Minireview

Revival of the identification of cytotoxic T-lymphocyte epitopes for immunological diagnosis, therapy and vaccine development

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Abstract

Immunogenic T-cell epitopes have a central role in the cellular immunity against pathogens and tumors. However, in the early stage of cellular immunity studies, it was complicated and time-consuming to identify and characterize T-cell epitopes. Currently, the epitope screening is experiencing renewed enthusiasm due to advances in novel techniques and theories. Moreover, the application of T-cell epitope-based diagnoses for tuberculosis and new data on epitope-based vaccine development have also revived the field. There is a growing knowledge on the emphasis of epitope-stimulated T-cell immune responses in the elimination of pathogens and tumors. In this review, we outline the significance of the identification and characterization of T-cell epitopes. We also summarize the methods and strategies for epitope definition and, more importantly, address the relevance of cytotoxic T-lymphocyte epitopes to clinical diagnoses, therapy and vaccine development.

Keywords: T-cell epitope, MHC, method, immunological diagnosis, therapy, vaccine development

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Introduction

Distinguishing self and non-self is one of the most important issues for our immune system when encountering pathogens. Generally, the immune system first recognizes the pathogen as a whole, the surface molecules of the pathogens, or the surface features of these molecules (pattern recognition). Once non-self antigens are recognized, our immune system triggers responses, such as neutralization, killing and immediate clearance.[1–4]

However, pathogens have evolved to elude these immune attacks. The surface proteins of these pathogens, especially the amino acids on the outside of the proteins, are prone to mutation. If some of the mutations are favorable, these mutants will avoid recognition by the host memory immunity. Thus, the pathogens escape from this kind of host recognition strategy.[5,6]

In general, the pathogen's intracellular proteins are not prone to mutation, because such mutations more easily alter the physiological function(s) of the proteins.[7] Considering the static nature of these internal proteins sequences, how does the host's immune system recognize these parts of the pathogen and eliminate the pathogens?

To make use of these shortcomings of the pathogens, humans have evolved a sophisticated strategy to recognize the pathogens. The pathogen's proteins are processed in antigen presenting cells (APCs) and cut into short peptides.[8] Consequently, these non-self peptides are presented on the surface of the infected cells or presentation cells through the loading of a special type of molecule named the major histocompatibility complex (MHC) or human leukocyte antigen (HLA) in humans. These peptide and MHC complexes (pMHCs) are recognized by specific T-cells that perform immune responses, such as cytotoxicity (in the case of class I MHC). The peptides that act as the markers of the pathogens are called T-cell epitopes, and this is similar to the myth that humans discern Satan through his symbol: the cloven hoofs that are always hidden under his clothes. The entire process of T-cell immunity is MHC-restricted and was initially defined by Zinkernagel and Doherty.[9]

Since their discovery, T-cell epitopes have been considered as potential reagents for humans to conquer the war against pathogens, especially against viruses such as HIV and influenza viruses, whose genomes easily mutate and possess strong and efficient strategies to escape immune recognition.[10,11] These T-cell epitopes often remain relatively conserved. Therefore, vaccine may be developed to stimulate the immune response against these viruses according to the identified T-cell epitopes.[12,13]

Initially, the identification and characterization of T-cell epitopes was tedious and inefficient but important for our understanding of antigen processing.[11,14] After many epitopes were identified and characterized, it was hoped that they might aid in new vaccine development, and indeed, this technique has been used for HIV vaccine studies.[10,15–17] Unfortunately this approach has not been as beneficial as we have expected. Thus, epitope identification work has declined recently.[18,19] However, the application of T-cell epitope-based diagnoses for tuberculosis[20–22] and new data on epitope-based vaccine development[12,15,23,24] have revived the field. In this review, we outline the significance of identifying and characterizing T-cell epitopes. We also introduce methods and strategies for epitope definition and, more importantly, the relevance of cytotoxic T-lymphocyte (CTL) epitopes to disease diagnoses, vaccine development and therapy.

Biology of peptide antigen presentation

Antigen processing and presentation by class I MHC (MHC I) has been well studied, and we now know this process in detail;[8,25] a brief introduction of the process is illustrated in Figure 1. Proteins from non-self (pathogens) or altered self (e.g. malignancy) are processed in the cytoplasm and presented by MHC I. Prior to processing, proteins are covalently linked to ubiquitin molecules in an ATP-dependent manner. A protease–proteasome complex cleaves these endogenously synthesized proteins by recognition of polyubiquitin targeting. However, some of the peptides require further trimming by cytosolic aminopeptidases before fitting into the MHC I groove.

Consequently, peptides of the optimal length are translocated by the transporters associated with antigen processing (TAPs) into the endoplasmic reticulum (ER). Peptides are loaded into the MHC I heavy chain, which is retained by some molecular chaperones in the ER. Then, an antigen-derived peptide, MHC I heavy chain, and another small molecule, β_2-microglobulin, assemble into a heterotrimeric complex and further transit through the Golgi to the cell surface.

The MHC I heavy chain forms an antigen-binding groove (like a 'bed' with two side protectors) that can accommodate a peptide of the optimal length (8–11 residues). The polymorphism of MHC molecules is mainly dependent on the amino acids that line the peptide binding groove. Therefore, MHC I molecules with different allele restrictions select different specific peptides comprising conserved amino acids in particular positions. These antigenic peptides are recognized by T-cell receptors (TCRs) on the surface of CTLs in an MHC allele-specific manner. Consequently, T-cells are activated by the signal transversed by TCR and its co-receptors and other co-stimulating molecules,[26,27] and they perform functions such as secreting cytokines (e.g. interleukin 2 [IL-2] or interferon γ [IFN-γ]), killing target cells, etc.

Techniques to identify CTL epitopes

According to the processing and presentation progress of T-cell epitopes and their ability to specifically stimulate T-cells in an MHC allele-restricted manner, there are three essential elements for a random peptide to be considered a T-cell epitope (Figure 1). First, the peptide must be naturally processed into an optimal length via a proteasome-dependent or independent pathway. Second, the peptide should have the affinity to bind to the corresponding MHC molecule. Finally, and perhaps most importantly, the epitope must have the ability to induce a T-cell-specific response during its presentation on an MHC molecule. These three elements comprise the most significant features of an epitope. Therefore, when defining a peptide as a specific MHC-restricted epitope, one must evaluate all three features of a given peptide. Thus, the techniques to identify an epitope can be classified into three corresponding types.

MHC-binding-based methods

Thus far, many complex structures of peptides and MHC molecules of different alleles from different species have been determined, and their binding modes and features have been clearly demonstrated. Accordingly, diverse methods have been developed to illustrate the binding affinity of peptides to MHC molecules.

Standard peptide binding inhibition assay is a common method used to quantitatively measure the binding affinity of target peptides.[28–32] The essential factor of this technique is the preparation of a fluorescein- or radio-labeled standard peptide known to bind to a particular MHC with medium affinity. The standard peptide-loaded purified MHC I molecules are then incubated with serial dilutions of the test peptides, which competitively inhibit the binding of the labeled standard probe peptide. Consequently, the mixture is separated and analyzed through gel filtration with fluorescence or a radioisotope detector. The binding affinity of the tested peptides is measured by the concentration of peptide yielding 50% inhibition of the binding of the labeled probe peptide (IC_{50}). Commonly, peptides with IC_{50} <500 nmol/L are defined as high-affinity bound peptides.

Similarly, the MHC transfected cell line binding assay is utilized by many laboratories for its simplicity, repeatability and quantifiability for determining peptide and MHC-binding affinities.[33–37] The crux of this method is the cell lines (e.g. TAP-deficient T2 cells,[33] T2-A24 cells[38] and HLA-A24-expressing RMA-S cells[37]), which are transfected with MHC-expressing genes. These cells are defective in genes involved in endogenous antigen processing. The

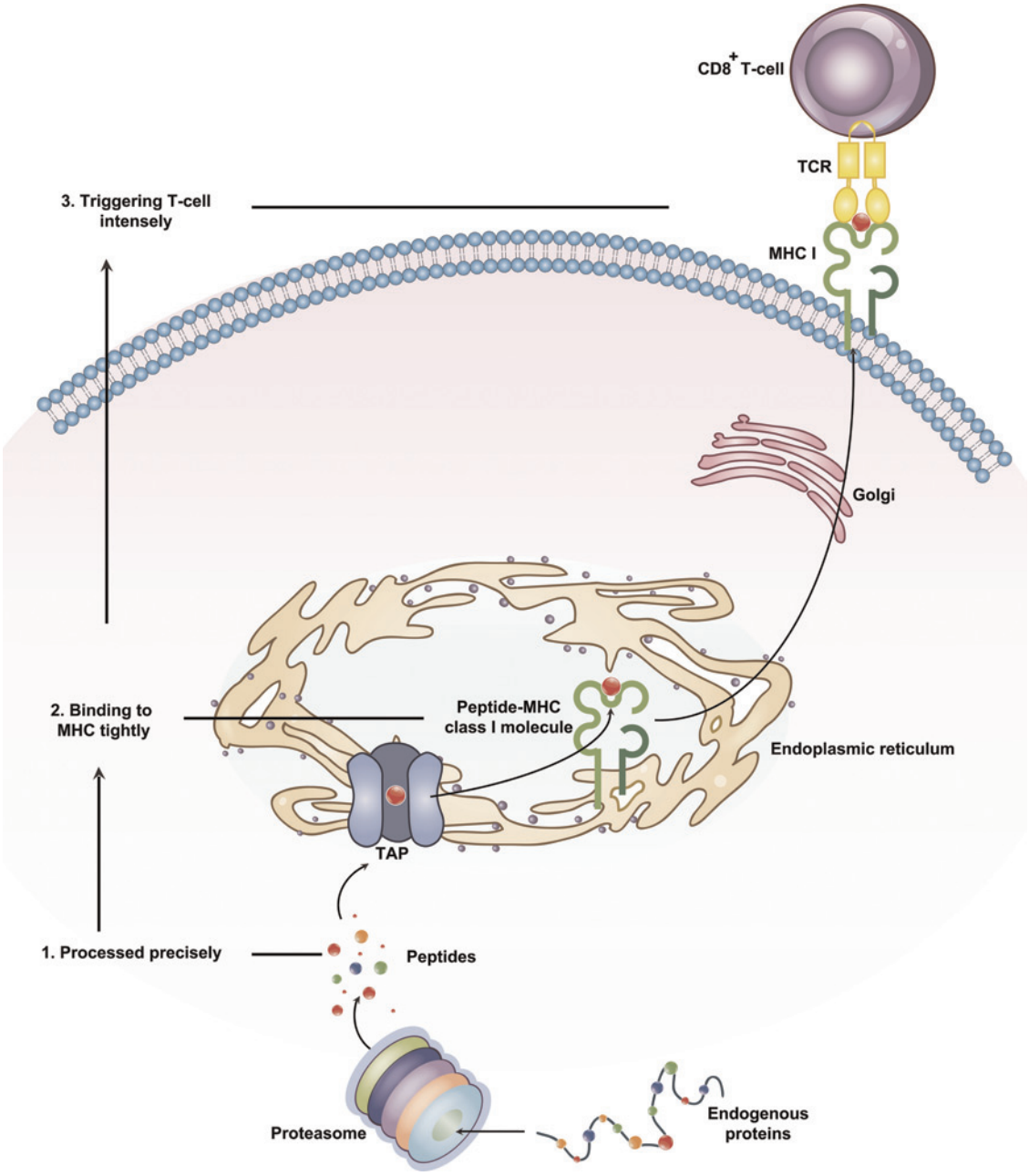

Figure 1 The processing and presentation of major histocompatibility complex I (MHC I) epitopes and their three essential elements. The pathway of MHC I eptiope presentation has been exhibited and the process comprise three main steps: first, endogenous proteins are digested into short peptides by proteasomes; second, special epitopes (red) are selected to load onto MHC I molecules (green) within the endoplasmic reticulum; finally, the peptide MHC complexes are transferred to cell surface and recognized by specific T-cell receptors. These three steps also reflect the three essential elements of a T-cell epitope. All these elements must be confirmed together when identifying a T-cell eptiope. TAP, transporters associated with antigen processing; TCR, T-cell receptor

peptides under consideration, together with the negative and positive control peptides, are then incubated with these cells. It is presumed that MHC-binding peptides increase the amount of stably expressed MHC molecules on the surface of the cells, which can be detected by fluorescence-conjugated anti-MHC antibodies. Peptides with artificially defined high-binding affinity generally cause a 100% increase in the mean fluorescence index over the negative peptide control.

Peptide–MHC co-refolding was first developed by Professor Don C Wiley and his colleagues for the purpose of studying the structures of recombinant MHC molecules that are expressed by *E. coli* in inclusion bodies.[39] They found that the denatured MHC heavy chain only reassembles efficiently in the presence of specific MHC-restricted peptides. This means that the ability of a peptide to aid in MHC refolding reflects the binding affinity of the peptide to its corresponding MHC molecules. We developed a refolding method in our lab to semiquantitatively evaluate the binding affinity of peptides of interest.[35,36,40,41] Dozens of peptides were successfully screened with high binding affinity to diverse MHC alleles from

different species, such as HLA-A2, SLA-1 from swine, BL21 from chicken and fish MHC.[35,36,41] Further, the target pathogens from which the peptides were derived included influenza virus, severe acute respiratory syndrome-associated coronavirus (SARS-CoV), the hepatitis B virus (HBV), *Mycobacterium tuberculosis* (MTB), etc. Generally, the peptide and inclusion bodies (containing MHC heavy chain and β_2m) slowly assemble through a system of gradient dilution. The refolding results are then measured by gel filtration chromatography (Figure 2). Correctly folded MHC complexes are eluted at a volume that corresponds to an absorbing peak in the detector. If we hold the amount of peptide and inclusion body used fixed, the amount of the peak area semiquantitatively reflect the binding affinity of the peptide to the related MHC; the negative control peptide has no absorbing peak in the corresponding volume. Another refolding efficiency evaluation assay (currently being assessed in our lab) uses ion exchange chromatography to compare the binding affinity of peptide to the MHC molecule. MHC complexed to peptides with low binding affinity may dissociate at a particular ionic strength, while complexes formed by peptides with high binding affinity will survive.

Enzyme-linked immunosorbent assays (ELISAs) of peptide–MHC complex formation are considered microassays for refolding.[42–44] The key ingredients in this assay are the two antibodies used. The capturing antibody is usually an anti-MHC heavy chain monoclonal antibody (e.g. w6/32 and bb7.2, etc.) that recognizes the conformational epitope. The other antibody acts as a detector, which is usually β_2m-specific. The mixture of peptide, purified recombinant MHC heavy chain (from inclusion bodies) and β_2m are incubated in a microrefolding system, and the amount of correctly reassembled MHC complex molecules is revealed by the anti-β_2m antibodies, which are usually horseradish peroxidase conjugated. The peptide MHC binding affinity is presented as an equilibrium dissociation constant (K_D), which is calculated based on ELISAs using various concentrations of the peptide.

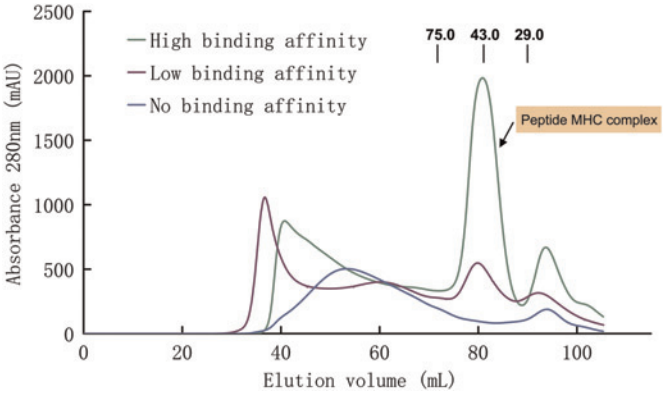

Figure 2 Co-refolding of peptides with human leukocyte antigen (HLA)-A2 heavy chain and β_2m to determine the binding affinity of peptides to HLA-A2 molecules. High HLA-A2 binding affinity peptides could help the HLA-A2 complexes refold. The peaks (green) of the complexes with the expected molecular mass 45 kDa were eluted at the estimated volume 80 mL on a Superdex^{TM} 200 column (GE Healthcare, Uppsala, Sweden). The profile was marked with approximate positions of the molecular mass standards of 75.0, 43.0 and 29.0 kDa. The much lower peak (purple) appeared when refolding HLA-A2 with low affinity peptide. For the peptide without any affinity to HLA-A2, no peak (blue) was observed between the positions of the molecular mass standards of 75.0 and 43.0 kDa

T-cell-specific antigenicity-based methods

The most significant step in the process of T-cell epitope identification is to determine the antigenicity of the peptides. Peptides with atypical anchoring residues for a related MHC allele always display very low binding affinity for MHC molecules tested *in vitro*. However, some of these peptides may possess high 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 (or other multimers, e.g. pentamer) staining,[45] enzyme-linked immunosorbent spot (ELISPOT) assays,[46] intracellular cytokine staining (ICS),[47] cytotoxicity assays[48,49] and T-cell proliferation assays.[50] These techniques focus on different aspects of specific T-cell function. For example, MHC tetramers are usually prepared to stain the specific TCRs that reflect the phenotypes of the specific T-cells. ELISPOT and ICS demonstrate the ability of the T-cells to specifically secrete cytokines such as IFN-γ, IL-2, TNF-α, etc. Cytotoxicity assays (e.g. chromium [^{51}Cr] release or carboxyfluorescein diacetate succinimidyl ester [CFSE]-labeled cell detection) are performed to directly detect specific cytotoxic effects of the T-cell on target cells and are commonly considered the gold standard to determine specific T-cell responses. Proliferation assays are designed based on the fact that T-cells display specific reduplication under the stimulation of related antigenic peptides. These methods are commonly used and described in detail elsewhere.[45–50] Thus, we only introduce novel techniques here which illustrate the T-cell-specific antigenicity of peptides.

Microarrays have been used as artificial APCs to detect functional T-cell responses and screen specific epitopes.[51] To mimic the surface of APCs, recombinant peptide MHC complexes and co-stimulatory molecules are immobilized on dry chips. Cytokine-capture antibodies are then spotted onto the chips to adhere to specific T-cells and evaluate specific T-cell responses. This technique enables the rapid screening of specific T-cell epitopes among the genome of pathogens. Artificial APCs constructed using dry chips or beads may be extensively applicable in specific T-cell detection and epitope identification.[51,52]

A high throughput T-cell functional assay has been developed by using a microculture and an array approach to quantify secreted cytokines.[53] Specific T-cell lines are diluted into autologous irradiated peripheral blood monocytes with a very low ratio of effector cells/APCs. Micro-well ELISAs are used to confirm the detection of a specific functional signal from a single T-cell derived from a T-cell line. This assay can be automated with high throughput processing to screen pathogen-derived peptides.

John Frelinger and his colleagues have described a novel methodology (named the T-cell antigen discovery assay [T-CAD]) to identify T-cell epitopes, which exploits the

phenomenon of cross-presentation.[54] The key component of the technique is inducible lacZ hybridoma cells, which are created by fusing specific T-cells to BWZ.36 partners, which comprises the β-galactosidase gene under the control of the IL-2 promoter NF-AT regulatory elements. When T-cells are specifically activated, the IL-2 synthesis within the cell is induced, and therefore, the expression of β-galactosidase is up-regulated by coupling the IL-2 promoter elements. The specific T-cells are then assessed by the β-galactosidase substrate X-gal. This assay allows for direct monitoring of specific T-cell activation by the linkage of specific T-cells to effector signals.

Verification of natural processing of the epitope

Some artificially synthesized peptides have the immunogenicity to trigger specific T-cell responses and the high binding affinity to bind to the related MHC molecules *in vitro*. However, these peptides cannot be generated through natural processing and presentation steps.[44] Therefore, demonstrating that a given peptide is processed naturally is a prerequisite of epitope identification. However, as mentioned above, peptide processing is very complicated, and thus the verification of an epitope as naturally processed requires confirmation by many aspects.

There are two conventional techniques using reversed phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry to analyze if a peptide is naturally processed. One method is to manipulate the peptide through the following procedures: (1) target cells transfected with special MHC alleles are infected with a virus of interest; (2) the cells are harvested, and the MHC molecules expressed on the surface of the cells are purified; and (3) the peptides bound to the MHC molecules are acid-eluted, purified and finally analyzed by MS. Identifying peptides in this way is thought to be direct evidence of natural processing.[29,55] The other technique is to cleave the longer peptides that include the short target peptide with purified proteasome complexes *in vitro*.[28] Production of peptides by the digestion is analyzed by HPLC and MS. 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.

One quantitative method to confirm a naturally processed peptide is the direct detection of the peptide–MHC complexes on the surface of APCs. This can be realized either through multivalent TCRs obtained from specific T-cells or through using monoclonal antibodies (mAbs) that mimic the specific TCRs.[56,57] The TCR-mimic mAbs are specific for a particular peptide–MHC complex in a peptide-dependent and MHC-restricted manner. Therefore, they can be used to visualize the naturally, intracellularly processed peptides on the surface of APCs by flow cytometry or by visualizing the cells *in situ*. These techniques utilize marker molecules to detect particular APCs, which is similar to tetramer staining for specific T-cells.

The specific recognition of endogenously synthesized antigens by peptide-specific T-cells is another important way to indirectly demonstrate peptide processing. We consider these methods as indirect, because they do not directly detect the processed peptides or visualize the presented peptides on MHC molecules. For example, if peptide-specific T-cells can be screened out of a mouse infected with viruses, or immunized with reconstructed vaccines expressing whole proteins comprising the target peptides, these peptides will be identified as naturally processed.[35,36,41] Another method is to test whether the peptide-specific CTL lines or clones can lyse cells infected with viruses or transfected with cDNA plasmids expressing the epitope-containing protein.[58,59] Furthermore, tumor cells can also serve as the target cells when analyzing tumor-specific antigen-derived peptides. This method is manipulated *in vitro* and is easier to perform than mouse immunization. Currently, a novel method has been developed using a combination of MHC tetramer and ICS to reveal antigenic epitopes naturally presented on tumor cells.[60] After activation by culturing with tumor cells that process and present specific T-cell epitopes, the specific T-cells are directly identified as tetramer-positive and cytokine-producing cells. This method does not require the generation of specific T-cell lines or clones, and thus it eliminates the time requirements of long-term T-cell culture.

Strategies for screening CTL epitopes

Because there are many diverse 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. Different laboratories have developed distinct strategies, but there is one outstanding principle that should be fully followed: all three elements discussed above should be determined for a fully identified epitope.

Screening through the *in silico* prediction

As increasing numbers of T-cell epitopes have been identified and because hundreds of three-dimensional (3D) structures of peptide–MHC complexes have been determined, the binding mode of the peptides to MHC molecules has been well demonstrated. Therefore, it is possible to predict the potential epitopic peptides in the antigenic protein and even the entire proteome of the pathogen. Many computer algorithms have been developed to predict MHC-binding peptides,[61,62] and screening T-cell epitopes with MHC restrictions beginning with such predictions is the most popular strategy.[63] Because most of the software depends on the binding mode of the peptides and MHC molecules, this strategy actually belongs to the binding-affinity identification category. Based on this strategy, our group has developed an efficient standard platform (Figure 3) to screen pathogen-derived antigenic epitopes with diverse MHC allele restrictions.[35,36,41] First, the entire sequence of the target antigenic protein is scanned for potential T-cell epitopes through *in silico* prediction and our empiricism. Following the synthesis of peptides with high purity, we screen for high MHC binding affinity peptides through *in vitro* refolding and MHC transgenic cell line binding assays. After several rounds of screening, a limited

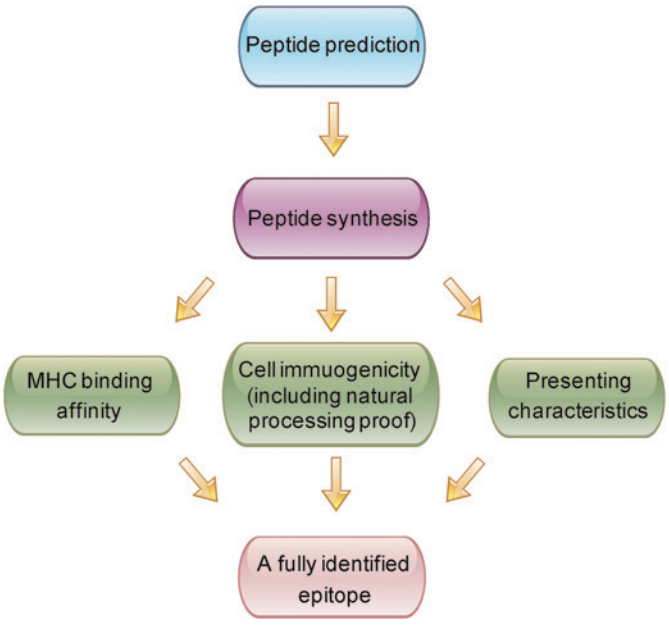

Figure 3 Major histocompatibility complex (MHC) epitope identification strategy, starting with bioinformatics prediction of peptide binding affinity to MHC. After the *in silico* prediction, potential MHC binding peptides are synthesized and analyzed for their features to be T-cell epitopes. A peptide cannot be defined as an epitope unless it has binding affinity to MHC molecules, cell immunogenicity and can be processed naturally. It is more convincing if the presenting characteristics can be illuminated through the structural determination of the pMHC molecules

number of peptides with high MHC binding affinity have been selected. Consequently, the immunogenicity of the peptides can be evaluated through a series of *in vivo* tests described above, e.g. specific T-cell monitoring among antigen-immunized mice or virus-infected patients and recovered donors. Moreover, our group has developed a rapid peptide–MHC complex structure determining platform that can help to further confirm the epitopes identified and visualize the peptide presentation features on the MHC in a 3D form.

Antigenic analysis through sequence walking

No matter how accurately computer algorithms predict potential peptide candidates, there are a considerable number of epitopes that are ignored by the first round of screening after *in silico* prediction. In order to evaluate the antigenicity of all parts of the target antigen, a sequence walking assay can be undertaken for the entire length of the antigen, and there are different strategies that use this principle. One is concerned with the protein level, using long, synthesized peptides that overlap each other.[64–66] The peptides are divided into different peptide pools as stimulators to trigger specific T-cell responses. The individual peptides within the positive pools are then tested for antigenicity through another round of T-cell functional assays. Truncated peptides within the identified long peptides are synthesized and utilized to identify the naturally processed epitopes.

Another strategy to systematically screen antigenic epitopes along the sequence of a target protein is to construct deletion cDNA fragments in an expressing vector.[54,67–69] If a one-step deletion leads to a loss in reactivity of the specific T-cell, the deleted part of the construct may possess an antigenic epitope with related MHC allele restrictions (Figure 4). After determining the antigenic region on the cDNA of the protein, the following steps to determine the natural epitope are the same as in the overlapping peptide synthesis strategy. Britten and colleagues[70] utilized this strategy to generate truncated antigens expressed in HLA-A1.1-expressing APCs, which are recognized by specific T-cells from healthy donors. In their experiments, they used mRNA fragments from cytomegalovirus pp65 protein, which were electroporated directly into APCs.

Screening through MHC–peptide exchange

Schumacher and colleagues[71] designed a method for high-throughput screening of T-cell epitopes with a combination of the MHC binding and T-cell antigenic features of an epitope. They use epitope-mimic ligands to conditionally stabilize the MHC binding. These ligands can be self-cleaved into fragments under photolysis. When the conditionally stabilized MHC molecules are exposed to a large molar excess of selected peptides during photocleavage, the self-cleaved ligand in the peptide-binding groove is exchanged for peptide candidates with high MHC-binding affinity. Consequently, the newly generated

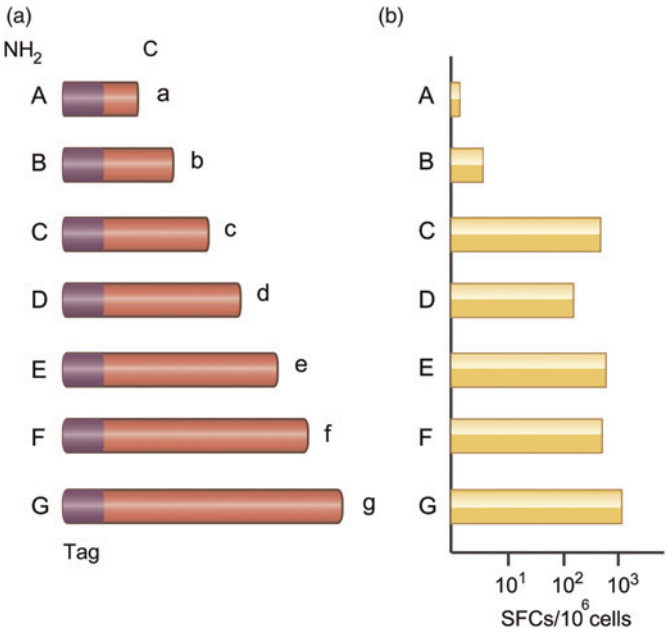

Figure 4 Antigenicity evaluation of protein deletion constructs for epitope identification. (a) Different deletion cDNA fragments (fragment A–F) and the full-length (fragment G) sequence of the antigenic protein are constructed in expression vectors. The N-terminal tag (in purple) is used for the detection and purification of the target protein. (b) Deletion constructs expressing different protein fragments are tested for antigenicity using specific T-cell lines or clones. The loss of responses of the T-cells to deletion fragments B indicates that the sequence from b to c comprises specific T-cell epitopes. SFC, spot-forming cells

MHC complexes are prepared for tetramer assays to test the specific T-cell antigenicity of the peptides with high MHC binding affinity. This technology simultaneously produces large amounts of peptide–MHC complexes and enables high-throughput T-cell epitope analysis. New HLA-A2-restricted epitopes from influenza virus and H-2K^b- and H-2D^b-restricted epitopes from *Chlamydia trachomatis* have been identified utilizing this epitope-discovering strategy.[71–73]

Every T-cell epitope screening strategy described here has its own features and advantages, and thus far, no strategy has been developed to substitute other screening methods. Further, the fact that different strategies can independently identify the same naturally presented epitopes derived from the same pathogen enhances the confidence to use diverse strategies for T-cell epitope screening.[74,75]

Peptide–MHC structural studies in epitope identification

MHC-restricted epitopes usually bind to MHC molecules stably *in vitro*. Peptide–MHC complexes prepared *in vitro* can be crystallized, and their structures can be solved by X-ray diffraction. This provides a unique chance to visualize the peptide presentation by certain MHC molecules, though it is restricted to researches with access to crystallography reagents and equipments.

Confirmation of typical anchoring residues and peptide conformation

Different MHC molecules bind peptides with different types of residues, which bind to the MHC surface in different manners, though usually two amino acids act as anchor residues. For example, the anchor residues of HLA-A2-restricted epitopes are usually two amino acids with hydrophobic side chains that stretch into the hydrophobic peptide binding clefts of HLA-A2 (Figure 5).[76,77] In contrast, HLA-A33-restricted epitopes utilize different residues, especially at C-termini. It is known that arginine (R) is always used. With its positive charge, the side chain of arginine inserts into the electronegative F pocket of the HLA-A33 molecule.[78,79] If the structure of the peptide–MHC complex is defined, typical anchor residues in the peptide binding grooves can be consequently observed.

When presenting peptides, different MHC molecules present their respective characteristic conformations. For instance, the previously solved structure of the HLA-A11 molecule adopts an M-shape when presenting peptides, while chicken BL21-restricted epitopes demonstrate an alpha-helix conformation in the peptide-binding groove.[80,81] Moreover, different conformational features are exhibited when identical MHC molecules present peptides of different lengths. Octamers and nonamers appear to be flat when presented by MHC, while the longer decamers and undecamers appear with a bulge protruding from the peptide-binding groove in the middle (Figure 5).[40,80,82] Therefore, if a short peptide presents a typical conformation as an octamer or nonamer with a related MHC allele restriction, and a longer peptide of interest adopts a classical bulged conformation, one can conclude that the peptide in question is a typical MHC-restricted epitope.

Confirmation of minimal (optimal) epitopes

The peptide-binding grooves of MHC I molecules are enclosed at each end, which limits the length of T-cell epitopes to 8–11 amino acids. Typically, anchor residues of an epitope, which bind to the MHC I molecules, are located at the N-terminus and C-terminus, respectively (Figure 5). Thereby, the structural determination of the antigenic peptides complexed to MHC molecules can help to confirm whether the peptides are optimal epitopes in length. If the typical anchor residues of a target peptide are not at the two ends of the sequence represented in the structure and the peptide extends from the end of MHC I-binding site, there may be a truncated epitope contained within the peptide under consideration. If the middle amino acids of the shorter peptide present a different conformation in comparison to the longer peptide, these two peptides may function as independent epitopes and correspond to different specific T-cell repertoires.

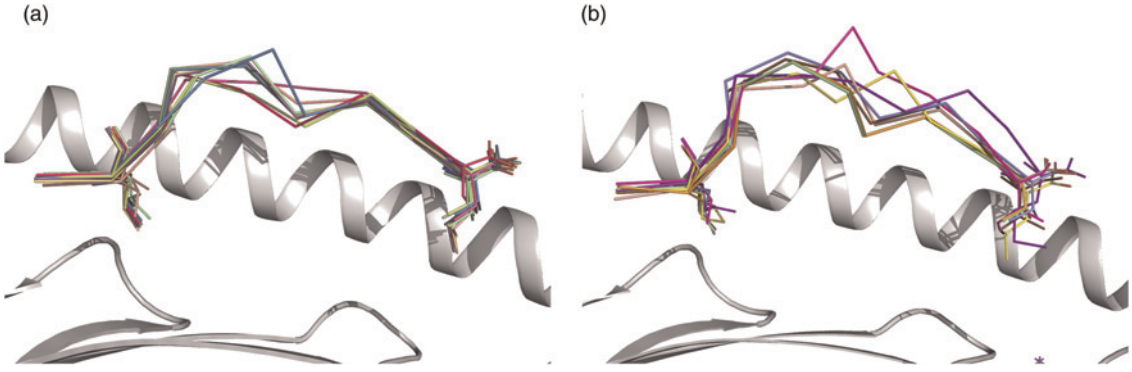

Figure 5 The typical conformation of human leukocyte antigen (HLA)-A2-restricted epitopes. Nonamer (a) and decamer (b) epitopes of HLA-A2 are presented on the peptide-binding groove seen from the α2-helix of the HLA-A2 heavy chain. The amino acids in position 2 of the N-terminus and the amino acids of the C-terminus act as primary anchoring residues of the peptides. The decamer epitopes (b) possess a more bulged conformation compared with the nonamer epitopes (a)

Confirmation of immunodominance

Different T-cell epitopes elicit immune responses of different frequencies and intensities among individuals of the corresponding HLA types. Thus, the concept of immunodominance to describe epitopes that elicit highly frequent and intense immune responses has been introduced.[83–85] The immunodominant epitopes have their own unique characteristics in 3D structures when presented by MHC molecules. Typically, peptides with a featured conformation (generally believed to possess amino acids with long side chains in the middle of the peptide protruding from the MHC molecule surface) can be recognized universally by TCRs in the T-cell repertoire and elicit relatively strong immune responses. However, TCR repertoires that recognize peptides with conformations that are too flat or bulged are limited, which easily leads to immune escape when mutations occur in the peptide sequence.[86,87] With the determination of the structures of objective epitopes and MHC molecule complexes, one can thus preliminarily predict the immunogenicity of these peptides and provide rational explanations for the divergence between their immunogenicity.

MHC structure and the design of altered peptide ligands

In cancer treatment, it has become popular to design altered peptide ligands (APLs) with higher antigenicity according to the structures of the peptides presented on MHC molecules.[88–91] The wide application of APLs is due to the elimination of T-cells through negative selection in the thymus, which are specific for naturally presented self-epitopes with high avidity to MHC molecules.

There are two main strategies for the designation of APLs. First, primary and secondary anchor residues are substituted by preferred anchor residues of the corresponding MHC molecules in order to elevate the avidity between the peptide and MHC. An example of this strategy is the modification of the T-cell epitope gp100 209–217 (ITDQVPFSV) derived from a melanoma-specific antigen gp100. A mutation from T to M at position 2 of the N-terminus results in elevated avidity between the epitope and the HLA-A2 molecule and thus intensifies the immunogenicity of this epitope.[92–94] The second strategy is to modify the amino acids with a side chain protruding out of the peptide binding cleft of the MHC molecule, because these amino acids may participate in TCR recognition. If they are substituted for by amino acids with other properties, a mutated epitope may bind more efficiently to TCRs or elicit a more diverse TCR reservoir.[95,96]

The clinical applications of T-cell-specific epitopes

The widespread clinical use of antibody-based diagnosis and therapy is a reflection of the enormous contribution of immunological studies to human health. During the early stages of cellular immunity studies, the application of T-cell immunity-related techniques was not as widely used as antibody-based assays, partially due to the complexity and expense of the determination of T-cell-specific epitopes. Currently, with the development of techniques for screening and identification of T-cell epitopes, the immunological methods correlated with T-cell epitopes have been applied extensively in disease diagnosis, therapy and vaccine development.

The application in the diagnosis of MTB

MTB infects nine million people and kills more than one million of them annually around the world.[97] It is estimated that one-third of the world's population is infected with MTB, and 10% of them will develop the active disease during their lives.[98]

Diagnosis during the early phase of MTB infection is essential for recovery from the disease and control of its transmission. Traditional diagnosis relies on a tuberculin skin test (TST), which is limited by the high frequency of false-positive reactions from previous vaccination by Bacillus Calmette-Guérin (BCG) and previous contact with environmental Mycobacteria. In addition, TST sensitivity in HIV co-infected cases is lower due to the anergy caused by immunosuppression.[99]

Research into MTB-specific cell-mediated immunity and detection of specific $CD4^+$ and $CD8^+$ T-cells has given rise to a new era for MTB infection diagnosis.[100] Comparisons of the genome of BCG strains and MTB reveal that the region of deletion-1 (RD-1) is completely deleted from all BCG strains and most common environmental Mycobacteria.[101] Therefore, detection of RD-1-encoded antigen-specific T-cells in a human reveals whether he is infected with MTB or not. Two of the RD-1-encoded proteins, early secreted antigenic target 6-kDa protein (ESAT-6) and culture filtrate protein 10 (CFP-10), can evoke robust $CD4^+$ and $CD8^+$ T-cell responses.[100] Taking advantage of these two antigens resulted in the development of two commercially available IFN-γ release assays: QuantiFERON-TB Gold and T-SPOT.TB. In the QuantiFERON-TB Gold assay, specific $CD4^+$ and $CD8^+$ T-cells in lymphocytes are quantified by the release of IFN-γ via ELISA after incubation with ESAT-6- and CFP-10-overlapping peptides.[102] T-SPOT.TB takes advantage of the ELISPOT assay to quantify specific $CD4^+$ and $CD8^+$ T-cells in lymphocytes after incubation with ESAT-6 and CFP-10 proteins.[103] The specificity of these assays is greatly improved compared with TST (97% for QuantiFERON-TB Gold and 92% for T-SPOT.TB versus 66% for TST), and their results are unaffected by prior BCG vaccination.[99] Further, both of the assays have been approved for diagnosis of MTB infection. A summary of the T-cell epitopes in ESAT-6 and CFP-10 identified to date reveals that both of the antigens are rich in $CD4^+$ and $CD8^+$ T-cell epitopes.[104–115] More specifically, the N- and C-termini are strong targets for T-cell responses for ESAT-6, and epitopes are widespread throughout CFP-10 (Figure 6).

Considering the treatment of MTB and control of its transmission, actively infected subjects are in more urgent need of health care. However, the above-mentioned

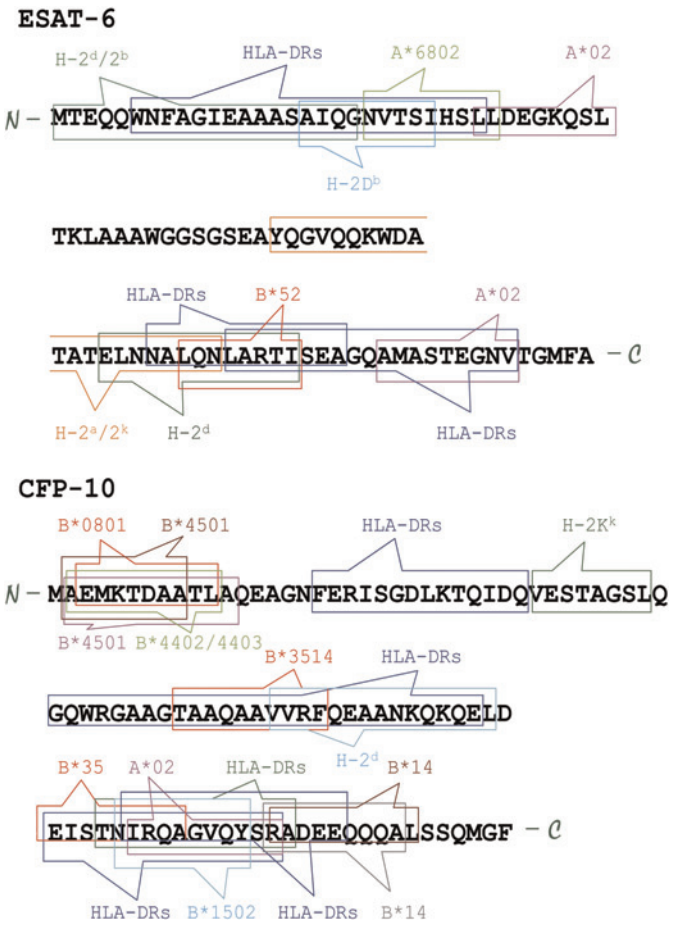

Figure 6 Summary of the T-cell epitopes in *Mycobacterium tuberculosis* (MTB)-specific antigens early secreted antigenic target 6-kDa protein (ESAT-6) and culture filtrate protein 10 (CFP-10). ESAT-6 and CFP-10 are MTB-specific antigens used in the diagnosis of tuberculosis infection with QuantiFERON-TB Gold and T-SPOT.TB assays. Both antigens contain $CD4^+$ and $CD8^+$ T-cell epitopes in mouse models and humans. Epitope restrictions with HLA-A/B alleles are mainly HLA-A*02, A*68; HLA-B*08, B*14, B*15, B*35, B*45, B*44, B*52. Promiscuous HLA-DR-restricted $CD4^+$ T-cell epitopes are present on the N- and C-terminus of ESAT-6 and throughout CFP-10. H-$2D^b$, H-$2K^k$-restricted $CD8^+$ T-cell epitopes and H-$2^a/2^b/2^d/2^k$ promiscuous restricted CD4+ T-cell epitopes are found in mouse models

two assays cannot discriminate latent tuberculosis infection and active infection.[108] Based on a selection of five promiscuous HLA-DR-restricted epitopes from ESAT-6 and CFP-10, Goletti *et al.*[105,108] have reported a whole-blood ELISPOT assay that has a much higher specificity for active MTB infection over latent infection compared with T-SPOT.TB and QuantiFERON-TB Gold. These results indicate that some epitopes may play an important role in a particular stage during the course of the infection. In this consideration, some shorter $CD8^+$ T-cell epitopes contained within these long epitopes may be responsible for the difference in T-cell responses through the course of the disease.

Further identification of epitopes related to the course of infection and deeper research into T-cell responses throughout the course of the disease will contribute to a more specific diagnosis of active MTB infection and latent infection. By uncovering more HLA-restricted epitopes, a 'specific epitope cocktail' (which would result in the highly accurate diagnosis of TB infection) may be developed and could be of great interest to realize the goal of eliminating MTB as a global problem before 2050.[98]

Immunotherapy of melanoma

With an incidence rate of ~20 cases/100,000 people and a death rate of 2.6 cases/100,000 people each year in the USA according to a National Cancer Institute Surveillance, Epidemiology and End Results report,[116] malignant melanoma is one of the most fatal cancers. When detected and treated at an early stage, melanoma is by no means incurable, but patients in stage IIB, III and IV are either incurable or have a high reoccurrence rate after surgery due to immune escape mechanisms.

Apart from primary treatments for cancers, adjuvant therapies such as radiotherapy, chemotherapy and immunotherapy are always adopted as additional cancer treatments.[117] Immunotherapy for melanoma can be categorized into two groups, active immunotherapy and passive immunotherapy, or alternatively classified into two groups in terms of specificity.[118]

In non-specific immunotherapies, melanoma-specific recognition is not involved. Active non-specific immunotherapy uses cytokines, such as IL-2 and IFN, or other antigens like BCG, to boost immune responses, while current passive non-specific immunotherapy includes the transference of lymphokine activated killer cells (LAKs), which has been used since 1982.[119]

Specific immunotherapies are more related to known epitopes, which simplify immunological surveillance. Active specific immunotherapy elicits the host's immune response with immunizations of specific antigens, such as inactivated tumor cells or cell lysate derived from melanoma cell lines or purified tumors associated antigens (TAAs), in other words, TAAs or identified tumor epitopes with or without adjuvant. Since the discovery of the first TAA for melanoma antigen gene (MAGE) in 1991,[120] an increasing number of TAAs (e.g. melanoma differentiation antigens gp100, tyrosinase and MART-1, and cancer-testis antigens NY-ESO-1 and the MAGE family) and epitopes have been found. These TAAs and epitopes provide the foundation and stimulus for melanoma immunotherapy. Commonly used TAAs for melanoma treatment and the quantity of confirmed epitopes are listed in Table 1. For more detailed information, please refer to http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm.

There are essentially three approaches for the immunization of tumor peptides. The first, and the most commonly applied method, is to inject peptides consistent with the host's HLA type alone or with an adjuvant like alum, incomplete Freund's adjuvant or immune stimulating complexes (ISCOMS) to enhance the immune response or cross-presentation. A clinical trial for a peptide vaccine was initially conducted by the Rosenberg group[94] in 1998, with a high rate of T-cell response but a low rate of objective response. Reviewed by the Rosenberg group in 2004, it was shown that the peptide vaccine immunization had an overall objective response rate of 2.9% among 381 patients

Table 1 Major $CD8^+$ T-cell-specific antigens of melanoma

Antigen classification	Tumor-associated antigens	Epitope quantity	HLA types
Melanoma differentiation antigen	gp100	21	A2, A3, A11, A24, A32, A68, B7, B35, Cw8
	Tyrosinase	12	A1, A2, A24, A26, B35, B38, B40,B44
	TRP-2	5	A2, A31, A33, Cw8
	MART-1	5	A2, B35, B45, Cw7
Cancer-testis antigen	MAGE-A1	12	A1, A2, A3, A68, B7, B35, B37, B53, B57, Cw2, Cw3, Cw16
	MAGE-A3	12	A1, A2, A24, B18, B35, B37, B40, B44, B52, Cw7
	NY-ESO-1	11	A2, A31, A68, B7, B35, B49, B51, Cw3, Cw6
	MAGE-A6	5	A34, B35, B37, Cw7, Cw16
	LAGE-1	5	A2, A31, A68, B7
	MAGE-A2	4	A2, A24, B37, Cw7
	MAGE-A4	4	A1, A2, A24, B37
	MAGE-A12	3	A2, Cw7
	MAGE-C2	3	A2, B44
	MAGE-A10	2	A2, B35
Over-expressed antigen	RAGE-1	3	A2, B7
	MUC1	2	A2
	WT1	2	A1, A24

HLA, human leukocyte antigen

with metastatic cancer.[121] Thousands of patients have participated in peptide vaccination trials like this, and recent studies have resulted in similar findings.[122] The second approach for immunization is to activate autologous dendritic cells with specific epitopes *ex vivo* and subsequently re-inject them back into the host, which aims to elicit the immune response more efficiently while using equal levels of peptides for immunization.[123] The third method for tumor peptide immunization is to inject plasmid vectors or viral vectors encoding the objective peptides. The most widely used viral vectors include recombinant vaccinia virus, fowlpox, canarypox and adenovirus. Carried by these vectors, the expressed peptides are thought to function longer and more broadly, but the overall objective response rate of a peptide in viral vectors is even lower than that of peptide injection.[121]

Passive specific immunotherapy includes infusion of antibodies and specific lymphocytes. The latter is termed adoptive cell transfer, which is currently the most effective method for cancer treatment.[124] The procedure entails *ex vivo* culture and re-injection of tumor infiltration lymphocytes (TILs) and results in a significantly higher response rate compared with other immunotherapies.[121] However, effective TILs are not always available. Therefore, the transfer of genetically engineered T lymphocytes expressing tumor epitope-specific TCRs is another promising approach for cancer treatment. Initially conducted by the Rosenberg group in 2006, the alpha and beta chains of a TCR from an effective TIL clone (which recognizes a melanoma epitope MART-1:27–35 presented by HLA-A2) were cloned into a retroviral vector. After transduction of the vector into peripheral blood lymphocytes, these cells were subsequently infused into lympho-depleted $HLA\text{-}A2^+$ melanoma patients, resulting in an objective response rate of 13%.[125]

Though the objective response rate of each approach mentioned above appears to be unsatisfactory, for late-stage melanoma patients, these approaches have brought some hope. Further, with more research into the enhancement of cancer vaccine efficacy and the improvement of immunotherapies, better outcomes are expected.

Epitope-based vaccine development for HIV

Since the emergence of acquired immunodeficiency syndrome in the 1980s, the HIV vaccine has been in development for over 20 years. However, no safe and effective vaccine has been clinically applied thus far, which may be partly due to the high genetic variability of the virus (allowing it to escape from virus-specific immunity), the lack of standard animal models to deliver research results into humans, and the dearth of knowledge of anti-infection responses, including immunity. It has been demonstrated that $CD8^+$ T-cells provide correlates of immune protection against HIV and are critical to transiently control HIV viremia.[126–128] One of the direct proofs for the irreplaceable role of $CD8^+$ T-cells in antiviral immunity is the widespread correlation between viral amino acid sequence polymorphisms at certain positions and particular HLA alleles.[129] One of the major driving forces of HIV genetic variability is $CD8^+$ T-cells.[128,130] Another proof is the association of the $HLA\text{-}B^*5701$ class I allele with long-term non-progression.[131] Recent studies indicate that the effects of thymic selection of the T-cell repertoire contribute to this correlation.[132]

Currently, several ongoing T-cell-based vaccine developments utilizing novel vaccination strategies have been reported to elicit long-lasting cellular immune responses in human or primate animal models.[133–136] Fused multi-epitope DNA vaccines have been developed as a potential strategy to elicit $CD8^+$ T-cell responses.[15–17] Recently, a novel HIV-1 candidate vaccine expressing $HLA\text{-}B^*5101$-restricted minimal epitopes has been preclinically evaluated among BALB/c mice using an $H\text{-}2^d$-restricted epitope as a facilitating ingredient.[12] It was determined that this vaccine protects mice from chimaeric ecotropic HIV-1 challenge. *In vitro*-synthesized lipopeptides that comprised $CD8^+$ T-cell specific epitopes were also explored as potential vaccines against HIV and have been demonstrated to induce robust

HIV-specific CD8$^+$ T-cell responses in humans.[24,137] Ultimately, peptide-based vaccines may be developed and applied widely for their special advantages: i.e. safety without any infectious material, drug-like features that allow non-natural design, and stability for production, storage and distribution.[23]

Conclusions and perspectives

Current studies on T-cell immunity against pathogens and the clinical applications of T-cell epitopes in immunological diagnosis, vaccine development and therapy manifest the significant role of T-cell-specific immunity in the elimination of pathogens and tumor cells. However, compared with humoral immunity, the situation seems more complicated for a T-cell-specific response. The recognition of the same pathogen is different in individuals with diverse HLA alleles and even within individuals with the same HLA alleles. The typical example is that rarely T-cell epitopes have been identified thus far that can be recognized in 100% of individuals with the same HLA allele.

However, the scenario is even more complicated. Some recently identified epitopes can be cross-presented by different HLAs and stimulate robust T-cell responses in different HLA allele-restricted individuals. These HLA alleles can come from the same HLA super-type or from different super-types. Functional and structural studies on these cross-presented peptides may help to design T-cell-related vaccines that can be used in individuals with diverse HLA alleles.[138,139] Further, post-translationally modified ligands for MHC molecules have been demonstrated in tumor and pathogen specific T-cell responses.[140] Recent studies report that these post-translationally modified peptides, when compared with common peptides, may have distinct modes of binding with MHC heavy chains and of triggering TCR.[141,142] Therefore, it is important to explore and design techniques to screen and identify modified T-cell epitopes. Moreover, the phenomenon of T-cell epitope clustering has been described in different pathogens.[36,143,144] This clustering defines that different epitopes are derived from the same region of the antigens, and these epitopes may have different MHC restrictions. This phenomenon may be utilized to screen T-cell epitopes more effectively within an antigenic region of the antigens.

Elucidating the mechanisms behind these phenomena may help to rapidly and efficiently identify T-cell epitopes. After discerning these cloven hoofs of Satan, epitope-related immunological strategies may be developed to assist humans in the war against pathogens and tumors.

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