Antigens for CD4 and CD8 T Cells in Tuberculosis

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Tuberculosis (TB), caused by infection with *Mycobacterium tuberculosis* (MTB), represents an important cause of morbidity and mortality worldwide for which an improved vaccine and immunodiagnostics are urgently needed. CD4⁺ and CD8⁺ T cells play an important role in host defense to TB. Definition of the antigens recognized by these T cells is critical for improved understanding of the immunobiology of TB and for development of vaccines and diagnostics. Herein, the antigens and epitopes recognized by classically HLA class I- and II-restricted CD4⁺ and CD8⁺ T cells in humans infected with MTB are reviewed. Immunodominant antigens and epitopes have been defined using approaches targeting particular TB proteins or classes of proteins and by genome-wide discovery approaches. Antigens and epitopes recognized by classically restricted CD4⁺ and CD8⁺ T cells show extensive breadth and diversity in MTB-infected humans.

Mycobacterium tuberculosis (MTB), the intracellular bacterium that causes tuberculosis (TB), was discovered in 1882 by Robert Koch and is responsible for more human deaths than any other single pathogen today (WHO 2011). One-third of the world's population is infected by MTB, each year more than 1.5 million people die of TB, and more than 9 million develop TB (WHO 2011). Conquering this staggering problem is further complicated by the increasing prevalence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) MTB strains (Gandhi et al. 2010) and recently virtually untreatable totally drug-resistant (TDR) strains (Velayati et al. 2009).

T-cell responses are essential for TB immunity, primarily because of the intracellular lifestyle of MTB. Both CD4⁺ Th1 cells and CD8⁺ T cells produce IFN- γ , which has been shown to be critical for protection in the murine TB model and for immune control in MTB-infected humans (Grotzke and Lewinsohn 2005; Flynn 2006; Winslow et al. 2008). A key role for IFN- γ in the control of TB is also clearly shown by increased susceptibility to TB in mice with a disrupted IFN- γ gene and in humans with mutations in genes involved in the IFN- γ and IL-12 pathways (Cooper et al. 1993; Flynn et al. 1993; de Jong et al. 1998; Dorman and Holland 2000). Because of this, IFN- γ production by T

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cells has been a critical criterion for antigen discovery.

Many antigens have been identified and characterized, both classically HLA class I- and II-restricted (Blythe et al. 2007), as well as restricted by nonclassical molecules CD1 (Sieling et al. 1995), MR1 (Gold et al. 2010; Gold and Lewinsohn 2013), and HLA-E (Heinzel et al. 2002). MTB has evolved many strategies that subvert and evade the host adaptive response (Baena and Porcelli 2009). Because of the complexity of TB disease and diversity of donors, it is challenging to find antigens that are recognized by the majority of MTB-infected humans.

Defining the repertoire of antigenic targets is central to understanding the immune response against TB, and it has been vigorously pursued. Identification of novel epitopes and antigens from MTB is important because they can be used for identification and design of new vaccine candidates, diagnostics (including diagnostics to assess vaccine take), and markers to follow treatment response. Here we discuss discovery approaches and describe TB antigens and epitopes recognized by human classically restricted CD4⁺ and CD8⁺ T cells.

ROLE OF CD4⁺ T CELLS IN CONTROLLING TUBERCULOSIS

Early murine studies and evidence from HIV infection have proved an essential role for CD4⁺ T cells in the control of MTB infection. This was shown by antibody depletion of CD4⁺ T cells (Muller et al. 1987), by adoptive transfer of CD4⁺ T cells (Orme and Collins 1983, 1984), and in gene-disrupted mice (Caruso et al. 1999). In the case of HIV infection, loss of CD4⁺ T cells results in progressive primary TB infection, reactivation of latent TB infection (LTBI), and enhanced susceptibility to reinfection (Barnes et al. 1991; Hopewell 1992; Ravigliione et al. 1995). Strikingly, the risk for HIV⁺TST⁺ (tuberculin skin test) subjects to develop TB disease is 8%–10% annually compared with a 10% lifetime risk for HIV[−]TST⁺ individuals (Selwyn et al. 1989). Because these early experiments showed a dominant role for CD4⁺ T cells in controlling TB infection, CD4 antigens have been more exten-

sively characterized than CD8 antigens (Skjot et al. 2001; Reed and Lobet 2005).

PROTEIN-BASED ANTIGEN DISCOVERY

In the early 1990s attempts were made to dissect the secreted MTB proteome (Nagai et al. 1991). Traditional biochemical methods for separation and antigen discovery identified many immunodominant antigens from complex mycobacterial protein mixtures, abundant or easily purified proteins (Boesen et al. 1995; Covert et al. 2001; Andersen and Doherty 2005). A short-term culture filtrate was defined that was enriched in secreted antigens (Andersen et al. 1991). These secreted antigens were shown to offer a CD4⁺ T-cell-dependent protective effect following vaccination in mice and guinea pigs (Hubbard et al. 1992; Pal and Horwitz 1992; Andersen 1994). This in turn led to a focus on antigen discovery in culture filtrates also strengthened by the notion that in contrast to live BCG (Bacillus Calmette–Guérin) vaccine, immunization with killed BCG confers little or no protection against infection with MTB, thus giving rise to the theory that active secretion of antigenic proteins induces appropriate protective T-cell responses (Orme et al. 1993; Horwitz et al. 1995). Recently it was shown that mycobacteria secrete membrane vesicles rich in antigens (Prados-Rosales et al. 2011). It is now clear, however, that T-cell antigens are also found among nonsecreted antigens. Several of the antigens identified early are now pursued as candidates for a TB vaccine, such as one of the most studied antigens from the mycobacterial secretome, antigen 85A, part of the antigen 85 complex of proteins (Ag85 A, B, and C) (Andersen and Doherty 2005).

Traditional methods rely on isolation of antigens from protein mixtures, offer limited proteome coverage, and depend on relative quantity and amenability to standard purification techniques. The completion of the MTB genome sequence (Cole et al. 1998) made more rational antigen discovery methods possible. Several studies have used bioinformatical approaches to select a subset of genes as antigen candidates (Bertholet et al. 2008; Chegou et al.

2012) or have screened MTB expression libraries in *Escherichia coli* with T-cell clones derived from individuals with LTBI (Coler et al. 2009). In only a few cases have techniques allowing ex vivo detection and/or characterization of MTB-specific T cells before any in vitro expansion and manipulations been used (Lalvani et al. 2001; Pathan et al. 2001; Lindestam Arlehamn et al. 2012, 2013).

EPITOPE DISCOVERY BY TARGETED APPROACHES

Several studies have reported the identification of T-cell epitopes from MTB in a wide variety of cohorts including individuals with TB disease, LTBI, BCG vaccination, or exposure to MTB and/or nontuberculosis mycobacteria (NTM, also known as environmental mycobacteria, atypical mycobacteria, and mycobacteria other than tuberculosis) (Vordermeier et al. 2003; Hammond et al. 2005; McMurry et al. 2005, 2007; Mustafa and Shaban 2006; Vani et al. 2006; Roupie et al. 2007; Lindestam Arlehamn et al. 2012). These studies have used either a classical approach using overlapping peptide pools covering a specific protein (Brock et al. 2004; Mustafa et al. 2005; Axelsson-Robertson et al. 2012) or reverse immunology based on bioinformatics tools followed by in vitro confirmation by demonstration of peptide-epitope-expanded T cells (Hammond et al. 2005; McMurry et al. 2007). However, similarly to antigen discovery efforts, these studies are restricted to a limited number of genes or HLA molecules resulting in a limited coverage of the MTB proteome (Blythe et al. 2007; Axelsson-Robertson et al. 2012). For the majority of antigens only very limited number of epitopes have been identified, whereas for others, such as ESAT-6 and CFP10 (Arend et al. 2000; Mustafa et al. 2000), a large number (>20) of epitopes have been described (Blythe et al. 2007; Axelsson-Robertson et al. 2012).

PROTEIN CATEGORIES OF DESCRIBED ANTIGENS

T-cell antigens have been described from all main MTB protein categories, indicating that

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protein function or cellular location per se does not determine which proteins can be recognized. Two important protein categories are the PE/PPE proteins and the Esx protein family, which have both been shown to elicit humoral and cellular immune responses (Akhter et al. 2011; Sampson 2011). PE/PPE proteins are found exclusively in pathogenic mycobacteria. The function(s) of PE/PPE proteins are not fully understood, but data indicates that they influence bacterial attachment to host cells, immunomodulation, antigen presentation, and host cell apoptosis (Akhter et al. 2011; Sampson 2011). The PE/PPE genes encode almost 200 proteins and constitute almost 10% of the TB genome coding capacity (Cole et al. 1998). They are categorized based on their proline-glutamic acid (PE) and proline-proline-glutamic acid (PPE) motifs near their amino termini (Sampson 2011). PE/PPE proteins are mainly located within the bacterial cell wall and cell surface; however, some are also secreted, thus readily accessible to the immune system (Abdallah et al. 2009).

The PE/PPE genes are closely related to the Esx regions. Esx regions encode Type VII secretion systems (or ESX systems) and include well-known antigens such as ESAT-6 (early secretory antigenic target-6, EsxA) and CFP10 (culture filtrate protein 10, EsxB) (Gey van Pittius et al. 2006). Secretion of some PE/PPE proteins is dependent on ESX systems. All ESX systems, five complete and six incomplete systems in TB, carry a pair of genes that encode homologs of ESAT-6 and CFP10, and most of them also encode PE/PPE proteins (Gey van Pittius et al. 2001). Despite these similarities these systems do not complement each other and play distinct roles in TB virulence and physiology (Gey van Pittius et al. 2001).

Attenuation of BCG resulted in major deletions of 129 open reading frames (ORFs), among which were the RD1 region containing ESAT-6 and CFP10. The genes in this region play an important role in virulence, as shown by attenuation of TB with a knockout in the RD1 region (Andersen and Doherty 2005). The antigens from RD1 are strongly recognized by a majority of MTB-infected individuals, and

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commercial tests for LTBI (QuantiFERON and T-SPOT.TB) use ESAT-6 and CFP10 as antigens (Lalvani et al. 1998, 2001; Ulrichs et al. 1998; Ravn et al. 1999; Arend et al. 2000; Mustafa et al. 2000; Olsen et al. 2000; Pathan et al. 2000; van Pinxteren et al. 2000; Aagaard et al. 2004; Brock et al. 2004; Mori et al. 2004; Shams et al. 2004). These tests marked a major advance in diagnosis of LTBI compared with the classically used TST, which is not able to distinguish between LTBI and BCG vaccination. In addition to BCG, ESAT-6 and CFP10 are also absent from the majority of NTM, providing an ability to distinguish LTBI not only from BCG vaccination but also some of the NTM diseases (Harboe et al. 1996; Lalvani et al. 1998; Behr et al. 1999).

METABOLIC STATE-SPECIFIC ANTIGENS

During infection, MTB alters its metabolic state from active replication to slow or nonreplication, which is accompanied by changes in the gene expression profile and thus protein expression and antigens available to the immune system (Schnappinger et al. 2003). Expression of early secreted antigens decrease, whereas antigens encoded during starvation and dormancy increase (Leyten et al. 2006; Rustad et al. 2008; Schuck et al. 2009). Furthermore, the MTB genome encodes a large number of regulatory proteins (Cole et al. 1998), which indicates an ability to adapt to different environments by altered gene expression (Yuan et al. 1996; Triccas and Gicquel 2000; Florczyk et al. 2001; Honer zu Bentrup and Russell 2001; Mehrotra and Bishai 2001). Other antigens have been described as “resuscitation antigens” (Commandeur et al. 2011b), which are small bacterial proteins that promote proliferation of dormant mycobacteria, and are therefore believed to be involved in the reactivation of MTB. The discovery of these metabolic state-specific antigens provided the hypothesis that these might be preferentially recognized by certain stages of infection.

DISEASE STAGE-SPECIFIC ANTIGENS

The identification of disease stage-specific antigens is difficult (Honer zu Bentrup and Rus-

sell 2001), but there is evidence of stage-specific antigens in humans (Wilkinson et al. 1998; Leyten et al. 2006). Initial studies used MTB grown under conditions that mimic the environmental stresses believed to be encountered in granulomas such as hypoxia, low pH, NO, nutrient deprivation, and host immune pressure (Yuan et al. 1996; Florczyk et al. 2001; Monahan et al. 2001). Under these conditions MTB is believed to be in a dormant state and genes encoded by the DosR regulon are up-regulated. Several antigens encoded by this regulon have been described as preferentially recognized by individuals with LTBI (Black et al. 2009; Schuck et al. 2009), but other studies failed to detect this correlation (Gideon et al. 2010). The resuscitation antigens also do not distinguish LTBI from TB disease. The difficulty in identifying disease stage-specific antigens is complicated by the spectrum of infection ranging from sterilizing immunity, to subclinical active disease to fulminant active disease (Young et al. 2009). Furthermore, hypoxic lesions are found in both LTBI and TB disease (Barry III et al. 2009; Young et al. 2009). The relative scarcity of data relating to disease stage-specific antigens may also be related to the lack of a genome-wide screen of MTB reactivity in individuals with different stages of MTB infection and disease.

GENOME-WIDE ANTIGEN DISCOVERY

MTB has a very large genome with around 4000 ORFs (Cole et al. 1998), and identification of T-cell antigens/epitopes from such a large genome is especially challenging, yet necessary for vaccine development, vaccine trials, and disease monitoring. Several investigations have tried to identify immunodominant antigens through genomics and proteomics (Covert et al. 2001; Malen et al. 2008; Deenadayalan et al. 2010), comparative genomics (Cockle et al. 2002), or bioinformatics (Zvi et al. 2008), none of which covered the entire genome. The majority of the MTB genome was covered in an investigation for in vivo expression of MTB genes during MTB infection in the lungs of mice (Commandeur et al. 2013). Identified antigens were confirmed in humans with TB disease and may

represent antigen candidates for vaccine development.

Advances in high-throughput peptide synthesis had made it possible to perform a genome-wide analysis for MTB antigens (Lindestam Arlehamn et al. 2013). Taking advantage of computational prediction methods and high-throughput assays to identify immunodominant epitopes and antigens, a synthetic peptide library was constructed following predictions for binding affinity to common DR, DP, and DQ HLA class II alleles representative of those most commonly expressed in the general population, using a consensus approach based on three prediction methods (Wang et al. 2008). The advantage of this approach is that it identifies the optimal set of peptide candidates for immunogenicity testing, eliminating the necessity of synthesizing a large number of overlapping peptides and, more importantly, circumvents the need to test each one of them for binding to numerous HLA class II molecules in vitro. Circulating T cells from individuals with LTBI were then directly tested against the peptide library using high-throughput IFN- γ ELISPOT (enzyme-linked immunosorbent spot [assay]), which is in contrast to other studies in which in vitro expansion is typically performed. The results published recently identified hundreds of new CD4 epitopes and several antigens. Analysis of responses revealed that natural immunity to MTB is multiantigenic and enriched for responses to secreted and cell wall-associated proteins such as PE/PPE and ESX-associated proteins, consistent with earlier studies as described above. However, both secreted and nonsecreted proteins were shown to induce a strong immune response. A large fraction of the identified PE/PPE proteins were novel, which implies that there is still a lot to discover. The prevalence of responses to PE/PPE and ESX proteins could represent cross-reactive epitopes shared by multiple homologous family members (Sayes et al. 2012). It has been shown that T-cell responses were greater to peptide pools representing the conserved amino-terminal region of PE/PPE proteins than to peptide pools representing other regions of the protein (Vordermeier et al. 2012).

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The same phenomenon has not yet been shown for ESX proteins. In addition, the genome-wide screen identified three distinct antigenic regions all containing Esx protein pairs and two containing type VII secretion systems ESX-1 and ESX-3.

High-throughput approaches do not capture all immunodominant antigens. In this respect, it is important to highlight that a number of studies have shown that many peptides with highly promiscuous binding capacity are frequently recognized by immune individuals (Alexander et al. 1994; Lamonaca et al. 1999; Doolan et al. 2000; Wilson et al. 2001; Tangri et al. 2005), and that promiscuous recognition in the context of multiple HLA class II molecules may be a mechanism significantly contributing to epitope immunodominance (Lindestam Arlehamn et al. 2012). Studies have showed that bioinformatics predictions directed toward selection of the most promiscuous binding peptides can allow identification of a dominant fraction of the pathogen-specific response (Assarsson et al. 2008; Oseroff et al. 2010, 2012a,b).

Two studies have used analysis of serum antibody responses, as a surrogate for CD4⁺ T-cell responses, to interrogate the entire MTB proteome for antigens (Kunnath-Velayudhan et al. 2010; Li et al. 2010). These studies relied on the assumption that there is a strong linkage between targets of antibodies and of the CD4⁺ Th cells involved in their generation (Sette et al. 2008). In these studies ~6%–10% of the proteome was found to be immunogenic and also enriched for secreted and cell wall-associated proteins. In particular these studies also identified a prominence of responses to PE/PPE and ESX protein families, emphasizing the immunodominance of these proteins.

The comprehensive proteome-wide screens for epitopes and serum antibody responses revealed striking levels of heterogeneity of responses to MTB in humans. This might reflect differences in MTB strains, bacillary load, and metabolic state, resulting in qualitative or quantitative differences in antigen expression.

Once defined, T-cell epitope responses can be characterized by several different approaches, including determination of the secreted cyto-

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kine patterns by intracellular cytokine staining, characterization of the specific T-cell subsets generated in response to the epitopes by the use of tetramer staining reagents, and characterization of memory phenotypes. Importantly, definition of T-cell epitopes allows in-depth characterization and tracking of responding T-cell subsets. The genome-wide screen for CD4⁺ T-cell epitopes documented the novel observation that T cells in individuals with LTBI are confined to a recently described CXCR3⁺ CCR6⁺ phenotype (Acosta-Rodriguez et al. 2007; Gosselin et al. 2010; Lindestam Arlehamn et al. 2013).

ROLE OF CD8⁺ T CELLS IN TB HOST DEFENSE

As discussed above, control of MTB infection relies heavily on the cellular immune system—that is, the interaction of lymphocytes and MTB-infected macrophages and dendritic cells (DCs) (Flynn and Chan 2001; North and Jung 2004). Although the essential role of CD4⁺ T cells in TB host defense was defined first, ample experimental evidence in the mouse TB model now suggests a protective role for CD8⁺ T cells as well. Adoptive transfer or in vivo depletion of CD8⁺ cells showed that this subset could confer protection against subsequent challenge (Muller et al. 1987; Orme 1987; Silva et al. 1994). β 2-microglobulin-deficient mice, deficient in expression of major histocompatibility complex (MHC) class I (Flynn et al. 1992), and CD8-deficient mice (Sousa et al. 2000) are more susceptible to MTB infection than their wild-type littermates. As reviewed above, because IFN- γ is an important TB host defense effector molecule and CD8⁺ T cells represent a cellular source of this cytokine, this set of experimental evidence also supports the importance of CD8⁺ T cells. Finally, evidence in both mice and non-human primates suggests that CD8⁺ T cells may play a unique role in the immune surveillance of chronic infection (Cooper 2009). In humans, MTB-specific CD8⁺ T cells have been identified in MTB-infected individuals and include CD8⁺ T cells that are classically, HLA class Ia-restricted, and nonclassically, HLA class Ib,

restricted by HLA-E (Lewinsohn et al. 1998; Heinzel et al. 2002) and by CD1 (Beckman et al. 1996; Rosat et al. 1999; Moody et al. 2000). CD8⁺ T cells share some redundant effector mechanisms with CD4⁺ T cells, such as IFN- γ secretion and cytolytic functions (Lewinsohn et al. 2011). However CD8⁺ T cells can also play a unique role in TB host defense. CD8⁺ T cells preferentially recognize and destroy heavily infected macrophages (Lewinsohn et al. 2003). This ability to distinguish heavily infected cells suggests that the magnitude of the CD8⁺ T-cell response may represent a sensor of intracellular burden. In support of this, the magnitude of the CD8⁺ T-cell response decreased in adults effectively treated for pulmonary TB (Nyendak et al. 2013), Rosat et al. showed higher CD8⁺ T-cell response in adults with pulmonary TB than with extrapulmonary TB and with extrapulmonary TB than with LTBI (Rosat et al. 1999), and the presence of detectable CD8⁺ T-cell responses in young children distinguished those with pulmonary TB from asymptomatic household contacts of adults with infectious TB (Lancioni et al. 2012). CD8⁺ T cells are also able to eliminate infected HLA class II-negative cells such as epithelial cells, endothelial cells, and fibroblasts, as well as inhibit mycobacterial growth and induce apoptosis in infected cells (Lazarevic and Flynn 2002). The role of apoptosis as a host defense mechanism has been highlighted by Behar and colleagues (Behar et al. 2010; Divangahi et al. 2010). Furthermore, components of the cytotoxic granule, such as granzysin, may play a direct role in the inhibition of MTB growth (Stenger et al. 1998). Taken together, studies of mice and humans support an important role for CD8⁺ T cells in TB immunity.

PROCESSING AND PRESENTATION OF TB ANTIGENS TO CLASSICALLY RESTRICTED CD8⁺ T CELLS

CD8⁺ T cells typically recognize short peptides of 9–11 amino acids in length derived from cytosolic proteins, such as viral proteins, that are presented by HLA class I molecules. That MTB antigen does get into the class I processing

pathway is evidenced by the induction of classically restricted CD8⁺ T cells in MTB-infected individuals. Yet how this occurs is not straightforward. Rather, MTB antigen likely accesses the class I processing pathway through multiple mechanisms, which include both pathways that include and do not include trafficking of antigenic peptide through the cytosol. Noncytosolic pathways include a TAP (transporter for antigen presentation)- and proteasome-independent MHC-I recycling pathway (Pfeifer et al. 1993; Song and Harding 1996) and cross-presentation through the uptake of exosomes including MTB lipids and proteins (Beatty et al. 2000, 2001; Beatty and Russell 2000) or of apoptotic bodies (Schaible et al. 2003). However, most evidence supports that antigenic peptide accesses the cytosol (Behar 2013). Specifically, evidence suggests that in primary human DCs, MTB phagosomes contain class I processing machinery, and antigens contained in the phagosome are retrotranslocated into the cytosol, processed in a proteasome- and TAP-dependent manner, and egress to the cell surface through the endoplasmic reticulum Golgi apparatus (Grotzke et al. 2009, 2010).

DEFINITION OF CD8 TB ANTIGENS

As described above, substantial effort has been dedicated to the definition of TB CD4 antigens and, more recently, epitopes. In contrast, much less effort has been expended to date on TB CD8 antigens, perhaps because the importance of CD8⁺ T cells in host defense to TB has been more recently defined. In fact, many investigations defining CD8 antigens have focused on MTB proteins already well characterized as CD4 antigens (Smith et al. 2000; Lewinsohn et al. 2007; Commandeur et al. 2011a). For example, Smith et al. investigated recognition of CD4 antigens Ag85A, Ag85B, and ESAT-6 by CD8⁺ T cells. Peripheral blood mononuclear cells (PBMCs) from BCG-vaccinated individuals or individuals with TB disease were expanded in vitro with MTB and then CD8⁺ T cells responding to antigen-presenting cells (APCs) expressing Ag85A, Ag85B, or ESAT-6 were measured using intracellular cytokine staining (ICS)

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with analysis by flow cytometry (Smith et al. 2000). CD8⁺ T cells from individuals with TB disease recognized all three antigens, whereas CD8⁺ T cells from healthy BCG-vaccinated individuals recognized only Ag85A and Ag85B. Lewinsohn et al. (2007) compared ex vivo CD8⁺ T-cell responses in individuals with LTBI, TB disease, or no infection using autologous DC displaying overlapping 15-mers representing each protein and CD8⁺ T cells positively selected from PBMC in an IFN- γ ELISPOT assay. In this study, CD8⁺ T cells recognizing known CD4 antigens Mtb39, CFP10, ESAT-6, Ag85, EsxG, 19 kDa, and Mtb 9.9a were detected only in MTB-infected individuals (LTBI and TB disease). Commandeur et al. (2011a) focused on another TB protein group of interest, proteins expressed from the DosR-regulon (Rv1733 and Rv2029c), and compared the CD8⁺ T-cell responses with those directed toward Ag85B and HspX (hsp 16, α -crystallin, Rv2031c) in a unique group of elderly individuals with LTBI who had never received TB therapy. Using ICS with analysis by flow cytometry and overlapping 20-mers as a source of antigen, they showed stronger IFN- γ /TNF- α producing CD8⁺ T cells to the DosR-regulon encoded proteins and HspX as compared with Ag85, and no CD8⁺ T-cell responses in MTB-uninfected individuals. In summary, study of CD8⁺ T-cell responses to entire MTB proteins has been fairly limited and shows that both BCG and MTB infection can induce CD8⁺ T cells to proteins shared by BCG and TB, and, as might be expected, only MTB infection induces CD8⁺ T-cell responses to proteins found only in MTB, such as CFP10 and ESAT-6.

DEFINITION OF CD8 TB EPITOPES: TARGETING MTB PROTEINS OF INTEREST

By far the preponderance of studies defining CD8 epitopes focus on MTB proteins of interest and use a “reverse immunogenetics” approach—that is, the MTB protein of interest is interrogated for peptides predicted to bind to a particular HLA allele, and then a CD8⁺ T-cell response restricted by this HLA allele is shown

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in MTB-infected individuals. In these studies MHC restriction is showed using tetramers or, as in the case of HLA-A*02:01, by recognition of T2 cells or THP-1 cells only expressing A*02:01. In fact, perhaps because of the availability of these reagents, and that A*02 is a commonly expressed allele with a well-defined motif, HLA-A*02:01 has been the most thoroughly studied. HLA-A*02:01-restricted CD8⁺ T-cell responses within MTB-infected individuals have been shown in epitopes contained within a variety of proteins including secreted proteins Ag85B (Weichold et al. 2007), ESAT-6 (Lalvani et al. 1998; Axelsson-Robertson et al. 2013), TB10.4 (Axelsson-Robertson et al. 2010), Mtb19 (Mohagheghpour et al. 1998), SodA (Dong et al. 2004), AlaDH (Dong et al. 2004), Glns (Dong et al. 2004), 38-kDa antigen (Shams et al. 2003), and 28-kDa hemolysin (Shams et al. 2003); PE/PPE family members PE_PGRS33, PE_PGRS62, and PPE46 (Chaitra et al. 2008); and proteins expressed during hypoxia, the 16-kDa antigen (Caccamo et al. 2002), and Rv1733c (Commandeur et al. 2011a). Additional HLA-A alleles have been interrogated for MTB proteins of interest and CD8 epitopes defined. These include A*24:02, A*30:01, A*30:02, A*68:01, and A*68:02-restricted epitopes in ESAT-6 (Pathan et al. 2000; Axelsson-Robertson et al. 2013), A*01:01, A*03:01, A*11:01, A*24:02, A*30:01, A*30:02, and A*68:01-restricted epitopes in TB10.4 (Axelsson-Robertson et al. 2011, 2013), and A*01:01, A*11:01, A*24:02, A*30:01, A*30:02, and A*68:01-restricted epitopes in Ag85B (Weichold et al. 2007; Axelsson-Robertson et al. 2013). HLA-B alleles have been less frequently interrogated by this approach. However, several HLA-B-restricted CD8 epitopes have been defined, including B*52, B*07:02, and B*58:01-restricted epitopes in ESAT-6 (Lalvani et al. 1998; Axelsson-Robertson et al. 2013), B*07:02, B*08:01, B*15:01, and B*58:01-restricted epitopes in TB10.4 (Axelsson-Robertson et al. 2010, 2013), and B*07:02, B*08:01, B*15:01, and B*58:01-restricted epitopes in Ag85B (Weichold et al. 2007; Axelsson-Robertson et al. 2013).

One measure of immunodominance is the detection of strong (high-magnitude) CD8⁺ T-

cell responses directly ex vivo in MTB-infected individuals. In this regard, using the “reverse immunogenetics” approach, several studies report strong ex vivo CD8⁺ T-cell responses as have been observed for common viruses (Yewdell and Bennink 1999), in MTB-infected individuals using IFN- γ ELISPOT (Lalvani et al. 1998; Pathan et al. 2000), ICS with analysis by flow cytometry (Caccamo et al. 2002), or tetramer analysis (Weichold et al. 2007; Axelsson-Robertson et al. 2010, 2011, 2013). However, in many studies ex vivo CD8⁺ T-cell responses were not performed (Mohagheghpour et al. 1998; Shams et al. 2003; Dong et al. 2004; Chaitra et al. 2008) requiring establishment of T-cell lines or clones in vitro to define the epitope, suggesting that these do not represent immunodominant epitopes.

Although all studies reported here isolated CD8⁺ T cells recognizing these epitopes from MTB-infected individuals, only a few studies looked at specificity—that is, whether or not epitope-specific T cells could be detected in individuals without MTB infection—and all showed the absence of epitope-specific CD8⁺ T-cell responses in uninfected individuals (Mohagheghpour et al. 1998; Pathan et al. 2000; Caccamo et al. 2002; Dong et al. 2004). However, the majority of studies only looked at MTB-infected individuals. This is especially problematic for the interpretation of the studies by Axelsson-Robertson et al. (2011, 2012, 2013) who reported particularly broad and numerous epitopes within a few MTB proteins, some of which were detected at relatively low frequencies. Using tetramer analysis, these studies did not look at uninfected individuals, and either did not study MTB-infected individuals with HLA-type mismatched for the tetramer HLA allele, or when MTB-infected individuals with mismatched HLA alleles were investigated, reactivity in these HLA-mismatched individuals was detected, although attributed to promiscuous epitopes. Nonetheless, when taking the literature in aggregate, CD8⁺ T cells are readily detected in MTB-infected individuals and CD8 epitopes are present in almost every MTB protein investigated.

DEFINITION OF CD8 TB EPITOPES: GENOME-WIDE APPROACH

With the sequencing of the MTB genome completed (Cole et al. 1998), two more recent studies have interrogated the entire MTB genome for CD8 epitopes. Caccamo et al. (2009) evaluated the MTB genome for 9-mer peptide sequences predicted to bind to A2*01 and then showed binding of peptides predicted to bind with the highest affinity. Six peptides showing the highest binding affinity for A2*01 were tested using tetramers in individuals with TB and LTBI. Using this approach they reported two new CD8 epitopes restricted by A*02:01 in Rv1490 and Rv1614. Moreover, these epitopes were observed in those with TB and LTBI but not in uninfected individuals. In addition, Tang et al. (2011) screened the entire H37Rv genome for 9-mers predicted to bind to A*02:01, A*03:01, and B*07:02, evaluating predicted affinity as well as processing and presentation. Screening more than 400 peptides in individuals with LTBI, the highest frequencies of responses were found within secreted proteins and within MTB proteins commonly used in TB vaccines. From these data 18 tetramers were selected and 16 of 18 tetramers detected epitope-specific CD8⁺ T cells in 16 distinct MTB proteins, many of which have unknown function.

DEFINITION OF CD8 TB EPITOPES: USING T CELLS RECOGNIZING THE MTB-INFECTED CELL

An alternative approach to CD8 epitope discovery uses CD8⁺ T cells derived from MTB-infected individuals that recognize an MTB-infected cell. The advantage of this approach is that it is unbiased with regard to the HLA restricting allele. First, to identify the MTB protein recognized, CD8⁺ T-cell clones, derived from individuals with LTBI and isolated using MTB-infected DC, were screened against either a limited synthetic peptide library comprised of 15-mers overlapping by 11 amino acids representing known CD4 antigens (Lewinsohn et al. 2001, 2007) or a more comprehensive peptide library representing 10% of the MTB proteome select-

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ed for potential antigenicity (Lewinsohn et al. 2013). Peptide pools were deconvoluted to identify the 15-mer recognized, and then nested peptides within the 15-mer were used to identify the minimal epitope. Using this approach, 17 epitopes were defined located in several previously defined CD4 antigens (CFP10, EsxG, Mtb8.4, and Ag85), as well as in EsxJ and members of the PE/PPE family of MTB proteins (PE9 and PE_PGRS). Strikingly, using this approach unbiased for HLA alleles, all but one of these epitopes are restricted by a diverse set of HLA-B alleles. Thus, further delineation of TB epitopes will require careful evaluation of HLA-B alleles. Finally, these epitopes were recognized at high frequency in individuals from whom they were recognized, suggesting that this approach was efficient at identifying immunodominant epitopes.

CONCLUDING REMARKS

For both CD4⁺ and CD8⁺ T-cell responses in TB, there is extensive diversity of immunodominant responses in infected individuals. For both CD4 and CD8 antigens, secreted antigens are important. Moreover, there is extensive overlap between defined CD4 and CD8 antigens. However, this is likely biased by the CD8 discovery process, which at least initially, focused on previously defined CD4 antigens.

Several important gaps remain to be addressed in the definition of CD4 and CD8 TB antigens, particularly in regard to the spectrum, magnitude, and phenotypes of T-cell responses elicited by different disease stages and vaccination strategies. First, given the breadth and diversity of immunodominant antigens defined thus far, and the relatively limited number of studies using genome-wide approaches, there likely remain more CD4 and CD8 TB antigens and epitopes to be defined. In addition, studies comparing the antigenic specificity, magnitude, and phenotype of T-cell responses in different stages of MTB infection, from LTBI to subclinical disease to advanced TB disease, remain limited. Thus far, neither CD4 nor CD8 antigen recognition can distinguish those with LTBI from those with TB disease. In this regard, there

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may be antigens and/or epitopes that are specifically recognized in a particular disease stage or a particular phenotype associated with a disease stage. Moreover, different disease stages may show distinct composite immunologic signatures. Regardless, more extensive definition of T-cell antigens and epitopes are likely to inform immunologic definition of disease stages and may be used to develop accurate correlates of protective immunity to use in TB vaccine studies and/or immunodiagnostics to identify individuals who may benefit from early therapy.

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