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Antigenic Variation and Immune Escape in the MTBC

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Abstract

Microbes that infect other organisms encounter host immune responses, and must overcome or evade innate and adaptive immune responses to successfully establish infection. Highly successful microbial pathogens, including *M. tuberculosis*, are able to evade adaptive immune responses (mediated by antibodies and/or T lymphocytes) and thereby establish long-term chronic infection. One mechanism that diverse pathogens use to evade adaptive immunity is antigenic variation, in which structural variants emerge that alter recognition by established immune responses and allow those pathogens to persist and/or to infect previously-immune hosts. Despite the wide use of antigenic variation by diverse pathogens, this mechanism appears to be infrequent in *M. tuberculosis*, as indicated by findings that known and predicted human T cell epitopes in this organism are highly conserved, although there are exceptions. These findings have implications for diagnostic tests that are based on measuring host immune responses, and for vaccine design and development.

Keywords

Antigenic variation; Immune Escape; T lymphocytes; Epitopes; Antibodies; Tuberculosis

9.1 Immune Evasion by Pathogens

All host organisms possess mechanisms to defend themselves from foreign microbial invaders, especially those that have detrimental effects on the hosts. Bacteria and archaea possess nucleic acid restriction-modification systems (Makarova et al. 2013) and CRISPR systems (Abudayyeh et al. 2016; Jinek et al. 2012) to defend against invaders such as bacteriophages, while higher organisms have evolved progressively more complex systems of immunity to balance their interactions with beneficial and pathogenic microbes in their environments, to remain fit and capable of reproduction.

In metazoans, immune responses are categorized as innate or adaptive, although the boundaries between these sometimes blur (Cerwenka and Lanier 2016; Cheng et al. 2014; Netea et al. 2016; Saeed et al. 2014). Innate immune responses do not require genetic rearrangements in host somatic cells to respond to a pathogen, and as such are rapidly

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available and provide broad defense against diverse pathogens (Murphy and Weaver 2016). Innate immune responses are initiated by host molecular recognition of foreign molecular targets, including structural components of bacteria and fungi, certain microbial signalling molecules, and viral nucleic acids. In contrast, adaptive immune responses, represented by antibodies and by T lymphocytes, require genetic rearrangements of immunoglobulin or T cell antigen receptor genes in individual host cells, followed by clonal expansion of antigen-specific B and T lymphocytes and production of antibodies or T cell effector molecules, in response to the individual host's encounter with a microbe bearing a specific repertoire of antigens. Because of the requirement for clonal expansion and differentiation of lymphocytes after specific antigen exposure, initial adaptive immune responses are less rapid than innate immune responses, but a specialized property of adaptive immunity is that of immune memory, which allows for more rapid and higher quality responses when a host encounters a given antigen for a second (or third, or more) time (Murphy and Weaver 2016).

If innate and/or adaptive immune responses operated successfully all of the time, pathological infections would not happen, so the high frequency and diverse nature of infections indicates that host immune responses either fail, or can be evaded by specific mechanisms.

The range of mechanisms of immune evasion by pathogens is vast, and discovery of those mechanisms has yielded considerable understanding of the pathogenesis of specific infections and the roles of specific arms of immunity in control of certain pathogens. Representative mechanisms of immune evasion by pathogens include: (1) masking or modification of pathogen molecules that are typically recognized by innate pattern recognition receptors (Cambier et al. 2014; Vladimer et al. 2012), (2) manipulation (enhancing or inhibiting, depending on the pathogen) of host cell entry, to occupy optimal compartments for replication (Fu and Galan 1999; Hardt et al. 1998); (3) manipulation of intracellular signalling (Xu et al. 2014) or vesicle trafficking (Martinez et al. 2016; Newton et al. 2014) (Mordue et al. 1999; Mordue and Sibley 1997); (4) resistance to or evasion of host microbicidal mechanisms (LaRock et al. 2015; Potter et al. 2012); (5) molecular mimicry, where pathogen molecules resemble host molecules and are not recognized as foreign (Elde and Malik 2009; Hill et al. 2010); (6) antigenic variation, where structural variants of pathogen molecules develop to escape recognition by pre-existing antibodies or T lymphocytes (Deitsch et al. 2009).

9.2 Antigenic Variation in Human Pathogens

Antigenic variation is a common mechanism of immune evasion in diverse pathogens. In a typical example, a pathogen with a given antigenic composition infects a population of hosts that respond by developing adaptive immune responses (antibodies and/or T cells). In many cases, those adaptive immune responses provide protective immunity, which prevents members of that population from subsequent infection. However, since antibodies and T cells have restricted specificities, pathogen variants can develop that are not recognized by the antibodies or T cells in immune hosts, and those pathogen variants can then cause infection in hosts that are immune to the original pathogen (Fig. 9.1). Sequential rounds of immune escape can generate populations of antigenically diverse pathogens. The

consequences of this process of successive generation of pathogen antigenic variants are illustrated by the examples described below.

9.2.1 Influenza

Infection with influenza virus generates T cell and antibody responses, both of which contribute to protective immunity, although it is thought that antibody responses make the more important contribution to preventing reinfection after immunization or infection with a given influenza viral strain. The major target of protective antibody responses to influenza virus is the viral hemagglutinin (HA) protein (Ohmit et al. 2011; Virelizier 1975), one of the two viral proteins exposed on the viral surface. The HA protein is responsible for virus binding to host cells, a requirement for viral entry and replication, and the domain of HA that directly mediates viral binding to host cells is termed the globular head (Tsibane et al. 2012).

The HA globular head is also the predominant target of antibodies generated in response to influenza vaccination or infection (Wrammert et al. 2008). Since the globular head can tolerate a large number and broad range of mutations while retaining its cell-binding function, the virus mutates to escape recognition by anti-HA antibodies that act by blocking virus-host cell binding (Smith et al. 2004; Thyagarajan and Bloom 2014). In addition to mutations in HA that allow escape from antibody recognition, recent evidence indicates that influenza virus can also mutate to escape CD8 T cell recognition of matrix and nucleoproteins (Valkenburg et al. 2013, 2016). Antigenic variation in influenza virus is the result of point mutations in the viral genome, which happen with high frequencies, due to the lack of proofreading and error correction by the viral RNA polymerase. Antigenic variation in influenza virus can also result from development of reassortant viruses, in which a novel HA antigenic type is acquired from a virus that has not previously circulated in the human population.

The effect of escape mutations and antigenic variation in influenza viruses is significant for human and public health, as it contributes to the need to generate and administer new influenza vaccines nearly every year (Grohskopf et al. 2015), and it allows for global pandemics, when new antigenic variants are generated that are not recognized by antibodies generated by prior vaccination or infection (Bautista et al. 2010).

9.2.2 Human Immunodeficiency Virus (HIV)

Like other pathogens, HIV induces adaptive immune responses that include CD4 and CD8 T cells, as well as antibodies. However, unlike influenza, HIV establishes a chronic infection, because adaptive immune responses are unable to clear the virus and cure the infection. Antigenic variation, which happens at an exceptionally high frequency in HIV, is a major reason that antibodies and T cells are unable to clear the virus.

Although antibodies and CD8 T cell responses are both directed against multiple HIV proteins, these two mechanisms of immunity have distinct effects, depending on the antigenic target: antibody responses drive antigenic variation of the HIV surface envelope glycoprotein (Hraber et al. 2015), while CD8 T cell responses drive antigenic variation of the HIV internal gag protein (Crawford et al. 2007, 2009; Goepfert et al. 2008).

Neutralizing antibodies to HIV develop early after infection, but most of these are specific for the initial infecting virus, and do not cross-react with other viral strains (Li et al. 2009). Antibodies to the HIV envelope glycoprotein are directed at surface-exposed loops of the protein (Pantophlet and Burton 2006), and specific do mains within these loops represent hotspots of variation during evolution of HIV (Korber and Gnanakaran 2009). Together, these data indicate that HIV develops escape mutants that allow it to evade the predominant neutralizing antibodies that develop after infection, thus generating considerable sequence and antigenic diversity in the viral envelope.

CD8 T cell responses in HIV-infected individuals bearing HLA-B27, HLA-B57, or HLA-B58 alleles, are especially efficacious in limiting viral replication and progression to AIDS (Kaslow et al. 1996; Lazaryan et al. 2010). Intensive studies have identified several dominant epitope targets of CD8 T cells that are presented by these HLA alleles, and revealed that recognition of those epitopes in the HIV gag protein by CD8 T cells contributes to limiting the HIV viral load in plasma. The same studies have revealed a high frequency of escape mutations in those epitopes, with concomitant decrease in viral fitness, which also contributes to limiting the level of the HIV viral load (Crawford et al. 2007, 2009; Goepfert et al. 2008). That the observed mutations represent escape from CD8 T cell recognition is supported by a high rate of reversion to the ancestral sequence in the involved epitopes when HIV is transmitted to an individual lacking a protective HLA allele.

Although antigenic diversity in HIV can develop as the consequence of viral recombination (Vuilleumier and Bonhoeffer 2015), the extremely high mutation rate of HIV (3×10^{-5} per base per round of replication from polymerase errors (Roberts et al. 1988) and approximately 100-fold higher rates due to host cytidine deaminases (Cuevas et al. 2015) indicates that most antigenic variation in HIV is attributable to point mutations.

9.2.3 *Streptococcus pneumoniae*

Streptococcus pneumoniae is a gram-positive bacterial pathogen that inhabits respiratory mucosal surfaces and can cause potentially-fatal invasive infections including pneumonia, bacteremia and sepsis, and meningitis. Immunity to *S. pneumoniae* depends on specific antibodies, antibody-mediated phagocytosis (Hosea et al. 1981) or netosis (Beiter et al. 2006), and intracellular or extracellular killing of the bacteria, respectively.

Antigenic variation in *S. pneumoniae* has been known for more than 100 years (Dochez and Gillespie 1913), and its significance in protective immunity has been known for nearly as long (Cooper et al. 1932). As a predominantly extracellular pathogen, the most important mechanism of adaptive immunity to *S. pneumoniae* is mediated by antibodies: individuals deficient in anti-pneumococcal IgG are especially susceptible to invasive infection, and administration of anti-pneumococcal IgG reduces the risk (Chapel et al. 1994). The dominant target of protective antibodies against *S. pneumoniae* is the bacterial surface polysaccharide capsule (Avery and Heidelberger 1925), which exists in ~90 antigenically distinct forms.

Studies performed to evaluate the efficacy of vaccination with *S. pneumoniae* capsular polysaccharide antigens have provided strong evidence for antigenic variation, driven by

population immunity. Introduction of a pneumococcal polysaccharide conjugate vaccine containing seven antigenic types markedly reduced the frequency of invasive infections due to the antigenic variants included in the vaccine in several distinct populations (Feikin et al. 2013; Hsu et al. 2009; Pichon et al. 2013; Richter et al. 2013). This indicates that the vaccine was highly efficacious in producing protection against infection, and thus exerted strong immunological selection pressure on the bacterial population. However, each of these studies also revealed increases in the frequency of infections due to *S. pneumoniae* expressing polysaccharide antigens that were not included in the vaccine, indicated that antigenic variation allows escape from vaccine-induced immunity (Feikin et al. 2013; Hsu et al. 2009; Pichon et al. 2013; Richter et al. 2013).

Unlike in influenza virus or HIV, antigenic variation in *S. pneumoniae* is not predominantly due to immune selection of point mutants, but is the result of horizontal gene transfer, in which a given virulent bacterial strain acquires a genomic locus for biosynthesis of an antigenically distinct capsular polysaccharide from another strain (Wyres et al. 2013). Although capsular antigen switching by horizontal gene transfer is characteristic of *S. pneumoniae*, selection pressure exerted by vaccine-induced immunity amplifies the effect and impact of this mechanism of antigenic variation (Croucher et al. 2011).

9.2.4 Antigenic Variation in Other Major Global Pathogens

Although the mechanisms and significance of antigenic variation are best characterized for the examples described above, antigenic variation is a common strategy in multiple other pathogens (Table 9.1). The importance of antigenic variation to pathogen survival, replication, and transmission is demonstrated by its employment by a broad range of pathogens, including viruses, intracellular and extracellular bacteria, and intracellular and extracellular eukaryotic parasites. Moreover, multiple molecular mechanisms underlie antigenic variation, including nucleotide substitution (point mutation), gene conversion, recombination to place different copies of a multigene family into a transcriptionally-active site, alternative transcription without recombination, and transcriptional selection by phase variation. Finally, antigenic variation involves targets of antibody, CD4, and CD8 T cell recognition. It is also likely that additional examples and mechanisms for this biologically important pathogen strategy remain to be discovered.

9.3 *M. tuberculosis* Immune Evasion

As a highly successful pathogen, *M. tuberculosis* possesses numerous mechanisms for manipulating and modulating immune responses to optimize its survival, replication, and transmission. Unlike viruses, bacteria such as *M. tuberculosis* can respond to distinct environmental conditions and signals to optimize their gene expression and deploy mechanisms that optimally suit the bacteria in a given context. For example, during certain parts of the infection cycle, the bacteria may benefit from going undetected by host mechanisms, while during other parts of the infection cycle, *M. tuberculosis* may gain most by inducing vigorous inflammatory and immune responses (Ernst 2012). While a comprehensive review of mechanisms of immune evasion is beyond the scope of this

chapter, selected examples are given below, to provide context for the main points of the chapter.

9.3.1 *M. tuberculosis* Manipulation of Innate Immunity

Innate immune responses provide rapidly-available responses to the presence of diverse pathogens, including *M. tuberculosis*. In particular, innate immune responses to bacteria, including *M. tuberculosis*, include production of proinflammatory and regulatory cytokines, as well as chemotactic cytokines (also known as chemokines). Chemokines and their receptors govern the types and numbers of cells recruited to a site of infection or inflammation, and contribute to early responses to mycobacterial infection. As one example, C-C Chemokine Receptor-2 (CCR2), which is expressed by mononuclear cells and is important for their trafficking to sites of inflammation, plays a complex role in innate immune responses to *M. tuberculosis*, as it is essential for control of infection but also paradoxically contributes to pathogenesis. In the former case, CCR2-dependent mononuclear cell recruitment to the lungs after *M. tuberculosis* infection is crucial for accumulation of mononuclear cell-derived dendritic cells in the lungs (Peters et al. 2001, 2004), which in turn become infected and are required for activation of antigen-specific CD4 T cells that ultimately control infection (Wolf et al. 2007, 2008). In the latter case, *M. tuberculosis* infection uses CCR2-dependent monocyte recruitment to generate a population of monocyte-derived cells that actively supports intracellular bacterial growth and spread (Antonelli et al. 2010). The factors that determine whether the host-beneficial or the pathogen-beneficial effects of CCR2-dependent cell trafficking predominate remain to be determined. Together, these data indicate that innate immune responses induced by *M. tuberculosis* that involve CCR2-dependent cell recruitment are critical determinants of the course of infection. It is not surprising, then, that pathogenic mycobacteria possess mechanisms to manipulate cell recruitment to their own advantage.

Mycobacterial manipulation of monocyte/macrophage recruitment is mediated by masking of bacterial Toll-like receptor agonist molecules by the lipoglycan, pthiocerol dimycoceroserate (PDIM), thus reducing recruitment of macrophages with mycobactericidal potential (Cambier et al. 2014). While using PDIM to reduce recruitment of mycobactericidal macrophages, mycobacteria use surface phenolic glycolipid (PGL) for CCR2-dependent recruitment of monocyte/macrophages that support intracellular growth of the bacteria and thereby promote infection (Cambier et al. 2014).

In an additional mechanism to manipulate innate immune responses for its own benefit, *M. tuberculosis* uses a cytoplasmic signalling pathway involving the DNA sensor, cyclic GMP-AMP synthase (cGAS) and its downstream signalling molecule, stimulator of interferon genes (STING) to induce expression of type I interferon (Watson et al. 2015; Wiens and Ernst 2016). Type I interferons act as regulatory cytokines, and are implicated in promoting progressive infection with *M. tuberculosis* at least in part by suppressing expression of the proinflammatory cy-tokine, interleukin-1 (Mayer-Barber et al. 2011, 2014). Type I interferons regulate a large number of genes, and an interferon-responsive transcriptional signature is found in the context of active tuberculosis in humans (Berry et al. 2010; Bloom et al. 2012; Maertzdorf et al. 2011; Ottenhoff et al. 2012).

9.3.2 *M. tuberculosis* Manipulates the Intersection of Innate and Adaptive Immunity

In experimental infections with *M. tuberculosis*, the bacterial population expands in the lungs until the onset of adaptive (e.g., T cell) responses, which are able to arrest progressive growth of the bacteria (Wolf et al. 2008). A remarkable characteristic of tuberculosis in humans and in experimental animals is that the time required for development of T cell responses is much longer than for T cell responses to other infections. In humans with close, but limited (≤24 h), exposure to a person with active tuberculosis, the average time to develop a detectable cellular immune response is 6 weeks (Poulsen 1950); this contrasts with an average of 7 days for humans to generate T cell responses after experimental exposure to a live virus (the vaccine strain of yellow fever virus) (Miller et al. 2008). The onset of CD4 and CD8 T cell responses after aerosol infection with *M. tuberculosis* is similarly delayed, and averages approximately 17 days (Chackerian et al. 2002; Wolf et al. 2008), compared with 3–5 days after experimental influenza infection (Lawrence and Braciale 2004; Lawrence et al. 2005). The rate-limiting step in priming naive antigen-specific CD4 T cells after aerosol infection of mice with *M. tuberculosis* is the acquisition of bacteria by dendritic cells in the lungs, which happens 8–10 days after aerosol infection (Blomgran et al. 2012; Blomgran and Ernst 2011; Wolf et al. 2007). This is followed by dendritic cell transport of the bacteria from the lungs to the local lymph node (Wolf et al. 2008) and transfer of bacterial antigens (but not the bacteria) (Samstein et al. 2013; Srivastava and Ernst 2014) to resident dendritic cells, which process and present the mycobacterial antigens to antigen-specific CD4 T cells. The latter step (transfer of bacterial antigens from migratory to resident dendritic cells) compensates for *M. tuberculosis* inhibition of MHC class II antigen presentation by the infected cells themselves, but the compensation is incomplete (Srivastava et al. 2016). In mice, the long delay before appearance of antigen-specific T cells in the lungs allows ~40,000-fold expansion of the bacterial population, indicating that *M. tuberculosis* benefits from delaying the onset of T cell responses.

The mechanisms of immune evasion and manipulation by *M. tuberculosis* given above are only a partial sampling of the numerous mechanisms employed by this persistent pathogen. Additional mechanisms, and a comprehensive review of mechanisms of immunity to *M. tuberculosis* in humans and experimental animals are found in (O'Garra et al. 2013).

9.4 Antigenic Variation in *M. tuberculosis*

The well-documented ability of *M. tuberculosis* to survive in immunocompetent hosts and reactivate to cause active disease decades later (Lillebaek et al. 2002), together with evidence that infection with *M. tuberculosis* does not reliably confer protection from reinfection (Caminero et al. 2001; du Plessis et al. 2001; Interrante et al. 2015), indicates that this pathogen has a very effective armamentarium for evading the human immune responses that successfully eradicate other pathogens. Considering the numerous precedents provided by pathogens that evade immunity through antigenic variation, it is important to understand the role of antigenic variation in the success of *M. tuberculosis* as a pathogen.

9.4.1 Initial Studies of Antigen Sequence Variation in *M. tuberculosis*

Early evidence that antigenic diversity may be low in *M. tuberculosis* was derived from sequence analysis of 24 antigen-coding genes in 16 strains of *M. tuberculosis* (Musser et al. 2000). That study found that 19 of the 24 genes studied lacked any sequence variation; this included 5 of the 8 PE or PPE genes selected for analysis. Although the study was limited in its scope and it did not distinguish antigens recognized by antibodies or by T cells, or focus on specific epitopes in the proteins analyzed, it provided valuable information regarding the sequence diversity of the *M. tuberculosis* antigens that were known at that time.

Among the unique insights gained from the genome sequence of *M. tuberculosis* H37Rv was the discovery of the genes encoding proteins of the PE/PPE family, including the PE_PGRS family (Cole et al. 1998). Based on precedents in other pathogens, the presence of this large family of proteins suggested that one of their functions was in antigenic variation. This hypothesis prompted several analyses of sequence variation in individual members of the *pe_pgrs* family. Analysis of *pe_pgrs33* in 123 strains revealed a higher frequency of variants than in other *M. tuberculosis* genes studied. Using the H37Rv reference sequence, variants of *pe_pgrs33* were found in 84 of the 123 strains, and represented 25 distinct variants (Talarico et al. 2005). Of the 25 variants, 13 involved insertions or deletions (collectively termed indels), and 12 were single nucleotide polymorphisms (SNPs). All of the insertions or deletions were found in the PRGS domain, as were 9 of the 12 SNPs; synonymous and nonsynonymous SNPs were equally frequent. Similar observations were obtained from sequencing the *pe_pgrs16* and *pe_pgrs26* genes in 200 *M. tuberculosis* isolates (Talarico et al. 2008). While these genes were similar to each other and to *pe_pgrs33* in their frequency of sequence variants (indels and SNPs), they differed in the effect of their indels: in 40% of the strains, the indels in *pe_pgrs16* caused frameshifts, while the indels in *pe_pgrs26* caused frameshifts in only 2% of the strains. Together, these studies provided evidence for a high frequency of sequence variants in members of the *pe_pgrs* gene family, although they did not reveal whether the sequence variants altered immune recognition of the proteins by either antibodies or T cells. A subsequent analysis of sequence variants of the *ppe18* gene, combined with independently-reported T cell epitope mapping data, predicted that some, but not all, of the nSNPs were contained in epitope domains of PPE18 (Hebert et al. 2007).

9.4.2 Genomewide Evaluation of Antigenic Variation in *M. tuberculosis*

Two major advances have facilitated comprehensive, high-resolution analysis of antigenic variation in *M. tuberculosis*, at least for human T cell recognition. The first advance is the wide availability of economical genome sequencing and bioinformatic analyses, which is dealt with in depth in other chapters in this volume. The other advance is the development and readily-accessed Immune Epitope Database (IEDB; www.iedb.org), funded by the U.S. National Institutes of Health. The IEDB identifies, stores, and provides analytical tools for T cell and B cell epitopes from a variety of sources of antigens, including infectious agents such as *M. tuberculosis*. As of August, 2016, the IEDB contains information on 2148 human T cell epitopes of *M. tuberculosis*, derived from 440 antigens. The sources of epitope data in the IEDB include published papers, meeting abstracts, and the results of several separately-funded epitope discovery projects. The availability of information on *M. tuberculosis* T cell

epitopes in the IEDB has markedly facilitated studies revealing the frequency and nature of antigenic variation in this organism. The paucity of information on B cell antigens and epitopes of *M. tuberculosis* limits the availability of this knowledge in the IEDB, so the preponderance of analyses have focused on T cell epitopes. T cell epitopes are short (8–20 amino acids) peptide fragments of proteins (in the present case, from *M. tuberculosis*) generated by intracellular (host) proteolysis, bound to specific MHC (HLA in human) class I or class II alleles, for recognition by CD8 or CD4 T cells, respectively.

The first genomewide analysis of human T cell epitope sequence diversity examined 491 epitopes (from 78 antigens) available in the IEDB in 21 strains representative of the six major global lineages of the *M. tuberculosis* complex (MTBC) known at the time (Comas et al. 2010). Generation and comparison of the genome sequences of these strains (plus that of *M. canettii*) revealed an expected result: that essential genes (as defined in Sassetti et al. 2003; Sassetti and Rubin 2003) are more conserved than are nonessential genes. The unexpected result was that the great majority (468 of 491; 95%) of the experimentally-verified human T cell epitopes analyzed are completely conserved, with no amino acid sequence variants in these representative strains of the MTBC. Additional analyses revealed that the ratio of the rates of nonsynonymous SNPs (nSNPs) to synonymous SNPs (sSNPs) (dN/dS) is lower for the identified T cell epitopes than for nonessential genes, and is lower for the epitope than for the nonepitope domains in their source protein. These findings indicate that the known human T cell epitopes of *M. tuberculosis* are not under diversifying selection, implying that little, if any, selection pressure is exerted on these epitopes by human T cell recognition. This unique finding, that antigenic variation is rare in *M. tuberculosis*, provides evidence that the nature of the interaction of this pathogen with its human hosts is distinct from that of other pathogens, and that principles established with other pathogens may not reliably apply to *M. tuberculosis*.

9.4.3 PE_PGRS Proteins as Variable Antigens

A limitation of the aforementioned study is that the sequencing technology used generates short reads, and this prohibited analysis of epitopes located in protein members of large families with long stretches of sequence identity, such as the PE/PPE genes. As a consequence, it remained possible that the PE/PPE family is a source of antigenic (and epitope) diversity that could not be identified with the genome sequencing approach that was used. This possibility was especially plausible, in light of the evidence discussed above that certain members of the PE_PGRS family exhibit a high frequency of sequence variation.

To determine whether PE_PGRS proteins are a source of antigenic variation in *M. tuberculosis*, we used Sanger dideoxynucleotide chain termination sequencing to analyse 27 *pe_pgrs* genes in 94 phylogenetically diverse strains of *M. tuberculosis* (Copin et al. 2014). These analyses revealed that the nucleotide diversity in the *pg_pgrs* gene family is higher than the genome average in the MTBC, consistent with the results of the studies of *pe_pgrs16*, *pe_pgrs26*, and *pe_pgrs33* (Talarico et al. 2005, 2008). However, the comparison of 27 *pe_pgrs* genes revealed previously-unsuspected properties of this gene family. First, the individual members of the *pe_pgrs* family differ greatly in their nucleotide diversity, with some members (e.g., *pe_pgrs38*) much more diverse ($\pi > 0.0015$) than the genomewide

average for the MTBC ($\pi \sim 0.0003$), while other members are even more conserved than the genome as a whole (e.g., *pe_pgrs23*; $\pi < 0.00006$). By this analysis, which does not take indels into account, the previously-characterized *pe_pgrs26* gene is among the group with the highest nucleotide diversity ($\pi \sim 0.00089$), while *pe_pgrs33* is close to the genome average. The vast range of nu cleotide diversity extended to a vast range of ratios of substitution rates at nonsynonymous and synonymous sites (dN/dS): as extreme examples, *pe_pgrs59* exhibited evidence of exceptionally strong purifying selection (dN/dS ~0.12), while *pe_pgrs23* is under potent diversifying selection (dN/dS ∞; no sSNPs identified). In a similar manner, individual members of the *pe_pgrs* family exhibited a wide range of frequencies of indels; there was little concordance between nucleotide diversity and indel diversity in these genes. Notably, nSNPs and indels were clustered in the PGRS domains of the proteins, while the N-terminal (~110 amino acid) PE domains were significantly more conserved.

To directly determine whether the PE_PGRS proteins are a source of antigenic variation in *M. tuberculosis*, we used bioinformatic analyses to predict human T cell epitopes, using HLA alleles found at the highest frequencies in six major human population groups. This revealed that the great majority of the predicted epitopes are found in the PE domain, with very few in the PGRS domain of any of the proteins (Fig. 9.2). The validity of the epitope predictions was confirmed using synthetic peptides designed from the sequences of the predicted epitopes in the PE domains, which activated T cells from blood of patients with active pulmonary tuberculosis (Copin et al. 2014). Together the sequencing and epitope prediction efforts revealed that, while structural variants of PE_PGRS proteins are common, they are clustered in the PGRS domains, while the human T cell epitopes are clustered in the PE domains.

These findings indicated that the PE_PGRS proteins are not a major source of variation of human T cell epitopes, in accord with the findings that human T cell epitopes in *M. tuberculosis* are highly conserved. However, the findings are consistent with the possibility that the PE_PGRS proteins are a source of antigenic variation for antibody recognition, as other studies have determined that the PGRS domains are targets of antibodies in humans infected with *M. tuberculosis* (Cohen et al. 2014; Delogu and Brennan 2001; Koh et al. 2009).

9.4.4 Esx Proteins as Variable Antigens

The short-read genome sequencing technology used in the original study of epitope diversity also necessitated exclusion of certain *Esx* genes, which encode closely-related immunogenic low molecular weight secreted proteins (Comas et al. 2010). An independent study of these proteins in 108 phylogenetically-diverse strains used Sanger dideoxynucleotide chain termination sequencing to identify sequence variants in 23 genes of the *Esx* family (Uplekar et al. 2011). This revealed an overall SNP frequency similar to the *M. tuberculosis* genomewide average, and identified a total of 109 unique SNPs (50 sSNPs and 59 nSNPs) in th 23 genes. *Esx* genes in the ESX-1 to ESX-4 secretion system loci contained few SNPs, while genes that belong to the Mtb9.9 and QILSS subfamilies, including those in the ESX-5 secretion system locus, contained the majority of the variants. Coincidentally, these latter

subfam ilies exhibit an especially high degree of amino acid sequence similarity (93–98%) in paralogous protein members. Comparison of the identified nSNPs with previously-identified human T cell epitopes in Esx proteins revealed one amino acid substitution in an epitope in EsxB (CFP-10); otherwise there were no sequence variants found in the 80 identified epitopes in EsxA (ESAT-6) or EsxB. In contrast, of the 13 epitopes identified in the other members of the Esx protein family, 9 contained one or more amino acid substitutions, including 1 in EsxH, which was also identified in the initial genomewide epitope analysis (Comas et al. 2010). This study provided valuable insight into sequence variation in the Esx proteins that was not revealed by the initial genomewide epi-tope analysis. It confirmed that the high level of epitope conservation extends to epitopes in the immunodominant EsxA and EsxB proteins, and revealed the presence of potentially-important sequence variants in epitopes of other members of the Esx protein family.

9.4.5 Population genomics and computational and experimental immunology to identify and quantitate human T cell epitope sequence variation in the *M. tuberculosis* complex

An additional limitation to the first genomewide analysis of human T cell epitope variation is that it used a database of experimentally-verified peptide epitopes, and was thus subject to factors that might skew epitope discovery toward conserved epitopes. For example, studies of *M. tuberculosis* antigen or epitope discovery typically use the H37Rv strain (from Lineage 4) as the source of antigen or sequences for preparation of synthetic peptides or recombinant antigens. If studies of T cell recognition or epitope mapping are performed with cells from individuals infected with bacteria from a different lineage (e.g., Lineage 6), then antigens or epitopes that are conserved in Lineages 4 and 6 are most likely to be discovered.

To circumvent the potential for epitope discovery favoring conserved epitopes, we took an alternative approach, using comparative genomics to identify the most diverse regions of the *M. tuberculosis* genome, reasoning that antigens or epitopes undergoing diversifying selection by T cell recognition would exhibit nucleotide diversities and dN/dS ratios higher than the genomewide average.

To identify and enumerate potential epitopes undergoing diversifying selection by human T cell recognition, we first analyzed 3774 coding regions in newly-generated whole genome sequences of 216 human-adapted strains representative of the seven main lineages of the *M. tuberculosis* complex (Coscolla et al. 2015), and identified loci with the highest nucleotide diversity in the 216 genomes. We then focused our analyses on coding regions exhibiting high rates of nSNPs. For this, we selected the most variable 5% of the genes (N = 189) and determined that the dN/dS in these genes ranged from 0 to 4.21; 88 of the 189 genes showed a dN/dS > 1, indicating evidence of diversifying selection. From these 88 genes, we selected those with at least one nSNP present in an entire MTBC lineage; this narrowed the analysis to seven genes, which are associated with various functional categories, though none of the protein products are known or predicted to be secreted.

To explore whether sequence diversity in the seven genes of interest could be related to human T cell recognition, we first computationally predicted human CD4 and CD8 T cell epitopes in the protein products of these genes using HLA class I and class II alleles that are prevalent in diverse human populations. This predicted a mean of 207 high-affinity epitopes

per protein for HLA class I and 150 epitopes per protein for HLA class II; comparison of the locations of the predicted epitopes and the nSNPs in the seven genes of interest revealed that 51 of the 56 (91%) nSNPs identified coincided with predicted CD4 and/or CD8 T cell epitopes. We then examined the impact of the identified nSNPs (and corresponding amino acid substitutions) on the capacity of the predicted epitope peptides to bind to selected HLA alleles. On average, a given naturally-occurring amino acid substitution decreased the binding of 25% and 18% of the predicted peptide:HLA class I and peptide:HLA class II interactions, respectively. The naturally occurring sequence variation could also lead to an increase in the number of HLA alleles capable of binding the corresponding epitope peptide, indicating that the naturally-occurring sequence variants in predicted epitopes in the protein products of the seven genes of interest could result in either loss or gain of recognition by human T cells.

Since computational prediction of epitopes has greater sensitivity than specificity, it was essential to determine whether the new predicted T cell epitopes we identified with naturally-occurring sequence variants are genuinely recognized by T cells of humans with tuberculosis. Of the putative epitopes we identified, we selected those predicted to bind to HLA class I and class II alleles prevalent in the Gambia, and studied their ability to stimulate interferon gamma production in a modified diluted whole blood assay, performed in the Gambia using cells from 82 individuals with active pulmonary tuberculosis. For this experimental verification, we used 30 peptides corresponding to 14 ancestral and 16 variant sequences from the seven proteins of interest. Fifty-two (63%) of the subjects responded to at least one candidate epitope peptide; cells from individual subjects responded to an average of 3 of the 30 candidate epitope peptides (ancestral and/or variant form), although cells of some subjects responded to as many as 22 of the peptides. An especially important finding is that an average of 72% of the responding subjects for a given candidate epitope exhibited differential responses to the ancestral compared with the variant sequences of each of the 14 candidate epitopes, indicating that the naturally-occurring amino acid substitutions in these epitopes have a functional impact on human T cell recognition and activation.

The most important conclusions from this study are the confirmation that the majority of human T cell epitopes in *M. tuberculosis* are conserved, and that a small number of antigens and epitopes in this pathogen exhibit evidence of diversifying selection and antigenic variation. Therefore, unlike other pathogens that employ antigenic variation as a mechanism to evade human adaptive immune responses, antigenic variation appears to be exceptional in *M. tuberculosis*.

9.5 Practical Implications

The findings that antigenic variation is unusual, and has little if any effect on the widely-studied immunodominant protein antigens of *M. tuberculosis*, has practical implications that differ in specific contexts.

In the context of immunodiagnostic assays, whether they are designed to detect the antigens or responses to the antigens, antigen and epi-tope conservation implies that specific diagnostic reagents can be considered universal. That is, antigenic variation has little, if any,

effect on the sensitivity of assays that employ a single antigenic sequence. While this can be a confident conclusion for assays involving T cell recognition and/or antigens identified specifically as T cell antigens, studies to date are insufficient to conclude that the same rule would apply for assays that detect antibody responses.

The identification of rare antigens and epitopes that do exhibit naturally-occurring sequence variation may provide opportunities to enhance studies of TB epidemiology and transmission, especially in regions where strains from several bacterial lineages coexist. For example, it is currently only possible to detect reinfection with *M. tuberculosis* if a person progresses to active disease and the disease-causing isolate can be identified and found to be distinct from the isolate that caused an initial episode of disease. It is possible that use of the newly-identified antigens and epitopes with sequence variants can be used in T cell assays to detect reinfection with a new strain based on T cell responses to distinct epitope variants, even in the absence of progression to active disease. Development and validation of such assays will require close collaborations between individuals with expertise in optimizing conditions and readouts in T cell assays and experts in TB epidemiology.

The practical implications of antigen and epi-tope conservation for TB vaccine development are complex. On the surface, that there is little sequence variation in the immunodominant antigens of *M. tuberculosis* that are included in current candidate TB vaccines could be regarded as a favorable finding. That is, the results imply that it may not be necessary to develop distinct TB vaccines for different populations, and that a polyvalent TB vaccine is unnecessary, to account for bacterial strain-dependent antigenic variation. However, the finding that most currently-favored TB vaccine antigens are not under diversifying selection pressure from human T cell recognition may be interpreted as evidence that *M. tuberculosis* has effective mechanisms to disarm T cell responses to these antigens, potentially limiting the efficacy of vaccine-induced T cells. Whether this should be taken into account and given significant weight in TB vaccine development deserves in-depth consideration and experimental study. One consideration is that one or more of the seven newly-discovered antigens that exhibit naturally- occurring sequence diversity might actually be superior TB vaccine antigens, in light of the evidence that their recognition is sufficient to select escape mutants. In that vein, the finding that none of the identified epitopes in those antigens has more than three sequence variants implies that the bacteria have limited options for immune escape by further mutation and antigenic variation.

The suggestion that antigen and epitope conservation prevails in *M. tuberculosis* because human T cell recognition of these antigens provides an evolutionary advantage to the bacteria also deserves consideration and in-depth study, to avoid pursuing TB vaccine strategies that induce T cell responses that benefit the bacteria (for example, by promoting transmission).

9.6 Remaining Questions and Future Directions

Although studies of antigenic variation and immune escape in the genomic era of tuberculosis research have revealed considerable valuable and unexpected information, many important matters are only partially understood.

First, it is of utmost importance to determine whether the low frequency of antigenic variation in the majority of *M. tuberculosis* antigens is truly due to little diversifying selection pressure exerted by human T cell recognition. If this is so, then it will be especially important to identify the most important mechanisms used by *M. tuberculosis* to evade effector T cell responses and to develop ways to overcome or circumvent them.

Second, it is important to determine whether human T cell responses to *M. tuberculosis* antigens that do exhibit evidence of escape mutation are more advantageous to the host than are responses to conserved antigens. This may be studied in at least two ways. One is through prospective studies of individuals with latent TB, to determine whether T cell responses to the variable antigens are more advantageous than T cell responses to conserved antigens, as manifest by differential association with subsequent progression to active TB disease. Another is through experimental vaccine studies in relevant model systems, in which protection conferred by responses to vaccines containing different antigens can be assessed.

Third, it is currently difficult to reconcile *M. tuberculosis* T cell epitope conservation with the vast diversity of HLA alleles and differential HLA allele frequencies in distinct populations. On the surface, it would seem that a given epi-tope might be conserved in a population with a given prevalent HLA allele, but that same epitope would be subject to genetic drift in a population where that HLA allele is rare or absent. One potential explanation for this is that conservation of epitope sequences is attributable to mechanisms other than T cell recognition, that is, that *M. tuberculosis* epitopes have low inherent mutational tolerance (Thyagarajan and Bloom 2014). While there is currently no direct evidence that allows exclusion of this as a predominant mechanism for epitope sequence conservation, several observations weigh against it: *M. tuberculosis* T cell epitopes are not concentrated in essential genes, nor are they disproportionately located in or near functional protein domains such as enzyme active sites (Comas et al. 2010); and *M. tuberculosis* T cell epitopes are found in diverse structural domains of proteins, including beta sheets, alpha helices, and loops, implying that their ability to tolerate sequence variants is not constrained by protein structural requirements (J. Ernst, unpublished observation). One alternative possibility is that *M. tuberculosis* epitopes are more promiscuous for binding diverse HLA alleles than are epitopes in other pathogens (McKinney et al. 2013; Paul et al. 2015), and that HLA allele diversity has little influence on responses to *M. tuberculosis* T cell epitopes. This latter possibility is consistent with the observation that no single HLA class I or class II locus or allele is reproducibly associated with susceptibility or resistance to tuberculosis.

Fourth, rigorous studies of the importance of antibody responses in the immune control of tuberculosis are needed, to determine whether the principles recently established for *M. tuberculosis* T cell epitopes also apply to B cell/antibody epitopes, and whether TB vaccines that induce antibody responses should be a high priority for development. The finding that the PGRS domains of certain PE_PGRS proteins are under potent diversifying selection by a process other than T cell recognition, coupled with the evidence that PGRS domains are targets for antibody recognition, suggests that certain of these proteins are worthy of greater investigation as potential TB vaccine antigens.

In conclusion, *M. tuberculosis* differs from other successful pathogens in the rarity of its employment of antigenic variation. Further investigation is needed to understand the basis of antigen and epitope conservation in *M. tuberculosis*, and to exploit the resulting knowledge to impact the global epidemic of human TB.

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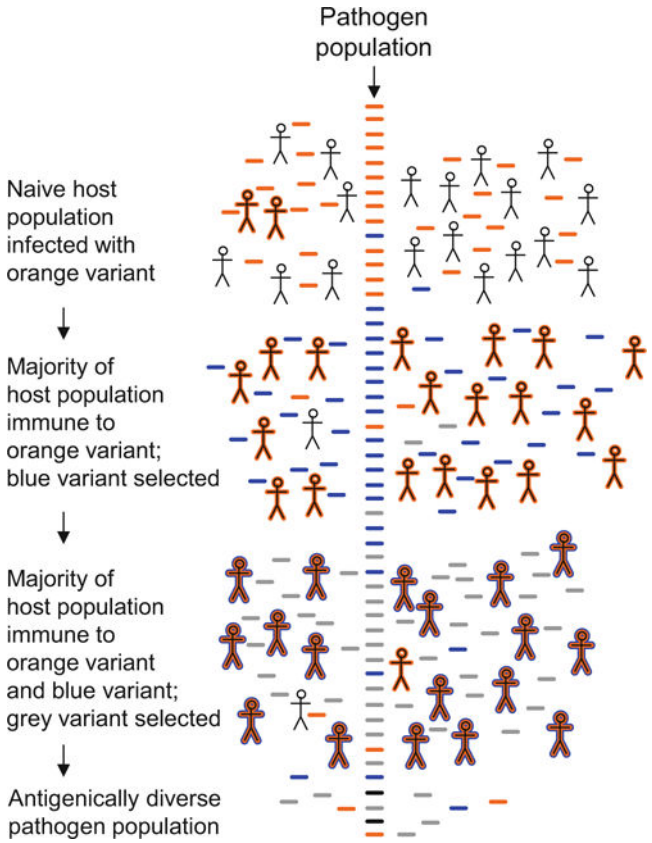

Fig. 9.1.

Antigenic variation allows a pathogen to infect hosts immune to original pathogen variant. In this example, a pathogen (*orange*) is able to infect immunologically naive hosts, but not hosts immune to the orange pathogen variant. Blue pathogen variant arises, and can infect host population largely immune to the orange pathogen variant. Infection with blue variant causes additional immunity to blue variant, yet allows infection with grey variant. In each population of hosts, a minor subset of hosts may remain nonimmune, and these can sustain minor variants in the pathogen population (indicated by the mix of colored variants after three rounds of infection and antigenic variation)

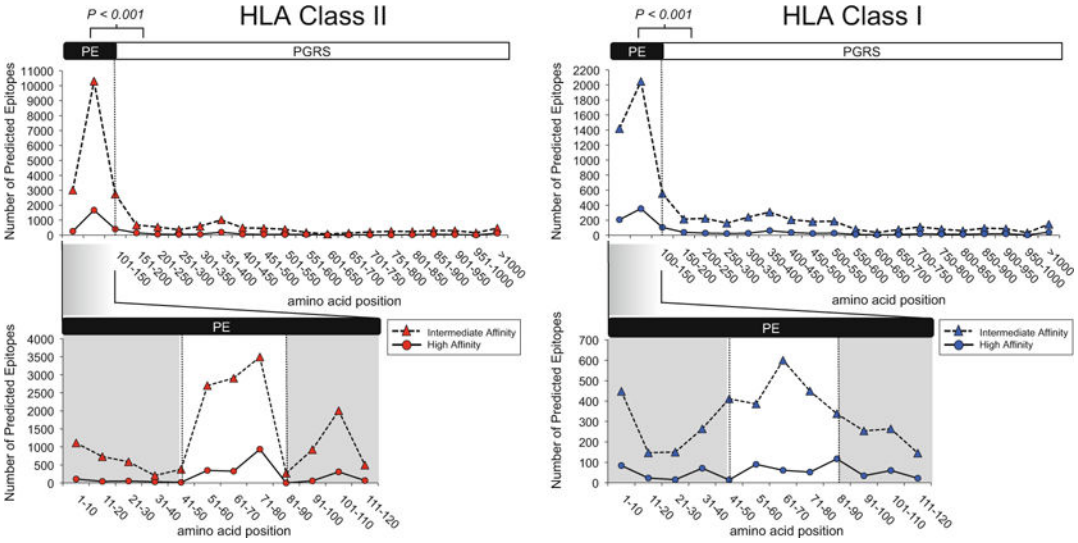

Fig. 9.2.

Human T cell epitopes are concentrated in the PE domain of PE_PGRS proteins. The amino acid sequences of all 64 annotated PE_PGRS were used for in silico epitope prediction. The graph represents the number of high binding affinity (IC50 <50 nM; *solid line*) and intermediate binding affinity (IC50 <500 nM; *dashed line*) epitopes for the selected CD4 (MHC class II and CD8 (MHC class I alleles, by amino acid position of the proteins). The lower part of each panel shows an expanded view of the PE domain containing a high density of predicted epitopes (Adapted from MBio 5 (1):e00960–00913)

Table 9.1

Antigenic variation in other human pathogens

Pathogen	Mechanism of protective immunity	Antigenic target(s)	Mechanism of antigenic variation	References
Hepatitis C virus	CD8 T cells	Multiple structural and nonstructural proteins	Point mutations	Erickson et al. (2001), Kasprowicz et al. (2010), and Nivarthi et al. (2014)
Neisseria gonorrhoeae	Antibodies	Lipooligosaccharide (LOS)	Phase variation	Yang and Gotschlich (1996)
		Opacity (Opa) proteins	Multiple loci with phase variation	Bhat et al. (1991)
		Pilin	Recombination	Segal et al. (1986)
Streptococcus pyogenes	Antibodies	M protein	Point mutations	Lannergard et al. 2011; Persson et al. (2006)
		SMEZ protein		Hoe et al. 1999; Proft et al. (2000)
Borrelia relapsing fever agents (e.g., *B. hermsii*)	Antibodies	Vlp and Vsp lipoproteins	Gene conversion; alternative transcription of multicopy genes	Barbour et al. (2006), Plasterk et al. (1985), and Stoenner et al. (1982)
Borrelia Lyme disease agents (e.g., *B. burgdorferi*)	Antibodies	VlsE lipoprotein	Gene conversion	Lawrenz et al. (2004) and Liang et al. (2004)
Anaplasma phagocy- tophilum/marginale	Antibodies, CD4 T cells	MSP2	Segmental gene conversion	Brown et al. (2003)
Trypanosoma brucei	Antibodies	Surface glycoprotein	Recombination; transcription switching	Morrison et al. (2009)
Plasmodium falciparum	Antibodies	Pfemp1	Multicopy genes with transcriptional switching	Claessens et al. (2014), Peters et al. (2002), and Ralph et al. (2005)