

Characterization of the Aspergillus-Specific T-Cell Response by Using Crf1 and Catalase1 Overlapping Peptides

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The development of adoptive T-cell therapy for invasive aspergillosis is one of the possible strategies to improve the outcome of aspergillus infections after allogeneic stem cell transplantation. Here we show that low frequencies of Crf1- and Catalase1-specific T-cells, recognizing a broad variety of T-cell epitopes, are present in healthy individuals. By stimulating with overlapping peptide pools generated from low cost PepTrack™ – Research Track Peptide Libraries of several *A. fumigatus* proteins, a T-cell product with a broad repertoire and known specificity, suitable for adoptive T-cell therapy, can be generated.

Introduction

Aspergillosis is a common infectious complication in patients with hematological malignancies and in patients after allogeneic stem cell transplantation (SCT) (1;2). There is increasing evidence that impaired T-cell immune reconstitution is an important risk factor for invasive aspergillosis (3-5). To improve the outcome of patients after SCT and reduce the morbidity and mortality of aspergillus infections adoptive T-cell therapy for aspergillosis may be beneficial.

In several murine studies (4;6) and one study in humans (7), it was shown that the adoptive transfer of CD4+ T-cells which were obtained after stimulation with *A. fumigatus* crude culture antigens, resulted in a better prognosis. However, when generating a T-cell product by stimulating with *Aspergillus* crude extract or recombinant protein the specificity of the T-cell line is difficult to establish. Furthermore, variation between different batches of crude extracts and recombinant proteins can be expected. To avoid these complicating factors, another option for the generation of an *Aspergillus*-specific T-cell product is by stimulating with synthetic peptides of *A. fumigatus* proteins.

To get more insight into which *A. fumigatus* peptides should be used to generate a T-cell product with immunological activity against *A. fumigatus*, it is important to study the diversity of the *Aspergillus*-specific T-cell repertoire.

Materials & Methods

Overlapping peptides (PepTrack™ – Research Track Peptide Libraries) of the *Aspergillus* proteins Crf1 and Catalase1, consisting of 15mer peptides with an 11 amino acid overlap, were synthesized by JPT Peptide Technologies (Berlin, Germany). Peptides were divided in a master pool, matrix pools of 8 to 12 peptides, and 96 individual peptides for Crf1 and 180 individual peptides for Catalase1.

PBMC from healthy donors (0.5x10⁶) were stimulated with the Crf1 or Catalase1 complete pool (10-6M) in a 96-well plate and cultured in IMDM, supplemented with 5% inactivated fetal calf serum (FCS, Gibco), 5% inactivated human serum and 100 IU/ml IL-2 (Chiron) (T-cell medium), and restimulated with non-loaded or peptide-pulsed autologous PBMC (0.5x10⁶) after 7 days. Subsequently, cells were either stained intracellular for analysis of the frequency of activated and cytokine producing T-cells by flowcytometry (8) or stained for the surface marker CD137 to select *Aspergillus*-specific T-cells and generate T-cell clones (8). For identification of the T-cell epitopes, clones were stimulated with autologous PBMC (R:S 1:10) loaded with matrix pools (10-6M) of the overlapping peptides. To confirm the identified epitopes the clones were tested with the individual peptide.

Results

To investigate the presence of *Aspergillus*-specific T-cells in healthy individuals PBMC of healthy donors were stimulated with the overlapping peptides of the *A. fumigatus* proteins Crf1 and

Catalase1 and analyzed by flowcytometric analysis. The frequencies of *Aspergillus*-specific T-cells were too low to detect directly *ex vivo* on the basis of IFN γ production or expression of the activation markers CD154 or CD137. To increase the frequency, we *in vitro* expanded the *Aspergillus*-specific T-cells using a restimulation protocol. In **figure 1A and 1B** representative examples of the flow cytometric analyses of a donor with a high frequency and a donor with a low frequency of *Aspergillus*-specific T-cells are shown. Overall, IFN γ + CD154+ T-cells specific for Crf1 or Catalase1, with a frequency ranging from 0.05% to 0.4% of CD4+ T-cells, were demonstrated in 18 of the 24 analyzed healthy donors.

Figure 1: Flow cytometry of CD4+ T-cells specific for Crf1 and Catalase1. Examples of donors with (A) high frequency of *Aspergillus*-specific T-cells and (B) low frequency of *Aspergillus*-specific T-cells. PBMC of healthy individuals after 7 days of *in vitro* stimulation with overlapping peptides of Catalase1 or Crf1 were restimulated with non-pulsed (no pep), Catalase1 peptide mixture-pulsed (Cat1), or Crf1 peptide mixture-pulsed (Crf1) autologous PBMC. T-cells were analyzed by FACS after 5 hours of stimulation. FACS plots are gated on CD4+ T-cells.

To identify the recognized T-cell epitopes of the Crf1- and Catalase1-specific T-cells, *Aspergillus*-specific CD4+ T-cells were single cell sorted on the basis of CD137 expression using the restimulation protocol. The recognized epitopes were identified by measuring IFN γ or IL-4 production after stimulating the T-cell clones with the matrix pools of overlapping Crf1- or Catalase1 peptides, which have been composed in a matrix (**figure 2A**). Every peptide was only present in 2 subpools, which enabled us to identify the target peptide directly from the matrix pool analysis. An example is shown in **figure 2B**. T-cell clone ZBE5 recognized the Crf1 matrix pools 4, 5 and D, and therefore recognized the peptides D4 (aa 157-171) and D5 (aa 161-175), as can be derived from the matrix in **figure 2A**. The T-cell epitope is the 11 amino acid overlap between these two peptides, aa 161-171 (HTYIDWTKDA), which was confirmed by stimulating the clone with the single peptide (**figure 2C**). In this way we identified 7 Crf1 epitopes and 30 Catalase1 epitopes.

Figure 2: Identification of the recognized epitopes by matrix pool analysis. (A) Composition of the matrix pools. Every peptide is only present in 2 matrix pools, to be able to identify the target peptide directly from the analysis. (B) IFN γ production of T-cell clone 5 from individual ZBE after stimulation with the matrix pools of Crf1. The target peptides for clone ZBE5 are peptide D4 (aa 157-171, QETFHTYIDWTKDA) and D5 (aa 161-175, HTYTIDWTKDAVTWS). (C) The T-cell epitope recognized by this clone consists of the overlap between the two recognized peptides (aa 161-171).

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Discussion & Conclusions

In this work, we show by stimulating with overlapping peptides of Crf1 and Catalase1 that low frequencies of both Crf1- and Catalase1-specific T-cells are present in healthy individuals, directed against a broad variety of T-cell epitopes. Therefore it will be difficult to select the appropriate T-cells for adoptive transfer on the basis of one single peptide specificity. However, by stimulating with overlapping peptide pools of several *A.fumigatus* proteins, a T-cell product with the desired broad repertoire and known specificity can be generated.

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