung tissue from Mtb infected mice. Tissue from microdissection has also been used for qPCR, and immunohistochemistry from M. bovis induced granulomas in cattle.

Discussion

This review has described a variety of in vitro experimental models available that can help in understanding the complex host–pathogen relationship that takes place within the TB granuloma. An understanding of the pathophysiology of granulomas is critical for the design of new TB drugs and vaccines. A major caveat in the in vitro granuloma model is whether in vitro models of Mtb complex granulomas are truly representative of human pulmonary granulomas or just the cell aggregates. We propose that these PBMC models are representative of human pulmonary granulomas in many ways. Both natural and in vitro Mtb granulomas contain basic elements, such as macrophages, T cells and bacilli, and are multilayered.
with multicellular structures [123–124,131]. In vitro
granuloma models can help us understand the
early stages of cell recruitment and immune
responses over multiple environmental condi-
tions in a manner that is not feasible with animal
models or in clinical studies. Human infections
and in vitro models also share common immune
responses. In humans, patients show increased
mRNA and protein expression of IL-8, IL-12,
IFN-γ and TNF-α in granulomatous lymph
nodes [136], IL-8 and TNF-α in PBMCs [137],
and IL-6 and TNF-α in bronchealveolar lavage
fluid [138–140]. The production of these cytokines,
as well as time-dependent variability in cytokine
production has also been described within
in vitro granuloma models. Some in vitro granu-
loma models may also mimic the surrounding
lung tissue environment where the granuloma
is anchored by extracellular matrix proteins,
including osteopontin and fibronectin [140].

However, some questions about the exact
nature of in vitro granulomas still remain unan-
swered. For example, the exact role of Mtb factors
in granuloma formation is unclear. In addition,
questions still exist about the role of the Mtb RD1
region in the creation of granulomas in vitro and
in vivo [118]. We still do not completely under-
stand how host responses drive granuloma forma-
tion. Hopefully some of these questions can be
further elucidated by a variety of experimental
models, each with their own peculiar strengths
and weaknesses [118,130].

Conclusion & future perspective
This review identifies the reasons why in vitro
granuloma models have a practical purpose for
the study of TB pathophysiology, host immune
responses and mycobacterial responses to anti-
mycobacterial agents. In vitro granuloma mod-
els allow for the study of defined cellular and
soluble host factors, as well as specific natural
strains, acellular mycobacterial components
and other species. This ability to work with
simpler systems involved in granuloma forma-
tion, while still maintaining a sense of com-
plexity, allows for the creation of well-focused
scientific hypotheses in a model that is more
representative of the natural host. It is clear that
the health status of the donor can impact the
formation and recruitment of the cells to the
granuloma. In vitro granuloma models allow
the exploration of genetic and immunologic
variables contributing to TB immunopatho-
genesis, including the metabolic state of myco-
bacteria contained inside human granulomas.
In doing so, our understanding of the patho-
physiology of the human Mtb granulomatous
response will be greatly improved, thus ena-
bling new ideas for biomarkers, therapy and
development of effective vaccines against TB
infection.

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EXECUTIVE SUMMARY
- Tuberculosis remains a major human health threat, with one in three individuvals in the world infected and
  approximately 1.5 million deaths worldwide each year.
- Treatment and prevention options are limited and there is an urgent need for new immunotherapeutic vaccine
  and new antimycobacterial agents. These will require an improved understanding of infections within a granuloma
  environment, as well as host correlates of effective immune responses to infection.
- The outcome of Mycobacterium tuberculosis infection in the lung depends on the initial environment encountered by
  the bacterium and the genetics of the host.
- Multiple models have been created that allow us to understand the interaction of Mycobacterium tuberculosis and the
  host within the granuloma. Each has its strengths and weaknesses.
- In vitro granuloma models allow for the systematic determination of bacterial and host factors that drive granuloma
  formation and pathophysiology in ways that are not possible with culture-based methods or animal infections.
- Newer 3D in vitro granuloma models may allow for new studies as these models can be used to mimic events, such as
dormancy and resuscitation.
Understanding the pathophysiology of the human TB lung granuloma using in vitro granuloma models

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• This manuscript identifies the key role that an extracellular matrix plays in Mtb pathophysiology.

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Understanding the pathophysiology of the human TB lung granuloma using in vitro granuloma models


Mechanisms of mycobacterial transmission: how does *Mycobacterium tuberculosis* enter and escape from the human host

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In 1882, while lecturing on his discovery of the cause of tuberculosis, Robert Koch said “If the importance of a disease for mankind is measured by the number of fatalities it causes, then tuberculosis must be considered much more important than those most feared infectious diseases, plague, cholera and the like.” Although Koch’s identification of *Mycobacterium tuberculosis* (Mtb) as the cause of tuberculosis heralded many future discoveries and treatments, Mtb remains responsible for 1.5 million deaths annually. Therefore, more research is needed on all stages of Mtb infection. This commentary provides a framework for understanding two central questions in Mtb biology at the extremes of the infectious life cycle: how does Mtb enter the body and how does it escape?

**How is tuberculosis spread?**

The tubercle bacillus is spread person-to-person almost exclusively by aerosolized particles. The size of infectious droplets from Mtb in infected patients ranges from 0.65 (small) to >7.0 μm (medium–large) [1]. While small Mtb aerosol particles can be trapped in the upper airway or oropharynx where they can potentially lead to tuberculosis of the oropharynx or cervical lymph nodes [2]. Despite the apparent ease with which Mtb is spread – how else could nearly 2 billion humans be infected currently? – and the strong epidemiologic evidence linking airborne transmission to close personal contacts, directly demonstrating aerosol transmission of Mtb has been exceedingly difficult [3]. Indeed, Riley et al. needed to expose hundreds of Mtb-susceptible guinea pigs to air from a ward of Mtb-infected patients continuously for months to demonstrate airborne transmission of Mtb [4], indicating that aerosolization and transmission of Mtb is a rare event. This observation is corroborated by epidemiologic and modeling data indicating that the likelihood of transmission of Mtb is proportional to the duration of exposure to an infectious person and inversely proportional to the volume of space within which exposures occur [5].

**What causes active disease?**

The simplest model of human interaction with Mtb is represented by the binary outcome ensuing
from exposure of a naive individual to Mtb: development of either asymptomatic latent infection or active disease characterized by fever, weight loss and a bloody cough. Latently infected individuals can either remain asymptomatic throughout their lifetime or develop active disease at a remote time from the primary infection in a process known as reactivation. Epidemiologic studies have shown that only 5% of otherwise healthy individuals will develop active disease when first exposed to Mtb, thus leading to the fundamental question in Mtb biology of why so few acute infections prove symptomatic? One straightforward explanation relates to inoculum dose: multiple studies have demonstrated a direct correlation between the number of bacilli in an infected person’s sputum and the likelihood that contacts will develop symptomatic active tuberculosis. Likewise, animal studies show that a lower initial Mtb inoculum in wild-type animals results in a quiescent but persistent infection, whereas a higher inoculum leads to active disease, pneumonia and death. Since a coughing, actively infected person typically produces only a few aerosolized bacteria [3], his or her contacts likely will inhale a low number of bacteria, skewing the probability to development of latent infection rather than active disease. As well as the importance of inoculum size, environmental factors such as proximity and duration of the contact as well as micronutrient or relative vitamin D deficiency have been associated with susceptibility to tuberculosis. Furthermore, being immunocompromised at the time of infection due to either host genetics or acquired deficiencies can also increase a person’s likelihood of developing active disease from an initial infection. In addition, it has been recently proposed [2] that trapping of larger particles in the upper airway may induce a protective immune response. Finally, as described below, direct translocation of bacteria across the epithelium overlying mucosa-associated lymphatic tissue (MALT) might also dictate whether acute or latent infection occurs.

How does tuberculosis cross the airway mucosa?

The current paradigm of primary tuberculosis infection is that small airborne particles distribute to the terminal alveoli, where resident alveolar macrophages or tissue dendritic cells ingest the airway bacteria. Subsequently, infected macrophages or dendritic cells migrate to draining lymph nodes, activate adaptive immunity and then return to the initial site of infection where a granuloma forms [6]. However, prior to reaching the terminal alveoli, some small and large particles likely become trapped along the mucosa, where they can interact with noninnate immune cells between and above MALT. Oropharyngeal MALT, widespread in childhood but regressed in adults, includes nasal-associated lymphatic tissue, the tonsils and adenoids of Waldeyer’s ring, and bronchus-associated lymphatic tissue. Interestingly, MALT is prevalent during a vulnerable period of early childhood and adolescence when tuberculosis manifests in more severe and disseminated forms. Overlying MALT are both primary epithelial cells and specialized cells called microfold cells (M cells), the latter whose function in both the airway and GI tract is to ingest and transcytose foreign antigens [7,8]. It has previously been demonstrated that airway epithelial cells can mediate dissemination of Mtb from the airway [9], and a functional role for M cells in Mtb entry was recently established [10], extending observations made over 15 years ago [11]. M cell depletion prior to airway infection results in fewer Mtb recovered from draining lymph nodes during nasal or airway infection and protection from long-term Mtb-mediated mortality in mice [10]. Not tested in these experiments is the impact of Mtb transcytosis by M cells on adaptive immunity, nor how inoculum dose affects the immune response. Since M cells targeting has recently been proposed as a method for inducing vaccine responses in MALT as well as systemically [12], M cells are prominent in nasal-associated lymphatic tissue [13] and intranasal vaccination with BCG provides enhanced protection from Mtb infection [14], it is also possible that low level or repeated infection (i.e., with <10 bacteria) in the upper airway results in direct delivery of Mtb to APCs in MALT in a pathway that stimulates a more robust protective immune response than when Mtb are ingested by alveolar macrophages directly. Thus, both inoculum dose and particle size could influence the outcome of disease such that individuals whose M cells transcytose either paucibacillary or larger Mtb particles develop latent disease or even remain completely uninfected. However, if the initial dose of Mtb transcytosed by M cells in the upper airway mucosa overwhelms the primary local response, then drainage of Mtb to local lymph nodes could result in lymphatic disease, as is seen in scrofula. Likewise, if the particles are very small, they might bypass epithelial cells altogether and reach the terminal alveoli to be ingested by airway phagocytes.

“The success of any bacterial pathogen ultimately depends on its ability to multiply and infect new hosts.”
How does tuberculosis escape from the lung?
The success of any bacterial pathogen ultimately depends on its ability to multiply and infect new hosts. Many pathogenic bacteria interact with hosts by secreting virulence molecules like proteins and lipids. A classic example is the production of cholera toxin by *Vibrio cholerae*, which enhances the pathogen’s spread from person-to-person by promoting profuse watery diarrhea. *Mtb* also employs sophisticated means of dissemination, including mediating caseation, access to the bronchial tree and cough-mediated airborne transmission.

Role of tissue destruction & caseation
Recently, it was proposed that the transition of airway tuberculosis from the initial, asymptomatic infection to symptomatic, necrotic and cavitary disease occurs in three stages [15]. In this model, the first stage reflects early infection of alveolar macrophages by *Mtb* and activation of cell-mediated immunity, followed by immune control with granuloma formation. In some individuals, a second stage ensues, represented by accumulation of *Mtb* antigens and host lipids in the airway, leading to bronchial obstruction and obstructive lipid pneumonia. Finally, depending on the host response, the third stage is characterized by either cavitary or fibrocaseous disease with communication to the outside world. The mechanisms accounting for each stage, and in particular, the formation of obstructive, lipid pneumonia are not well known, though some studies have identified critical roles for host matrix metalloproteinases in tissue destruction and dissemination [16].

Cough in tuberculosis transmission
Although cough is a well-known hallmark feature of active pulmonary tuberculosis, very little is known about the pathogenesis of infectious cough [17]. Cough may be a natural consequence of lung inflammation and host production of prostaglandins, bradykinin and other inflammatory mediators that activate afferent neuronal C-fibers in the lung mucosa [18]. In addition, cavity or cyst formation itself might induce mechanical activation of either rapidly adapting receptors or slowly adapting stretch receptors that can sensitize the lungs to cough triggers [19-20]. Conversely, cough itself, perhaps triggered by secreted mycobacterial factors, could lead to *Mtb* aerosolization and/or cavity formation. In this model, a granuloma or region of caseous necrotic pneumonia could be induced to form a cavity by very high mechanical forces (intrathoracic pressures as high as 300 mm Hg and expiratory velocities as high as 800 km/h) generated by a strenuous cough [18], forcing weakened extracellular matrix and elastic tissue [16] to stretch into a cavity. Thus, while cough may be a major route of aerosolization and spread, it may precede and/or overlap with the cavitary disease stage.

Conclusion
Though significant progress has been made to understand the innate and adaptive responses to *Mtb* infection since Koch’s discovery of tuberculosis, both how *Mtb* penetrates the mucosa to initiate infection and how it reverses the process to escape remain poorly understood. Novel approaches are needed to further elucidate these critical events in the pathogenesis of tuberculosis.

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The new WHO End TB Strategy sets ambitious 2035 targets and emphasizes targeted treatment of the *Mycobacterium tuberculosis* infected at risk of developing tuberculosis (TB). New short-course treatment regimens have shown promise for better compliance; however, the available diagnostic tests are insufficient to guide treatment.

This editorial discusses current state of art and future developments in the field of latent TB diagnostics, with emphasis on Quantiferon-TB Gold® Plus (Qiagen), IP-10 release assays, specific skin tests and transcriptomic signatures that suggests a brighter future with more accurate predictive tools.

With 7.3 million annual cases and 1.3 million deaths, TB remains among the most significant infectious killers [1]. Each year, close to half a million new cases of multidrug-resistant cases emerge and HIV-associated TB affects more than a million people [1]. Before diagnosis and treatment, every TB patient will have passed on the bacteria to on average 11 contacts [2], hereby feeding the huge reservoir of *M. tuberculosis* (*Mtb*) infected at risk of developing active TB disease. The implementation of direct observed therapy and roll out of PCR-based diagnostics and drug susceptible testing have prevented millions of lives; however, it is clear that radical means are required to curb the epidemic [1].

A central pillar in the new WHO End TB Strategy is a recommendation of target treatment of *Mtb* infection among at-risk populations in upper-middle and high-income countries with an incidence <100 per 100,000 population [1,3]. This is an extension of the global recommendations for targeted treatment of children [4] and people living with HIV [5], and is among the core activities expected to facilitate the achievement of the ambitious targets of 90% reduction in TB incidence and 95% reduction in TB deaths by 2035 [1].

**KEYWORDS**
- correlate of risk • IGRA • IP-10
- prediction of progression • specific skin test • transcriptomics

“The new WHO End TB Strategy sets ambitious 2035 targets and emphasizes targeted treatment of the *Mycobacterium tuberculosis* infected at risk of developing tuberculosis.”

“...to meet these ambitious goals there is a need for shorter treatment regimens and better diagnostics to guide the treatment.”
The recommendations are supported by models suggesting a significant impact of targeted treatment [6]; however, to meet these ambitious goals there is a need for shorter treatment regimens and better diagnostics to guide the treatment. The introduction of the 3-month weekly isoniazid and rifapentine ensures better compliance and higher treatment success [7,8], however, the currently available toolbox of tests to guide preventive treatment is a bottleneck.

**Current diagnostic toolbox to guide targeted treatment**

The tuberculin skin test (TST) has been the standard method of determining whether a person is infected with *Mtb* [6] for over a century. The test is performed by intradermal injection of purified protein derivative tuberculin, a precipitate of species-non-specific antigens [7]. A major limitation to the use of TST, is false-positive reactions occurring in persons infected with nontuberculous mycobacteria and in persons vaccinated with Bacille Calmette–Guérin (BCG) resulting in lower test specificity and compromised potential to guide targeted treatment [9].

The IFN-γ release assays (IGRAs) is an *in vitro* diagnostic alternative to TST [8]. These tests are based on the *Mtb*-specific antigens ESAT-6, CFP10 and TB7,7, and provide an objective read-out and solve the problem of false-positive TST results. Two IGRA tests are commercially available, the whole blood-based Quantiferon Gold® In-Tube test (QFT, Qiagen, Germany) and the purified peripheral blood mononuclear cells-based test T-SPOT.TB® test (Oxford Immunotec, UK). Compared with the TST, IGRAs are more complex and labor intensive, requiring laboratory infrastructure and skilled staff [9]. Although IGRAs have solved the problem of false-positive reactions in BCG vaccinated, both IGRA and TST are unable to differentiate latent infection from active disease [9,10]. Thus, current tests have low-positive predictive value for the development of active TB and require treatment of >30 contacts to prevent one case of TB [11-13].

**Imminent future**

In 2015, Qiagen launched the fourth generation Quantiferon-TB Gold® Plus (QFT 4G), as a test with improved diagnostic sensitivity for infection in immunosuppressed [14]. QFT 4G builds on the QFT 3G in-tube design and comprises two blood collection tubes, TB1 and TB2, instead of the single TB-Ag tube known from QFT 3G. TB1 contains a cocktail of peptides from ESAT-6 and CFP10 identical to the predecessor [15] but without the TB7,7 antigen [9,14]. TB2 comprise the same ESAT-6 and CFP10 peptides as TB1, and – in addition – an unknown number of shorter peptides intended to activate the CD8-positive T cells. The rationale for the TB2 tube is to boost diagnostic sensitivity for infection, for example, in people living with HIV (PLHIV) with low CD4 T cells. QFT 4G is interpreted positive if either TB1 or TB2 induces an antigen-specific IFN-γ release ≥0.35 IU/ml, a modification of the algorithm that in itself will increase the positivity rate by doubling the chance for samples with low IFN-γ levels (and very high analytical variability [16]) of becoming positive.

Registration studies and the first independent evaluations found that the QFT 4G provides a marginal and nonsignificant increase in the detection rates in TB patients and contacts [14,17-18]. The million-dollar question yet to be answered experimentally is whether the extra detected individuals are found among those that progress to TB – hereby truly improving the test; or whether it picks up more in the group who never progress to TB and, therefore, will be a detriment to the predictive value. Nevertheless, the fourth tube adds cost and complexity to an already laborious test, wherefore it is difficult to appreciate QFT 4G as the panacea enabling the End TB Strategy targets.

**Further ahead**

Many activities are underway aiming to improve the IGRA. Several groups have explored the use of alternative readout markers expressed in higher levels compared with IFN-γ. It is now established that cell-mediated immune responses can be detected with other cytokines and chemokines, among which IP-10 is the leading alternative [19]. IP-10 is a chemokine expressed in 100-fold higher levels than IFN-γ, allowing for simpler detection technology, such as the lateral flow quick test format or even extraction from dried blood spots [19,20]. IP-10-based IGRAs are highly concordant with IFN-γ-based IGRA, but the high magnitude of response allows for improved sensitivity in children and PLHIV with confirmed *Mtb* infection [19]. Of particular relevance for the implementation of the End TB strategy, the lateral flow-based IP-10 IGRA would enable implementation of a rapid
and simple IGRA like diagnostic with minimal equipment and training.

Another approach to improving the predictive potential of the IGRA is alternative antigens associated with controlled infection. One example is heparin-binding hemagglutinin, a protein expressed on the surface of several mycobacterial species. Heparin-binding hemagglutinin-specific cellular immune responses are associated with 

\( \text{Mt}_b \) containment suggesting a not yet fully explored predictive potential when combined with specific antigens, such as ESAT-6 and CFP10 [21], in a two-tube test vis-a-vis QFT 4G.

**The era of specific skin tests?**

Specific skin tests are another promising and field friendly alternative to IGRA. C-Tb developed by Statens Serum Institut (Copenhagen, Denmark) is a skin test based on a recombinant double ESAT-6 protein and single CFP10 monomer expressed in *Lactococcus lactis*. Phase II data suggest that C-Tb drives induration sizes on level with TST and delivers positivity rates in TB cases and unexposed controls on par with QFT [22,23]. As C-Tb is unaffected by previous BCG vaccination, the responses seen in infected are more clearly separated from uninfected, and the cutoff can be lower and universal (at ≥5 mm) irrespective of HIV infection or age [18,19]. Phase III trial data recently shown at conferences suggest that C-Tb is safe and delivers positivity rates highly concordant with QFT 3G in recent contacts, but also that C-Tb appears more robust than QFT in PLHIV with active TB and low number of circulating CD4 T cells [24] (Ruhwald, Agerbaek, Pers. Comm.). Diaskintest developed by Pharmstandard (Moscow, Russia) using recombinant ESAT-6 and CFP10 dimer expressed in *Escherichia coli* is another specific skin test. Despite having been on the market in several former soviet republics since 2005, few results of this test has emerged in the international scientific literature. Diaskintest seems to deliver IGRA like diagnostic performance in BCG vaccinated, but reports suggest a relatively high number of adverse events associated with this test [25].

**Prediction beyond the IGRA**

Outside the IGRA concept, a recent landmark paper from Zak *et al.* suggested that whole-blood transcriptomic mRNA expression signatures accurately predict risk of progression in latently infected individuals [26]. A correlate of risk was identified by mining RNA-sequencing data from large prospective cohorts of *Mt*_b-infected adolescents from South Africa, and a 63 mRNA transcript from 16 genes was migrated to a chip-based PCR assay for high-throughput analysis. Validation in independent prospective cohorts from South Africa and Gambia demonstrated sensitivity of 54% and specificity of 83% for progression to active disease within a year [26]. The mRNA signature was not in itself specific for infection, but indicates a future two-step approach with a doorstep IGRA followed by a PCR-based risk prediction in IGRA-positive cases.

In conclusion, the field of immunodiagnostics is in transition. Innovative new specific skin tests and field-friendly IGRA like test formats will enable specific and likely cheaper diagnostics. Transcriptomic signatures could be a game changer, guiding rational use of targeted treatment. Encouraging, the predictive potential of transcriptomic signatures could also provide a revolution in TB vaccine development, enabling much smaller and cost-effective efficacy trials in high-risk populations to triage vaccine candidates [27]. Advances on all fronts are required to meet the ambitious 2035 targets of the End TB strategy, but without a new and efficacious TB vaccine and drastic improvements in living conditions and global access to healthcare, the targets still seem a high bar to meet.

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