

MINI-REVIEW

Immunological consequences of strain variation within the *Mycobacterium tuberculosis* complex

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In 2015, there were an estimated 10.4 million new cases of tuberculosis (TB) globally, making it one of the leading causes of death due to an infectious disease. TB is caused by members of the *Mycobacterium tuberculosis* complex (MTBC), with human disease resulting from infection by *M. tuberculosis sensu stricto* and *M. africanum*. Recent progress in genotyping techniques, in particular the increasing availability of whole genome sequence data, has revealed previously under appreciated levels of genetic diversity within the MTBC. Several studies have shown that this genetic diversity may translate into differences in TB transmission, clinical manifestations of disease, and host immune responses. This suggests the existence of MTBC genotype-dependent host-pathogen interactions which may influence the outcome of infection and progression of disease. In this review, we highlight the studies demonstrating differences in innate and adaptive immunological outcomes consequent on MTBC genetic diversity, and discuss how these differences in immune response might influence the development of TB vaccines, diagnostics and new therapies.

Keywords: Adaptive and innate immunity · Genetic diversity · Host response · *Mycobacterium tuberculosis* complex · Translational implications

Introduction

Tuberculosis (TB), caused by members of the *Mycobacterium tuberculosis* complex (MTBC), is a chronic disease that affects humans and animals [1]. The MTBC includes strictly human pathogens *M. tuberculosis sensu stricto* (Mtb) and *M. africanum* [2] that are clas-

sified into different lineages according to their phylogeographic structure, which is hypothesized to reflect adaptation to the human populations in which they cause disease. Animal-adapted MTBC strains include *M. bovis*, a pathogen of cattle and badgers [3]; *M. microti*, found in rodents [4]; *M. pinnipedii* infects sea lions [5]; *M. caprae* in goats [6]; the recently described *M. orygis* which

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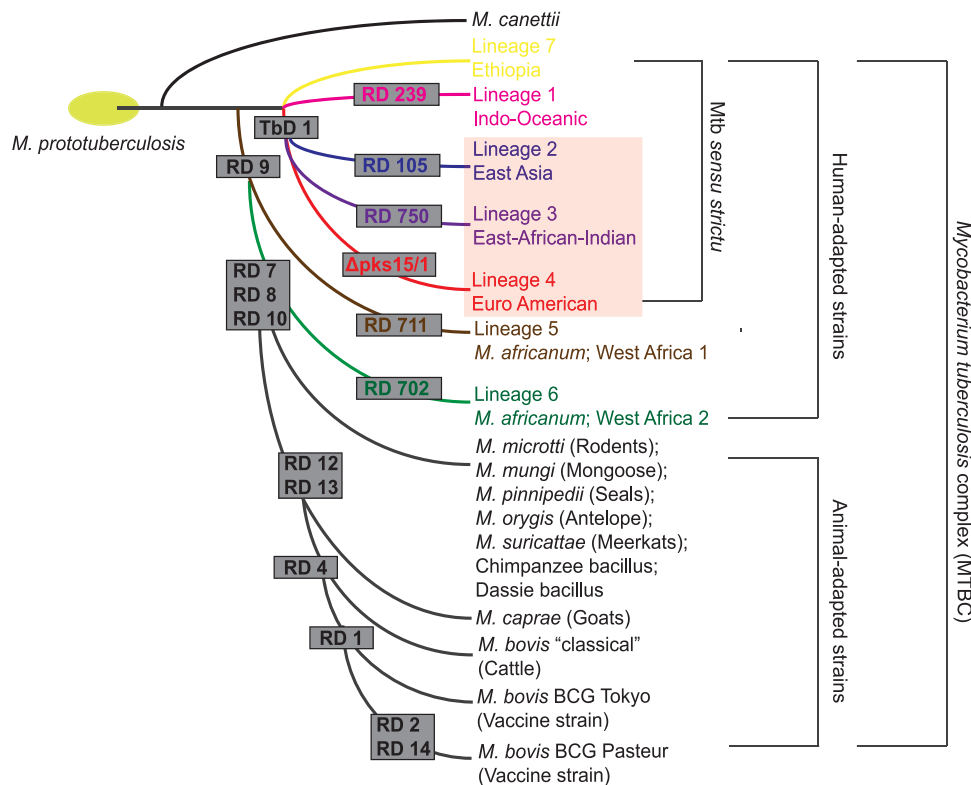


Figure 1. Schematic diagram illustrating the evolutionary relationship between selected members of the MTBC [1, 84, 111, 112]. Human-adapted and animal-adapted members of the MTBC can be classified according to the absence or presence of deletions known as regions of difference (RD), which were originally defined by [1]. Grey boxes indicate the specific deletion event that has occurred on the branches leading to a lineage. Human-adapted strains of the MTBC are grouped into 7 lineages and are associated with specific geographic regions. In the main text of the article, we refer to these lineages using the numerical nomenclature; however, the alternative nomenclature reflecting the geographic region wherein strains are prevalent is indicated below the numerical name. The presence or absence of the TbD1 regions discriminates modern (highlighted in a pink box) and ancient human-adapted MTBC strains. Animal-adapted members of the MTBC, together with the host that is infected by that strain, are indicated on the figure.

infects wild buck [7]; *M. mungi*, which was isolated in mongoose populations [8] and *M. suricattae* in meerkats (Fig. 1). Zoonotic transmission of MTBC to humans mainly involves *M. bovis*. Other animal adapted members have rarely been isolated from humans, thereby making the humans the only significant natural reservoir responsible for the global TB burden.

With the development of genotyping techniques a phylogeographic genetic structure for the MTBC began to emerge [1] (Table 1). Large insertions and deletions within the genome, referred to as large sequence polymorphisms (LSPs), were initially used to characterize the evolution and phylogeography of the MTBC [1]. The association of lineages with specific host populations led to a nomenclature for MTBC lineages which mirrors geographic regions where lineages predominate [9] (Fig. 1). MTBC phylogenies subsequently constructed using whole genome sequence (WGS) data were congruent with those generated using LSPs [10], and are used in this review. Human-adapted MTBC strains are grouped into seven lineages classified as “ancient” (lineages 1, 5, 6, and 7) or “modern” (2, 3, and 4) according to the presence or absence of the tuberculosis-specific deletion (TbD1) region (Fig. 1). While modern MTBC lineages (i.e. lineage 2 and 4) have spread globally, most ancient lineages (i.e. lineage

5 and 6 (West Africa) and lineage 7 (Ethiopia)) remain geographically restricted to specific regions/countries where they cause disease [11]. Moreover, WGS data has been used to demonstrate significant intra-lineage diversity in lineage 2 [12] and lineage 4 groups [13]. In a recent study, Stucki et al. show that certain lineage 4 sublineages are more geographically distributed than others [13].

Differences in virulence between MTBC strains were reported as early as the 1960s, with the observation that MTBC isolated in India were attenuated in the guinea pig model when compared with those isolated in the UK [14]. When genotyping techniques became available in the 1990s, outbreaks caused by specific MTBC strains could be delineated and these strains have been characterized in a range of models [15]. Two of the most highly characterized outbreak strains are Mtb CDC1551 and HN878. The CDC1551 strain, a member of lineage 4, was responsible for a TB outbreak, that exhibited an unusually high rate of tuberculin skin test (TST) conversion in contacts [16]. Subsequently it was shown that CDC1551 induced high levels of IL-12 and IFN- γ in mice, which may explain the observation of high levels of TST conversion [17]. The HN878 Mtb, a lineage 2 strain, was implicated in multiple outbreaks in Houston, Texas, USA, and was shown to be

Table 1. Common genotyping techniques for MTBC [111, 113, 114]

Technique	Principle	Advantages	Disadvantages
Spoligotyping	<ul style="list-style-type: none"> Polymorphism at a direct repeat (DR) locus is used to discriminate strains. The locus is in fact made up of clustered regularly interspaced short palindromic repeats (CRISPRs), and in other bacteria are associated with bacterial immunity to foreign DNA. PCR and reverse hybridization is applied to detect the presence or absence of 35 – 41bp “spacer” regions, which occur between 36bp repeat sequences. 	<ul style="list-style-type: none"> Inexpensive Fast Minimal DNA required therefore samples do not need to be cultured prior to typing. 	<ul style="list-style-type: none"> Low resolution Can't effectively discriminate between MTBC strains, particularly Lineage 2 strains, all of which lack spacers 1 – 34.
IS6110-Restriction fragment length polymorphism (RFLP)	<ul style="list-style-type: none"> IS6110 is a 1361bp IS3-family mobile genetic element which can be found in the varying frequency and loci within the genome. The number of copies and the site of IS6110 insertion are detected via restriction digest followed by blotting and labelling. 	<ul style="list-style-type: none"> Discriminatory power is better than spoligotyping and can resolve differences between MTBC lineages. 	<ul style="list-style-type: none"> Can't be used to accurately type isolates with fewer than 5 IS6110 bands. Requires large amounts of good quality DNA.
Variable-number tandem-repeat typing (VNTR)	<ul style="list-style-type: none"> Repetitive regions (40 – 100bp in length) that are found at 41 loci throughout the chromosome, with varying number of repetitive units. The regions are also known as mycobacterial interspersed repetitive units (MIRUs) and therefore the technique can be referred to as MIRU-VNTR. Polymorphism is detected via PCR using primers specific for the flanking regions of the repeats. Can be performed on 15- or 24-loci, with 24-loci providing better resolution. 	<ul style="list-style-type: none"> Better discriminatory power than both RFLP and spoligotyping. Minimal DNA required therefore samples do not need to be cultured prior to typing. 	<ul style="list-style-type: none"> Automation requires a sequencer and specialized software.
Whole genome sequencing (WGS)	<ul style="list-style-type: none"> The full genome sequence of the isolate is determined. The most common technique applied is shotgun sequencing, generating the sequence of short reads (25 – 450bp) after fragmentation of the genome. Reads are then aligned to a reference genome. Newer technologies, such as the Nanopore and PacBio systems, can sequence single molecules of DNA to generate long reads [115]. These have not been widely applied to MTBC due to cost and availability of technology. 	<ul style="list-style-type: none"> Unrivalled resolution and discriminatory power. Can provide lineage and drug resistance information. Can provide information about specific SNP differences at specific loci, allowing investigation of the functional implications of genetic diversity. 	<ul style="list-style-type: none"> Short read sequencing data can't be used to determine the sequence of repetitive regions. This is major disadvantage for Mtb given that ~10% of the genome comprises repeat regions of which the PE/PPE genes being significant for the proposed role in host-pathogen interactions. Expensive Specialized software and skilled personnel required for data analysis. Standard techniques require large amounts of good quality DNA.

highly virulent in mice [18] and rabbits [19]. The HN878 strain, and most other members of the lineage 2 strain family, produces a phenolic glycolipid that dampens the proinflammatory response, suggesting one mechanism for the increased virulence of these strains [20]. In addition to transmission, associations between specific MTBC lineages and the site of disease [21], clinical presentation, and the duration of disease [22] have been observed.

Evidence of significant differences in the transmission and manifestations of disease of MTBC lineages worldwide (reviewed in [23]), and variable evidence for host genetic polymorphism and susceptibility to MTBC [24], suggests that genetic variation within the MTBC may influence the host–pathogen interaction and outcome of the infection. In this review, we specifically consider the immunological consequences of MTBC lineage diversity on the innate and adaptive response and discuss the potential translational implications of differences in the immunological response to different MTBC strains.

The impact of MTBC lineage diversity on the interaction with innate immune cell receptors

Innate immune cell receptors are the first point of contact with MTBC bacilli, and thus play an important role in downstream immune response to MTBC. A wide range of pathogen recognition receptors (PRRs) are involved in the initial interaction between MTBC and host cells, including toll like receptors (TLRs), C-type lectin receptors (CLRs), nod like receptors (NLRs) and scavenger proteins [25, 26]. The cell wall structure of MTBC is complex, including glycolipids and lipoproteins that are uniquely found in pathogenic mycobacterial species and can vary between lineages [27]. Together with secreted effector proteins, cell wall components act as pathogen-associated molecular patterns (PAMPs) that are recognized by host immune cells. Subtle differences in cell wall components can impact the immune response [28] and in addition to activating the immune response, many cell wall components have immunomodulatory functions, which can modulate the immune response in favor of the bacterium [25]. In the following sections we highlight experimental evidence that demonstrates variation in PAMPs between MTBC lineages that may influence interaction with PRRs.

C-type lectins

C-type lectins are one important class of receptor that function in innate immunity to TB. Alveolar macrophages highly express Mannose-receptor (MR, CD206), while dendritic cell specific intercellular-adhesion-molecule-3 grabbing non-integrin (DC-SIGN, CD209) is mostly highly expressed on dendritic cells (DC) [29]. MTBC cell wall products such as lipoarabinomannan (LAM) and mannosylated-lipoarabinomannan (Man-LAM) [29, 30] are recognized by these receptors. Phagocytosis of MTBC via this pathway leads to a reduced inflammatory response thereby presenting an important route of entry from the perspective of the bacil-

lus [27, 30]. Structural variation in LAM between virulent and attenuated Mtb laboratory strains results in lower macrophage uptake in vitro of the attenuated Mtb H37Ra strain when compared with more virulent H37Rv or Erdman strains [31]. Similar differences have been reported amongst clinical MTBC. A set of lineage 2 isolates were identified as being deficient in uptake by human monocyte-derived-macrophages (MDMs) in vitro when compared with the Erdman Mtb strain (a lineage 4 strain) [32]. While links with diversity in lipid metabolizing genes were not uncovered, biochemical characterization revealed that ManLAMs in these strains were truncated and more highly branched, leading to reduced surface exposure and thus decreased engagement with the MR [32].

Toll-like receptors (TLRS)

Toll-like receptors (TLRs) are another set of PRRs that are important for sensing MTBC infection [25]. Differential recognition of MTBC lineages may impact the response of phagocytes and cytokine production [33]. Lipid and protein antigens from MTBC can be recognized by TLR4 and human polymorphism in TLR9 is associated with TB susceptibility; however, due its affinity for lipids, TLR2 is thought to be the dominant TLR for recognition of MTBC [25, 34]. Lipid fractions from a lineage 2 isolate, the H37Rv laboratory strain, and *M. canettii* have also been shown to differentially affect TLR2 and TLR4 expression on macrophages. Human MDMs produced higher levels of TNF- α and IL-10, and expressed lower levels of TLR2 and TLR4 when stimulated with lipid fractions from a lineage 2 strain when compared with H37Rv and *M. canettii* [35]. In a subsequent study characterizing the ability of lineage 2 strains to activate TLR2 and TLR4 receptors in mice, Carmona et al. (2013) show that for most strains studied, cytokine production by macrophages was induced via TLR2 activation [33]. However, a specific lineage 2 strain activated the TLR4 receptor on bone marrow-derived macrophages, leading to production of IFN- β , increased bacterial burden and lung pathology during the early stages of mouse infection [33]. A study in mice demonstrated that the lineage 2 HN878 Mtb strain induced lower levels of mRNA for Th1 cytokines, TNF- α , IL-6, IL-12, and IFN- γ , and higher levels for mRNA encoding type 1 IFNs, potentially explaining the increased virulence of this strain in mice [18]. While this study did not directly address the role of TLRs, preferential activation of TLR4 may partially explain this phenotype. However, Carmona et al. (2013) also demonstrated that activation of TLR4 was not specific to lineage 2 strains, with the Mtb Haarlem sub lineage strain, a member of lineage 4, also able to activate TLR4 with concomitant production of IFN- β [33]. In addition, the production of IL-17 via a TLR2-dependent pathway was shown to be protective for mice infected with HN878 but not H37Rv [36]. These studies demonstrate that immunological differences associated with MTBC strain variation are likely to arise from the interaction between multiple factors and immune receptors.

An interaction between host and bacterial genotype has been observed with respect to TLRs and susceptibility to disseminated TB disease [37] (Table 2). In particular, lineage 2 isolates were

Table 2. Studies describing the impact of both MTBC genotype and host genotype on immunological outcomes of TB infection

Geographic location	Variation in immune component	Methods used to genotype MTBC strains	Key finding	Reference
Ghana	5-lipoxygenase (ALOX5)	<ul style="list-style-type: none"> Clinical MTBC strains were genotyped by spoligotyping and IS6110 fingerprinting 	<ul style="list-style-type: none"> Heterozygous “5/non 5” promoter mutation that results in lower levels of 5-LO was associated with TB susceptibility regardless of MTBC lineage. The exonic variant G760A in males was associated with TB caused by infection by lineage 6 strains. 	[96]
Ghana	Autophagy related human immunity-related GTPase M (IRGM)	<ul style="list-style-type: none"> Clinical MTBC strains that were genotyped by spoligotyping, MIRU-VNTR, IS6110 fingerprinting and <i>pks1-15</i> region typing. 	<ul style="list-style-type: none"> The IRGM genotype –261TT was associated with relative protection against TB caused by <i>Mtb</i> lineage 4 but not <i>M. africanum</i>. Stratification of MTBC strains showed that protection was specific against TB caused by lineage 4 strains with a disrupted <i>pks1-15</i> gene. 	[116]
Ghana	Mannose Binding Lectin (MBL)	<ul style="list-style-type: none"> Clinical MTBC strains genotyped using LSP typing, IS6110 fingerprinting and typing of the <i>pks1-15</i>. 	<ul style="list-style-type: none"> MBL2 G57E variant was associated with protection against TB caused by <i>M. africanum</i> but not <i>Mtb sensu stricto</i>. 	[42]
Ghana	Macrophage Chemoattractant Protein 1 (MCP-1)	<ul style="list-style-type: none"> Clinical MTBC strains genotyped IS6110 fingerprinting, spoligotyping and MIRU-VNTR. 	<ul style="list-style-type: none"> MCP-1 genotypes variants were associated with resistance to tuberculosis and this was not affected by MTBC lineage differences. 	[117, 118]
The Gambia/ Guinea-Bissau	Epiregulin (EREG) and V-ATPase (TCIRG1)	<ul style="list-style-type: none"> Clinical MTBC strains genotyped using spoligotyping 	<ul style="list-style-type: none"> Marginal association between rs11228127 in TCIRG1 and patients infected with <i>M. africanum</i> in The Gambia. 	[119]
South Africa	HLA class I	<ul style="list-style-type: none"> Clinical MTBC strains genotyped by spoligotyping and IS6110 fingerprinting. 	<ul style="list-style-type: none"> Associations between several lineages and specific HLA haplotypes e.g. A*03 was associated with infection by lineage 4 strains. 	[120]
Vietnam	TLR2 and TIRAP	<ul style="list-style-type: none"> Clinical MTBC strains genotyped by IS6110 fingerprinting, large sequence polymorphism and MIRU-VNTRs. 	<ul style="list-style-type: none"> C allele of TLR-2 T597C was associated with TB disease caused by lineage 2 MTBC strains. 	[37]
Vietnam	Macrophage receptor with collagenous structure (MARCO)	<ul style="list-style-type: none"> Clinical MTBC genotyping by IS6110 fingerprinting, MIRU and <i>pks1-15</i> region typing. 	<ul style="list-style-type: none"> Two heterozygous (AG) genotypes (rs2278589 and rs6751745) were associated with impaired phagocytosis of MTBC and increased susceptibility to lineage 2 strains but not lineage 4 and lineage 3 <i>Mtb</i> strains. 	[121]
Indonesia	SLC11A1 gene (also known as “NRAMP1”)	<ul style="list-style-type: none"> Clinical MTBC strains genotyped by spoligotyping. 	<ul style="list-style-type: none"> Lineage 2 strains were significantly associated with the G allele and the GG phenotype of the D543N polymorphism compared to non-lineage 2 strains. 	[122]
Russia	CD209 (DC-SIGN)	<ul style="list-style-type: none"> Clinical MTBC strains genotyped by spoligotyping and MIRU-VNTR. 	<ul style="list-style-type: none"> Strong association between the G allele of CD209 -336A/G and increased mortality from TB caused by lineage 2 strains. 	[123]

more likely to cause meningeal tuberculosis in individuals with the C allele of the T597C polymorphism in TLR2. While the implications for this allele on TLR2 function is not known, this study suggests an important interaction between TLR2 polymorphism and bacterial genotype in TB susceptibility [37].

Mannose binding lectin (MBL)

Mannose Binding Lectin (MBL), a member of the collectin family, acts as an opsonin by binding pathogen cell surface carbohydrates [38, 39]. While some studies have reported that patients with high serum MBL are more susceptible to TB (reviewed in [40]), others suggest the opposite (reviewed in [41]). A study conducted in Ghana suggests that diversity between MTBC lineages might partially explain this difference. The study showed that a relatively high proportion of the population studied had an MBL polymorphism [42] that results in lower serum availability of functional MBL by disrupting formation of oligomers [43]. The polymorphism was associated with a protective effect against infection with strains of the *M. africanum* lineage but not strains from *Mtb sensu stricto* lineages. Furthermore, *M. africanum* strains were better able to bind to wild-type MBL than the H37Rv *Mtb* strain in vitro, suggesting that *M. africanum* uses this receptor as an entry ligand thereby promoting infection. This potentially explains why polymorphisms in MBL are protective against *M. africanum* but not *Mtb sensu stricto* [42].

These studies highlight the need to take human genetic variation into account when considering differential immunological outcomes associated with MTBC strain variation. Studies investigating association between polymorphism and human susceptibility to TB disease have yielded discrepant results (reviewed in [24, 44]), and relatively few studies have taken MTBC genetic diversity into account (highlighted in Table 2). Discrepant associations between polymorphism in host and TB susceptibility might be partially explained by MTBC variation. Matching bacterial and host genotypes presents a challenge to the field. To produce generalizable insights going forward, these studies should involve more than one population and be large enough to be well powered.

The response of innate immune cells to diverse MTBC lineages

The early response to MTBC infection is characterized by a systemic inflammatory response that is mediated by macrophages, neutrophils, DC, and innate T cells [26, 34]. The observation that a proportion of people heavily exposed to MTBC do not develop evidence of sensitization suggests that the innate immune system may completely clear MTBC before infection is established, highlighting the importance of these mechanisms [45]. The innate immune response also activates and augments adaptive responses to MTBC infection. MTBC has developed various mechanisms to interfere with effective innate immune function and variation in

the capability of various MTBC strains and lineages to do this may significantly influence the outcome of MTBC infection.

Macrophages

Monocyte and macrophage cell populations have been widely used to study differences in virulence between MTBC lineages. In the following section we highlight some studies that have investigated the macrophage and macrophage-like cell response to different MTBC lineages.

A study comparing the capacity of MTBC strains, to grow and stimulate cytokine production in primary human monocytes and THP-1 macrophage-like cells, that were either transmitted within households or not transmitted within households suggested that transmitted MTBC isolates grew more rapidly than strains that were not transmitted, in both kinds of cells [46]. In addition to growth patterns, cytokine production by monocytes infected with different MTBC isolates has been widely studied. Using representative strains for each lineage, Portevin et al. (2011) showed that modern strains (lineage 2, 3, and 4) of MTBC elicited a lower early inflammatory response, as characterized by cytokine production in human peripheral blood MDMs than ancient MTBC strains (lineage 1, 5, and 6) [47]. This suggests that modern MTBC strains have adapted to cause more rapid disease progression and therefore transmission [47]. In support of this, mice infected with more evolutionary modern sublineages of lineage 2 MTBC strains, isolated in Brazil and Mozambique, had greater lung pathology and lower survival than mice infected with lineage 2 strains that were more evolutionarily ancient [48]. MTBC lineages seem to exhibit a lineage-specific transcriptional pattern in macrophages [49]; however, within the modern MTBC group, sublineage-specific patterns of cytokine production by macrophages have also been observed [50] suggesting that significant intra-lineage variation also occurs with regards to transcriptional patterns.

Differences in monocyte activation have been shown to be partially responsible for the differences in virulence of the well-known CDC1551 and HN878 outbreak MTBC strains [51]. In particular, human peripheral blood mononuclear cells (PBMC) infected with CDC1551 produced higher levels of pro-inflammatory cytokines, IL-1 α / β ; IL-1 α , and MIP3- α , indicative of Th1 type immunity and immune protection. In contrast, HN878 stimulated high levels of IL-4 and IL-13, which are characteristic of the Th2 type immune response [51]. A study published around the same time demonstrated that production of pro-inflammatory cytokines in murine bone-marrow-derived macrophages was inhibited by a phenolic glycolipid produced by HN878. Deletion of *pks1-15*, the genes encoding the polyketide synthase enzymes that produce this lipid, led to abrogation of this phenotype directly linking a mycobacterial genetic determinant to an immunological phenotype [20]. A lineage 3 strain responsible for a large TB outbreak in Leicester, UK, stimulated higher levels of the anti-inflammatory cytokine IL-10 and lower pro-inflammatory IL-12p40 from human MDMs, suggesting phagocyte deactivation [52]. By systematically complementing

deletions specific to this MTBC strain, the authors showed that the phenotypic reaction in macrophages was related to the specific deletion of *Rv1519*. However, the precise function of this gene in MTBC is unknown [52].

Neutrophils

Neutrophils are the first innate cells to migrate to the site of infection and represent the most highly infected phagocytes in the airways of active pulmonary TB patients [53]. Moreover, a whole blood neutrophil driven type I interferon transcriptional profile characterizes active from latent TB [54]. Studies have shown that TNF- α -activated neutrophils can phagocytose and kill MTBC via α -defensins and proteases rather than ROI and RNI [55, 56]. Outbreaks of multidrug resistant (MDR)-Mtb caused by strains belonging to the Harlem and LAM family of lineage 4 were shown to induce a differential respiratory burst from human neutrophils [57]. In particular, apoptosis of neutrophils was more strongly induced by strains of the LAM family, leading to concomitant production of ROI. By contrast the Haarlem family strain, which has been responsible for an ongoing MDR-TB outbreak in Argentina, induced significantly less neutrophil apoptosis, suggesting that this ability might be one of the reasons for its success [58]. This phenotype may be due to differences in the structure of α -glucans present on the cell wall of the Haarlem strain, which promotes non-apoptotic neutrophil pathways, allowing the bacteria to sequester and replicate [57].

Dendritic cells

DC make up a large proportion of MTBC-infected cells, and play an important role in the response to MTBC by transporting antigen to the lymph nodes where it is presented to and activates naïve T cells [26, 45]. The function of DC can be inhibited by MTBC and murine bone-marrow-derived DC stimulated with soluble extracts from the HN878 lineage 2 Mtb strain expressed lower levels of surface MHC class II molecules, and led to less activation of CD4⁺ T cells than less virulent H37Rv and BCG strains [59]. In a separate study, exposure of human monocyte-derived DCs to clinical Mtb isolates from India, whose genotypes were not defined, resulted in reduced migration of DCs towards a chemokine ligand important to guide DCs to lymphoid tissue, CCL21, compared to LPS stimulation of DCs [60]. A study profiling a relatively large number of clinical Mtb isolates using semi-global protein arrays and ELISA-arrays showed that human monocyte-derived DCs infected with lineage 2 Mtb isolates generally produced lower levels of TNF, IL-6 and IL-10 than the H37Rv strain, suggesting that this might be a mechanism of increased virulence of these strains [61]. Contrasting results were found in a separate study investigating the virulence of different MTBC strains isolated from TB meningitis patients. In this study, there was no difference in cytokine production by murine bone-marrow-derived DC in vitro; however, lineage 2 and 3 isolates displayed higher virulence in mice than lineage

4 strains, with more rapid growth and increased dissemination to the blood and lung pathology [62].

Variation in the adaptive immune response to MTBC lineages

There is a considerable delay in the onset of the adaptive immune response to MTBC, which primarily consists of a T helper 1 (Th1) T cell response. CD4⁺ T cells differentiate into Th1 cells producing IFN- γ , TNF- α and GM-CSF to activate infected macrophages. These macrophages in turn restrict intracellular MTBC by initiating the formation of RNI and ROI, and by autophagy [63]. Th1 cells also produce IL-2 that promotes proliferation and increased cytokine synthesis by T cells and NK cells and LT- α (lymphotoxin- α), which is necessary for recruitment and activation of neutrophils. CD8⁺ T cells also have a role in protection against MTBC. Production of granzysin and serine esterase containing granzymes may directly kill MTBC [64], while the production of cytokines such as IFN- γ and TNF- α activates phagocytes, and the production of IL-2 promotes the proliferation of NK and T cells, indirectly contributing to the control of MTBC [65]. While the T cell response has been widely studied, less is known about variation in the B-cell response in TB. However, recent work characterizing antibodies from humans with active and latent TB suggests that B cells may play a significant role in protection against TB [66, 67]. Ultimately adaptive immunity results in the formation of a granuloma comprised of uninfected macrophages that confine infected macrophages, epithelioid cells and neutrophils. This barrier is then surrounded by T and B cells, thus providing a physical barrier to contain MTBC [45].

T-cell-mediated immunity

Characterization of the adaptive response to whole cell MTBC has revealed differences in the T cell response to different lineages. A low dose aerosol guinea pig model of infection was applied to examine differences in virulence of a set of lineage 2 MTBC isolates selected for their differences in transmission [68]. All strains elicited an appreciable Th1 response, with increased mRNA levels for IFN- γ , IL-12p40, and TNF- α in the lungs, and the presence of activated T cells by day 30 of the infection. However, two strains, which caused a significantly greater number of secondary cases, elicited higher levels of Foxp3 and TGF- β mRNA in the lungs at day 60, coinciding with a drop in the number of activated CD4⁺ T cells, suggesting expansion of regulatory T cells [68]. A study applied a mouse model to extensively characterize the virulence and T cell response to the K strain, a lineage 2 Mtb isolate responsible for a TB outbreak in a Korean school [69]. The K strain was more virulent in mouse lungs, with rapid growth and more extensive pathology, when compared the H37Rv or H37Ra laboratory strains. In addition to differences in the innate response in the mouse lung, the study demonstrated differences in the kinetics of T cell subtypes in animals infected with different MTBC strains. In

the lungs of mice infected with all strains, Th1 T-bet expressing CD4⁺ T cells had accumulated by 14 days, however, at 28 days, these cells began to decrease in the lungs of mice infected with the K strain compared to the H37Rv and H37Ra laboratory strains. Moreover, CD4⁺ T cells expressing ROR γ t, thought to represent a Th17 subset, were not present in the lungs of mice infected with the K strain while they were present in appreciable numbers in mice infected with laboratory strains, H37Rv and H37Ra. Similarly to the guinea pig study discussed above, higher numbers of CD4⁺CD25⁺Foxp3⁺CD223⁺IL-10⁺ T-regulatory (Treg) cells were observed in K Mtb strain infected mice when compared with H37Rv or H37Ra strains [69]. In a separate study, in mice infected with HN878, higher numbers of Treg cells were observed within 21 days of infection when compared with H37Rv, suggesting a potential contribution of this cell type to the virulence of this strain [70]. While the genetic determinants driving the mechanisms for these observations are unclear, one possible explanation is that some Mtb strains express yet to be discovered antigens that specifically stimulate the expansion of regulatory T cells [69].

Epitope variation in MTBC

Unlike many bacterial pathogens, several genomics studies have revealed that MTBC T cell epitopes appear to be genetically conserved [10, 71]. However, almost all of the WGS data generated thus far utilizes short-read sequencing platforms, which are not able to efficiently determine sequence in highly repetitive genetic regions, some of which are antigenic [72]. One of the most variable regions of the MTBC genome – the PE/PPE gene family – comprises highly repetitive sequences that are routinely filtered out of WGS datasets. Several of the PE proteins have been shown to elicit both cellular and humoral responses in a wide range of models studied [73]. This observation has led to the hypothesis that these regions may be a source of antigenic variation as yet undetected by WGS. However, a study characterizing the immunogenicity of a relatively large set of PE proteins in cattle infected with *M. bovis*, or PBMC from humans infected with Mtb, revealed that both the proportion of responders and the breadth of response, as measured by IFN- γ production, were positively correlated with sequence conservation [74]. The study did not characterize the highly variable PE.PGRS subfamily of these genes; however, computational prediction of epitopes in the PE.PGRS subfamily indicated that epitope encoding regions mostly occurred in conserved PE domain of these proteins [71]. Furthermore, the impact of non-synonymous SNPs occurring in these regions in 94 clinical Mtb isolates was found to be negligible for predicted HLA class I and II binding, and epitope prediction [71]. While these findings suggest that epitopes are conserved in MTBC even in genomic regions thought to be variable, a small subset of MTBC epitopes that are highly variable were recently uncovered by comparative genomics [75]. The immunogenicity of a selection of these epitopes was determined by measuring IFN- γ responses in whole blood from HIV-uninfected TB patients in the Gambia, revealing differential responses to ancestral and variant epitope pep-

tides [75]. These findings have important potentially implications for vaccine development, discussed in more detail below.

Translational implications of MTBC lineage associated variation in host immunity

Implications of MTBC lineage variation for vaccine development

The translational implications of MTBC lineage variation is perhaps most apparent for the design of effective new TB vaccines. Escape from BCG vaccination is one of the factors proposed to have driven the wide geographic distribution of the lineage 2 genotype [76, 77]. A recent reconstruction of the evolutionary history of Mtb lineage 2 strains, by genetic characterization of more than one thousand isolates, suggests that BCG vaccination had minimal impact on the population size of lineage 2, suggesting vaccination was insufficient to prevent lineage 2 dissemination [12]. To determine whether this is specific to the lineage 2 genotype or due to general variability in BCG efficacy would require similar characterization of MTBC lineages. Data from BALB/c mice and a rabbit model suggest that BCG vaccination provides lower levels of protection against challenge with lineage 2 isolates [78, 79]; however, these findings were not reproduced in C57BL/6 mice [80]. A subsequent study conducted in C57BL/6 mice and guinea pigs suggested that BCG conferred acceptable levels of protection to lineage 2 strains isolated in South Africa, but not against those isolated in the USA [81]. The authors suggest that variable BCG protection is a function of strain fitness, rather than lineage diversity; however, in all of these studies only a few isolates of lineage 2 strain were investigated [81].

In terms of new vaccines, the recombinant BCG candidate (VPM1002) expressing listeriolysin but defective in urease production, was shown to confer better protection than standard BCG in BALB/c mice, hypothesized as a result of increased antigen translocation into the cytoplasm of infected macrophages and resultant antigen cross-priming [82]. Moreover, this vaccine conferred a similar level of protection against a lineage 2 strain as H37Rv [82]. VPM1002 is currently in Phase II trials as a BCG replacement candidate vaccine in HIV-infected infants, and is proposed for Phase III efficacy trials [83].

As previously discussed, several comparative genomics studies have shown that in MTBC, unlike other bacteria such as *Streptococcus pneumoniae*, T cell epitopes are evolutionarily conserved [10, 75]. While the biological consequences are not completely understood, these findings imply that these epitopes are conserved because they fulfill an important biological role, and that immune recognition of MTBC may favor the pathogen [10, 84]. One of the proteins in a polypeptide candidate TB vaccine, M72/AS0E1, which recently yielded acceptable safety results in a phase II trial [85], contains a sequence from a PE protein discussed in previous sections. High sequence variability among MTBC clinical isolates has been observed in these specific proteins [86, 87]. Although the direct effects of the observed sequence variability on

the proteins' immunogenicity has not yet been established, these findings may warrant further investigation.

Impact of MTBC lineage diversity on TB diagnostics

Currently available tools for detection of MTBC infection rely on immunological sensitization and thus could theoretically be influenced by MTBC lineage associated immunological variation. The TST has traditionally been used to detect latent TB infection (LTBI). The test, which requires subcutaneous injection of purified protein derivative (PPD) cannot differentiate MTBC infection from BCG vaccination or exposure to environmental nontuberculous mycobacteria (NTMs), and thus may yield false positive results [88]. IFN- γ release assays (IGRA) are a set of newer generation tests that measure in vitro IFN- γ production in whole blood or PBMC in response to MTBC antigen stimulation, such as CFP10 and ESAT-6 (and p38-55 of Rv2654c) [89]. Although IGRA are still unable to discriminate active from latent infection, the absence of the CFP10 and ESAT-6 in BCG and most NTMs confers on IGRA increased specificity to detect MTBC sensitization [89]. However, the attenuated T cell response to ESAT-6 stimulation in *M. africanum* infected individuals has been shown to affect the interpretation of IGRA results [90]. In addition, a number of studies have reported that activated T cell markers [91] and soluble cytokines [92] can be used as reliable biomarker for monitoring the anti-TB treatment response. It has been shown that the production of 27 proinflammatory and regulatory cytokines in response to ESAT-6/CFP-10 stimulation was not significantly different between *M. africanum* and *Mtb*-infected patients pre-treatment. However, the kinetics of production of these cytokines following treatment was significantly different between the two groups, with IFN- γ and GM-CSF significantly higher in *Mtb* compared with *M. africanum* infected patients post-treatment [93]. These studies reveal that the interpretation of cytokine production-based diagnostic tests for monitoring anti-TB treatment response may need to account for MTBC lineage variation in West Africa where *M. africanum* is prevalent.

The influence of MTBC lineage diversity on novel host-directed therapies

MTBC lineage diversity may also constitute an important factor to consider in the development of host-directed therapies (HDT). Some HDT aim to augment the host defense against TB by modulating host pathways involved in the elimination of MTBC [94]. Due to both host and MTBC genetic variation, the efficacy of an HDT targeting a specific pathway might differ in specific populations. For example, a combination of zileuton and prostaglandin E2 (PGE2) as an HDT, which abrogates the detrimental type 1 interferon response and boosts eicosanoid levels, may find utility in TB therapy [95]. The exonic g.760A allele in *ALOX5*, a key mediator of the type 1 IFN response to TB infection, is associated with protection against TB caused by *M. africanum* strains but not *Mtb sensu stricto* [96] (Table 2). While the functional implications of

this variant are not known, the association could influence individual responses to the aforementioned HDT. In another example, the virulent lineage 2 HN878 isolate was shown to stimulate a high level of type 1 Interferon signaling in mice [18, 97], suggesting that higher levels of HDT that modulate eicosanoids might be required to be effective against infection with lineage 2 strains, or conversely that the HDT may actually be more effective in persons infected with lineage 2 strains.

The impact of HIV co-infection on immunological outcomes associated with different MTBC lineages

Due to dysregulated host immunity, it has been suggested that HIV-1 co-infection may facilitate the survival of less "fit" MTBC lineages within a population [2]. It has been observed that TB is not transmitted efficiently from HIV-1 co-infected individuals, thus transmission predominantly occurs from HIV-1 uninfected individuals to HIV-1 co-infected individuals [98, 99]. A recent study modelling the impact of HIV-1 on a large MDR outbreak in South America found no evidence of association between HIV-1 and the mutation rate of *Mtb*, or MDR-TB transmission [100].

MTBC creates an inflammatory milieu that exacerbates HIV-1 infection and increases viral replication [26]. Activation of nuclear factor (NF)- κ B pathway and positive transcription elongation factor (P-TEF β), loss of CCAAT/enhancer-binding protein and other chemokine and cytokine dependent pathways underlie this phenomenon [26]. Clinical isolates of MTBC have been shown to differentially affect the replication of HIV-1 in PBMC [101]. For example, the lineage 4 CDC1551 strain induced higher levels of viral replication in human PBMCs than the lineage 2 HN878 strain [101]. The HN878 strain contains an intact *pk1-15* gene cluster resulting in production of a virulence associated phenolic glycolipid [20]. Viral replication in PBMC co-infected with a mutant HN878 *pk1-15* deletion strain, which no longer produces the phenolic glycolipid, was similar to levels observed in PBMC co-infected with the CDC1551 strain. This suggests that strain specific effects on HIV-1 replication are associated with cell wall components of MTBC [101]. MTBC dependent enhancement of HIV-1 replication was subsequently shown to be a result of direct binding of transcription factor nuclear factor of activated T cells 5 (NFAT5) to the HIV-1 LTR promoter [102]. NFAT5 expression was induced in macrophages after MTBC infection in THP-1 cells in a TLR2 dependent manner. While the authors did not examine the influence impact of MTBC strain variation in this study [102], the discussion above highlights the differential effects MTBC might have on TLRs. Together these findings suggest that MTBC strain variation may have important implications for HIV-1 replication, and disease progression during HIV/TB co-infection [102].

Conclusion

The studies discussed above indicate that genetic diversity within the MTBC can influence the host response to infection and during

TB disease. However, the use of in vitro systems and animal models that do not completely recapitulate human TB makes it difficult to assess whether immunological variation associated with MTBC genetic diversity appreciably impacts clinical outcome, or whether TB control approaches should be tailored according to the infecting MTBC strain. The use of techniques such 2-deoxy-2-[¹⁸F]-fluoro-D-glucose (FDG) positron emission tomography/computed tomography (PET/CT) [103] and transcriptional profiling [54] during TB infection have recently provided important insights into pathophysiology within the human host and could be applied to investigate the impact of MTBC strain diversity on clinical outcome.

In addition to technical considerations associated with the immunological readout, many studies have characterized only a few representatives of a lineage, in many cases those that have been associated with well-known outbreaks. Significant intra-lineage diversity exists [12, 13] making extrapolation of phenotypes associated with a few representatives to an entire lineage difficult. Moreover, as previously discussed, almost all MTBC WGS data have thus far been generated via short read sequencing techniques. Sequence in highly repetitive regions cannot reliably be determined using these techniques and are usually removed from downstream analysis [72]. Short read data are also not useful for detecting genomic changes such as deletions and duplications, particularly those that span larger regions of the genome. These changes in genetic architecture, as well as epigenetic changes such as methylation patterns, may have important implications for MTBC physiology and associated host–pathogen interactions [104]. Newer technologies, such as the PacBio system and hybrid sequencing strategies, which combine short-read sequencing with single molecule sequencing [105], will provide the opportunity to investigate these genomic alterations and their associated immunological phenotypes.

In terms of childhood TB, studies suggest that associations between particular MTBC genotypes and TB in children is likely to reflect ongoing transmission from adults [106–108]. However, variation in immunological outcomes due to MTBC genetic diversity in childhood TB specifically has not been extensively investigated. Given the immunobiology of TB disease in children is distinct from that of adults and varies with age [109], this presents an important area for further research.

Finally, definitively establishing genotype–phenotype relationships may benefit from allelic exchange type experiments that facilitate an understanding of cause and effect between a polymorphism and a particular phenotype [110]. However, genetic manipulation of clinical isolates is likely to be challenging and complex phenotypes may result from epistatic interactions between multiple SNPs.

The aforementioned caveats notwithstanding, the studies discussed here have uncovered important variation in innate and adaptive immune responses to different MTBC strains, that warrants further investigation. The use of new immunological, clinical, and genomics techniques provides the opportunity to pursue and throw greater light on their significant in tuberculosis response.

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