The handle http://hdl.handle.net/1887/47911 holds various files of this Leiden University dissertation

**Author:** Jolink, H.  
**Title:** Human T-cell responses to Aspergillus fumigatus: In healthy individuals and patients with Aspergillus-related disease  
**Issue Date:** 2017-03-07
Chapter 3

Induction of *A. fumigatus*-specific CD4+ T-cells in patients recovering from invasive aspergillosis

Hetty Jolink, Renate S. Hagedoorn, Ellen L. Lagendijk, Jan W. Drijfhout, Jaap T. van Dissel, J.H. Frederik Falkenburg and Mirjam H.M. Heemskerk

Haematologica. 2014 Jul;99(7):1255-63
Abstract

Patients after allogeneic stem cell transplantation are at risk for invasive aspergillosis, especially during neutropenia. Recent data suggest that an impaired T-cell immune reconstitution post-transplantation plays an important role in this increased risk. In this study we investigated whether *Aspergillus*-specific T-cells are involved in the recovery from invasive aspergillosis by analyzing the *Aspergillus*-specific T-cell response in patients with invasive aspergillosis. In 9 patients with improvement of their aspergillus infection, we identified Crf1- or Catalase1-specific T-cells on the basis of CD154 expression and IFNγ production by stimulating with overlapping peptides of the *A. fumigatus* proteins Crf1 and Catalase1. These *Aspergillus*-specific T-cells were induced at the moment of regression of the aspergillus lesions. Crf1- and Catalase1-specific T-cells, sorted on the basis of CD154 expression at the peak of the immune response, had a Thelper1 phenotype and recognized a variety of T-cell epitopes. In contrast, in 2 patients with progressive invasive aspergillosis we were not able to identify any Crf1- or Catalase1-specific T-cells. These data indicate that the presence of *Aspergillus*-specific T-cells with a Thelper1 phenotype correlates with the clearance of aspergillus infection.
Introduction

Invasive aspergillosis is a common and life-threatening complication in recipients of allogeneic stem cell transplantation. Patients are most at risk in the neutropenic phase, however a substantial number of patients are diagnosed with invasive aspergillosis in a later phase after SCT at the time the neutrophil granulocyte counts have recovered to normal. It is hypothesized that the impaired T-cell mediated immunity after SCT plays a role in the increased risk of aspergillus infection in these patients.

In murine studies, vaccination with Aspergillus recombinant proteins or the adoptive transfer of Aspergillus-pulsed dendritic cells or Aspergillus-specific CD4+ splenocytes decreased the fungal burden and improved the survival after experimental aspergillus infection. In humans, it was shown that aspergillus infections after haploidentical SCT were cleared more often in patients who were treated with T-cell lines generated by stimulation with Aspergillus crude extracts, than in patients with aspergillosis not receiving adoptive immunotherapy.

In healthy individuals the presence of Aspergillus-specific T-cells in peripheral blood was demonstrated by stimulating peripheral blood mononuclear cells (PBMC) with Aspergillus crude extracts or conidia, A. fumigatus recombinant proteins or overlapping peptides of A. fumigatus proteins. We previously identified Crf1- and Catalase1-specific T-cells in healthy individuals using overlapping peptides. Crf1- and Catalase1-specific T-cells recognizing a broad variety of T-cell epitopes were identified in the majority of healthy individuals and Aspergillus reactivity was shown by stimulating the Crf1- and Catalase1-specific T-cells with Aspergillus protein extract or recombinant protein. This method to study the Aspergillus-specific T-cell response demonstrated to be efficient and reliable, can also be used to study the T-cell mediated immune response in patients with invasive aspergillosis. Previously, the Aspergillus-specific immune response in patients with aspergillosis was studied by stimulating PBMC with Aspergillus crude extracts. In patients with regression of aspergillus lesions a lymphoproliferative response was identified, however, the kinetics of the Aspergillus-specific T-cell response were not studied in detail. When Aspergillus-specific T-cells are important in the prevention or clearance of aspergillus infection, there can be a role for adoptive immunotherapy in the management of invasive aspergillosis.

In this study in patients with invasive aspergillosis, we demonstrate the induction of Crf1- or Catalase1-specific CD4+ T-cells coinciding with the regression of aspergillus lesions. These data give further support to the hypothesis that Aspergillus-specific T-cells with a Thelper1 phenotype play an important role in the prevention and/or clearance of aspergillus infection.
Methods

Aspergillus antigens and synthetic peptides

Overlapping peptides of the Aspergillus proteins Crf1 and Catalase1 were synthesized by JPT Peptide Technologies (Berlin, Germany). To confirm specificity of the T-cell clones synthetic peptides were made at the Leiden University Medical Center (LUMC, Leiden, Netherlands). For the production of Catalase1 recombinant protein three Catalase1 fragments were generated with a 12aa overlap. For the in-house preparation of Aspergillus crude extract we used A. fumigatus strain CBS144.89. Furthermore, commercially available crude extracts of strain CBS192.65 (HAL Allergy, Leiden, Netherlands) and strain CBS545.65 (Allergon, Ängelholm, Sweden) were used (see Supplementary Methods for details).

Flow cytometry

All studies were conducted with approval of the institutional review board of the LUMC. After informed consent, peripheral blood samples were obtained from patients before and at regular intervals after alloSCT and cryopreserved until further use. PBMC (0.5x10^6) were stimulated with the overlapping peptide pools (10^{-6} M) in 96-well plates, cultured for 7 days in 150 μl T-cell medium consisting of Iscove's Modified Dulbecco's Medium (IMDM, Lonza, Breda, Netherlands), supplemented with 5% fetal calf serum (Gibco, Invitrogen, Bleiswijk, Netherlands), 5% human serum and 100 IU/ml IL-2 (Novartis, Emeryville, CA), and restimulated with non-loaded or peptide-pulsed autologous PBMC (0.5x10^6). 1 hour after restimulation 10 μg/ml Brefeldin-A (BFA, Sigma-Aldrich, Zwijndrecht, Netherlands) was added to promote intracellular accumulation of cytokines. 5 hours after restimulation, cells were stained with PerCP-labeled anti-CD4 (BD/Pharmingen, Breda, Netherlands), fixated with paraformaldehyde 1% (pharmacy LUMC) and permeabilised with saponin 0.1% (Sigma-Aldrich). PE-labeled anti-CD154 (Beckman Coulter, Woerden, Netherlands) and APC-labeled anti-IFNγ (BD/Pharmingen) were added for intracellular staining of IFNγ production and the activation marker CD154. Cells were collected and analyzed on the Calibur II (BD, Breda, Netherlands).

Generation of T-cell clones

PBMC (0.5x10^6) were stimulated with the overlapping peptide pools (10^{-6} M) in a 96-well plate, cultured for 7 days in 150 μl T-cell medium and restimulated with peptide-pulsed autologous PBMC (0.5x10^6). Anti-CD40 antibody (1 μg/ml) was added and 48 hours after restimulation Aspergillus-specific T-cells were selected on the basis of CD154 expression, by sorting 1 cell per well by fluorescence activated cell sorting (FACS) after staining with PE-labeled anti-CD154 (Beckman Coulter) and FITC-labeled anti-CD4 (BD/Pharmingen).
Epitope identification and determination of \textit{Aspergillus} reactivity and HLA-restriction

For epitope identification, T-cell clones were stimulated with autologous Epstein-Barr virus-transformed B lymphoblastoid cell lines (EBV-LCL, Responder:Stimulator ratio 1:4) loaded with subpools ($10^{-6}$M) of the overlapping peptides. To confirm the identified epitopes the clones were tested against the single peptides.

To determine \textit{Aspergillus} reactivity, peptide specific clones were stimulated with autologous EBV-LCL (Responder:Stimulator ratio 1:4) loaded with recombinant protein (100 μg/ml) or with autologous monocyte derived dendritic cells preloaded with \textit{Aspergillus} crude extract (Responder:Stimulator ratio 1:4) (see Supplementary Methods for details). To determine the HLA-restriction, we used HLA-blocking antibodies, an HLA-typed EBV-LCL panel and HLA-class II negative Hela cells transduced with relevant HLA-DR, -DQ or -DP molecules\textsuperscript{14} (see Supplementary Methods for details).

Results

Clinical characteristics of the patients

For inclusion in this study, we screened 33 patients who were diagnosed with probable or proven invasive aspergillosis after allogeneic stem cell transplantation, according to the revised definitions of invasive fungal disease from the EORTC/MSG consensus group\textsuperscript{15}. Twenty-two patients could not be included in the study. The median survival period of these 22 patients was 4 weeks, varying from 1 week to 3 months. Due to the short survival period no peripheral blood samples could be collected from 13 of these patients. Nine patients had no T-cells in this short follow-up period after invasive aspergillosis, and therefore analysis of the presence of \textit{Aspergillus}-specific T-cells was not possible. Of the patients not included in the study, 15 had progressive invasive aspergillosis, 6 patients had stable aspergillus infection and in 1 patient aspergillus infection was improving.

For the remaining 11 patients blood samples after aspergillosis were available with sufficient T-cells for analysis, and they were included in this study. The median survival period for patients included in the study was 9 months, varying from 4 months to more than 6 years. Characteristics of the 11 analyzed patients are summarized in table 3.1. For microbiological diagnosis \textit{A. fumigatus} culture and Galactomannan in serum or broncho-alveolar lavage fluid were used; \textit{A. fumigatus} PCR and β-Glucan were not available. One patient (FBV) underwent a lung biopsy and was diagnosed with proven invasive aspergillosis. Nine patients with a probable invasive aspergillosis had a positive Galactomannan in serum, broncho-alveolar lavage fluid or both, and one patient (ESF) was diagnosed with probable invasive aspergillosis on the basis of repeated positive sputum cultures for \textit{A. fumigatus}. All patients had typical CT-findings. \textit{Aspergillus} infection was diagnosed 2 weeks to 10 months after allogeneic stem cell transplantation and antifungal treatment
### Table 3.1 Characteristics of patients with invasive aspergillosis

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Gender</th>
<th>Hem dis</th>
<th>SCT</th>
<th>Immunosup at time of IA</th>
<th>EORTC category IA</th>
<th>EORTC criteria IA</th>
<th>Antifungal medication¶</th>
<th>Occurence of Asp-specific T-cells</th>
<th>Clinical course IA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLF</td>
<td>41</td>
<td>female</td>
<td>MM</td>
<td>NMA</td>
<td>no</td>
<td>probable</td>
<td>BAL</td>
<td>BAL</td>
<td>Vori, 8 mo</td>
<td>yes</td>
</tr>
<tr>
<td>FBV</td>
<td>40</td>
<td>male</td>
<td>CML</td>
<td>MA</td>
<td>ciclo, pred</td>
<td>proven</td>
<td>sputum biopsy</td>
<td>neg</td>
<td>AmfoB, 6 w ltra, 2 mo</td>
<td>yes</td>
</tr>
<tr>
<td>NKB</td>
<td>43</td>
<td>male</td>
<td>MM</td>
<td>NMA</td>
<td>no</td>
<td>probable</td>
<td>BAL</td>
<td>BAL</td>
<td>Vori, 8 mo</td>
<td>yes</td>
</tr>
<tr>
<td>CSS</td>
<td>45</td>
<td>female</td>
<td>NHL</td>
<td>MA</td>
<td>ciclo, pred</td>
<td>probable</td>
<td>neg</td>
<td>BAL</td>
<td>Vori, 1 w AmfoB, 2 w</td>
<td>yes</td>
</tr>
<tr>
<td>MPK</td>
<td>62</td>
<td>female</td>
<td>MM</td>
<td>NMA</td>
<td>ciclo</td>
<td>probable</td>
<td>sputum</td>
<td>BAL</td>
<td>Vori, 8 w</td>
<td>yes</td>
</tr>
<tr>
<td>TPA</td>
<td>62</td>
<td>female</td>
<td>NHL</td>
<td>NMA</td>
<td>pred</td>
<td>probable</td>
<td>BAL</td>
<td>BAL</td>
<td>Vori, 15 mo</td>
<td>yes</td>
</tr>
<tr>
<td>ACD</td>
<td>55</td>
<td>female</td>
<td>MM</td>
<td>NMA</td>
<td>pred</td>
<td>probable</td>
<td>sputum</td>
<td>serum</td>
<td>Vori, 5 w Vori*, 5 mo</td>
<td>yes</td>
</tr>
<tr>
<td>JKJ</td>
<td>73</td>
<td>male</td>
<td>CLL</td>
<td>NMA</td>
<td>pred</td>
<td>probable</td>
<td>neg</td>
<td>BAL</td>
<td>Vori, 8 mo</td>
<td>no</td>
</tr>
<tr>
<td>JHF</td>
<td>48</td>
<td>female</td>
<td>MM</td>
<td>NMA</td>
<td>no</td>
<td>probable</td>
<td>neg</td>
<td>serum</td>
<td>Caspo, 4 w Vori, 8 w</td>
<td>yes</td>
</tr>
<tr>
<td>MST</td>
<td>37</td>
<td>male</td>
<td>NHL</td>
<td>NMA</td>
<td>ciclo, pred</td>
<td>probable</td>
<td>sputum</td>
<td>serum</td>
<td>Posa, 4 w Vori, 2 w</td>
<td>no</td>
</tr>
<tr>
<td>ESF</td>
<td>42</td>
<td>male</td>
<td>CML</td>
<td>MA</td>
<td>ciclo, pred, MMF</td>
<td>probable</td>
<td>sputum</td>
<td>neg</td>
<td>AmfoB, 4 d Vori*, 4 mo</td>
<td>no</td>
</tr>
</tbody>
</table>

¶ consecutively prescribed antifungal medication, starting from day of diagnosis
Hem dis haematological disease, SCT stem cell transplantation, IA invasive aspergillosis, MM multiple myeloma, CML chronic myeloid leukemia, NHL non-Hodgkin lymphoma, CLL chronic lymphoid leukemia, NMA non myeloablative, MA myeloablative, MSD matched sibling donor, MUD matched unrelated donor, Ciclo ciclosporin, Pred prednisolone, MMF mycofenolate-mofetil, BAL bronchoalveolar lavage, Vori voriconazole, Vori* voriconazole dose adjusted according to serum levels, ltra itraconazole, AmfoB liposomal amphotericin B, Caspo caspofungine, Posa posaconazole
was started with voriconazole in 8 patients, liposomal amphotericin B in 2 patients, and caspofungin in 1 patient. In case of lack of improvement of the aspergillus infection, azole-resistance was tested, voriconazole serum levels were measured and antifungal treatment was optimized by increasing the dose or changing antifungal medication (table 3.1).

All patients received a T-cell depleted allogeneic stem cell transplantation because of a hematological malignancy. Four patients were transplanted with a matched sibling donor (MSD), 7 with a matched unrelated donor (MUD). Myeloablative conditioning regimen consisted of total body irradiation (9 Gy), non myeloablative conditioning therapy consisted of fludarabine (150 mg/m²), busulfan (6.4 mg/kg body weight) and either anti-thymocyte globulin (20 mg/kg body weight) or alemtuzumab (30 mg). Nine patients needed systemic immunosuppression for graft-versus-host disease (GvHD) or auto-immune phenomena post-transplantation (table 3.1).

**Crf1- and Catalase1-specific T-cells develop in patients with invasive aspergillosis, coinciding with the regression of aspergillus lesions**

The frequencies of *Aspergillus*-specific T-cells at several time points before and after the diagnosis of invasive aspergillosis were analyzed by flow cytometry. IFNγ-production and CD154-expression in CD4+ T-cells were measured after stimulating and restimulating PBMC with Crf1 or Catalase1 overlapping peptides.

FACS-plots, imaging studies and graphs summarizing the kinetics of *Aspergillus*-specific T-cells of two representative patients with improvement of invasive aspergillosis are shown in figure 3.1. In patient MLF a steep increase in the frequencies of Crf1- and Catalase1-specific T-cells was seen, within 3 weeks after the diagnosis of invasive aspergillosis (figure 3.1A). At the moment of regression of the aspergillus lesion on the CT-scan (figure 3.1D) the frequencies of Crf1- and Catalase1-specific T-cells peaked, and after clearance of the infection decreased to undetectable levels (figure 3.1A-C). The rise and decline of Crf1- and Catalase1-specific T-cells showed a similar pattern, when analyzed on the basis of CD154-expression (figure 3.1B) or IFNγ-production (figure 3.1C). During the course of the aspergillus infection patient MLF used topical immunosuppressive medication because of GvHD, and did not experience leucopenia or granulocytopenia (figure 3.1B-C).

Patient TPA was treated for invasive aspergillosis for more than a year because of a prolonged period of leucopenia and granulocytopenia (figure 3.1F-G). During this period patient TPA was alternately treated with systemic and topical immunosuppression for GvHD (figure 3.1F-G). More than one year after the diagnosis of invasive aspergillosis, 8 months after recovery of the leucocyte and granulocyte count, a peak in both Crf1- and Catalase1-specific T-cells was identified on the basis of CD154-expression (figure 3.1E and F) and IFNγ-production (figure 3.1E and G), coinciding with the regression of aspergillus lesions (figure 3.1H).
Figure 3.1 Aspergillus-specific T-cell responses and imaging studies in patients with improvement of invasive aspergillosis.
Figure 3.1 Continued
Aspergillus-specific T-cells were also identified in the 7 other analyzed patients with improvement of their aspergillus infection (figure 3.2). The majority used systemic immunosuppression in the course of the aspergillus infection. In 2 patients only Crf1-specific T-cells were present (patient JHF and FBV), in 1 patient only Catalase1-specific T-cells (patient JKJ), and in 4 patients (NKB, MPK, ACD and CSS) both Crf1- and Catalase1-specific T-cells were identified (table 3.1). Peak frequencies of Crf1- and Catalase1-specific T-cells were present around the time of regression of aspergillus lesions, and varied between 0.1% and 2.3% of CD4+ T-cells on the basis of IFNγ-production.

Two patients had progressive aspergillus infection. Patient MST was diagnosed with a probable invasive aspergillosis 1 week before allogeneic stem cell transplantation and was treated with antifungal medication. At that moment sputum cultures were positive for \textit{A. fumigatus}, but serum Galactomannan was negative. Despite the recovery of the leucocyte and granulocyte count (figure 3.3B), patient developed new nodular lesions on the CT-scan 4 months later, with a positive serum Galactomannan (figure 3.3C). In a sputum culture \textit{A. fumigatus} and \textit{Rhizomucor} were grown. At this moment the patient had GvHD of skin, liver and colon for which he was treated with prednisolone and ciclosporin. Patient died of respiratory insufficiency and refractory shock due to progressive fungal infection and suspected bacterial superinfection. No Crf1- or Catalase1-specific T-cells could be identified on the basis of CD154-expression or IFNγ-production (figure 3.3A and B). In patient ESF possible invasive aspergillosis was diagnosed 2 weeks after allogeneic stem cell transplantation. This patient was treated for 4 months until pulmonary X-ray had normalized. Seven months later, he had a progressive, probable aspergillosis with positive \textit{A. fumigatus} sputum cultures and new lesions on the CT-scan (figure 3.3F), despite normal leucocyte and granulocyte counts (figure 3.3E). The patient had been treated with topical and systemic immunosuppression because of skin GvHD, which eventually worsened...
Figure 3.2 Kinetics of Aspergillus-specific T-cells in patients with improvement of aspergillosis infection. Percentages of Crf1- (■) and Catalase1-specific (●) IFNγ+ CD4+ T-cells (left y-axis) and absolute leukocyte (□) and granulocyte counts (△) (right y-axis) in 7 patients before and after diagnosis (▼) and improvement (▲) of aspergillosis infection. Use of topical (■) and systemic (□) immunosuppression and lowering of the dose (△) during the study period are depicted in the graphs. Percentages of Aspergillus-specific IFNγ+ CD4+ T-cells were calculated as % IFNγ+ of CD4+ T-cells after restimulation with peptide-pulsed PBMC minus % IFNγ+ of CD4+ T-cells after restimulation with unpulsed PBMC.
Figure 3.3 Aspergillus-specific T-cell responses and imaging studies in patients with progressive invasive aspergillosis. FACS-analyses, imaging studies and kinetics of the Aspergillus-specific T-cell responses of patient MST (A-C) and ESF (D-F).

(A and D) FACS-analyses were performed on PBMC after restimulation with Crf1- or Catalase1-pulsed PBMC or with unpulsed PBMC (no pep). T-cells were analyzed 5 hours after restimulation and FACS-plots are gated on CD4+ T-cells. (B and E) Percentages of Crf1- (■) and Catalase1-specific (●) IFNγ+ CD4+ T-cells (left y-axis) and absolute leukocyte (□) and granulocyte counts (▲) (right y-axis) after diagnosis (▼) of aspergillus infection. Use of topical (□) and systemic (▲) immunosuppression during the study period are depicted in the graphs. Percentages of Aspergillus-specific IFNγ+ CD4+ T-cells were calculated as % IFNγ+ of CD4+ T-cells after restimulation with peptide-pulsed PBMC minus % IFNγ+ of CD4+ T-cells after restimulation with unpulsed PBMC. Pulmonary CT-scans of patient MST (C) and ESF (F) show progressive disease with new nodular lesions at later timepoints. Aspergillus lesions on the CT-scans are indicated with an arrow. The timepoints indicated by Roman numerals in the top of the graphs in figure B and E correspond to the timepoints of FACS-analyses and CT-scans in respectively figure A and C, and figure D and F. In figure A and D the corresponding timeperiod in months after invasive aspergillosis (Mo after IA) is given for every timepoint.
with development of GvHD of the liver. Shortly after, he deteriorated and died of brain herniation due to an intracerebral bleeding. Retrospectively, very low frequencies of Catalase1-specific T-cells could be identified, 0.04% of CD4+ T-cells, on the basis of IFNγ-production 3 months before his death (figure 3.3D and E). No samples were available from the last period of his life. To exclude that the absence of Aspergillus-specific T-cells was caused by the limited number of antigens used for stimulation, we stimulated PBMC with peptide pools of 4 different Aspergillus proteins. After stimulation and restimulation with Aspf1, Aspf2, Aspf3 and Aspf4 no Aspergillus-specific T-cells were identified in patient MST and ESF (figure S3.1).
The mean percentage of Crf1-specific T-cells of all analyzed patients around diagnosis was 0.03% (range 0-0.2%) and 0.02% (range 0-0.09%) for Catalase1-specific T-cells. At the first timepoint with normal leukocyte counts percentages of Crf1- and Catalase1-specific T-cells had not changed. However, around the moment of improvement of aspergillus infection, the mean percentage of Crf1-specific T-cells was significantly higher (p<0.01) with a percentage of 0.35% (range 0.03-1.2%). For Catalase1-specific T-cells a trend towards increase (p=0.07) was seen with a mean percentage of 0.42% (range 0-2.28%) (figure 3.4).

Overall, in 9 of 11 analyzed patients who had an improvement of their aspergillus infection, we identified Aspergillus-specific T-cells on the basis of CD154-expression and IFNγ-production, with peak frequencies present around the time of regression of the aspergillus lesions. In 2 patients with progressive disease no or very low frequencies – below 0.05% – of Aspergillus-specific T-cells were detected.

**Figure 3.4 Increases of Crf1- and Catalase1-specific T-cells at the moment of improvement of aspergillus infection.** Percentages of Crf1- (■) and Catalase1-specific (●) IFNγ+ CD4+ T-cells around diagnosis of aspergillus infection (11 patients), at the first timepoint with normal leukocyte counts (11 patients) and at the moment of improvement of aspergillus infection (9 patients). * Significant difference between compared groups (p<0.01, two-tailed Student T test) Horizontal line indicates the mean percentage of Crf1- or Catalase1-specific T-cells among all analyzed patients at this timepoint.
**Aspergillus-specific T-cell clones from patients with invasive aspergillosis mainly have a Thelper1 phenotype**

In previous studies it was suggested that *Aspergillus*-specific Thelper1 cells are beneficial in invasive aspergillosis, whereas Thelper2 cells could be detrimental.\(^2\)\(^-\)\(^5\);\(^16\)

To analyze the cytokine profiles of the *Aspergillus*-specific T-cells in the patients with improvement of their aspergillus infection, we single cell sorted CD4+ T-cells of 6 patients at the peak of the immune response on the basis of CD154-expression, after stimulation and restimulation with the overlapping peptide pools of Crf1 and Catalase1.

From the patients we isolated 49 T-cell clones specific for either Crf1 or Catalase1, of which 19 were unique clones based on Vβ usage and specificity (supplementary table S3.1). To analyze the cytokine profiles of the clones, T-cell clones were stimulated with the overlapping peptide pools and production of IFNγ, IL-4, IL-10 and IL-17 was measured using standard Elisa. Of the 19 T-cell clones, 16 produced high amounts of IFNγ and a minority of the IFNγ-producing T-cell clones produced also low amounts of IL-4, IL-10 or IL-17. Two clones only produced IL-17, and one only produced IL-4 (supplementary table S3.1). T-cell clones with different cytokine profiles can be present in one patient; in patient ACD we identified 4 IFNγ producing T-cell clones and 1 IL-17 producing T-cell clone and in another patient (FBV) we identified 2 IFNγ producing T-cell clones and 1 IL-4 producing clone. Epitope specificity did not correlate with cytokine profile, since we identified 2 T-cell clones directed against the same peptide with a different cytokine profile (aa 57-67, presented in HLA-DQB1*0301) (supplementary table S3.1).

To exclude that the Thelper1 phenotype that we found was due to the type of antigens used, we performed an additional analysis of patient TPA as well as some healthy controls, using intracellular staining for IFNγ, IL-4 and IL-5. After stimulation and restimulation with 4 other *A. fumigatus* proteins in addition to Crf1 and Catalase1, we found in part of the healthy controls a Thelper1 response against these proteins. In patient TPA we again observed a Thelper1 response against Crf1 and Catalase1, whereas no response against Aspf1, Aspf2, Aspf3 and Aspf4 was observed (data not shown).

**Aspergillus-specific T-cells that develop in patients recovering from invasive aspergillosis recognize a diverse repertoire of Crf1 and Catalase1 epitopes**

Previously, we have shown that the immune response against *A. fumigatus* in healthy individuals was directed against a broad variety of T-cell epitopes.\(^14\) To study the diversity of the *Aspergillus*-specific immune response in patients in more detail, we determined the epitope-specificity of the T-cell clones by stimulating the T-cells with EBV-LCL loaded with the subpools of Crf1 or Catalase1 overlapping peptides, which have been composed in a matrix, as described previously.\(^14\) As every peptide is only present in 2 subpools, we could identify the recognized epitopes directly from the subpool analysis. A representative example is shown in
figure S3.2A. T-cell clone ACD3 recognized the subpools 1G and 11, and therefore recognized the peptide 1G11 (aa 333-347, DPTKIVPEEFVPITK). The T-cell epitopes were confirmed by stimulating the T-cell clones with newly synthesized peptides, an example is shown in figure S3.2B.

HLA-restrictions of the majority of the T-cell clones were determined by using HLA-class II monoclonal antibodies and an HLA-typed EBV-LCL panel. In case of unclear results, HLA-restriction was established by using HLA-transduced Hela cells. An example is shown in figure S3.2C. On the basis of HLA-class II monoclonal antibodies and the HLA-typed EBV-LCL panel the HLA-restriction of clone ACD3 was determined to be HLA-DPB1*0401 and HLA-DPB1*0402 (data not shown). This was confirmed by stimulating the T-cell clone with peptide-loaded Hela cells transduced with different HLA-DR-, DP- or DQ-molecules expressed by individual ACD. IFNγ-production was seen after stimulation with the peptide-loaded HLA-DPB1*0401 and HLA-DPB1*0402 transduced Hela cells (figure S3.2C). In the majority of patients, T-cell clones directed against several different epitopes of the Crf1 or Catalase1 protein were identified. Eight of the 16 epitopes identified in these patients were identified previously in healthy individuals (supplementary table S3.1).

To confirm that the Crf1- and Catalase1-specific T-cells are Aspergillus-specific and developed in the immune response against A. fumigatus, we tested the Aspergillus-reactivity of the T-cell clones. All Crf1-specific T-cell clones were reactive to the Aspergillus crude extract (figure S3.2D). Five of the 7 Catalase1-specific T-cell clones recognized Catalase1 recombinant protein, and three of these also recognized Aspergillus protein extract (figure S3.2E).

Discussion

In this study we demonstrate the induction of Crf1- and Catalase1-specific T-cells in 9 patients with invasive aspergillosis. The appearance of these Aspergillus-specific T-cells coincided with the regression of aspergillus lesions, indicating that Aspergillus-specific T-cells are important in the clearance of aspergillus infection. In two patients with progressive aspergillus infection, we were not able to identify Crf1- or Catalase1-specific T-cells. In the patients recovering from invasive aspergillosis, Aspergillus-specific T-cells were directed against various different epitopes derived from Crf1 or Catalase1 and preferentially exerted a Thelper1 phenotype.

In previous studies low frequencies of Aspergillus-specific T-cells have been identified in peripheral blood of healthy individuals by stimulating with conidia of Aspergillus fumigatus, A. fumigatus crude extract, A. fumigatus recombinant proteins, or overlapping peptides of A. fumigatus proteins. Aspergillus-specific T-cell responses were equivalent when stimulated with Catalase1 or Crf1 recombinant protein compared to conidia or crude extracts, and we
have previously demonstrated that T-cells directed against *A. fumigatus* can be identified by the use of overlapping peptides of Crf1 and Catalase1. Whether these T-cells play a role in the defense against *Aspergillus fumigatus* was however not investigated.

In this study we were able to follow in time 11 patients after allogeneic stem cell transplantation who were diagnosed with invasive aspergillosis. In the 9 patients recovering from invasive aspergillosis we could show the induction of CD4+ T-cells directed against the *A. fumigatus* proteins Crf1 and Catalase1 at the time of aspergillus regression. For only 2 patients with progressive aspergillus infection blood samples with sufficient T-cells were present and could be analyzed. In these 2 patients no Crf1- or Catalase1-specific T-cells were identified. The small size of the group of patients with progressive aspergillosis that could be analyzed for the presence of *Aspergillus*-specific T-cells, precluded a statistical analysis to compare between recovering and progressive patients. However, from the 9 patients with lymphopenia that were screened but not included in this study, 8 patients also had progressive invasive aspergillosis, suggesting that in these patients the total absence of T-cells may have played a role. Based on the amount and size of aspergillus lesions we expect no difference in the load of *A. fumigatus* between patients who cleared the aspergillus infection and patients who did not. Thus, the presence or absence of *Aspergillus*-specific T-cells cannot be explained by a difference in the amount of *A. fumigatus* antigen present in these patients. However, in the progressive patients, as well as in 5 of the 9 patients recovering from invasive aspergillosis, levels of systemic immunosuppression were high during aspergillus infection, whereas at the moment of recovery of invasive aspergillosis doses of systemic immunosuppression were lowered. Thus, in these patients the high levels of immunosuppression probably have led to the absence of *Aspergillus*-specific T-cells, contributing to the increased risk of invasive aspergillosis. Furthermore, when we compared the frequencies of Crf1- and Catalase1-specific T-cells at the moment of regression of aspergillus lesions with the moment of diagnosis or recovery of leukocyte count we found a significant difference in frequencies between these timepoints, further pointing to a role for *Aspergillus*-specific T-cells in the recovery of invasive aspergillosis.

Previously, Hebart et al. studied the T-cell response to *Aspergillus* crude extracts in patients with invasive aspergillosis. For the majority of patients in that study only one time point after stem cell transplantation was analyzed, and therefore no correlation between the induction of *Aspergillus*-specific T cells and the regression of aspergillus lesions could be demonstrated. Furthermore, many patients in that cohort were diagnosed as possible invasive aspergillosis, in which certainty of the diagnosis is more open to doubt. Finally, the T-cell immune responses identified by Hebart et al. may have been overestimated due to reactivity to other components than *A. fumigatus* proteins in the crude extract.

In previous studies *Aspergillus*-specific T-cells from patients with a favorable response had a high IFNγ/IL-10 ratio, whereas high amounts of IL-10 production were documented in patients with invasive aspergillosis before adoptive
immunotherapy with *Aspergillus*-specific T-cells, and in patients with progressive disease. In the present study, similar to what was demonstrated in healthy individuals, the majority of T-cell clones from patients recovering from invasive aspergillosis were directed against a broad diversity of Crf1 and Catalase1 epitopes and had a Th1 phenotype, producing high amounts of IFNγ. A selection of clones also produced IL-4, IL-10 or IL-17, but only 3 T-cell clones solely produced IL-4 or IL-17, indicating that Tregs and Thelper2 cells do not seem to have played a major role in these patients. Although this can be a consequence of the culture procedure or the selection method based on CD154-expression, it fits the previous data indicating that Thelper1 cells are associated with improvement of aspergillus infection, whereas Thelper2 and Thelper17 cells are associated with increased fungal growth. From the progressing patients we were not able to select T-cell clones, due to lack of CD154-positive T-cells after stimulation with the overlapping peptides. This suggests the absence of *Aspergillus*-specific T-cells in these patients rather than a Thelper2-skewed cytokine profile, because we could not detect an increase in CD154-expressing, non-IFNγ producing T-cells by flow cytometry. Furthermore, an additional analysis of a subset of patients using intracellular cytokine staining after stimulation with 4 other *A. fumigatus* proteins could not detect any IL-4 or IL-5 producing T-cells, not in patients recovering from invasive aspergillosis nor in patients with progressive disease.

Although the peaks of *Aspergillus*-specific T-cells were present around the time of regression of the aspergillus lesions, the sequence of events could not be established. No validated, sensitive, non-invasive diagnostic method is available to diagnose invasive aspergillosis, in contrast to, for example, CMV-infection that can be confirmed reliably and quantitatively by determining the plasma viral load. In CMV it could be demonstrated that the clearance of CMV-infection was preceded by the occurrence of CMV-specific T-cells in the peripheral blood. Invasive aspergillosis is diagnosed when a patient fulfills the EORTC criteria for a possible, probable or proven diagnosis of aspergillosis, based on clinical, microbiological and radiological data. The exact time of improvement of the aspergillus infection is hard to establish. Non-culture based methods can be used, but are not sensitive, like serum Galactomannan or not validated, like *Aspergillus* PCR. Radiological improvement can often only be observed on a CT-scan, a diagnostic test that is not performed on a regular basis. However, it seems likely that the induction of *Aspergillus*-specific T-cells preceded the improvement of aspergillus infection, indicating a role for *Aspergillus*-specific CD4+ T-cells in the clearance of invasive aspergillosis. This is also in line with the positive results obtained in several murine studies and a human study on adoptive immunotherapy for invasive aspergillosis, which demonstrated a beneficial effect of the administration of *Aspergillus*-specific T-cells.

In summary, we analyzed the natural course of *Aspergillus*-specific T-cell immunity in 11 patients with invasive aspergillosis. In patients recovering from invasive aspergillosis IFNγ-producing Crf1- or Catalase1-specific CD4+ T-cells,
directed against several T-cell epitopes, were identified at the moment of regression of aspergillus lesions, whereas no Aspergillus-specific T-cells were present in patients with progressive aspergillus infection. These data indicate that CD4+ T-cells specific for A. fumigatus proteins play an important role in the recovery from invasive aspergillosis in patients with normal neutrophil granulocyte counts, and suggest that adoptive immunotherapy might be a valuable additional tool in the management of invasive aspergillosis after allogeneic stem cell transplantation.

**Acknowledgements**

The authors would like to thank Guido de Roo, Menno van der Hoorn and Erwin de Haas for expert technical assistance, and Arend Mulder for providing the monoclonal antibodies used for the HLA-blocking experiments.
Reference List


(17) Chai LY, van de Veerdonk F, Marijnissen RJ et al. Anti-Aspergillus human host defence relies on type 1 T helper (Th1), rather than type 17 T helper (Th17), cellular immunity. *Immunology* 2010;130:46-54.


Supplementary methods

Aspergillus antigens and synthetic peptides

Overlapping peptides 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 complete peptide pool consisting of all overlapping peptides, subpools of 8 to 12 peptides, and 96 single peptides for Crf1 and 180 single peptides for Catalase1. For additional analyses we used overlapping peptides of the *Aspergillus* proteins Aspf1, Aspf2, Aspf3 and Aspf4, also synthesized by JPT Peptide Technologies. To confirm specificity of the T cell clones synthetic peptides were made at the Leiden University Medical Center (LUMC, Leiden, Netherlands).

For the production of Catalase1 recombinant protein three Catalase1 fragments were generated with a 12aa overlap. The fragments were cloned into pDon221 and following sequencing cloned into Gateway (Invitrogen) bacterial expression vector pDEST17. Vectors were transformed into *Escherichia coli* BL21 and protein expression was induced by 1 mM IPTG (Promega). Recombinant protein was isolated by purification of inclusion bodies.

For the preparation of *Aspergillus* crude extract, conidia of *A. fumigatus* strain CBS 144.89 (10^6/ml) were cultured in 70 ml of liquid complete medium, and gently shaken at 37°C overnight. The mycelium was ground in the presence of liquid nitrogen and protein extraction buffer was added. Protein content was measured by the Bio-Rad technique. Furthermore, commercially available crude extracts of the *A. fumigatus* strains CBS 192.65 (HAL Allergy, Leiden, Netherlands) and *A. fumigatus* strain CBS 545.65 (Allergon, Ängelholm, Sweden) were used.

Flow cytometry

In some patients we performed intracellular staining with PerCP-Cy5.5 labeled anti-IL-4 (BD/Pharmingen), PE-labeled anti-IL-5 (BD/Pharmingen) and Brilliant Violet 605-labeled anti-IL-17 (ITK Biolegend, Uithoorn, Netherlands), combined with fluorescein isothiocyanate (FITC)-labeled anti-CD154 (BD/Pharmingen). In those experiments, cells were after restimulation analyzed on the LSR II (BD).

Determination of Aspergillus reactivity and HLA-restriction

To determine *Aspergillus* reactivity, peptide specific clones were stimulated overnight with autologous EBV-LCL (R:S ratio 1:4) loaded with recombinant protein (100 μg/ml) or with autologous monocyte derived DC preloaded with *Aspergillus* crude extract (R:S ratio 1:4). Briefly, for the generation of immature DC we isolated monocytes from PBMC by MACS using anti-CD14 coated magnetic beads (Milteny Biotec, Bergisch Gladbach, Germany) and CD14+ cells (1x10^6) were cultured in 24-well plates in 1 ml of IMDM supplemented with 10% human
serum, 500 IU/ml IL-4 (Schering-Plough) and 100 ng/ml GM-CSF (Novartis). After 2 days, immature DC were preloaded with *Aspergillus* crude extract (50 μg/ml) for 4 hours, and then matured by adding 100 ng/ml GM-CSF, 10 ng/ml TNFα (Cellgenix, Freiburg, Germany), 10 ng/ml IL1-β (Cellgenix), 10 ng/ml IL-6 (Cellgenix), 1 μg/ml prostaglandin E₂ (Sigma-Aldrich) and 500 IU/ml IFNγ (Boehringer-Ingelheim, Alkmaar, Netherlands) for 3 days.

To determine the HLA-restriction of the T cell clones, autologous EBV-LCL or autologous PBMC were loaded with the single peptide (10⁻⁶M) and incubated for 2 hours. After washing the stimulator cells, saturating concentrations of mAbs specific for HLA-class II (PdV5.2), HLA-DR (B8.11.2), HLA-DQ (SPVL3) or HLA-DP (B7.21) were added and incubated for 1 hour. Subsequently, T cells were added in a R:S ratio of 1:4 and incubated ON. Supernatant was collected and analyzed using standard ELISA. Further determination of the HLA-restriction was analyzed using the HLA-typed EBV-LCL panel, containing partially matched allogeneic EBV-LCL. The T cell clones and EBV-LCL (R:S ratio 1:4) were incubated ON with the complete overlapping peptide pool (10⁻⁶M) and the supernatant was tested for cytokine production in ELISA. In case of unclear results, the HLA-restriction of the T cell clones was determined by loading the peptide pool on HLA-class II negative Hela cells that were transduced with the HLA-DR, -DQ or -DP molecules expressed by the individuals the T cell clones were derived from.
Supplemental figure S3.1 part 1

Figure S3.1  FACS-analyses of T-cell responses specific for Aspf1, Aspf2, Aspf3 and Aspf4 in patients with progressive invasive aspergillosis.

FACS-analyses were performed on PBMC of patient MST (A and C) and patient ESF (B and D) after restimulation with Aspf1- Aspf2- Aspf3- or Aspf4-pulsed PBMC or with unpulsed PBMC (no pep). T-cells were analyzed 5 hours after restimulation and FACS-plots are gated on CD4+ T-cells. CD154-expression and IFNγ-production are depicted in figure A and B, CD154-expression and IL-5 production are depicted in figure C and D. In the top of the graphs the timepoints are indicated by Roman numerals and the corresponding timeperiod in months after invasive aspergillosis (Mo after IA) is given for every timepoint. The timepoints in figure A and C correspond to the timepoints in the graph of figure 3.3B and the timepoints in figure B and D correspond to the timepoints in the graph of figure 3.3E.
Supplemental figure S3.1 part 2

### C

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Mo after IA</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pep</td>
<td></td>
<td>1.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Aspf1</td>
<td></td>
<td>1.5</td>
<td>0.1</td>
</tr>
<tr>
<td>Aspf2</td>
<td></td>
<td>1.6</td>
<td>0.1</td>
</tr>
<tr>
<td>Aspf3</td>
<td></td>
<td>1.5</td>
<td>0.1</td>
</tr>
<tr>
<td>Aspf4</td>
<td></td>
<td>1.5</td>
<td>0.1</td>
</tr>
</tbody>
</table>

### D

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Mo after IA</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pep</td>
<td></td>
<td>0.1</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Aspf1</td>
<td></td>
<td>0.1</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Aspf2</td>
<td></td>
<td>0.1</td>
<td>0.5</td>
<td>1.2</td>
</tr>
<tr>
<td>Aspf3</td>
<td></td>
<td>0.1</td>
<td>0.3</td>
<td>1.5</td>
</tr>
<tr>
<td>Aspf4</td>
<td></td>
<td>0.1</td>
<td>0.4</td>
<td>1.5</td>
</tr>
</tbody>
</table>

CD154 vs IL-5
Supplemental figure S3.2

A

IFN\(\gamma\) pg/ml

subpool

ACD 3

B

no pep

ACD 3 (aa 333-347 Cat1)

DPB1*0402

DPB1*0401

DQB1*0501

DQB1*0301

DRB1*1101

IFN\(\gamma\) pg/ml

C

no pep

pep

D

Cytokine production (pg/ml)

MLF3 MLF77 ACD4 ACD11 NKB6 NKB14 FBV1 FBV16 JHF21 JHF22 JHF25 JHF31

unloaded

Asp extract

Cat1 rec prot

Cat1 pep

E

Cytokine production (pg/ml)

unloaded

Asp extract

Cat1 rec prot

Cat1 pep

TPA 6 ACD 1 ACD 3 ACD 5 NKB 10 JHF 5 FBV 9
**Figure S3.2** Determination of epitope-specificity, HLA-restriction and Aspergillus reactivity.

(A) IFNγ production by T-cell clone 3 from individual ACD after ON stimulation with the subpools of Catalase1 loaded on autologous PBMC. Every peptide is only present in 2 subpools, to be able to identify the target peptide directly from the subpool analysis. The target peptide for clone ACD3 is peptide 1G11 (aa 333-347, DPTKIVPEEFVPITK). (B) The T-cell epitope recognized by the clone was confirmed by stimulating the clone with newly synthesized peptide. (C) HLA-restriction of clone ACD3 was confirmed by testing with peptide-loaded HLA class II transduced Hela cells. The HLA-restriction molecules used for presentation of the epitope are underlined. T-cell clone ACD3 was isolated from an individual with HLA type DRB1*01, DRB1*1101, DRB3*02, DQB1*0301, DQB1*0501, DPB1*0401 and DPB1*0402. (D) Crf1-specific T-cell clones were stimulated with autologous monocyte-derived dendritic cells that were preloaded with Aspergillus protein extract for 4 hours and then matured for 3 days. (E) Catalase1-specific T-cell clones were stimulated with unloaded autologous dendritic cells or with autologous dendritic cells preloaded with Aspergillus protein extract, dendritic cells loaded with Catalase1 recombinant protein or dendritic cells loaded with Catalase1 overlapping peptide pool. IFNγ production was measured after ON stimulation.
### Supplemental table S3.1

*Identified Crf1 and Catalase1 epitopes in patients with invasive aspergillosis*

<table>
<thead>
<tr>
<th>Epitope</th>
<th>aa sequence</th>
<th>Clone</th>
<th>Cytokine production</th>
<th>Vβ§</th>
<th>HLA-restriction</th>
<th>Aspergillus extract</th>
<th>Catalase1 rec protein*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Crf1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aa 57-67</td>
<td>EVTAKVPVGP</td>
<td>MLF 77</td>
<td>IFNy</td>
<td>7.2</td>
<td>DRB1*11</td>
<td>yes</td>
<td>n/a</td>
</tr>
<tr>
<td>aa 89-99</td>
<td>FFFGKAEEVVMK</td>
<td>ACD 11, JHF 31</td>
<td>IL-17, IFNy</td>
<td>17, 13.2</td>
<td>DQ81*0301</td>
<td>yes</td>
<td>n/a</td>
</tr>
<tr>
<td>aa 122-132</td>
<td>VLESDDLDEVD</td>
<td>NKB 14</td>
<td>IFNy</td>
<td>2</td>
<td>DQ81*0202</td>
<td>yes</td>
<td>n/a</td>
</tr>
<tr>
<td>aa 161-175</td>
<td>HYTIDWTKDAVTWS</td>
<td>FBV 16</td>
<td>IFNy</td>
<td>u.k.</td>
<td>DRB3*01</td>
<td>yes</td>
<td>n/a</td>
</tr>
<tr>
<td>aa 165-179</td>
<td>IDWTKDAVTWSIDGA</td>
<td>FBV 1</td>
<td>IFNy</td>
<td>2</td>
<td>DPB1*0101</td>
<td>yes</td>
<td>n/a</td>
</tr>
<tr>
<td>aa 169-183</td>
<td>KDAVTWSIDGAVRT</td>
<td>JHF 21</td>
<td>IFNy</td>
<td>u.k.</td>
<td>DRB1*03</td>
<td>yes</td>
<td>n/a</td>
</tr>
<tr>
<td>aa 217–231</td>
<td>GTIEWAGGLTDYSAG</td>
<td>TPA 6</td>
<td>IL-17</td>
<td>22</td>
<td>DRB1*15</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>aa 233-243</td>
<td>YTVMKSVRVE</td>
<td>NKB 6, JHF 22</td>
<td>IFNy, IFNy</td>
<td>8, 13.1</td>
<td>DQ81*0701</td>
<td>yes</td>
<td>n/a</td>
</tr>
<tr>
<td>aa 237-247</td>
<td>VKSVREIIS</td>
<td>JHF 25</td>
<td>IFNy</td>
<td>u.k.</td>
<td>DRB4*0103</td>
<td>yes</td>
<td>n/a</td>
</tr>
</tbody>
</table>

| **Catalase1** |             |       |                     |     |                 |                     |                       |
| aa 117-127 | DFSNITAAASFL | NKB 10 | IFNy                | 2   | DRB1*0701       | no                  | no                    |
| aa 137-147 | FVRSTVAGSR | JHF 5  | IFNy                | 3   | DRB1*0408       | no                  | yes                   |
| aa 333-347 | DPTKIVPEFVIPKT | ACD 3 | IFNy                | 3   | DPB1*0401/DPB1*0402 | yes | yes |
| aa 345-355 | GKMQLNRPN | ACD 1, ACD 5 | IFNy, IFNy | 12, u.k. | DRB3*02 | yes | yes |
| aa 497-507 | IRFENANVKSP | TPA 6  | IL-17               | 22  | DRB1*15       | no                  | yes                   |
| aa 705-719 | DDFANDLKEGLRTFK | FBV 9 | IL-4                | 2   | DRB1*0301     | no                  | no                    |

* n/a: not applicable

§ Vβ analysis by TCR Vβ monoclonal antibodies

§ u.k.: Vβ unknown when not present in TCR Vβ kit

*Bold:* epitopes previously identified in healthy individuals
Aspergillus-specific T-cells in aspergillosis