19

Identification of CD8$^+$ T Cell Epitopes Against *Mycobacterium tuberculosis*

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1. Introduction

Although the effective immune response against *Mycobacterium tuberculosis* (*M. tuberculosis*, Mtb) is primarily due to cell-mediated immunity, the mechanisms by which T cells participate in the control of infection are still not completely understood. The importance of CD8$^+$ T cells in the immune response to tuberculosis has been recognized by many researchers [1]. Experimental evidence from the murine model indicated that CD8$^+$ T cells could be important to the control of *Mycobacterium tuberculosis* infection *in vivo* [2-5]. The isolation of *M. tuberculosis*-specific CD8$^+$ T cells from infected mice and human clearly showed that this subset could be induced during infection [6-8]. Reports on epitope-specific *M. tuberculosis* reactive CD8$^+$ T cells, which are present at very high frequencies in the peripheral blood of PPD positive individuals and patients with active tuberculosis, support the importance of CD8$^+$ T cells to the immunity of *M. tuberculosis* and emphasize that the CD8$^+$ T cell subset should be considered to the design of new anti-tuberculosis vaccines [9, 10]. CD8$^+$ T cells may contribute to the control of *M. tuberculosis* infection through four mechanisms: (1) Cytokine release, such as IFN-γ and TNF-α; (2) Cytotoxicity via granule-dependent exocytosis pathway; (3) Cytotoxicity mediated through Fas/Fas ligand interaction; (4) Direct microbicidal activity.

The pathogen's antigens are processed by antigen presenting cells (APCs) and digested into short peptides. Consequently, these non-self peptides are presented to the surface of the infected cells or APCs through the loading of the major histocompatibility complex (MHC) or called human leukocyte antigen (HLA) in human. These peptide/MHC complexes are recognized by specific T-cells that perform immune responses, such as cytotoxicity (in the case of class I MHC). These peptides which act as the markers of the pathogens are called T cell epitopes [11]. The identification of novel cytotoxic T lymphocyte (CTL) epitopes is important to the analysis of the involvement of CD8$^+$ T cells in *M. tuberculosis* infection as well as the anti-Mtb vaccine development. However, only a small number of MHC-I-restricted CTL epitopes have been identified within a few of *M. tuberculosis* proteins. The step to develop epitope-based vaccines include: (1) antigen selection; (2) epitope prediction; (3) epitope identification; (4) vaccine prototype engineering; (5) immunization and challenge studies. In this chapter we mainly focused on the strategy to identify CD8$^+$ T cell epitopes against *Mycobacterium tuberculosis*. It

includes: selection of the antigens; Prediction the $CD8^+$ T cell epitopes; Synthesis and modification of the epitopes; Identification of epitopes by *in vitro* and *in vivo* assays; Development of $CD8^+$ T cell epitope-based vaccines.

2. Selection of antigens from *Mycobacterium tuberculosis*

An essential step towards the development of novel vaccines against Mtb is gaining more information on the antigenic architecture of *Mycobacterium tuberculosis* to identify T-cell epitopes responsible for eliciting protective immune responses. Determination of the complete genome sequence of *Mycobacterium tuberculosis* facilitated this step considerably. The H37Rv strain of *Mycobacterium tuberculosis* has been found extensive, worldwide application in biomedical research because it has retained full virulence in animal models. Cole et al reported the complete genome sequence of H37Rv in 1998 [12]. This progress in genomics makes it possible that we can find corresponding protein sequences through submitting to publicly accessible sequence databases such as GenBank and TB database [13].

The Mtb genome contains about 4,203 open reading frames, nearly half of which are categorized as 'hypothetical proteins' with unknown function. Although secreted proteins are generally associated with protective immune responses, no proteins or protein families have yet been shown to fully protect against the active Mtb infection. Screening each protein from these 4,023 open reading frames by using human serum and gel electrophoresis would be time-consuming and costly method of identifying new vaccine candidates. Bioinformatics tools provide more practical means to rapidly identify potential epitopes and antigens for Mtb vaccines development. A range of approaches have been utilized to select proteins for analysis prior to epitope mapping. For example, whole genome analysis can be performed in order to identify candidate vaccine components, or alternatively, bioinformatics tools that select proteins according to their secretion characteristics can be used to narrow the number of proteins evaluated in the next step [14-16]. Frieder et al used pepmixes created by Micro-Scale SPOT™ to map T cell epitopes in 389 proteins of *Mycobacterium tuberculosis* [17]. These proteins are sorted by their known or likely known function such as PPE/PE, cell wall, cell processes, virulence, detoxification, and adaptation. Some $CD8^+$ T cell epitopes identified by researchers were listed in **Table 1.**

So far, antigens in *Mycobacterium tuberculosis* from which $CD8^+$ T cell epitopes were identified are mainly focused on secretory proteins, lipoproteins, such as Ag85-complex, ESAT-6, CFP10, 16kDa protein, 19kDa lipoprotein and so on. But there are few studies focused on the membrane proteins like the drug efflux pumps which play important role in the drug-resistant of *Mycobacterium tuberculosis.* Researchers in our lab tried to identify novel $CD8^+$ T cell epitopes from these antigens and found several novel $CD8^+$ T cell epitopes which could elicit potent immune responses both *in vitro* and *in vivo* [32].

3. Prediction of the $CD8^+$ T cell epitopes

The lack of simple methods to identify relevant T-cell epitopes, the high mutation rate of many pathogens, and restriction of T-cell response to epitopes due to human lymphocyte

Protein name	Gene description	Amino acid position	HLA allele	Frequency	References
Ag85A	mycolyltransferases	48-56 242-250	HLA-A2 HLA-A2	1/20,693PBMCs (Elispot) 1/3,300 PBMCs (tetramer) 1/23,779 PBMCs (Elispot) 1/3,750 PBMCs (tetramer)	[18]
Ag85B	mycolyltransferases	143–152 199–207 264–272	HLA-A2 HLA-A2 HLA-B35	ND Undetectable (tetramer) ND	[19, 20]
Ag85C	mycolyltransferases	204–212	HLA-B35	1/13,700–1/22,200 PBMCs	[20]
ESAT-6	Early secretory antigen target	69–76 82–90 21–29	HLA-B53 HLA-A2 HLA-A6802	1/700 $CD8^+$ 1/2,500 PBMCs 1/2,100 $CD8^+$	[21, 22]
CFP10	10kDa culture filtrate antigen	2–9 2–12 3–11 75–83 2–11 49–58 85–94 71–79 76–85	HLA-B4501 HLA-B4501 HLA-B0801 HLA-B1502 HLA-B44 HLA-B44 HLA-B3514 HLA-B14 HLA-B3514	1/101 $CD8^+$ 1/125 $CD8^+$ 1/645 $CD8^+$ 1/145 $CD8^+$ 1/700 $CD8^+$ 1/7,000 $CD8^+$ 1/2,100 $CD8^+$ 1/438–1/1,602 PBMCs 1/437–1/2,427 PBMCs	[11, 23, 24]
CFP21	cutinase precursor	134-142	HLA-A2	3/5000-1/10000 PBMCs ((Elispot))	[25]

Protein name	Gene description	Amino acid position	HLA allele	Frequency	References
Mtb8.4	low molecular weight T-cell antigen	5–15 32–40	HLA-B1501 HLA-B3514	1/10,416 $CD8^+$ 1/1,190 $CD8^+$	[23]
Mtb9.8	Hypothetical protein	3–11 53–61	HLA-A0201 HLA-B0801	<1/25,000 $CD8^+$ 1/2,840 $CD8^+$	[23]
Mtb39	PPE family protein	144–153 346–355	HLA-B44 HLA-B44	ND ND	[26]
16kDa protein	heat shock protein	21–29 120–128	HLA-A2 HLA-A2	1/1,000 $CD8^+$ 1/800 $CD8^+$	[19]
19kDa lipoprotein	19 kDa lipoprotein antigen precursor	88–97	HLA-A2	ND	[27]
38kDa protein	periplasmic phosphate-binding lipoprotein	8–17	HLA-A0201	1/331 $CD8^+$	[28]
Hsp65	chaperonin	369–377	HLA-A2	ND	[29]
Rv0341	isoniazid inductible gene protein	33–42 33–44 33–45	HLA-A2 HLA-A2 HLA-A2	ND ND ND	[30]
Rv2903c	signal peptidase	201–209	HLA-B35	Undetectable	[31]
Rv1410c	aminoglycosides/tetracycline -transport integral membrane protein	510-518	HLA-A0201	3/2500 PBMCs	[32]
GlnA1	glutamine synthetase	308–316	HLA-A2	ND	[33]
SodA	superoxide dismutase	160–168	HLA-A2	ND	[32]
AlaDH	delta-aminolevulinic acid dehydratase	160–169	HLA-A2	ND	[32]
Hemolysin	cytotoxin/hemolysin	73–82	HLA-A0201	1/353 $CD8^+$	[28]

Table 1. $CD8^+$ T cell epitopes identified in *Mycobacterium tuberculosis*

antigen (HLA) polymorphism have significantly hindered the development of cytotoxic T-lymphocyte (CTL) epitope-based or epitope-driven vaccines. As the development of bioinformatics, computer-driven algorithms methods for predicting $CD8^+$ T cell epitopes are used as important tools to the vaccine design. These tools offer a significant advantage over other methods of epitope selection because high-throughput screening can be performed in silico, followed by consequent immunological experiments *in vitro* and *in vivo* [34]. Traditionally, the identification of T cell epitopes required the synthesis of overlapping

peptides that spanned the entire length of a protein, followed by experimental assays for each peptide, such as *in vitro* intracellular cytokine staining, to determine the T cell activation. This method is economically viable only for single protein or pathogen that consists of fewer proteins. As a result, alternative computational approaches have been developed for the prediction of T cell epitopes, which have significantly decreased the experimental burden that is associated with epitope identification. Computer-driven algorithms are now routinely employed to sort through protein sequences for linear strings of amino acids that confirm to previously established patterns known to be associated with the binding to antigen presenting molecules (such as MHC) and stimulation of T cells. These peptide strings, once proven to stimulate T cells *in vitro* or *in vivo*, are considered as T cell epitopes.

A lot of computational algorithms have been developed to predict CTL epitopes in pathogen protein sequences (some of these are listed in **Table 2**). In reality, using only one computational algorithm to predict CTL epitopes may lead to large number of false positives and false negatives. Later and more comprehensive validations using data from several different prediction softwares are accurate and of high value in reducing the cost for epitope identification. Researchers in our lab used several prediction servers and identified novel CTL epitopes successfully [25, 32].

4. Synthesis and modification of the epitopes

Solid phase peptide synthesis (SPPS) offers important advantages over the synthesis in solution, in which coupling reactions can be carried out more rapidly and nearly to completion using an excess of the activated amino acid derivative, which can be removed at the end of the reaction by simple washing operations. The introduction of Fmoc protecting group into SPPS by Carpino in 1970 allowed the entire process of SPPS to be carried out under milder reactive conditions [35]. As a result of this chemical progress, nowadays the synthesis of many peptides can be smoothly accomplished by manual or automate-assisted SPPS and even longer proteins can be synthesized by coupling protected segments or more efficiently by chemical ligation with unprotected peptide segments [36].

The immunogenicity of the native epitope is often weak. Substitutions at main anchor positions to increase the complementarity between the peptide and HLA binding cleft constitute a common procedure to improve the binding capacity and immunogenicity of the native epitope. In 1993, Ruppert et al determined that the 'canonical' HLA-A2.1 motif could be defined as leucine (L) or methione (M) at position 2 (P2) and leucine (L), valine (V), or isoleucine (I) at position 9 (P9) [37]. In 2000, Tourdot et al demonstrated that residue tyrosine (Y) at position 1 (P1) could enhance the affinity of epitope to HLA-I molecule [38]. These results suggested that an altered peptide ligand (APL) might be used to exploit a latent capacity of the T cell repertoire to respond more effectively to the native epitope. We also used this strategy to modify the related positions and found the binding capacity and immunogenicity of some epitopes improved [25, 32]. The second strategy is to modify the amino acids with a side chain protruding out of the peptide binding cleft of the HLA molecule, because these amino acids may participate in T cell receptor (TCR) recognition. If they are substituted by amino acids with other properties, a mutated epitope may bind more efficiently to TCR or elicit a more diverse TCR reservoir [39, 40].

ID	server	Abbreviation	Prediction algorithm
1	BIMAS	BIMAS	Matrix[a]
2	HLA Ligand	HLA_LI	Matrix[a]
3	IEDB (ANN)	IEDB_ANN	ANN[b]
4	IEDB (ARB)	IEDB_ARB	Matrix[a]
5	IEDB (SMM)	IEDB_SMM	Matrix[a]
6	MAPPP (Bimas)	MAPPP_B	Matrix[a]
7	MAPPP(SYFPEITHI)	MAPPP_S	Matrix[a]
8	MHC Binder Prediction	MHC_BP	Matrix[a]
9	MHC-BPS	MHC-BPS	SVM[c]
10	MHC-I (Multiple matrix)	MHCI_MM	Structure-based model
11	MHC-I (Single matrix)	MHCI_SM	Structure-based model
12	MHCPred (Interactions)	MHCP_I	Partial least square
13	MHCPred (Amino Acids)	MHCP_AA	Partial least square
14	MULTIPRED (ANN)	MULTI_ANN	ANN[b]
15	MULTIPRED (HMM)	MULTI_HMM	HMM[d]
16	MULTIPRED (SVM)	MULTI_SVM	SVM[c]
17	NetMHC (ANN)	NETM_ANN	ANN[b]
18	NetMHC (Weight Matrix)	NETM_WM	Matrix[a]
19	nHLAPred (ANNPred)	NHP_ANN	ANN[b]
20	nHLAPred (ComPred)	NHP_CP	ANN[b] and Matrix[a]
21	PepDist	PEPDIST	distance function
22	PeptideCheck (Matrix)	PEPC_M	Matrix[a]
23	Predep	PREDEP	Structure-based model
24	ProPred1	PROPRED	Matrix[a]
25	Rankpep	RANKPEP	Matrix[a]
26	SMM	SMM	Matrix[a]
27	SVMHC (MHCPEP)	SVMHC_M	SVM[c]
28	SVMHC (SYFPEITHI)	SVMHC_S	SVM[c]
29	SVRMHC	SVRMHC	SVM[c]
30	SYFPEITHI	SYFPEITHI	Matrix[a]

a: binding matrices
b: artificial neural networks
c: support vector machines
d: hidden Markov models

Table 2. Some epitope prediction servers

5. Identification of epitopes by *in vitro* and *in vivo* assays

The immunogenicity of epitopes predicted through appropriate prediction softwares should be identified by a series of *in vitro* and *in vivo* assays. Only epitopes which are proven to stimulate T cells *in vitro* or *in vivo* can be used as potential subunit components for the design of vaccines against tuberculosis. Because there are various techniques to identify specific T-cell epitopes, the question arises about how to select and combine these techniques to screen peptides more efficiently and rapidly.

According to the processing and presenting progress of T cell epitopes and their ability to specifically stimulate T cells in an MHC allele-restricted manner. The pathway of MHC-I eptiope presentation has been exhibited and the process comprise three main steps: firstly, endogenous proteins are digested into short peptides by proteasomes; secondly, special epitopes are selected to load onto MHC-I molecule within the endoplasmic reticulum; finally, the peptide/MHC complexes are transferred to cell surface and to stimulate specific T-cell receptors on T cells. Therefore, there are three essential elements for a random peptide to be considered as MHC-I restricted T cell epitope. First, the peptide must be naturally processed into an optimal length via a proteasome dependent pathway. Second, the peptide should have the affinity to bind to the corresponding MHC-I molecule. Finally, and perhaps the most important, the peptide must have the ability to induce a T-cell-specific response after its presentation on an MHC-I molecule [11]. Thus, the techniques to evaluate the three features of a given peptide can be classified into three corresponding types: MHC-binding assay; T cell specific antigenicity assay; Verification of the natural processing of the peptide.

5.1 MHC binding assay

Various methods have been developed to evaluate the binding affinity of peptides to MHC-I molecule. Standard peptide binding inhibition assay is a common method used to quantitatively measure the binding affinity of the target peptides [41]. The MHC-I transfected cell line binding assay was utilized in many labs for its simplicity, repeatability and quantifiability for determining peptide and MHC-binding affinities [25, 32].

5.2 T cell specific antigenicity assay

Peptides with atypical anchoring residues for a related MHC-I allele always display very low binding affinity for MHC-I molecule tested *in vitro*. However, some of these peptides may possess potent antigenicity to induce robust and specific T cell responses. Therefore, it is commonly considered that the most definitive means of defining an epitope is to test peptide-specific T cell responses. Numerous techniques are currently utilized in peptide specific T cell determination, including MHC tetramer staining, enzyme-linked immunospot (ELISPOT) assay, intracellular cytokine staining (ICS), cytotoxicity assay (such as lactate dehydrogenase release assay and ^{51}Cr release assay) and T-cell proliferation assay [18-33].

5.3 Verification of the natural processing of the peptide

Some artificially synthesized peptides have the potent immunogenicity to trigger specific T cell responses and binding affinity to the related MHC molecules *in vitro*. However, these

peptides cannot be generated through natural processing and presenting steps [42]. Therefore, demonstrating that a given peptide is processed naturally is a prerequisite to epitope identification. There are two conventional techniques by using reversed phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry (MS) to analyze if a peptide is naturally processed. One method is to manipulate the peptide through the following procedures: (1) Target cells are transfected with specific MHC-I allele; (2) The cells are harvested, and the MHC molecules expressed on the surface of the cells are purified; (3) The peptides bound to the MHC molecules are acid-eluted, purified and finally analyzed by MS [43]. The other technique is to cleave the longer peptides which include the potential epitopes with purified proteasome complexes *in vitro*. Then, production of peptides by the digestion is analyzed by RP-HPLC and MS [44]. However, the results of this method must be analyzed carefully, because proteasome digestion can only generate the proper C terminus of the MHC-loaded peptides [11].

6. Development of $CD8^+$ T cell epitope-based vaccines

One of the approaches to find effective and safe vaccines is epitope-based DNA vaccination that enables focusing of the immune response on important and highly conserved epitopes [45]. This provides the opportunity to use specific epitopes to shift the immune system toward a Th1 or a Th2-mediated immune response and eliminate the unwanted responses. Besides that, CTL epitope-based immunization has the advantage of eliciting immune response only against the protective epitope and avoidance of epitope drift in *Mycobacterium tuberculosis* infection [46].

In many cases, a single CTL epitope-based DNA vaccine could not be fully protective against the Mtb infection. Development of epitope-based vaccines has been hampered for its relatively low antigenicity. Thus, different vaccine patterns and delivery methodologies have been developed, which include synthetic multivalent peptide vaccine, recombinant protein vaccine, DNA vaccine, viral vector, protein carrier, and adjuvant, to solve the problem. Studies of Mtb in human indicate that the induction of broad T-cell mediated immunity to Mtb and Type 1 cytokines including interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumor necrosis factor α (TNF-α) are essential for Mtb vaccine design [47]. Vaccines based on CTL epitopes represent a logical approach to generate effective cellular immunity in both the prophylactic and therapeutic settings because multiple epitopes can be incorporated into the vaccine design with the goal of inducing broadly reactive responses composed of multiple CTL clones directed against different epitopes. DNA vaccines derived from multiple epitopes have been reported to induce broad CTL responses against HIV, HBV, and SARS-CoV [48-50]. Although epitope-based vaccines are often thought to be limited with respect to HLA polymorphism and population coverage, the use of supertype-restricted epitopes, those capable of binding with significant affinity to multiple related HLA alleles, provides a means to address this problem [51]. There are several problems which need to be solved in the future studies of epitope-based Mtb vaccines, such as the safety of the DNA vaccine, the development of better adjuvants, the difference and mutation among the Mtb strains, complete clearance of the Mtb, the results from challenge models in rats or guinea pigs inconsistent with that of clinical trials.

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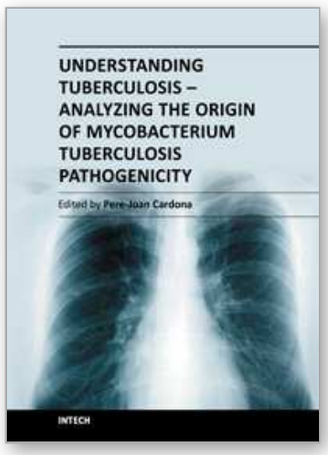

Understanding Tuberculosis - Analyzing the Origin of Mycobacterium Tuberculosis Pathogenicity

Edited by Dr. Pere-Joan Cardona

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Mycobacterium tuberculosis in an attempt to understand the extent to which the bacilli has adapted itself to the host and to its final target. On the other hand, there is a section in which other specialists discuss how to manipulate this immune response to obtain innovative prophylactic and therapeutic approaches to truncate the intimal co-evolution between Mycobacterium tuberculosis and the Homo sapiens.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

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