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

Hetty Jolink,[1,2] Renate S. Hagedoorn,[1] Ellen L. Lagendijk,[3] Jan W. Drijfhout,[4] Jaap T. van Dissel,[2] J.H. Frederik Falkenburg,[1] and Mirjam H.M. Heemskerk[1]

[1]Department of Hematology, Leiden University Medical Center; [2]Department of Infectious Diseases, Leiden University Medical Center; [3]Department Molecular Microbiology and Biotechnology, Institute of Biology, Leiden University; and [4]Department of Immunohematology and Blood transfusion, Leiden University Medical Center, the Netherlands

ABSTRACT

After allogeneic stem cell transplantation patients are at risk of invasive aspergillosis, especially during the period of neutropenia. Recent data suggest that impaired T-cell immune reconstitution after 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 nine patients whose *Aspergillus* infection improved, we identified Crf1- or Catalase1-specific T cells on the basis of CD154 expression and interferon-γ production following stimulation 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 T helper-1 phenotype and recognized a variety of T-cell epitopes. In contrast, in two patients with progressive invasive aspergillosis, no Crf1- or Catalase1-specific T cells were identified. These data indicate that the presence of *Aspergillus*-specific T cells with a T helper-1 phenotype correlates with the clearance of aspergillus infection.

Introduction

Invasive aspergillosis is a common, life-threatening complication in recipients of allogeneic stem cell transplants.[1] Patients are most at risk in the neutropenic phase, although a substantial number of patients are diagnosed with invasive aspergillosis in a later phase after the transplant at a time when neutrophil granulocyte counts have recovered to normal. It is hypothesized that impaired T-cell mediated immunity after stem cell transplantation plays a role in the increased risk of aspergillus infection in these patients.

In murine studies, vaccination with *Aspergillus* recombinant proteins or adoptive transfer of *Aspergillus*-pulsed dendritic cells or *Aspergillus*-specific CD4$^+$ splenocytes decreased fungal burden and improved survival after experimental aspergillus infection.[2-5] In humans, it was shown that aspergillus infections after haploidentical stem cell transplantation were cleared more often in patients who were treated with T-cell lines generated by stimulation with *Aspergillus* crude extracts than in patients who did not receive adoptive immunotherapy.[6]

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.[5,7-13] We have 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 T cells with *Aspergillus* protein extract or recombinant protein.[14] This method of studying *Aspergillus*-specific T-cell responses, which has been 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. A lymphoproliferative response was identified in patients whose aspergillus lesions regressed; however, the kinetics of *Aspergillus*-specific T-cell responses were not studied in detail.[5] When *Aspergillus*-specific T cells are important in the prevention or clearance of aspergillosis, there could be a role for adoptive immunotherapy in managing this invasive infection.

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 support the hypothesis that *Aspergillus*-specific T cells with a T helper 1 phenotype play an important role in the prevention and/or clearance of aspergillus infection.

Methods

Aspergillus *antigens and synthetic peptides*

Overlapping peptides of *Aspergillus* proteins Crf1 and Catalase1 were synthesized by JPT Peptide Technologies (Berlin, Germany). To confirm the specificity of the T-cell clones, synthetic peptides were

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Correspondence: mhmheemskerk@lumc.nl

made at the Leiden University Medical Center (LUMC, Leiden, The Netherlands). For the production of Catalase1 recombinant protein, three Catalase1 fragments were generated with a 12-amino acid overlap. We used the *A. fumigatus* strain CBS144.89 for the in-house preparation of *Aspergillus* crude extract. Commercially available crude extracts of strain CBS192.65 (HAL Allergy, Leiden, The Netherlands) and strain CBS545.65 (Allergon, Ängelholm, Sweden) were also used (see the *Online Supplementary Methods* for details).

Flowcytometry

All studies were conducted with the approval of the institutional review board of the LUMC and after obtaining informed consent from the patients.

Peripheral blood samples were obtained from patients before and at regular intervals after allogeneic stem cell transplantation and cryopreserved until further use. PBMC ($0.5x10^6$) were stimulated with 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, The Netherlands), supplemented with 5% fetal calf serum (Gibco, Invitrogen, Bleiswijk, The Netherlands), 5% human serum and 100 IU/mL interleukin (IL)-2 (Novartis, Emeryville, CA, USA), and restimulated with non-loaded or peptide-pulsed autologous PBMC ($0.5x10^6$). One hour after restimulation, 10 μg/mL brefeldin A (Sigma-Aldrich, Zwijndrecht, The Netherlands) was added to promote intracellular accumulation of cytokines. Five hours after restimulation, cells were stained with peridinin chlorophyll-labeled anti-CD4 (BD/Pharmingen, Breda, The Netherlands), fixed with paraformaldehyde 1% (pharmacy LUMC) and permeabilized with saponin 0.1% (Sigma-Aldrich). Phycoerythrin-labeled anti-CD154 (Beckman Coulter, Woerden, The Netherlands) and allophycocyanin-labeled anti-interferon (IFN)γ (BD/Pharmingen) were added for intracellular staining of IFNγ production and the activation marker CD154. Cells were collected and analyzed on a Calibur II (BD, Breda, The Netherlands).

Generation of T-cell clones

PBMC ($0.5x10^6$) were stimulated with 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 h after restimulation *Aspergillus*-specific T cells were selected on the basis of CD154 expression, by sorting one cell per well by fluorescence activated cell sorting (FACS) after staining with phycoerythrin-labeled anti-CD154 (Beckman Coulter) and fluorescein isothiocyanate-labeled anti-CD4 (BD/Pharmingen).[14]

Epitope identification and determination of Aspergillus reactivity and HLA-restriction

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

To determine *Aspergillus* reactivity, peptide-specific clones were stimulated with an autologous EBV-LCL (responder:stimulator ratio 1:4) loaded with recombinant protein (100 μg/mL) or with autologous monocyte-derived dendritic cells preloaded with *Aspergillus* crude extract (responder:stimulator ratio 1:4) (see the *Online 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[14] (see the *Online 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 revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer and the Mycoses Study Group (EORTC/MSG) consensus group.[15] Twenty-two patients could not be included in the study. The median survival period of these 22 patients was 4 weeks (range, 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 analysis of the presence of *Aspergillus*-specific T cells was not, therefore, possible. Of the patients not included in the study, 15 had progressive invasive aspergillosis, six had stable aspergillus infection and in one patient the aspergillus infection was improving.

For the remaining 11 patients blood samples with sufficient T cells for analysis were available after aspergillosis and these patients were included in this study. The median survival period of the patients included was 9 months (range, 4 months to more than 6 years). The characteristics of the 11 patients analyzed are summarized in Table 1. The microbiological diagnosis was made on the basis of *A. fumigatus* culture and Galactomannan in serum or broncho-alveolar lavage fluid; *A. fumigatus* polymerase chain reaction analysis and information on 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 test for 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 *A. fumigatus*. All patients had typical computed tomography (CT) findings. Aspergillus infection was diagnosed 2 weeks to 10 months after allogeneic stem cell transplantation and antifungal treatment was started with voriconazole in eight patients, liposomal amphotericin B in two patients, and caspofungin in one patient. In the case of lack of improvement of aspergillus infection, azole-resistance was tested, voriconazole serum levels were measured and antifungal treatment was optimized by increasing the dose or changing the antifungal medication (Table 1).

All patients had received a T-cell depleted allogeneic stem cell transplant because of a hematologic malignancy. In four cases the graft was from a matched sibling donor, whereas in seven cases it was from a matched unrelated donor. The myeloablative conditioning regimen consisted of total body irradiation (9 Gy), whereas non-myeloablative conditioning therapy consisted of fludarabine (150 mg/m^2), busulfan (6.4 mg/kg body weight) and either antithymocyte globulin (20 mg/kg body weight) or alemtuzumab (30 mg). Nine patients needed systemic immunosuppression for post-transplantation graft-*versus*-host disease (GvHD) or auto-immune phenomena (Table 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

Table 1. Characteristics of the patients with invasive aspergillosis.

Patient	Age (year)	Gender	Hemat. disease	SCT	Systemic IS at time of IA	EORTC category IA	EORTC criteria for IA			Antifungal medication[¶]	Occurrence of Asp-specific T-cells		Clinical course of IA
							A. fum culture	Galactomannan	CT		Crf1	Cat1	
MLF	41	female	MM	NMA MSD	no	probable	BAL, sputum	BAL	nodules	Vori, 8 mo	yes	yes	clearance
FBV	40	male	CML	MA MUD	cyclo, pred	proven	biopsy, sputum	neg	nodules, cavitation	AmB, 6 w Itra, 2 mo	yes	no	clearance
NKB	43	male	MM	NMA MUD	no	probable	BAL	BAL, serum	nodules	Vori, 8 mo	yes	yes	clearance
CSS	45	female	NHL	MA MUD	cyclo, pred	probable	neg	BAL	nodules, cavitation	Vori, 1 w AmB, 2 w, Vori, 6 mo	yes	yes	clearance
MPK	62	female	MM	NMA MUD	cyclo	probable	sputum	BAL, serum	nodules	AmB, 6 w Vori*, 6 mo	yes	yes	clearance
TPA	62	female	NHL	NMA MUD	pred	probable	BAL	BAL	nodules	Vori, 15 mo	yes	yes	clearance
ACD	55	female	MM	NMA MUD	pred	probable	sputum	serum	nodules	Vori, 5 w Vori*, 5 mo	yes	yes	regression
JKJ	73	male	CLL	NMA MUD	pred	probable	neg	BAL	nodules, halo	Vori, 8 mo	no	yes	regression
JHF	48	female	MM	NMA MSD	no	probable	neg	serum	nodules, halo	Caspo, 4 w Vori, 8 w Posa, 4 w	yes	no	stable
MST	37	male	NHL	NMA MSD	cyclo, pred	probable	sputum	serum	nodules	Vori, 4 mo Caspo, 3 d AmB, 4 d	no	no	progressive
ESF	42	male	CML	MA MSD	cyclo, pred, MMF	probable	sputum	neg	nodules, halo	Vori, 4 mo No antifung, 7 mo Vori, 2 w	no	no	progressive

[¶]Consecutively prescribed antifungal medication, starting from day of diagnosis. Hemat disease: hematologic 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; cycle: cyclosporine; Pred: prednisolone; MMF: mycofenolate-mofetil; BAL: bronchoalveolar lavage; Vori: voriconazole; Vori voriconazole dose adjusted according to serum levels, Itra: itraconazole; AmB liposomal amphotericin B: Caspo:caspofungine; Posa: posaconazole; mo:months; w: weeks; d: days.*

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 from two representative patients with improvement of invasive aspergillosis are shown in Figure 1. In patient MLF a steep increase was seen in the frequencies of Crf1- and Catalase1-specific T cells, within 3 weeks after the diagnosis of invasive aspergillosis (Figure 1A). The frequencies of Crf1- and Catalase1-specific T cells peaked at the moment of regression of the aspergillus lesion on the CT scan (Figure 1D) and, after clearance of the infection, decreased to undetectable levels (Figure 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 1B) or IFNγ production (Figure 1C). During the course of her aspergillus infection patient MLF used topical immunosuppressive medication because of GvHD, and did not experience leukopenia or granulocytopenia (Figure 1B,C).

Patient TPA was treated for invasive aspergillosis for more than a year because of a prolonged period of leukopenia and granulocytopenia (Figure 1F,G). During this period she was treated alternately with systemic and topical immunosuppression for GvHD (figure 1F,G). More than 1 year after the diagnosis of invasive aspergillosis, 8 months after recovery of leukocyte and granulocyte counts, peaks in both Crf1- and Catalase1-specific T cells were identified on the basis of CD154 expression (Figure 1E,F) and IFNγ production (Figure 1E,G), coinciding with regression of the aspergillus lesions (Figure 1H).

Aspergillus-specific T cells were also identified in the seven other analyzed patients with improvement of aspergillus infection (Figure 2). The majority received systemic immunosuppression during the course of their aspergillus infection. In two patients (JHF and FBV) only Crf1-specific T cells were present, one patient (JKJ) had only Catalase1-specific T cells, and in four patients (NKB, MPK, ACD and CSS) both Crf1- and Catalase1-specific T cells were identified (Table 1). Peak frequencies of Crf1- and Catalase1-specific T cells occurred around the time of regression of the 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 *A. fumigatus*, but serum Galactomannan was negative. Despite recovery of leukocyte and granulocyte counts (Figure 3B), the patient developed new nodular lesions on the CT scan 4 months later, with positive serum Galactomannan (Figure 3C). *A. fumigatus* and *Rhizomucor* were grown from a sputum culture. At this moment the patient had GvHD of the

skin, liver and colon for which he was treated with prednisolone and cyclosporine. The patient died of respiratory failure 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 3A,B). In patient ESF possible invasive aspergillosis was diagnosed 2 weeks after allogeneic stem cell transplantation. This patient was treated for 4 months until chest X-rays normalized. Seven months later, he had a progressive,

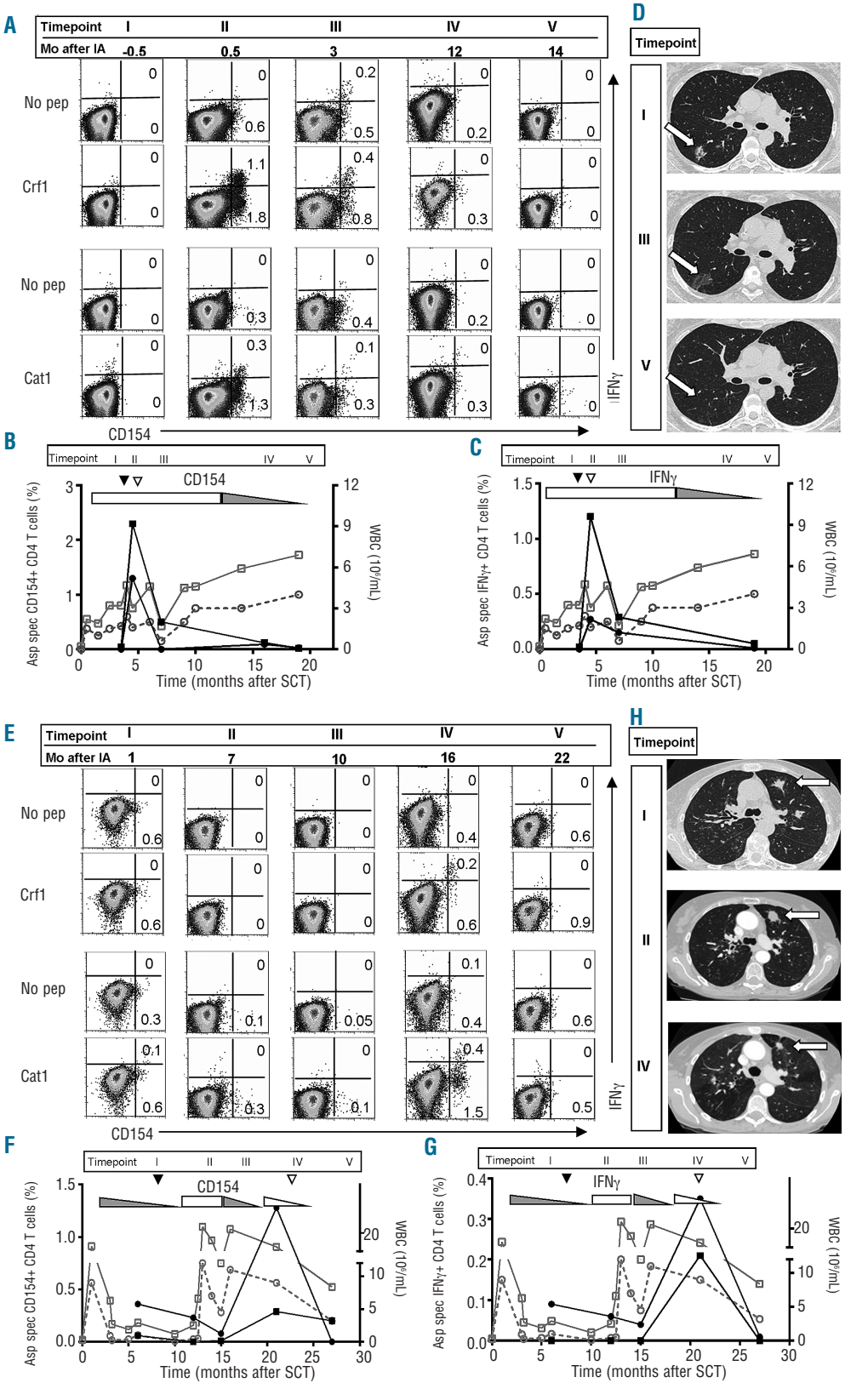

Figure 1. *Aspergillus*-specific T-cell responses and imaging studies in patients with improvement of invasive aspergillosis. FACS-analyses, imaging studies and kinetics of the *Aspergillus*-specific T-cell responses of patient MLF (A-D) and patient TPA (E-H). (A and E) FACS analyses were performed on PBMC that were stimulated with Crf1 or Catalase1, and restimulated with Crf1- or Catalase1-pulsed PBMC or with unpulsed PBMC (no pep). T cells were analyzed 5 h after restimulation and FACS-plots are gated on CD4⁺ T cells. The kinetics of the percentages of Crf1- (■) and Catalase1-specific (●) CD154⁺ CD4⁺ T-cells (left y-axis, B and F) and IFNγ⁺ CD4⁺ T cells (left y-axis, C and G) and absolute leukocyte (□) and granulocyte counts (○) (right y-axis) are shown before and after diagnosis (▼) and improvement (▽) of aspergillus 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 CD154⁺ CD4⁺ T cells were calculated as % CD154⁺ of CD4⁺ T cells after restimulation with peptide-pulsed PBMC minus % CD154⁺ of CD4⁺ T cells after restimulation with unpulsed PBMC. The same method of calculation was used for IFNγ⁺ CD4⁺ T cells. Pulmonary CT scans of patient MLF (D) and patient TPA (H) at different time points after diagnosis show the changes in the size of aspergillus lesions during the course of infection. Aspergillus lesions on the CT scans are indicated with an arrow. The timepoints indicated by Roman numerals in the top of the graphs in (B) and (C) and in (F) and (G) correspond to the time points of FACS analyses and CT scans in respectively (A) and (D), and (E) and (H). In figure (A) and (E) the corresponding period in months after invasive aspergillosis (Mo after IA) is given for every timepoint. SCT: stem cell transplantation; WBC: white blood cells.

probable aspergillosis with positive *A. fumigatus* sputum cultures and new lesions on a CT scan (Figure 3F), despite normal leukocyte and granulocyte counts (Figure 3E). The patient had been treated with topical and systemic immunosuppression because of skin GvHD, which eventually worsened with development of GvHD of the liver. Shortly after, he deteriorated and died of brain herniation due to an intracerebral bleed. Retrospectively, very low frequencies of Catalase1-specific T cells were identified, 0.04% of CD4$^+$ T cells, on the basis of IFNγ production, 3 months before his death (Figure 3D,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 four different *Aspergillus* proteins. After stimulation and restimulation with Aspf1, Aspf2, Aspf3 and Aspf4 no *Aspergillus*-specific T cells were identified in patients MST and ESF (*Online Supplementary Figure S1*).

The mean percentage of Crf1-specific T cells of all analyzed patients around diagnosis was 0.03% (range, 0-0.2%), while that of Catalase1-specific T cells was 0.02% (range, 0-0.09%). The percentages of Crf1- and Catalase1-specific T cells were not changed at the first time point with normal leukocyte counts. However, around the moment of improvement of aspergillus infection, the mean percentage of Crf1-specific T cells was significantly higher (0.35%; range, 0.03-1.2%) (P<0.01). For Catalase1-specific T cells a trend towards an increase (P=0.07) was seen, with the mean percentage being 0.42% (range, 0-2.28%) (Figure 4).

Overall, in nine 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 occurring around the time of regression of the aspergillus lesions. In two patients with progressive disease no or very low frequencies (below 0.05%) of *Aspergillus*-specific T cells were detected.

Aspergillus-specific T-cell clones from patients with invasive aspergillosis mainly have a T helper 1 phenotype

It has been suggested in the past that *Aspergillus*-specific T helper 1 cells are beneficial in invasive aspergillosis, whereas T helper 2 cells could be detrimental.[2-5,16] To analyze the cytokine profiles of *Aspergillus*-specific T cells in patients with improvement of their aspergillus infection, we sorted single CD4$^+$ T cells from six patients at the peak of the immune response on the basis of CD154 expression, after stimulation and restimulation with 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 (*Online Supplementary Table S1*). To analyze the cytokine profiles of the clones, the T-cell clones were stimulated with overlapping peptide pools and production of IFNγ, IL-4, IL-10 and IL-17 was measured using standard enzyme-linked immmunosorbent assays. Of the 19 T-cell clones, 16 produced large amounts of IFNγ and a minority of the IFNγ-producing T-cell clones also produced small amounts of IL-

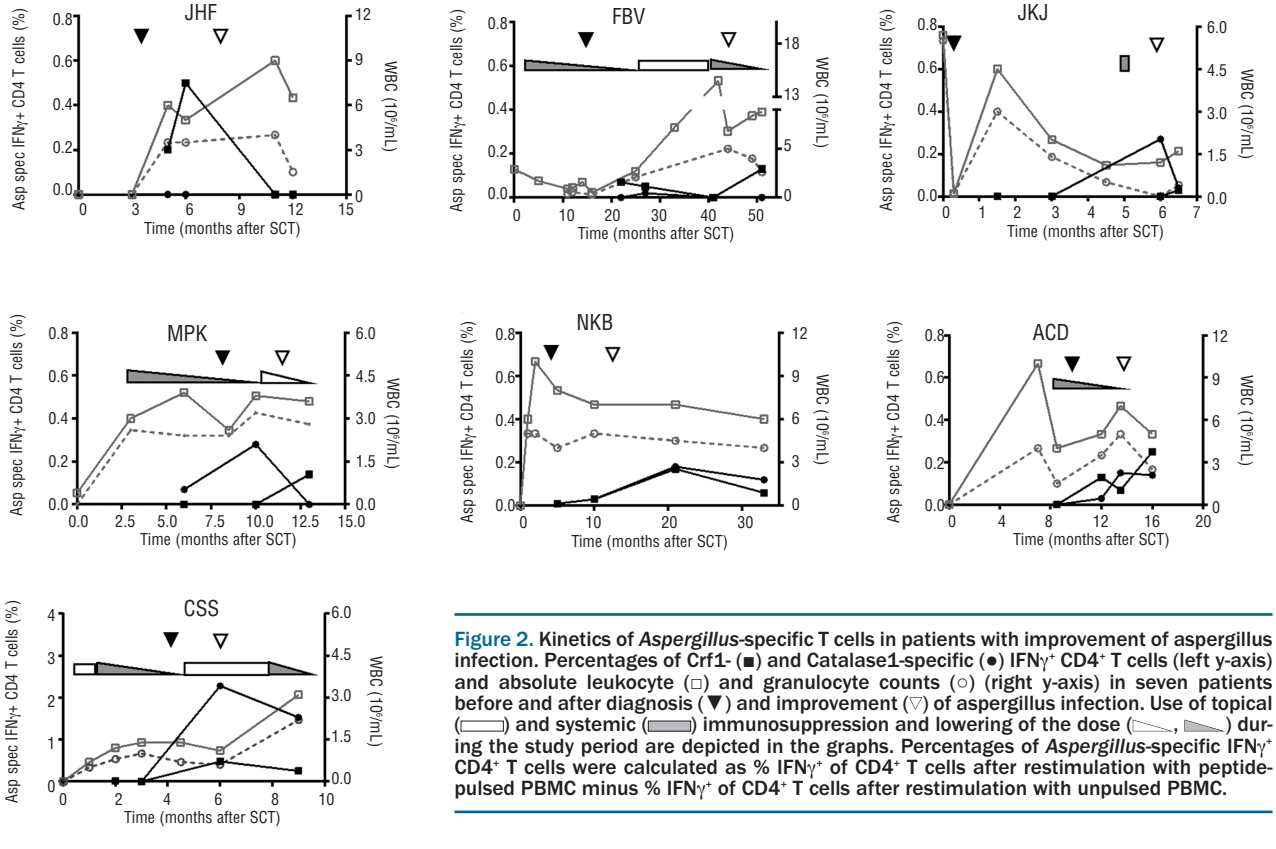

Figure 2. Kinetics of *Aspergillus*-specific T cells in patients with improvement of aspergillus infection. Percentages of Crf1- (■) and Catalase1-specific (●) IFNγ$^+$ CD4$^+$ T cells (left y-axis) and absolute leukocyte (□) and granulocyte counts (○) (right y-axis) in seven patients before and after diagnosis (▼) and improvement (▽) of aspergillus 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.

4, IL-10 or IL-17. Two clones only produced IL-17, and one only produced IL-4 (*Online Supplementary Table S1*). T-cell clones with different cytokine profiles can be present in one patient; for example, in patient ACD we identified four IFNγ-producing T-cell clones and one IL-17-producing T-cell clone and in another patient (FBV) we identified two IFNγ-producing T-cell clones and one IL-4-producing clone. Epitope specificity did not correlate with cytokine profile, since we identified two T-cell clones directed against the same peptide with a different cytokine profile (amino acids 57-67, presented in HLA-DQB1*0301) (*Online Supplementary Table S1*).

To exclude that the T helper 1 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 four other *A. fumigatus* proteins in addition to Crf1 and Catalase1, we found a T helper 1 response against these proteins in some of the healthy controls. In patient TPA we again observed a T helper 1 response against Crf1 and Catalase1, whereas no response against Aspf1, Aspf2, Aspf3 or 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 showed that the immune response against *A. fumigatus* in healthy individuals is 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 subpools of Crf1 or Catalase1 overlapping peptides, which have been composed in a matrix, as described previously.[14] As every peptide is only present

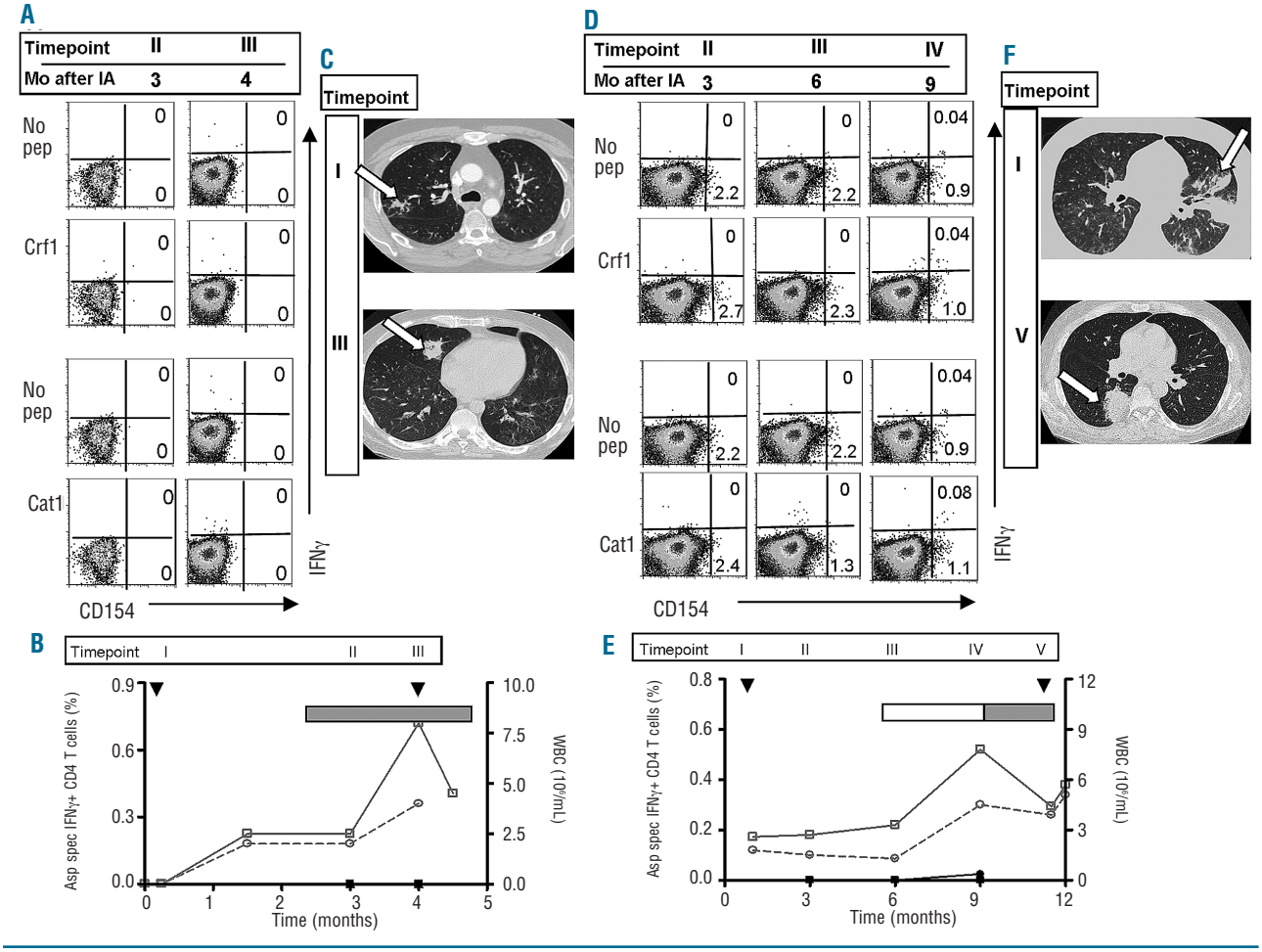

Figure 3. *Aspergillus*-specific T-cell responses and imaging studies in patients with progressive invasive aspergillosis. FACS analyses, imaging studies and kinetics of the *Aspergillus*-specific T-cell responses of patient MST (A-C) and ESF (D-F). (A and D) FACS analyses were performed on PBMC after restimulation with Crf1- or Catalase1-pulsed PBMC or with unpulsed PBMC (no pep). T cells were analyzed 5 h 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 is 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 time points. *Aspergillus* lesions on the CT scans are indicated with an arrow. The time points indicated by Roman numerals in the top of the graphs in (B) and (E) correspond to the time points of FACS analyses and CT scans in, respectively, (A) and (C), and (D) and (F). In (A) and (D) the corresponding period in months after invasive aspergillosis (Mo after IA) is given for every time point.

in two subpools, we could identify the epitopes recognized directly from the subpool analysis. A representative example is shown in *Online Supplementary Figure S2A*. T-cell clone ACD3 recognized subpools 1G and 11, and therefore recognized peptide 1G11 (amino acids 333-347, DPTKIVPEEFVPITK). The T-cell epitopes were confirmed by stimulating the T-cell clones with newly synthesized peptides: an example is shown in *Online Supplementary Figure S2B*.

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 the case of unclear results, HLA restriction was established using HLA-transduced Hela cells. An example is shown in *Online Supplementary Figure S2C*. 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 (*Online Supplementary Figure S2C*). T-cell clones directed against several different epitopes of Crf1 or Catalase1 protein were identified in the majority of patients. Eight of 16 epitopes identified in these patients had been previously identified in healthy individuals (*Online Supplementary Table S1*).

To confirm that the Crf1- and Catalase1-specific T cells were *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 *Aspergillus* crude extract (*Online Supplementary Figure S2D*). Five of the seven Catalase1-specific T-cell clones recognized Catalase1 recombinant protein, and three of these also recognized *Aspergillus* protein extract (*Online Supplementary Figure S2E*).

Discussion

In this study we demonstrated the induction of Crf1- and Catalase1-specific T cells in nine patients with invasive aspergillosis. The appearance of these *Aspergillus*-specific T cells coincided with regression of the 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 patients recovering from invasive aspergillosis, *Aspergillus*-specific T cells were directed against various different epitopes derived from Crf1 or Catalase1 and preferentially exerted a T helper 1 phenotype.

In previous studies low frequencies of *Aspergillus*-specific T cells were identified in the peripheral blood of healthy individuals stimulated with conidia of *A. fumigatus*,[5,7,10] *A. fumigatus* crude extract,[5,8,10] *A. fumigatus* recombinant proteins[5,7,8] or overlapping peptides of *A. fumigatus* proteins.[9,11-14] *Aspergillus*-specific T-cell responses were equivalent when stimulated with Catalase1 or Crf1 recombinant protein compared to conidia[7] or crude extracts,[5] and we have previously demonstrated that T cells directed against *A. fumigatus* can be identified by using overlapping peptides of Crf1 and Catalase1.[14] It was not, however, investigated whether these T cells play a role in the defense against *A. fumigatus*.

In this study we were able to follow, over time, 11 patients who were diagnosed with invasive aspergillosis after allogeneic stem cell transplantation. In the nine patients recovering from invasive aspergillosis we were able to show the induction of CD4+ T cells directed against the *A. fumigatus* proteins Crf1 and Catalase1 at the time of regression of the aspergillus lesions. There were blood samples with sufficient T cells for analysis from only two patients with progressive aspergillus infection. No Crf1- or Catalase1-specific T cells were identified in these two patients. The small size of the group of patients with progressive aspergillosis who could be analyzed for the presence of *Aspergillus*-specific T cells precluded a statistical analysis of the comparison between patients whose infection was progressing or recovering. However, among nine patients with lymphopenia who were screened for this study, but not included in it, eight 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 whose infection cleared and those in whom the infection progressed. 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 patients with progressive infection, as well as in five of the nine 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 led to the absence of *Aspergillus*-specific T cells, contributing to the increased risk of invasive aspergillosis. Furthermore, when comparing the frequencies of Crf1-

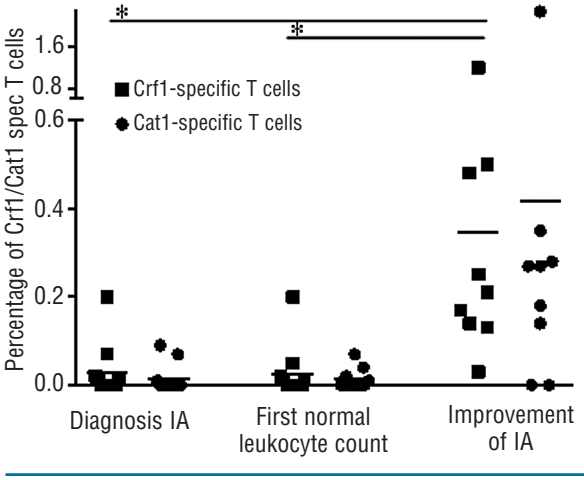

Figure 4. Increases of Crf1- and Catalase1-specific T cells at the time of improvement of aspergillus infection. Percentages of Crf1- (■) and Catalase1-specific (●) IFNγ+ CD4+ T cells around diagnosis of aspergillus infection (11 patients), at the first time point with normal leukocyte counts (11 patients) and at the time of improvement of aspergillus infection (9 patients). *Significant difference between compared groups (*P*<0.01, two-tailed Student T test) Horizontal lines indicate the mean percentage of Crf1- or Catalase1-specific T cells among all analyzed patients at this time point.

and Catalase1-specific T cells at the moment of regression of aspergillus lesions and the frequencies at the moment of diagnosis or recovery of leukocyte count, we found a significant difference in frequencies between these time points, further pointing to a role for *Aspergillus*-specific T cells in the recovery from invasive aspergillosis.

Previously, Hebart *et al.* studied the T-cell response to *Aspergillus* crude extracts in patients with invasive aspergillosis.[5] 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 having possible invasive aspergillosis, so the diagnosis was 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,[5] whereas high levels of IL-10 production were documented in patients with invasive aspergillosis before adoptive immunotherapy with *Aspergillus*-specific T cells,[6] and in patients with progressive disease.[5] In this study, similarly to what was demonstrated in healthy individuals,[14] 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 T helper 1 phenotype, producing large amounts of IFNγ. A selection of clones also produced IL-4, IL-10 or IL-17, but only three T-cell clones produced only IL-4 or IL-17, indicating that T regulatory cells and T helper 2 cells probably did not play a major role in these patients. Although this could be a consequence of the culture procedure or the selection method based on CD154 expression, it fits with previous data indicating that T helper 1 cells are associated with improvement of aspergillus infection,[2-5,7,17] whereas T helper 2 and T helper 17 cells are associated with increased fungal growth.[5,6,16,17] We were not able to select T-cell clones from the patients with progressive infection because of the lack of CD154$^+$ T cells after stimulation with the overlapping peptides. This suggests the absence of *Aspergillus*-specific T cells in these patients rather than a T helper 2-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 four other *A. fumigatus* proteins did not detect any IL-4- or IL-5-producing T cells, either in patients recovering from invasive aspergillosis or in patients with progressive disease.

Although the peaks of *Aspergillus*-specific T cells occurred 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, cytomegalovirus infection, which can be confirmed reliably and quantitatively by determining the plasma viral load.[18] In cytomegalovirus infection it has been demonstrated that the clearance of the viral infection is preceded by the occurrence of CMV-specific T cells in the peripheral blood.[19] Invasive aspergillosis is diagnosed when a patient fulfills the EORTC criteria for a possible, probable or proven diagnosis of aspergillosis,[15] 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, such as serum Galactomannan, or not validated, like *Aspergillus* polymerase chain reaction analysis. 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 the 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[2-4,7] and a human study[6] 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 time 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.

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Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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

Hetty Jolink,[1,2] Renate S. Hagedoorn,[1] Ellen L. Lagendijk,[3] Jan W. Drijfhout,[4] Jaap T. van Dissel,[2] J.H. Frederik Falkenburg,[1] and Mirjam H.M. Heemskerk[1]

[1]Department of Hematology, Leiden University Medical Center; [2]Department of Infectious Diseases, Leiden University Medical Center; [3]Department Molecular Microbiology and Biotechnology, Institute of Biology, Leiden University; and [4]Department of Immunohematology and Blood transfusion, Leiden University Medical Center, the Netherlands

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Correspondence: mhmheemskerk@lumc.nl

Online 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.

Flowcytometry

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 (Miltenyi 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 E_2 (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^{-6}M) 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

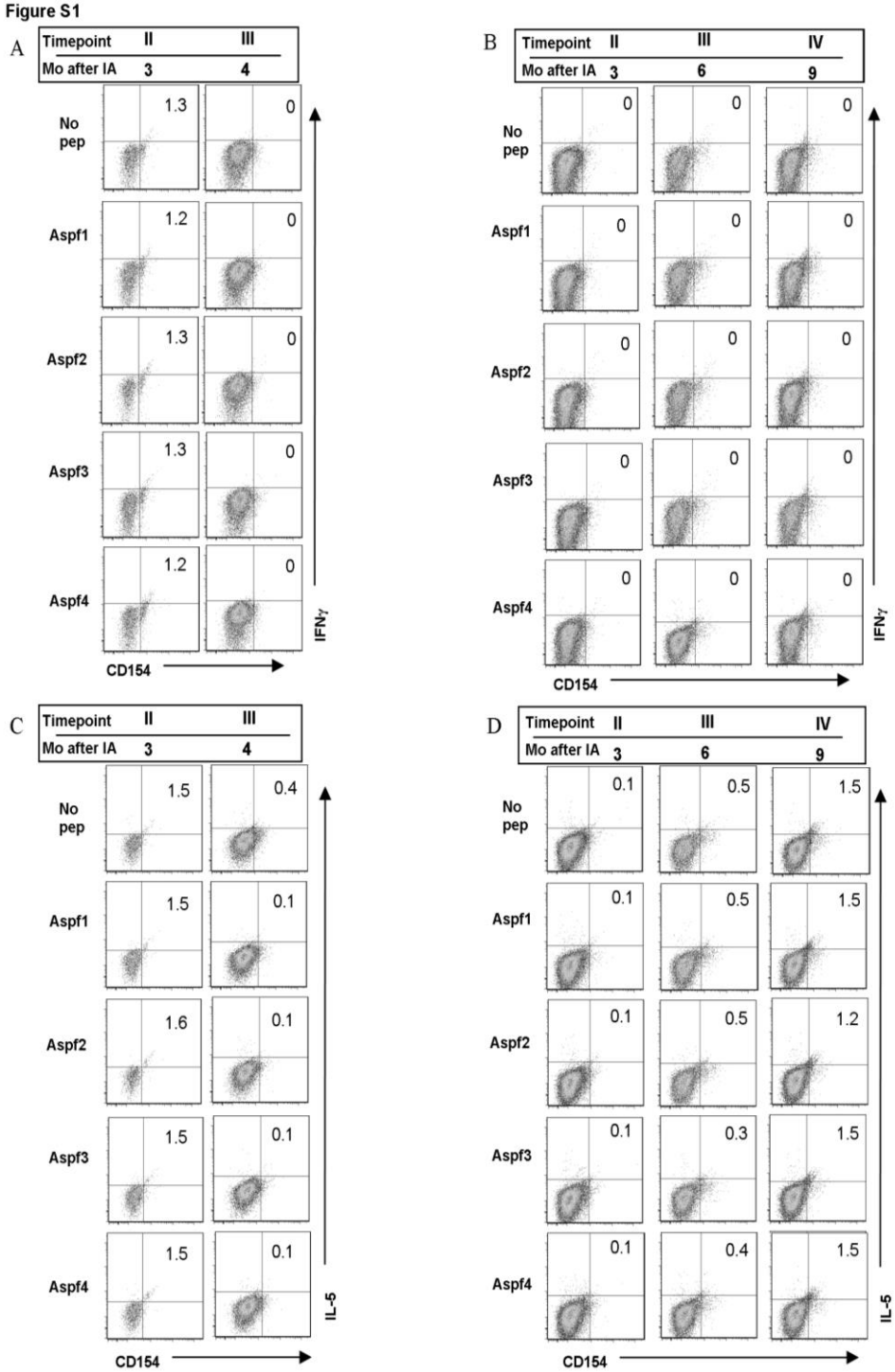
hour. Subsequently, T cells were added in a R:S ratio of 1:4 and incubated ON. Supernatant was collected and analyzed using standard ELISA. Further determination of the HLA-restriction was analyzed using the HLA-typed EBV-LCL panel, containing partially matched allogeneic EBV-LCL. The T cell clones and EBV-LCL (R:S ratio 1:4) were incubated ON with the complete overlapping peptide pool (10^{-6}M) 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.

Supplementary table S1. Identified Crf1 and Catalase1 epitopes in patients with invasive aspergillosis

Epitope	aa sequence	Clone	Cytokine production	Vβ‡§	HLA-restriction	*Aspergillus* extract	Catalase1 rec protein*
Crf1							
aa 57-67	EVTAGKVPVGP	MLF 77	IFNγ	7.2	DRB1*11	yes	n/a
aa 57-67	EVTAGKVPVGP	ACD 11, JHF 31	IL-17, IFNγ	17, 13.2	DQB1*0301	yes	n/a
aa 89-99	**FFFGKAEVVMK**	ACD 4	IFNγ	u.k.	DRB1*0101	yes	n/a
aa 122-132	VLESDDLDEVD	NKB 14	IFNγ	2	DQB1*0202	yes	n/a
aa 161-175	**HTYTIDWTKDAVTWS**	FBV 16	IFNγ	u.k.	DRB3*01	yes	n/a
aa 165-179	**IDWTKDAVTWSIDGA**	FBV 1	IFNγ	2	DPB1*0101	yes	n/a
aa 169-183	KDAVTWSIDGAVVRT	MLF 3	IFNγ	u.k.	DRB1*03	yes	n/a
aa 217–231	GTIEWAGGLTDYSAG	JHF 21	IFNγ	2	DQB1*0301	yes	n/a
aa 233-243	**YTMYVKSVRIE**	NKB 6, JHF 22	IFNγ, IFNγ	8, 13.1	DRB1*0701	yes	n/a
aa 237-247	VKSVRIENANP	JHF 25	IFNγ	u.k.	DRB4*0103	yes	n/a
Catalase1							
aa 117-127	**DFSNITAASFL**	NKB 10	IFNγ	2	DRB1*0701	no	no
aa 137-147	FVRFSTVAGSR	JHF 5	IFNγ	3	DRB1*0408	no	yes
aa 333-347	DPTKIVPEEFVPITK	ACD 3	IFNγ	3	DPB1*0401/DPB1*0402	yes	yes
aa 345-355	**GKMQLNRNPRN**	ACD 1, ACD 5	IFNγ, IFNγ	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

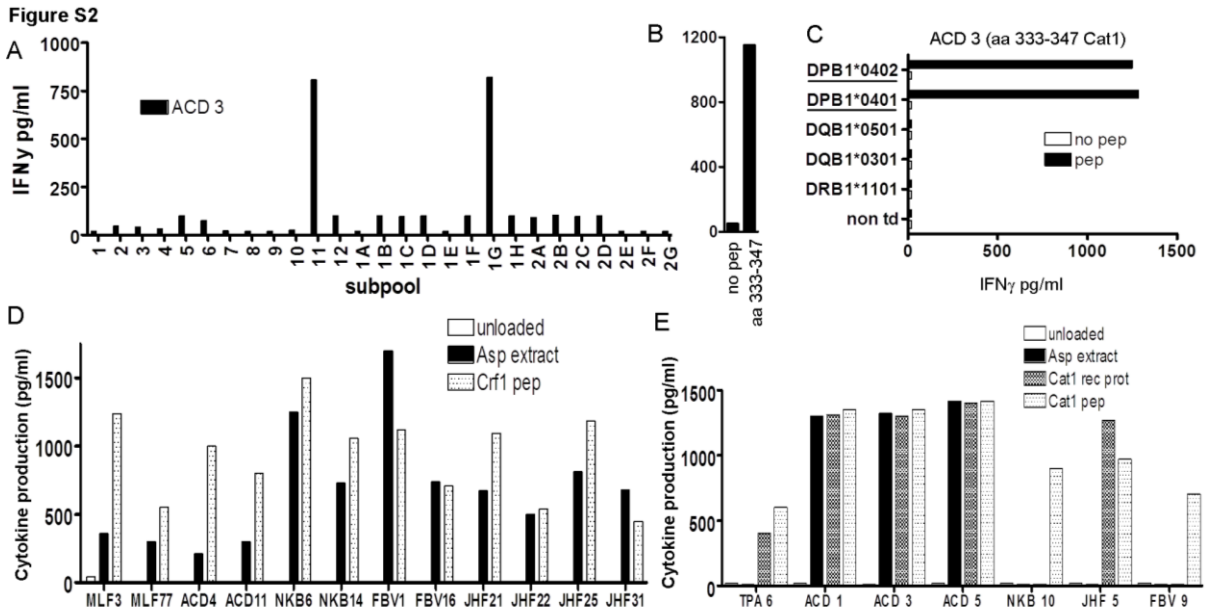
* n/a: not applicable
‡ Vβ analysis by TCR Vβ monoclonal antibodies
§ u.k.: Vβ unknown when not present in TCR Vβ kit
Bold: epitopes previously identified in healthy individuals

Figure S1



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 3B and the timepoints in figure B and D correspond to the timepoints in the graph of figure 3E.



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.