Strategy for Identification of CD8 T-cell Epitopes in a Viral Protein

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Knowledge of the antigenicity repertoire of a pathogen is a prerequisite for the development of antimicrobial interventions. Thus, identification of the CD8 T-cell immunome of murine cytomegalovirus (mCMV) was the key for the establishment of cytoimmunotherapeutic approaches in infected hosts. CD8 T-cell epitopes can be identified by searching for MHC class-I binding motifs. This approach fails, when the amino acid (aa) sequence of the antigenic peptide does not fit to these motifs. This limitation can be circumvented using a library of overlapping peptides covering the complete aa sequence of the antigen. Here we describe the application of a PepTrack™ peptide library for the identification of a CD8 T-cell epitope in a viral protein.

Introduction

Immune control of mCMV infection is dominated by CD8 T cells. The first CD8 T-cell epitope of mCMV was described already in 1989 (1). A break through in the identification of further CD8 T-cell epitopes was the discovery of Rammensee`s group in that MHC-bound peptides display defined binding motifs (2). Based on these motifs we identified 8 further CD8 T-cell epitopes of mCMV in haplotype H-2^d (3). Screening of an mCMV-open reading frame (ORF) library indicated that there exists at least another antigenic peptide in the viral protein M54.

In a first attempt to identify the M54 encoded CD8 T-cell epitope(s) we used computational algorithms which are based on MHC class-I binding motifs, a strategy we have already applied successfully. Peptides with the highest scores were synthesized and used to stimulate CD8 T cells from mCMV-infected BALB/c mice in an ELISPOT assay. This approach failed as no significant numbers of CD8 T cells could be activated by the predicted peptides.

Therefore, we performed a M54-protein screen using a **PepTrack™ Fast Track micro-scale peptide library** consisting of overlapping decamers. Stimulating CD8 T cells from infected mice with this library resulted in the identification of 3 antigenic decamers. The exact CD8 T-cell epitope was identified by an Alanine (Ala)-walk through the candidate peptides, followed by confirmation with purified synthetic peptides.

Materials & Methods

M54-peptide library. A PepTrack™ Fast Track micro-scale peptide library covering the complete aa-sequence of the mCMV-protein M54 was synthesized by JPT Peptide Technologies, Berlin (Germany). The library consisted of 549 unpurified 10-mer peptides, each with an amine at the N-terminus and an individual aa at the C-terminus. Peptides were delivered freeze-dried (50-100nmol each). Lyophilisates were resolved in 5µl DMSO 100% (v/v) per well and diluted with 95µl PBS resulting in an approximate concentration of 5x10^{-4}M of each peptide. The final concentration of 1x10^{-5}M for usage in the ELISPOT assay was achieved by further dilution with PBS in polypropylene (pp) tubes. 20µl per peptide were seeded in duplicates for exogenously loading of target cells in the ELISPOT assay.

Ala-peptide library. Another PepTrack™ Fast Track micro-scale Ala-peptide library was synthezised for the antigenic 12-mer M54$_{81-92}$ and all possible 11-mers, 10-mers and 9-mers derived thereof (JPT Peptide Technologies). Therefore, every aa-position of each of the 10 peptides was replaced by Ala (figure 2A). Conditions of synthesis and delivery were the same as for the M54-peptide library with a total peptide amount of ca. 20nmol per peptide. Lyophilisates were resolved and diluted as described for the M54-peptide library.

Stimulation of CD8 T cells with the peptide libraries.

CD8 T cells from spleens of mCMV-infected BALB/c mice were immunomagnetically enriched using anti-CD8 MicroBeads (Miltenyi Biotec). P815 cells were used as antigen presenting cells (APC) exogenously loaded with the synthetic peptides of the libraries for 1h at room temperature. IFNγ secretion of activated CD8 T cells was monitored in an IFNγ-based ELISPOT assay.

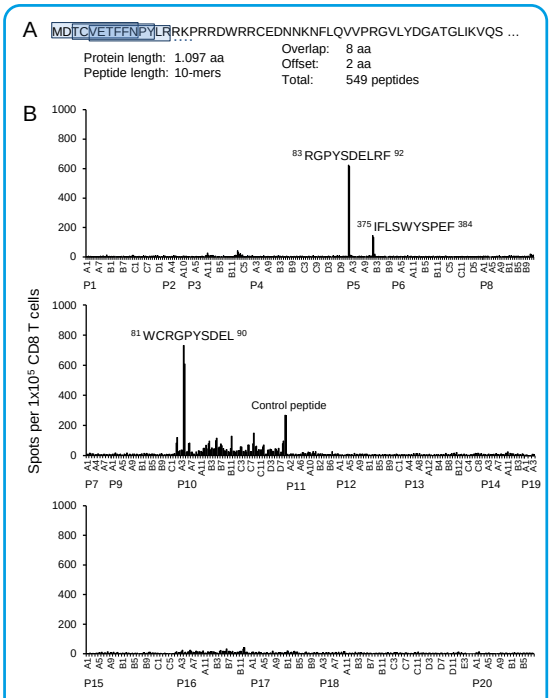

Figure 1: M54 peptide library screening. CD8 T cells were isolated from the spleens of BALB/c mice 1 week after mCMV infection and stimulated with APC exogenously loaded with the M54-peptide library in a final concentration of 10^{-6}M of each peptide in duplicates. (A) Design of the mCMV-M54 peptide library. (B) Frequencies of IFNγ-producing CD8 T cells stimulated with the peptides contained in well numbers A1-A12, B1-B12 etc. of the peptide plate (P) indicated.

Results

Screening the mCMV-specific CD8 T-cell immunome in mouse haplotype H-2^d indicated ORF M54 to code for at least one CD8 T-cell epitope. To identify the corresponding peptide(s) we applied different bioinformatic algorithms (e.g. SYFPEITHI (5), RANKPEP (6)). The top scoring peptides were synthesized but failed in stimulating a sufficent number of CD8 T cells in the ELISPOT assay and in generation of cytotoxic T-cell lines. Therefore we decided to apply a peptide library covering the complete aa-sequence of the M54 protein, consisting of 10-mers with an offset of 2 aa (**figure 1A**).

Stimulation of *ex vivo* isolated CD8 T cells from mCMV-infected BALB/c mice with the M54-peptide library resulted in 3 candidate peptides activating a significant number of CD8 T cells, (**figure 1B**). Two of them were consecutive peptides with an overlap of 8 aa making it highly probable that one antigenic peptide covered by the corresponding 12-mer stimulated the CD8 T cells. Bioinformatic search for MHC class-I L^d, D^d or K^d peptides with high

MHC-I binding scores resulted in three 9-mers as candidate peptides encoded by the 12-mer $M54_{81-92}$ and two 9-mers encoded by the 10-mer $M54_{375-384}$. These peptides were synthesized (JPT, purity >80%) and used for stimulation of CD8 T cells from mCMV-infected mice in an IFNγ-based ELISPOT assay. Peptides $M54_{82-90}$ and $M54_{83-91}$ proofed to be CD8 T-cell epitopes with comparable antigenicity.

To identitfy the minimal epitope, the antigenic 12-mer $M54_{81-92}$ and all possible 11-, 10- and 9-mers derived thereof were synthesized. In addition, an Ala-walk through all of these peptides was performed (**figure 2A**). Stimulation of CD8 T cells from mCMV-infected mice with this library resulted in recognition patterns, exemplified for the unmodified peptides and the Ala-walk through the 12-mer (**figure 2B**). This screening revealed the 10-mer $M54_{83-92}$ as the peptide with the highest antigenicity. The Ala-walk further disclosed the impact of every single aa for the antigenicity of the peptide (**figure 2C**), a strategy which was already successfully applied for the first CD8 T-cell epitope of mCMV described (4).

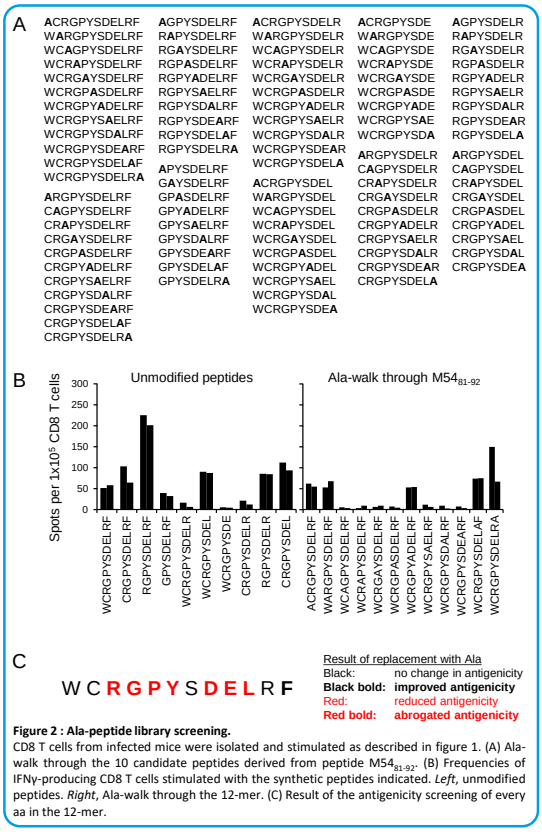

Figure 2 : Ala-peptide library screening.
CD8 T cells from infected mice were isolated and stimulated as described in figure 1. (A) Ala-walk through the 10 candidate peptides derived from peptide $M54_{81-92}$. (B) Frequencies of IFNγ-producing CD8 T cells stimulated with the synthetic peptides indicated. *Left*, unmodified peptides. *Right*, Ala-walk through the 12-mer. (C) Result of the antigenicity screening of every aa in the 12-mer.

Discussion

Screening approaches to identify CD8 T-cell epitopes of a pathogen are a prerequisite for analysing the antigenicity repertoire of the microbe. Based on this knowledge, the protective potential of the corresponding CD8 T-cell specificities primed in the infected host can be evaluated. These informations are fundamental for the development of cytoimmunotherapeutic approches (3).

If the aa-sequence of an antigenic protein coding for a CD8 T-cell epitope is known, bioinformatic algorithms are valuable tools for predicting MHC class-I binding peptides (5,6). These computational programs use position specific scoring matrices or profiles, considering also potential proteasomal cleavage sides (6), or motif matrices considering the aa in the anchor and auxiliary anchor positions (5).

Nevertheless, these predictions may fail and PepTrack™ Fast Track peptide libraries are useful and cost-efficient tools for antigenicity screening of the complete aa-sequence of a given protein or polypeptide, in particular if the presenting MHC class-I molecule is unknown. Peptide length as well as the overlap of 2 consecutive peptides depend on different factors, taking into account also the potential presenting MHC molecules. Using libraries of peptides with appropriate length (8-11 aa for MHC class I presented peptides) and an offset of 1-2 aa minimizes the probability to miss an epitope. This saves time in particular as the probability to identify the epitope using bioinformatic algorithms is significantly lower.

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Research topic of Prof. Dr. Rafaela Holtappels is the study of the immune control of mCMV infection. She identified and characterized the antiviral CD8 T-cell immunome in mouse haplotype H-2^d and thereby laid the foundation to investigate mCMV immune control mechanisms by many groups worldwide. Understanding the principles of cytoimmunotherapy of CMV disease after haematopoietic cell transplantation and its improvement is the merit of her scientific work.

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